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Roles of TH2 and TH17 CD4+ T-Helper Cell Cytokines in the Pathogenesis of Experiemental Cytomegalovirus Retinitis

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ROLES OF TH2 AND TH17 CD4+ T-HELPER CELL CYTOKINES IN THE PATHOGENESIS OF EXPERIMENTAL CYTOMEGALOVIRUS RETINITIS

by

EMILY LAUREN BLALOCK

Under the Direction of Richard D. Dix

ABSTRACT

Human cytomegalovirus (HCMV) is a betaherpesvirus that infects up to 80% of the population worldwide, and establishes latency in monocytes and bone marrow cells. Reactivated HCMV can become an opportunistic pathogen in individuals who are immunocompromised, such as those with acquired immunodeficiency syndrome (AIDS). HCMV infection of AIDS patients causes a sight-threatening retinitis that leads to vision loss and blindness in up to 46% of this population without antiretroviral treatment. Because untreated HIV-infected individuals exhibit the loss of cell-mediated immunity and alterations in CD4+ T-helper (Th) cell cytokines, including elevation of interleukin-4 (IL-4), IL-10, and IL-17, we sought to test the hypothesis that these cytokines play key roles in governing the susceptibility to AIDS-related HCMV retinitis. This hypothesis was tested utilizing a clinically relevant mouse model of experimental murine cytomegalovirus (MCMV) retinitis that occurs in C57BL/6 mice immunosuppressed by mouse retroviruses (MAIDS). Studies revealed that MAIDS progression was associated with increased lev-

els of IL-4 and IL-10, cytokines whose production has been associated with diminished CD8+ T-cell-mediated immunity during HIV infection. However, MCMV–infected eyes of retinitis-susceptible IL-$4^{-/-}$ or IL-$10^{-/-}$ MAIDS mice exhibited frequency and severity of retinitis and viral titers equivalent to MCMV-infected eyes of wild-type MAIDS animals. These studies indicated that neither IL-4 nor IL-10 alone play key roles in increased susceptibility to MCMV retinitis. In comparison, IL-17, an inflammatory cytokine associated with the ocular autoimmune disease uveitis, was systemically increased during the progression of MAIDS, but MCMV-infected eyes of retinitis-susceptible MAIDS mice exhibited a significant reduction in IL-17. These findings suggested that IL-17 plays no direct role in the pathogenesis of experimental MCMV retinitis. However, these results also suggested the remarkable possibility that MCMV downregulates IL-17 production, a hypothesis supported by the observation that systemic MCMV infection of healthy and MAIDS mice resulted in the downregulation of IL-17. Mechanistic studies revealed that knockdown of IL-10 resulted in a partial recovery IL-17 levels during MCMV infection. We conclude that MCMV-induced IL-17 downregulation occurs via the stimulation of IL-10 and the suppressor of cytokine signaling (SOCS)-3. Taken together, our results add new information to the immunobiology of HCMV and to our basic understanding of the pathogenesis of AIDS-related HCMV retinitis.

INDEX WORDS: Cytomegalovirus, Acquired immunodeficiency syndrome (AIDS), Retinitis, Murine acquired immunodeficiency syndrome, Murine cytomegalovirus, CD4+ T-helper 17 cells, CD4+ T-helper 2 cells, Interleukin-10, Interleukin-4, Interleukin-17

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DEDICATION

I dedicate this dissertation to my family and loved ones. To my parents, Colin and Jessica Blalock, your unconditional love, encouragement, and support have provided me with the perseverance to achieve my life goals. Thank you for always being there for me when I needed you both. To my bother Michael and his family, Leigh and Hudson, you all have brought so much joy and laughter into my life. To the love of my life, Sturla, you have been my rock as well as a soft place to fall, my patience when I had none, and my laughter when I have been at my lowest. I will love you always. To my all of my GSU friends, past and present, thank you for all of your support and camaraderie; I have truly made some life-long friends during this journey. Without each of you, none of this would have been possible; I am truly indebted to you all. Thank you again from the bottom of my heart.

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LIST OF ABBREVIATIONS

Cytomegalovirus (CMV)

Human cytomegalovirus (HCMV)

Murine cytomegalovirus (MCMV)

Human Immunodeficiency Virus (HIV)

Acquired Immune Deficiency Syndrome (AIDS)

Murine AIDS (MAIDS)

Open reading frame (ORF)

Immediate Early genes (IE)

Delayed-early genes (DE)

Late genes (L)

Unique long (UL)

Unique short (US)

Murine (m)

Glycoprotein (gp)

Phosphoprotein (pp)

Major histocompatibility complex I (MHCI)

Major histocompatibility complex II (MHCII)

Antiretroviral therapy (ART)

Central nervous system (CNS)

Retinal pigment epithelium (RPE)

Natural killer cells (NK)

T-helper cells (Th)

Antigen presenting cells (APCs)

Interferon-gamma (IFN-γ)

Interleukin-2 (IL-2)

Delayed-typed hypersensitivity (DTH)

Interleukin-4 (IL-4)

Interleukin-6 (IL-6)

Interluekin-10 (IL-10)

Immunoglobulin E (IgE)

Interleukin-17 (IL-17)

Fas-ligand (FasL)

Transforming growth factor beta (TGF-β)

Interleukin-23 (IL-23)

Murine AIDS (MAIDS)

Tumor necrosis factor-alpha (TNF-α)

Simian immunodeficiency virus (SIV)

Suppressor of cytokine signaling-1 (SOCS-1)

Suppressor of cytokine signaling-3 (SOCS-3)

Plaque forming unit (PFU)

Intraperitoneal (i.p.)

Post-infection (p.i)

1 INTRODUCTION

1.1 Classification and Clinical Significance of Herpesviruses

Herpesviruses are widely distributed in nature, with at least one herpesvirus found within a majority of animal species. The family *Herpesviridae* is characterized as viruses with large double-stranded DNA genomes [120-240 kilobase pairs (kbp)], viruses that replicate and assemble within the nucleus, and viruses that establish life-long latent infection (Arvin et al., 2007; Knipe and Howley, 2007; Wildy et al., 1960). The *Herpesviridae* family is divided into three subfamilies: *Alphaherpesvirinae*, *Gammaherpesvirinae,* and *Betaherpesvirinae* (Arvin et al., 2007; Knipe and Howley, 2007; Matthews, 1979; Melnick, 1982; Roizman et al., 1981). The *Alphaherpesvirinae* subfamily is neurotropic in nature and includes herpes simplex virus type-1 (HSV-1), herpes simplex virus type-2 (HSV-2), and varicella-zoster virus (VZV) (Davison et al., 2009; Enquist et al., 1998; Evans and Melnick, 1949; Knipe and Howley, 2007; Schneweis and Brandis, 1961; Smith and Melnick, 1962; Weller et al., 1958). Alphaherpesviruses initially infect epithelial cells via direct contact, and the virus quickly disseminates to sensory ganglia where latent infection is established (Cohrs and Gilden, 2011; Cohrs and Gilden, 2001; Knipe and Howley, 2007; Melnick, 1982). Viral reactivation is triggered through stress and/or other environmental factors (Cohrs and Gilden, 2011; Cohrs and Gilden, 2001; Knipe and Howley, 2007). Epstein-Barr virus (EBV) and *Human herpesvirus* 8 [(HHV-8), also known as Kaposi's sarcoma-associated herpesvirus (KSHV)] are found within the lymphotropic *Gammaherpesvirinae* subfamily (Antman and Chang, 2000; Burkitt and O'Conor, 1961; Epstein et al., 1964). Like alphaherpesviruses, gammaherpesviruses initially infect epithelial cells, but gammaherpesviruses dis-

seminate through and establish latency in B cells (Antman and Chang, 2000; Davison et al., 2009; Henderson et al., 1977; Knipe and Howley, 2007; Rivas et al., 2001). Lastly, viruses found within the subfamily *Betaherpesvirinae* are lymphotropic in nature and include *Human herpesvirus* 6 (HHV-6), *Human herpesvirus* 7 (HHV-7), and human cytomegalovirus (HCMV) (Caselli and Di Luca, 2007; Davison et al., 2009; Frenkel et al., 1990; Knipe and Howley, 2007; Salahuddin et al., 1986). Betaherpesviruses initially infect epithelial cells found within mucosal surfaces and disseminate via leukocytes where latency is established. HCMV-infected cells become enlarged and display a pathology known as cytomegalia (Goodpasture, 1921; Knipe and Howley, 2007; Melnick, 1982).

HCMV was first noted in the 1900's to cause an owl-eye pathology in the salivary gland, pancreas, liver, lungs, and kidneys of infants at autopsy (Farber and Wolbach, 1932; Goodpasture, 1921; Ribbert, 1904). This pathology was later coined cytomegalic inclusion disease (CID) (Knipe and Howley, 2007; Wyatt et al., 1950). HCMV was first isolated from human salivary glands in 1955 by Margaret Smith (Smith, 1956); two other independent laboratories soon followed suit with the isolation of HCMV as well (Craig et al., 1957; Rowe et al., 1956). Prior to the isolation of HCMV, Smith also isolated the mouse-specific virus, murine cytomegalovirus (MCMV), from mouse salivary glands (Knipe and Howley, 2007; Smith, 1954). While CMVs infect numerous animal species, and while CMVs share genomic structure and organization, they diverge from one another based upon their host genes and are therefore species-specific (Davison et al., 2003; Hudson, 1979; Jordan, 1983; Knipe and Howley, 2007; Reddehase, 2006). HCMV infects up to 80% of the population worldwide (Knipe and Howley, 2007; Reddehase, 2006). HCMV infection is typically asymptomatic, and the virus establishes a life-

long infection in monocytes and bone marrow cells (Knipe and Howley, 2007; Mendelson et al., 1996; Reddehase, 2006; Sinclair, 2008; Slobedman and Mocarski, 1999; Taylor-Wiedeman et al., 1991). HCMV can become pathogenic, however, in individuals who are immunocompromised. Additionally, HCMV is able to cross the placenta during pregnancy to cause disease in developing neonates known as congenital cytomegalovirus infection (Crough and Khanna, 2009; Knipe and Howley, 2007; Reddehase, 2006).

1.2 Human Cytomegalovirus Genome and Virion Structure

The HCMV genome consists of linear double-stranded DNA. The genome is divided into two regions: the unique long (UL) region and the unique short (US) region (Kilpatrick and Huang, 1977; Spaete and Mocarski, 1985a; Weststrate et al., 1980). The regions' ends are flanked by terminal repeats, and the regions are divided by a series of internal repeats (Figure 1.1) (Knipe and Howley, 2007; Spaete and Mocarski, 1985a; Weststrate et al., 1980). The genome of approximately 235 kbp consists of 200 open reading frames (ORFs) that encode approximately 70 proteins (Crough and Khanna, 2009; Davison et al., 2003; Dunn et al., 2003b; Reddehase, 2006).

