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SUPPRESSOR OF CYTOKINE SIGNALING (SOCS)1 AND SOCS3 STIMULATION
DURING EXPERIMENTAL CYTOMEGALOVIRUS RETINITIS: VIROLOGIC,
IMMUNOLOGIC, OR PATHOLOGIC MECHANISMS

by

CHRISTINE I. ALSTON

Under the Direction of Richard D. Dix, PhD

ABSTRACT

AIDS-related human cytomegalovirus (HCMV) retinitis remains the leading cause of blindness among untreated HIV/AIDS patients worldwide. Understanding the pathogenesis of this disease is essential for developing new, safe, and effective treatments for its prevention or management, yet much remains unknown about the virologic and immunologic mechanisms contributing to its pathology. To study such mechanisms, we use a well-established, reproducible, and clinically relevant animal model with retrovirus-induced murine acquired immunodeficiency syndrome (MAIDS) that mimics in mice the symptoms and progression of AIDS in humans. Over 8 to 12 weeks, MAIDS mice become susceptible to experimental murine

cytomegalovirus (MCMV) retinitis. We have found in this model that MCMV infection significantly stimulates ocular suppressor of cytokine signaling (SOCS)1 and SOCS3, host proteins which dampen immune-related signaling by cytokines, including antiviral interferons. Herein we investigated virologic and/or immunologic mechanisms involved in this stimulation and how virally-modulated SOCS1 and/or SOCS3 proteins may contribute to MCMV infection or experimental MAIDS-related MCMV retinitis. Through pursuit of two specific aims, we tested the central hypothesis that MCMV stimulates and employs SOCS1 and/or SOCS3 to induce the onset and development of MCMV retinal disease. MCMV-related SOCS1 and SOCS3 stimulation *in vivo* occurred with intraocular infection, was dependent on method and stage of immune suppression and severity of ocular pathology, was associated with stimulation of SOCS-inducing cytokines, and SOCS1 and SOCS3 were differentially sensitive to antiviral treatment. *In vitro* studies further demonstrated that SOCS1 and SOCS3 stimulation during MCMV infection occurred with expected immediate early kinetics, required viral gene expression in cell-type-dependent and virus origin-dependent patterns of expression, and displayed differential sensitivity to antiviral treatment. These data suggest that SOCS1 and SOCS3 are stimulated by divergent virologic, immunologic, and/or pathologic mechanisms during MCMV infection, and that they contribute to the pathogenesis of retinal disease, revealing new insights into the pathophysiology of AIDS-related HCMV retinitis.

INDEX WORDS: Cytomegalovirus retinitis, human cytomegalovirus (HCMV), HIV/AIDS, suppressors of cytokine signaling (SOCS), murine cytomegalovirus (MCMV), murine AIDS (MAIDS)

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CHRISTINE I. ALSTON

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2016

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Christine Iris Alston
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December 2016

DEDICATION

This dissertation is dedicated to my loving and supportive family: to my husband, Kenny, whose tireless sacrifice and encouragement have made this work possible; to my parents Phil and Mary; my parents-in-law John and Kathy; my sister Audrey; my sister-in-law Holly; to my son, Philip Conan, whose miraculous birth and life I count as the greatest and most fulfilling triumph of my life thus far; and to the rest of my family, all of whom, by their presence, prayers, and legacies, bear witness and minister accountability as constant reminders of the significant purpose of my life and work on this earth.

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	v
LIST OF TABLES	x
LIST OF FIGURES	xi
1 Introduction	1
1.1 The Eye.....	2
1.1.1 Anatomy of the Eye.....	2
1.1.2 Retina and Retinal Pigmented Epithelium (RPE)	3
1.1.3 Immune Cells of the Retina.....	5
1.1.4 Ocular Immune Privilege	6
1.2 Pathogenesis of AIDS-Related Cytomegalovirus Retinitis	8
1.3 Cytomegalovirus.....	10
1.3.1 Historical Context	10
1.3.2 HCMV and MCMV Classification, Structure, and Genome.....	10
1.3.3 HCMV and MCMV Replication Cycle.....	17
1.3.4 Host Response to Cytomegalovirus	22
1.3.5 Diseases of Cytomegalovirus Etiology.....	25
1.4 Human Immunodeficiency Virus and AIDS.....	30
1.4.1 Historical Context	30
1.4.2 HIV-1 Classification, Structure, and Genome	30

1.4.3	HIV-1 Epidemiology, Tropism, and Transmission.....	31
1.4.4	HIV-1 Pathology and Immune Response to Infection	32
1.5	MAIDS-Related Murine Cytomegalovirus Retinitis.....	33
1.5.1	Murine Acquired Immunodeficiency Syndrome (MAIDS)	33
1.5.2	Experimental Murine Cytomegalovirus Retinitis.....	34
1.6	Suppressors of Cytokine Signaling (SOCS).....	39
1.6.1	SOCS Family, Structure, and Function.....	39
1.6.2	SOCS1 and SOCS3	42
1.6.3	Viral Exploitation of SOCS1 and SOCS3.....	44
1.7	Goals of this Dissertation.....	45
2	Materials and Methods	49
2.1	Cell Lines and Stocks	49
2.2	Viruses	50
2.3	Animals.....	54
2.4	Histopathology.....	56
2.5	Quantification of Infectious MCMV	57
2.6	Enzyme-Linked Immunosorbent Assay (ELISA)	58
2.7	Immunofluorescent (IF) Staining	59
2.8	Western Blot Assays.....	59
2.9	RNA Extraction and Real-Time RT-PCR	60

2.10	Statistical Analyses	63
3	Specific Aim 1 SOCS1 and SOCS3 during MCMV Infection <i>In Vivo</i>	64
3.1	SOCS1 and SOCS3 Expression during Systemic MCMV Infection	64
3.1.1	Systemic MCMV in Immunologically Normal Mice without MAIDS	64
3.1.2	Systemic MCMV during MAIDS-4 or MAIDS-10	69
3.2	Subretinal MCMV during Late-Stage MAIDS Progression	75
3.3	Subretinal MCMV during Drug-Induced Immune Suppression	80
3.4	Ganciclovir Treatment during Subretinal MCMV in MAIDS Mice.....	86
4	Specific Aim 2 SOCS1 and SOCS3 during MCMV Infection <i>In Vitro</i>	88
4.1	Expression Kinetics with SG-MCMV or TC-MCMV in IC-21 Cells.....	88
4.2	UV-Inactivated MCMV in IC-21 Mouse Macrophages or MEF Cells	99
4.3	MCMV-Stimulated SOCS3 in IC-21 Cells is Sensitive to Ganciclovir	107
4.4	Infection of IC-21 Cells with EGFP-Tagged MCMV RM4503.....	109
5	Discussion and Conclusions	111
5.1	Specific Aim 1: SOCS1 and SOCS3 during MCMV Infection <i>In Vivo</i>	112
5.1.1	Systemic MCMV Infection in Mice without or with MAIDS	114
5.1.2	Subretinal MCMV during the Progression of Late-Stage MAIDS	117
5.1.3	Subretinal MCMV during Drug-Induced Immunosuppression.....	119
5.1.4	Antiviral Treatment during Subretinal MCMV in MAIDS Mice.....	123
5.2	Specific Aim 2: SOCS1 and SOCS3 during MCMV Infection <i>In Vitro</i>	125

5.2.1	Expression Kinetics with SG-MCMV or TC-MCMV in IC-21 Cells ...	127
5.2.2	UV-Inactivated MCMV in IC-21 or MEF Cells.....	130
5.2.3	MCMV-Stimulated SOCS3 is Sensitive to Ganciclovir.....	132
5.2.4	Infection of IC-21 Cells with SG-MCMV RM4503	133
5.3	Future Directions.....	136
5.4	Clinical Significance.....	140
5.5	Summary	143
5.5.1	Summary of Specific Aim 1.....	143
5.5.2	Summary of Specific Aim 2.....	144
5.6	Conclusions	146
	References	147

LIST OF TABLES

Table 1.1: Human Herpesviruses in the Family Herpesviridae.	12
Table 1.2: Taxonomies of Human and Murine Cytomegalovirus.	13
Table 2.1: Histologic Grading Criteria for MCMV Retinitis, Posterior Segment.	57
Table 2.2: Real-Time RT-PCR Primer Sequences or Designations.	62
Table 3.1: Frequency and Severity of Retinitis and Ocular Viral Load in Two Different Models of Experimental MCMV Retinitis.	83
Table 5.1: Ocular Specifications during Systemic or Subretinal MCMV Infection of C57BL/6 Mice with MAIDS or Corticosteroid-Induced Immune Suppression.	113

LIST OF FIGURES

Figure 1.1. Schematic section of the human eye.....	3
Figure 1.2. Histology of the layers of a normal C57BL/6 mouse retina.....	4
Figure 1.3. Fundus photograph of human retina during AIDS-related HCMV retinitis.	8
Figure 1.4. Structure of the cytomegalovirus virion.....	14
Figure 1.5. Genomic structures of HCMV and MCMV.....	17
Figure 1.6. HCMV or MCMV replication cycle within a host cell.....	18
Figure 1.7. Schematic mouse eye with subretinal site of injection.	35
Figure 1.8. Ocular histopathology of experimental MAIDS-related MCMV retinitis.....	38
Figure 1.9. SOCS family proteins homologous domains.	40
Figure 1.10. Schematic representation of cytokine signaling by the JAK/STAT pathway, with induction and function of SOCS proteins.	41
Figure 1.11. Intraocular SOCS1 and SOCS3 mRNA and proteins are highly stimulated in the MCMV-infected eyes of retinitis-susceptible MAIDS-10 mice, but not retinitis- resistant MAIDS-4 mice.	46
Figure 1.12. Ocular F4/80⁺ macrophages express SOCS1 and SOCS3 during MAIDS- related MCMV retinitis.....	47
Figure 2.1. Molecular construction of MCMV RM4503.	52
Figure 3.1. SOCS1 and SOCS3 expression in whole splenic cells is moderately up-regulated following systemic MCMV infection in immunologically normal C57BL/6 mice.....	66
Figure 3.2. Differential mRNA expression of cytokine inducers of SOCS1 and SOCS3 in whole splenic cells following systemic MCMV infection of immunologically normal C57BL/6 mice.	68

Figure 3.3. SOCS1 and SOCS3 expression in whole splenic cells is not stimulated following systemic MCMV infection in MAIDS-4 or MAIDS-10 mice.	70
Figure 3.4. Splenic cytokines known to induce SOCS1 and SOCS3 are not highly stimulated following systemic MCMV infection in MAIDS-4 or MAIDS-10 mice... ..	71
Figure 3.5. SOCS1 and SOCS3 expression in whole eyes is not stimulated following systemic MCMV infection of MAIDS-4 or MAIDS-10 mice.	73
Figure 3.6. Ocular cytokines known to induce SOCS1 and SOCS3 are differentially stimulated following systemic MCMV infection in MAIDS-4 or MAIDS-10 mice... ..	74
Figure 3.7. MCMV-related stimulation of SOCS1 and SOCS3 mRNA expression in MCMV-infected eyes decreases during late-stage progression of MAIDS.....	76
Figure 3.8. Ocular SOCS1 and SOCS3 protein are not significantly stimulated during subretinal MCMV infection of MAIDS-12 mice.....	77
Figure 3.9. Ocular SOCS1 and SOCS3 mRNA expression in MAIDS-10 subretinally-injected eyes compared with uninjected MAIDS-10 eyes.	79
Figure 3.10. SOCS1 and SOCS3 are not highly stimulated during experimental MCMV retinitis of corticosteroid-immunosuppressed C57BL/6 mice.....	81
Figure 3.11. Cytokine inducers of SOCS1 and SOCS3 are stimulated during experimental MCMV retinitis in corticosteroid-immunosuppressed C57BL/6 mice.	85
Figure 3.12. MCMV-stimulated SOCS1 or SOCS3 expression is differentially sensitive to systemic GCV treatment during MAIDS.	87
Figure 4.1. Infection of mouse macrophages with MCMV stimulates SOCS1 and SOCS3, but not SOCS5, mRNA transcripts at early time points.	90

Figure 4.2. MCMV Infection of mouse macrophages causes phosphorylation of STAT proteins at early time points.....	92
Figure 4.3. SG-MCMV, but not TC-MCMV, stimulates SOCS1 and SOCS3 mRNA transcripts in IC-21 mouse macrophages.	94
Figure 4.4. SG-MCMV, but not TC-MCMV, highly stimulates type II IFN expression in IC-21 mouse macrophages.	96
Figure 4.5. Viral gene expression and growth curves in IC-21 mouse macrophages are delayed during SG-MCMV infection compared with TC-MCMV infection.	98
Figure 4.6. UV-inactivation of MCMV reduces MCMV-related stimulation of SOCS1 and SOCS3 mRNA transcripts in mouse macrophages.....	100
Figure 4.7. UV-inactivation of MCMV reduces MCMV-related stimulation of SOCS1 and SOCS3 protein in mouse macrophages.....	101
Figure 4.8. Infection of mouse macrophages with UV-inactivated MCMV reduces MCMV-related stimulation of cytokines known to induce SOCS1 and SOCS3.	103
Figure 4.9. MCMV infection stimulates mRNA expression of SOCS1 and SOCS3 mRNA in MEF cells.	105
Figure 4.10. SOCS-inducing cytokines are transcriptionally stimulated during MCMV infection of MEF cells.	106
Figure 4.11. GCV treatment of MCMV-infected mouse macrophages reduces MCMV-related stimulation of SOCS3 mRNA at 72 hpi.....	108
Figure 4.12. SOCS1 and SOCS3 protein expression during MCMV infection in IC-21 mouse macrophages occurs in uninfected bystander cells.	110

1 INTRODUCTION

“The only thing worse than being blind is having sight but no vision.” – Helen Keller

Despite the development of combination antiretroviral therapy (cART) to treat human immunodeficiency virus (HIV) infection, AIDS-related human cytomegalovirus (HCMV) retinitis remains a major sight-threatening disease worldwide (reviewed in [1-6]). Understanding the pathogenesis of this disease is essential for developing new, safe, and effective treatments for its prevention or management in the clinical setting, yet much remains unknown about the virologic and immunologic mechanisms contributing to its pathology. To study such mechanisms, we use a well-established, reproducible, and clinically relevant animal model of murine cytomegalovirus (MCMV) retinitis in mice with retrovirus-induced immune suppression (murine AIDS, or MAIDS [7-9]) to elucidate the role of potential candidates contributing to the progression of this disease. One such candidate is the family of host proteins known as suppressors of cytokine signaling (SOCS) which, among their other functions, negatively regulate signaling pathways induced by antiviral and inflammatory cytokines (reviewed in [10-12]). Possible virologic, immunologic, and/or pathologic mechanisms involved in SOCS production or function during cytomegalovirus infection and/or retinal disease are unclear.

The pathogenesis of AIDS-related HCMV retinitis involves the complex orchestration of cytomegalovirus infection during AIDS-mediated progressive destruction of the immune system, within the context of the cells in the retina of the eye. These components are discussed in greater detail to follow, along with a brief introduction of host SOCS proteins as pertinent to the specific aims of this study.

1.1 The Eye

Sight is facilitated by a complex visual system whose gross anatomy, microanatomy, biophysical, and biochemical properties are critical to its function. Disruption of any one of thousands of components of this system could lead to visual impairment or blindness. Herein we provide only a brief introduction to the general anatomy of the ocular compartment and neurosensory retina.

1.1.1 Anatomy of the Eye

The eye is a spherical organ designed to capture, focus, and project light onto specialized light-sensing cells that line the inside of the eye. It is anatomically arranged in many distinct and increasingly complex layers within layers. The three general layers or tunics are the external layer, the intermediate uveal layer, and the internal sensory layer [13]. These layers are specialized depending on their general location in the anterior or posterior segments, which are delineated by the posterior side of the crystalline lens and ciliary body. The anterior portion of the external layer is the transparent cornea protruding from the conjunctiva, and the posterior portion is the sclera [13]. The intermediate layer is the uveal tract, which on the anterior side forms the iris and ciliary body, and on the posterior side becomes the choroid. The internal layer is present only in the posterior segment and is the neurosensory retina, which contains the rod and cone photoreceptors critical for translating photons of light into neurochemical signals interpretable by the brain.

Light first encounters the cornea, which acts as a powerful lens to focus light through the liquid-filled anterior chamber, through the aperture of the pupil, and into the crystalline lens. The crystalline lens focuses light with greater precision through the viscous vitreous gel and onto the parfait-like layers of the neurosensory retina at the back of the eye. Photoreceptors in the retina

detect photons of light and transmit signals through first-order, second-order, and third-order neurons into ganglion cell axons that exit the eye as the optic nerve (**Figure 1.1**) [14].

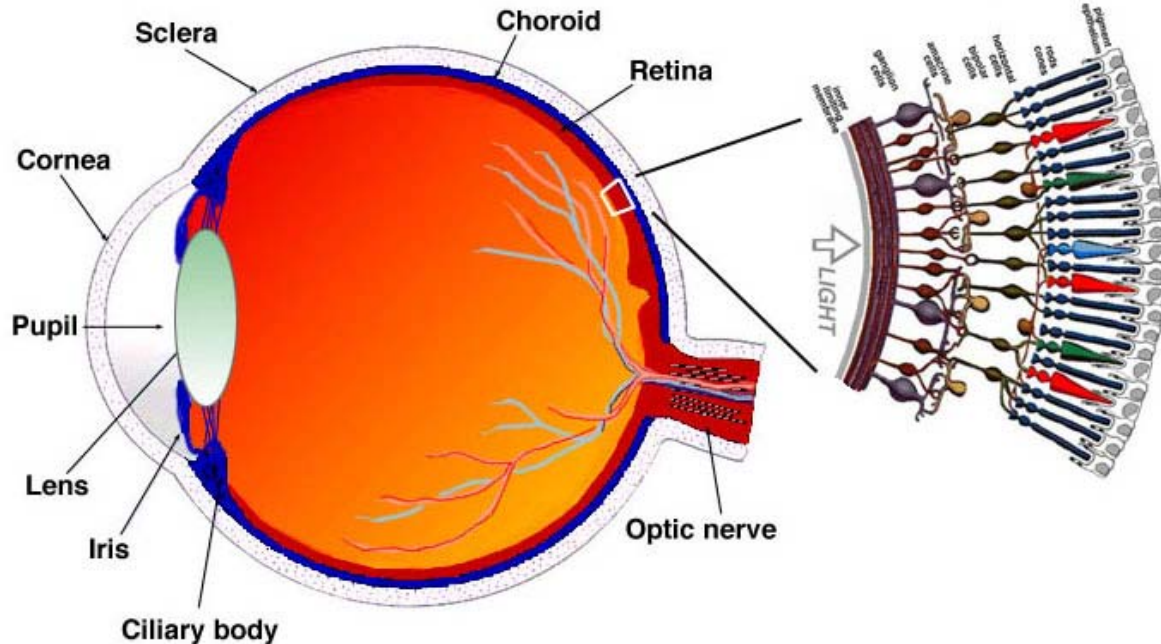


Figure 1.1. Schematic section of the human eye.

Depiction of a transverse section of a human eye, with schematic detail of the retina, including retinal layers and cell types, from [14].

1.1.2 Retina and Retinal Pigmented Epithelium (RPE)

Photons encountering the retina from the vitreous gel travel through the layers of the retina: inner limiting membrane, nerve fiber layer, ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, external limiting membrane, photoreceptor inner and outer segments, and retinal pigmented epithelium (RPE) (**Figure 1.2**).

The inner limiting membrane separates the vitreous cavity from the rest of the retina. The nerve fiber layer consists of third-order ganglion cell axons that run from their individual ganglion cells along the inside of the retina toward the optic nerve where they exit the retina. The nuclei of

these ganglion cells comprise the ganglion cell layer. They receive neural signals from synapses from many types of specialized bipolar and amacrine cells, and these synapses make up the inner plexiform layer. The nuclei of the bipolar and amacrine cells, as well as those of specialized horizontal cells, are in the inner nuclear layer, and their dendrites extend into the outer plexiform layer, where they synapse with photoreceptor neurons. Photoreceptor cell nuclei comprise the outer nuclear layer, and their specialized inner and outer segments project through the external limiting membrane toward the RPE to form the photoreceptor layer (reviewed in [13-15], see **Figure 1.1** and **Figure 1.2**).

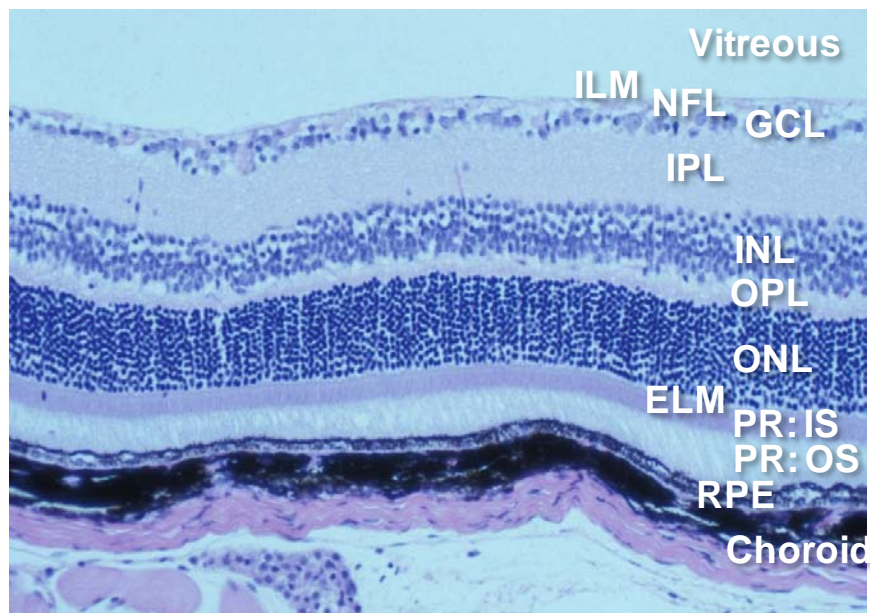


Figure 1.2. Histology of the layers of a normal C57BL/6 mouse retina.

The normal mouse retina, which retains layers of the human retina, is frequently used in experimental models to study ocular histopathology. Light focused from the cornea and crystalline lens would approach through the vitreous cavity from the direction of the top of the image and encounter the retinal layers in this order: inner limiting membrane (ILM), nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), external limiting membrane (ELM), photoreceptor inner segments (PR: IS) and outer segments (PR: OS). Retinal pigmented epithelium (RPE) and choroid are also labeled for reference to orientation. Photomicrograph courtesy of Drs. Dix and Cousins, Bascom Palmer Eye Institute, Miami, FL.

Photoreceptors are considered first-order sensory neurons. Their outer segments contain thousands of tightly-packed photopigment proteins that house the machinery to absorb photons and initiate cellular changes that coordinate neurotransmitter signals to second-order neurons including different types of bipolar and horizontal cells. These neurons innervate amacrine and ganglion cells, and signals are sent through ganglion cell axons through the optic nerve to synapses in the brain. Thus, the path of a single photon into the eye toward its final destination in the photopigment of a photoreceptor outer segment flows in the opposite direction of the propagated neural signal that it initiates [15].

Photoreceptor outer segments are highly metabolic and are constantly refreshed. They are embedded in the RPE, a specialized layer of phagocytic, multifunctional epithelial cells responsible for photoreceptor waste disposal, ion and nutrient exchange, protection from light-induced oxidative stress, recycling of light-sensing chromophores, and secretion of crucial growth factors and cytokines (reviewed in [15, 16]). In addition, because RPE cells are connected to each other by tight junctions, the RPE also provides a physical barrier between the retina and the choroid, comprising the choroidal blood-retinal-barrier (reviewed in [17]).

1.1.3 Immune Cells of the Retina

The specialized neuronal cells of the retina are supported by networks of three basic types of glial cells: Müller cells and astrocytes, which together comprise the retinal macroglia, and the microglia (reviewed in [18, 19]). Müller cells traverse the depth of the retina in both directions from their cell bodies in the inner nuclear layer toward their termination points in the inner and outer limiting membranes [19]. Their physical and biochemical support of other retinal cells involves metabolic regulation, extracellular ion composition, neurotransmitter recycling, and mitigation of oxidative stress (reviewed in [20]). Müller cells express high amounts of glutamate

synthetase (GS), a common immunological marker for these cells [21]. Glial fibrillary acidic protein (GFAP) is also abundantly found in activated Müller cells as well as astrocytes [22, 23]. Müller cells and retinal astrocytes both putatively contribute to the blood-retinal barrier between the retinal ganglia cells and the retinal blood vessels [19, 20]. These macroglia are generally distinguished from each other based on their embryologic origins, morphologies, and locations within the retinal layers. Whereas Müller cells are embryologically derived from neuroprogenitor cells of the retina and are found throughout all of the retinal layers, astrocytes migrate during development from the optic nerve into the retina and function to support the retinal ganglia cells [24]. Retinal astrocytes span radially along the nerve fiber and ganglion cell layers and are rarely found in any of the other retinal layers [24]. Microglia are phagocytic, macrophage-like, resident antigen presenting cells of the retina which dramatically change their morphology upon activation [19]. Ionized calcium-binding adapter molecule 1 (Iba-1) is an immunoreactive marker for retinal microglia [25].

1.1.4 Ocular Immune Privilege

The retina, as part of the posterior segment of the eye and an extension of the brain, is considered an immune-privileged site [26] primarily because it does not elicit a typical inflammatory immune response to the introduction of antigens (reviewed in [27, 28]). This immune privilege is provided, first, by the blood-retinal barrier. For the retina, this means that nutrients from the blood delivered by each of the two major blood supply routes to the retina must filter through a secondary cell before they reach retinal neural tissues. For the choroidal blood supply to the photoreceptors from the choriocapilaris, the blood-retinal barrier is maintained by the RPE. For the retinal blood vessels that enter into strata of the retina through the center of the optic nerve, this barrier is made by tight junctions between blood vessel

endothelia and is believed to be maintained by pericytes, retinal astrocytes, and Müller cells. These barriers facilitate a tightly-controlled microenvironment that drives immune cells into anti-inflammatory phenotypes characteristic of immune privilege [27]. Thus, irreplaceable neuronal tissue is somewhat protected from the damaging effects of inflammation and immunopathogeneses.

Systemic immune tolerance is another important characteristic of ocular immune privilege [28]. This feature is illustrated by the phenomenon that introduction of a foreign antigen into a compartment of the eye, such as the anterior chamber, followed by systemic challenge for the antigen, will not elicit a systemic inflammatory response. Such deviation from a normal immune response was named anterior chamber-associated immune deviation (ACAID) upon its discovery [29, 30].

A major molecular mechanism of this immune suppressive microenvironment and systemic immune tolerance is the anti-inflammatory cytokine transforming growth factor (TGF)- β 2. TGF- β 2 is highly abundant in sites of immune privilege and performs immunosuppressive functions on antigen presenting cells and on T cells by mechanisms that include increasing the percentage of regulatory T cells (T_{reg}) compared with effector T cells, decreasing the functional capacity of effector T cells, or inducing apoptosis on effector T cells [31-35].

1.2 Pathogenesis of AIDS-Related Cytomegalovirus Retinitis

When the immune privilege of the ocular compartment fails, inflammation causing severe immunopathogenesis and permanent, sight-threatening damage may occur, as in the case of AIDS-related HCMV retinitis. Prior to the era of antiretroviral therapies, this progressive necrosis of the retina occurred in approximately 30% of HIV/AIDS patients [1]. This disease is clinically diagnosed by ophthalmoscope examination through a fully-dilated pupil with the characteristic appearance of dense retinal whitening that tends to follow retinal blood vessels and may be accompanied by hemorrhage [36]. Fundus photographs show the retina at the back of the normal eye (**Figure 1.3A**), or an eye with AIDS-related HCMV retinitis (**Figure 1.3B**) with areas of dense, white retinal necrosis and hemorrhage.

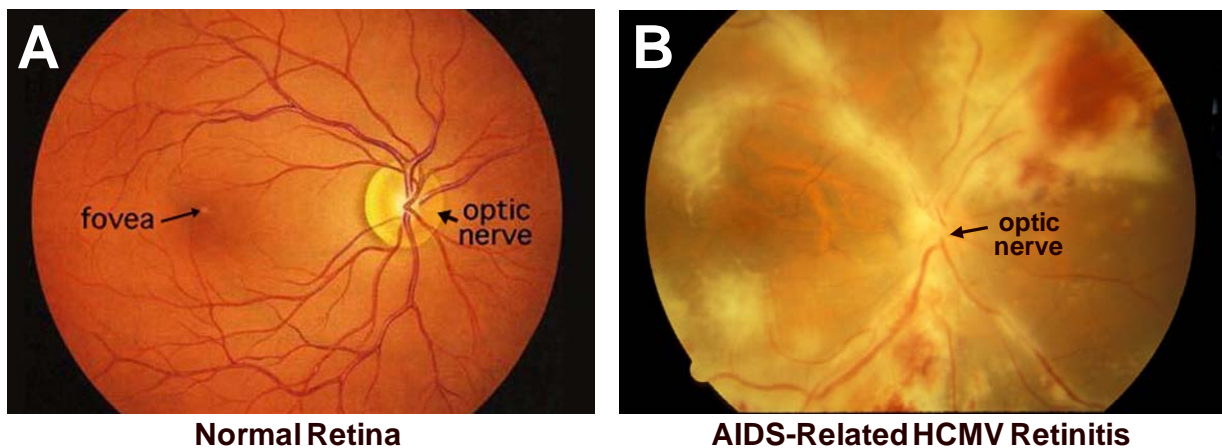


Figure 1.3. Fundus photograph of human retina during AIDS-related HCMV retinitis.

Fundus photographs of normal human retina (**A**) from [14], or during AIDS-related HCMV retinitis (**B**) showing dense, white areas of retinal necrosis (light areas) and hemorrhage (red areas), from Drs. Dix and Cousins, Bascom Palmer Eye Institute, Miami, FL.

The mechanisms of blindness caused by this disease may involve destruction of the retina itself, retinal detachment, or a uveitis that can occur with reconstitution of the immune system associated with well-tolerated antiretroviral therapies (immune recovery uveitis, IRU) [1, 36].

Although currently available treatments only prevent further vision loss, failure to treat this disease results in blindness of most or all of the affected eye, usually followed within one year by vision loss in the contralateral eye. The era of cART (formerly HAART, highly active antiretroviral therapy) to treat HIV infection [1, 36] has greatly reduced but failed to eliminate the number of new cases of AIDS-related HCMV retinitis in the United States [37]. HCMV replication generally can be controlled by lifelong administration of antiviral drugs (ganciclovir, cidofovir, foscarnet), but these drugs require frequent dosing, cause harmful side-effects, do not eradicate the virus, and merely slow the progression of HCMV-caused ocular or neuronal damage without reversing it [38-42]. Vaccination has been one of the most effective methods for controlling other problematic infectious diseases, but three decades of attempts to engineer an effective vaccine against HCMV so far have been unsuccessful [43, 44].

1.3 Cytomegalovirus

1.3.1 *Historical Context*

Pathologic details of a congenital disease now associated with HCMV were first reported at a natural science society meeting in Germany in 1881 by pathologist Dr. H. Ribbert, who described microscopic cytopathology and inclusion bodies in the nuclei of kidney cells from a stillborn infant (reviewed in [45-47]). Jesionek and Kiolemenoglou later described the same disease in tremendous detail, including “owl eye” cytopathology characteristic of cytomegaly [47]. Filterable viruses causing similar lesions to those first described in humans were later discovered for guinea pigs [48], mice [49], and other mammals, including non-human primates, rats, dogs, pigs, horses, cows, and bats (reviewed in [6]).

Significant strides in our understanding of the pathologic and molecular mechanisms of the known cytomegalovirus sequelae have been made since its first description. Much of what we now know is attributable to small animal models using their respective cytomegalovirus species. The mouse-specific salivary gland virus now called murine cytomegalovirus (MCMV) was first isolated by Dr. Margaret Smith [50], who also successfully isolated HCMV from human lymphatic tissue, published simultaneously with other laboratories [51, 52].

1.3.2 *HCMV and MCMV Classification, Structure, and Genome*

All viruses contain nucleic acid packaged into a protein capsid, collectively known as the nucleocapsid. Viral genetic material may be either single-stranded (ss) or double-stranded (ds) DNA or RNA, with ssRNA viruses either having plus-sense or minus-sense orientation. Some viruses additionally contain a host-derived membrane called the envelope that surrounds the nucleocapsid. In these cases, the space between the nucleocapsid and the envelope, known as the matrix or tegument, often contains many virus-encoded proteins which help to establish viral

takeover of the host cell during early infection. Commonly protruding from the nucleocapsid or envelope are structural glycoproteins which specifically recognize receptors present on the host and thus promote entry into the host cell. Virus taxonomic organization has traditionally been based on physiologic and morphologic similarities, and phylogenetics have more recently deepened our understanding of the relation of viruses to each other and to their hosts [46].

Cytomegaloviruses belong to the subfamily *Betaherpesvirinae* in the *Herpesviridae* family of the relatively new taxonomic order *Herpesvirales* [53]. Admittance into the *Herpesviridae* family traditionally is based upon the virus structure: dsDNA within an icosahedral capsid surrounded by an amorphous tegument between the host cell-derived envelope encrusted with viral glycoproteins (reviewed in [54]). Members of this family share the biological characteristics of replication within host cell nuclei, the establishment of latency, and ultimate destruction of lytically infected host cells [46, 53]. To date, there are eight known human herpesviruses (HHV)-1 through HHV-8 (**Table 1.1**), with a recently-recognized ninth member in the division of HHV-6 into HHV-6A and HHV-6B [55] as distinct herpesvirus species (reviewed in [46]).

Table 1.1: Human Herpesviruses in the Family Herpesviridae.

Subfamily	Formal Name	Acronym	Informal Name
<i>Alphaherpesvirinae</i>	<i>Human herpesvirus 1</i>	HHV-1	Herpes simplex virus type 1 (HSV-1)
	<i>Human herpesvirus 2</i>	HHV-2	Herpes simplex virus type 2 (HSV-2)
	<i>Human herpesvirus 3</i>	HHV-3	Varicella-zoster virus (VZV)
<i>Betaherpesvirinae</i>	<i>Human herpesvirus 5</i>	HHV-5	Human cytomegalovirus (HCMV)
	<i>Human herpesvirus 6A</i>	HHV-6A	Human herpesvirus 6 (HHV-6)
	<i>Human herpesvirus 6B</i>	HHV-6B	
	<i>Human herpesvirus 7</i>	HHV-7	Human herpesvirus 7 (HHV-7)
<i>Gammapherpesvirinae</i>	<i>Human herpesvirus 4</i>	HHV-4	Epstein-Barr virus (EBV)
	<i>Human herpesvirus 8</i>	HHV-8	Kaposi's sarcoma-associated herpesvirus (KSHV)

Adapted from [46, 53]

Alphaherpesvirinae. The α -herpesviruses are characterized by their ability to establish latency in neurons, to infect a variety of host species, to replicate and spread relatively quickly, and to destroy infected host cells [46]. This subfamily consists of two genera infecting mammals: *Simplexvirus* and *Varicellovirus*. Common pathologies of *Simplexvirus* herpes simplex virus type 1 (HSV-1) and/or HSV-2 include oropharyngeal lesions (cold sores), herpes simplex keratoconjunctivitis, herpes simplex encephalitis, and genital herpes. Very rarely, the *Simplexvirus Macacine herpesvirus 1* (McHV-1), or B Virus, will stray from its native host the macaque monkey to infect a human, causing a high rate of mortality in untreated patients within a few weeks [56-58]. Varicella-zoster virus (VZV) of the *Varicellovirus* genus is the etiological agent of varicella (chickenpox) and herpes zoster (shingles).

Betaherpesvirinae. The β -herpesviruses generally replicate more slowly than other herpesviruses and display host species specificity, with a propensity to establish latency in lymphoid cells of hematopoietic origin [46]. The genus *Roseolavirus* comprises HHV-6 and

HHV-7, of which HHV-6B and HHV-7 have been shown to cause exanthem subitum (roseola) [55, 59]. In the *Proboscivirus* genus is *Elephantid herpesvirus 1*, or elephant endotheliotropic herpesvirus [46, 53]. Of particular importance to this study are the genera *Cytomegalovirus*, which contains HCMV, and *Muromegalovirus*, which includes *Murid herpesvirus 1* (MuHV-1), or murine cytomegalovirus (MCMV) [6] (see **Table 1.2** for classification of these two genera). As these viruses represent a central subject of this report, they are therefore discussed in greater detail in following sections. Along with the four genera included in this subfamily, *Beta-herpesvirinae* also contains several unassigned species, one of which is the cytomegalovirus infecting guinea pigs (*Caviid herpesvirus 2*) [46, 53].

Table 1.2: Taxonomies of Human and Murine Cytomegalovirus.

Taxon	Name	Acronym	Common Name
Order	<i>Herpesvirales</i>		
Family	<i>Herpesviridae</i>		
Subfamily	<i>Betaherpesvirinae</i>		
Genus	<i>Cytomegalovirus</i>		
Species	<i>Cercopithecine herpesvirus 5</i>	CeHV5	African green monkey cytomegalovirus
	<i>Human herpesvirus 5</i>	HHV5	Human cytomegalovirus (HCMV)
	<i>Macacine herpesvirus 3</i>	McHV3	Rhesus cytomegalovirus
	<i>Panine herpesvirus 2</i>	PnHV2	Chimpanzee cytomegalovirus
Genus	<i>Muromegalovirus</i>		
Species	<i>Murid herpesvirus 1</i>	MuHV1	Murine cytomegalovirus (MCMV)
	<i>Murid herpesvirus 2</i>	MuHV2	Rat cytomegalovirus (RCMV)

Adapted from [46, 53]

Gammaherpesvirinae. The γ -herpesvirus subfamily contains viruses that are species-specific, generally prefer B or T lymphocytes for replication, and establish latency within lymphoid tissue [46]. This subfamily contains four genera, of which *Lymphocryptovirus* contains

Epstein-Barr virus (EBV), and *Rhadinovirus* includes Kaposi's sarcoma-associated herpesvirus (KSHV) [46].

Cytomegalovirus Structure. At 200-230 nm in diameter including the envelope, cytomegaloviruses are generally the largest of all herpesviruses, which all share similar virion structures. HCMV contains a dense, pressurized core of linear dsDNA (~236 kilobases, kb) located within a 130-nm, 162-capsomere icosahedral nucleocapsid of T = 16 symmetry. This is surrounded by a relatively thick tegument contained within an envelope embedded with virus-encoded glycoproteins (see **Figure 1.4** from [60]). At least 66 known virally-encoded proteins comprise the structure of the infectious HCMV virion (reviewed in [6, 61]).

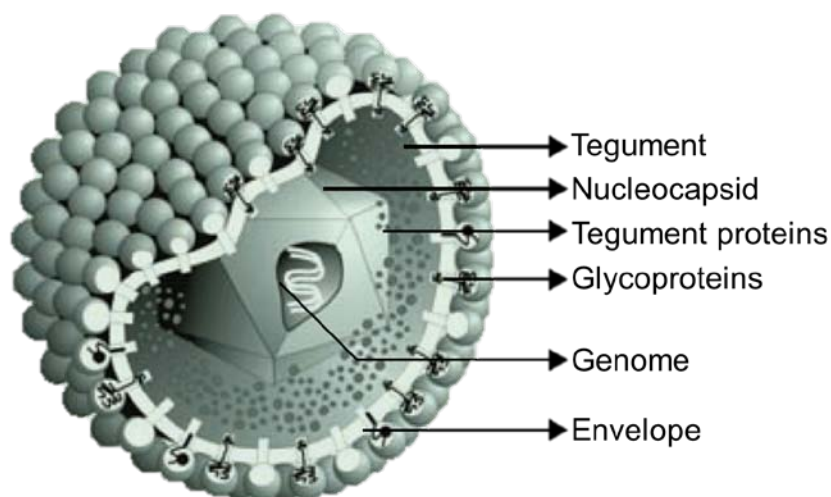


Figure 1.4. Structure of the cytomegalovirus virion.

Schematic representation of components of a prototypical cytomegalovirus (HCMV or MCMV) virion. Figure modified from [60].

The structure of the HCMV capsid consists of four virus-encoded proteins that are all essential for growth: major capsid protein (MCP), triplex subunits 1 (TRI1), TRI2, and smallest capsid protein (SCP). MCP comprises the bulk of the capsid, forming hexons and pentons that

are held together by strategic placement of the remaining three core proteins and other structural proteins. A single pentameric side of the icosahedron contains a specialized pore made of the portal protein (PORT), facilitating packaging and release of the virion genome [6].