HCMV virions are approximately 250 nm in diameter, spherical, and enveloped (Wildy et al., 1960; Wright et al., 1964). The virion envelope surrounds the viral tegument proteins and the viral DNA-containing nucleocapsid. The tegument consists of a majority of the virion's proteins, making up 40% of the virion's mass (Kalejta, 2008; Knipe and Howley, 2007). In addition, the tegument area houses both viral and host cell RNA (Bresnahan and Shenk, 2000; Greijer et al., 2000; Knipe and Howley, 2007; Terhune et al., 2004). Viral tegument proteins have a wide array of functions during HCMV infection including mediating virion delivery to the host cell nu-

cleus, activating immediate early (IE) gene expression, and evading the host cell immune response (Davison and Bhella, 2007; Kalejta, 2008; Miller-Kittrell and Sparer, 2009). The 125-nm icosahedral nucleocapsid, which houses the viral DNA, is embedded in the tegument and is composed of 5 proteins: major capsid protein (MCP), minor capsid protein (TRI1), minor capsid protein binding protein (TRI2), smallest capsid protein (SCP), and the portal protein (PORT) (Butcher et al., 1998; Chee et al., 1989; Dittmer and Bogner, 2005; Gibson et al., 1996; Irmiere and Gibson, 1985; Knipe and Howley, 2007).

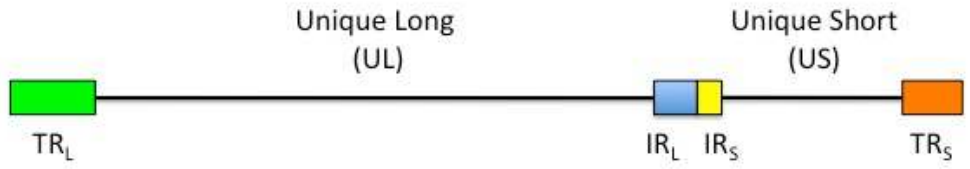

Figure 1.1. Schematic representation of HCMV genome. The HCMV genome is divided into two regions of sequences, the unique long (UL) and unique short (US) sequence regions. The UL and US are flanked by terminal repeats: terminal repeat long (TR_L) and terminal repeat short (TR_S). The UL and US are divided by a series of internal repeats: internal repeat long (IR_L) and internal repeat short (IR_S). Adapted from (Kotenko et al., 2000).

1.3 Cytomegalovirus Replication Cycle

HCMV is able to infect numerous cell types including epithelial cells, endothelial cells, fibroblasts, myeloid cells, and retinal pigment epithelial (RPE) cells (Bodaghi et al., 1999; Knipe and Howley, 2007; Miceli et al., 1989; Reddehase, 2006; Sinzger, 2008). The replication and assembly of HCMV occurs within the nucleus of the host cell (Figure 1.2). HCMV genes are transcribed in a regulated cascade beginning with immediate early (IE, α) genes, followed by delayed-early or early (DE or E, β) genes, and lastly late (L, γ) genes (Reddehase, 2006; Stinski, 1978). Entry of the virion into the cell is mediated through the interactions of glycoprotein B (gB), the glycoprotein H (gH): glycoprotein L (gL) complex, and the glycoprotein M (gM): glycoprotein N (gN) complex with heparan sulfate and/or other unidentified receptor(s) on the host cell surface (Carlson et al., 1997; Huber and Compton, 1997; Kari and Gehrz, 1993; Knipe and Howley, 2007; Mach et al., 2000; Mach et al., 2005; Reddehase, 2006). Additionally, HCMV has been noted to enter endothelial cells through fusion with endocytic vesicles (Bodaghi et al., 1999; Knipe and Howley, 2007; Sinzger, 2008). Once in the cytoplasm, microtubules assist with the transport of the HCMV nucleocapsid to the host cell nucleus (Knipe and Howley, 2007; Ogawa-Goto et al., 2003). The viral DNA and viral tegument proteins are released into the nucleus. The expression of IE genes is initiated through viral tegument proteins phosphoprotein-71 (pp71) and UL35 (Knipe and Howley, 2007; Liu and Stinski, 1992; Schierling et al., 2005; Spaete and Mocarski, 1985b; Stinski and Roehr, 1985). IE gene transcription, which occurs within 2-4 hours after infection, results in the production of viral regulatory proteins that lead to the activation of DE or E genes (Knipe and Howley, 2007; Vancikova and Dvorak, 2001). DE genes encode for viral DNA replication machinery and proteins necessary for capsid maturation

(Knipe and Howley, 2007; Vancikova and Dvorak, 2001). DE gene products initiate L gene expression approximately 36 to 48 hours post infection; L genes encode for the structural proteins of the virion (Knipe and Howley, 2007; Vancikova and Dvorak, 2001). Accumulation of L-gene protein products within the host cell nucleus results in capsid assembly and viral DNA encapsidation (Knipe and Howley, 2007). The tegument proteins UL50 and UL53, as well as non-structural viral proteins, assist with the egress of the nucleocapsid from the nucleus; this is a two-step process, wherein the nucleocapsid buds through the inner nuclear membrane and gains an envelope (Knipe and Howley, 2007). The enveloped nucleocapsid then loses its envelope as it is released from the outer nuclear membrane (Knipe and Howley, 2007). The viral nucleocapsid is then re-enveloped in the cytoplasm at the endoplasmic reticulum golgi intermediate compartment (ERGIC) (Knipe and Howley, 2007). These mature virions are transported to the cell surface and exocytosed (Knipe and Howley, 2007).

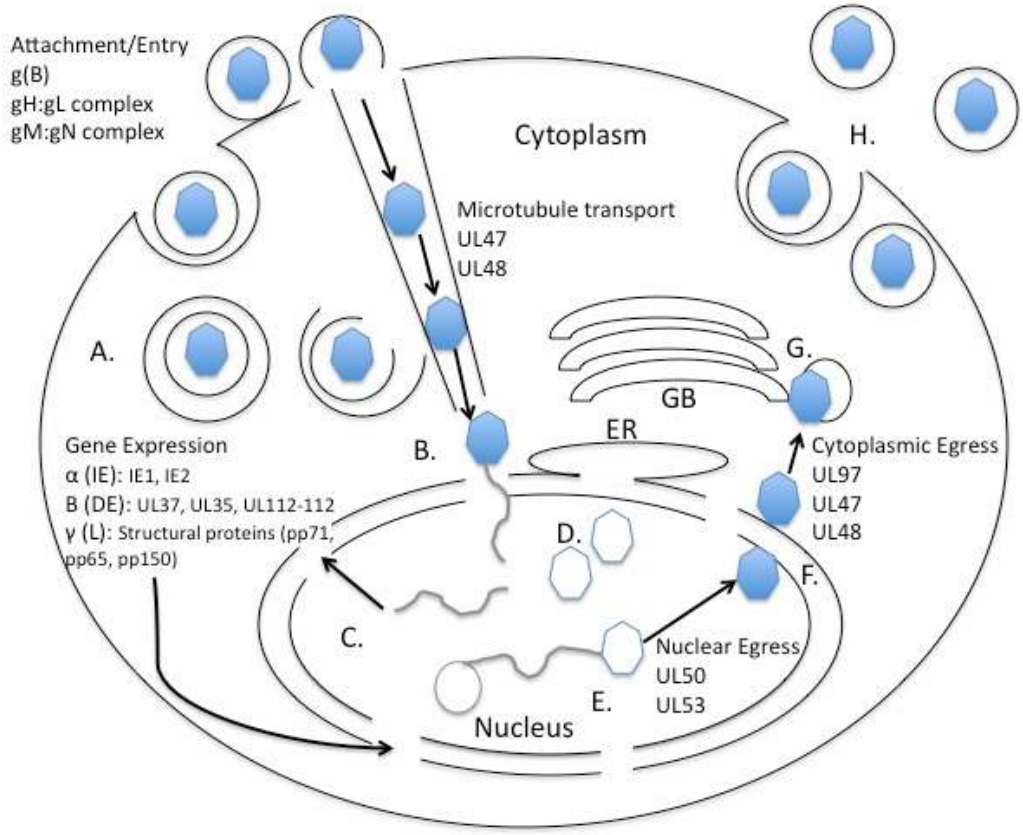

Figure 1.2. Human Cytomegalovirus replication cycle. (A) Virion attachment, entry and uncoating of viral nucleocapsid. (B) Transport of viral nucleocapsid to the nucleus via microtubules and release of viral DNA and tegument proteins into nucleus. (C) DNA replication, transcription, and translation of viral proteins under cascade regulation: IE, DE, and L genes. (D) Capsid protein assembly. (E) Viral DNA encapsidation. (F) Nuclear egress of the viral nucleocapsid. (G) Secondary envelopment at endoplasmic reticulum golgi intermediate compartment (ERGIC). (H) Release of viral progeny. ER, endoplasmic reticulum, and GB, golgi body. Based on (Knipe and Howley, 2007; Mocarski Jr, 2007).

1.4 HCMV Clinical Disease

HCMV is transmitted through bodily secretions including urine, saliva, and breast milk (Arvin et al., 2007; Britt, 2008; Crough and Khanna, 2009; Knipe and Howley, 2007; Vancikova and Dvorak, 2001). Primary infection typically occurs in young children in the daycare setting but can also occur in adolescents and adults (Britt, 2008; Knipe and Howley, 2007; Pass, 1985; Staras et al., 2008; Vancikova and Dvorak, 2001). Fifty to 90% of adults are seropositive for HCMV (Britt, 2008; Gandhi and Khanna, 2004; Staras et al., 2006; Vancikova and Dvorak, 2001). Similar to other herpesviruses, HCMV persists in the host for life (Gandhi and Khanna, 2004; Knipe and Howley, 2007; Slobedman and Mocarski, 1999; Vancikova and Dvorak, 2001). Following primary infection, HCMV becomes latent in monocytes and bone marrow cells (Knipe and Howley, 2007; Mendelson et al., 1996; Slobedman and Mocarski, 1999; Taylor-Wiedeman et al., 1991; Vancikova and Dvorak, 2001).

1.4.1 HCMV Infection of the Immunocompetent

In the immunocompetent host, acute infection is typically asymptomatic and self-limited. Though rare, symptoms in the immunocompetent include fever, fatigue, adenopathy, and splenomegaly, the compilation of these symptoms being HCMV infectious mononucleosis (heterophile antibody negative) (Britt, 2008; Klemola, 1973; Klemola and Kaariainen, 1965; Reddehase, 2006). HCMV-related symptoms are more common and more severe in the immunocompromised due to high, uncontrolled HCMV replication (Crough and Khanna, 2009; Kano and Shiohara, 2000; Reddehase, 2006; Rowshani et al., 2005). Additionally, immunocompetent individuals that receive blood transfusions can develop HCMV infectious mononucleosis

(heterophile antibody negative) (Lang, 1972; Monif et al., 1976; Vancikova and Dvorak, 2001). The risk for developing HCMV mononucleosis is approximately 3%; this risk increases with the number of blood units transfused and whether the individual receiving blood is HCMV sero-negative (Armstrong et al., 1976; Kane et al., 1975; Vancikova and Dvorak, 2001). Symptoms that present in immunocompetent patients during HCMV infection typically resolve within 1-4 weeks without treatment (Knipe and Howley, 2007; Vancikova and Dvorak, 2001). However, chronic HCMV infection in the immunocompetent population has been linked to atherosclerotic coronary artery disease, coronary restenosis, malignant gliomas, and inflammatory bowel disease (Adam et al., 1997; Cobbs et al., 2002; Melnick et al., 1996; Rahbar et al., 2003; Reddehase, 2006; Speir et al., 1994; Streblow et al., 2008). In addition, chronic HCMV infection has recently been established as a co-factor in age-related macular degeneration (Cousins et al., 2012).