The HCMV tegument is sandwiched between the capsid and the envelope, and it contains at least 32 known virally-encoded tegument proteins along with host proteins [62] and host RNA [63]. The individual functions of these tegument proteins are highly diverse, influencing nearly all intracellular steps of the replication cycle from entry to egress [6]. Many tegument proteins are phosphorylated (phosphoprotein, pp) and induce immunogenic reactivity in the host. Of the virus-encoded tegument proteins, HCMV pp65 (product of the UL83 gene) and its MCMV counterpart M84 [64], are targeted by CD8⁺ T cells, and pp65 is the most abundant viral protein in infectious HCMV particles, noninfectious dense bodies that do not contain nucleocapsids, and infected host cells [6]. Although dispensable for growth in cell culture [65], pp65 seems to modulate interferon (IFN)-like cellular responses in the host cell nucleus. The virion transactivator (VTA) tegument protein pp71 (UL82 gene product) is another important virus-encoded tegument protein that translocates to the nucleus very quickly after viral entry and recruits host cell machinery to initiate immediate early (IE) gene transcription [6].

The current paradigm is that HCMV derives its envelope from the host cell endoplasmic reticulum (ER), the ER-Golgi intermediate compartment (ERGIC), or other endosomes [6, 66, 67]. Mature virions contain three major glycoprotein complexes of importance: a trimer of glycoprotein B (gB) and the gH:gL complex are required for viral entry, and the gM:gN complex is implicated in maturation. Regardless of their roles, many viral glycoproteins are readily recognized by the immune system. The gH:gL:gO complex of MCMV has recently shown a promising potential antigenic target for immunization [68]. In addition to infectious virus

particles, HCMV [69] and MCMV [70] also produce noninfectious dense bodies [71], which lack nucleocapsids and scaffolding, and noninfectious enveloped particles, which lack nucleocapsids but retain scaffolding (reviewed in [6, 66, 67]).

Cytomegalovirus Genome. Nomenclature of herpesvirus genomes and gene products are not universally standardized, although virus proteins are frequently noted by their common protein names and gene loci. For HCMV, these loci designations are derived from the general genome structure, containing two unique sets of linear, dsDNA that are attached together and are designated the unique long (U_L) and unique short (U_S) sequences. Each of these is flanked by inverted repeat sequences, and they are connected with an internal redundancy sequence (**Figure 1.5**) (reviewed in [46]). Although each HCMV virion contains only one copy of the entire genome, the structure of this genome generates the possibility of four different isomers during replication, depending on the orientations of each unique sequence in relation to the other (reviewed in [6, 46]). Unlike HCMV, the MCMV genome lacks internal repeats [72, 73] and therefore the ability to form isomeric genome structures (**Figure 1.5**). For HCMV, genes are numbered according to their U_L or U_S loci. MCMV nomenclature is determined by numbering open reading frames (ORFs) from 5' to 3' of the single unique long (U_L) sequence, with “M” designating those genes of HCMV homology and “m” preceding those genes that lack HCMV homologues [74, 75].

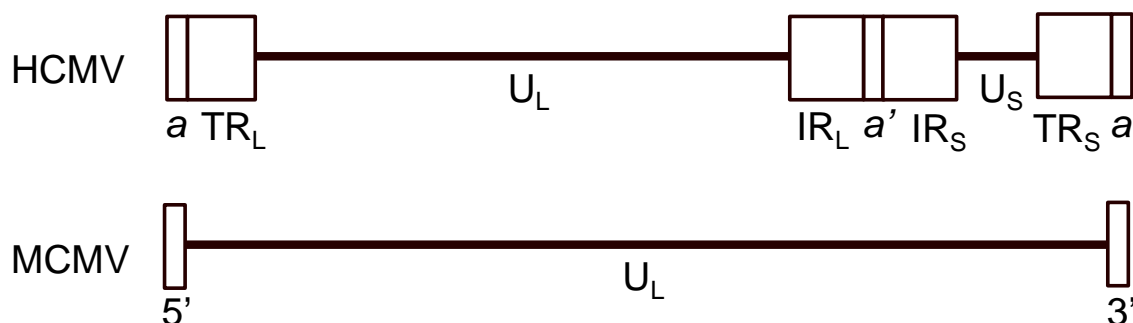


Figure 1.5. Genomic structures of HCMV and MCMV.

HCMV genome from [46]. MCMV genome adapted from [75]. Thin, dark lines represent the genomes (not to scale), while open boxes denote inverted repeats. U: unique, L: long, S: short, TR: terminal repeat, IR: internal repeat, *a*: terminal redundancy, *a'*: internal redundancy. The MCMV genome contains several short (~30-bp) internal repeat sequences [75] not shown in this schematic representation.

The HCMV genome is approximately 236 kb long and has the capacity to code for at least 167 known gene products [76], including structural and nonstructural proteins, 32 tegument proteins, and as many as 23 glycoproteins. The viral genome codes for multiple alternative splicing mRNA sites, noncoding RNA, and 23 micro-RNA (miRNA) sequences [77, 78] (reviewed in [6, 79, 80]). The MCMV genome (Smith strain) has a length of approximately 230 kb and is predicted to contain at least 170 gene products [75]. Although the structure of MCMV differs somewhat from HCMV and contains only a single long segment of DNA, it is highly comparable to that of HCMV [72-75]. Sequences from these two cytomegalovirus species contain roughly 180 kb of overlap representing approximately 78 homologous ORFs [75]. All β -herpesviruses, including cytomegaloviruses, share a conserved DNA synthesis origin for lytic infection (*oriLyt*) that is located between the UL57 and UL69 genes in the HCMV genome [6].

1.3.3 HCMV and MCMV Replication Cycle

Like all herpesviruses, HCMV and MCMV undergo a temporal, step-wise viral gene expression and replication cascade involving the synchronized expression of three genetic

classes of genes: immediate early (IE) or α genes, early (E) or β genes (also called delayed early, DE), and late (L) or γ genes (reviewed in [6, 46, 54, 61]). The sequence of expression of these genes is highly regulated, but steps may occur more quickly or may be delayed depending on host cell type and the stage of the cell cycle upon infection [6]. An overview of the replication cycle of HCMV or MCMV is represented schematically in **Figure 1.6**.

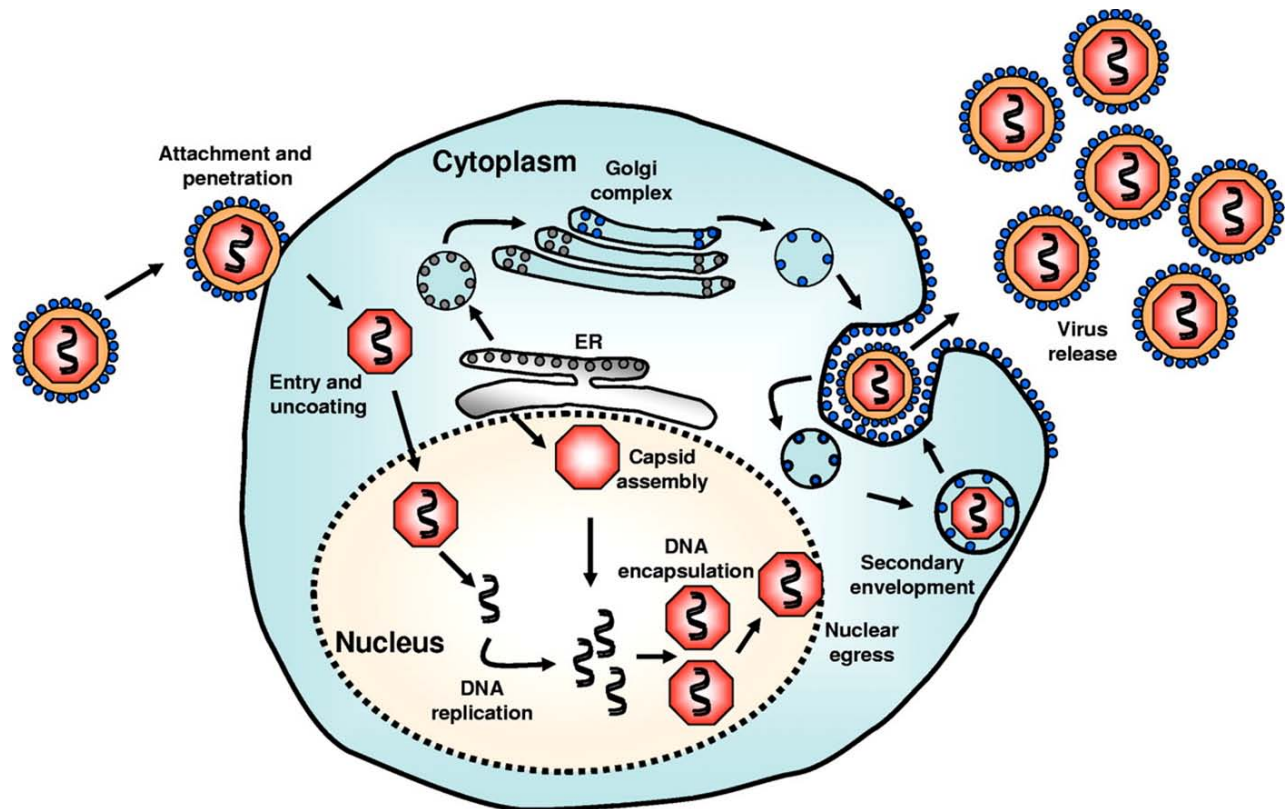


Figure 1.6. HCMV or MCMV replication cycle within a host cell.

Attachment, adsorption, uncoating, release of tegument proteins into the cytoplasm, shuttling of nucleocapsid to the nucleus, IE gene expression, E gene expression, DNA replication, L gene expression, capsid assembly, envelopment, egress, and release from the cell. Figure from [81].

Initial interaction of an infectious HCMV or MCMV virion with a permissive host cell involves immediate recognition and attachment and adsorption of glycoprotein complexes in the viral envelope to complementary receptors on the host cell surface. Cellular heparin sulfate is a major participant in initial adsorption interactions. Although specific cellular receptors for many

of the HCMV glycoproteins remain unknown, several proposed receptors have been studied and seem to play a role in entry under various conditions. HCMV gB, for instance, is a major fusion protein that facilitates binding to host heparin sulfate proteoglycans, and although the cellular receptor specific for gB is yet to be elucidated [6, 66, 67] it has been shown to bind cellular integrins [82]. Epidermal growth factor receptor (EGFR) [83] and platelet-derived growth factor receptor (PDGFR) [84] have also been investigated as putative cellular receptors for HCMV entry.

Fusion is facilitated in HCMV and MCMV by gB and gH:gL for all cell types, and different combinations of the gH:gL complex with other glycoproteins drive host cell tropism. The gH:gL complex of HCMV can either form a trimeric complex with gO or a pentameric complex with UL128 gene products, dictating epithelial, endothelial, lymphoid, and/or myeloid (macrophage) cell tropisms [85, 86]. MCMV homologues also form the trimeric gH:gL:gO complex [87] and an alternative gH:gL:MCK-2 complex [88] that similarly governs cell tropisms, particularly in macrophages [89]. Cell tropisms therefore exist between strains or variants of strains of HCMV or MCMV. Although they are capable of infecting many different types of cells, both HCMV and MCMV establish latency in circulating monocytes and bone marrow cells [90].

Entry into the host cell immediately follows attachment and adsorption, with receptor-mediated endocytosis occurring in endothelial and epithelial cells, or fusion of the viral envelope with the host membrane occurring in fibroblasts [6]. This results in the uncoating and release of the nucleocapsid and components of the tegument into the host cell cytoplasm. As nucleocapsids are trafficked to the nucleus, tegument proteins hitchhike on cellular cytoskeleton networks to various regions of the host cell where they perform a variety of duties, from dampening host cell

defense mechanisms to initiating transcription of IE viral genes [6]. The nucleocapsid interacts with nuclear pores and injects viral DNA into the nucleus [46].

Transcription of the viral IE genes commences once the viral genome is inside the cell nucleus and peaks between 8-12 hours post-infection (hpi) during HCMV infection. It involves tegument proteins (e.g., pp71) and cellular RNA polymerase II transcription machinery. Transcripts for the two major IE proteins, IE1 (p72, UL122) and IE2 (p86, UL123), are alternatively spliced from a single major IE promoter region (MIEP) [6, 91]. For MCMV, the IE1 and IE3 genes are the alternatively-spliced locational [92] and functional homologues of HCMV IE1 and IE2, respectively [93]. MCMV IE2 has no sequential or functional homologue in the HCMV genome and is dispensable for growth *in vitro* [94] and *in vivo* [95]. Transcription of MCMV IE genes IE1 (m123), IE2 (m128), and IE3 (M122) occurs between 1-4 hpi in fibroblast cells [96, 97], with IE2 mRNA up-regulation by 1 hpi in mouse macrophages during infection with tissue culture-passaged MCMV [98]. Translation of IE transcripts, and of all viral proteins, requires host cell ribosomes. IE protein functions range from continuing dysregulation of cellular functions in favor of viral production to initiating and enabling next steps in the replication cascade [54].

The presence of viral IE proteins enables the transcription of E genes, occurring in HCMV-infected cells around 8-12 hpi and continuing until around 18-24 hpi [6]. MCMV E gene transcripts are detected as early as 2 hpi until about 16 hpi in fibroblast cells [97]. E gene products modulate IE gene expression. Among these gene products is the virus-encoded DNA polymerase (HCMV UL54), which appears by 8 hpi and drives viral DNA replication [54].

HCMV genome replication in fibroblasts occurs between 14 and 48 hpi, peaking around 24 hpi. It involves *oriLyt* promoter activity, virus-encoded DNA polymerase (HCMV UL54),

and the E gene product ppUL84 complexing with IE2-p86 (reviewed in [6, 54, 99]). DNA synthesis occurs in a large nuclear inclusion in the host cell. Host cell nuclei thus infected usually have a kidney bean shape rather than circular appearance [6, 54].

Viral E proteins also enable expression of L genes, which occurs during HCMV infection of fibroblasts around 24 hpi. Expression of MCMV L genes in fibroblast cells commences between 8-16 hpi [97, 100] and has been reported to continue for 36 hpi or longer in these cells [100]. Most tegument and glycoproteins are coded by L genes. General functions of these gene products involve capsid assembly, virion maturation, and egress from the host cell. It is currently believed that nucleocapsids are most likely enveloped and de-enveloped through the nuclear membranes and are ushered into large cytoplasmic inclusions for further maturation [6, 54]. Final envelopment of nucleocapsids with teguments occurs at an ER-Golgi intermediate compartment (ERGIC) of endosomes and/or exosomes. Mature virions then egress by exocytosis.

There are several experimental or pharmaceutical strategies for inhibiting certain steps of the cytomegalovirus replication cycle. Exposure to DNA-damaging ultraviolet (UV) light (UV-inactivation) allows the virus to undergo its first kinetic steps of infection, including attachment, adsorption, uncoating, and release of viral-associated tegument proteins into the host cell, but prevents transcription of viral genes, viral replication, and the formation of progeny virus [101]. Several effective antiviral drugs like ganciclovir (GCV), cidofovir, and foscarnet, are available for the treatment of cytomegaloviruses. These target viral DNA polymerase and therefore DNA synthesis [6, 54]. GCV in particular acts a guanosine analog [102], preferentially inhibits HCMV and MCMV DNA polymerases [103] and therefore viral replication, and allows viral IE and E gene expression without L gene expression [104].

1.3.4 Host Response to Cytomegalovirus

The balance between virulence and the host immune response sways the outcome of any viral infection. Just as the host has an arsenal of mechanisms for sensing, stopping, and clearing viral infection, viruses have as many mechanisms for evading, escaping, and producing productive infections in the host. HCMV and MCMV modulate their host cells by interfering with signaling pathways important to the innate or adaptive immune response, and by temporarily inhibiting cell death pathways [6].

Integral to the first-responding innate immunity is the vast family of pattern recognition receptors (PRR) which are capable of detecting common non-self, pathogen-associated molecular patterns (PAMPs) (reviewed in [105]). PAMPs are highly-conserved molecules which are usually indispensable to the pathogens with which they are associated [105-107]. Many types of PRRs have been identified so far, including toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), and absent in melanoma 2 (AIM2)-like receptors [108, 109]. In general, activation of any of these PRRs leads to one or more well-characterized cell signaling pathways responsible for the upregulation of proinflammatory cytokines, including type I IFNs [108]. Among these pathways are nuclear factor κ B (NF- κ B), mitogen activated protein kinase (MAPK) signaling pathways through phosphorylation of c-Jun N-terminal kinases (JNKs) [110, 111], as well as inflammasome/caspase-1-dependent IL-1 β maturation [112]. Infection with HCMV or MCMV has the capacity to stimulate and/or to modulate several of these PRRs [6]. For instance, MCMV infection of monocytes and other cell types stimulates TLR2-MyD88 [113], TLR3-TRIF, and TLR9-MyD88 [114] signaling.

The importance of macrophages and macrophage progenitor cells (monocytes, bone marrow cells) for viral dissemination and latency during systemic HCMV or MCMV infection has been demonstrated [90, 115-119]. Macrophages play critical and sometimes contradictory roles during MCMV infection, dependent partly on their reaction to cytokines such as type I and type II IFNs [115, 116, 118, 120-122]. It has been demonstrated by others that macrophages infected with MCMV become resistant to IFN- γ -driven activation in a manner partially dependent upon antiviral type I IFN [121, 122], and/or viral inhibition of the promoter assembly for IFN- γ [123].

Macrophages exhibit divergent activation phenotypes in response to various stimuli. These have very generally been categorized into classically-activated (M1) macrophages and alternatively-activated (M2) macrophages [124], so called for their association with CD4⁺ T_H1 or T_H2 polarization, respectively. In general, M1 macrophages are activated via exposure to IFN- γ alone or together with TNF- α , PAMPs such as TLR4-recognized lipopolysaccharide (LPS), or other stimuli; they express TNF- α , IL-6, IL-1, and IL-12 upon activation; and through production of these pro-inflammatory cytokines and nitric oxide (NO), they exhibit a pro-inflammatory phenotype (reviewed in [125]). Alternatively-activated M2 macrophages have grown to include all non-classically-activated macrophages and therefore display a diverse range of activation phenotypes. An M2 phenotype is generally induced by exposure to IL-4 or corticosteroids, results in the production of anti-inflammatory IL-10 and IL-1 receptor antagonist, and participates in anti-inflammatory or pro-angiogenic activities (reviewed in [125]). These macrophage polarizations exhibit extreme plasticity, however, and are not as clearly defined as originally thought. Monocytes infected with HCMV, for instance, display a hybrid M1/M2

activation phenotype, simultaneously showing pro-inflammatory and pro-angiogenic properties, but with a propensity mostly toward the M1 phenotype [126-129].

Natural killer (NK) cells are granulocytic and highly effective at destroying cells that fail to display sufficient amounts of major histocompatibility complex (MHC) class I (MHC-I), which presents intracellularly-derived antigens to MHC-I-restricted immune cells such as CD8⁺ T cells (reviewed in [130]). The cytotoxic effector function of NK cells also requires signaling by activating receptors and/or signaling by cytokines such as type I IFN or IL-12 [131]. Activated NK cells produce high amounts of IFN- γ and use an arsenal of cytotoxic molecules like perforin or granzyme B to fulfill their cytotoxic functions [132]. NK cells play a protective role in response to systemic HCMV and MCMV infection [6, 133] and are primarily responsible for immediate control of infection.

In addition to the immediate response of NK cells of the innate immune system, large numbers of MHC-II-restricted CD4⁺ T cells as well as MHC-I-restricted CD8⁺ T cells of the adaptive immune system specifically target HCMV or MCMV antigens during viral infection (reviewed in [6, 132, 134]). More so than the HCMV- or MCMV-specific antibody response of B cells, T cells keep the virus in check throughout the life of the host and play a role in the balance between persistent infection and latency [131, 132]. The role of the immune system, and particularly of CD4⁺ and CD8⁺ T cells, in controlling lifelong HCMV or MCMV infection is underscored by the profound susceptibility to cytomegalovirus-derived pathologies that occur during depletion or dysfunction of these cells [2, 6, 8, 36, 54, 61, 135-141].

1.3.5 Diseases of Cytomegalovirus Etiology

HCMV disease in the immunocompetent host. Despite the relatively high prevalence of HCMV-seropositive individuals in the worldwide population (45-100%) [142-144], most immunocompetent individuals fail to present acute symptoms. Mild mononucleosis, different from that of EBV etiology, has been occasionally associated with primary HCMV infection in immunologically normal persons [145]. Very rarely, immunocompetent individuals may develop severe diseases of cytomegalovirus etiology (reviewed in [5, 144-147]), including colitis [148-152], vascular thrombosis [153], myocarditis [154, 155], pneumonia [156-158], hepatitis [159-167], encephalitis [168-172], and retinitis [173-175]. It must be noted that many of these case studies report “immune competent” individuals who also harbor some other malady or circumstance that may impact the ability of HCMV to cause disease [5]. For instance, several cases of HCMV retinitis have occurred in immunocompetent individuals following intraocular injection of corticosteroids [176-182].

Congenital HCMV. In pregnant women, HCMV can cross the placenta causing congenital HCMV disease at a rate between 0.3% and 4% of worldwide births [6], with primary HCMV infection during the first trimester of pregnancy producing a much higher incidence and risk of congenital HCMV disease than primary infection during the second or third trimesters, or recurrent HCMV. Congenital HCMV causes sensorineural diseases in 12-25% of infected infants and, more rarely, can cause fatal cytomegalic inclusion disease (CID) [183]. Infants with CID usually experience one or more neurological symptoms including microcephaly, enlargement of the ventricles, and cerebral atrophy. Those with CID who survive past infancy will usually experience lifelong neurological deficits ranging from hearing or vision loss to mental retardation [184]. Transmission of HCMV vertically during childbirth or through breastmilk is

associated with less severe disease in infants, consistent with resistance to HCMV pathogenesis with the development of immune system functionality [6].

HCMV disease in the immunocompromised host. Solid-organ and bone marrow transplant recipients must be kept immunocompromised to prevent rejection of their donor organs. Life-threatening HCMV pneumonitis is the most common (80%) opportunistic HCMV disease in this patient population [6], followed by gastrointestinal disease. These patients may alternatively develop graft-versus-host disease (GVHD), and acute or chronic HCMV infection may contribute to this disease [185]. More rarely, HCMV retinitis can also develop in solid-organ or bone marrow transplant recipients, with a ~5% incidence [186].

Patients with HIV/AIDS also are at risk for many HCMV associated diseases because of their immune compromised state. HCMV retinitis accounts for 80-90% of HCMV-related diseases in HIV/AIDS patients [1-4], particularly those not receiving or resistant to cART. HCMV retinitis is the most common clinical manifestation of HCMV-related disease in the HIV/AIDS patient community, with esophagitis and colitis following, and, more rarely, HCMV-related encephalitis, peripheral neuropathy, pneumonitis, gastritis, and hepatitis [6, 187].

HCMV and chronic inflammation. In addition to cases of known HCMV etiology, it has been hypothesized that chronic HCMV may play an unforeseen role in many more diseases than are currently known or reported. Because cytomegaloviruses establish persistent infection in their hosts and modulate cells of the immune system toward chronic inflammation, the potential exists for its involvement in other diseases characterized by chronic inflammation, including age-related macular degeneration (AMD), cardiovascular disease, premature aging associated with co-infection with chronic HIV, and oncogenesis. The involvement of HCMV in any or all of these chronic diseases is unclear and controversial, but the capacity of HCMV to contribute to

similar environment(s) as those found during these diseases has been demonstrated. Therefore, it may be that HCMV contributes to one or more of these chronic diseases, if only in an indirect, exacerbating capacity or as a co-factor.

AMD is a disease affecting mainly the central visual field of the retina and is associated with chronic inflammation and, in the later-stage wet form of AMD, choroidal neovascularization connected with increased levels of vascular endothelial growth factor (VEGF) [188]. The precise etiology of AMD is unclear, but it is associated with chronic inflammation and immune components [188, 189], including a putative role for macrophages in the choroidal neovascularization that generally precedes wet AMD [190]. HCMV infection is associated with chronic inflammation and atypical macrophage activation, and populations of patients with wet AMD have a high incidence of HCMV seropositivity [191]. Subsequent studies in collaboration with our laboratory reported that choroidal neovascularization increases during chronic MCMV infection in an experimental mouse model, that MCMV-infected macrophages produce high amounts of pro-angiogenic VEGF, and that MCMV-infected macrophages are driven toward the M2-like pro-angiogenic phenotype [192]. Chronic HCMV infection may therefore belong on the list with other co-factors such as smoking, diet, and genetics as likely contributors to the onset and/or progression of wet AMD.

Because HCMV has the propensity to increase angiogenesis and neovascularization [193], it also has a putative involvement in the severity of atherosclerosis [194] and cardiovascular disease. Although this speculation remains controversial because of conflicting reports correlating disease severity with HCMV seropositivity (reviewed in [6]), HCMV nevertheless possesses the capacity to generate dysfunctional vascular endothelia leading to the pro-inflammatory environment associated with atherosclerosis. HCMV infects and disseminates

in monocytes/macrophages and can persistently infect vascular endothelial cells, driving a state of chronic inflammation and leukocyte transmigration, production of coagulation-promoting thrombin, and accumulation of arterial plaque-associated lipids (reviewed in [195]). In experimental mouse models of myocarditis, pathogenesis is exacerbated by MCMV infection, and antiviral treatment with cidofovir or ganciclovir decreases this disease severity [196].

Both HCMV and MCMV employ multiple mechanisms to generate an environment favorable for viral reproduction that may also nurture oncogenesis [197-200], including modulation of cell cycle regulation, apoptosis, genetic instability, angiogenesis, chronic inflammation, and evasion of immune surveillance [61, 101, 197-204]. Although the potential role that HCMV may have in tumorigenesis remains a controversial subject of debate, studies suggest that HCMV has the capacity to infect tumor cells or surrounding tissue and contribute indirectly to malignant properties without directly transforming the cells [205]. Positive immunostaining for HCMV proteins has been reported for many cancer types, including glioblastoma [202, 206-208] medulloblastoma [209], colon cancer [210], prostate cancer [211], and carcinoma of the cervix [212]. Although a high incidence of HCMV in human glioblastoma multiforme surgical resection samples has been reported for many cases [202, 206-208], other reports [212-215] demonstrate low or no correlation with HCMV genome or protein in brain tumor samples. Discrepancies in these opposing findings have been attributed to the different techniques used for detecting the low quantities of HCMV genome and protein found in the positive reports [207], but more research is needed to determine what role, if any, HCMV may play during oncogenesis.

The phenomenon of “immunosenescence” occurs in the elderly and is associated with several molecular markers such as decreased telomere lengths, altered or inverted CD4⁺ to CD8⁺

T-cell population ratios (i.e., normally there are more CD4⁺ than CD8⁺ T cells, but this ratio is reversed during immunosenescence), and increased production of the cytokines TNF- α and IL-6 (reviewed in [216]). There has recently been increased interest in the potential involvement of HCMV with immunosenescence, and some evidence suggests that there may be a correlation between HCMV seropositivity and markers for this premature aging phenomenon [217].

Although there are noted benefits of latent or persistent HCMV or MCMV infection in youthful populations, the long-term effects of chronic HCMV or MCMV infection on elderly humans or mice are destructive [216]. Recent studies have outlined a role for T-cell senescence during chronic HCMV or MCMV infection in part because HCMV-specific CD8⁺ T cells dominate the adaptive response and are driven to proliferate until this senescence is reached [216]. The addition of chronic HIV-1 infection, even if it is well-controlled by cART, exacerbates this ageing phenomenon, resulting in the appearance of age-related markers many years prior to their appearance in the HIV-1-negative elderly population [216, 218]. Although evidence suggests that HIV-1 infection contributes to this phenotype, the possibility also remains that cART itself may further contribute to the premature ageing phenomenon observed in patients harboring HIV-1 and HCMV infections [216, 218].

1.4 Human Immunodeficiency Virus and AIDS

1.4.1 Historical Context

In 1980-1981, physicians and the Centers for Disease Control and Prevention (CDC) noticed and reported an epidemic of severe immunodeficiency occurring disproportionately in populations of young, homosexual men in progressive United States cities. This disease was accompanied by lymphadenopathy, the presentation of usually rare opportunistic infections, and a sharp decline in the number of circulating CD4⁺ T cells (reviewed in [219, 220]). No more than three years later, the etiologic retrovirus of AIDS was discovered and isolated [221-223]. The retrovirus associated with AIDS in the United States, Europe, and central Africa was eventually named human immunodeficiency virus (HIV) (reviewed in [220]), and later HIV-1, with the discovery of the similar but immunologically and pathogenically distinct species HIV-2 [224].

1.4.2 HIV-1 Classification, Structure, and Genome

As a fellow member of the family *Retroviridae* and subfamily *Orthoretrovirinae*, HIV-1 shares several characteristics with other retroviruses. It has an enveloped nucleocapsid containing two copies of its ssRNA, highly associated with viral proteins. Its long terminal repeat (LTR)-flanked genome consists of *gag*, *pol*, and *env* regions which are translated as single polypeptide chains and then cleaved by proteases [219]. As a member of the *Lentivirus* genus, HIV-1 contains several additional unique coding regions for Tat (p15) (transactivator of transcription), Rev (p19), Vpr (p14), Vif (p23), Vpu (p16), and Nef (p27) [219, 220]. HIV-1 protease (PR), reverse transcriptase (RT), and integrase (IN) are contained within the 160-kD Gag-Pol precursor, which is also cleaved by viral protease. HIV-1 Env precursor is glycosylated and cleaved by a cellular protease into surface (SU) gp120 and transmembrane (TM) gp41 [219, 220].

1.4.3 HIV-1 Epidemiology, Tropism, and Transmission

Since its discovery, HIV-1 has spread into a global pandemic, infecting individuals on all continents [220, 225, 226]). It is estimated that approximately 40 million people worldwide are currently infected with HIV-1, with about 4 to 5 million new infections per year [225]. Until the development of HAART, and now cART, HIV-1 infection was almost universally fatal after progression into AIDS [226].

One of the contributing factors to its devastating pathology is that HIV-1 not only targets cells of the immune system but also destroys them or their immune function, leaving the host susceptible to life-threatening opportunistic secondary infections. Macrophages, dendritic cells, and CD4⁺ T cells are the primary targets of HIV-1, and the viral surface gp120 demonstrates high-affinity binding to cellular CD4 on these cells [219, 220]. Binding of gp120 to cellular CD4 elicits a conformational change in glycoprotein, exposing gp41 to bind its co-receptor and facilitate fusion of the viral envelope with the cellular membrane. Cellular chemokine receptors CXCR4 and CCR5 act as co-receptors for HIV-1 attachment, and viral strains are sub-categorized based on their co-receptor affinity: X4 for those strains which bind CXCR4, R5 strains correspond to CCR5, and those strains with an affinity for both are designated R5X4 [219, 220].

A cell-free viremia is a significant factor in HIV-1 infection, and direct cell-to-cell contact is not required for efficient transmission of HIV-1 from person to person. Infected individuals shed infectious virus in their bodily fluids and secretions, and transmission may therefore occur by direct exchange of bodily fluids such as blood, semen, vaginal secretions, saliva, urine, or breast milk [219, 220].

1.4.4 HIV-1 Pathology and Immune Response to Infection

Primary infection with HIV-1 usually includes generalized symptoms of malaise, fever, swollen lymph nodes, diarrhea, and rash, which may account for any number of diseases without specificity [226]. This stage is associated with a prolific viremia in which HIV replicates exponentially within circulating lymphocytes and may be found in high titers in the blood [220, 226]. After this acute primary onset of HIV is a usually lengthy period of asymptomatic disease, in which the virus mutates at an extremely high rate, probably somewhat contributable to selective pressures of the immune system. During this time, lymph node architecture begins to change, and the blood will contain circulating anti-HIV immunoglobulins, indicative of an immune response against the virus that fails to clear it completely from the infected individual. The determining factor for when HIV has progressed into AIDS is a drop in the count of CD4⁺ T cells from the normal range of between 1,000 and 1,500 cells/ μ L of peripheral blood to fewer than 200 cells/ μ L [226]. Susceptibility to HCMV retinitis generally occurs at or below levels of 50 cells/ μ L of peripheral blood.

1.5 MAIDS-Related Murine Cytomegalovirus Retinitis

1.5.1 *Murine Acquired Immunodeficiency Syndrome (MAIDS)*

A popular small animal experimental model for investigating retrovirus-induced acquired immunodeficiency is the MAIDS (murine AIDS) model. Retrovirus-induced immunodeficiency in this model is accomplished by intraperitoneal injection of a mixture of murine leukemia viruses (MuLV) designated lymphoproliferative bone marrow 5 (LP-BM5) [227]. Induction of MAIDS by this retrovirus mixture is dependent upon mouse strain, with T_H1 -prominent C57BL/6 or C57BL/10 mice being more susceptible to the induction of MAIDS by the LP-BM5 retrovirus mixture than the T_H2 -prominent BALB/c strain [228]. In these susceptible strains, the progression of MAIDS follows a kinetically reproducible pattern that is designated in weeks following injection with the retrovirus mixture. Early-stage MAIDS occurs between the time of retrovirus injection at week 0 (MAIDS-0) through week 3 (MAIDS-3), the transitional phase takes place between MAIDS-3 and MAIDS-6, mid-stage disease happens between MAIDS-6 and MAIDS-8, and late-stage progression occurs between MAIDS-8 and MAIDS-12.

AIDS of humans and MAIDS of mice are both caused by species-specific retroviruses and share many immunologic and pathologic features [9, 229]. Both syndromes are characterized by progressive generalized lymphadenopathy, polyclonal B-cell activation [230], diminished $CD4^+$ T-cell and $CD8^+$ T-cell functions [231], and a cytokine shift from a T_H1 origin to T_H2 -associated cytokines [228, 232, 233]. Commencement of this T_H1 to T_H2 cytokine shift begins by MAIDS-3 [228, 232, 233] and occurs prior to complete T-cell dysfunction in these animals [9, 231, 234]. Although profound splenomegaly also occurs in MAIDS mice, this overall increase in splenic cell counts is associated with dysfunctional immune cells [235]. By MAIDS-

10, B cells [236, 237], CD4⁺ and CD8⁺ T cells [231, 238, 239], NK cells [240], and neutrophils [241] are dysfunctional, and macrophage phenotypes are irregular [236, 239, 242].

As during HIV-1 infection, macrophages are also targets of retroviral infection during MAIDS [236, 242]. Whereas some reports demonstrate that MAIDS causes reduced Mac1⁺ (CD11b⁺) macrophage population percentages and activation frequencies (MAIDS-4) [229, 243], others demonstrate increased macrophage numbers between MAIDS-8 and MAIDS-12 [239]. Macrophage populations in MAIDS mice are driven mostly toward a phenotype consistent with the alternatively-activated pro-angiogenic M2 phenotype but retain some M1 characteristics (i.e., decreased TNF- α and IFN- α production following LPS stimulation, but increased IL-1 β and IL-6 production) [236, 242].

1.5.2 Experimental Murine Cytomegalovirus Retinitis

The species-specificity of HCMV precludes its ability to establish productive infection in animal models or cells [244]. MCMV is therefore commonly substituted in research laboratories to investigate cytomegalovirus infection and pathogenesis in mouse models [61, 245]. As with humans and HCMV, immunologically normal mice are generally resistant to MCMV retinitis [8, 139, 246, 247], depending on mouse strain [248, 249], viral load, and route of viral inoculum [250-252]. Establishment of an immune-suppressed state with delivery of a substantial amount (10⁴ PFU) of MCMV into the subretinal (supraciliary) space of the eye (**Figure 1.7**) overcomes this resistance, consistently manifesting high frequencies (75–100%) of experimental MCMV retinitis [7, 136, 252] in a manner dependent upon viral load [252] and mouse strain [8, 136, 248, 249, 252, 253]. Two successful immunosuppression strategies to achieve susceptibility to MCMV retinitis include systemic delivery of corticosteroid drugs [136, 252, 254] or a mixture of mouse-specific retroviruses (LP-BM5) [227, 232] that induce MAIDS after 8–12 weeks in

C57BL/6 mice [9, 229, 255], thereby conferring susceptibility to MCMV retinitis. The combination of MAIDS and subretinal MCMV injection is unique to our laboratory [7, 8].

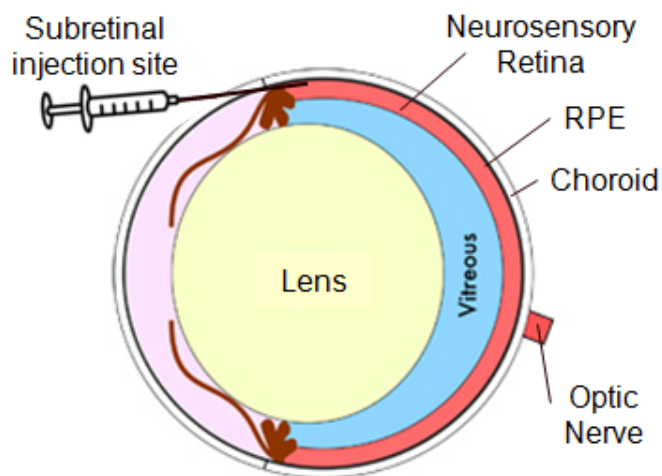


Figure 1.7. Schematic mouse eye with subretinal site of injection.

The mouse eye contains a much larger crystalline lens than the human eye. Shown is a representation of the subretinal or supraciliary injection site for experimental MCMV retinitis. A 30-gauge needle is carefully inserted into the subretinal space, and a 2- μ L volume is slowly injected, followed by air bubbles to prevent backflow. Figure modified from [256].

The strain of mouse used during experimental MCMV retinitis studies impacts susceptibility to either MCMV or to the LP-BM5 retrovirus mixture. BALB/c mice are more susceptible than C57BL/6 mice to systemic MCMV infection [249, 250, 257-260]. During corticosteroid (drug)-induced immune suppression, the frequency of MCMV retinitis in BALB/c mice (90%, [136]) appears to be greater than in C57BL/6 mice (50%, [253]), with the exception of an adoptive transfer study [261] suggesting that both mouse strains appear to be 80-100% susceptible to retinitis during drug-induced immune suppression. BALB/c mice, however, are more resistant than C57BL/6 mice to the induction of MAIDS by LP-BM5 [9, 262], requiring one year or longer to progress to late-stage MAIDS. For these reasons, although BALB/c mice

are usually used for experimental MCMV retinitis models in drug-induced immune suppression, C57BL/6 mice are used for MAIDS-induced immune suppression.

In the absence of MCMV infection, these two different techniques to accomplish immune suppression also differ in their types of dysfunctional immune cells, the timing of immune cell demise, and the mechanisms by which these immune cells are rendered defective. One of the major differences between these models is the number and function of macrophages. MAIDS, without MCMV infection, causes reduced Mac1⁺ (CD11b⁺) macrophage population percentages and activation frequencies at MAIDS-4 [229, 243], with increased macrophage numbers between MAIDS-8 and MAIDS-12 [239]. Macrophage populations in MAIDS mice are driven toward an alternatively-activated pro-angiogenic phenotype that is between classically-activated M1 and alternatively-activated M2. They have decreased TNF- α and IFN- α production but increased IL-1 β and IL-6 production in response to LPS [236, 242]. By contrast, corticosteroids such as methylprednisolone acetate, in the absence of MCMV infection, poison nearly all aspects of the innate and adaptive immune system within days, including macrophages [263]. Whatever macrophages remain tend to be driven toward the M2 alternatively-activated phenotype, in a similar manner as macrophages exposed to IL-4, and they avidly produce IL-10, but not TNF- α , IL-1, or IL-6 [124, 125]. Therefore, whereas MAIDS mice experience a functional change in macrophage phenotype at later stages of disease [236, 239, 242], drug-induced immune suppression very quickly results in significant loss of macrophages [263].

Corticosteroids also decrease the overall number and function of CD4⁺ and CD8⁺ T cells (~93% depletion, [254, 263, 264]) and generally dampen the immune response by suppressing the expression, release, and/or function of inflammatory cytokines such as IFN- γ TNF- α , and IL-2 (reviewed in [264]). This rapid, acute decline of the immune system is not observed during

MAIDS, which slowly progresses through distinct phases of immune cell dysfunction. Whereas corticosteroid treatment causes apoptosis in leukocytes and lymphocytes therefore decreasing the overall number of these populations [263, 264], MAIDS causes aberrant proliferation of B and T lymphocytes [230, 231] that results in increases in these cell populations coupled with retrovirus-induced cellular dysfunction [9, 231, 234]. By late-stage MAIDS, NK cells [240] and neutrophils [241] are also dysfunctional, and macrophage phenotypes are irregular [236, 239, 242]. These two different methods of immune suppression therefore differently affect immune cell populations, particularly macrophage populations, and cytokine responses to infection.