1.4.2 Congenital and Perinatal HCMV Infection

HCMV is able to cross the placenta and cause congenital abnormalities in the auditory and visual organs of a growing fetus (Arvin et al., 2007; Knipe and Howley, 2007; Malm and Engman, 2007; Stagno et al., 1982). In developing countries, HCMV infection is the leading cause of congenital cytomegalovirus disease, with primary HCMV infection occurring in 2% of all pregnancies (Britt, 2008; Malm and Engman, 2007; Pass et al., 2006; Vancikova and Dvorak, 2001). The transmission rate from mother to fetus after primary infection is 40% (Britt, 2008; Malm and Engman, 2007; Vancikova and Dvorak, 2001). If primary infection of the mother occurs prior to the second trimester, the sequelae are much more severe than if primary infection

of the mother occurs during the third trimester (Pass et al., 2006; Vancikova and Dvorak, 2001). At birth, 7-10% of these infected neonates exhibit symptoms such as pneumonitis, liver disease, encephalitis, and clotting disorders (Malm and Engman, 2007; Vancikova and Dvorak, 2001). Of the neonates that survive, approximately 90% will have sensorineural hearing loss (Fowler and Boppana, 2006; Fowler et al., 1992; Vancikova and Dvorak, 2001).

Because HCMV can be transmitted through cervical secretions as well as through breast milk, it is possible for a healthy infant to become infected if the mother is HCMV-infected (Hayes et al., 1972; Reynolds et al., 1973). Perinatal infection that occurs in infants is typically asymptomatic and self-limited; however, symptoms that can occur include lymphadenopathy, pneumonitis, and hepatitis (Granstrom and Leinikki, 1978; Leinikki et al., 1978; Vancikova and Dvorak, 2001; Whitley et al., 1976). Depending on the severity of symptoms, treatment of perinatal infection includes administration of the antiviral ganciclovir (Nigro et al., 1997).

1.4.3 HCMV Infection of the Immunocompromised

When cell-mediated immunity is suppressed, as seen in solid-organ transplant recipients, bone-marrow transplant patients, and in human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) patients, persistent HCMV infection can lead to disease development due to the inability of CD4+ and CD8+ T cells to control viral replication and dissemination (Reddehase, 2000; Reddehase et al., 1985; Riddell et al., 1992; Steffens et al., 1998). The immunocompromised population, especially solid-organ transplant and bone-marrow transplant patients, is at risk for developing colitis and pneumonitis as a result of HCMV infection (Boeckh et al., 2003; Britt, 2008; Reddehase, 2006; Rowshani et al., 2005; Vancikova and

Dvorak, 2001). Less than 1% of allograft recipients experience HCMV infection of ocular structures (Britt, 2008). HCMV infection of the donor or of the transplant organ can contribute to organ rejection in transplant patients due to the reactivation of latent virus and ultimately uncontrolled virus replication in the transplanted organ (Britt, 2008; Cainelli and Vento, 2002; Grattan et al., 1989; Lopez et al., 1974; Rubin, 1989; Vancikova and Dvorak, 2001). In addition, HCMV-infected donor organs have been shown to express high levels of cellular adhesion molecules, leading to inflammatory cell infiltration and damage to the donor tissues (Cainelli and Vento, 2002; Steinhoff et al., 1996; Waldman and Knight, 1996). In contrast to allograft recipients, HCMV infection of late-stage HIV/AIDS patients who are not on antiretroviral therapy (ART) frequently causes a sight-threatening retinal necrosis in addition to gastrointestinal diseases and neurological sequelae (Britt, 2008; Dix and Cousins, 2004a; Reddehase, 2006; Vancikova and Dvorak, 2001); however, HCMV retinitis does not appear in AIDS patients until CD4+ T cell counts fall below 50 cells/mm^3 of blood, although late-stage HIV patients become susceptible to other opportunistic infections when CD4+ T cell counts fall below 200 cells/mm^3 of blood (AIDS) (Gerard et al., 1997; Palella et al., 1998; Salmon-Ceron et al., 2000).

1.5 The Anatomy of the Eye

The eye is divided into two segments: the anterior and posterior segments (Figure 1.3) (Cousins, 1997; Kiel, 2010; Kolb, 1995a; Newell, 1992). The sclera, comprised of connective tissue, encompasses the outer portion of the eye to provide integrity to the visual organ (Kolb, 1995a; Newell, 1992). The anterior segment consists of the ocular components necessary for focusing light onto the neurosensory retina: the crystalline lens and the cornea (Kiel, 2010;

Kolb, 1995a). In addition, the anterior segment contains the conjunctiva, iris, pupil, ciliary body, aqueous humor, and anterior uvea (Cousins, 1997; Kolb, 1995a). The conjunctiva is comprised of stratified-squamous epithelium that is interspersed with goblet cells and functions to secrete mucins that comprise the mucus of the tear film that helps to protect the ocular surface of the eye (Gipson, 2007; Inatomi et al., 1996). The pupil is the portion of the eye that allows light to enter, and the colored iris acts to control the size of the pupil, allowing more or less light to enter depending upon external conditions (Kardon, 1995; Kolb, 1995a; Wilhelm, 2008). The aqueous humor, a fluid that is produced by the non-pigmented epithelial cells of the ciliary body, bathes the crystalline lens and helps to maintain proper ocular pressure; the aqueous humor also contains growth factors and other nutrients (Brubaker, 1991; Chowdhury et al., 2010; Cousins, 1997; To et al., 2002). The uvea, located between the sclera and the retina, is the pigmented vascular portion of the eye (Caspi, 2010; Commodaro et al., 2011). The anterior uvea is comprised of the iris and the ciliary body, while the posterior uvea consists of the retinal choroid (Caspi, 2010; Commodaro et al., 2011).

The posterior segment consists of the vitreous cavity, the neurosensory retina, the retinal pigment epithelium (RPE), and the choroid (posterior uvea) (Cousins, 1997; Kolb, 1995a). The vitreous cavity comprises a majority of the posterior segment of the eye and is filled with a gel-like substance that is composed of hylauronic acid, collagen, water, proteins, and regulatory cytokines (Bishop, 2000; Scott, 1992). The neurosensory retina, the area of light processing, contains rods and cones (photoreceptors) as well as ganglion cells, bipolar cells, horizontal cells, amacrine cells, microglia and Müller cells (Dowling, 1987; Kolb, 1995b). Cones are concentrated in the foveal area of retina, where light is received, while rods dominate the remainder

of the retina with cones interspersed (Brown and Wald, 1964; Kolb, 1995c; Marks et al., 1964). Rod and cone cells are attached to the cells of the RPE. The RPE functions to absorb light, regulate the transport of metabolites in and out of the neurosensory retina, phagocytose damaged photoreceptor segments, and secrete cytokines such as transforming growth factor beta (TGF-β) (Strauss, 1995). Horizontal cells and amacrine cells are interneurons that mediate signaling between the photoreceptors and the retinal bipolar cells (Boycott, 1988; Boycott and Kolb, 1973; Gallego, 1971; Kolb, 1995b; Poche and Reese, 2009). Signals received by the bipolar cells are transmitted to the ganglion cells of the retina (Kolb, 1995b; Mariani, 1982). Both microglia and Muller cells work to maintain homeostasis of the neurosensory retina (Bringmann et al., 2006; Chen et al., 2002). Microglia are widely distributed throughout the neurosensory retina, and are located within the outer and inner plexiform layers, the ganglion cell layer, and in the nerve fiber layer (Boycott and Hopkins, 1981; Chen et al., 2002). Microglia act as both antigen presenting cells (APCs) as well as phagocytic cells when activated during infection and/or retinal damage (Chen et al., 2002). Müller cells span the entire thickness of the retina from the ganglion cell layer to the retinal blood vessels; hence, Müller cells possess numerous ion channels and transmembrane transporters that enable them to maintain retinal glucose metabolism, regulate blood flow within the retina, and maintain water and ion balance (Bringmann et al., 2006). In addition, in times of retinal injury and/or infection, Müller cells function as immunomodulators by releasing proinflammatory cytokines as well as phagocytosing foreign substances and damaged retinal cells (Bringmann et al., 2006). The actions of Müller cells during infection and/or retinal assault involve the cooperation of retinal microglia, ultimately resulting

in the alteration of retinal blood flow, which allows for the infiltration of leukocytes into the retina (Bringmann et al., 2006).

The eye is supplied by two vasculatures. The first vasculature originates from the ophthalmic artery, which branches into the central retinal artery and enters the eye through the optic nerve (Lang and Kageyama, 1990; Raviola, 1977). This artery then branches to supply blood to the neurosensory retina (Kolb, 1995d; Newell, 1992). The second blood supply originates from orbital arteries that penetrate the sclera to provide blood to the anterior segment, the uvea, the RPE, and the outer retina (Kur et al., 2012; Morrison et al., 1996; Raviola, 1977). Divisions of this vasculature supply blood to the choroidal vessels and ciliary body (Kur et al., 2012; Morrison et al., 1996). The lens and cornea are avascular during health; however, lacrimal glands located in the corner of each eye secrete tears that supply nutrients and protection to these structures (Cousins, 1997).