Immunologically normal C57BL/6 mice and MAIDS-4 C57BL/6 mice are resistant to MCMV retinitis (0% frequency). Mice with MAIDS-8 to MAIDS-12, however, are susceptible (80-100%) to MCMV retinitis following subretinal [8, 246, 247], but not systemic [251], MCMV inoculation. Importantly, retinitis susceptibility does not correlate with ocular viral titers, because MCMV replication in the ocular compartment at 6–10 days after subretinal inoculation reaches equivalently high levels ($\sim 3 \times 10^4$ PFU/eye) in retinitis-resistant MAIDS-4 mice as those in retinitis-susceptible MAIDS-10 mice [247, 265]. By comparison, immunologically normal mice receiving the same amount of subretinally-injected MCMV typically produce only $\sim 10^2$ PFU/eye [8]. High intraocular MCMV titers alone are therefore insufficient for retinitis, and susceptibility to intraocular MCMV replication precedes susceptibility to retinitis in this model [247]. Mice with MAIDS-8 to MAIDS-12 develop a retinitis 8-10 days following subretinal MCMV injection that exhibits histopathologic features similar to those found in AIDS-related HCMV retinitis [8, 81], including full-thickness retinitis, cytomegalic cells, and transition zones of histologically normal to diseased retina (**Figure 1.8**). MAIDS-related MCMV retinitis is therefore a clinically-relevant, reproducible model for studying the pathogenesis of AIDS-related HCMV retinitis.

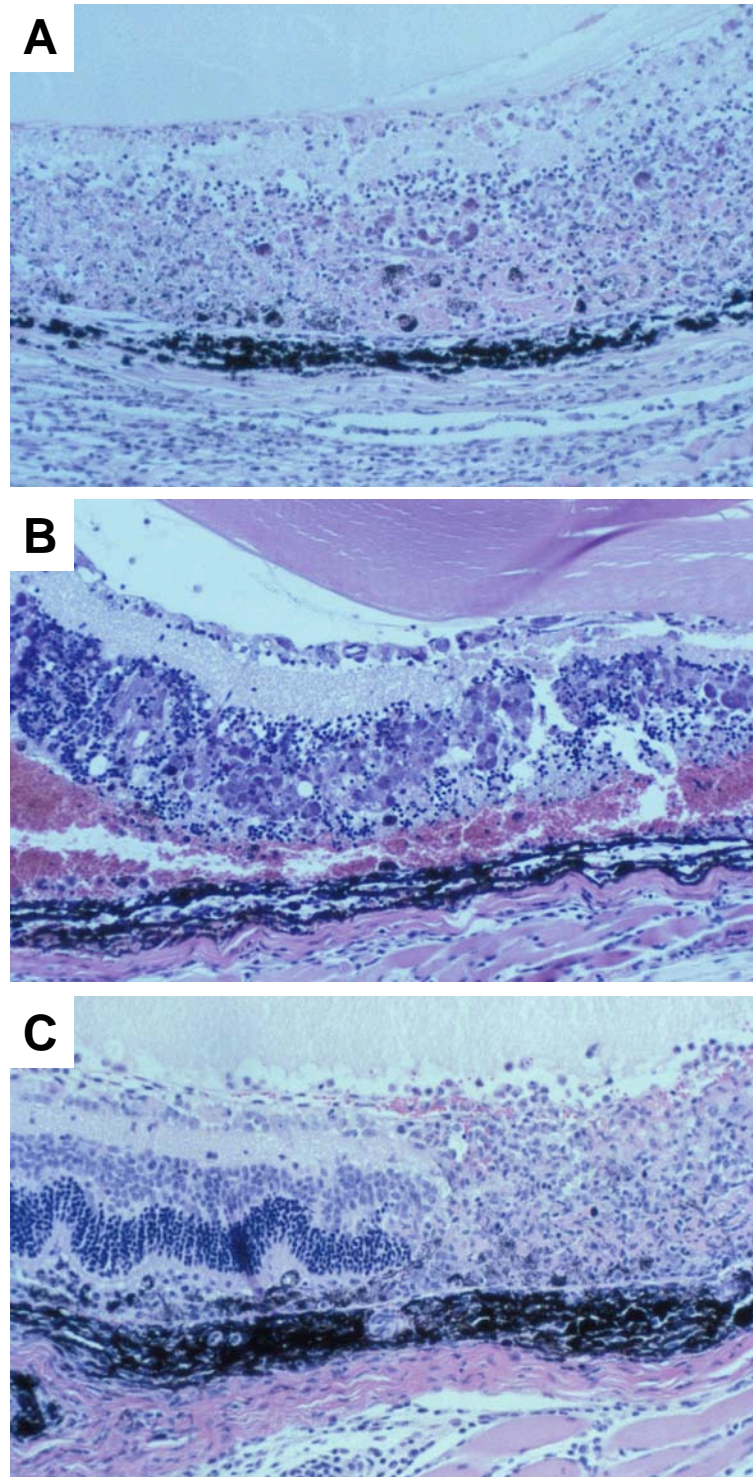


Figure 1.8. Ocular histopathology of experimental MAIDS-related MCMV retinitis.

Hematoxylin and eosin (H&E)-stained cross-sections of eyes from retinitis-susceptible MAIDS mice at day 8 following subretinal MCMV infection show full-thickness retinal necrosis (A), with cytomegalic cells and hemorrhage (B), and transition zones between intact retina (with folding) and full-thickness retinitis (C). Photomicrographs from [8], original magnification 200 \times .

1.6 Suppressors of Cytokine Signaling (SOCS)

Cells of innate and adaptive immune responses secrete cytokines and chemokines to orchestrate a coherent, integrated immune response to protect the host against pathogens. During infection, cytokines initiate, execute, and resolve inflammatory responses, such that cytokine signaling is the crucial control switch between the initiation of the immune response and the maintenance of homeostasis in the periphery. Therefore, cellular negative feedback loops play an important role in maintaining the tight balance of cytokine secretion and cytokine inhibition, and suppressor of cytokine signaling (SOCS) proteins function in such a capacity.

1.6.1 SOCS Family, Structure, and Function

The first SOCS protein was discovered in the mid-1990s as a cytokine-induced inhibitor of signal transducers and activators of transcription (STAT) cell signaling pathways [266-269]. Current consensus is that the SOCS protein family contains eight members, SOCS1 through SOCS7 and the cytokine-inducible SH2 containing domain protein (CIS), which are selectively up-regulated in response to multiple cell signaling pathways [270] and subsequently function intracellularly as negative regulators of cell signaling (reviewed in [12]). SOCS family proteins are expressed in many different organs [268] and cell types, but they are most abundantly associated with cells of hematopoietic origin [271] of the innate and adaptive immune systems (reviewed in [12, 272]). Some of these SOCS-expressing cell types include monocytes [273], macrophages [267, 274], dendritic cells (DC) [275, 276], microglia [277], neutrophils [278], NK cells [279], CD4⁺ and CD8⁺ T cells [280, 281], and Müller cells [282].

All SOCS proteins characteristically contain an internal SH2 domain, a C-terminal SOCS box, and a variable length N-terminal region. SOCS1 and SOCS3 additionally possess an N-terminal kinase inhibitory region (KIR) which can act as a pseudosubstrate to block the kinase

activity of such proteins as Janus kinases (JAKs) (**Figure 1.9**) [267, 283, 284]. These SOCS proteins negatively regulate intracellular signaling pathways by several mechanisms, including competitive binding of phosphotyrosine residues with various recruited STAT proteins, KIR-mediated inhibition of JAK activity, or SOCS box-mediated ubiquitination and degradation of SOCS-bound components [10, 12].

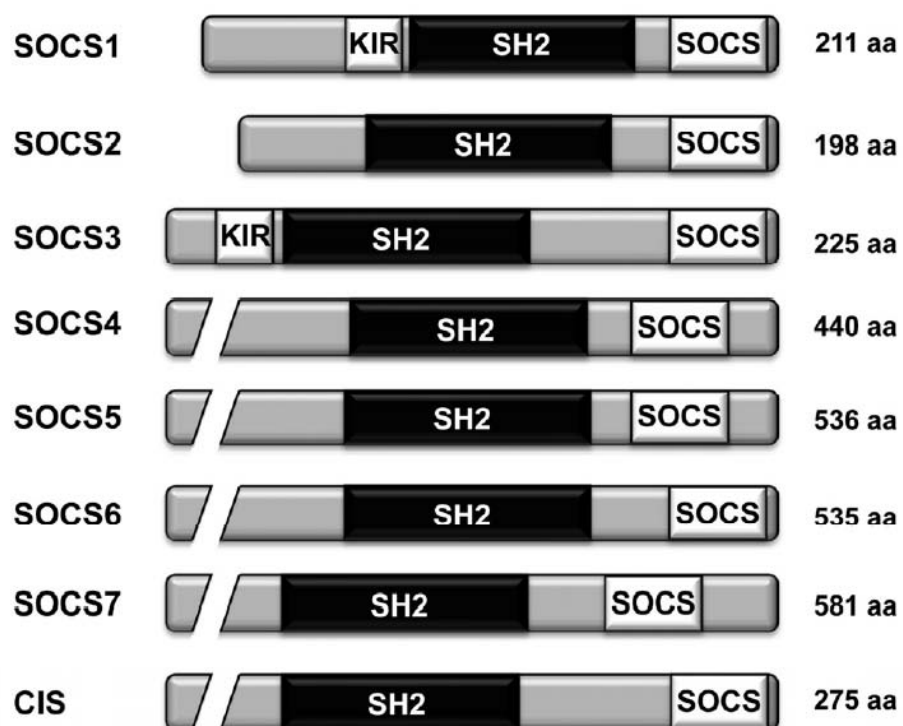


Figure 1.9. SOCS family proteins homologous domains.

KIR = kinase inhibitory region: unique to SOCS1 and SOCS3, this domain allows for direct, functional inhibition of JAKs. **SH2** = sequence homology 2 domain, which recognizes phosphorylated tyrosines flanked by specific amino acid sequences, such as those found on intracellular domains of cytokine receptors. **SOCS** = SOCS box domain, which recruits cellular ubiquitinating machinery. Figure modified from [284].

Under normal physiological conditions in host cells, extracellular cytokines recognized by their specific transmembrane receptors on target cell surfaces initiate an intracellular signaling cascade that stimulates the production of dozens of gene products (reviewed in [285-287]),

including SOCS family proteins. Although many cell signaling pathways have been shown to be capable of inducing SOCS [288-291], JAK/STAT pathways are major transcriptional stimulators of SOCS proteins (reviewed in [12]). Once induced, SOCS family proteins act intracellularly to regulate signaling by JAK/STAT pathways driven by antiviral IFNs and other cytokines such as interleukin (IL)-6 (**Figure 1.10**) [12, 268, 272, 292, 293].

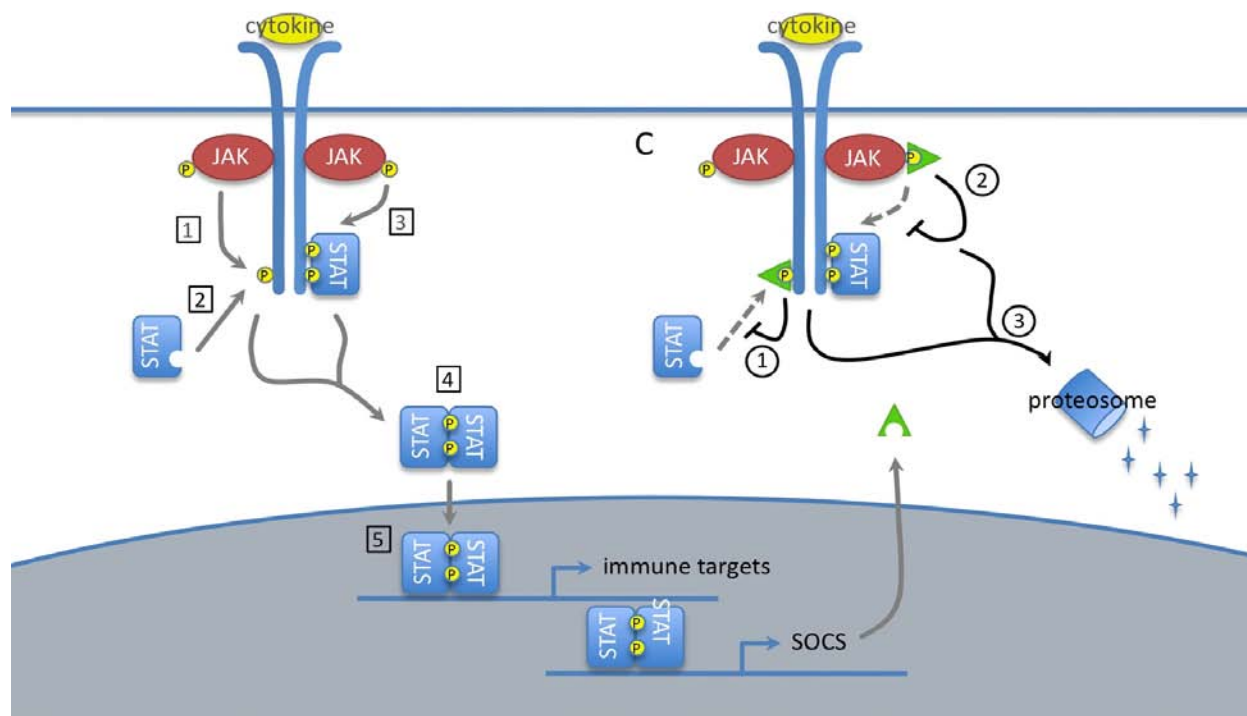


Figure 1.10. Schematic representation of cytokine signaling by the JAK/STAT pathway, with induction and function of SOCS proteins.

Left side: Extracellular cytokines (yellow ovals) are recognized by their transmembrane receptors on target cells. These receptors dock members of the JAK family of proteins (red ovals), avid kinases. Upon coupling of receptor components, (1) JAKs phosphorylate (P) each other and tyrosine residues on intracellular domains of cytokine receptors, creating docking sites (2) for STAT proteins, which are (3) phosphorylated (activated) by JAKs. (4) Activated pSTATs dimerize and translocate to the nucleus (5) to act as transcription factors, stimulating dozens of gene products, including negative regulators such as SOCS proteins. **Right side:** SOCS transcripts are translated and act intracellularly to inhibit JAK/STAT signaling by (1) competitive inhibition of STAT docking sites on internal receptor domains through the SOCS SH2 domain, (2) inhibiting JAKs via the SOCS KIR domain (SOCS1 and SOCS3), or (3) with recruitment of cellular ubiquitination machinery to the SOCS Box domain, tagging JAKs, STATs, and/or receptors for proteasome degradation. Figure from [10].

In particular, SOCS1 and SOCS3 have been implicated in the pathogenesises of several viral infections (reviewed in [10]), as viral up-regulation of these host proteins may dysregulate host antiviral strategies and thereby assist virally-infected cells in evading immune destruction.

1.6.2 SOCS1 and SOCS3

The importance of SOCS1 and SOCS3 in modulating immune responses is emphasized in knockout mice, as SOCS1-deficient mice die within 3-4 weeks of birth from massive IFN-related inflammation [294-296], and deletion of the SOCS3 gene is embryonically lethal [297]. SOCS1 proteins are able to limit the surface expression of molecules that mediate the immune response, suppress inflammation by dampening expression of cytokines and chemokines, inhibit pathogen infiltration and replication, and prevent central nervous system demyelination. SOCS1 is quickly induced by IFN signaling and inhibits the specific JAK and STAT proteins involved during IFN signaling [298, 299]. In addition to its primary role in the regulation of components of the JAK/STAT pathway, SOCS1 has also been shown to be capable of regulating other cellular signaling pathways such as TLR signaling and macrophage activation [289]. SOCS1 also plays a dual role in CD4⁺ T-helper (T_H) cell differentiation [280, 300-302]. As a key attenuator of IFN- γ signaling, SOCS1 can inhibit IFN- γ -mediated STAT1 activation by targeting JAK2, thus suppressing the differentiation of the T_H1 lineage in CD4⁺ T cells [299, 303]. SOCS1 is alternatively able to inhibit interleukin-4 (IL-4) signaling, thereby driving differentiation toward a T_H1 phenotype [280, 304].

By comparison, SOCS3 is classically up-regulated as a consequence of signaling by the IL-6 family of cytokines [268]. Once induced, a major function of SOCS3 is then to inhibit the signaling of IL-6 family cytokines by targeting their common gp130 receptor [272, 305, 306]. Furthermore, SOCS3 is a key regulator of IL-23-mediated STAT3 [301, 307] and of IL-12-

mediated STAT4 activation [306], such that SOCS3 is also able to inhibit the development of CD4⁺ T_H1 and T_H17 cells [308], thereby promoting differentiation to the T_H2 lineage.

Both SOCS1 and SOCS3 have demonstrated transcriptional induction by type I IFNs, which are recognized as key immune regulators in mounting an antiviral response [309, 310]. These cytokines play a role in the activation of NK and T cells, and they induce cell death in virus-infected cells [107, 311]. The type I IFN family consists of the many subtypes of IFN- α , as well as IFN- β , IFN- ϵ , IFN- κ , and IFN- ω [312]. Almost all cell types are capable of producing type I IFNs in response to various stimuli [310, 311, 313]. Plasmacytoid dendritic cells (pDC) in particular are one of the highest contributors to the secretion of type I IFNs [311]. Type I IFNs signal through the heterodimerization of the type I IFN receptors, IFNAR-1 and IFNAR-2, which signal through the JAK/STAT pathway, mediated specifically by the JAKs Tyk2 and JAK1, and by STAT1 and STAT2 [311, 314]. Unlike most dimerized STATs, the STAT1/STAT2 heterodimer must bind to an additional protein, interferon regulatory factor 9 (IRF9), and form the interferon-stimulated gene factor 3 (ISGF3), before they are able to recognize the interferon-stimulated response element (ISRE) and begin transcription of ISGs [311]. More than 300 ISGs have been identified to date [315], including SOCS proteins, particularly SOCS1, and, to a lesser extent, SOCS3.

In addition to this classical induction by cytokine signaling via the JAK/STAT pathway, SOCS proteins have also shown to be simulated by alternative cell signaling pathways. Among these pathways are NF- κ B and MAPK signaling pathways through phosphorylation of JNKs [110, 111]. SOCS proteins can also be induced by stimulation of TLRs [290, 316, 317], which are expressed by many cell types, including the RPE [318, 319] and Müller cells [320] of the eye. In macrophages and DCs, non-TLR sensor dectin-1 induces SOCS1 by MAPK/ERK, and

SOCS1 modulates TLR9 signaling by inhibiting NF- κ B [321]. Stimulation of these pathways therefore may induce the production of SOCS proteins directly or indirectly by the production of SOCS-inducing cytokines such as type I IFN.

1.6.3 Viral Exploitation of SOCS1 and SOCS3

SOCS1 and/or SOCS3 are also induced and exploited by viruses, including HIV-1 [322-325], HSV-1 [326-328], VZV [329], KSHV [330], hepatitis B virus [331], hepatitis C virus [332, 333], Semliki forest virus [292], and respiratory syncytial virus [334]. Stimulation of SOCS1 and/or SOCS3 during viral infection generally facilitates events that are beneficial for the virus, including increased viral replication and immune evasion, ultimately enhancing pathogenesis (reviewed in [10]).

Virus-induced expression of SOCS proteins may be an indirect consequence of pathogen-induced cytokines, or SOCS may be directly stimulated by viral components. Although evidence suggests that SOCS1 and SOCS3 are stimulated by HCMV infection of monocytes [335] and MCMV infection of macrophages [336], the effect of HCMV or MCMV infection on SOCS expression remains uncharacterized. Both HCMV and MCMV encode functional proteins to evade host immune clearance, and both viruses have the ability to hijack host-encoded immune-modulating proteins that allow for enhanced viral replication, dissemination, and the establishment of latency [337-340]. MCMV infection also induces the expression of IL-6 as well as type I and type II IFNs [341-348]. In addition, deficiency of IFN- γ or IFN-correlated signaling molecules increases susceptibility to MCMV infection [336, 348, 349]. Ocular MCMV infection via the subretinal route also up-regulates IFN- γ mRNA expression [247]. Therefore, if SOCS protein expression is driven by MCMV-induced cytokines during infection, then IFNs and IL-6 may be likely SOCS-inducing candidates, particularly for the production of SOCS1 and SOCS3.

1.7 Goals of this Dissertation

We found that SOCS1 and SOCS3 are significantly stimulated in the eyes of mice during MAIDS-related MCMV retinitis ([350, 351] and **Figure 1.11**), and that intraocular, infiltrating F4/80⁺ mouse macrophages are significant cellular sources of SOCS1 and SOCS3 during experimental MCMV retinitis ([352] and **Figure 1.12**). Elucidating possible virologic, immunologic, and/or pathologic mechanisms involved in this stimulation is the focus of this study. SOCS proteins are induced by many innate or adaptive immune factors, but the most prominent induction is through JAK/STAT pathways stimulated by type I and II IFNs for SOCS1 and the IL-6 family of cytokines for SOCS3. Alternatively, MCMV itself may contain viral proteins able to bind directly to SOCS gene promoters, initiating SOCS transcription. Therefore, MCMV infection may induce SOCS proteins directly or indirectly, and it may do either or both of these by one or more mechanisms that requires active viral replication, that does not require viral replication, or by multifaceted virologic mechanisms specific to certain parameters such as post-infection kinetics or cell type. Once induced, SOCS proteins in the cytoplasm interfere with JAK/STAT pathways of host cell signaling, but SOCS1 and SOCS3 are also capable of interfering with other signaling pathways, in a cell-type-dependent manner.

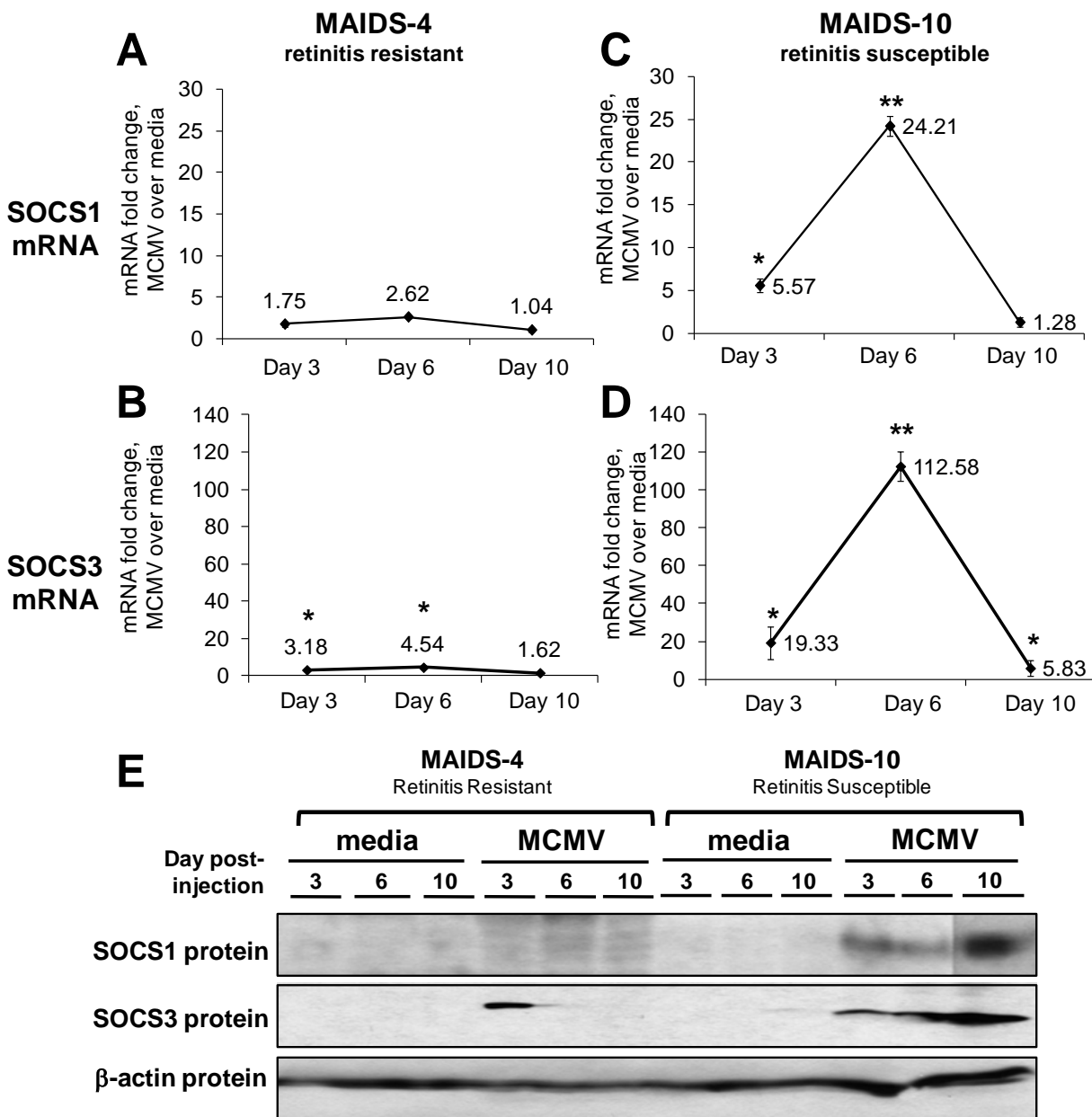


Figure 1.11. Intraocular SOCS1 and SOCS3 mRNA and proteins are highly stimulated in the MCMV-infected eyes of retinitis-susceptible MAIDS-10 mice, but not retinitis-resistant MAIDS-4 mice.

C57BL/6 mice with MAIDS-4 (A and B) or MAIDS-10 (C and D) were subretinally injected with 10^4 PFU of MCMV (left eyes) or media (right eyes) as in Materials and Methods. Whole eyes were assessed for SOCS1 (A and C) or SOCS3 (B and D) mRNA transcripts. Means \pm SD of $n = 5$ mice per group are shown. * $p < 0.05$ and ** $p < 0.01$ for MCMV-infected eyes compared with media-injected controls. Western blotting (E) of SOCS1 and SOCS3 proteins was performed to assess ocular SOCS1 and SOCS3 proteins, with β -actin used as a loading control. These experiments were performed by Dr. Hsin Chien under the direction of Dr. Richard D. Dix, Georgia State University [351].

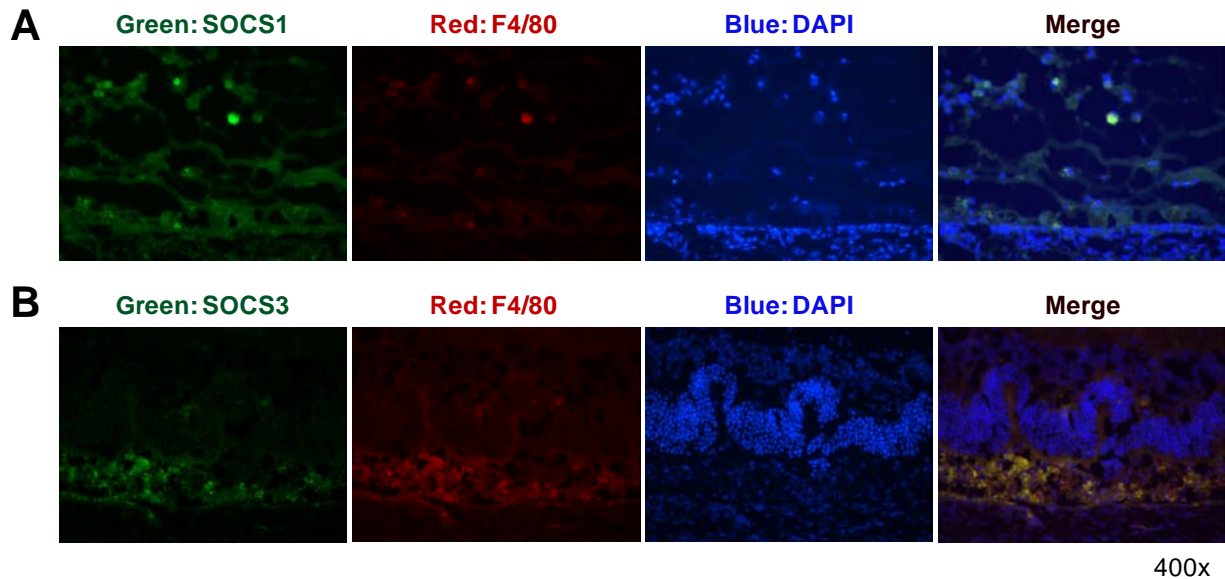


Figure 1.12. Ocular F4/80⁺ macrophages express SOCS1 and SOCS3 during MAIDS-related MCMV retinitis.

Retinitis-susceptible MAIDS-10 eyes at day 10 following subretinal MCMV were formalin-fixed, sectioned, and immunofluorescently stained with antibodies against SOCS1 (A) or SOCS3 (B) (green) and macrophage cell marker F4/80 (red). Nuclei counterstained with DAPI. Original magnification, 400 \times . These experiments were performed by Dr. Hsin Chien under the direction of Dr. Richard D. Dix, Georgia State University [352].

Through pursuit of two specific aims, herein we test the **central hypothesis that MCMV stimulates and employs SOCS1 and/or SOCS3 to induce onset and development of MCMV retinal disease.**

Specific Aim 1: Test the hypothesis that SOCS1 and/or SOCS3 stimulation *in vivo* is affected by virologic, immunologic, and/or pathologic events during MCMV infection.

SOCS1 and SOCS3 mRNA and protein are significantly up-regulated during experimental MAIDS-related MCMV retinitis ([350] and **Figure 1.11**). Because the *in vivo* parameters under which MCMV may cause stimulation of SOCS1 and SOCS3 remain unclear, herein we determined whether stimulation of these proteins is correlated with cytokine inducers and/or pathologies under the following conditions: (a) in the spleens and/or eyes of mice with systemic MCMV infection in the absence of retinal disease, with or without MAIDS, (b) in the MCMV-infected eyes of mice during the progression of late-stage MAIDS, (c) in experimental MCMV retinitis during corticosteroid-induced immune suppression, and (d) intraocularly following antiviral inhibition of MCMV replication during MAIDS-related experimental MCMV.

Specific Aim 2: Test the hypothesis that SOCS1 and/or SOCS3 stimulation *in vitro* is affected by virologic and/or immunologic events during MCMV infection. SOCS1 and SOCS3 are significantly stimulated during experimental MAIDS-related MCMV retinitis, and F4/80⁺ macrophages are a significant cellular source of SOCS1 and SOCS3 in this model (**Figure 1.12**). We therefore tested whether stimulation of SOCS1 and/or SOCS3 occurs during: (a) infection of IC-21 mouse macrophages with salivary gland-derived MCMV or cell culture-derived MCMV, (b) inhibition of viral replication by UV inactivation in macrophage or fibroblast cells, (c) antiviral inhibition of MCMV replication, (d) direct MCMV infection and/or in uninfected bystander cells.

2 MATERIALS AND METHODS

2.1 Cell Lines and Stocks

C57BL/6 mouse embryonic fibroblast (MEF) cells from ATCC (Manassas, VA, No. SCRC-1002) were grown in Dulbecco's modified eagle media (DMEM, Corning Life Sciences, Manassas, VA, #10-013) supplemented with 15% fetal bovine serum (FBS, Atlas Biologicals, Fort Collins, CO, #FR-0500-A), 4 mM L-glutamine, 1% penicillin/streptomycin, and 0.1 mg/mL gentamicin. These cells were used for titration of MCMV stocks and experimental tissue homogenates, and, in some experiments, for propagation of MCMV through tissue culture passage (TC-MCMV).

SC-1 fibroblasts (ATCC #CRL-1404) and SC-1/MuLV LP-BM5 cells [232] provided by the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (Germantown, MD) were maintained in DMEM containing 10% FBS, 24 mM L-glutamine, 1% penicillin/streptomycin, and 0.1 mg/mL gentamicin. These cell lines were used for propagation of the mouse retrovirus mixture used to induce MAIDS.

IC-21 mouse macrophages (ATCC #TIB-186) were maintained in RPMI-1640 media supplemented with 10% FBS, 1% penicillin/streptomycin, and 0.1 mg/mL gentamicin. These are a simian virus 40 (SV40)-transformed cell line of macrophages derived from T_H1-dominant C57BL/6 mice [353]. Macrophages support the full MCMV replication cycle and are important for viral dissemination and latency [354], and IC-21 mouse macrophages are commonly used in cell culture studies with MCMV infection [98, 121, 336, 349, 355], whereupon they are driven mostly toward a pro-angiogenic M2-like phenotype [192]. Furthermore, IC-21 cells infected with lacZ-expressing MCMV tracer virus RM427 [117], when intravenously injected into MAIDS mice, disseminate lacZ-positive signals to the spleen and eye (Dix, unpublished findings). IC-21

macrophages and primary peritoneal macrophages display similar phagocytic and lysosyme activities [353]. Differences between murine primary bone marrow macrophages (BMMs) and IC-21 macrophages were studied by others in the context of the ability of MCMV epitope-specific cytotoxic T-lymphocytes (CTLs) to lyse macrophages infected with tissue culture-derived MCMV clones. Although BMMs are more sensitive to CTL lysis than IC-21 cells in a manner dependent on MCMV m04 gene, the two types of macrophages express identical levels of MHC I upon TC-MCMV infection [98]. Unless otherwise indicated, experiments with IC-21 macrophages were performed with IC-21 media containing 5% FBS, and media in all experimental wells was refreshed every 24 hrs.

2.2 Viruses

Propagation of mouse retrovirus mixture for MAIDS induction. MuLV LP-BM5 stocks to induce MAIDS were made as previously described [8], with modifications. SC-1/MuLV LP-BM5 cells were seeded in a 1:1 ratio with uninfected SC-1 cells into 16, T-150 flasks and maintained with 12 mL per flask of complete SC-1 media for 6 days. Cells were scraped into 8 mL/flask of their own media, pooled, aliquoted, and frozen at -80°C . Fresh aliquots thawed for each experiment were clarified by centrifugation to remove cell debris before injection.

Propagation of salivary gland-derived MCMV (SG-MCMV). MCMV (Smith strain) was propagated through salivary gland passage in BALB/c mice (Harlan/Envigo, Frederick, MD) as previously described [8, 356, 357]. In brief, at least 20 mice were injected intraperitoneally with between 10^2 and 10^3 plaque forming units (PFU) of MCMV; animals were euthanized 2 weeks later, and salivary glands were harvested and homogenized together in a 15-mL Tenbroeck tissue grinder with 1.5 mL DMEM. Virus stock preparations were aliquoted and stored in liquid nitrogen, and a fresh tube was thawed and used for each experiment. Each virus

stock was quantified by plaque assay in MEF cells. *In vivo* and *in vitro* experiments were performed with this salivary gland-derived MCMV (SG-MCMV) from at least six different stock preparations. For animal studies herein, Smith strain SG-MCMV is referred to as MCMV and was used for all *in vivo* experiments and for most *in vitro* experiments, unless otherwise stated.

Construction and propagation of the MCMV RM4503 IE2-deficient mutant. HCMV IE1 and IE2 are encoded by overlapping regions of the HCMV genome and are alternatively spliced into several gene products [91]. For MCMV, the IE1 and IE3 genes are the alternatively-spliced locational [92] and functional homologues of HCMV IE1 and IE2, respectively [93]. MCMV IE2 has no sequential or functional homologue in the HCMV genome and is dispensable for growth *in vitro* [94] and *in vivo* [95]. MCMV mutant RM4503 [358, 359] expresses enhanced green fluorescent protein (EGFP) under the control of a fragment of the HCMV promoter-enhancer adjacent to the MCMV IE2 enhancer in the MCMV genome. This mutant was previously constructed by others [358] and was a gift from the laboratory of Dr. Tim Sparer, Department of Microbiology, University of Tennessee, Knoxville. MCMV RM4503 was originally constructed by the Mocarski lab by insertion of the EGFP construct into the MCMV genome to disrupt the MCMV IE2 gene, and it therefore does not express IE2 [358] (see **Figure 2.1**). Instead, EGFP is expressed with IE2 kinetics [358-360]. Before use in experiments, we propagated this tissue culture-derived mutant virus three times sequentially through the salivary glands of female BALB/c mice as described for MCMV Smith strain.

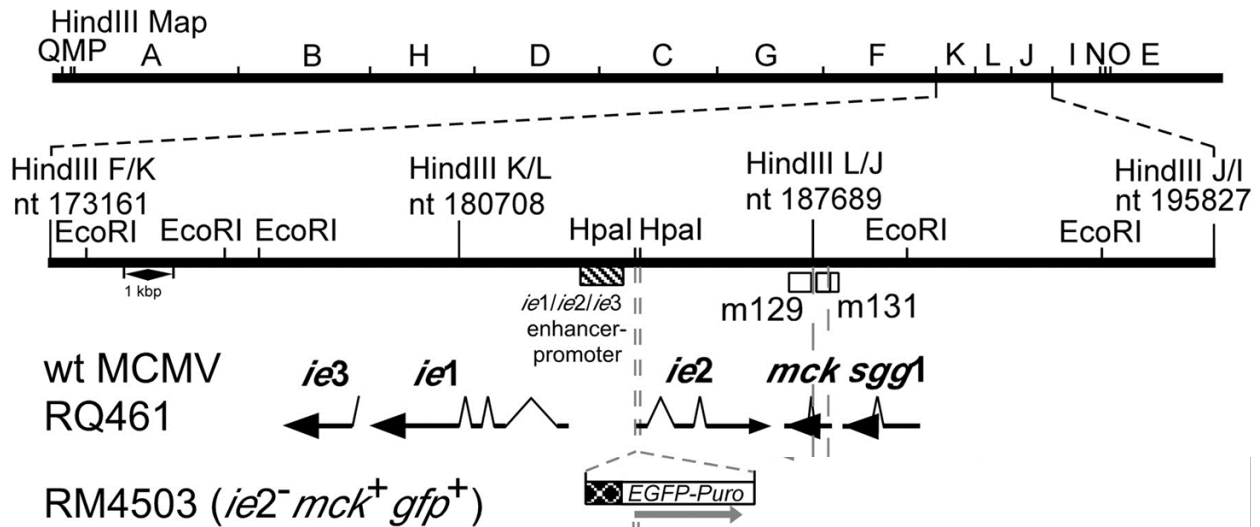


Figure 2.1. Molecular construction of MCMV RM4503.

MCMV RM4503 is an IE2-deficient mutant in which the EGFP gene is inserted into the IE2 gene [358]. Because the EGFP gene is under the control of the MCMV *ie1/ie2/ie3* promoter-enhancer, this mutant expresses EGFP with IE2 kinetics, but without IE2 mRNA or protein expression [358]. Figure modified from [360].

Propagation of tissue culture-derived MCMV (TC-MCMV). For some *in vitro* experiments, MCMV (Smith) parent strains derived from salivary gland passage were propagated through cell culture (tissue culture) passage and are designated TC-MCMV with a passage (p) number indicating the number of serial passages through cell culture (e.g., TC-MCMV p4 indicates four serial stock preparations through MEF cell culture). Propagation of TC-MCMV was first achieved by inoculating at least 3, T75 flasks of MEF cells (designated TC-MCMV) or BALB/3T3 cells (designated TC-MCMV/BALB) with a low multiplicity of infection (MOI) of 0.001–0.01 PFU/cell of parent SG-MCMV and incubating the flasks for 3–4 days until cells reached a cytopathic effect (CPE) of 3+, when between 75% and 100% of cells show CPE. Cells were then harvested by scraping into their own media, pooled, aliquoted, and frozen in liquid nitrogen. The first passage through cell culture from SG-MCMV inoculum was

designated as TC-MCMV p1, and all subsequent serial passages through cell culture were increased by one per serial passage (i.e., TC-MCMV p2, TC-MCMV p3, etc.).

Purification of TC-MCMV stocks by ultracentrifugation. Because TC-MCMV titers were consistently too low ($\sim 10^4$ PFU/mL) to achieve an experimental MOI of 3 PFU/cell in some studies, aliquots of TC-MCMV or TC-MCMV/BALB were further propagated through their respective cell lines contained within a large number of flasks (12 to 16, T150 flasks or 24 to 32, T75 flasks) and purified and concentrated by ultracentrifugation over a discontinuous (20-50%) sucrose gradient in Tris-buffered saline (TN, 0.05 M Tris, 0.1 M NaCl, pH 7.4). Purified virus pellets were resuspended in 1 mL TN, aliquoted, and frozen in liquid nitrogen. Virus stocks thus prepared consistently achieved titers between 5×10^6 and 1×10^7 PFU/mL.