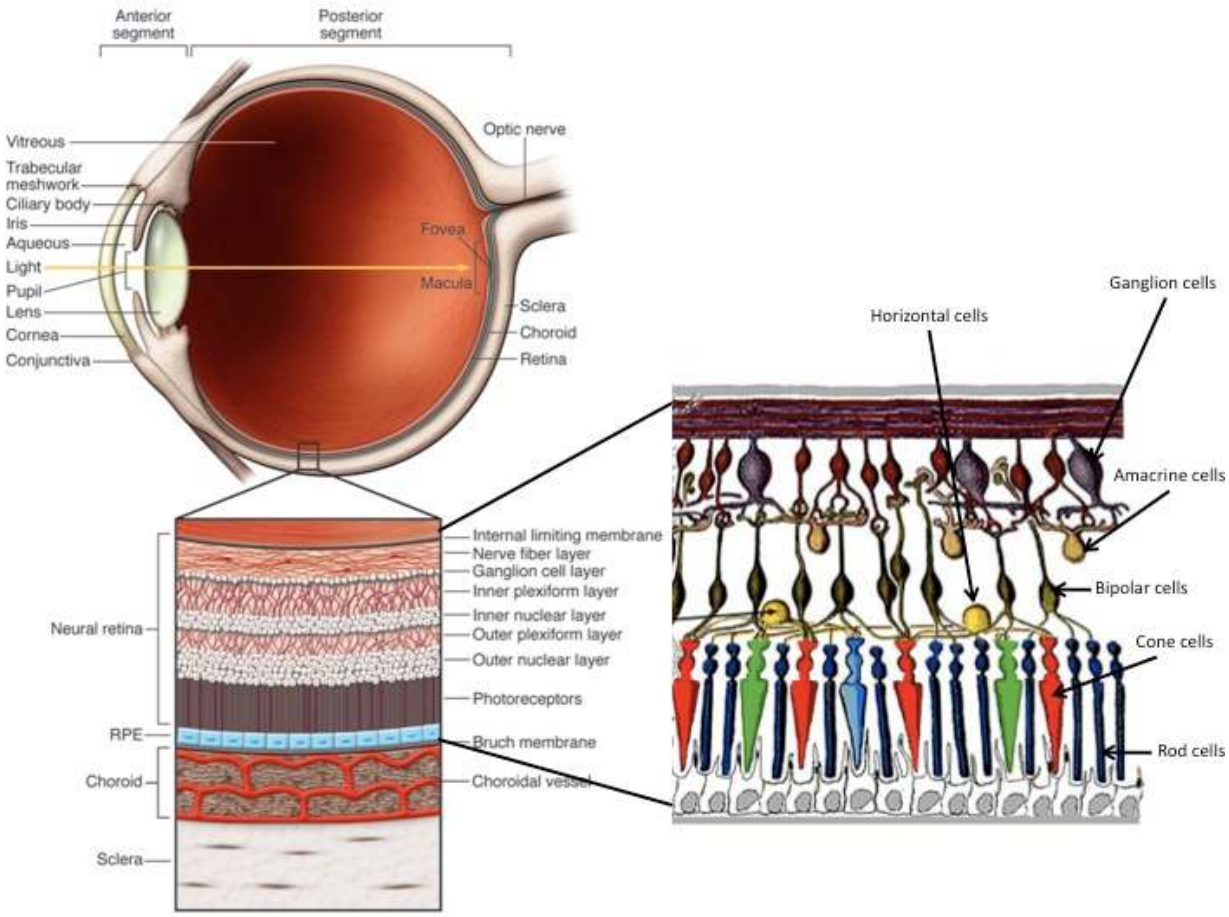

Figure 1.3. Structure of the eye and neurosensory retina. The eye is divided into two segments, the anterior and posterior segments. The anterior segment consists of the cornea and crystalline lens, structures necessary for receiving and focusing light onto the posterior segment of the eye. The posterior segment receives light at the central point of the retina, the fovea, and is then processed by the cells of the neurosensory retina (photoreceptors: rods and cones). Enlarged sections depict the cells and divisions of the neurosensory retina. (Caspi, 2010; Kolb, 1995d)

1.6 Ocular Immune Privilege

The eye is a site of immune privilege similar to the central nervous system (CNS) in that if foreign tissue were transplanted into the cornea, anterior chamber, the vitreous cavity, or the subretinal space, the tissue would survive indefinitely, whereas if the same foreign tissue were transplanted into another area of the body, it would be rejected by the recipient (Hazlett and Hendricks, 2010; Kaplan and Streilein, 1977; Medawar, 1948; Niederkorn, 1990; Streilein, 2003b). The eye is compartmentalized in a similar fashion to the blood-brain barrier of the central nervous system and is called the blood-retinal barrier (Ashton and Cunha-Vaz, 1965; Cunha-Vaz, 1979; Streilein, 2003b). In the posterior segment, the RPE and the retinal endothelial cells are non-fenestrated and have tight junctions (Shakib and Cunha-Vaz, 1966; Shiose and Oguri, 1969; Smith and Rudt, 1975). These two cell types physically regulate molecules entering and exiting the retina; for example, dye delivered to the vasculature is excluded from this portion of the eye that possesses non-fenestrated cells with tight junctions (Bellhorn, 1980, 1981; Cousins, 1997).

The blood-retinal barrier differs between the neurosensory retina and the uveal tract. While the passage of macromolecules into the retinal vasculature is tightly regulated, molecules freely pass through the barrier in the uveal tract via a concentration gradient (Cunha-Vaz, 1979; Cunha-Vaz et al., 1966; Raviola, 1977). Thus, the uveal tract typically contains numerous leukocytes including macrophages and dendritic cells (Bellhorn, 1980, 1981; Cousins, 1997). The barrier of the ciliary body is regulated similarly to the retinal vasculature so not to permit free flow of molecules into the aqueous humor (Cunha-Vaz, 1979; Shiose and Oguri, 1969; Smith, 1971).

However, the blood-retinal barrier is not absolute and when disrupted, unchecked immune effector cells can enter to cause tissue destruction, vision loss, and even blindness.

In addition to a physical barrier, both the aqueous humor and the cells of the neurosensory retina produce a number of immunoregulatory molecules, including TGF-β2, vasoactive intestinal peptide (VIP), somatostatin (SOM), and alpha-melanocyte stimulating hormone (α-MSH) that inhibit T cell activation in order to prevent extensive inflammation and ultimately harm to the visual organ (Streilein, 2003b; Taylor, 2009; Taylor and Lee, 2010; Taylor and Yee, 2003). A similar suppression of T cell activation also occurs when antigens are introduced into the anterior chamber. Anterior chamber-associated immune deviation (ACAID) occurs when macrophages (Lin et al., 2005; Wilbanks and Streilein, 1992a, b) within the anterior chamber of the eye process foreign antigens and/or alloantigens, migrate from the eye through the blood to the spleen, and initiate the production of suppressor T cells through the interaction with splenic B cells (D'Orazio and Niederkorn, 1998; Kaplan and Streilein, 1977; Streilein, 2003b). The resulting immune response leads to the suppression of the T-helper cell responses, including delayed-type hypersensitivity (DTH), to the eye-derived antigen (Streilein, 2003a, b). This non-inflammatory response provides the eye another line of defense against damaging inflammatory insults.

1.7 AIDS-related HCMV Retinitis

HIV/AIDS infection is characterized by the progressive loss of cell-mediated immunity. CD4+ T cells are the targets of HIV; therefore, HIV-infected patients exhibit a gradual loss of CD4+ T cells numbers and function in both the blood and mucosal sites of the body (Williams

and Burdo, 2009). While innate immune cells including macrophages, neutrophils, and natural killer cells, as well as adaptive immune CD8+ T cells, remain intact during HIV infection (Pantaleo et al., 1993; Poli et al., 1993; Williams and Burdo, 2009), the alteration of CD4+ T-helper cell signaling from a type 1 response to a type 2 response ultimately leads to the loss of cellular immunity. When CD4+ T cell numbers fall below 200 cells/mm^3 of blood, HIV-infected individuals become susceptible to a number of opportunistic infections, however, AIDS-related HCMV retinitis does not appear in this population until CD4+ T cell counts fall below 50 cells/mm^3 of blood (Dix and Cousins, 2004a; Gerard et al., 1997; Holland, 2008; Palella et al., 1998; Salmon-Ceron et al., 2000).

Among these is the slowly progressive retinal disease AIDS-related HCMV retinitis. First noted in the early 1980's to be affiliated with late-stage HIV infection, AIDS-related HCMV retinitis caused vision loss and blindness in up to 46% of HIV/AIDS patients prior to the advent of antiretroviral therapy (ART) (Dix and Cousins, 2004a; Holland, 2008). While ART has significantly reduced the number AIDS-related HCMV retinitis cases in the United States, incidence in the HIV/AIDS population remains high in developing countries such as Thailand and Africa where these drugs are not readily available (Heiden et al., 2007; Stewart, 2010).

HCMV retinitis is characterized by a progressive retinal necrosis, hemorrhage, viral inclusions, and cytomegalic cells (Dix and Cousins, 2004a). HCMV retinitis can occur in one or both eyes of the HIV/AIDS patient. HCMV disseminates through hematogenous spread of infected monocytes that enter the retinal vasculature to become activated macrophages (Dix and Cousins, 2004a).

1.8 MAIDS Animal Model of MCMV Retinitis

Murine AIDS (MAIDS) infection in C57BL/6 mice results in progressive changes in immune cell phenotype and function that closely resembles HIV/AIDS (Dix and Cousins, 2004a) (Table 1.1). MAIDS is caused by a mixture of mouse retroviruses collectively known as LP-BM5 (Mosier et al., 1985). The LP-BM5 mixture is comprised of a non-pathogenic helper virus that is replication-competent and a pathogenic virus that is replication-deficient (Chattopadhyay et al., 1991; Morse et al., 1995). The *gag* region products p15 and p12 of the defective, pathogenic virus have been shown to be essential to induce MAIDS in C57BL/6 mice (Kubo et al., 1994). The *gag* products of this region are expressed on B cells and act as a superantigen, which results in extensive T cell activation (Hugin et al., 1991) and perhaps diminishes the ability of T cells to respond to other pathogens as MAIDS infection progresses. While the primary targets of LP-BM5 infection are B cells, the retrovirus mixture is able to infect both T cells and macrophages (Kanagawa et al., 1994; Kim et al., 1994). In addition, mice devoid of mature B cells or that lack CD4+ T cells do not develop MAIDS (Kim et al., 1994; Yetter et al., 1988).

During the first 3 weeks of retrovirus infection, C57BL/6 mice present with a persistent generalized lymphadenopathy that is associated with early polyclonal B cell activation, expansion, and hypergammaglobulinemia (Gazzinelli et al., 1992; Klinman and Morse, 1989; Morse et al., 1995; Mosier et al., 1985). As MAIDS progresses, B cells become defective and are unable to respond to antigen due to compromised immunoglobulin-receptor signaling (Klinman and Morse, 1989; Selvey et al., 1995). Sixteen-weeks post retrovirus infection, B cell proliferation in MAIDS mice slows and numbers of antibody-secreting B cells decline, possibly due to bone mar-

row precursor exhaustion or to the alteration in CD4+ T cell cytokine production that occurs following week 4 of infection (Klinman and Morse, 1989).

Alterations in the CD4+ T-helper (Th) cell cytokine profile and profound dysfunction of cellular immunity in MAIDS mice become evident 3 to 4-weeks after retrovirus infection. During this time CD4+ T cells exhibit a shift in cytokine profile in the absence of antigen stimulation, such that the levels of type-1 (Th1) cytokines including IFN-γ and IL-2 become decreased and levels of the type-2 (Th2) cytokines IL-4, IL-6, and IL-10 are increased (Gazzinelli et al., 1992). In addition, concanavalin A stimulated CD4+ T cells isolated from MAIDS mice following this shift in cytokine profile fail to produce IL-2 or IFN-γ (Gazzinelli et al., 1992). However, the impaired ability of CD4+ T cells to respond to antigen or mitogen stimulation during MAIDS is not due to a decline in CD4+ T cell numbers (Mosier et al., 1985), but rather the induction of anergy in these CD4+ T cells (Muralidhar et al., 1992).