UV-inactivation of MCMV. For experiments utilizing UV-inactivation of the virus, a portion of MCMV from the same stock per experiment was exposed to DNA-damaging UV light for 1 hr. This UV inactivation allows the virus to undergo its first kinetic steps of infection, including attachment, adsorption, uncoating, and release of viral-associated tegument proteins into the host cell, but prevents transcription of viral genes, viral replication, and the formation of progeny virus [101]. All UV-inactivated inocula were tested by back-titration to ensure complete inactivation, such that a 0.1-mL sampling failed to produce any plaques in MEF cells after 2 weeks.

Antiviral inhibition of MCMV replication *in vitro*. To test whether SOCS1 and/or SOCS3 expression is sensitive to antiviral inhibition of MCMV replication later during infection, some monolayers were treated with various concentrations of the antiviral drug GCV (Sigma-Aldrich, St. Louis, MO). GCV in particular acts a guanosine analog [102], preferentially inhibits HCMV and MCMV DNA polymerases [103] and therefore viral replication, and allows viral IE

and E gene expression without L gene expression [104]. For these studies, GCV was dissolved in dimethyl sulfoxide (DMSO) at 10X concentrations and diluted to indicated final concentrations in media containing 5% FBS. Daily-refreshed media was supplemented with the appropriate concentrations of GCV or DMSO vehicle control per well for each group.

2.3 Animals

Adult female BALB/c mice (8-12 weeks old) used for MCMV propagation were purchased from Harlan Laboratories (Indianapolis, IN, USA). Wild type female C57BL/6 mice were used for all *in vivo* experiments and were purchased from Charles River Labs (Raleigh, NC, USA), from Jackson Laboratory (Bar Harbor, ME, USA), or from Taconic Farms (Germantown, NY, USA) over a period of six years. Mice used in MAIDS studies were 3–4 weeks old upon MAIDS induction, and 6–10-week-old mice were used for studies in immunologically normal mice or mice with corticosteroid-induced immune suppression (without MAIDS). Animals were housed in the Georgia State University vivarium in 12-hr light/dark cycles and given unrestricted access to food and water. All animal procedures were conducted in compliance with Georgia State University Institutional Animal Care and Use Committee (IACUC) protocols and with the Association for Research in Vision and Ophthalmology (ARVO) statement for Use of Animals in Ophthalmic and Vision Research.

Induction of MAIDS. MAIDS was induced in C57BL/6 mice by intraperitoneal injection of 1 mL of the LP-BM5 murine leukemia retrovirus mixture into 3-week-old C57BL/6 mice as previously described such that each mouse received approximately 5×10^3 – 10^4 of infectious retroviruses [8, 232]. The retrovirus mixture was allowed to progress to MAIDS for 4 weeks (MAIDS-4), for 8 weeks (MAIDS-8), for 10 weeks (MAIDS-10), or for 12 weeks (MAIDS-12). MAIDS-4 mice display a mid-stage progression and remain retinitis-resistant,

while mice with late-stage MAIDS (MAIDS-8 through MAIDS-12) are susceptible to retinitis, as previously described by us [8, 350, 361].

Corticosteroid-Induced Immunosuppression. Drug-induced immunosuppression of C57BL/6 mice was achieved as previously described by others [136, 253, 254, 362, 363] via intramuscular injection of the corticosteroid methylprednisolone acetate (2 mg/mouse, ~40 mg/kg) every 3 days, beginning at day -2 relative to subretinal MCMV injection at day 0.

Systemic MCMV Infection. Adult C57BL/6 mice without MAIDS (immunologically normal), with MAIDS-4, or with MAIDS-10 were injected intraperitoneally with 0.1 mL DMEM (media, controls) or with $\sim 10^4$ PFU of MCMV contained within a 0.1-mL volume. At indicated days following infection, mice from each group were euthanized in an atmosphere of isoflurane, and whole spleens and/or whole eyes were harvested. Tissues were stored in RNAlater for subsequent processing and analysis of mRNA expression by real-time RT-PCR assay, or were frozen in liquid nitrogen for protein analyses and/or quantification of tissue MCMV by plaque assay.

Subretinal MCMV Injections. Eyes were dilated with atropine and tropicamide ophthalmic drops, and mice were deeply anesthetized by intramuscular injection of 0.1 mL xylazine (1.72 mg/mL) and 0.1 mL acepromazine (0.28 mg/mL), followed by intraperitoneal injection of 0.1–0.2 mL ketamine (8.58 mg/mL). Approximately 10^4 PFU of MCMV contained within 2 μ L were injected subretinally into the left eyes of all mice, and the right (contralateral) eyes were subretinally injected with the same volume of DMEM. Mice were anesthetized under an atmosphere of isoflurane before euthanasia at 3, 6, or 10 days following subretinal injection. Because subretinally-injected MCMV does not travel to the contralateral eye within this time [8, 364], the right eye of each mouse served as its own control (within-subject experimental design).

Systemic GCV Treatment. For some studies, systemic administration of the antiviral drug GCV was used to test the necessity of intraocular MCMV replication on SOCS1 and/or SOCS3 production. The 50% effective dose (ED_{50}) of GCV is much higher for MCMV than for HCMV, particularly *in vivo* when mice are immune suppressed [365, 366]. Mice were injected with ~40 mg/kg once daily beginning at day -1 relative to subretinal injection at day 0. This dose was chosen because it was used in previous studies and inhibited or reduced MCMV-related pathogenesis [192, 196]. Others have demonstrated that a daily subcutaneous dose of 80 mg/kg of GCV inhibits MCMV replication by 100-fold in the ocular compartment of immune compromised mice [366], and that systemic GCV does not begin to show toxicity until dosages above 75 mg/kg [367].

2.4 Histopathology

For scoring frequency and severity of retinitis, mice ($n = 5$) were euthanized 10 days following subretinal injection with MCMV (left eyes) or maintenance media (right eyes), and whole eyes were harvested and stored in 10% buffered formalin solution at 4°C. Eyes were embedded in paraffin, cut into 5- μ m-thick transverse sections, and stained for hematoxylin and eosin by the Pathology Department of the Emory Eye Center. Every sixth section of each eye was evaluated for frequency (presence or absence) of retinitis and scored for severity of retinitis as previously described [8] (**Table 2.1**).

Table 2.1: Histologic Grading Criteria for MCMV Retinitis, Posterior Segment.

0 =	Normal or injection artifact	
½ =	Mild atypical retinopathy	Absence of cytomegaly plus retinal folds and/or vascular cuffing involving less than ¾ of the section.
1 =	Moderate atypical retinopathy	Mild atypical retinopathy involving more than ¾ of the section; <i>or</i> photoreceptor degeneration and mild retinal infiltration involving more than ¼ of the section without necrosis or cytomegaly.
2 =	Mild necrotizing retinitis	Focal cytomegaly within retina associated with partial-thickness necrosis extending beyond ⅛ of distance to the injection site; <i>or</i> full-thickness retinal necrosis with cytomegaly extending beyond ⅛ section from injection site but less than ¼ section; <i>or</i> optic nerve inflammation plus cytomegaly with peripapillary retinal involvement; <i>or</i> massive infection and cytomegaly of RPE involving greater than ¾ section with partial-thickness necrosis of overlying retina.
3 =	Moderate necrotizing retinitis	Cytomegaly plus full-thickness retinal necrosis involving ¼ to ¾ of the section.
4 =	Severe necrotizing retinitis	Cytomegaly with full-thickness necrosis involving the entire retina in that section.

Modified from [8, 136]

The scoring guide describes a 0–4 scale on which scores of 1.0 or less indicate the absence of frank retinitis, and scores above 1.0 represent increasingly severe cytomegalic retinitis. This technique is frequently and successfully used to score MCMV retinitis in MAIDS mice [246, 247, 261, 265, 361, 368].

2.5 Quantification of Infectious MCMV

Whole eyes and/or whole spleens were harvested and stored in liquid nitrogen. Frozen tissues were homogenized on ice in a 2-mL Tenbroeck tissue grinder (Wheaton, Millville, NJ) in a 1-mL total volume of phosphate buffered saline (PBS) per organ. Homogenates were clarified by centrifugation and immediately titered in MEF cells by plaque assay.

Plaque Assay. MEF cells seeded in 6-well plates were used at ~90% confluency, when media was removed and replaced with 0.9 mL fresh media per well. Serial log₁₀ dilutions of MCMV stock aliquots or clarified tissue homogenates were plated in 0.1-mL volumes per well in duplicate wells and placed at 37°C for 1 hr without centrifugal enhancement. Wells were then overlaid with 1–2% methylcellulose in complete media and incubated for an additional 5 to 6 days. Plaques were counted under an inverted microscope, and quantification of PFU per mL was calculated as: [n PFU/(0.1 mL × *dilution factor*)], where n = the average number of plaques found in duplicate wells wherein 0.1 mL of the *dilution factor* yielded between 10 and 99 plaques per well. The detection limit for this assay was therefore calculated to be: [10 PFU/(0.1 mL × 10⁰) × 1 mL/eye], or 100 PFU/eye.

2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

Tissues frozen and stored in liquid nitrogen were homogenized on ice in 1 mL total volume per organ of PBS containing protease inhibitors (Roche, Indianapolis, IN). Homogenates were frozen overnight, sonicated, and clarified by centrifugation. SOCS1 and SOCS3 proteins from clarified homogenates were quantified in duplicate per eye using commercially-available sandwich ELISA kits (Antibodies Online, Atlanta, GA) according to the manufacturer's instructions. SOCS1 or SOCS3 concentrations (ng/mL or pg/mL) were derived from polynomial standard curves per the manufacturer's instructions. Total protein per eye (mg/mL) was determined using Bio-Rad protein dye reagents and protocol based on the Bradford assay [369] against a standard curve derived from known concentrations of bovine serum albumin (Bio-Rad, Hercules, CA, USA). The SOCS1 and SOCS3 protein amounts (ng/mL or pg/mL) of each eye were divided by total protein amounts per eye (mg/mL) for normalized values of SOCS1 or SOCS3 protein per total protein (ng/mg or pg/mg) in each eye.

2.7 Immunofluorescent (IF) Staining

IC-21 mouse macrophages were grown on German glass cover slips (Electron Microscopy Sciences, Hatfield, PA) in 24-well dishes or in NuncTM Lab-Tek II Chamber Slide Systems (ThermoFisher Scientific, Waltham, MA, USA) and infected with MCMV (MOI = 3 PFU/cell), UVi-MCMV from the same stock, or control media. In a separate experiment, IF staining was performed on IC-21 monolayers infected with EGFP-expressing MCMV RM4503. SG-MCMV Smith strain served as a positive control for MCMV-stimulated SOCS1 and SOCS3 expression, with media treatment negative control.

At 3 hpi, cover slides or chamber wells from each group were fixed in ice-cold methanol, blocked in 5% bovine serum albumin, and probed for rabbit-anti-mouse SOCS1 or SOCS3 primary antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX). For experiments comparing SG-MCMV infection with UVi-MCMV treatment, primary SOCS1 or SOCS3 antibodies were detected with goat-anti-rabbit IgG Fab' fragment antibody conjugated with FITC (green) (Jackson ImmunoResearch, West Grove, PA). For experiments with EGFP-expressing MCMV RM4503 (green), SOCS1 or SOCS3 primary antibodies were detected using goat-anti-rabbit IgG Fab' fragment antibody conjugated with Cy3 (red) (Jackson ImmunoResearch). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) in Vectashield mounting solution (Vector Laboratories, Burlingame, CA), and cover slides fixed to microscope slides were observed under a Nikon Eclipse fluorescent microscope.

2.8 Western Blot Assays

To assess whether STAT proteins are tyrosine-phosphorylated during MCMV infection of IC-21 cells, western blot assays were performed on MCMV-infected or media-treated IC-21 macrophages at 2 and 4 hpi. Because growth factor components of FBS may be capable of

inducing cellular signaling pathways including JAK/STAT, two media controls were used: serum-starved cells without (-) FBS, or cells treated with (+) FBS (10%). For this experiment, MCMV-infected wells were serum-starved and infected in media without (-) FBS. Intracellular protein was extracted from 6-well plates of IC-21 cells by lysis in radioimmunoprecipitation assay (RIPA) buffer [10 mM Tris-Cl at pH 8.0, 140 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium deoxycholate, 1% Triton X-100] containing protease inhibitor (Roche, Indianapolis, IN). Samples were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a 0.2- μ m-pore polyvinylidene fluoride membrane, blocked in 5% bovine serum albumin, and probed with rabbit-anti-mouse primary antibodies: glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:1000, Sigma-Aldrich, St. Louis, MO), p-Tyr701-STAT1 (1:1000, Cell Signaling Technologies, Danvers, MA), p-Tyr690-STAT2 (1:1000, EMD Millipore, Billerica, MA), p-Tyr705-STAT3 (1:1000, Cell Signaling). Goat-anti-rabbit IgG (1:2000, ThermoFisher) conjugated with horseradish peroxidase was used as a secondary antibody. SuperSignal™ West Pico Chemiluminescent Substrate (ThermoFisher) was used for detection of bands exposed to HyBlot film (Denville, Holliston, MA).

2.9 RNA Extraction and Real-Time RT-PCR

RNA extraction from tissue samples. At indicated time points following intraperitoneal or subretinal injection of MCMV or DMEM (media, controls) into adult C57BL/6 mice, mice were euthanized and whole spleens and/or whole eyes were harvested and stored 4°C in RNAlater reagent (Ambion/ThermoFisher). Individual eyes or spleens were homogenized in a 2-mL Tenbroeck tissue grinder (Wheaton, Millville, NJ) in TRIzol® reagent (Ambion/ThermoFisher), and total RNA was isolated and purified over PureLink® RNA Mini

Kit spin cartridge filters per the manufacturer (Ambion/ThermoFisher). RNA was stored frozen at -80°C until use in downstream applications.

RNA extraction from cell monolayers. Cell monolayers experimentally treated as specified were harvested at indicated time points in TRIzol® reagent (Ambion/ThermoFisher). Total RNA was isolated by chloroform extraction, purified over PureLink® RNA Mini Kit spin cartridge filters according to the manufacturer's instructions (Ambion/ThermoFisher), and stored frozen at -80°C until use in downstream applications.

Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). RNA was reverse-transcribed (RT) into cDNA with SuperScript™ III First-Strand Synthesis Kit reagents using random hexamers according to the manufacturer (Invitrogen/ThermoFisher). Real-time RT-PCR was achieved with Applied Biosystems 7500 Fast Real-Time PCR System hardware and software using Power SYBR Green Master mix (Applied Biosystems, Foster City, CA) and QuantiTect Primer Assays (**Table 2.2**) for mouse-specific SOCS1, SOCS3, SOCS5, IFN- α_2 , IFN- β , IFN- γ , IL-6, and GAPDH obtained from QIAGEN (Valencia, CA). MCMV IE1 and MCMV gH primers (**Table 2.2**) were obtained from Integrated DNA Technologies (IDT, Redwood City, CA). Thermocycling parameters for all primer sets were as follows: 10 min at 95°C , followed by 40 cycles consisting of 15 s at 94°C , 31 s at 55°C , and 35 s at 70°C . Cycles to threshold (C_T) for each target gene were determined, and each sample was normalized to its own endogenous housekeeping gene (GAPDH) by ΔC_T analysis ($\Delta C_T = C_{T \text{ target gene}} - C_{T \text{ GAPDH}}$).

Table 2.2: Real-Time RT-PCR Primer Sequences or Designations.

QIAGEN QuantiTect Primer Assays ^a		
Target Gene	QIAGEN Catalogue No.	QIAGEN Product Designation
SOCS1	QT01059268	Mm_Socs1_1_SG
SOCS3	QT00100331	Mm_Socs3_1_SG
SOCS5	QT00132083	Mm_Socs5_1_SG
IFN- α_2	QT00253092	Mm_Ifna2_1_SG
IFN- β	QT00249662	Mm_Ifnb1_1_SG
IFN- γ	QT01038821	Mm_Ifng_1_SG
IL-6	QT00098875	Mm_Il6_1_SG
GAPDH	QT01658692	Mm_Gapdh_3_SG

Sequences of Integrated DNA Technology-Synthesized Primers		
Target Gene	Sense Primer Sequence	Antisense Primer Sequence
MCMV IE1	5'- TCA GCC ATC AAC TCT GCT ACC AAC -3'	5'- ATC TGA AAC AGC CGT ATA TCA TCT TG -3'
MCMV gH	5'- GAC ACG GTC GAG TTC TTC TT-3'	5'- AGC AGC ACG AAA TGC CGT CT-3'

^a: Specific primer sequences of QuantiTect Primer Assays are proprietary and not provided by the manufacturer.

For experiments with systemic MCMV, ΔC_T values of target gene mRNA expression from MCMV-injected groups were compared back to values of media-injected groups per day by the $2^{-\Delta\Delta C_T}$ method, yielding a relative fold change in mRNA expression of MCMV over media for each day. For subretinal experiments, ΔC_T values of target gene mRNA expression from each MCMV-injected eye was compared back to its own contralateral media-injected eye by the $2^{-\Delta\Delta C_T}$ method, yielding a relative fold change in mRNA expression for each eye, unless otherwise stated. Data points represent mean fold changes \pm standard deviations (SD) of duplicate independent experiments, each consisting of at least 3 mice per group. For *in vitro* experiments, all time-course studies were analyzed by comparing each sample back to the media control group at 0 hour postinfection (hpi). Unless otherwise specified, data points represent mean fold changes \pm SD of at least duplicate experimental repeats.

2.10 Statistical Analyses

All statistical analyses were performed with a significance level (α) set to 0.05. P-values of < 0.05 were considered statistically significant, and were denoted in figures where appropriate by asterisks as: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Statistical tests were performed as appropriate for each study, as specified below.

Between-subjects *in vivo* studies of immunologically normal mice, MAIDS-4 mice, or MAIDS-10 mice. Tissues from MCMV-infected mice were compared with tissues from media-injected control mice of similar immune status (immunologically normal, MAIDS-4, or MAIDS-10) at the same time points by unpaired, two-sided Student's *t* test.

Within-subjects *in vivo* studies of experimental MCMV retinitis during MAIDS progression or during corticosteroid-induced immunosuppression. MCMV-infected eyes were compared with their respective media-injected controls at the same time points by paired, two-sided Student's *t* test or Wilcoxon signed-rank test.

Between-subjects *in vivo* studies during antiviral inhibition of MCMV replication. MCMV-infected eyes were compared with their respective contralateral media-injected eyes at the same time points by the $2^{-\Delta\Delta C_t}$ method, yielding a relative fold change for each mouse. These values from groups of mice receiving intraperitoneal injections of GCV were compared with the values of vehicle control mice at the same time points by unpaired, two-sided Student's *t* test.

***In vitro* studies comparing two or more groups to a control.** Statistical analyses of *in vitro* experiments were performed using GraphPad Prism[®] v6.07 software. Experimental groups were compared with respective control groups at the same time points by two-way analysis of variance (ANOVA) with Tukey's post-hoc analysis (time course experiments) or by one-way ANOVA with Dunnett's multiple comparisons test (GCV experiments).

3 SPECIFIC AIM 1

SOCS1 AND SOCS3 DURING MCMV INFECTION *IN VIVO*

Specific Aim 1: Test the hypothesis that SOCS1 and/or SOCS3 stimulation *in vivo* is affected by virologic, immunologic, and/or pathologic events during MCMV infection.

SOCS1 and SOCS3 mRNA and protein are significantly up-regulated during experimental MAIDS-related MCMV retinitis. Because the *in vivo* parameters under which MCMV may cause stimulation of SOCS1 and SOCS3 remain unclear, we determined whether stimulation of these proteins is correlated with cytokine inducers and/or pathologies under the following conditions: (a) in the spleens and/or eyes of mice with systemic MCMV infection in the absence of retinal disease, with or without MAIDS, (b) in the MCMV-infected eyes of mice during the progression of late-stage MAIDS, (c) in experimental MCMV retinitis during corticosteroid-induced immune suppression, and (d) intraocularly following antiviral inhibition of MCMV replication during MAIDS-related experimental MCMV.

3.1 SOCS1 and SOCS3 Expression during Systemic MCMV Infection

3.1.1 Systemic MCMV in Immunologically Normal Mice without MAIDS

Splenic SOCS1 and SOCS3 are moderately stimulated during acute, systemic MCMV infection in immunologically normal C57BL/6 mice without MAIDS. SOCS1 and SOCS3 mRNA transcripts are stimulated by MCMV infection of macrophages *in vitro* [336] and in whole eyes subretinally injected with MCMV during MAIDS-related experimental MCMV retinitis [350], but it is unclear whether systemic MCMV infection *in vivo* stimulates SOCS1 and/or SOCS3 expression. Intraperitoneally-injected MCMV at sub-lethal doses causes a self-

limited systemic infection which disseminates through the blood to replicate in various organs, including the spleen [245, 370, 371]. SOCS1 and SOCS3 are most abundantly associated with cells of the innate and adaptive immune systems (reviewed in [12, 272]), and the spleen is a major center for these immune cells. We therefore assessed SOCS1 and SOCS3 mRNA and protein expression in whole splenic cells between days 1-10 following systemic MCMV infection compared with media-inoculated control groups per day.

In immunologically normal mice without MAIDS, systemic MCMV caused moderate, transient up-regulation of SOCS1 mRNA and protein and SOCS3 protein (**Figure 3.1**). Splenic SOCS1 and SOCS3 mRNA expression peaked on day 2, with mRNA transcripts for SOCS1 (**Figure 3.1A**), but not SOCS3 (**Figure 3.1B**), reaching statistical significance ($p < 0.05$) in MCMV-infected mice compared with media-injected controls at this time. Immunoblots of splenic SOCS1 and SOCS3 proteins (**Figure 3.1C**) demonstrated moderate up-regulation at days 2, 3, and 10 following systemic MCMV infection compared with splenic protein from media-injected control groups.

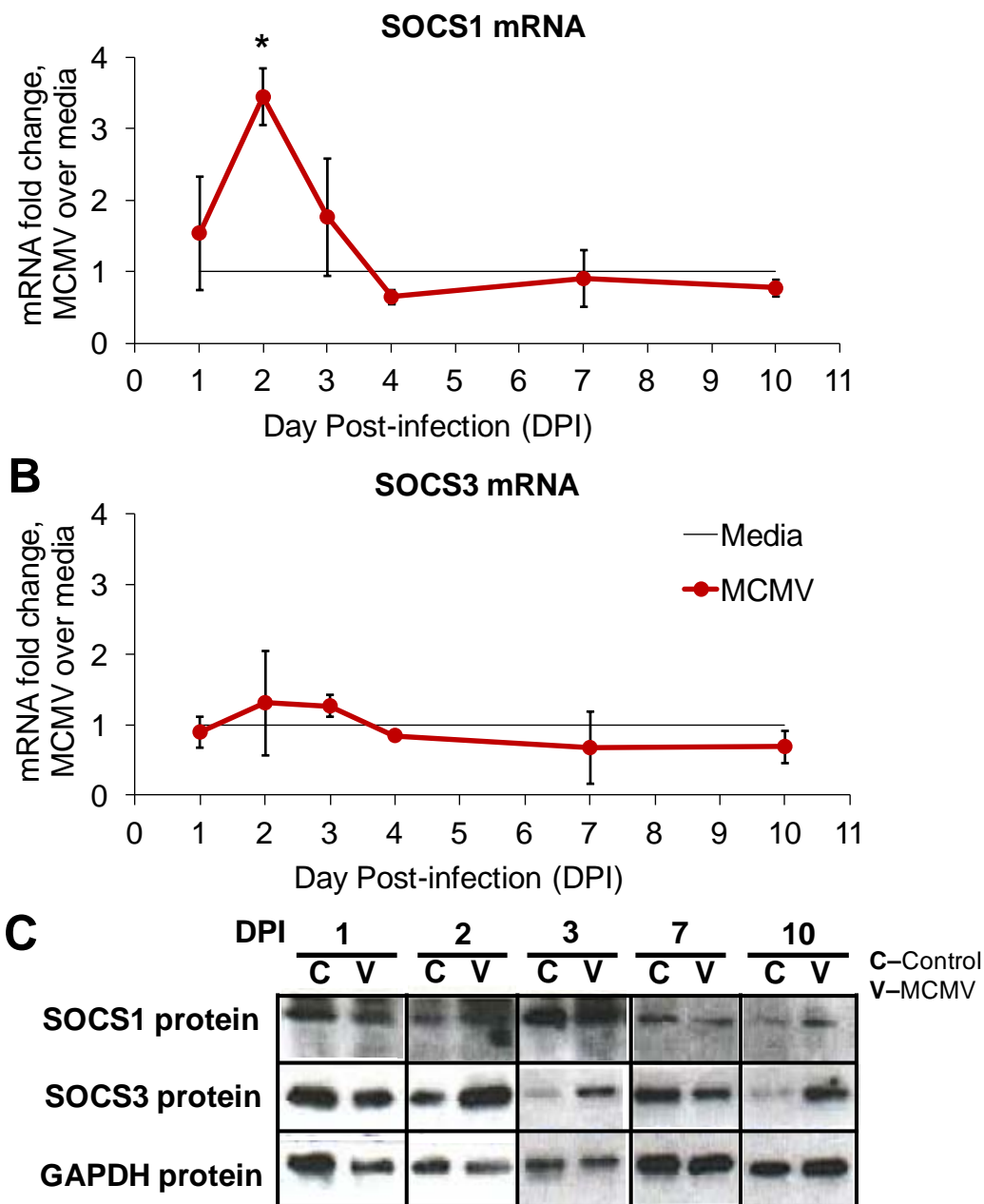


Figure 3.1. SOCS1 and SOCS3 expression in whole splenic cells is moderately up-regulated following systemic MCMV infection in immunologically normal C57BL/6 mice.

Whole spleens were collected at 1, 2, 3, 4, 7, and 10 days post-infection (dpi) from groups ($n = 3-5$) of immunologically normal C57BL/6 mice without MAIDS, injected intraperitoneally with 10^4 PFU of MCMV or with DMEM (Media). Homogenized spleens were assessed for SOCS1 (A) and SOCS3 (B) mRNA, with MCMV samples compared to their respective media controls, per day. Western blot analysis (C) was performed to assess splenic SOCS1 and SOCS3 proteins, with GAPDH used as a loading control. * $p < 0.05$, MCMV groups compared with media controls at the same time points.

Splenic cytokines known to induce SOCS1 and SOCS3 are differentially expressed during acute, systemic MCMV infection in immunologically normal C57BL/6 mice. SOCS proteins are among the dozens of gene products that are rapidly induced by signaling of cytokines such as antiviral type I IFN (IFN- α and IFN- β), type II IFN (IFN- γ), and IL-6 [272, 372] through the JAK/STAT pathway [11, 12, 268, 270], and MCMV infection causes up-regulation of many of these cytokines such as IFN- γ [247, 258] and IL-6 [373]. Because SOCS1 and SOCS3 proteins were moderately, transiently stimulated during acute, systemic MCMV infection of immunologically normal mice (**Figure 3.1**), we therefore asked whether such SOCS-inducing cytokines are concurrently stimulated with SOCS1 and SOCS3 expression during acute, systemic MCMV infection of immunologically normal C57BL/6 mice. We reasoned that if the moderate stimulation of splenic SOCS1 and SOCS3 during systemic MCMV infection can be attributed, even in part, to an indirect consequence of viral stimulation of SOCS-inducing cytokines, then transcripts for these cytokines would also be up-regulated, likely preceding stimulation of the SOCS transcripts. The same total cDNA samples used in **Figure 3.1** were therefore again probed by real-time RT-PCR assay with primers specific for antiviral type I IFN (IFN- α and IFN- β), type II IFN (IFN- γ), and IL-6.

Splenic type I IFN mRNA transcripts were not significantly stimulated at any time point evaluated during acute, systemic MCMV infection but were significantly down-regulated at day 4 post-infection (**Figure 3.2A and B**). By contrast, splenic IFN- γ (**Figure 3.2C**) and IL-6 (**Figure 3.2D**) mRNA expression levels were significantly up-regulated ($p < 0.05$) between 2 and 3 days following MCMV infection, but returned to media-injected control levels between days 4 and 10. Therefore, the putative SOCS inducers IFN- γ and IL-6 followed similar, albeit more robust, splenic mRNA expression patterns as those seen with SOCS1 mRNA in MCMV-infected

mice, but splenic IFN- α and IFN- β mRNA expression from MCMV-infected mice transiently showed significant down-regulation compared with media-injected controls.

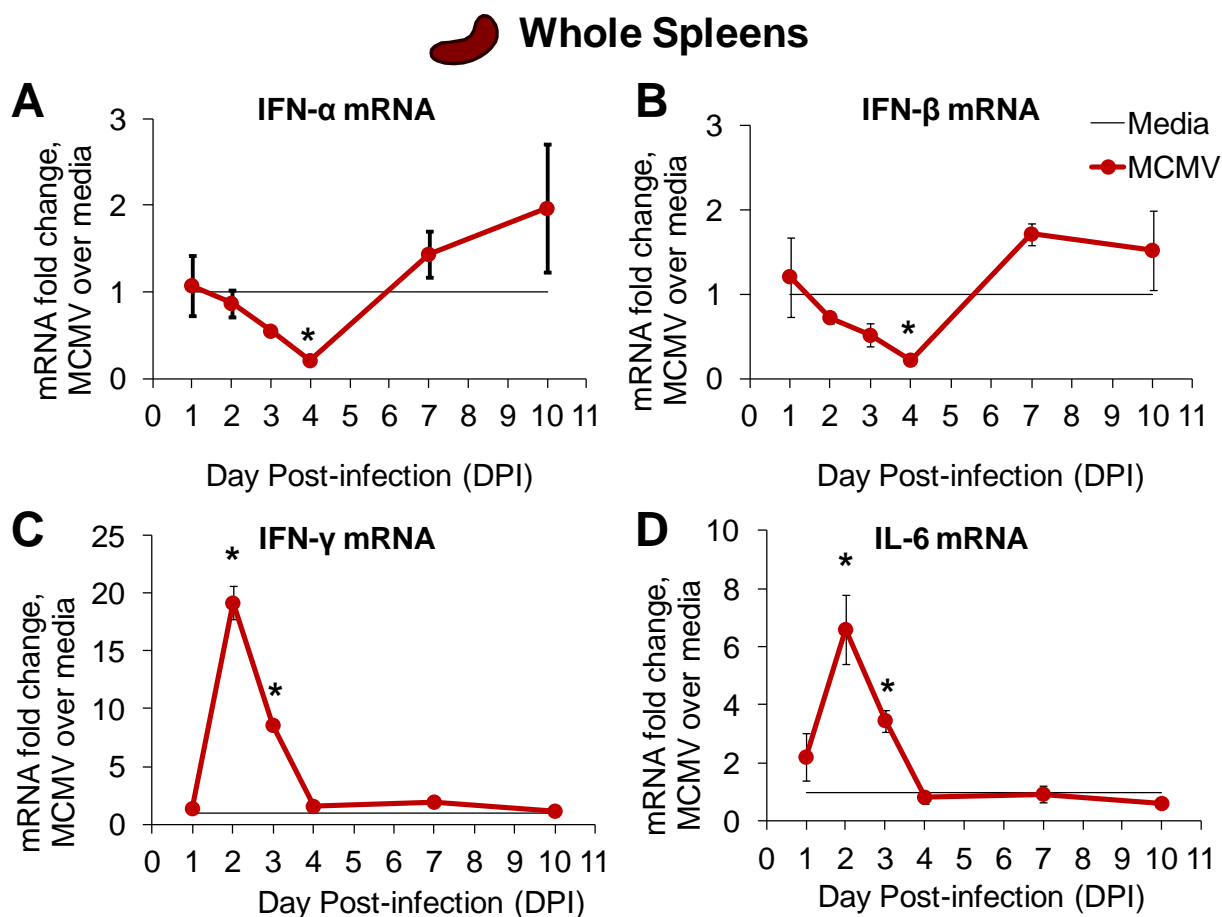


Figure 3.2. Differential mRNA expression of cytokine inducers of SOCS1 and SOCS3 in whole splenic cells following systemic MCMV infection of immunologically normal C57BL/6 mice.

Homogenized spleens from $n = 3-5$ mice per group (same samples as in **Figure 3.1**) were assessed at 1, 2, 3, 4, 7, and 10 dpi for IFN- α (**A**), IFN- β (**B**), IFN- γ (**C**), and IL-6 (**D**) mRNA transcripts, with MCMV samples compared back to their respective media controls, per day. * $p < 0.05$, MCMV groups compared with media controls at the same time points.

3.1.2 Systemic MCMV during MAIDS-4 or MAIDS-10

Splenic SOCS1 and SOCS3 mRNA transcripts are not stimulated during acute, systemic MCMV infection in MAIDS-4 or MAIDS-10 C57BL/6 mice. Because ocular SOCS1 and SOCS3 mRNA expression following subretinal MCMV infection becomes more highly up-regulated during MAIDS progression from MAIDS-4 to MAIDS-10, we next investigated whether systemic MCMV infection would induce greater amounts of splenic SOCS1 and SOCS3 mRNA during MAIDS progression from MAIDS-4 to MAIDS-10. Systemic MCMV did not alter splenic SOCS1 or SOCS3 mRNA levels over media-injected controls during MAIDS-4 (**Figure 3.3A and B**) or MAIDS-10 (**Figure 3.3C and D**).

Splenic cytokines known to induce SOCS1 and SOCS3 are not stimulated during acute, systemic MCMV infection in MAIDS-4 or MAIDS-10 C57BL/6 mice. Splenic mRNA expression of type I IFNs and IL-6 were unaffected by systemic MCMV infection of MAIDS-4 (**Figure 3.4A, B, D**) or MAIDS-10 mice (**Figure 3.4E, F, H**). IFN- γ (type II IFN) mRNA expression was modestly but significantly up-regulated during systemic MCMV infection in the spleens of MAIDS-4 mice (**Figure 3.4C**) but not MAIDS-10 mice (**Figure 3.4G**).

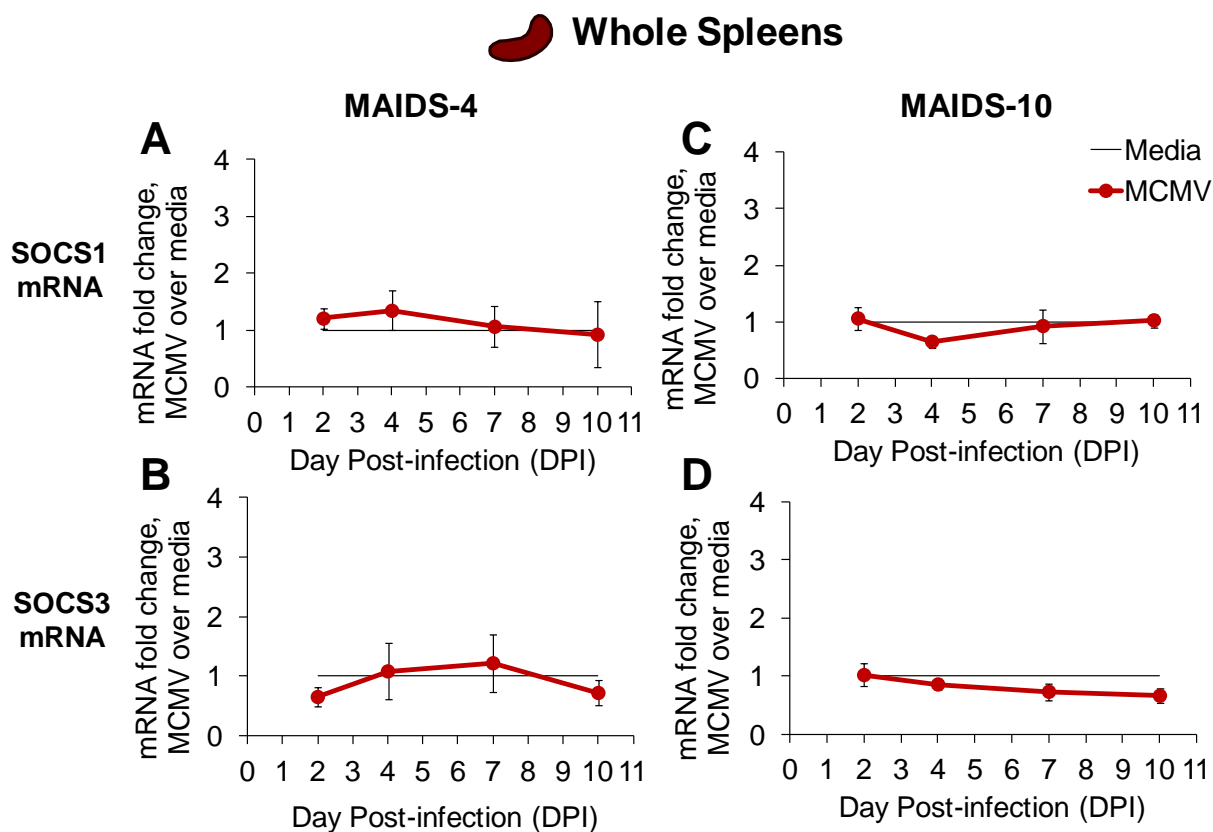


Figure 3.3. SOCS1 and SOCS3 expression in whole splenic cells is not stimulated following systemic MCMV infection in MAIDS-4 or MAIDS-10 mice.

Whole spleens were collected at 2, 4, 7, and 10 dpi from groups (n = 3–5) of C57BL/6 mice with MAIDS-4 or MAIDS-10 injected intraperitoneally with 10^4 PFU of MCMV or with media. Homogenized spleens were assessed for SOCS1 (A) and SOCS3 (B) mRNA, with MCMV samples compared back to their respective media controls, per day.

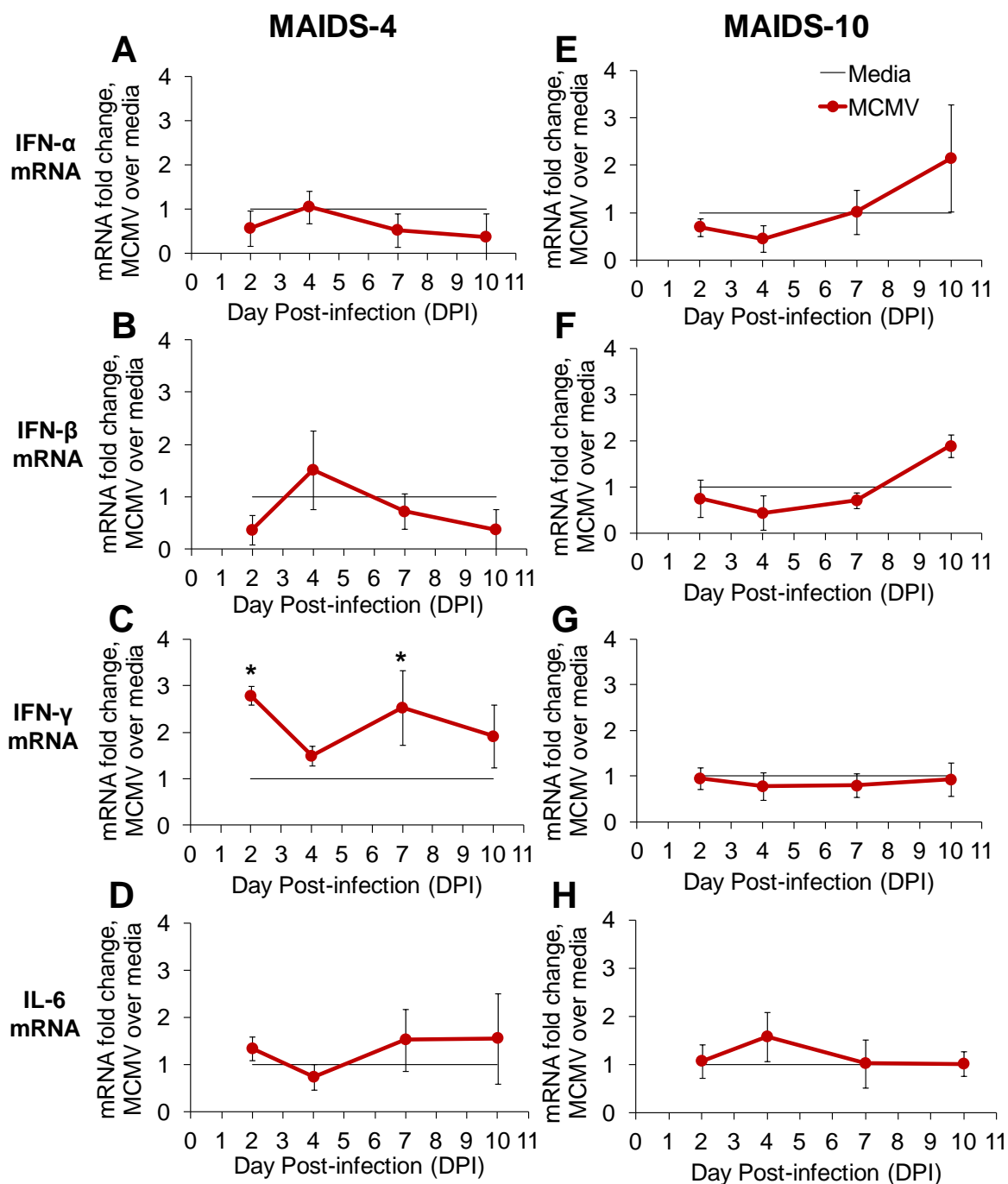
 Whole Spleens


Figure 3.4. Splenic cytokines known to induce SOCS1 and SOCS3 are not highly stimulated following systemic MCMV infection in MAIDS-4 or MAIDS-10 mice.

Homogenized MAIDS-4 spleens (A-D) and MAIDS-10 spleens (E-H) (same samples as in **Figure 3.3**) were assessed at 2, 4, 7, and 10 dpi for IFN- α (A and E), IFN- β (B and F), IFN- γ (C and G), and IL-6 (D and H) mRNA, with MCMV samples compared back to media controls, per day. * $p < 0.05$, MCMV groups compared with media controls at the same time points.

Ocular SOCS1 and SOCS3 mRNA transcripts are not stimulated during acute, systemic MCMV infection in MAIDS-4 or MAIDS-10 C57BL/6 mice. Although systemic MCMV can travel to the ocular compartment and is found in the uveal tract and RPE, systemically-inoculated MCMV fails to produce retinitis, even during immune suppression with corticosteroids [374] or with MAIDS [251]. We therefore sought to determine whether systemic MCMV infection of MAIDS mice alters ocular SOCS1 and SOCS3 in the absence of ocular pathogenesis. Neither SOCS1 nor SOCS3 mRNA was significantly altered during systemic MCMV infection of MAIDS-4 or MAIDS-10 mice (**Figure 3.5**).

Ocular cytokines known to induce SOCS1 and SOCS3 are differentially stimulated during acute, systemic MCMV infection in MAIDS-4 or MAIDS-10 C57BL/6 mice. As with whole spleens, ocular mRNA expression of type I IFNs was not stimulated by systemic MCMV infection in MAIDS mice at any times investigated (**Figure 3.6A, B, E, F**). IFN- γ mRNA was significantly up-regulated in the eyes of MAIDS-4 and MAIDS-10 mice with systemic MCMV (**Figure 3.6C and G**). IL-6 mRNA was elevated in the eyes of MAIDS-4 mice with systemic MCMV but there was no change in IL-6 expression in MAIDS-10 eyes following systemic MCMV infection (**Figure 3.6D and H**).

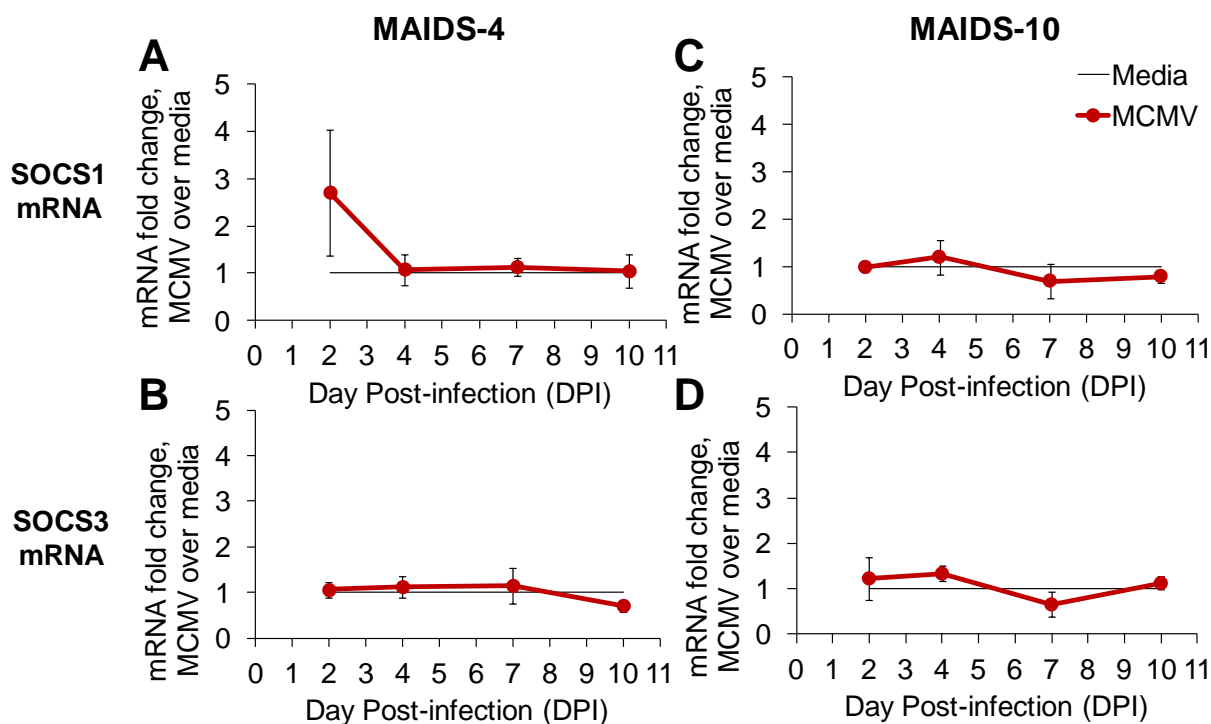
 Whole Eyes


Figure 3.5. SOCS1 and SOCS3 expression in whole eyes is not stimulated following systemic MCMV infection of MAIDS-4 or MAIDS-10 mice.

Whole eyes were collected at 2, 4, 7, and 10 dpi from groups ($n = 3-5$) of C57BL/6 mice with MAIDS-4 (**A** and **B**) or MAIDS-10 (**C** and **D**) injected intraperitoneally with 10^4 PFU of MCMV or with DMEM (Media). Homogenized eyes were assessed for SOCS1 (**A** and **C**) and SOCS3 (**B** and **D**) mRNA, with MCMV samples compared back to their respective media controls, per day.

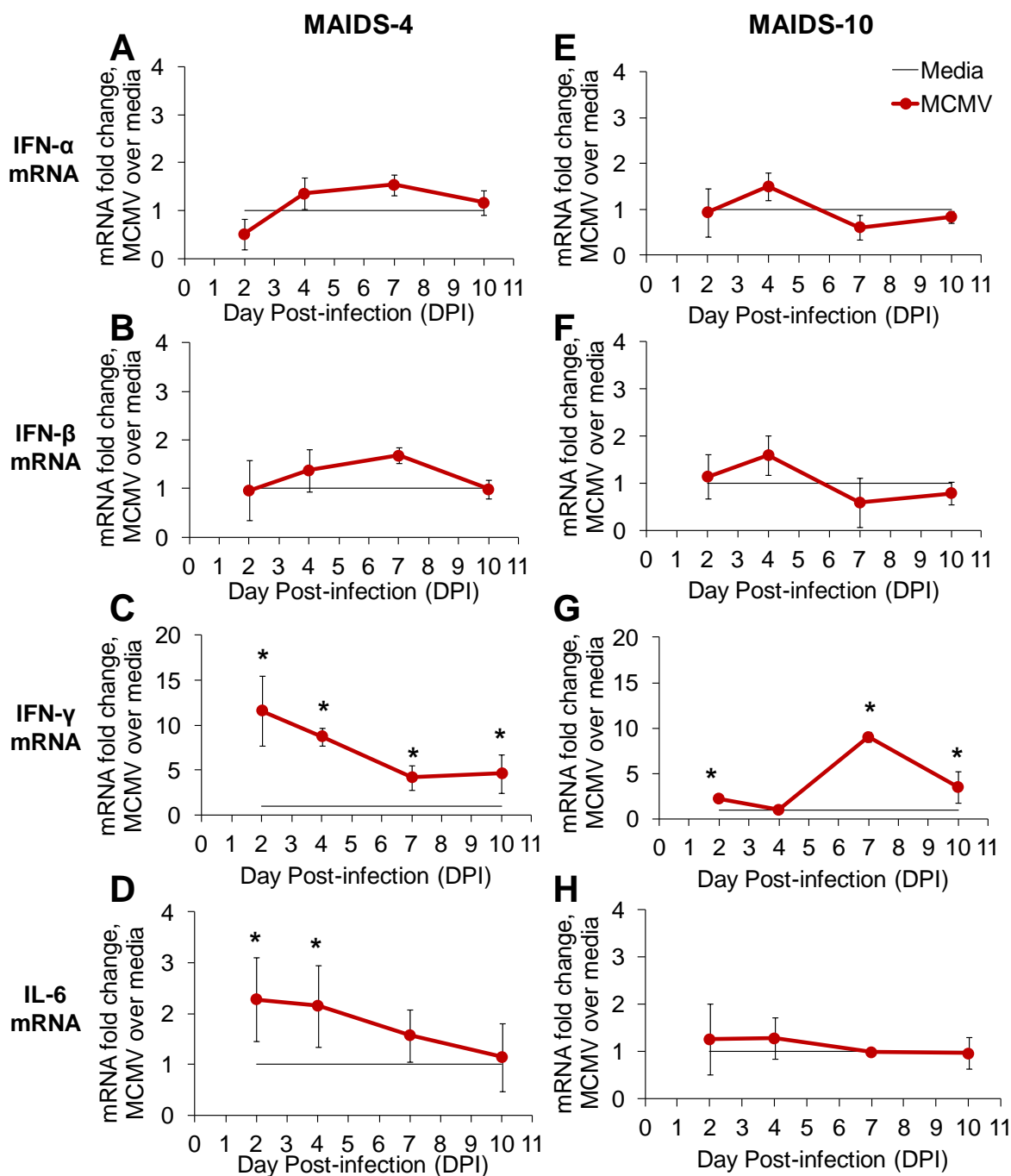
 Whole Eyes


Figure 3.6. Ocular cytokines known to induce SOCS1 and SOCS3 are differentially stimulated following systemic MCMV infection in MAIDS-4 or MAIDS-10 mice.

Homogenized MAIDS-4 eyes (A-D) and MAIDS-10 eyes (E-H) (same samples as in **Figure 3.5**) were assessed at 2, 4, 7, and 10 dpi for IFN- α (A and E), IFN- β (B and F), IFN- γ (C and G), and IL-6 (D and H) mRNA, with MCMV samples compared to media controls, per day. * $p < 0.05$, MCMV groups compared with media controls at the same time points.

3.2 Subretinal MCMV during Late-Stage MAIDS Progression

MCMV-related stimulation of ocular SOCS1 and/or SOCS3 expression progressively declines as late-stage MAIDS progresses. During different weeks of MAIDS progression, different types of immune cells become dysfunctional [235]. We previously found robust stimulation of SOCS1 and SOCS3 mRNA expression in the MCMV-infected eyes of MAIDS-10 mice, when B cells [236, 237], CD4⁺ and CD8⁺ T cells [231, 238, 239], NK cells [240], and neutrophils [241] are dysfunctional and macrophage phenotypes are irregular [236, 239, 242], but not in MAIDS-4 mice, during commencement of the T_H1 to T_H2 cytokine shift which occurs prior to complete T-cell dysfunction in these animals [9, 231, 234]. We therefore sought to quantify SOCS1 and SOCS3 mRNA expression in the MCMV-infected eyes of late-stage MAIDS mice, when NK cells [240], and neutrophils [241] become dysfunctional and proangiogenic macrophages are numerous [236, 239, 242]. During MAIDS-8, subretinal MCMV infection highly stimulated ocular SOCS1 (**Figure 3.7A**) and SOCS3 (**Figure 3.7B**) mRNA transcripts over contralateral media-injected control eyes. The amplitude of this stimulation declined as MAIDS progressed to 10 weeks (**Figure 3.7C and D**), until no significant MCMV-related stimulation of SOCS1 or SOCS3 mRNA transcripts (**Figure 3.7E and F**) or protein (**Figure 3.8**) could be found in the eyes of MAIDS-12 mice.

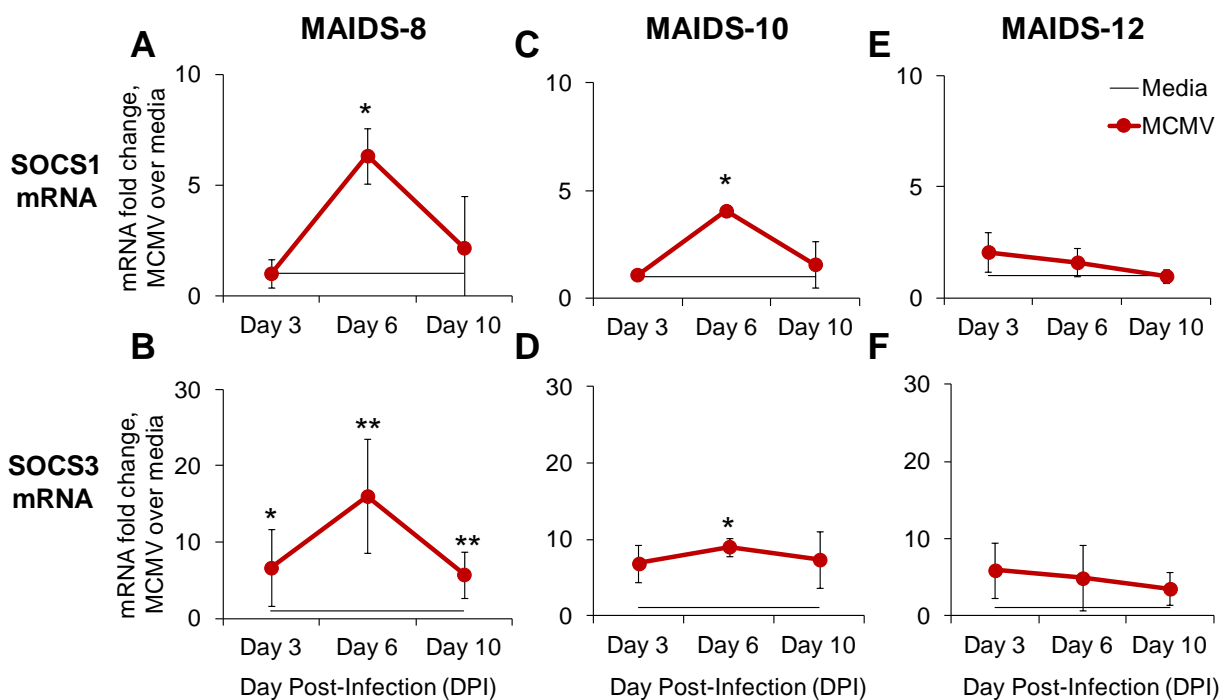
 **Whole Eyes**


Figure 3.7. MCMV-related stimulation of SOCS1 and SOCS3 mRNA expression in MCMV-infected eyes decreases during late-stage progression of MAIDS.

Mice with MAIDS-8 (A and B), MAIDS-10 (C and D), or MAIDS-12 (E and F) were subretinally injected with MCMV (left) or media (right). Whole eyes ($n = 5-8$ mice/group) were harvested at 3, 6, or 10 dpi, homogenized, and quantified for SOCS1 (A, C, and E) and SOCS3 (B, D, and F) mRNA, with each MCMV-infected eye expressed as relative fold change over its respective contralateral media control, per day. * $p < 0.05$, ** $p < 0.01$, MCMV groups compared with media controls at the same time points.

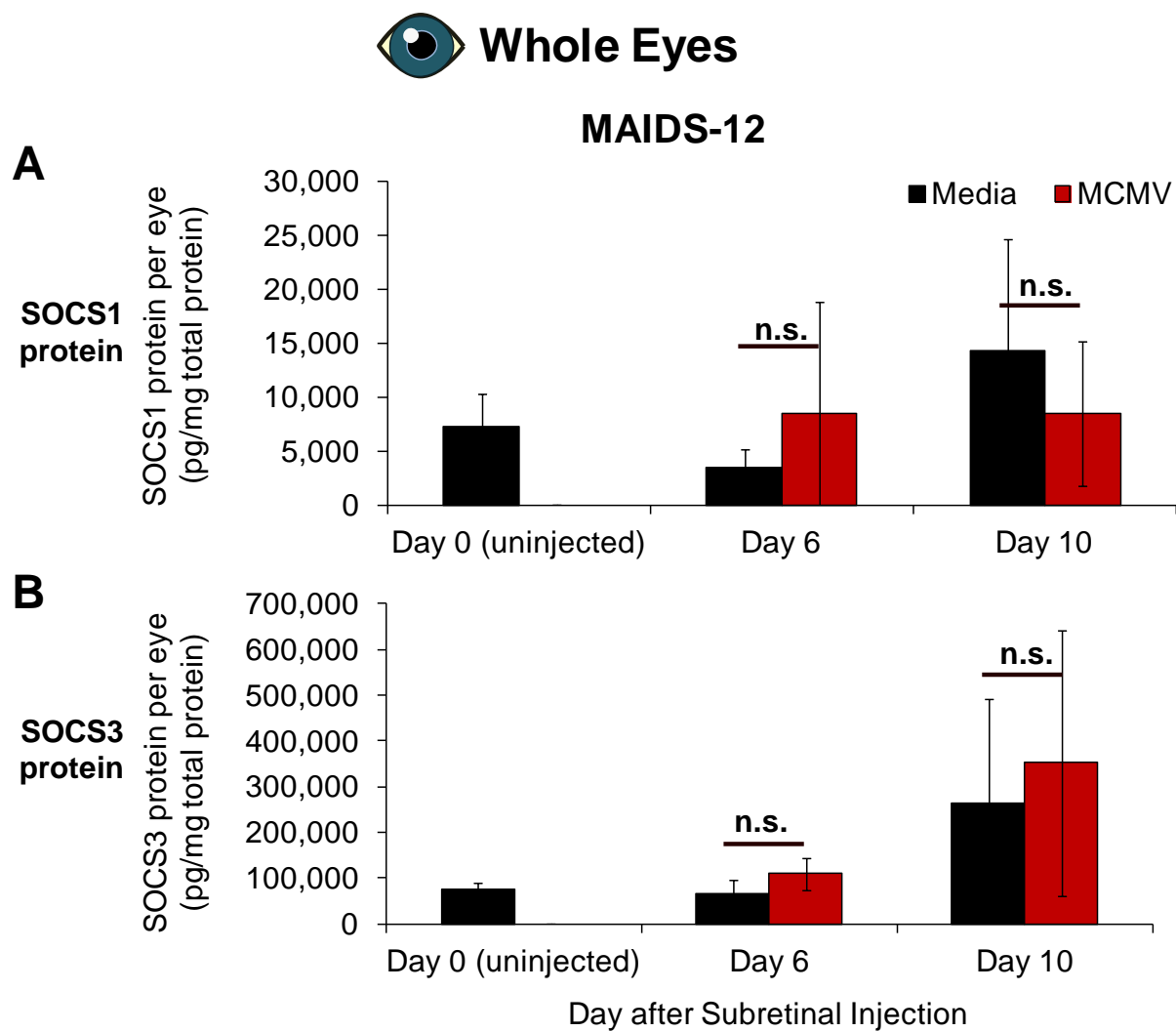


Figure 3.8. Ocular SOCS1 and SOCS3 protein are not significantly stimulated during subretinal MCMV infection of MAIDS-12 mice.

Mice with MAIDS-12 were subretinally injected with MCMV (left) or media (right). Whole eyes ($n = 6$ per group) were harvested at 6 or 10 dpi, homogenized, and quantified for SOCS1 (A) and SOCS3 (B) protein by ELISA. SOCS1 and SOCS3 protein were normalized to total protein per eye. **n.s.** = not significant, MCMV compared with media controls, per day.

Ocular SOCS3 mRNA expression in MAIDS-10 mice is stimulated following subretinal injection of MCMV or media when compared with uninjected eyes from MCMV-naïve MAIDS-10 mice. Because mRNA transcripts for MCMV-related SOCS1 and SOCS3 stimulation were assessed as a fold-change value relative to the contralateral eye per day (**Figure 3.7**), stimulation of SOCS1 or SOCS3 in the media control eyes could mask potential stimulation in the MCMV-injected eyes. To test this possibility, we reanalyzed SOCS1 and SOCS3 mRNA expression in MCMV-infected and media-injected eyes from a single experimental repeat of the MAIDS-10 mRNA data (n = 5), back to a group of their MAIDS-10 littermates whose eyes had remained uninjected and were therefore MCMV-naïve (n = 3). We found, compared with uninjected eyes of MCMV-naïve MAIDS-10 mice, that both media-injected contralateral control eyes and MCMV-infected eyes contained elevated SOCS3 mRNA levels (**Figure 3.9B**), although this phenomenon was not observed for SOCS1 (**Figure 3.9A**).

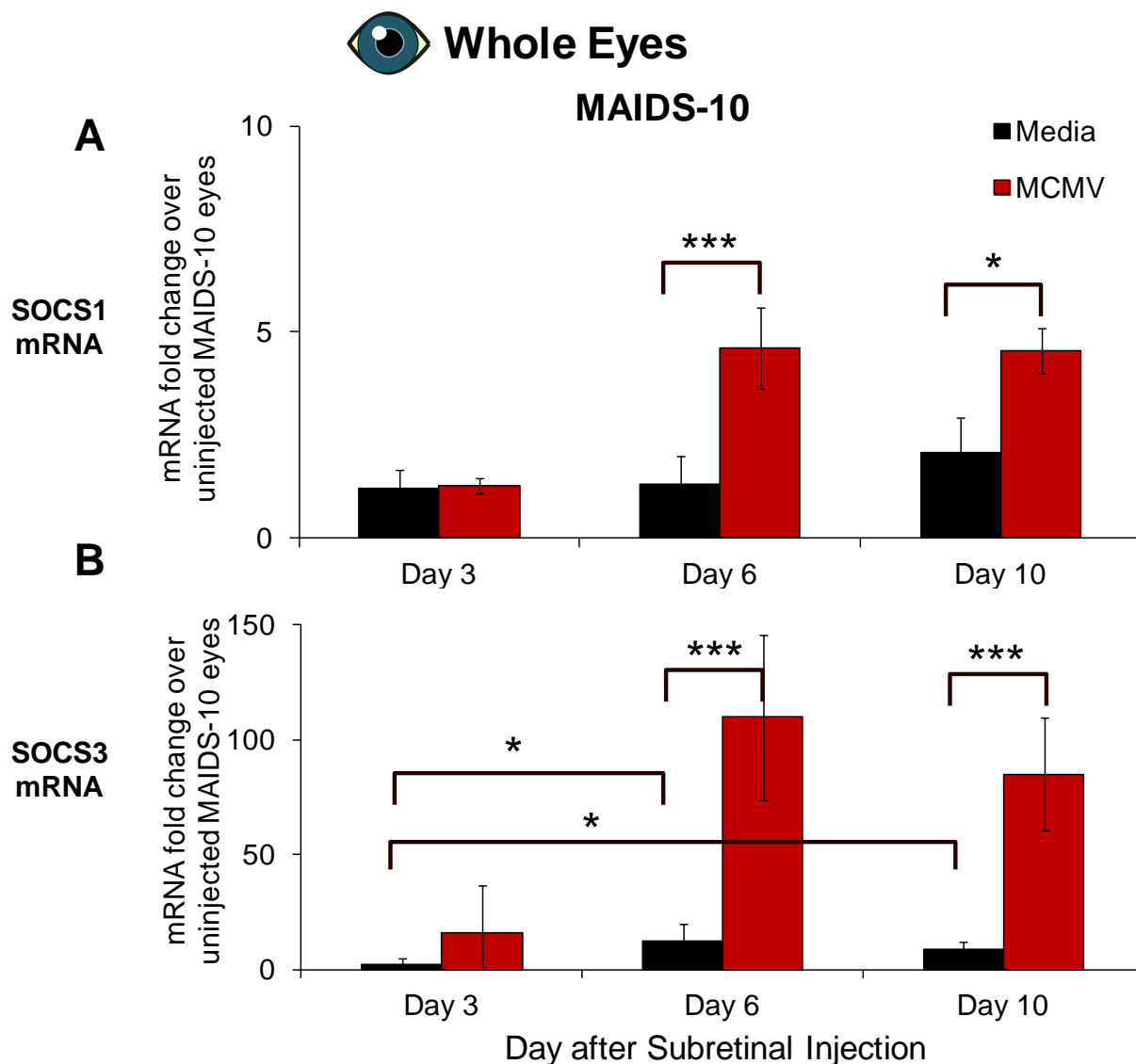


Figure 3.9. Ocular SOCS1 and SOCS3 mRNA expression in MAIDS-10 subretinally-injected eyes compared with uninjected MAIDS-10 eyes.

Fold-change analysis comparing SOCS1 (A) and SOCS3 (B) mRNA values from subretinally-injected MAIDS-10 eyes (MCMV left eyes, Media right eyes) back to the values of uninjected, MCMV-naïve MAIDS-10 eyes ($n = 3-5$ mice/group). Note differences in y-axis scales between target genes. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, groups compared as indicated with brackets.

3.3 Subretinal MCMV during Drug-Induced Immune Suppression

Ocular SOCS1 and SOCS3 mRNA are not highly stimulated in MCMV-infected eyes of C57BL/6 mice with corticosteroid-induced immunosuppression. We found significant stimulation of SOCS1 and SOCS3 mRNA in MCMV-infected eyes of C57BL/6 mice during retinitis-susceptible MAIDS-10, but not at MAIDS-4, when mice are still resistant to retinitis. We sought to investigate whether these proteins are also stimulated in the MCMV-infected eyes of C57BL/6 mice during corticosteroid (drug)-induced immunosuppression, reasoning that if these host proteins play a role in the pathogenesis of experimental MCMV retinitis, then they should be stimulated in more than one model of this disease. Whereas MAIDS mice experience a functional change in macrophage phenotype at later stages of disease [236, 239, 242], corticosteroid-induced immune suppression very quickly poisons the immune system, resulting in significant loss of immune cells, including macrophages [263]. In contrast to the robust stimulation of SOCS1 and SOCS3 mRNA ([350] and **Figure 1.11C and D**) found during experimental MCMV retinitis of MAIDS-10 mice, SOCS1 mRNA was not significantly stimulated (**Figure 3.10A**) and SOCS3 mRNA was only mildly stimulated (**Figure 3.10B**) in MCMV-infected eyes during corticosteroid-induced immunosuppression. At day 10 following subretinal injection, no statistical significance could be found between MCMV (left eyes) and media (control, right eyes) groups for SOCS1 protein (**Figure 3.10C**) or SOCS3 protein (**Figure 3.10D**) by ELISA.

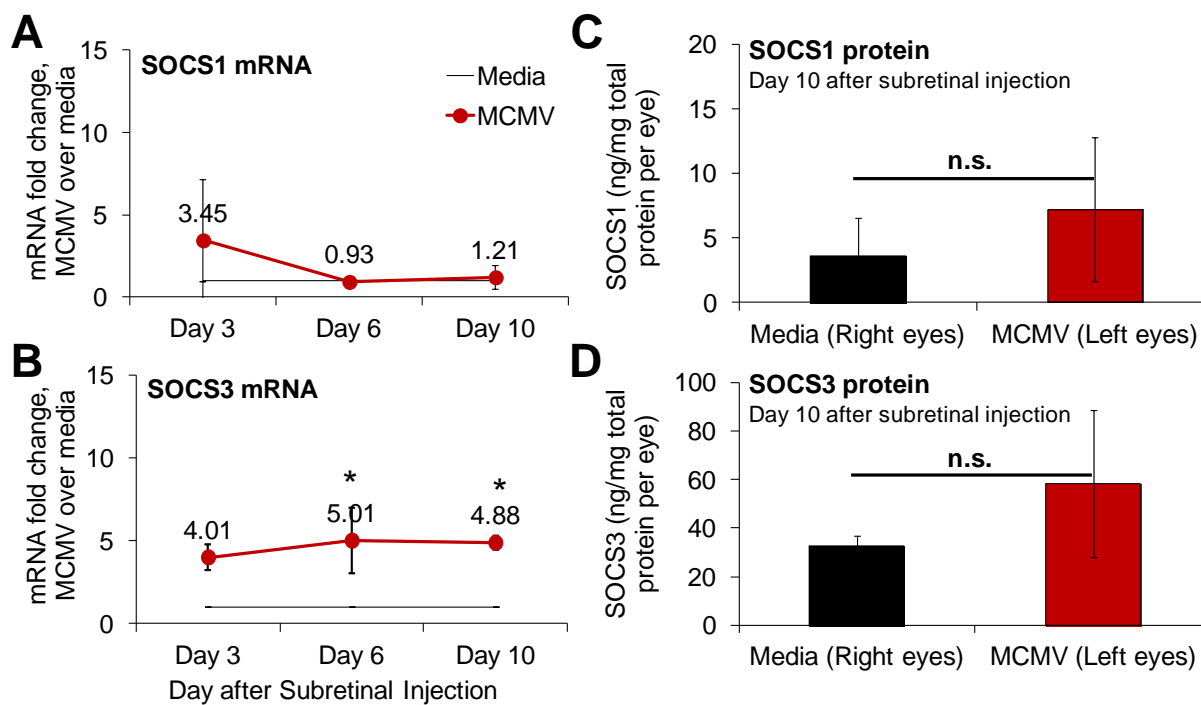


Figure 3.10. SOCS1 and SOCS3 are not highly stimulated during experimental MCMV retinitis of corticosteroid-immunosuppressed C57BL/6 mice.

Subretinally-injected whole eyes from mice with corticosteroid-induced immune suppression were assessed at 3, 6, and 10 dpi for SOCS1 (A) or SOCS3 (B) mRNA transcripts (n = 3–5 mice/group, two independent experiments), with each MCMV-infected eye compared back to its own contralateral media-injected eye. SOCS1 protein (C) and SOCS3 protein (D) were quantified at day 10 by ELISA (n = 4 mice/group). * p<0.05, **n.s.** = not significant, MCMV-infected eyes compared with media controls at the same time points.

Frequency and severity of MCMV retinitis and ocular viral titers during corticosteroid-induced immunosuppression in C57BL/6 mice are reduced compared with MAIDS. The low stimulation of MCMV-related ocular SOCS1 and SOCS3 expression during drug immunosuppression, as compared with our findings during MAIDS-related immune suppression, compelled us to seek possible explanations for this discrepancy between the two models of experimental MCMV retinitis. Because others have demonstrated that ~50% of drug-immunosuppressed C57BL/6 mice display full-thickness retinal necrosis during subretinal (supraciliary) MCMV infection [253], we sought to determine whether the failure to stimulate SOCS1 and SOCS3 is correlated with decreased frequency and severity of experimental MCMV retinitis during drug-induced immunosuppression of C57BL/6 mice compared with data previously published by us during MAIDS using a scoring guide on a 0–4 scale [8, 136] (**Table 2.1**) that has been used in previous publications to score frequency and severity of MCMV retinitis in mice with MAIDS [246, 247, 261, 265, 361, 368]. In agreement with previous findings for C57BL/6 mice [253], only 40% (2/5) of the MCMV-injected eyes of these drug-immunosuppressed mice achieved severity scores above 1.0, and the average severity of these retinitis-positive eyes was a relatively mild 2.0 (50% of maximum) (**Table 3.1**).

Table 3.1: Frequency and Severity of Retinitis and Ocular Viral Load in Two Different Models of Experimental MCMV Retinitis.

Method of Immune Suppression of C57BL/6 mice	Frequency (%) of Full-Thickness Retinal Necrosis	Retinitis Severity Score (% of max. possible score)	Ocular MCMV Titer (PFU/eye)
MAIDS-8 to MAIDS-12^a	80–100%	2–3.6 (50–90%)	$1-5 \times 10^4$
Corticosteroids^b	40%	1.4 (35%)	$\sim 5 \times 10^3$

All data are from whole eyes collected at day 10 following subretinal injection of $\sim 10^4$ PFU/eye of MCMV (Smith)

a: Data compiled from published MAIDS studies in C57BL/6 mice [8, 247, 265, 361]

b: Data from present study in C57BL/6 mice (n = 5) with corticosteroid-induced immunosuppression

During the progression of MAIDS, susceptibility to MCMV replication in the eye precedes susceptibility to retinitis [247], as retinitis-resistant mice with early-stage MAIDS and retinitis-susceptible mice with late-stage MAIDS harbor equivalent viral titers ($\sim 3 \times 10^4$ PFU/eye) [247, 265]. To determine whether the lack of SOCS1 and SOCS3 stimulation along with lower frequencies and severities of retinitis during drug-induced immunosuppression of C57BL/6 mice were correlated with a concurrent reduction in expected ocular viral titer, we quantified the amount of infectious virus in MCMV-infected eyes during drug-induced immunosuppression. Whereas retinitis-susceptible MAIDS animals consistently yield $\sim 1-5 \times 10^4$ PFU of MCMV per eye [8, 247, 265, 361] at 10 days following subretinal MCMV injection, in the current study we found an average of 5.3×10^3 PFU per eye in drug-immunosuppressed mice 10 days following the same amount of input subretinal MCMV (**Table 3.1**).

Cytokines known to induce expression of SOCS1 and SOCS3 are up-regulated in the eyes of corticosteroid-immunosuppressed mice following subretinal MCMV infection.

SOCS transcripts are classically induced by cytokine signaling [11, 12, 268, 270], and MCMV infection stimulates many cytokines such as IFN- γ [247, 258] and IL-6 [373]. We therefore asked whether such cytokines are stimulated in the MCMV-infected eyes of mice with drug-induced immunosuppression. We found that type II IFN (IFN- γ , **Figure 3.11C**) and IL-6 (**Figure 3.11D**), but not type I IFN (**Figure 3.11A and B**), are significantly stimulated in the MCMV-infected eyes of C57BL/6 mice during corticosteroid-induced immune suppression. Although IFN- γ and IL-6 mRNA values from MCMV-infected eyes reached statistical significance compared with their contralateral media-injected control eyes, we noted a surprisingly high variability between the individual eyes.

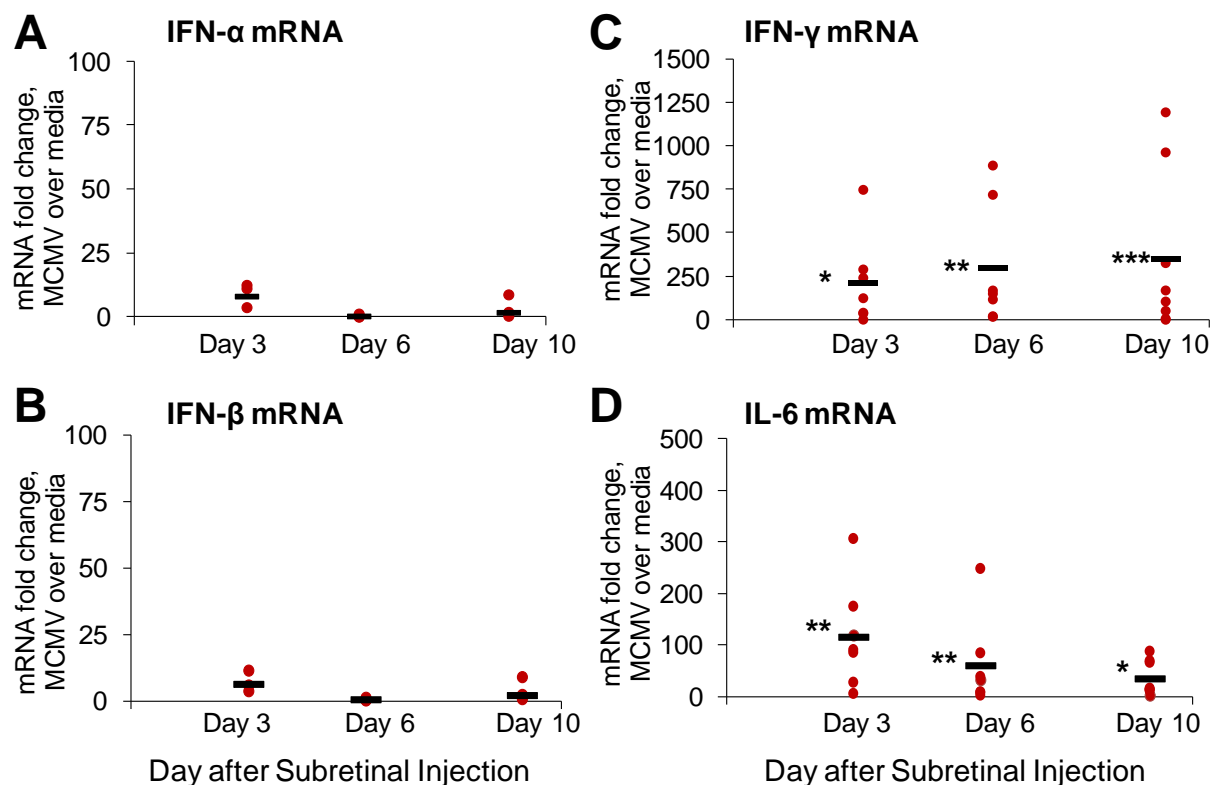


Figure 3.11. Cytokine inducers of SOCS1 and SOCS3 are stimulated during experimental MCMV retinitis in corticosteroid-immunosuppressed C57BL/6 mice.

At 3, 6, or 10 days following injection with 10^4 PFU of MCMV (left eyes) or media (right eyes), whole eyes were assessed for IFN- α (A), IFN- β (B), IFN- γ (C), or IL-6 (D) mRNA transcripts, with each MCMV-infected eye compared back to its own contralateral media-injected eye. Individual data points (circles) and arithmetic means (black lines) of $n = 8$ mice per group are shown. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, MCMV-infected eyes compared with contralateral media-injected controls.

3.4 Ganciclovir Treatment during Subretinal MCMV in MAIDS Mice

Antiviral Inhibition of MCMV Replication during MAIDS-Related Experimental MCMV Retinitis Inhibits Stimulation of Ocular SOCS1 and/or SOCS3. To test the hypothesis that MCMV replication is necessary for SOCS1 and SOCS3 mRNA stimulation during MAIDS-related MCMV retinitis, we injected MAIDS-10 or MAIDS-12 mice with the antiviral drug GCV (40 mg/kg/day) daily beginning at day -1 relative to subretinal injection at day 0. This dose was chosen because it was used in previous studies and inhibited or reduced MCMV-related pathogenesis [192, 196]. Others have demonstrated that a daily subcutaneous dose of 80 mg/kg of GCV inhibits MCMV replication by 100-fold in the ocular compartment of immune compromised mice [366], and that systemic GCV does not begin to show toxicity until dosages above 75 mg/kg [367]. GCV acts a guanosine analog [102], preferentially inhibits HCMV and MCMV DNA polymerases [103] and therefore viral replication, and allows viral IE and E gene expression without L gene expression [104]. We found sensitivity to systemic GCV treatment during MCMV stimulation of ocular SOCS1, but not SOCS3, in MAIDS-10 mice (**Figure 3.12A and B**) and during MCMV stimulation of ocular SOCS3, but not SOCS1, in MAIDS-12 mice (**Figure 3.12C and D**). Stimulation of ocular SOCS1 in MAIDS-10 mice was sensitive to GCV at day 6 following subretinal MCMV infection, but MCMV failed to stimulate ocular SOCS1 at MAIDS-12, and GCV treatment did not alter this lack of SOCS1 stimulation.

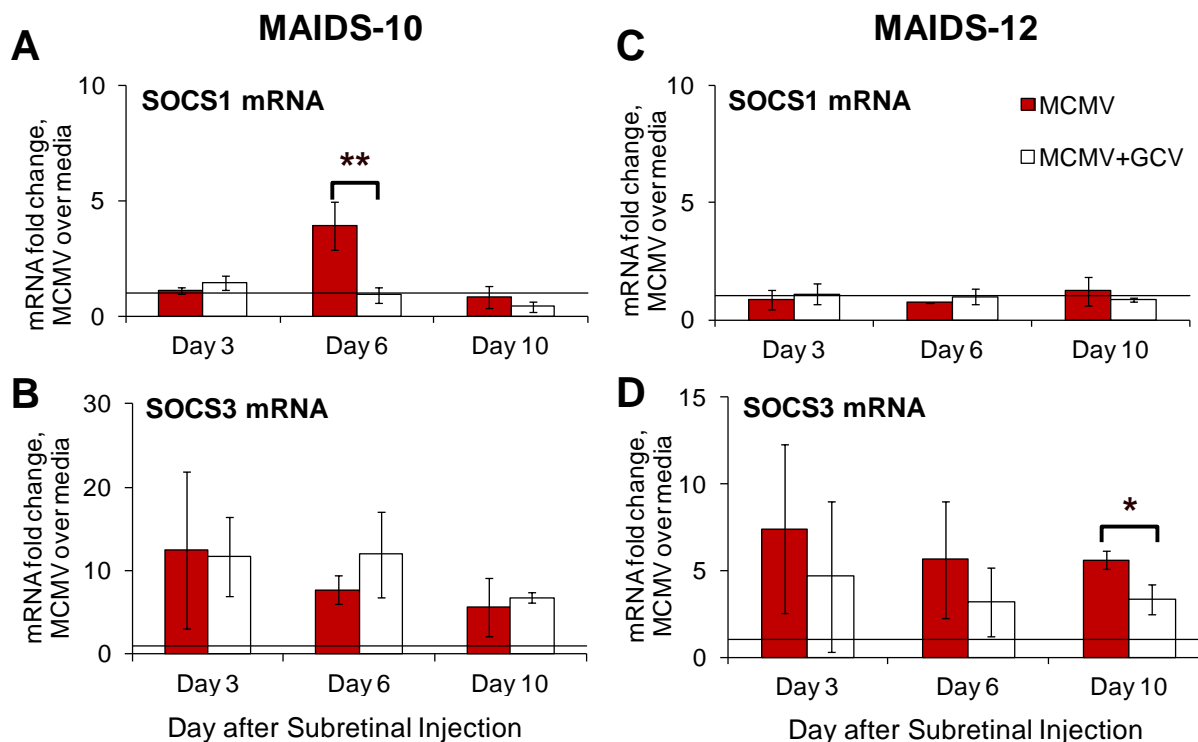


Figure 3.12. MCMV-stimulated SOCS1 or SOCS3 expression is differentially sensitive to systemic GCV treatment during MAIDS.