While innate immune responses from macrophages remain intact, this pivotal immunologic shift in CD4+ T cells leads to a progressive immunodeficiency characterized by abnormal CD8+ T cell and natural killer cell responses (Morse et al., 1989; Umemura et al., 2001) at approximately 8 weeks after retrovirus infection, which ultimately results in high susceptibility to several opportunistic diseases including murine cytomegalovirus (MCMV) retinitis. In fact, 100% of mice with MAIDS of 10 weeks' duration are susceptible to MCMV retinal disease by 10 days after subretinal MCMV inoculation (Dix and Cousins, 2004a). Moreover, the MAIDS-related MCMV retinal disease that develops presents with histopathologic features that closely resemble those observed in AIDS-related HCMV retinitis, specifically development of a striking full-thickness retinal necrosis that replaces the normal architecture of the retinal tissues within days

of virus infection in addition to hemorrhage, viral inclusions of the RPE, and cytomegalic cells (Dix and Cousins, 2004a). Therefore, the MAIDS model of experimental MCMV retinitis presents an attractive animal model to investigate the involvement of the CD4+ T cell cytokines IL-17, IL-4, and IL-10 in the context of AIDS-related HCMV retinal disease development.

Table 1.1. Mouse model of retrovirus-induced immunosuppression (MAIDS) development.

1 week	Polyclonal B cell activation
1-3 weeks	Generalized chronic lymphadenopathy
3 weeks	Th1 to Th2 shift in cytokine profile
4 weeks	Depressed T cell functions
8-10 weeks	Susceptible to opportunistic infections (MCMV retinitis)

1.9 Immunology of HCMV Infection

Both the innate and adaptive immune responses are key factors for host protection against HCMV-related diseases, and thus understanding the immune response to HCMV infection of the retina is of great importance for finding novel treatments of HCMV retinitis in HIV/AIDS patients. Because HCMV has strict species specificity, studying the pathogenesis of HCMV retinitis in HIV/AIDS patients has proved to be difficult. Thus MCMV, the mouse equiva-

lent of HCMV, has been utilized as it shares similar genomic structure and biological attributes with HCMV including pathogenesis, immunomodulation, and latency (Hudson, 1979; Jordan, 1983).

Firstly, like the HCMV genome, the MCMV genome consists of 230 kbps with an overall G+C content of 58% (Honess et al., 1989; Rawlinson et al., 1996). MCMV encodes for 170 genes, which are collinear with the central region of HCMV genome, spanning 180 kbps (Rawlinson et al., 1996). In addition, approximately 78 MCMV genes share significant homology with HCMV genes (Davison et al., 2003; Rawlinson et al., 1996). However, the ends of the MCMV genome encode for MCMV-specific glycoproteins (Rawlinson et al., 1996).

Secondly, MCMV can be transmitted between mice in a similar fashion that HCMV is transmitted between humans. MCMV transmission typically occurs through direct contact with saliva, as active MCMV replication is most prominent in the salivary glands of infected mice; in addition, MCMV can also be transmitted through sexual contact (Hudson, 1979; Staczek, 1990). Like HCMV, MCMV is able to infect numerous cells types *in vivo* including endothelial cells, epithelial cells, dendritic cells, and macrophages, the site of MCMV latency (Hsu et al., 2009; Jordan, 1983; Jordan and Takagi, 1983). MCMV infection of the immuocompetent mice is self-limited, but MCMV can become pathogenic in the immunocompromised mice to cause disease including myocarditis, pneumonitis, and retinitis (Craighead et al., 1992; Mutter et al., 1988; Shellam et al., 1985; Staczek, 1990). However, MCMV does not cross the placenta to cause cytomegalovirus-related congenital disease as is seen with HCMV infection of a developing fetus (Kashiwai et al., 1992; Tsutsui et al., 1993). Lastly, both HCMV and MCMV are susceptible to treatment with the antiviral ganciclovir (Shanley et al., 1985). Due to all of the similarities be-

tween MCMV and HCMV, MCMV presents an ideal model for studying HCMV-related disease pathogenesis and has begun to improve our understanding of the host immune response during HCMV infection, specifically HCMV retinitis (Dix and Cousins, 2004a; Knipe and Howley, 2007).

1.9.1 Innate Immune Response to HCMV Infection

As reviewed by Paul, 2008, the host defense against pathogens is divided into two types of responses: the innate immune response and the adaptive immune response. The innate immune response is the first line of defense against microbes and is comprised of specific components that perform distinct functions to protect the host from infection. Numerous immune cells including macrophages, neutrophils, and natural killer (NK) cells work to eliminate pathogens through direct phagocytosis of microbes or through the directed killing of infected cells. Virus-infected cells are able to secrete type I interferons, interferon-alpha (IFN-α) and interferon-beta (IFN-β), which turn on interferon-stimulated genes to induce an antiviral state in the infected cell, resulting in decreased virus replication. Secretion of IFN-α and IFN-β from virus-infected cells also results in the activation of macrophages and NK cells.

Macrophages, mononuclear leukocytes, are highly abundant and widely distributed throughout the lymphoidal and non-lymphoidal organs of the body. Circulating monocytes give rise to macrophages when they leave the peripheral blood and enter into the tissues (Gordon and Taylor, 2005; Van Furth et al., 1973). Once in the tissues, activated, heterogenic macrophages display numerous functions in order to maintain homeostasis in the host including the phagocytosis of infected cells, the secretion of pro- or anti-inflammatory cytokines, and the ac-

tivation of B and T cells (Gordon and Taylor, 2005). The activation of macrophages occurs via the classical or alternative pathway (Mosser and Edwards, 2008). Classically activated macrophages, also designated M1 macrophages, are induced through the secretion of IFN-γ and TNF-α from CD4+ T-helper type 1 cells and secrete pro-inflammatory cytokines including IL-6, IL-23, and IL-1 in response to viral or bacterial infections (Cassetta et al., 2011; Gordon, 2007; Mosser and Edwards, 2008; van Furth et al., 1972). Secretion of pro-inflammatory IL-6 and IL-23 are essential for the differentiation of the CD4+ Th17 lineage (Annunziato et al., 2010; Bettelli et al., 2007). Macrophages (M2) alternatively activated through the secretion of IL-4 and IL-13 secretion from CD4+ T-helper type 2 cells are anti-inflammatory and promote wound healing (Cassetta et al., 2011; Gordon, 2003; Mosser and Edwards, 2008; Sunderkotter et al., 1994).

During HCMV infection, macrophages are a double-edged sword in that they help to limit viral replication (Morahan et al., 1980), but are also the target of HCMV infection as well as the site of viral latency (Rice et al., 1984; Tegtmeyer and Craighead, 1968). In addition, activation of infected macrophages stimulates the replication of virus (Hanson et al., 1999), which contributes to viral dissemination and potentially disease (Blasi, 2004; Vliegen et al., 2004).

M1 Macrophages are also a major source of tumor necrosis factor-alpha (TNF-α), an inflammatory cytokine that initiates a broad range of cellular responses, ranging from the induction of cellular apoptosis to the activation of inflammatory genes to the induction of the adaptive immune response (Bacci et al., 2008; Vassalli, 1992). TNF-α signaling occurs through two receptors, TNF receptor 1 (TNFR1) and TNFR2 (Baud and Karin, 2001; Wajant et al., 2003). TNFR1 signaling results in the activation of caspases, including caspase-3 and -8, leading to cellular apoptosis (Bradley, 2008; Rahman and McFadden, 2006). On the other hand, TNFR2 sig-

naling results in cell survival and the production of inflammatory cytokines (Bradley, 2008; Rahman and McFadden, 2006). TNF-α is expressed in the eye of HIV/AIDS patients with HCMV retinitis, and M1 macrophages are thought to be the source of TNF-α in the ocular compartment of these individuals (Hofman and Hinton, 1992; Mondino et al., 1990). In addition, animal studies by Dix and colleagues have linked increased TNF-α production to experimental MCMV retinitis in MAIDS mice (Dix and Cousins, 2004b), potentially from M1 macrophages.

NK cells are granular lymphocytes that are able to spontaneously lyse virus-infected cells and tumor cells in a perforin-dependent manner (Altfeld et al., 2011; Paul, 2008). The lysing of target cells by NK cells is induced by the lack of MHC class I on and/or the overexpression of NK cell-activating ligands on the target cells' surfaces (Altfeld et al., 2011). NK cells produce antiviral cytokines such as IFN-gamma (IFN-γ) and TNF-α as well as interact with T cells in order to shape an effective adaptive immune response (Altfeld et al., 2011; Jackson et al., 2011).

Both the interferon response and NK cells a play crucial role in controlling early virus infection, including HCMV and MCMV infection. For example, mice treated with IFN-α and IFN-β antibodies exhibited increased susceptibility to MCMV infection and thus increased MCMV viral titers (Grundy and Melief, 1982; Grundy et al., 1982). Adoptive transfer of NK cells to neonate mice that lack NK cells or to severe combined immunodeficiency (SCID) mice provided protection against MCMV infection (Jackson et al., 2011; Tay et al., 1998). Mice with high levels of NK cells exhibited a 10-fold reduction in MCMV viral load (Knipe and Howley, 2007). When depleted of NK cells, mice were also susceptible to MCMV infection (Knipe and Howley, 2007). In clinical studies, patients with NK cell defects experience recurrent HCMV-related diseases (Biron et al., 1989; Gazit et al., 2004; Quinnan et al., 1982).

Other studies have established the protective role of NK cells in preventing MCMV retinal infection (Bigger et al., 1998; Inoue et al., 1993). Inoue and colleagues determined that MCMV was detected in the eyes of BALB/c mice depleted of NK following systemic MCMV infection (Inoue et al., 1993). Additionally, when compared to healthy mice, NK cell-depleted mice exhibited increased ocular MCMV viral titers and exhibited retinal damage during intravitreal MCMV infection (Inoue et al., 1993). The observations of Inoue and colleagues were further verified by Bigger and coworkers who showed that BALB/c mice depleted of NK cells were susceptible to MCMV retinitis (Bigger et al., 1998). In addition, Bigger and colleagues determined that poly(I-C)-activated NK cells were able to protect mice devoid of CD4+ T cells and CD8+ T cells from MCMV-related retinitis development (Bigger et al., 1998).

The importance of NK cells in the innate response to HCMV infection can also be inferred through the numerous HCMV viral proteins that inhibit the NK response. The HCMV UL16, UL40, UL140, UL141, and UL142 proteins downregulate NK cell activity. For example, UL16 directly downregulates the NK cell-activation ligand NKG2D (Dunn et al., 2003a; Rolle et al., 2003), UL40 binds to the host cell histocompatibility antigen E (HLA-E) and inhibits its expression (Tomasec et al., 2000), and UL141 downregulates the NK cell activation ligands CD155 and CD112 (Prod'homme et al., 2010; Tomasec et al., 2005). Similar to HCMV, MCMV viral proteins murine 138 (m138), m144, m145, and m155 also directly interact with and downregulate NK cell activation ligands (Farrell et al., 1997; Lenac et al., 2006; Lodoen et al., 2004).

1.9.2 Humoral Immune Response to HCMV Infection

As reviewed in Parham, 2005 and Paul, 2008, the adaptive immune response is comprised of T cells and B cells that possess a more potent and specific response against pathogen when compared to the innate immune response. Unlike the cells of the innate immune system, adaptive immune system cells must first be exposed to foreign antigens, which delays the immune response such that antigen-specific effector and memory cells can be differentiated. However, upon a secondary exposure to the same antigen, memory cells immediately respond via clonal expansion and effector actions in order to prevent disease development. The production of antigen-specific antibodies by B cells is called the humoral response.

The humoral immune response is important for limiting viral replication and spread during early HCMV infection. Studies conducted in guinea pigs revealed that animals were protected from disease development when either actively or passively immunized with guinea pig CMV glycoprotein B (gB) (Schleiss et al., 2004b). Additionally, when pregnant guinea pigs were vaccinated with guinea pig CMV gB or passive transfer of guinea pig immunoglobulin, fetal transmission of CMV was prevented (Adler and Nigro, 2008; Jackson et al., 2011; Schleiss et al., 2004b). A similar study conducted in pregnant women showed a reduction in fetal transmission of HCMV when mothers received HCMV hyperimmune serum (Nigro et al., 2005). In addition, maternal antibodies to HCMV were able to protect premature newborns against disease development (Fowler et al., 1992; Yeager et al., 1981), whereas mother-to-fetus transmission of HCMV was increased in women with poorly neutralizing HCMV antibodies (Schleiss et al., 2004a). Numerous HCMV proteins are immunogenic, including gB, gH, phosphoprotein 150

(pp150), and pp52 (Knipe and Howley, 2007). Thus, in addition to limiting viral replication, antibodies against HCMV may play a role in limiting HCMV mother-to-fetus transmission.

While HCMV-specific antibodies play are role in limiting viral replication, Dix and colleagues determined that the passive transfer of MCMV hyperimmune serum or anti-glycoprotein B (gB) MCMV monoclonal antibodies to mice with retrovirus-induced immunosuppression prior to ocular MCMV infection failed to reduce the frequency and severity of MCMV retinitis (Dix et al., 1997a). This work indicates that CMV-specific antibodies are not sufficient to prevent the development of HCMV retinitis, and that cell-mediated immunity, rather than humoral immunity, plays a critical role in protecting against CMV retinitis development.

1.9.3 Cell-mediated Immune Response to HCMV Infection

The cell-mediated immune response serves to protect the host against intracellular pathogens including viruses through the actions of CD4+ T-helper cells and cytotoxic CD8+ T cells. As reviewed by Paul, 2008, the actions of cytotoxic CD8+ T cells are carried out via two pathways: perforin-mediated cell death pathway and Fas-mediated cell death pathway. During perforin-mediated cytotoxicity, CD8+ T cells come into contact with infected target cells, and their granule mediators, perforin and granzyme B, are polarized toward the target cell. Perforin creates holes within the target cell membrane through which granzyme B is released (Liu et al., 1995). Granzyme B cleaves pro-caspase-3 into its active form, resulting in DNA fragmentation, leakage of mitochondrial proteins into the cytosol, and ultimately apoptosis of the infected target cell (Andrade et al., 1998; Ewen et al., 2012; Van de Craen et al., 1997). The Fas-mediated cytotoxic pathway involves interactions of the Fas ligand on effector CD8+ T cells with the Fas

protein expressed on target cells (Ju et al., 1995). This interaction signals the activation of caspases, which results in apoptosis of the infected target cell. In addition, cell death can be induced by the TNF-related apoptosis-inducing ligand (TRAIL) produced by CD8+ T cells (Almasan and Ashkenazi, 2003; Wolkers et al., 2011). The binding of TRAIL to the death receptors 4 or 5 that are expressed on cells results in the activation of caspase-8 and -10 and ultimately apoptosis (Almasan and Ashkenazi, 2003; Wolkers et al., 2011).

Cell-mediated immunity is the most prominent host defense against HCMV infection and thus HCMV-related diseases. Studies have shown that CD8+ T cells control HCMV viral replication and dissemination with assistance of NK cells and CD4+ T cells (Reddehase, 2000; Reddehase et al., 1985; Riddell et al., 1992; Steffens et al., 1998). The importance of both CD4+ and CD8+ T cells in protection against HCMV-related disease development can also be inferred by number of viral proteins that inhibit the actions of these cells during infection. For example, MCMV gp34, gp40, and gp48 are functional homologs of HCMV glycoproteins encoded by viral genes US2, US3, and US11, respectively (Kavanagh et al., 2001). These viral glycoproteins decrease MHC I protein expression on virus-infected cells (Kattenhorn et al., 2004; Kavanagh et al., 2001; Loewendorf and Benedict, 2010; Loewendorf et al., 2011; Mocarski, 2002; Wagner et al., 2002), leading to decreased CD4+ T cell and CD8+ T cell activation. In addition, the MCMV-specific m155 protein inhibits expression of the CD4+ T cell and CD8+ T cell stimulator protein CD40 in virus-infected monocytes/macrophages as well as in virus-infected dendritic cells (Elgueta et al., 2009; Loewendorf et al., 2011; Ma and Clark, 2009). These viral proteins lead to reduced CD4+ and CD8+ T cell activation during infection in order to delay virus recognition and promote virus replication and dissemination. In addition, other studies have noted that the

most severe HCMV-related diseases occur in individuals with intact antibody responses but who lack and/or have impaired T cell responses (Atherton et al., 1992; Boeckh et al., 2003; Cainelli and Vento, 2002; Holbrook et al., 2000; Jabs, 2011; Knipe and Howley, 2007; Lang, 1972; Rowshani et al., 2005; Rubin, 1989). The onset of HCMV retinitis occurs in HIV/AIDS patients following the loss of cellular immunity, specifically a reduction in CD4+ T cell numbers (Atherton et al., 1992; Dix and Cousins, 2004a; Holbrook et al., 2000; Jabs, 2011). These data suggest that CD4+ and CD8+ T cells play a crucial protective role in HCMV retinitis disease pathogenesis.

Mouse studies have begun to determine the exact roles of CD4+ and CD8+ T cells in protecting against HCMV-related diseases including retinitis (Dix and Cousins, 2004a). Atherton and colleagues noted that when BALB/c mice were treated with either anti-CD4+ or anti-CD8+ antibodies prior to subretinal MCMV injection, 80% of the CD8+ T cell depleted mice were susceptible to MCMV retinitis while 30% of the CD4+ T cell depleted mice were retinitis susceptible (Atherton et al., 1992). Additionally, adoptive transfer of MCMV-specific CD8+ T cells to drug immunosuppressed (methylprednisolone acetate) BALB/c mice prior to MCMV subretinal injection resulted in protection against retinitis development (Bigger et al., 1999). More specifically, Dix and colleagues determined that the CD8+ perforin-mediated pathway, but not the Fas/FasL-mediated pathway, is essential for protection against experimental MCMV retinitis due to the observation that only perforin knockout mice were susceptible to MCMV retinitis development (Dix et al., 2003a).

Our laboratory is currently investigating the involvement of CD4+ T cell cytokines expressed during HIV/AIDS, their influences over CD8+ T cell functions, and thus the potential contributions of CD4+ T cell cytokines to HCMV-related diseases specifically, retinitis. Dix and

coworkers investigated the ability of the CD4+ T-helper (Th) cell type 1 cytokine IL-2 or the CD4+ Th lineage activating cytokine IL-12 to govern susceptibility to MCMV retinitis during retrovirus immunosuppression (Dix et al., 1997b). IL-2 is a major immunoregulatory cytokine that affects T cell, B cell and NK cell activity, and IL-12 is an inducer of IFN-γ and has been shown to protective against parasitic infection as well as increase CD8+ T cell numbers during MCMV infection (Dix et al., 1997b). Administration of recombinant human IL-2, but not recombinant murine IL-12, to mice with retrovirus-induced immunosuppression prior to ocular MCMV infection resulted in a significant decrease in ocular MCMV titers as well as a significant reduction in the frequency and severity of MCMV retinitis (Dix et al., 1997b). Further studies of IL-2 immunotherapy during retrovirus-induced immunosuppression revealed that the resistance to MCMV retinitis was due to increased infiltration of perforin-expressing CD8+ T cells into the eyes of IL-2 treated mice during ocular MCMV infection (Dix and Cousins, 2003b; Dix et al., 2003b). We therefore wished to further investigate the potential of other CD4+ T cell cytokines including IL-4, IL-10, and IL-17 to govern susceptibility to experimental MCMV retinitis in mice during retrovirus-induced immunosuppression through the alteration of cellular immunity.

1.9.3.1 *CD4+ T-Helper Cell Cytokines*

CD4+ T cells, more specifically, T-helper (Th) cells, are involved in mediating the adaptive immune response. Three subsets of Th cells are involved in protecting the body against foreign invaders: Th1, Th2, and Th17 (Figure 1.4). The functions of these T cell subsets are correlated with the types of cytokines they secrete. Th1 cells are involved in cell-mediated immunity, protecting the body against intracellular pathogens, and inducing delayed-type hypersensitivity

(DTH) through the release of their respective cytokines IFN-γ and IL-2 (Mosmann et al., 1986; Mosmann and Sad, 1996). Th2 cells, on the other hand, mediate extracellular immunity and the allergy response including the production of immunoglobulin E (IgE) through IL-4, IL-6, and IL-10 production (Mosmann et al., 1986; Mosmann and Sad, 1996). Th17 cells regulate acute inflammation associated with autoimmune diseases and activate neutrophils through the secretion of IL-17A, IL-17F, TNF-α, and IL-6 (Bettelli et al., 2007; Furuzawa-Carballeda et al., 2007; Schmidt-Weber et al., 2007). CD4+ Th2 cell cytokines are able to suppress Th1 cell function and vice versa (Mosmann and Sad, 1996). Suppression of Th1 cell functions through increased production of Th2 cytokines leads to the suppression of cell-mediated immunity (Mosmann and Sad, 1996).

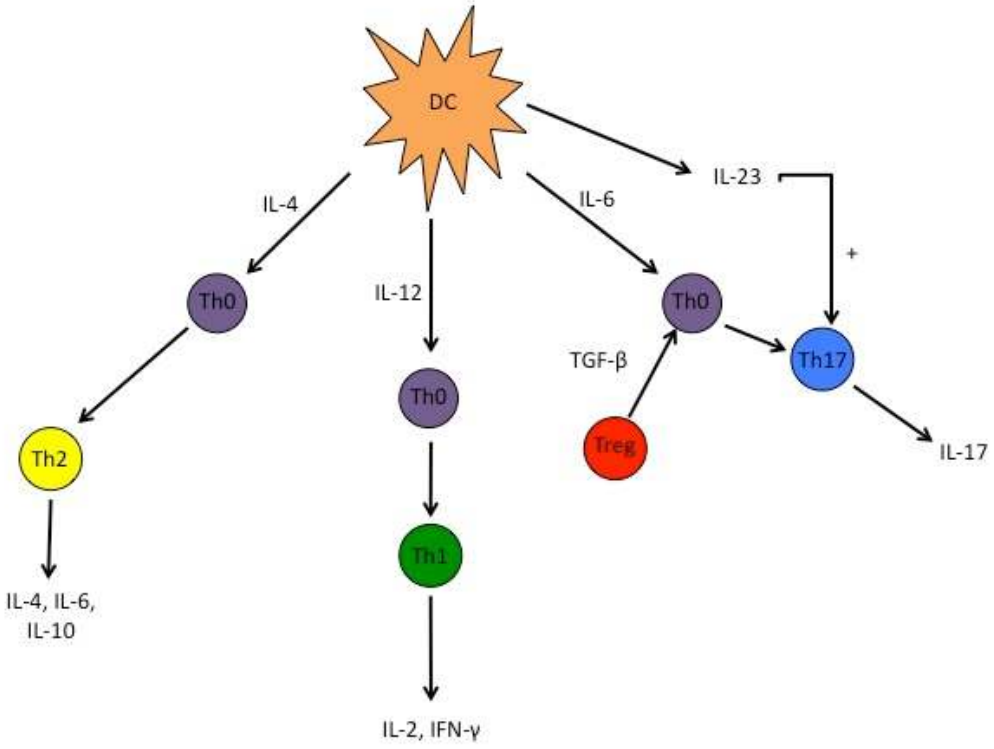

Figure 1.4. CD4+ T-helper cell differentiation. When exposed to pathogens, antigen-presenting cells (APCs) like dendritic cells (DC) secrete cytokines. The type of cytokines secreted will in turn cause the differentiation of particular CD4+ T-helper cell subsets. For example, IL-4 secretion leads to the development of Th2 helper subset from naïve T cells (Th0), which secretes IL-4, IL-6, and IL-10. An IL-12-dominated environment leads to Th1 lineage development, which secretes IL-2 and IFN-γ. Secretion of IL-6 from APCs and TGF-β from T regulatory cells (Treg) leads to the development of the Th17 subset, which is further stabilized by IL-23 secretion from APCs. The Th17 subset secretes IL-17. Based on (Afzali et al., 2007).