Groups of MAIDS-10 (A and B) or MAIDS-12 (C and D) mice (n = 5 mice/group for each day) were intraperitoneally injected with 40 mg/kg/day GCV or with an equal volume of vehicle control beginning at day -1 relative to subretinal injection at day 0 with MCMV (left eyes) or media (right eyes). Whole eyes were assessed at 3, 6, or 10 dpi for SOCS1 (A and C) and SOCS3 (B and D) mRNA, with each MCMV-infected eye expressed as relative fold change over its respective contralateral media control, per day. * p<0.05, ** p<0.01, relative fold changes of vehicle-treated groups compared with GCV-treated groups, per day.

4 SPECIFIC AIM 2

SOCS1 AND SOCS3 DURING MCMV INFECTION *IN VITRO*

Specific Aim 2: Test the hypothesis that SOCS1 and/or SOCS3 stimulation *in vitro* is affected by virologic and/or immunologic events during MCMV infection. SOCS1 and SOCS3 are significantly stimulated during experimental MAIDS-related MCMV retinitis, and F4/80⁺ macrophages are a significant cellular source of SOCS1 and SOCS3 in this model. We therefore tested whether stimulation of SOCS1 and/or SOCS3 occurs during: (a) infection of IC-21 mouse macrophages with salivary gland-derived MCMV or cell culture-derived MCMV, (b) inhibition of viral replication by UV inactivation in macrophage or fibroblast cells, (c) antiviral inhibition of MCMV replication, or (d) direct MCMV infection and/or in uninfected bystander cells.

4.1 Expression Kinetics with SG-MCMV or TC-MCMV in IC-21 Cells

SOCS1 and SOCS3 mRNA transcripts are transiently stimulated upon MCMV infection of IC-21 mouse macrophages. Because we previously found that subretinal MCMV infection stimulates SOCS1 and SOCS3 expression in F4/80⁺ macrophages within the eyes of MAIDS-10 mice (**Figure 1.12**), and macrophages are important participants during MCMV infection [115, 116, 118, 120-122], we tested whether MCMV infection stimulates SOCS1 and/or SOCS3 expression in IC-21 mouse macrophages. We found that SOCS1 (**Figure 4.1A**) and SOCS3 (**Figure 4.1B**), but not SOCS5 (**Figure 4.1C**), mRNA transcripts are up-regulated at early time points (2–6 hpi) following infection of IC-21 monolayers with salivary gland-derived MCMV (SG-MCMV). Treatment of IC-21 monolayers with BALB/c mouse salivary gland

homogenate (SG-homogenate) failed to stimulate SOCS1 or SOCS3 mRNA transcripts during the time points observed (**Figure 4.1**). Peak SOCS1 and SOCS3 mRNA stimulation occurred between 2–6 hpi, a relatively early time during productive MCMV infection that follows viral attachment, adsorption, and release of viral tegument proteins into the host cell and correlates with transcription and translation of MCMV IE genes in fibroblast cells [6, 54, 61, 96, 97].

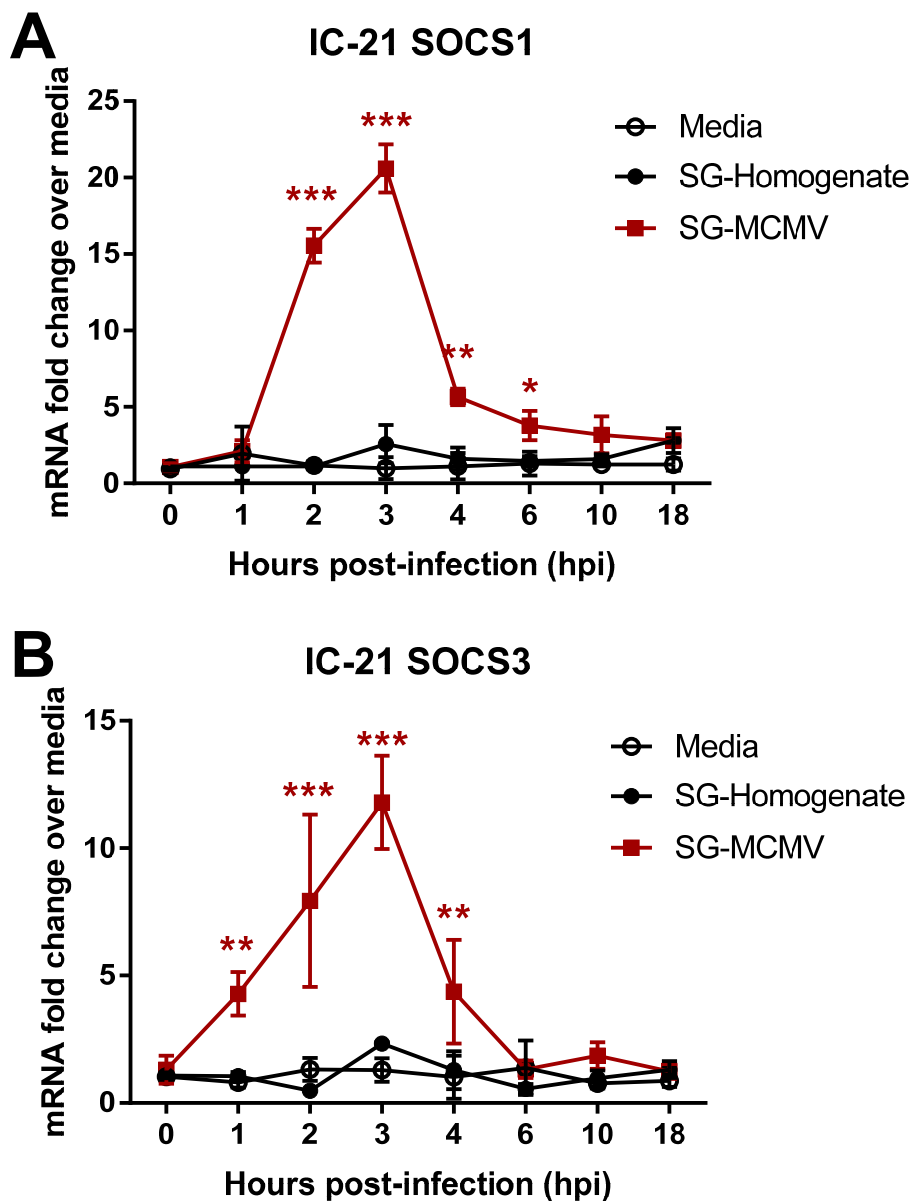


Figure 4.1. Infection of mouse macrophages with MCMV stimulates SOCS1 and SOCS3, but not SOCS5, mRNA transcripts at early time points.

IC-21 mouse macrophages were treated with media (control), uninfected salivary gland homogenate (SG-Homogenate) or infected with salivary gland-passaged MCMV (SG-MCMV), MOI = 3 PFU/cell. At 1, 2, 3, 4, 6, 10, and 18 hpi, cells were harvested and assessed for SOCS1 (A) or SOCS3 (B) mRNA transcripts, with all samples compared back to the media group at 0 hr postinfection (hpi). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ for SG-MCMV compared with media controls at the same time points. At no time points examined was there any statistically significant difference between the Media and SG-Homogenate groups.

SOCS-inducing STAT proteins are phosphorylated upon MCMV infection of IC-21 mouse macrophages. STAT proteins are transcriptional inducers of SOCS proteins, and SOCS1 and/or SOCS3 transcripts are classically induced by cytokine receptor JAK-mediated tyrosine phosphorylation of STAT1, STAT2 or STAT3 (reviewed in [11, 12, 270]). MCMV infection activates or interferes with components of this pathway in a time-dependent and cell-type-dependent manner [375-377]. Because MCMV-related stimulation of SOCS1 and SOCS3 mRNA expression could be an indirect consequence of viral stimulation of the JAK/STAT pathway, we investigated whether MCMV infection of IC-21 mouse macrophages causes activation of STAT proteins concurrently with significant stimulation of SOCS1 and SOCS3 transcripts. Tyrosine phosphorylation of STAT1, STAT2, and STAT3 occurred during early MCMV infection of IC-21 mouse macrophages at 2 and 4 hpi (**Figure 4.2**), time points corresponding with peak SOCS1 and SOCS3 transcript induction in these cells. Neither serum-starved media-treated control cells (-) nor cells treated with media containing 10% FBS (+) showed STAT phosphorylation at these early time points.

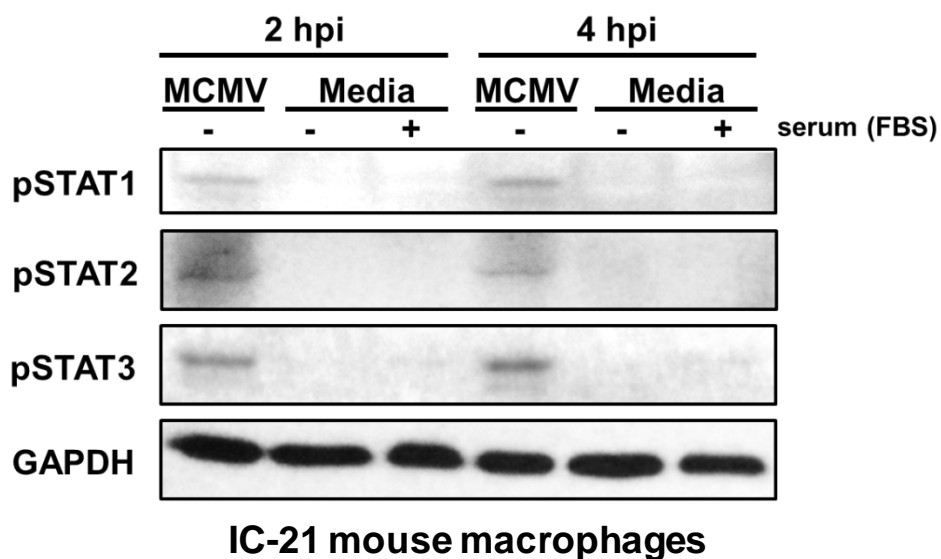


Figure 4.2. MCMV Infection of mouse macrophages causes phosphorylation of STAT proteins at early time points.

Western blots of serum-starved (-) MCMV-infected IC-21 mouse macrophages (MOI = 3) and media-treated control cells without (-) or with (+) 10% fetal bovine serum probed with antibodies against pSTAT1, pSTAT2, pSTAT3, or GAPDH proteins.

MCMV-related SOCS1 and SOCS3 stimulation in IC-21 mouse macrophages is dependent on virus passage origin (SG-MCMV vs. TC-MCMV). Because SG-MCMV and TC-MCMV display many virologic, immunologic, and pathologic differences *in vitro* and *in vivo* [378-381], particularly in relation to macrophage infection [248, 378-382], we tested whether MCMV-related stimulation of SOCS1 and/or SOCS3 is affected by viral passage origin (SG-MCMV or TC-MCMV). We found in monolayers of IC-21 mouse macrophages that SG-MCMV, but not TC-MCMV, highly stimulated SOCS1 and SOCS3 mRNA transcripts between 2-6 hpi (**Figure 4.3**).

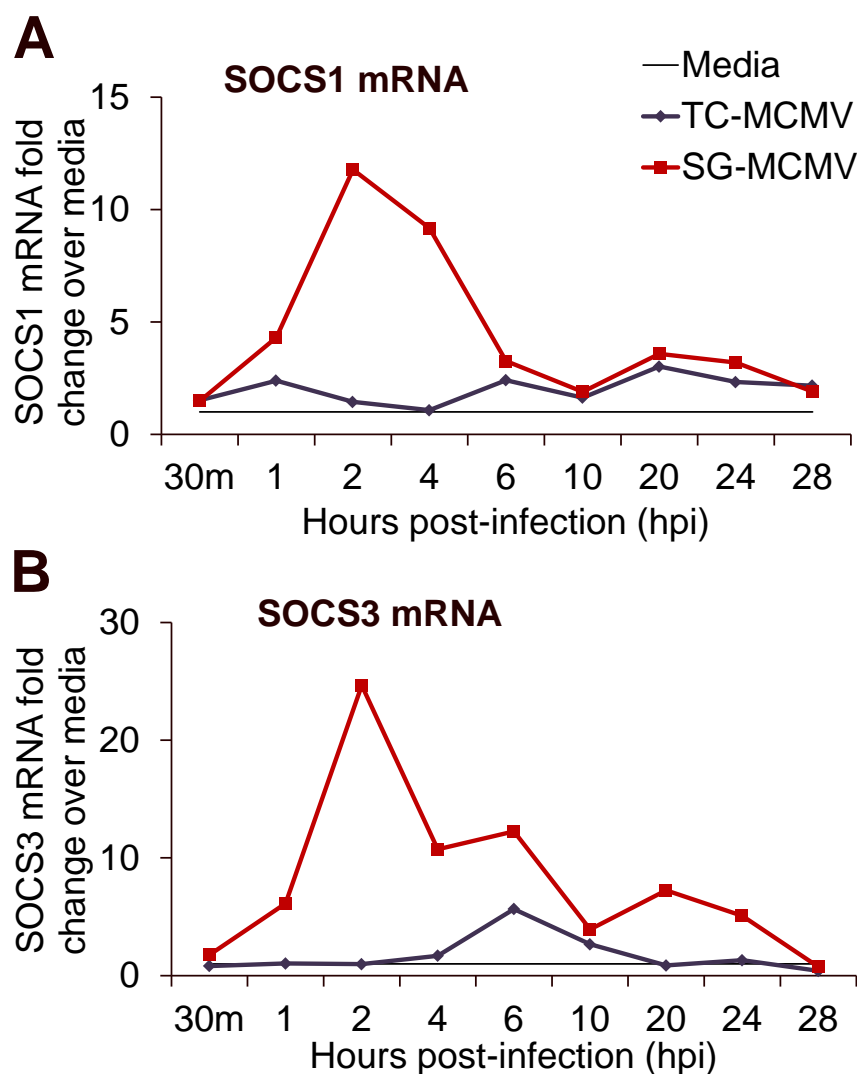


Figure 4.3. SG-MCMV, but not TC-MCMV, stimulates SOCS1 and SOCS3 mRNA transcripts in IC-21 mouse macrophages.

IC-21 mouse macrophages were treated with media (baseline controls), infected with MCMV passaged 3 consecutive times through tissue culture in MEF cells (TC-MCMV, p3), or infected with salivary gland-passaged MCMV (SG-MCMV), MOI = 3 PFU/cell. At 0.5 (30 m), 1, 2, 4, 6, 10, 20, 24, and 28 hpi, cells were harvested and assessed for SOCS1 (**A**) and SOCS3 (**B**) mRNA transcripts.

MCMV-related type II IFN stimulation in IC-21 mouse macrophages is dependent on virus passage origin (SG-MCMV vs. TC-MCMV). Because SOCS transcripts are induced by cytokine signaling through the JAK/STAT pathway [11, 12, 268, 270], and MCMV infection causes up-regulation of many cytokines such as IFN- γ [247, 258], we next asked whether stimulation of such cytokines during infection of IC-21 mouse macrophages is also dependent on MCMV passage origin. Whereas type I IFN (**Figure 4.4A, B**) was not highly stimulated by infection with MCMV from either passage origin, type II IFN was highly stimulated by SG-MCMV, but not TC-MCMV, infection (**Figure 4.4C**).

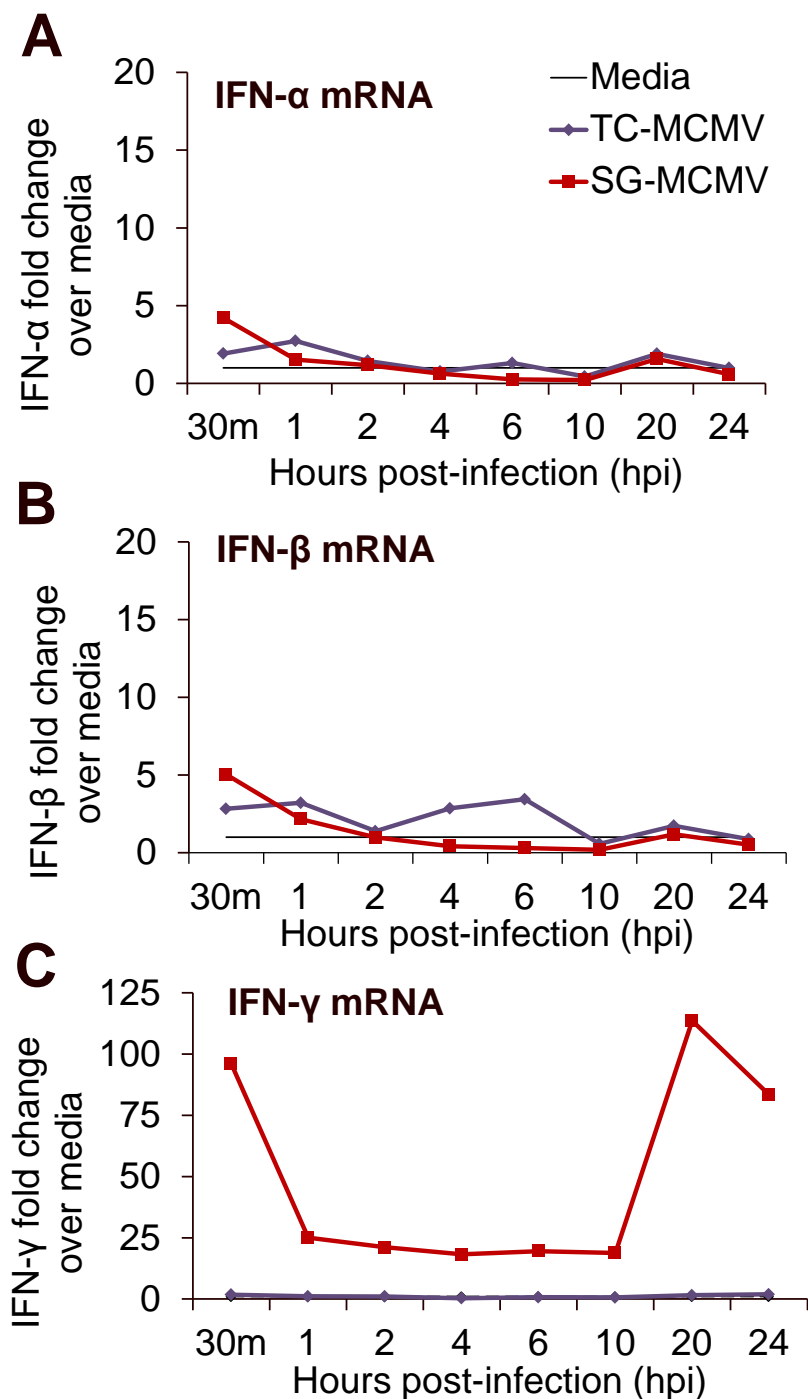


Figure 4.4. SG-MCMV, but not TC-MCMV, highly stimulates type II IFN expression in IC-21 mouse macrophages.

IC-21 mouse macrophages were infected TC-MCMV or SG-MCMV (MOI = 3 PFU/cell) or media control as in **Figure 4.3**. At 0.5, 1, 2, 4, 6, 10, 20, and 24 hpi, cells were harvested and assessed for IFN- α (**A**), IFN- β (**B**), and IFN- γ mRNA transcripts, compared with those in media-treated wells. Note the large differences in y-axis scales between type I and type II IFNs.

Viral growth curves and IE1 and gH mRNA expression are delayed during SG-MCMV infection of IC-21 mouse macrophages compared with expression kinetics during TC-MCMV infection. To determine whether this differential expression might be explained by virologic differences between passage sources, we assessed SG-MCMV-infected or TC-MCMV-infected IC-21 mouse macrophages for mRNA expression of MCMV-specific IE1 and L gene gH. We found, in agreement with previous findings in monocytes/macrophages [379, 381, 382], that MCMV gene expression (**Figure 4.5A, B**) and growth curves (**Figure 4.5C**) during SG-MCMV infection of IC-21 cells are delayed compared with TC-MCMV infection.

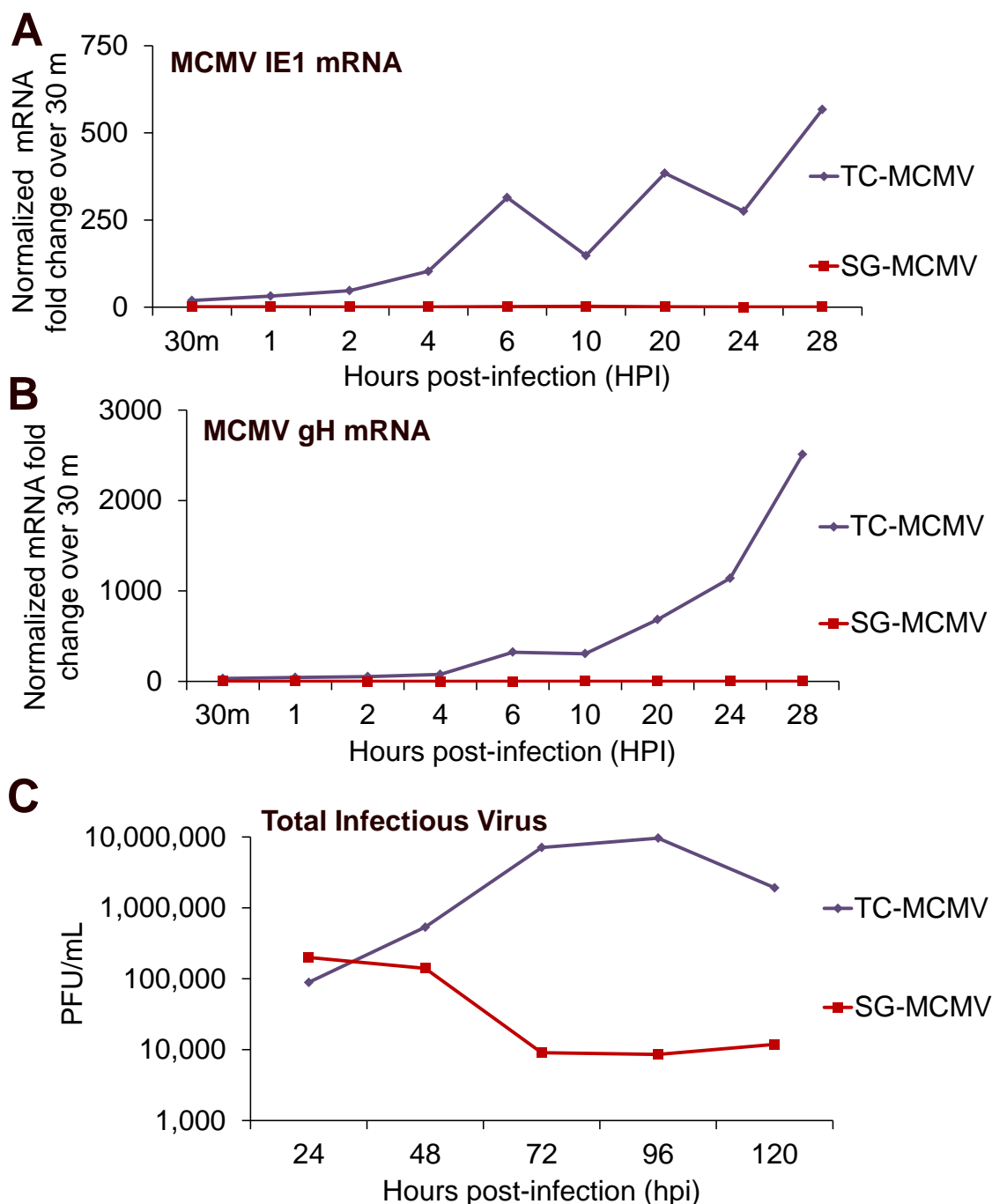


Figure 4.5. Viral gene expression and growth curves in IC-21 mouse macrophages are delayed during SG-MCMV infection compared with TC-MCMV infection.

IC-21 cells were infected with SG-MCMV or TC-MCMV (MOI = 3 PFU/cell) as in **Figure 4.3**. Cells were harvested at 0.5, 1, 2, 4, 6, 10, 20, 24, and 28 hpi and assessed for MCMV IE1 (**A**) or MCMV gH (**B**) mRNA gene expression over time, with all samples relative to SG-MCMV values at 0.5 hpi (30 m). Multistep growth curves of total infectious virus (**C**) for SG-MCMV and TC-MCMV in IC-21 cells at MOI = 0.1 PFU/cell were performed over 5 days (120 hpi).

4.2 UV-Inactivated MCMV in IC-21 Mouse Macrophages or MEF Cells

UV-inactivated MCMV does not significantly stimulate SOCS1 or SOCS3 expression in IC-21 mouse macrophages. To test the hypothesis that the early virologic events of attachment, adsorption, and/or release of tegument proteins are sufficient for SOCS1 and SOCS3 mRNA stimulation during MCMV infection, we infected monolayers of IC-21 macrophages with SG-MCMV exposed to DNA-damaging UV light (UV-inactivated MCMV, UVi-MCMV). This technique leaves any cellular or immunologic components of MCMV intact while rendering the virus deficient in viral gene expression and replication, therefore allowing attachment, adsorption, and release of tegument proteins into the infected cell [101]. In contrast with the significant stimulation of SOCS1 and SOCS3 mRNA transcripts in IC-21 mouse macrophages during productive MCMV infection, UVi-MCMV resulted in only a small trend toward transient stimulation of SOCS1 and SOCS3 that did not reach statistical significance when compared with media-treated control cells (**Figure 4.6**) and remained significantly lower than MCMV values (SOCS1: $p < 0.05$ for ≥ 2 hpi, and SOCS3: $p < 0.001$ for 2 and 4 hpi, UVi-MCMV compared with SG-MCMV at each time point). In IC-21 cells immunofluorescently labeled with anti-SOCS1 or anti-SOCS3 antibodies, treatment with media or UVi-MCMV resulted in basal to moderate SOCS1 or SOCS3 protein expression, while MCMV infection at 3 hpi caused stimulation of these proteins (**Figure 4.7**), found mostly in the cytoplasm.

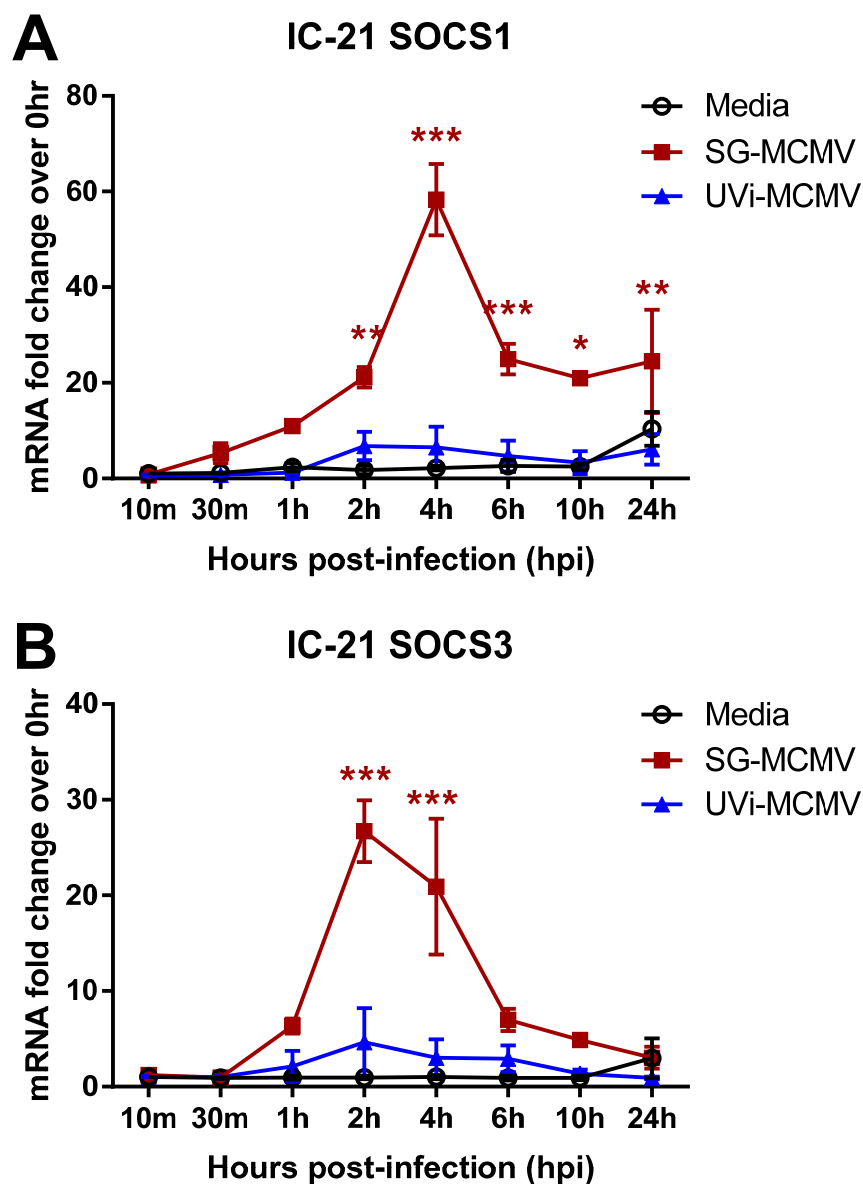


Figure 4.6. UV-inactivation of MCMV reduces MCMV-related stimulation of SOCS1 and SOCS3 mRNA transcripts in mouse macrophages.

IC-21 mouse macrophages treated with SG-MCMV (MOI = 3 PFU/cell), UV-inactivated SG-MCMV (UVi-MCMV), or media (control) were assessed for SOCS1 (A) or SOCS3 (B) mRNA, with all samples compared back to the media group at 0 hpi. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared with respective media controls at the same time points. No statistically significant differences were found between media and UVi-MCMV groups at any time point.

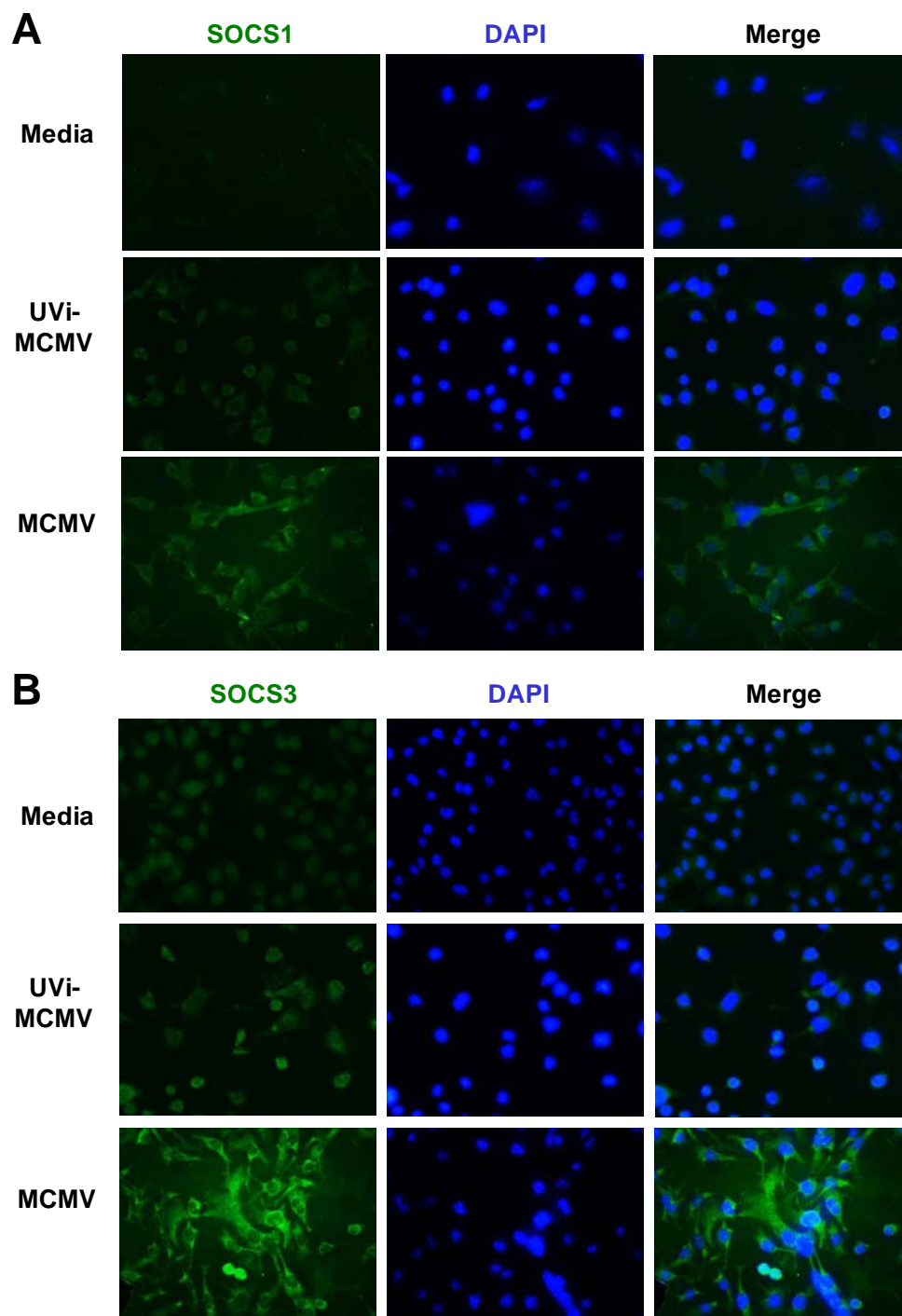


Figure 4.7. UV-inactivation of MCMV reduces MCMV-related stimulation of SOCS1 and SOCS3 protein in mouse macrophages.

IC-21 mouse macrophages grown on glass cover slides were treated with media, SG-MCMV (MOI = 3 PFU/cell), or UVi-MCMV. All groups were methanol-fixed at 3 hpi and assessed by immunofluorescent staining for SOCS1 (**A**) or SOCS3 protein (**B**) (green) and counterstained with DAPI (blue). Original magnification, 400 \times .

Cytokines known to induce expression of SOCS1 and SOCS3 are up-regulated during MCMV infection of IC-21 mouse macrophages. SOCS transcripts can be induced by cytokine signaling through the JAK/STAT pathway [11, 12, 268, 270], and MCMV infection causes up-regulation of cytokines such as IFN- γ [247, 258] and IL-6 [373]. We therefore investigated whether these cytokines are concurrently stimulated with SOCS1 and SOCS3 mRNA transcripts during MCMV infection of IC-21 mouse macrophages. We found transient stimulation of mRNA transcripts for antiviral type I IFN (IFN- α and IFN- β , **Figure 4.8A, B**) in agreement with previous findings by others [375], and prolonged stimulation of type II IFN (IFN- γ) (**Figure 4.8C**) and IL-6 (**Figure 4.8D**) mRNA. For all time points observed, infection of IC-21 cells with UVi-MCMV failed to stimulate these cytokines beyond the levels of the media-treated control wells (**Figure 4.8**).

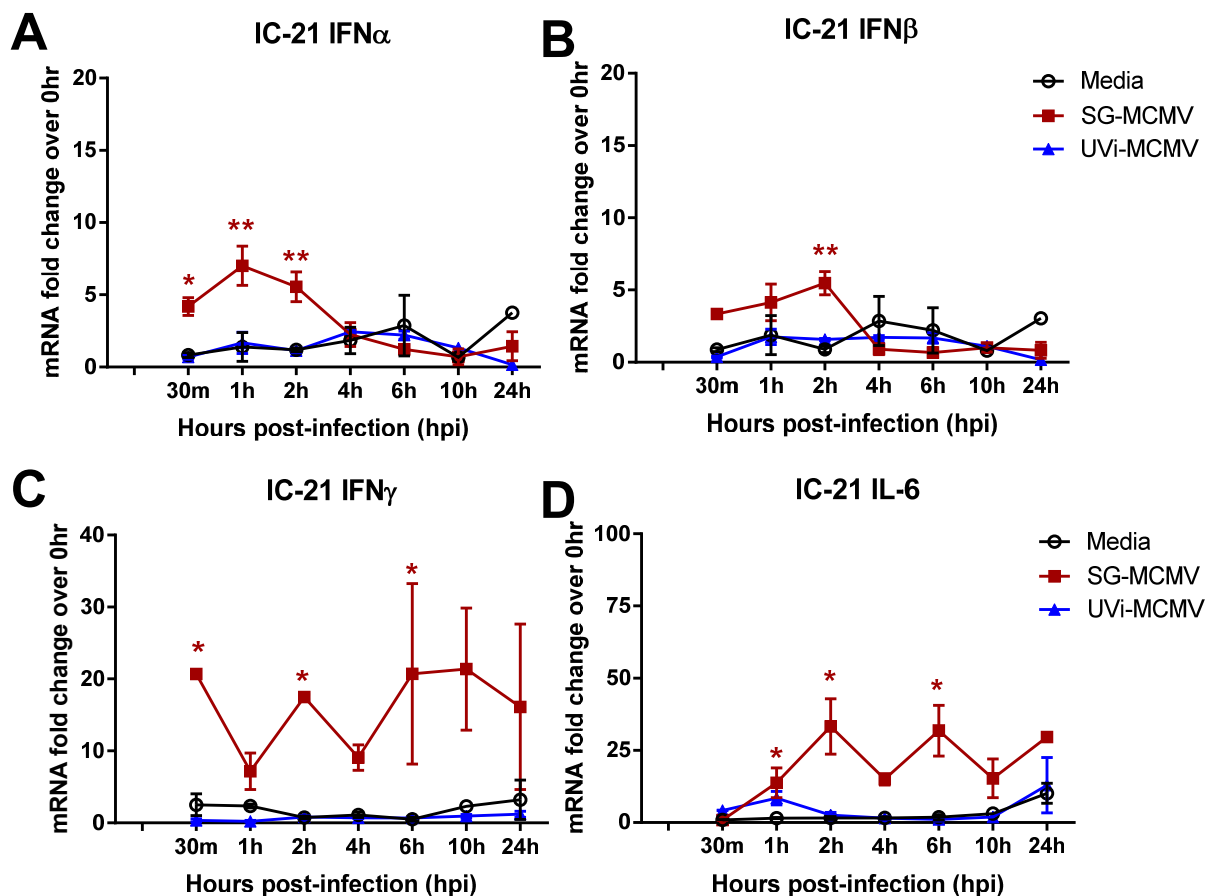


Figure 4.8. Infection of mouse macrophages with UV-inactivated MCMV reduces MCMV-related stimulation of cytokines known to induce SOCS1 and SOCS3.

IC-21 cells treated with media, SG-MCMV (MOI = 3 PFU/cell), or UVi-MCMV were harvested at indicated time points and assessed for IFN- α (A), IFN- β (B), IFN- γ (C), or IL-6 (D) mRNA, with all samples compared back to the media group at 0 hpi. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared with respective media controls at the same time points.

UV-Inactivated MCMV stimulates SOCS1 and SOCS3 expression in MEF cells.

Productive MCMV infection of MEF cells (**Figure 4.9**) produced similar temporal patterns of SOCS1 and SOCS3 mRNA expression to those found in IC-21 cells. Unlike UVi-MCMV infection of IC-21 cells, which produced no significant up-regulation of SOCS1 or SOCS3 transcripts during the time points observed in these macrophages, UVi-MCMV infection of MEF cells caused significant, albeit transient, stimulation of SOCS1 (**Figure 4.9A**) and SOCS3 (**Figure 4.9B**) mRNA expression at 2 hpi.