1.9.3.2 *Interleukin-4 (IL-4)*

The Th2 cytokine IL-4 is an immunomodulatory cytokine that has a broad range of cellular targets (Paul, 2008). Located on chromosome 5 within a group of cytokine genes, four exons encode the 129-amino acid secreted protein, which consists of four α-helices connected with short β-sheets (Chomarat and Banchereau, 1997, 1998; Paul, 2008). IL-4 is able to promote the CD4+ Th2 phenotype, alternatively activate macrophages, decrease the secretion of inflammatory cytokines, act as a B cell growth factor, promote immunoglobulin class switching (IgE), and induce MHC class II and CD23 expression (Paul, 2008). These biological functions are accomplished through the binding of IL-4 to its two-chain receptor, which is expressed at varying levels on both immune and non-immune cells (Brown and Hural, 1997).

Loss of cell-mediated immunity, during HIV infection for example, leads to increased production of Th2 cytokines such as IL-4 (Clerici and Shearer, 1993). Dix and colleagues noted a significant increase in IL-4 mRNA levels within eyes of mice with MAIDS that develop MCMV retinitis (Dix and Cousins, 2003a). Increased IL-4 production leads to increased Fas-ligand (FasL) expression and decreased perforin expression on T cells (Aung and Graham, 2000). Additionally, perforin knockout mice were susceptible to MCMV retinitis development (Dix and Cousins, 2003a; Dix et al., 2003a). Keinzle *et al.* determined that increased IL-4 production was associated with a decrease in granzyme B expression in CD8+ cytotoxic T cells (Kienzle et al., 2002; Kienzle et al., 2005). Based on these findings, we wished to investigate whether increased production of IL-4 during HIV infection governs susceptibility to AIDS-related HCMV retinitis through the direct downregulation of perforin and granzyme B from CD8+ cytotoxic T cells utilizing our MAIDS model of experimental cytomegalovirus retinitis.

1.9.3.3 *Interleukin-10 (IL-10)*

IL-10 is an anti-inflammatory cytokine secreted by numerous immune cells including macrophages, dendritic cells, CD4+ T cells, and CD8+ T cells (Couper et al., 2008; Paul, 2008). Located on chromosome 1, IL-10 consists of 160 amino acids, which fold into a non-covalently bonded homodimer (Moore et al., 2001; Ouyang et al., 2011; Paul, 2008). Actions of IL-10 are carried out via binding its two-chain IL-10 receptor, leading to the activation of the Janus kinase (Jak)/signal transducer and activator of transcription (STAT) pathway and ultimately to the activation of STAT-3, STAT-1, and STAT-5 (non-macrophage cells) (Moore et al., 2001; Ouyang et al., 2011). IL-10 acts to inhibit the production of IFN-γ as well as other cytokines secreted from Th1 cells (Ouyang et al., 2011; Paul, 2008).

IL-10 is able to regulate IL-17 secretion from CD4+ Th17 cells by binding its receptor expressed on Th17 cells (Chaudhry et al., 2011; Huber et al., 2011). The HIV Tat protein induces IL-10 production by monocytes (Ji et al., 2005; Leghmari et al., 2008). MCMV infection also induces expression of IL-10 in CD4+ T cells as well as in virus-infected macrophages (Akhtar et al., 2010; Humphreys et al., 2007; Redpath et al., 1999). Increased production of IL-10 during MCMV infection leads to reduction of MHC class II on infected cells and thus a decreased host response (Redpath et al., 1999).

In addition to controlling IL-17 secretion from Th17 cells, IL-10 production is able to alter CD8+ T cell mediated cytotoxicity, subsequently delaying pathogen clearance. For example, during HIV infection, IL-10-producing CD8+ T cells specific for HIV were associated with a decreased cytolytic response to HIV, as well as to other viruses including HCMV (Elrefaei et al., 2007). Depletion of these HIV-specific IL-10-producing CD8+ T cells, however, resulted in increased IL-2

production and increased cytolysis of virus-infected cells (Elrefaei et al., 2007). In addition, overexpression of IL-10, alone or in conjunction with IL-4, promoted the expression of FasL on CD4+ T cells (Dace et al., 2009; Furukawa et al., 2008). Due to the fact that previous studies suggest an association between increased IL-10 production and suppressed cellular immunity, especially cellular immunity involving perforin-mediated cytotoxicity, we investigated whether increased IL-10 during HIV/AIDS governs susceptibility to AIDS-related HCMV retinitis.

1.9.3.4 *Interleukin-17 (IL-17)*

A recently recognized CD4+ T-helper subset (Th17) uniquely secretes IL-17 (also known as IL-17A), in addition to IL-17F, TNF-α, and IL-6 (Furuzawa-Carballeda et al., 2007). The differentiation of the Th17 linage is dependent upon the secretion of IL-6 and TGF-β from APCs (Bettelli et al., 2007). The Th17 linage is then stabilized through the secretion of IL-23 from APCs (Bettelli et al., 2007). Located on chromosome 6, IL-17 consists of 155 amino acids that form a homodimer or a heterodimer with IL-17F. While IL-17F only shares 50% homology with IL-17A (IL-17) on the protein levels, it is a functional homolog of IL-17 (Weaver et al., 2007). Actions of IL-17 are carried out though binding to its receptor, IL-17RA (Weaver et al., 2007). IL-17 is a pro-inflammatory cytokine that is involved with recruitment of macrophages and neutrophils, as well as enhancement of the protective activities carried out by CD4+ T cells during the adaptive immune response (Stockinger et al., 2007). This pro-inflammatory cytokine, however, has been associated with cellular damage seen in various autoimmune diseases like rheumatoid arthritis and multiple sclerosis (Furuzawa-Carballeda et al., 2007). Additionally, recent work conducted by Luger and colleagues has linked increased IL-17 secretion to uveitis, an autoim-

mune disease of the eye that causes vision loss and blindness in up to 10% of the population in the Untied States (Luger et al., 2008). Though Th17 cells have been implicated to be involved in various autoimmune diseases, the role of IL-17 secretion HIV infection remains controversial, and its role in AIDS-related HCMV retinitis is unknown (Brenchley et al., 2008; Maek et al., 2007; Yue et al., 2008). It is possible that this pro-inflammatory cytokine is increased during HIV infection and that increased IL-17 production directly contributes to the pathogenesis of AIDS-related HCMV retinitis. We therefore focused our studies on the whether IL-17 governs susceptibility to experimental MCMV retinitis in MAIDS mice.

1.9.4 Suppressor of Cytokine Signaling 1 (SOCS-1) and SOCS-3

The nature and the length of the immune response to pathogens is determined by the responses initiated and received by effector T cells (Yoshimura et al., 2007). Therefore, negative-feedback loops are essential for controlling the immune response such that dysregulation and ultimately tissue damage does not occur. One negative-feedback system that is induced through immune cell cytokine secretion involves the intracellular proteins suppressor of cytokine signaling-1 (SOCS-1) and SOCS-3 (Yoshimura et al., 2007). Eight different SOCS proteins have been identified, but SOCS-1 and -3 proteins are unique proteins that contain a kinase inhibitory region (KIR) (Akhtar and Benveniste, 2011; Sabat et al., 2010; Yoshimura et al., 2007). The KIR acts to directly inhibit the activation of cytokine signaling through the Jak/STAT proteins associated with various cytokine receptors (Akhtar and Benveniste, 2011; Yoshimura et al., 2007).

1.9.4.1 *SOCS-1 and SOCS-3 in T cell Development, Differentiation, and Regulation*

SOCS-1 regulates the development of T cells in the thymus, specifically the positive and negative selection of T cells (Yoshimura et al., 2007). In addition, SOCS-1 is important in regulating the activation and proliferation of CD8+ T cells via cytokines IL-7 and IL-15 (Yoshimura et al., 2007). SOCS-1 acts to suppress Th1 and Th2 differentiation depending on the cytokine environment (IL-12 versus IL-4) and negatively regulates IFN-γ production (Yoshimura et al., 2007). Due to the fact that SOCS-1 can inhibit both Th1 and Th2 signaling, upregulation of SOCS-1 is thought to be a suppressor of both Th phenotypes (Yoshimura et al., 2007).

SOCS-3 inhibits IL-12 activation through directly binding to STAT4; overexpression of SOCS-3 leads to a predominately Th2 phenotype (Yoshimura et al., 2007). Additionally, high SOCS-3 levels correlate with high levels of IgE expression (Yoshimura et al., 2007). SOCS-3 is also able to negatively regulate Th17 cell differentiation through the suppression of STAT3 activation of IL-6 and IL-23 by directly binding to STAT3 on their receptors in Th17 cells (Yoshimura et al., 2007).

Viruses, including influenza, respiratory syncytial virus (RSV), HIV, and HSV-1 are able to exploit SOCS proteins in order to evade the immune system. For example, HSV-1, RSV, and HIV induction of SOCS-3 results in the inhibition of the antiviral interferon response and thus leads to increased viral replication increased during HIV infection (Akhtar et al., 2010; Yoshimura et al., 2007). Additionally, induction of SOCS-1 by HSV-1 inhibits STAT1 activation by IFN-γ resulting in increased viral replication (Frey et al., 2009). MCMV may or may not act in a similar manner to activate SOCS-1 and SOCS-3 proteins. We therefore wished to investigate whether

SOCS-1 and -3 govern the secretion of Th2 and Th17 cytokines and thus indirectly contribute to MCMV-related retinitis pathogenesis.

1.10 Goals of This Dissertation

1.10.1 Specific Aim 1: Test the Hypothesis that the increase in CD4+ T Cell Cytokines IL-4 or IL-10 downregulates cellular immunity to Increase Susceptibility to Experimental MCMV Retinitis in Mice with MAIDS.