UV-Inactivated MCMV in MEF cells stimulates expression of SOCS-inducing type I IFN, but not type II IFN or IL-6. In MEF cells, MCMV infection resulted in moderate, early stimulation of IFN- α (**Figure 4.10A**), but not IFN- β (**Figure 4.10B**), mRNA transcripts at 30 min post-infection, with subsequent dampening of these type I IFNs beyond 30 min. Interestingly, infection with UVi-MCMV, but not productive MCMV, caused significant up-regulation of these type I IFNs at later time points (10, 24 hpi). Transcripts of IFN- γ (**Figure 4.10C**) and IL-6 (**Figure 4.10D**) mRNA were highly stimulated in MEF cells following productive MCMV infection but not UVi-MCMV.

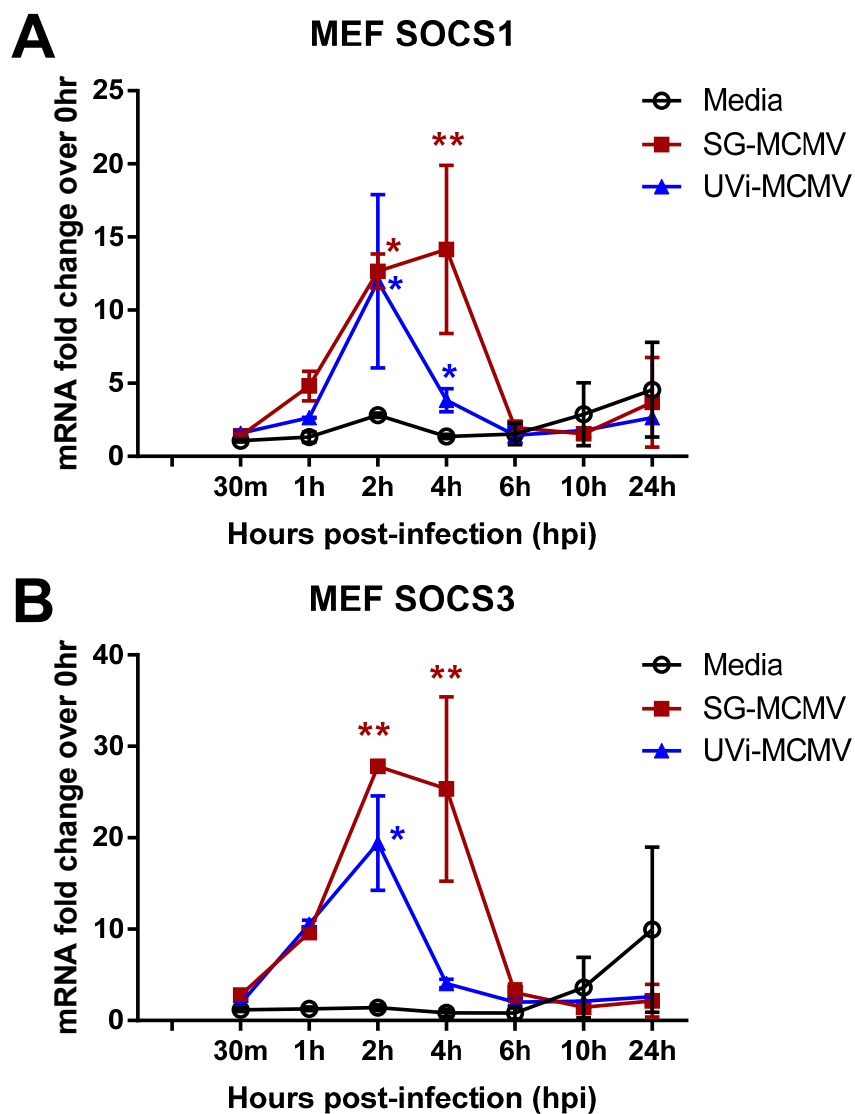


Figure 4.9. MCMV infection stimulates mRNA expression of SOCS1 and SOCS3 mRNA in MEF cells.

MEF cells treated with media, SG-MCMV (MOI = 3 PFU/cell), or UVi-MCMV were assessed at indicated time points for SOCS1 (A) or SOCS3 (B) mRNA, with samples compared back to the media group at 0 hpi. * $p < 0.05$ and ** $p < 0.01$, compared with media controls at same time points.

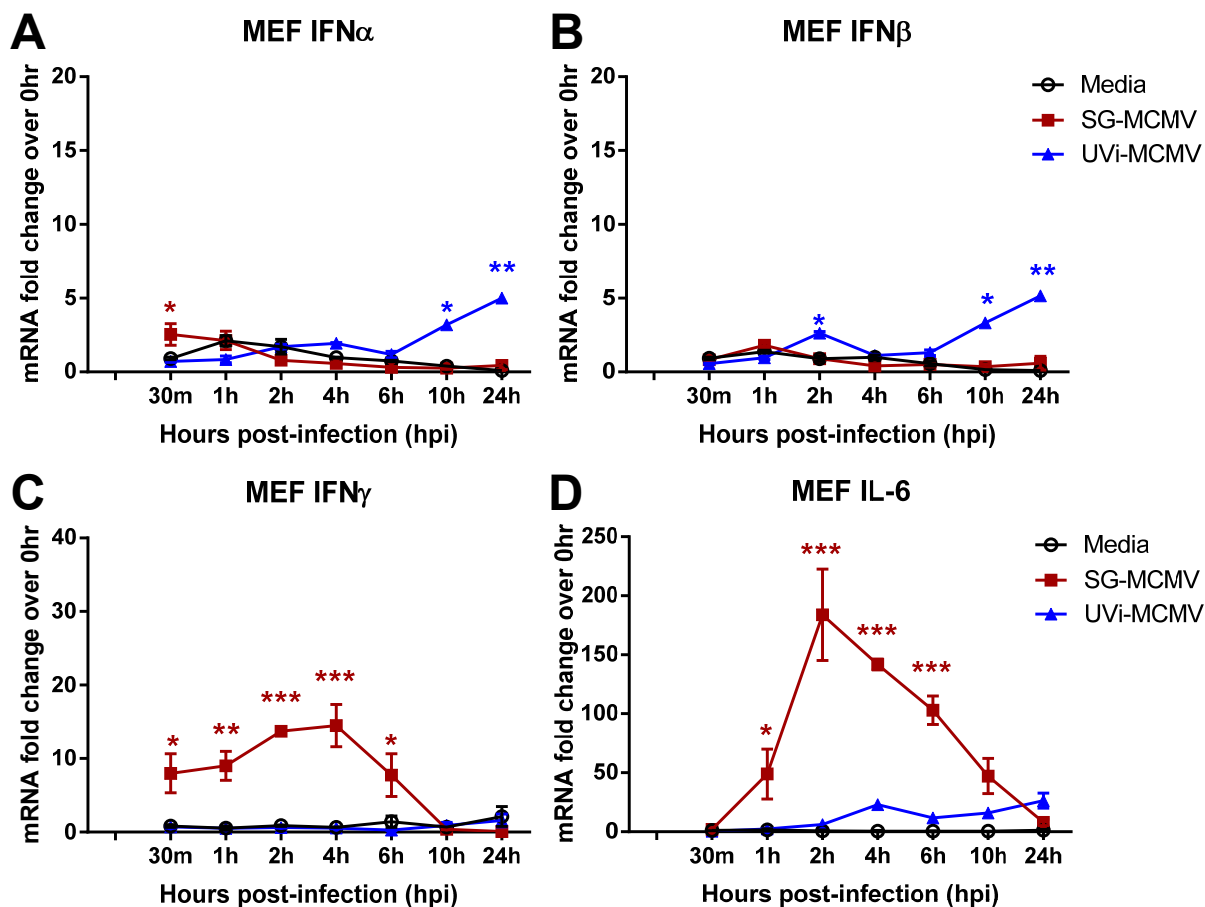


Figure 4.10. SOCS-inducing cytokines are transcriptionally stimulated during MCMV infection of MEF cells.

MEF cells treated as in **Figure 4.9** were assessed at indicated time points for IFN- α (**A**), IFN- β (**B**), IFN- γ (**C**), or IL-6 (**D**) mRNA, with all samples compared back to the media group at 0 hpi. Note differences in y-axis scales. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared with media controls at the same times.

4.3 MCMV-Stimulated SOCS3 in IC-21 Cells is Sensitive to Ganciclovir

GCV treatment decreases MCMV-stimulated SOCS3, but not SOCS1, production in IC-21 mouse macrophages. Because UV-inactivated MCMV failed to stimulate SOCS1 and SOCS3 expression at early time points following infection of IC-21 mouse macrophages, we next investigated SOCS1 and SOCS3 mRNA expression later during MCMV infection (72 hpi) with or without the antiviral drug GCV, which inhibits HCMV and MCMV replication and subsequent expression of late viral genes [104]. At 72 hrs following MCMV infection of IC-21 monolayers, GCV treatment significantly reduced MCMV-stimulated SOCS3 mRNA transcripts (**Figure 4.11B**), with SOCS1 mRNA expression displaying only a downward trend with GCV treatment that did not reach statistical significance (**Figure 4.11A**).

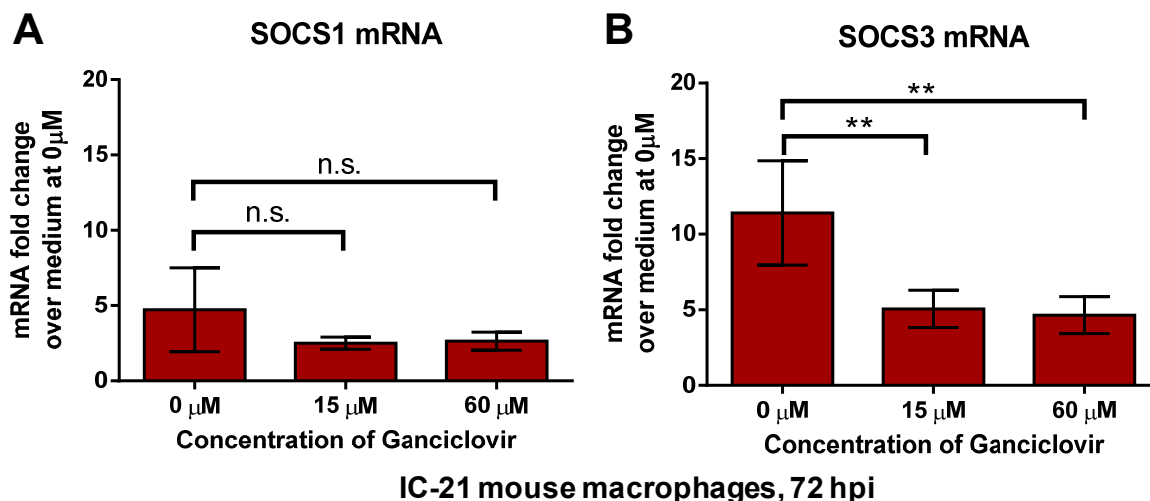


Figure 4.11. GCV treatment of MCMV-infected mouse macrophages reduces MCMV-related stimulation of SOCS3 mRNA at 72 hpi.

IC-21 cells were treated with media (baseline control) or infected with SG-MCMV (MOI = 3 PFU/cell). At 1 hpi, wells were treated with the antiviral drug GCV at the indicated final concentrations or vehicle control ($0\mu\text{M}$). At 72 hpi, cells were harvested and assessed for SOCS1 (**A**) or SOCS3 (**B**) mRNA, with all samples compared back to the vehicle-treated ($0\mu\text{M}$) media group. * $p < 0.05$, ** $p < 0.01$, and **n.s.** = not significant, for MCMV-infected GCV-treated groups compared with MCMV-infected vehicle controls.

4.4 Infection of IC-21 Cells with EGFP-Tagged MCMV RM4503

Uninfected bystander IC-21 mouse macrophages express SOCS1 and SOCS3 during MCMV infection. MCMV gene expression was necessary for viral-related stimulation of SOCS1 and SOCS3 mRNA and protein, and for transcripts of SOCS-inducing IFNs and IL-6, early after SG-MCMV infection of IC-21 mouse macrophages. Because MCMV infection potently stimulates transcription of host cell proteins [6], including SOCS-inducing cytokines [247, 258, 373], SOCS1 and/or SOCS3 stimulation may occur, in whole or in part, by an indirect effect on uninfected bystander cells. To test for this possibility, we infected IC-21 cells with the EGFP-expressing tracer virus MCMV RM4503 [358, 360] and assessed whether EGFP expression co-localizes with immunofluorescently-stained SOCS1 or SOCS3 proteins. SOCS1 and SOCS3 proteins were stimulated in IC-21 cells at 3 hpi by wild type SG-MCMV (Smith) or by SG-MCMV RM4503 compared with baseline expression in media-treated cells (**Figure 4.12**). At this time point, however, EGFP was not detected in IC-21 cells infected with MCMV RM4503, despite expected amounts of infectious virus and plaque-associated EGFP expression upon titration of the inoculum in MEF monolayers (data not shown).

EGFP expression during infection of IC-21 mouse macrophages with SG-MCMV RM4503 does not appear until 48 hpi. MCMV RM4503 expresses EGFP with IE2 kinetics and is detectable at 6 hpi in infected NIH/3T3 fibroblast cells [358]. We therefore expected to find IE2 promoter-driven EGFP expression very early during infection in IC-21 cells [98], but EGFP was undetected at 3 hpi. To determine the expression kinetics of IE2 promoter-driven EGFP in these cells, we infected a monolayer of IC-21 mouse macrophages with MCMV RM4503 and periodically screened the cells for EGFP expression under a fluorescent microscope. We did not detect EGFP from within MCMV RM4503-infected IC-21 cells until 48 hpi (data not shown).

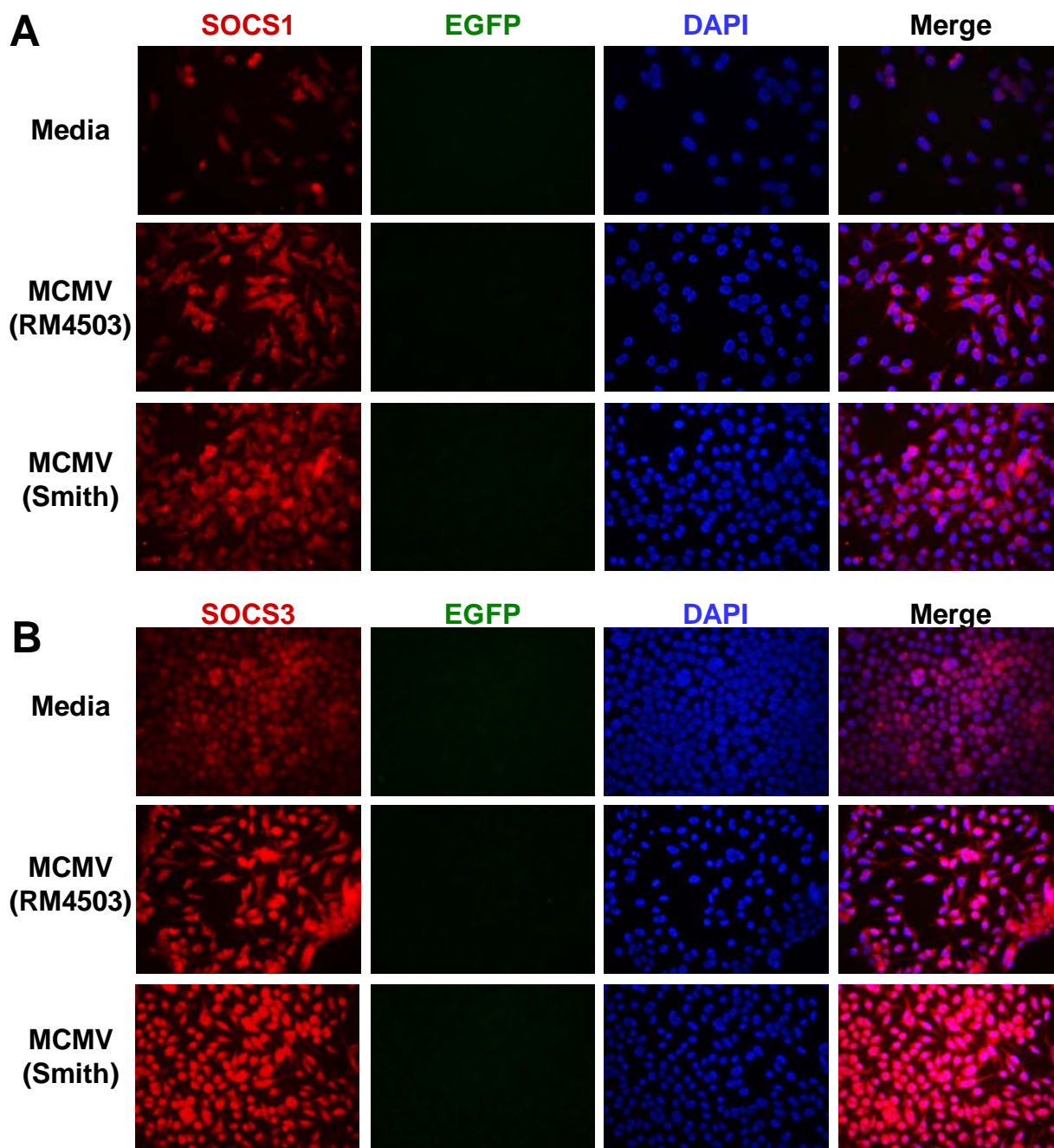


Figure 4.12. SOCS1 and SOCS3 protein expression during MCMV infection in IC-21 mouse macrophages occurs in uninfected bystander cells.

IC-21 cells grown in chamber slides were treated with media or infected (MOI = 3 PFU/cell) with wild type MCMV (Smith) or MCMV RM4503, which expresses EGFP under the control of the IE2 promoter. At 3 hpi, cells were methanol-fixed and stained with antibodies detecting SOCS1 (**A**) or SOCS3 (**B**) (red). No MCMV IE2-driven EGFP (green) is detectable in these cells at 3 hpi. Original magnification 400 \times .

5 DISCUSSION AND CONCLUSIONS

Herein we used *in vivo* model systems and *in vitro* techniques to investigate the virologic, immunologic, and/or pathologic mechanisms of SOCS1 and SOCS3 expression during MCMV infection. Through pursuit of two specific aims, we tested **the central hypothesis that MCMV stimulates and employs SOCS1 and/or SOCS3 to induce onset and development of MCMV retinal disease**. The results of this study suggest the following conclusions:

- (i) *In vivo* infection with MCMV induces SOCS1 and SOCS3 mRNA and protein expression under specific conditions that are related to severity of ocular disease,
- (ii) MCMV-stimulated ocular SOCS1 and/or SOCS3 fill a putative critical role upon initiation of late-stage MAIDS-8 and MAIDS-10, but this role becomes less critical as certain cell populations and/or functions decline during late-stage MAIDS-12 or during corticosteroid-induced immune suppression,
- (iii) MCMV replication in ocular tissues is required, but not sufficient, to stimulate ocular SOCS1 and/or SOCS3 during MAIDS-related MCMV retinitis,
- (iv) The virologic mechanism(s) of SOCS1 or SOCS3 expression during MCMV or HCMV infection depends on cell type and virus passage origin,
- (v) MCMV IE, E, or tegument-packaged host or viral RNA may govern biphasic SOCS1 and/or SOCS3 stimulation, and
- (vi) Direct MCMV infection is not required to stimulate SOCS1 and SOCS3 expression in uninfected bystander macrophages, implicating a role for SOCS-inducing cytokines.


Cytomegalovirus infection therefore stimulates SOCS1 and SOCS3 through divergent virologic or immunologic mechanisms in a cell-type-specific manner that reflects the complexity of the ocular compartment during the pathophysiology of retinal disease.

5.1 Specific Aim 1: SOCS1 and SOCS3 during MCMV Infection *In Vivo*

Specific Aim 1: Test the hypothesis that SOCS1 and/or SOCS3 stimulation *in vivo* is affected by virologic, immunologic, and/or pathologic events during MCMV infection.

Subretinal MCMV inoculation causes severe necrotizing retinitis in 80–100% of mice with late-stage MAIDS (MAIDS-8 to MAIDS-12), but 0% of MAIDS-4 mice [8, 246, 247]. Subretinal MCMV infection also highly stimulates ocular SOCS1 and SOCS3 expression in retinitis-susceptible MAIDS-8 and MAIDS-10 mice, but not retinitis-resistant MAIDS-4 mice [350]. Systemic MCMV infection of MAIDS mice, which does not cause retinitis (0% [251]), did not stimulate ocular SOCS1 or SOCS3. Ocular SOCS1 and SOCS3 were not highly stimulated during experimental MCMV retinitis of C57BL/6 mice with corticosteroid-induced immunosuppression, despite significant stimulation of SOCS-inducing IFN- γ and IL-6 transcripts. This model of experimental MCMV retinitis also resulted in less severe pathogenesis and reduced ocular titers compared with those of previous MAIDS studies. During MAIDS, MCMV-related intraocular SOCS1 and SOCS3 stimulation were both sensitive to GCV treatment at different stages of MAIDS. Our findings with ocular expression of SOCS1, SOCS3, and SOCS-inducing cytokines are summarized together with virologic, immunologic, and pathologic ocular findings from the current study and previous studies under various *in vivo* conditions in **Table 5.1**.

Table 5.1: Ocular Specifications during Systemic or Subretinal MCMV Infection of C57BL/6 Mice with MAIDS or Corticosteroid-Induced Immune Suppression.

 Whole Eyes	SOCS1 mRNA	SOCS3 mRNA	IFN- γ mRNA	IL-6 mRNA	Frequency (%) of Retinitis ^a	Severity Score (% of max) ^a	Ocular Titer (PFU/eye) ^a
Eyes of C57BL/6 Mice during SYSTEMIC MCMV Infection							
MAIDS-4	—	—	↑↑	↑	(<i>n.d.</i>)	(<i>n.d.</i>)	(<i>n.d.</i>)
MAIDS-10	—	—	↑	—	~ 0%	~ 0%	1–5 × 10 ²
Eyes of C57BL/6 Mice during SUBRETINAL MCMV Infection							
MAIDS-4 ^a	—	—	—	—	0–10%	0–25%	1–5 × 10 ⁴
MAIDS-8	↑	↑↑	↑↑	↑	80–100%	50–90%	1–5 × 10 ⁴
MAIDS-10	↑	↑	↑↑	↑↑	80–100%	50–90%	1–5 × 10 ⁴
MAIDS-12	—	—	↑	↑↑	80–100%	50–90%	1–5 × 10 ⁴
Corticosteroids	—	↑	↑↑↑	↑↑	40%	~ 35%	~ 5 × 10 ³

All data are from whole eyes collected at day 10 following subretinal injection of ~10⁴ PFU/eye of MCMV (Smith).
a: Data compiled from previous MAIDS studies [8, 247, 251, 265, 361].

— no significant difference in expression between MCMV-infected eyes and contralateral media-injected controls.
 ↑ p<0.05; ↑↑ p<0.01; ↑↑↑ p<0.001, up-regulation in MCMV-infected samples compared with media controls.
 (*n.d.*) = not done.

Taken together, the experiments of Specific Aim 1 suggest that:

- (i) *In vivo* infection with MCMV induces SOCS1 and SOCS3 mRNA and protein expression under specific conditions that are related to severity of ocular disease,
- (ii) MCMV-stimulated ocular SOCS1 and/or SOCS3 fill a putative critical role upon initiation of late-stage MAIDS-8 and MAIDS-10, but this role becomes less critical as certain cell populations and/or cellular functions decline during late-stage MAIDS-12 or during corticosteroid-induced immune suppression,
- (iii) MCMV replication in ocular tissues is required, but not sufficient, to stimulate ocular SOCS1 and/or SOCS3 during MAIDS-related MCMV retinitis.

5.1.1 Systemic MCMV Infection in Mice without or with MAIDS

Systemic MCMV infection moderately stimulates splenic SOCS1, SOCS3, and SOCS-inducing cytokines in C57BL/6 mice without MAIDS, but not with MAIDS.

Intraperitoneally-injected MCMV at sub-lethal doses causes a self-limited systemic infection which disseminates through the blood to replicate in various organs, including the spleen [245, 370, 371]. This results in mild, transient splenomegaly that involves relatively proportional expansion of leukocytes, and acute replication that peaks between 3–12 days post-infection [370, 383]. Herein we found that systemic MCMV infection in the absence of retinal disease or immune suppression caused mild, transient stimulation of splenic SOCS1 and SOCS3 protein that was not prolonged and did not reach the amplitude of stimulation found during intraocular MCMV infection in retinitis-susceptible MAIDS-10 mice.

In the spleens of immunologically normal mice without MAIDS, moderate stimulation of SOCS1 and SOCS3 occurred with peak IFN- γ and IL-6 mRNA expression. We therefore cannot eliminate the possibility that splenic SOCS1 and/or SOCS3 expression during systemic MCMV infection could be an indirect immunologic consequence of MCMV stimulating IFN- γ , IL-6, or other cytokines. Because systemic MCMV infection failed to stimulate splenic IFN- α and IFN- β mRNA transcripts at any time point investigated, it is unlikely that these type I IFNs are involved in SOCS1 and/or SOCS3 stimulation under these conditions, although we cannot rule out the possibility that these may be stimulated at time points not observed in this study.

The significant down-regulation of IFN- α and IFN- β mRNA expression at day 4 post-infection, which followed peak SOCS1 mRNA production, is consistent with previous findings *in vivo* [383] and *in vitro* in mouse fibroblast and macrophage cell lines [375]. Because SOCS1 has demonstrated an ability to interfere with signaling pathways that transcriptionally regulate

type I IFN, including NF- κ B [321], it is not unreasonable to hypothesize that the modest induction of SOCS1 found prior to type I IFN down-regulation may contribute to the down-regulation of type I IFN during systemic MCMV infection. Although MCMV also employs other virologic mechanisms that down-regulate type I IFN in a transcriptional or functional capacity (reviewed in [339]), SOCS1 and/or SOCS3 may contribute to the arsenal that MCMV utilizes to counteract these antiviral cytokines.

During the progression of MAIDS, systemic MCMV-related stimulation of SOCS1, SOCS3, and IL-6 in the spleen was abolished as early as MAIDS-4. Although splenic IFN- γ mRNA expression demonstrated intermediate stimulation at MAIDS-4, this effect was insufficient to stimulate SOCS1 or SOCS3 expression at that time point, and it was completely eliminated by MAIDS-10, suggesting a progressive decline in the ability of systemic MCMV to induce SOCS during the development of MAIDS. The phenomenon or cell type(s) responsible for modulation of splenic SOCS1, SOCS3, and/or SOCS-inducing cytokines during acute, systemic MCMV infection in immunologically normal mice, therefore, is progressively either abrogated or masked by the effects of MAIDS. It is possible that the MAIDS-inducing retrovirus mixture itself may interfere with the ability of MCMV to stimulate these host proteins. However possible, this is unlikely because of the robust stimulation of ocular SOCS1 and SOCS3 in the MCMV-infected eyes of MAIDS-10 mice. In addition, when compared with whole spleens from age-matched immunologically normal (healthy) control mice, mid-stage and late-stage MAIDS do not affect splenic SOCS1 mRNA and progressively increases SOCS3 mRNA in whole splenic cells [350]. Therefore, if the retrovirus mixture does interfere with the ability of MCMV to stimulate SOCS1 and/or SOCS3, it does so in a highly tissue-specific and/or cell-type-specific manner. Because different stages of MAIDS cause dysfunction or alteration of nearly all types of

splenic cells [235], including B and T lymphocytes [230, 231, 236-239], NK cells [240], neutrophils [241], and macrophages [236, 239, 242], it is possible that any of these cell types that might normally stimulate SOCS1 and/or SOCS3 expression fail to do so when rendered dysfunctional by MAIDS progression.

Alternatively, because MAIDS causes severe splenomegaly by aberrant proliferation of many splenic cell types, the primary splenic cells responsible for SOCS1 and/or SOCS3 expression during systemic MCMV infection without MAIDS could be underrepresented during MAIDS progression due to overpopulation of non-SOCS-expressing cells in the whole spleens of mice with MAIDS. This would further suggest that the splenic cell types undergoing aberrant proliferation as early as MAIDS-4 are not responsible for SOCS1 and/or SOCS3 expression during MCMV infection, and would therefore rule out B cells [230, 236, 237] as well as CD4⁺ and CD8⁺ T cells [231, 238, 239]. Reduced Mac1⁺ (CD11b⁺) macrophage population percentages and activation frequencies have been reported during MAIDS-4 [229, 243], positioning these cell types as potential candidates for SOCS1 and/or SOCS3 producers in whole splenic cells during MCMV infection of MAIDS-4 mice.

Systemic MCMV infection fails to stimulate ocular SOCS1, SOCS3, or SOCS-inducing cytokines in C57BL/6 mice during the progression of MAIDS. Although systemic MCMV infection in the absence of retrovirus-induced immune suppression induced moderate amounts of splenic SOCS1 and SOCS3 proteins, this MCMV-related up-regulation of splenic or ocular SOCS1 or SOCS3 did not occur during systemic MCMV infection of MAIDS-4 or MAIDS-10 mice in the absence of retinitis. The amplitude of SOCS1 and SOCS3 production during MCMV infection is therefore correlated with severity of MAIDS-related MCMV retinitis. Taken together, these data suggest that, similar to other viruses, MCMV may also induce and

exploit SOCS protein expression [326-328], but perhaps only under specific conditions that are related to ocular disease.

5.1.2 Subretinal MCMV during the Progression of Late-Stage MAIDS

MCMV replication in the ocular compartment is insufficient to stimulate ocular SOCS1 or SOCS3 during MAIDS-related MCMV retinitis. One of the most intriguing phenomena of the MAIDS model of experimental MCMV retinitis is that intraocular MCMV replication following subretinal inoculation reaches equivalent levels ($\sim 3 \times 10^4$ PFU/eye) in retinitis-resistant mid-stage MAIDS mice (MAIDS-4) as those in retinitis-susceptible late-stage mice (MAIDS-10) [247, 265]. Although this means that high MCMV titers alone are insufficient for retinal pathogenesis, it also suggests that susceptibility to intraocular MCMV replication precedes susceptibility to retinitis in this model [247]. MAIDS-4 mice with subretinal MCMV infection also fail to produce high amounts of SOCS1 and SOCS3 ([350], **Figure 1.11**). Therefore, intraocular MCMV titer alone is also insufficient to drive MCMV-related SOCS1 and SOCS3 stimulation, and this provides further evidence that SOCS1 and/or SOCS3 are involved in the pathogenesis of MAIDS-related MCMV retinitis.

MCMV-related stimulation of ocular SOCS1 and SOCS3 in retinitis-susceptible eyes of mice with late-stage MAIDS precedes the functional decline of NK cells, neutrophils, and macrophages. We found an inverse relation between the progression of late-stage MAIDS and the amplitude of ocular SOCS1 or SOCS3 mRNA up-regulation in MCMV-infected eyes compared with their contralateral controls. Late-stage MAIDS progression (MAIDS-8 to MAIDS-12) witnesses the complete dysfunction of B cells and T cells [231, 238, 239], the decline of function of NK cells [240] and neutrophils [241], and the appearance of irregular macrophage phenotypes [236, 239, 242]. It is possible that one of these or other

progressively-dysfunctional cell populations may represent a primary cellular source of ocular SOCS1 and/or SOCS3 during late-stage progression. If so, the gradual decrease in SOCS1 and SOCS3 stimulation could be due to the progressive decline in the cellular function or representative percentage of one or more SOCS-expressing cell types during late-stage MAIDS. If this were the case, we might expect that the severity of MAIDS-related MCMV retinitis would occur independently of such a cell type, because MCMV retinitis still occurs during MAIDS-12 regardless of this decline in SOCS1 and SOCS3 production and putative SOCS-expressing cell type.

Alternatively, perhaps MCMV-related retinal pathology could require the dysfunction or decline of such a putative SOCS-expressing cell type. In such a case, the over-expression of SOCS1 and/or SOCS3 at MAIDS-8 and MAIDS-10 could contribute to the dysfunction of this cell population by impeding its responsiveness to certain cytokines or other cell signaling pathways. Then by MAIDS-12, if this cell type has become underrepresented in the population, its scarcity or disappearance would still allow retinal disease without requiring SOCS1 or SOCS3 expression to render it dysfunctional. The presence of SOCS1 and/or SOCS3 could therefore contribute to MCMV-related retinal pathogenesis at certain times, such as upon initiation of late-stage MAIDS (MAIDS-8), but may be less critical as late-stage MAIDS further progresses (MAIDS-12). The existence or mechanism of such a cell type, however, is unknown and requires further study.

Subretinal MCMV infection during MAIDS affects SOCS3 mRNA expression in the contralateral media-injected control eye independently of virus replication. We performed thorough and extensive mRNA analysis using an alternative baseline control, comparing MAIDS-10 ocular mRNA from MCMV-infected eyes and their contralateral media-injected

control eyes back to the uninjected MAIDS-10 eyes of MCMV-naïve mice. While it was clear with both methods of analysis that MCMV-injected eyes produced significantly more SOCS1 and SOCS3 mRNA expression than their contralateral media-injected controls, SOCS3 mRNA fold change expression in MCMV-injected eyes was much greater when compared with uninjected MAIDS-10 eyes. This analysis revealed that the media-injected control eyes of MAIDS-10 mice mildly but perceivably also stimulated SOCS3 mRNA transcripts at days 6 and 10 following subretinal injection of MCMV into the left eyes and media into the right eyes. This stimulation was not due to accidental MCMV injection into the control eye, nor to MCMV traveling to the contralateral eye, because back-titers of the media inoculum and mRNA analysis for MCMV IE1 and MCMV gH genes were negative in these media-injected contralateral control eyes (data not shown). Perhaps this mild but significant SOCS3 stimulation in the media-injected contralateral control eye could be attributed to needle stick injury, which breaks the blood-ocular barrier, or to the presence of a small volume of media, which may increase intraocular pressure or introduce foreign antigens or growth factors. A more intriguing hypothesis is that subretinal MCMV infection during MAIDS may somehow “prime” the immune system to affect SOCS3 mRNA expression in the contralateral control eye, independently of virus migration, perhaps by a mechanism reminiscent of ACAID [27, 28, 384]. Future studies could test what effect this contralateral MCMV “priming” may have on the SOCS expression of media-injected or unmanipulated contralateral control eyes.

5.1.3 Subretinal MCMV during Drug-Induced Immunosuppression

Subretinal MCMV infection during corticosteroid-induced immune suppression fails to stimulate ocular SOCS1 or SOCS3, retinitis severity, or ocular titers to the levels achieved during MAIDS-related MCMV retinitis. Although MCMV highly stimulates SOCS1

and SOCS3 mRNA expression during experimental MCMV retinitis in retinitis-susceptible MAIDS-8 and MAIDS-10 mice, we were surprised to find that SOCS1 and SOCS3 mRNA and proteins were not as highly stimulated in another model of experimental MCMV retinitis during corticosteroid (drug)-induced immune suppression. Upon closer examination, however, we found a direct correlation between the amplitude of SOCS1 and SOCS3 stimulation and severity of retinal disease, further implicating these proteins in the pathogenesis of MCMV retinitis.

The stark difference in disease severities between these two models of experimental MCMV retinitis suggest that the MAIDS model is a more reproducible, and therefore more useful, model than the other, but it must be emphasized that mouse strain also is of great importance in these two disease models. BALB/c mice are generally more susceptible to MCMV infection than are C57BL/6 mice [249, 250, 257-260], and this is underscored in experimental MCMV retinitis during drug-induced immune suppression, where the frequency of MCMV retinitis in BALB/c mice (90%, [136]) is generally greater than in C57BL/6 mice (50%, [253]). By contrast, whereas C57BL/6 mice develop late-stage MAIDS between 8-12 weeks following injection with the LP-BM5 MuLV retrovirus mixture, BALB/c mice fail to develop MAIDS until one year or longer after injection [9, 262]. For this experiment, we chose to use C57BL/6 instead of BALB/c mice because C57BL/6 mice are used in MAIDS studies, and we wished to reduce the number of extraneous variables while exploring SOCS1 and SOCS3 production in these two models of experimental MCMV retinitis. Whether or not SOCS1 and/or SOCS3 production is highly produced during experimental MCMV retinitis in drug-immunosuppressed BALB/c mice remains to be seen, but such an occurrence would support our finding that SOCS1 and SOCS3 stimulation is directly correlated with severity of ocular pathogenesis.

Ocular SOCS1 and SOCS3 mRNA expression may be influenced by immune cell populations or activation and/or differentiation status, particularly macrophages. These two models also differ in the types of dysfunctional immune cells, the timing of immune cell demise, and the mechanisms by which these immune cells are rendered defective. One of the major differences between these models is the number and function of macrophages. MAIDS causes reduced Mac1⁺ (CD11b⁺) macrophage population percentages and activation frequencies at MAIDS-4 [229, 243], with increased macrophage numbers between MAIDS-8 and MAIDS-12 [239]. Macrophage populations in MAIDS mice are driven toward an alternatively-activated pro-angiogenic phenotype that is between classically-activated M1 and alternatively-activated M2. They have decreased TNF- α and IFN- α production but increased IL-1 β and IL-6 production in response to LPS [236, 242]. By contrast, corticosteroids such as methylprednisolone acetate, in the absence of MCMV infection, poison nearly all aspects of the innate and adaptive immune system within days, including macrophages [263]. Whatever macrophages remain tend to be driven toward the M2 alternatively-activated phenotype, in a similar manner as macrophages exposed to IL-4, and they avidly produce IL-10 but not TNF- α , IL-1, or IL-6 [124, 125]. Therefore, whereas MAIDS mice experience a functional change in macrophage phenotype at later stages of disease [236, 239, 242], corticosteroid-induced immune suppression very quickly results in significant loss of macrophages [263].

Corticosteroids also decrease the overall number and function of CD4⁺ and CD8⁺ T cells (~93% depletion, [254, 263, 264]) and generally dampens the immune response by suppressing the expression, release, and/or function of inflammatory cytokines such as IFN- γ TNF- α , and IL-2 (reviewed in [264]). It also alters the inflammatory functions of leukocytes such as macrophages. This rapid, acute decline of the immune system is not observed during MAIDS,

which slowly progresses through distinct phases of immune cell dysfunction. Whereas corticosteroid treatment causes apoptosis in leukocytes and lymphocytes therefore decreasing the overall number of these populations [263, 264], MAIDS causes aberrant proliferation of B and T lymphocytes [230, 231]. Although this increases the numbers of these cell populations, this is coupled with retrovirus-induced cellular dysfunction [9, 231, 234]. By late-stage MAIDS, NK cells [240] and neutrophils [241] are also dysfunctional, and macrophage phenotypes are irregular [236, 239, 242]. These two different methods of immune suppression therefore differently affect immune cell populations, particularly macrophage populations, and cytokine responses to infection. The difference in SOCS1 and/or SOCS3 production during subretinal MCMV infection in these models may therefore be driven by the different mechanisms that they employ to destroy immune cells, cytokines, and other components. The current study provides evidence that this outcome may be due, in particular, to quantitative differences in macrophage populations observed in the two mouse models of experimental MCMV retinitis.

Significantly high stimulation of the SOCS1- and SOCS3-inducing cytokines IFN- γ and IL-6 is insufficient to drive robust stimulation of SOCS1 and/or SOCS3 during corticosteroid-induced immune suppression. IFN- γ and IL-6 have previously been reported to induce SOCS1 and/or SOCS3 transcription (reviewed in [10-12]). We were therefore surprised to find that mRNA transcripts for these cytokines were highly stimulated during drug-induced immune suppression in the absence of robust SOCS1 and SOCS3 mRNA stimulation. While the reason for this is not clear, it must be pointed out that the variability of IFN- γ and IL-6 stimulation between each eye was inordinately high, and triplicate technical repeats performed in assaying each sample suggest that this large standard deviation was due rather to individual variability between mice than to technical error. We were perplexed by these data, because

SOCS1 and SOCS3 mRNA expression in the same samples yielded considerably lower variability between individual samples, and regression analyses did not reveal any correlation patterns between SOCS1 or SOCS3 expression and IFN- γ or IL-6 expression in individual eyes (all R^2 values were $< 50\%$ for linear, logarithmic, exponential, or polynomial regression curves, data not shown). It may be that the large standard deviation could be directly tied to the wide spectrum of retinal disease severities, but as histopathology and mRNA analyses were not performed on the same samples, we cannot perform regression analyses to explore this possibility. Future studies could employ laser-capture microdissection to test this hypothesis.

It is possible that the significant transcriptional stimulation of these cytokines does not result in translated or functional protein. Other possibilities are that the ocular cells bathed in these cytokines are severely dysfunctional, decreased in number, or rendered unresponsive to them. Whatever the case, it is clear that transcriptional stimulation of IFN- γ and IL-6 alone was insufficient to stimulate SOCS1 and/or SOCS3 mRNA or protein in this model. These data suggest that IFN- γ and IL-6 expression may not drive SOCS1 and/or SOCS3 production, particularly in an environment with such dramatic decreases in the numbers of immune cells such as macrophages.

5.1.4 Antiviral Treatment during Subretinal MCMV in MAIDS Mice

MCMV-related stimulation of ocular SOCS1 and SOCS3 mRNA transcripts during MAIDS-related MCMV retinitis is sensitive to antiviral GCV treatment. That ocular SOCS1 and SOCS3 both showed sensitivity to GCV treatment at some time during late-stage MAIDS suggests that viral replication is important for MCMV-related stimulation of these mRNA transcripts. The conditional differences in GCV sensitivity suggest different mechanisms by which MCMV stimulates SOCS1 and SOCS3 during MAIDS, and SOCS3 sensitivity to GCV

increases as MAIDS progresses. Stimulation of ocular SOCS1 in MAIDS-10 mice was sensitive to GCV, but MCMV infection without GCV failed to stimulate ocular SOCS1 at MAIDS-12, and GCV treatment did not alter this lack of SOCS1 stimulation. Rather than implying two completely separate mechanisms for SOCS1 or SOCS3 GCV sensitivity, this may simply be indicative of a delayed GCV sensitivity response from SOCS3 in relation to SOCS1 during MAIDS progression.

Although quality control mRNA and plaque assay analyses demonstrated expected ~10-fold to ~100-fold declines in MCMV IE1 and gH mRNA as well as ~10-fold to ~100-fold decreases in ocular MCMV titers with GCV treatment compared with vehicle-treated controls (data not shown), it cannot be ignored that GCV does not function only to inhibit HCMV or MCMV replication. Its most common side-effect is neutropenia [385], which may provide another possible mechanism for SOCS1 and/or SOCS3 modulation, particularly if a prominent cellular source of SOCS1 and/or SOCS3 originates from neutrophils. Unless systemic GCV causes different amounts of neutropenia in MAIDS-10 and MAIDS-12 mice, however, neutropenia is not a likely mechanism of action for SOCS3 GCV sensitivity at MAIDS-12. Otherwise, we would expect the MAIDS-10 neutropenia to affect SOCS3 expression to the same extent as during MAIDS-12. However unlikely, it also cannot be ruled out that the prominent cellular source of SOCS3 may change during this late stage of MAIDS from another cell type to neutrophils. The role of neutrophils, or other immune cell populations, in SOCS1 and/or SOCS3 expression during late-stage MAIDS and GCV sensitivity therefore requires further study.

5.2 Specific Aim 2: SOCS1 and SOCS3 during MCMV Infection *In Vitro*

Specific Aim 2: Test the hypothesis that SOCS1 and/or SOCS3 stimulation *in vitro* is affected by virologic and/or immunologic events during MCMV infection. Our previous investigations during experimental MCMV retinitis demonstrated that F4/80⁺ mouse macrophages are among the intraocular SOCS1- and SOCS3-expressing cell types during MAIDS-related MCMV retinitis. The importance of macrophages and macrophage progenitor cells for viral dissemination and latency during systemic MCMV infection has been demonstrated [90, 115-119]. Macrophages play critical and sometimes contradictory roles during MCMV infection, dependent partly on their reaction to cytokines such as type I and type II IFNs [115, 116, 118, 120-122]. It has been demonstrated by others that macrophages infected with MCMV become resistant to IFN- γ -driven activation in a manner partially dependent upon antiviral type I IFN [121, 122], and/or viral inhibition of the promoter assembly for IFN- γ [123]. SOCS family proteins are uniquely poised to influence this balance in a cell-type-dependent and time-dependent manner because they are inducible negative feedback regulators of cytokine signaling pathways that essentially act by reducing the effectiveness of certain secreted cytokines, and they act intracellularly only in those cells expressing them at any given time.

Herein we examined the effect of MCMV infection on SOCS1 and SOCS3 expression in IC-21 mouse macrophages [353] or MEF cells. We tested the hypothesis that MCMV stimulates host SOCS proteins in macrophages in a manner dependent on early steps of the viral replication cycle, and we used two approaches to disrupt this cycle: UV inactivation of the virus, and inhibition of viral DNA synthesis by GCV. UV inactivation allows viral attachment, adsorption, and release of tegument proteins into the host cell, but it impedes expression of viral genes and viral DNA replication [101]. GCV acts a guanosine analog [102], preferentially inhibits viral

DNA polymerases [103] and therefore viral replication, and allows viral IE and E gene expression without L gene expression [104]. We report that MCMV infection of mouse macrophages or MEF cells resulted in early, transient stimulation of SOCS1 and SOCS3 mRNA transcripts and SOCS-inducing cytokines, with similar temporal patterns between cell types. This stimulation was abrogated by UV inactivation of the virus in IC-21 mouse macrophages, but not in MEF cells. We also found that at 3 days following infection in IC-21 cells, MCMV stimulation of SOCS3 mRNA was significantly reduced by GCV.

We therefore conclude that viral gene expression is likely required for early MCMV-related SOCS1 and SOCS3 stimulation in IC-21 mouse macrophages, but not MEF cells, suggesting that one or more viral immediate early or early gene products in IC-21 cells may be responsible for SOCS1 and/or SOCS3 expression, and further suggesting cell type-dependent virologic mechanisms underlying early SOCS1 and SOCS3 stimulation. Furthermore, these data suggest possible biphasic stimulation of SOCS1 and/or SOCS3 during late MCMV infection of IC-21 cells that occur by divergent virologic and/or immunologic mechanisms.

Taken together, the experiments of Specific Aim 2 suggest that:

- (i) The virologic mechanism(s) of SOCS1 or SOCS3 expression during MCMV or HCMV infection depends on cell type and virus passage origin,
- (ii) MCMV IE, E, or tegument-packaged host or viral RNA may govern biphasic SOCS1 and/or SOCS3 stimulation, and
- (iii) Direct MCMV infection is not required to stimulate SOCS1 and SOCS3 expression in uninfected bystander macrophages, implicating a role for SOCS-inducing cytokines such as IFN- γ or IL-6.

5.2.1 *Expression Kinetics with SG-MCMV or TC-MCMV in IC-21 Cells*

Stimulation of SOCS1 and SOCS3 mRNA in SG-MCMV-infected IC-21 cells

follows IE gene expression kinetics. The MCMV replication cycle involves a temporal, step-wise viral gene expression and replication cascade (reviewed in [6, 54, 61]). Following initial attachment, adsorption, uncoating, and release of viral tegument proteins, viral IE gene expression occurs in fibroblast cells between 1–4 hpi [96, 97], E genes are seen 2–16 hpi [97], and DNA synthesis commences and L genes appear at 8–36 hpi [97, 100]. Peak stimulation of SOCS1 and SOCS3 in IC-21 macrophages and MEF cells therefore occurred at a time when expression of viral IE and/or E proteins during MCMV infection of fibroblasts has been reported [96, 97]. This is in agreement with a previous study in which primary macrophages infected with MCMV *in vitro* increased SOCS1 and SOCS3 mRNA expression levels from 2 to 24 hours after infection [336].

MCMV-related SOCS1, SOCS3, and type II IFN stimulation in IC-21 mouse macrophages is dependent on the cellular origin of virus stock preparation (SG-MCMV vs. TC-MCMV). Others have also demonstrated a lack of SOCS1 and SOCS3 stimulation in MCMV-infected fibroblasts at 24 hpi [376]. The discrepancy between this lack of SOCS1 and SOCS3 stimulation and the findings of the present study might be explained by one or more of the differing parameters of each study, such as different multiplicities of infection, host cell types, and/or viral passage origins of TC-MCMV vs. SG-MCMV. Indeed, we found that early (2–6 hpi) SOCS1 and SOCS3 stimulation in IC-21 mouse macrophages is entirely dependent on whether the stock originates from passage through salivary glands or cell culture. SG-MCMV and TC-MCMV display many virologic, immunologic, and pathologic differences *in vitro* and *in vivo* [378-381], particularly in relation to macrophage infection [248, 378-382], wherein SG-

MCMV infection replicates with delayed kinetics relative to TC-MCMV infection [379, 381, 382]. Our findings were in agreement with these. Despite the delayed replication kinetics of SG-MCMV, SOCS1 and SOCS3 mRNA expression were nevertheless stimulated very early during infection with SG-MCMV, but not TC-MCMV.

These data might suggest that during infection of macrophages, SOCS1 and SOCS3 stimulation are not dependent on viral load or gene expression, but we know from UVi-MCMV infection studies that this is not the case. Furthermore, SOCS1 or SOCS3 stimulation in IC-21 mouse macrophages could not be attributed to any immune or antigen factors in the salivary gland homogenates of uninfected mice, nor from any soluble factors found in MCMV-infected salivary gland stocks rendered noninfectious by UV inactivation. Although attenuation by viral gene mutation is possible, it is not likely that the viral gene(s) responsible for SOCS1 and/or SOCS3 expression in IC-21 mouse macrophages would be lost from this slowly-replicating DNA virus after only one to three passages through cell culture. Comparative genomic or proteomic analyses could be employed in future studies to determine whether this is the case.

Another explanation for this difference is that the stimulation of SOCS1 and SOCS3 during infection of IC-21 cells may require a very small amount of viral gene expression, so that in the presence of high amounts of viral gene expression, it does not occur. A more probable hypothesis stems from the finding that HCMV and MCMV readily package host proteins [62] and host RNA [63] into their teguments. This possibility could allow for a SOCS-inducing host-derived factor that SG-MCMV packages into the tegument but that is not present in cell cultures used for TC-MCMV stocks. Preliminary findings from our laboratory suggest that this transient MCMV-stimulated induction of SOCS1 and SOCS3 is dependent not merely on passage origin (TC-MCMV vs. SG-MCMV), but that it occurs in a manner dependent upon the host mouse

strain of the cell line used for the MCMV stock, with virus produced in BALB/c mice by salivary gland or through passage in BALB/c-derived cell lines (e.g., BALB/3T3 cells) generating higher amounts of SOCS1 and SOCS3 than MCMV stocks derived from cells of C57BL/6 origin (e.g., C57BL/6 MEF cells, SC-1 cells). Further studies are needed to determine what role any of these possibilities may play in the expression of SOCS1 and/or SOCS3. Nevertheless, this finding generates another level of parameters to consider for the development of a live, attenuated vaccine of HCMV.

Infection of IC-21 cells with SG-MCMV causes early phosphorylation of STAT proteins concurrently with SOCS1 and SOCS3 mRNA stimulation. SOCS1 and SOCS3 can be transcriptionally up-regulated by activation of the JAK/STAT pathway [11, 12, 268, 270], and because tyrosine phosphorylation of STAT proteins is required for their transcriptional activity [286, 386-389], this phosphorylation is commonly used as evidence of STAT activation and proper function. STAT1, STAT2, and/or STAT3 tyrosine phosphorylation has been demonstrated by others to occur in fibroblast cells [376] or macrophages [123] at various times following infection with TC-MCMV. Tyrosine phosphorylation of STAT1, STAT2, and STAT3 in IC-21 mouse macrophages occurred during SG-MCMV infection concurrently with SOCS1 and SOCS3 stimulation, suggesting that activated JAK/STAT pathways may facilitate this early SOCS1 and/or SOCS3 stimulation.

Others have recently demonstrated that 24 hrs following MCMV infection of fibroblasts, tyrosine phosphorylation of STAT1 and STAT3 does not necessarily confer transcriptional activation to these proteins, particularly during MCMV infection [376]. Although in the present study the downstream functional activity of these phosphorylated STAT proteins remains to be seen, it is nonetheless possible that early MCMV-related SOCS1 and SOCS3 stimulation in

IC-21 macrophages may be an indirect consequence of pSTAT1, pSTAT2, and/or pSTAT3 stimulation early during MCMV infection, possibly occurring through virally-mediated up-regulation of JAK/STAT-signaling cytokines. The use of mice deficient or conditionally deficient in STAT1, STAT2, or STAT3 may be useful in future *in vivo* or *ex vivo* studies to elucidate the role of these proteins in SOCS1 and/or SOCS3 induction during MCMV infection.

5.2.2 UV-Inactivated MCMV in IC-21 or MEF Cells

MCMV gene expression and/or replication are necessary for early stimulation of mRNA transcripts for SOCS1, SOCS3, and SOCS-inducing cytokines in IC-21 mouse macrophages, but not in MEF cells. SOCS1 or SOCS3 stimulation in IC-21 mouse macrophages could not be attributed to any immune or antigen factors in the salivary gland homogenates of uninfected mice, nor from any soluble factors found in MCMV-infected salivary gland stocks rendered noninfectious by UV inactivation. In agreement with these mRNA data, immunofluorescent staining of IC-21 macrophages for SOCS1 or SOCS3 revealed robust stimulation of these proteins at 3 hrs following infection with productive MCMV, with lesser or basal SOCS1 and SOCS3 up-regulation during exposure to UVi-MCMV compared with basal expression found in media-treated controls. SOCS1 and SOCS3 proteins following MCMV infection appeared mostly in the cytoplasm, where these proteins undergo their major suppressive functions (reviewed in [290]).

The transient, early stimulation of type I IFN in IC-21 mouse macrophages and MEFs is consistent with previous findings by others [375] for TC-MCMV infection of these cells at high MOIs (5 PFU/cell in fibroblasts, 15 PFU/cell in IC-21 cells). Given these data, we cannot ignore the possibility that stimulation of all or one of these cytokines could play a role in SOCS1 and SOCS3 up-regulation during MCMV infection of these cells, although it remains unclear

whether or to what extent this may be the case. As with SOCS1 and SOCS3 transcripts in IC-21 cells, viral gene expression was necessary for stimulation of type I and II IFN and IL-6 because UVi-MCMV failed to stimulate any of these SOCS-inducing cytokines at any time point examined, lending further support to the possibility that these cytokines may contribute to SOCS expression during productive MCMV infection.

Attachment, adsorption, and/or release of viral tegument proteins in the absence of viral gene expression (UVi-MCMV) were insufficient to cause significant stimulation of SOCS1 or SOCS3 in IC-21 mouse macrophages, but were sufficient to stimulate these transcripts in MEF cells. Taken together, these data may suggest a cell-type-specific putative role for one or more IE or E protein(s) in the expression of SOCS1 and SOCS3 during MCMV infection of mouse macrophages. It cannot be ignored, however, that MCMV gene expression kinetics in macrophages are likely different from those that have been reported for fibroblasts. MCMV replication curves, for example, are delayed in macrophages compared with fibroblasts [116, 118, 375]. Indeed, preliminary real-time RT-PCR data shows that SG-MCMV does not produce increasing amounts of MCMV IE1 mRNA transcripts in IC-21 mouse macrophages until 48 hpi. Furthermore, host or viral RNA or other genetic material packaged into the tegument would also be expected to be damaged during UV inactivation. Therefore, we have not yet eliminated the possibility that infection of IC-21 cells may stimulate SOCS1 and/or SOCS3 by one or more viral or host transcripts that are packaged into the tegument of SG-MCMV.

The unexpected up-regulation of type I IFN mRNA transcripts in MEF cells by UVi-MCMV, but not productive MCMV infection, at later time points (10, 24 hpi) might be explained by the presence of a cell-type-specific, virally-encoded inhibitor of type I IFN transcription, as observed by others [375]. These data suggest that the virologic mechanisms for

stimulation of SOCS-inducing cytokines are different depending on cell type, and the possibility cannot be excluded that SOCS1 and SOCS3 expression may be stimulated, in part or whole, as an indirect immunologic consequence of MCMV infection stimulating these or other cytokines.

This cell-type specific effect of UV inactivation on the expression of SOCS1 and SOCS3 was also observed by us during HCMV infection of human fibroblasts (MRC-5), RPE cells (ARPE-19), T cells (Jurkat), and peripheral blood mononuclear cells (PBMC) [390]. These findings underscore the clinical significance of the mouse model with its MCMV counterpart.

5.2.3 MCMV-Stimulated SOCS3 is Sensitive to Ganciclovir

We tested the dispensability of MCMV DNA replication and/or late gene expression on MCMV-stimulated SOCS1 or SOCS3 production in MCMV-infected IC-21 mouse macrophages at 72 hpi by assessing the sensitivity of SOCS1 or SOCS3 expression to increasing doses of GCV, which inhibits HCMV and MCMV replication and subsequent expression of late viral genes [104]. That SOCS3, but not SOCS1, is sensitive to GCV treatment suggests divergent mechanisms for stimulation of these proteins during late infection with MCMV. Because others have shown that MCMV-related tyrosine phosphorylation of STAT3 in fibroblasts is not sensitive to GCV [376], and pSTAT3 can induce the expression of SOCS1 as well as SOCS3 [12, 391], it is reasonable to hypothesize that the mechanism for GCV sensitivity of SOCS3, but not SOCS1, may occur independently of tyrosine-phosphorylated STAT3. It cannot be ignored that differences in cell types and MCMV stock origins between the previous work and the current study necessitate confirmation to test this hypothesis.

Because we found that SOCS3 was stimulated early during MCMV infection (2-6 hpi), but not at 24 hpi, and was then stimulated again at 72 hpi, this suggests a biphasic pattern of SOCS3 expression during MCMV infection of IC-21 macrophages. As the kinetics of this

expression may correspond with the timing of a complete viral replication cycle in this cell type at this MOI, perhaps MCMV infection utilizes similar mechanisms of SOCS3 stimulation at both the early (2-6 hpi) and the late (72 hpi) time points.

5.2.4 Infection of IC-21 Cells with SG-MCMV RM4503

Uninfected bystander IC-21 mouse macrophages express SOCS1 and SOCS3 during MCMV infection. SOCS1 and SOCS3 proteins were stimulated in IC-21 cells at 3 hpi with SG-MCMV RM4503, although at this time point no IE2-driven EGFP was detected. This was not due to photobleaching because EGFP was expressed in these cells at 48 hpi, and plaque-associated EGFP expression was detected upon titration of the inoculum in MEF monolayers. Taken together with the frequency of SOCS1- or SOCS3-positive immunofluorescently-stained cells approaching nearly 100% in SG-MCMV-infected IC-21 cells, these data provide strong evidence that SOCS1 and SOCS3 stimulation during MCMV infection of IC-21 mouse macrophages occurred in uninfected bystander cells. This finding is in agreement with previous *in vivo* studies during MAIDS-related MCMV retinitis, wherein cells double-stained by fluorescent in situ hybridization for SOCS1 or SOCS3 mRNA with MCMV IE1 mRNA show prolific SOCS1- or SOCS3-positive signals mostly from MCMV IE1-negative cells ([351] and [Chien et al., manuscript in preparation]).

Expression of EGFP was not detectable until 48 hrs following infection, much later than stimulation of SOCS1 and SOCS3 expression in these cells. By contrast, others have investigated the temporal kinetics of MCMV IE, E, and L gene mRNA transcripts following infection of TC-MCMV (BAC-derived strain MW97.01) parent virus in IC-21 mouse macrophages and showed significant mRNA expression of the IE2 gene (m128) as early as 1 hpi in these cells [98]. Possible explanations for this discrepancy in IE kinetics during MCMV infection of IC-21

macrophages may arise from the use of different viral strains or different passage sources of the MCMV stocks used, as the differences between TC-MCMV and SG-MCMV have already been discussed [378, 380-382]. Following its original construction by others [358], MCMV RM4503 was propagated through tissue culture passage before it was received by our laboratory [360]. Because infection with TC-MCMV (Smith) failed to stimulate SOCS1 and SOCS3 expression early after infection, we propagated MCMV RM4503 through three consecutive salivary gland passages in BALB/c mice. Although we did not assess the effect of a TC-derived MCMV RM4503 on SOCS1 and/or SOCS3 expression, if we assume that it would behave similarly to TC-MCMV Smith strain by exhibiting a failure to stimulate SOCS1 and SOCS3, then this salivary gland passage would appear to have restored the ability of the virus to stimulate SOCS1 and SOCS3 expression. This is consistent with the observation that MCMV is rapidly attenuated after cell culture passage, but virulence is restored after subsequent salivary gland passages [248, 392]. Because it is unlikely that putative viral genes may be lost and then restored within only a few passages, such a situation would further suggest that the mechanism driving some or all phenotypic differences between SG-MCMV and TC-MCMV passage origins is not due to genotypic differences [248, 392, 393] but may be found on a proteomic level, or perhaps with packaging of host cell proteins, RNA, or other cellular components into the tegument [6]. Taken together with our findings that UV-inactivation of SG-MCMV fails to stimulate SOCS1 or SOCS3 in IC-21 cells, however, this suggests that tegument proteins may not be involved, but perhaps viral or host RNA packaged into the tegument contributes to this stimulation. Further investigation is required to confirm whether this is the case.

MCMV IE2 is not necessary for SOCS1 and SOCS3 stimulation in IC-21 mouse macrophages. The temporal expression of SOCS1 and SOCS3 transcripts occurred with

previously-reported IE expression kinetics during infection of fibroblasts (0.5 to 4 hpi [96, 97, 394]). MCMV IE genes, including IE2, primarily function as transcriptional transactivators [95]. MCMV RM4503 contains the EGFP gene inserted into the MCMV genome to disrupt the MCMV IE2 gene, and it therefore does not express IE2 [358]. Instead, EGFP is expressed with IE2 kinetics [358-360] and is detectable at 6 hpi in NIH/3T3 fibroblasts [358]. MCMV-related stimulation of SOCS1 and SOCS3 occurred during infection of IC-21 mouse macrophages with the IE2-defective MCMV RM4503 [358, 359], providing strong evidence that IE2 is dispensable for this phenotype.

The significance and kinetics of IE1, IE3, and/or E gene expression in IC-21 mouse macrophages infected with SG-MCMV remain unclear; therefore, the effect(s) of IE gene expression, particularly that of IE3, on SOCS1 and SOCS3 production in IC-21 macrophages requires further investigation. Taken together, these data suggest that biphasic stimulation of SOCS1 and SOCS3 mRNA transcripts during MCMV infection in IC-21 mouse macrophages may be the result of several cumulative mechanisms and implicate a putative role for MCMV IE or E proteins, or perhaps for host or viral genetic material packaged into the tegument, in the direct or indirect stimulation of SOCS1 and/or SOCS3 expression in these cells.

5.3 Future Directions

Inhibition or overexpression of SOCS1 and/or SOCS3 *in vivo* could elucidate their mechanistic contribution to the severity of experimental MCMV retinitis. Although the studies reported herein provide substantial evidence that SOCS1 and SOCS3 play a role in the pathogenesis of MAIDS-related MCMV retinitis, experimentally inhibiting or enhancing SOCS1 or SOCS3 expression would confirm this and reveal the details of their involvement. As SOCS1-deficient mice die within 3–4 weeks of birth from massive IFN-related inflammation [294-296], and deletion of the SOCS3 gene is embryonically lethal [297], systemic genetic knockout of SOCS1 or SOCS3 cannot be used to achieve their inhibition in an experimentally useful way. When crossbred with mice deficient in IFN- α receptor 1 (IFNAR1) [292] or with IFN- γ -knockout mice [395], however, SOCS1-deficient mice survive beyond this 3-week lethality. These double-knockout mice therefore could be used, with wild type mice as well as IFNAR1 or IFN- γ single-knockout mice serving as controls, to examine the detailed mechanisms of SOCS1 on MAIDS-related MCMV retinitis. These SOCS1-knockout or double-knockout mice are not currently commercially available, however, and therefore would have to be acquired from collaboration with other laboratories or engineered in-house. SOCS3-floxed mice (B6;129S4-Socs3^{tm1Ayo}/J, Jackson Laboratory) [396] very recently have become commercially available and could be cross-bred with mice conditionally expressing cre-recombinase to produce conditional SOCS3-knockout mice. Both of these approaches present costly and time-consuming challenges, but they would be most useful in determining the detailed mechanistic contributions of SOCS1 or SOCS3 on experimental MCMV retinitis.

An alternative approach for inhibiting or overexpressing SOCS1 and/or SOCS3 *in vivo* or *in vitro* could involve the use of commercially-available molecular knockout, knock-in, or

knockdown techniques such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclease 9 (Cas9) [397] systems specifically designed to target SOCS1 or SOCS3 genes. CRISPR-Cas9 systems are derived from endogenous bacterial defenses against bacteriophages that potentially can be directed toward any known gene [397]. Target-specific CRISPR/Cas9 constructs have very recently been made commercially available in plasmid kits that specifically target any of a large number of genes for knockout, knock-in, or overexpression applications, including SOCS1 or SOCS3 (reviewed in [398-400]). Through the aid of transfection agents, CRISPR/Cas9 systems have been used successfully in model systems *in vivo* [401, 402] as well as *in vitro* (reviewed in [398-400]).

Other molecular knockdown techniques include the use of small interfering (si) RNA molecules that selectively bind and inhibit specific mRNA molecules. Although *in vivo*-optimized transfection agents may be needed, injection of gene-specific siRNA into the subretinal space of the eye has been shown to inhibit expression of targeted genes (in this case, VEGF) without the use of vectors or transfection agents in mice [403]. Another strategy for effective inhibition of SOCS1 and SOCS3 function is the use of a SOCS1- and SOCS3-sequestering peptide with an amino acid sequence analogous to a portion of tyrosine-phosphorylated JAK2, designated pJAK2(1001-1013) [283, 404, 405]. This peptide has conferred effective SOCS1 [405] and SOCS3 [406] functional inhibition in cell culture and animal models of infection. Because SOCS1 and SOCS3 proteins perform their functions intracellularly and are not secreted from the cells that express them, neutralizing antibodies would not be ideal for inhibition of these proteins.

The SOCS1-mimetic peptides Tkip [404] and SOCS-KIR target and inhibit pJAK2 and STAT activation, respectively, and they have been used in animal studies [407] to mimic the

inhibitory effects of SOCS1. Others have engineered cell-penetrating SOCS1 [384] and SOCS3 [408] peptides that show anti-inflammatory efficacy *in vitro* and *in vivo*. Through collaboration with these laboratories or in-house synthesis, these peptides could be used to study the effects of overexpression of SOCS1 or SOCS3 during experimental MCMV retinitis.

If MCMV exploits host SOCS1 and/or SOCS3 proteins to decrease cytokine production and/or signaling to increase viral load and pathogenesis during experimental MCMV retinitis, then knockdown of SOCS1 and/or SOCS3 during experimental MCMV retinitis would be expected to increase cytokine production and/or signaling, decrease viral load, and/or decrease frequency/severity of retinitis. In this case, overexpression of SOCS1 and/or SOCS3 would be expected to have the opposite effects. Furthermore, results from the current study suggest that this would occur in a tissue-specific or cell-type-specific manner.

Inhibiting expression of SOCS-inducing cytokines or STAT proteins could be used to determine their mechanistic contribution SOCS1 and/or SOCS3 stimulation during experimental MCMV retinitis. Systemic or conditional genetic knockout, genetic knockdown, or antibody-targeted sequestration of SOCS-inducing cytokines such as IFN- γ or IL-6 could be used to confirm whether SOCS1 and/or SOCS3 induction is dependent on these or other cytokine inducers of SOCS proteins. If MCMV infection stimulates SOCS1 and/or SOCS3 in some retinal cells as an indirect consequence of virally-stimulated cytokines, then inhibition of these SOCS-inducing cytokines would be expected to reduce SOCS1 and/or SOCS3 expression during MCMV-related retinitis. Although this might be expected to result in reduced severity of disease, it is possible that such cytokine inhibition may or may not occur with a change in the severity of retinitis. For instance, if SOCS1 and/or SOCS3 contribute to retinal pathogenesis by selectively inhibiting the effectiveness of these cytokines, then silencing of one or more of these cytokines

may produce a similar pathologic effect to what is observed during SOCS stimulation, even if SOCS proteins are not stimulated. It may be possible that dual-role or alternative mechanisms of SOCS modulation during MCMV infection could be due either to contributions from multiple pathways, or to compensatory mechanisms during knockout of any of these cytokines.

The use of mice deficient or conditionally deficient in STAT1, STAT2, or STAT3 may be useful in future *in vivo* or *ex vivo* studies to elucidate the role of these proteins in SOCS1 and/or SOCS3 induction during MCMV infection. STAT1 knockout mice (B6.129S(Cg)-*Stat1*^{tm1Dlv}/J), STAT2 knockout mice (B6.129-*Stat2*^{tm1Shnd}/J), and STAT3-floxed mice (B6.129S1-*Stat3*^{tm1Xyfu}/J) have only recently become commercially available (Jackson Labs) within the duration of the undertaking of the current studies. STAT1 knockout mice must be kept under specific pathogen free conditions due to their insensitivity to signaling by antiviral IFNs and concomitant susceptibility to viral infections [409]. STAT2 knockout mice may be useful in subsequent *in vivo* or *ex vivo* experiments to uncouple the roles of signaling by type I (STAT1 and STAT2) or type II (STAT1 only) IFNs. STAT3 knockout mice die during embryonic development [410], but STAT3-floxed mice are viable and display conditional STAT3 knockout when crossbred with mice containing conditionally-expressed cre-recombinase.

Expression vectors containing MCMV genes could be used to test whether exposure to these genes or gene products is sufficient for stimulating SOCS1 and/or SOCS3 *in vitro*. Whether MCMV IE or E genes are sufficient to stimulate SOCS1 and/or SOCS3 could be assessed *in vitro* or *in vivo* by introducing one or more viral genes into host cells by plasmid transfection or by CRISPR/Cas9 methods for gene knock-in [411]. Furthermore, the necessity of one or more of these viral genes for SOCS1 and/or SOCS3 expression could be assessed during MCMV infection by the addition of MCMV gene-specific targeting by CRISPR/Cas9 or siRNA.

5.4 Clinical Significance

AIDS-related HCMV retinitis remains the leading cause of blindness among untreated HIV/AIDS patients worldwide. Currently available treatments for this disease only prevent further vision loss, and failure to treat it results in blindness of most or all of the affected eye, usually followed within one year by vision loss in the contralateral eye (reviewed in [1-6]). HCMV replication generally can be controlled by lifelong administration of antiviral drugs (ganciclovir, cidofovir, or foscarnet), but these drugs cause harmful side-effects, do not eradicate the virus, and merely slow the progression of HCMV-caused ocular or neuronal damage without reversing it [38-42]. In addition, frequent administration of these drugs has led to an increase in drug-resistant strains of HCMV [412]. Vaccination has been one of the most effective methods for controlling other problematic infectious diseases, but three decades of attempts to engineer an effective vaccine against HCMV so far have been unsuccessful [43, 44]. AIDS-related HCMV retinitis may be indirectly prevented with cART to treat HIV infection [1, 36], but although this has greatly reduced the number of new cases of AIDS-related HCMV retinitis in the United States, it has failed to eliminate them [37].

Understanding the pathogenesis of this disease is essential for developing new, safe, and effective treatments for its prevention or management, yet much has remained unknown about the virologic and immunologic mechanisms contributing to its pathology. Because the species-specificity of HCMV precludes its ability to establish productive infection in animal models or cells [244], MCMV is commonly substituted in research laboratories to investigate cytomegalovirus infection and pathogenesis in mouse models [61, 245]. Such research with MCMV has significantly improved our collective understanding of HCMV characteristics and

pathogenesis, including the involvement of immune cell types such as CD8⁺ T cells and NK cells in controlling infection (reviewed in [6]).

To study the virologic and immunologic mechanisms of AIDS-related HCMV retinitis, we use a well-established, reproducible, and clinically relevant animal model with retrovirus-induced MAIDS that mimics in mice the symptoms and progression of AIDS in humans. AIDS of humans and MAIDS of mice are both caused by species-specific retroviruses and share many immunologic and pathologic features [9, 229]. Both syndromes are characterized by progressive generalized lymphadenopathy, polyclonal B-cell activation [230], diminished CD4⁺ T-cell and CD8⁺ T-cell functions [231], and a T_H1-to-T_H2 shift in cytokine profiles [228, 232, 233]. Although profound splenomegaly occurs in MAIDS mice but not in AIDS, this overall increase in the numbers of splenic cells is associated with dysfunctional immune cells [235]. Mice with late-stage MAIDS develop a retinitis 8-10 days following subretinal MCMV injection that exhibits histopathologic features similar to those found in AIDS-related HCMV retinitis [8, 81], including full-thickness retinitis, cytomegalic cells, and transition zones of histologically normal to diseased retina [8]. MAIDS-related MCMV retinitis is therefore a clinically-relevant, reproducible model for studying the pathogenesis of AIDS-related HCMV retinitis.

By using clinically relevant *in vivo* models of experimental MCMV retinitis and *in vitro* studies with MCMV infection, the work of this dissertation has contributed to the field of vision research by providing a better understanding of the basic virologic and/or immunologic mechanisms of retinal destruction that occur during the pathogenesis of AIDS-related HCMV retinitis. Together with the findings of the work presented herein and future experiments, SOCS1 and/or SOCS3 may reveal themselves as novel therapeutic targets to improve the management and/or prevention of AIDS-related HCMV retinitis. If SOCS1 and/or SOCS3 contribute to the

pathogenesis of this disease, then their inhibition in HIV/AIDS patients with HCMV retinitis could prevent further damage to affected eyes and/or protect the contralateral eye from vision loss. Strategies for targeting and inhibiting these proteins clinically could include SOCS1- and SOCS3-sequestering peptides such as pJAK2(1001-1013) [283, 404, 405], or gene therapy with CRISPR/Cas9 technology, or other techniques as they become clinically available. Because SOCS1 and SOCS3 dampen the ability of cytokines to propagate effective signals within their target cells, inhibition of SOCS1 and/or SOCS3 coupled with immunotherapy treatments such as antiviral interferons [413] could improve the efficacy of such treatments.

We cannot yet rule out the possibility that the immunosuppressive effect of SOCS1 and/or SOCS3 may play a protective role against a potential immunopathology of experimental MCMV retinitis or AIDS-related HCMV retinitis. This is the case for experimental autoimmune uveitis [414, 415], and SOCS1-mimetic peptides such as Tkip or SOCS-KIR reduce the severity of this disease in animal models [384, 416]. Further studies utilizing knockdown or overexpression of SOCS1 or SOCS3 would elucidate this possibility for experimental MCMV retinitis and/or AIDS-related HCMV retinitis. If overexpression of SOCS1 and/or SOCS3 results in less severe retinitis, this would suggest that SOCS1 and/or SOCS3 mimetics or overexpression treatment strategies might be used to combat this disease.

The results of this work therefore provide crucial basic knowledge that contributes to our understanding of the virologic, immunologic, and/or pathologic mechanisms of AIDS-related HCMV retinitis and, together with future studies, may contribute to the development of novel therapeutic targets that could improve the treatment or management of this sight-threatening disease.

5.5 Summary

5.5.1 Summary of Specific Aim 1

Systemic MCMV without MAIDS moderately stimulates splenic SOCS1 and SOCS3 proteins and SOCS-inducing cytokines type II IFN and IL-6, but this stimulation decreases in amplitude as MAIDS progresses. This does not appear to be due to any effect that MuLV itself has on SOCS1 and/or SOCS3 production as MAIDS progresses, because MAIDS progression does not affect SOCS1 mRNA and causes a progressive increase in SOCS3 mRNA in whole splenic cells [350]. A likely explanation is that the cell type(s) responsible for SOCS1 and/or SOCS3 production becomes dysfunctional or underrepresented as other cell populations increase. Systemic MCMV with MAIDS progression does not stimulate ocular SOCS1 or SOCS3, although SOCS-inducing type II IFN is transcriptionally up-regulated. Transcriptional stimulation of type II IFN in the eye during systemic MCMV infection without ocular pathogenesis is insufficient to stimulate ocular SOCS1 or SOCS3 mRNA transcripts.

Subretinal MCMV during late-stage MAIDS, when mice are susceptible to retinitis, highly stimulates intraocular SOCS1 and SOCS3 as well as type II IFN and IL-6, but this stimulation progressively declines as MAIDS progresses through weeks 10 and 12. There is also a decreased intraocular stimulation of SOCS1 and SOCS3 during experimental MCMV retinitis of C57BL/6 mice with corticosteroid-induced immune suppression. *In vivo* infection with MCMV therefore induces SOCS1 and SOCS3 mRNA and protein expression under specific conditions that are related to severity of ocular disease.

The transcriptional decline in SOCS1 and SOCS3 stimulation during the later weeks of MAIDS progression and during corticosteroid-induced immune suppression is likely due to the progressive dysfunction (during MAIDS) or decrease in number (during corticosteroids) of

SOCS-expressing cell types. Macrophages, NK cells, or retinal glial cells are strong cellular candidates for this hypothesis. MCMV-stimulated ocular SOCS1 and/or SOCS3 therefore putatively fulfill a crucial function(s) upon initiation of late-stage MAIDS-8 and MAIDS-10 that becomes unnecessary as cell populations or functions decline as late-stage MAIDS-12 progresses and as occurs with corticosteroid-induced immune suppression. Furthermore, MCMV replication in ocular tissues is required, but not sufficient, to stimulate ocular SOCS1 and/or SOCS3 during MAIDS-related MCMV retinitis.

5.5.2 Summary of Specific Aim 2

Stimulation of SOCS1 and SOCS3 mRNA in SG-MCMV-infected IC-21 mouse macrophages or MEF cells follows IE gene expression kinetics, but dependence on viral replication is different between cell types. This suggests a cell-type-specific driven dependence on MCMV gene expression and/or replication. In addition, MCMV-related SOCS1, SOCS3, and type II IFN stimulation in IC-21 mouse macrophages is dependent on the cellular origin of virus stock preparation (SG-MCMV vs. TC-MCMV).

MCMV replication is necessary for early stimulation of mRNA transcripts for SOCS1, SOCS3, and SOCS-inducing cytokines in IC-21 mouse macrophages, but not in MEF cells. Failure of UVi-MCMV to stimulate SOCS1 or SOCS3 in IC-21 mouse macrophages suggests that viral gene expression is necessary for stimulation of SOCS1, SOCS3, and SOCS-inducing cytokines. Host or viral RNA or other genetic material packaged into the tegument, however, would also be expected to be damaged during UV inactivation. Therefore, we have not yet eliminated the possibility that infection of IC-21 cells may stimulate SOCS1 and/or SOCS3 by one or more viral or host transcripts that are packaged into the tegument of SG-MCMV, but not TC-MCMV.

We furthermore provide evidence of a biphasic pattern of SOCS3 expression during MCMV infection of IC-21 macrophages. Direct infection is not necessary for early SOCS1 and SOCS3 protein stimulation in IC-21 cells. This suggests that MCMV infection stimulates SOCS-inducing factors that stimulate SOCS1 and SOCS3 in uninfected bystander cells in a MCMV IE2-independent manner. Taken together, these findings furthermore imply a putative role for other MCMV IE, E, or even tegument-packaged host or viral RNA, on SOCS1 and/or SOCS3 stimulation.

The virologic mechanism(s) of SOCS1 or SOCS3 expression during MCMV or HCMV infection therefore depend on cell type and virus passage origin. Furthermore, MCMV IE, E, or tegument-packaged host or viral RNA may govern biphasic SOCS1 and/or SOCS3 stimulation. Direct MCMV infection is not required to stimulate SOCS1 and SOCS3 expression in uninfected bystander macrophages, implicating a role for SOCS-inducing cytokines such as IFN- γ or IL-6.

5.6 Conclusions

Herein we tested **the central hypothesis that MCMV stimulates and employs SOCS1 and/or SOCS3 to induce onset and development of MCMV retinal disease.** The results of this study suggest the following conclusions:

- (i) *In vivo* infection with MCMV induces SOCS1 and SOCS3 mRNA and protein expression under specific conditions that are related to severity of ocular disease,
- (ii) MCMV-stimulated ocular SOCS1 and/or SOCS3 fill a putative critical role upon initiation of late-stage MAIDS-8 and MAIDS-10, but this role becomes less critical as certain cell populations and/or functions decline during late-stage MAIDS-12 or during corticosteroid-induced immune suppression,
- (iii) MCMV replication in ocular tissues is required, but not sufficient, to stimulate ocular SOCS1 and/or SOCS3 during MAIDS-related MCMV retinitis,
- (iv) The virologic mechanism(s) of SOCS1 or SOCS3 expression during MCMV or HCMV infection depends on cell type and virus passage origin,
- (v) MCMV IE, E, or tegument-packaged host or viral RNA may govern biphasic SOCS1 and/or SOCS3 stimulation, and
- (vi) Direct MCMV infection is not required to stimulate SOCS1 and SOCS3 expression in uninfected bystander macrophages, implicating a role for SOCS-inducing cytokines such as IFN- γ or IL-6.

Cytomegalovirus infection therefore stimulates SOCS1 and SOCS3 through divergent virologic or immunologic mechanisms in a cell-type-specific manner that reflects the complexity of the ocular compartment during the pathophysiology of retinal disease.

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