HCMV infection of the retina of untreated HIV/AIDS patients can cause the development of a severe sight-threatening retinal necrosis. The classic paradigm shift of CD4+ T cell cytokines from a Th1-dominant phenotype to a Th2-dominant phenotype during HIV infection results in the increase of Th2 cytokines including IL-4 and IL-10 (Clerici and Shearer, 1993; Ji et al., 2005; Leghmari et al., 2008). Studies conducted by Dix and colleagues noted a significant increase in IL-4 mRNA levels within the eyes of MAIDS mice that develop MCMV retinitis (Dix and Cousins, 2003a). Increased IL-4 production as well as increased IL-10 production leads to an increased Fas-ligand (FasL) expression and decreased perforin expression on T cells (Aung and Graham, 2000; Oshima et al., 2007; Saito et al., 1999). Additionally, perforin knockout mice are susceptible to MCMV retinitis (Dix and Cousins, 2003a; Dix et al., 2003a). Keinzle and coworkers also determined that IL-4 production was also associated with a decrease in granzyme B expression from CD8+ cytotoxic T cells (Kienzle et al., 2002; Kienzle et al., 2005). If IL-4 and/or IL-10 are involved in increased susceptibility to MCMV retinitis, then neutralization of IL-4 or IL-10 during MAIDS should result in resistance to MCMV retinitis. We therefore proposed a series of ex-

periments in order to determine whether loss of IL-4 or IL-10 during MAIDS results in resistance to MCMV retinitis, possibly through the restoration of granzyme B and perforin.

1.10.2 Specific Aim 2: Test the Hypothesis that increased production of IL-17 from CD4+ Th17 Cells Contributes to the Pathogenesis of Experimental MCMV Retinitis in Mice with MAIDS.

IL-17 has been associated with cellular damage seen in various autoimmune diseases like rheumatoid arthritis and multiple sclerosis (Furuzawa-Carballeda et al., 2007). The role of IL-17 secretion in HIV infection, however, remains unclear. Therefore, a series of experiments were performed to determine IL-17 levels in both whole splenic cells and enriched CD4+ T cells during MAIDS progression. Additionally, recent work conducted by Luger and colleagues has tied increased IL-17 expression to the ocular autoimmune disease uveitis (Luger et al., 2008). To further elucidate the role of IL-17 in MCMV-infected eyes of MAIDS mice, we performed a series of experiments correlating intraocular IL-17 expression with MCMV retinitis susceptibility.

2 METHODS AND MATERIALS

2.1 Animals

Wild-type female C57BL/6 mice, IL-4 -/- female mice on a C57BL/6 background, and IL-10 -/- female mice on a C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in pathogen-free conditions, allowed unlimited access to food and water, and maintained in alternating 12-hr light/dark cycles. All animal procedures were conducted in accordance with Georgia State University Institutional Animal Care and Use Committee (IACUC) policies as well as the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

2.2 Viruses

Stocks of MCMV (Smith strain, American Type Culture Collection, Manassas, VA) and the murine retrovirus mixture (LP-BM5) (kindly provided by the AIDS Research and Reference Reagent Program, Germantown, MD) were prepared and stored as previously described (Dix et al., 1994).

2.3 Induction of MAIDS

MAIDS was induced in 3-week-old wild-type mice, IL-4 -/- mice, and IL-10 -/- mice with the LP-BM5 retrovirus mixture by i.p. injection as described previously (Dix et al., 1994). Mice with MAIDS of 4-weeks duration (MAIDS-4), 8-weeks duration (MAIDS-8), and 10-weeks duration (MAIDS-10) were used throughout the investigation.

2.4 MCMV infection of mice

2.4.1 Systemic MCMV infection

Mice with MAIDS and healthy age-matched mice were systemically infected with a sub-lethal dose of MCMV [1 X10^4 plaque forming units (PFU)] by i.p. inoculation.

2.4.2 Subretinal MCMV infection.

Eyes of wild-type MAIDS-8 mice, IL-4 -/- MAIDS-8 mice, and IL-10 -/- MAIDS-8 mice were inoculated with MCMV as described previously (Dix et al., 1994). Briefly, 2 ul of maintenance medium containing 1 X 10^4 plaque-forming units (PFU) of MCMV was injected subretinally into the left eye of each mouse. The right eye of each mouse was injected subretinally with 2 ul of maintenance medium only and served as control for all investigations.

2.5 Splenic CD4+ T cell, macrophage, and Gr-1+ cell isolation and enrichment

Whole spleens were collected from euthanized MAIDS mice with or without systemic MCMV infection as well as from euthanized healthy, age-matched mice with or without systemic MCMV infection. Immediately after removal, whole spleens were placed in Dulbecco's minimal essential medium (DMEM) (Cellgro, Manassas, VA), teased apart, and pushed through a 210-μm nylon mesh screen. The resulting splenic cell suspension was treated with Gey's solution (Sigma Aldrich, St. Louis, MO) for lysis of red blood cells, and processed for enrichment of individual splenic cell populations according to the protocol provided by Miltenyi Biotec (Cambridge, MA). Enrichment of splenic CD4+ T cells was accomplished by the addition of CD4+ mi-

crobeads (Miltenyi Biotec) followed by the addition of anti-CD4+ FITC-labeled antibody (Miltenyi Biotec) to the splenic cell suspension. Enrichment of splenic macrophages was accomplished by the addition of anti-F4/80-PE antibody for 10 min in the dark at 4°C (eBioscience, San Diego, CA) followed by the addition of anti-PE microbeads (Miltenyi Biotec) to the splenic cell suspension. Enrichment of GR-1+ cells (including neutrophils) was accomplished by the addition of anti-Gr-1-PE antibody (Miltenyi Biotec) for 10 min in the dark at 4°C followed by the addition of anti-PE microbeads (Miltenyi Biotec) to the splenic cell suspension. Following incubation of all cell suspensions for 10 min in the dark at 4°C and an additional wash with 1 ml of MACS buffer [phosphate buffered saline (PBS) (Cellgro, Manassas, VA), 2 mM ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, St. Louis, MO), and 0.5% bovine serum albumin (BSA) (Fisher Scientific, Pittsburgh, PA)], all cell suspensions were applied to magnetic columns as described in the manufacturer's instructions (Miltenyi Biotec), and the resulting eluents containing the labeled CD4+ T cell and Gr-1+ cell populations were sorted via flow cytometry (BD FACS Aria III, BD Biosciences, San Jose, CA) to achieve an enrichment purity of > 90%.

2.6 Total RNA extraction

Total RNA was extracted from whole splenic cells, enriched populations of splenic cells, and whole eyes using Trizol (Invitrogen, Grand Island, NY) coupled with the PureLink RNA mini kit (Invitrogen) as per manufacturer's instructions. Extracted total RNA was stored at -80 °C.

2.7 Quantitative real-time RT-PCR assay

Total RNA was extracted from whole splenic cells and from whole eyes using Trizol (Invitrogen, Grand Island, NY) coupled with the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen) per manufacturer's instructions. Extracted total RNA was stored at -80C prior to processing. Extracted total RNA was subjected to reverse transcription following the SuperScript III First-Strand System for RT-PCR (Invitrogen) protocol. cDNA was stored at -20C prior to processing. cDNA from whole splenic cells and whole eyes from all animals was subjected to quantitative real-time RT-PCR to determine the amount of transcripts specific. Murine GAPDH cDNA (Qiagen) served as endogenous control. Briefly, 1.2 ul of cDNA was added to a reaction mixture of 15 ul of Power SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA), 3 ul of primers for each gene (see Table 2.1), 9.9 ul of double-distilled water, and 0.9 ul of dimethylsulfoxide (Sigma, St. Louis, MO) for a total volume of 30 ul per reaction. Parameters for each quantitative RT-PCR assay cycle were 15 min at 95C, 15 sec at 94C, 31 sec at 55C, and 35 sec at 72C for a total of 45 cycles. Transcription levels were determined utilizing the 7500 Fast Real-Time PCR System (Applied Biosystems), and average threshold cycles (Ct) were determined using the 7500 Fast Real-Time PCR System software (Applied Biosystems).

Table 2.1. Sequences, annealing temperature, and expected fragment size of primers used in real-time RT-PCR assay.

Gene (Accession Number)	Source	Primer Sequence	Annealing Temperature (°C)	AMP Length (bp)
IL-4 (NM_021283)	Qiagen	QuantiTect Primer Assay	55	104
IL-10 (NM_010548)	Qiagen	QuantiTect Primer Assay	55	103
Perforin (NM_011073)	Qiagen	QuantiTect Primer Assay	55	123
Granzyme B (NM_013542)	Qiagen	QuantiTect Primer Assay	55	149
Fas Ligand (NM_010177)	Qiagen	QuantiTect Primer Assay	55	99
Mm_IL-17A (NM_010552)	Integrated DNA Technologies	Sense- CCT GGC GGC TAC AGT GAA G Antisense- TTT GGA CAC GCT GAG CTT TG	55	63
Mm_IL-23 (p19) (NM_031252)	Integrated DNA Technologies	Sense- GGT TGA GCG GAA T Antisense- AGG GAG TGG GAA C	55	50
Mm_IL-6 (NM_031168)	Qiagen	QuantiTect primer assay	55	128
Mm_GAPDH (NM_008084)	Qiagen	QuantiTect primer assay	55	144

2.8 ELISA

Whole splenic cells and whole eyes from all animals were collected, individually stored in liquid nitrogen, thawed, individually homogenized in 1.0 ml of phosphate-buffered saline (PBS) containing a protease inhibitor cocktail (Sigma), and individually stored at -20C prior to performance of ELISA. At time of ELISA, homogenates were thawed, sonicated, clarified by centrifugation, and the resulting supernatants were subjected to ELISA for quantification of murine IL-4, IL-10, or IL17 protein using the commercially available kit provided by eBioscience (San Diego, CA) per manufacturer's instructions. Total protein for each sample was determined using the Bradford Protein Assay (BioRad, Hercules, CA).

2.9 Western blot analysis

Whole spleens and whole eyes were collected from uninfected MAIDS -4, MAIDS-8, and MAIDS-10 animals, individually stored in liquid nitrogen, thawed, and individually homogenized in 1 ml of PBS containing protease inhibitor cocktail (Sigma). Standard Western blot analysis was performed for detection of IL-17 and GAPDH proteins using rabbit-anti-human IL-17 antibody (1:200) (Santa Cruz Biotechnology) or rabbit-anti-mouse GAPDH antibody (1:3000) (Sigma) as primary antibodies, respectively. Following incubation with ImmunoPure goat anti-rabbit IgG antibody (heavy plus light chains [H+L]) (1:5000) (Thermo Scientific) secondary antibody, the resulting nitrocellulose membrane (BioRad) was treated with chemiluminescence (ECL) Western blot detection reagents (GE Healthcare, Piscataway, NJ) and exposed to BioMax light film (Kodak, Rochester, NY).

2.10 Histopathology

Whole eyes collected from all animals were immediately fixed in 10% buffered formalin (Electron Microscopy Sciences, Hartford, PA) for at least 48 hr at 4C, frozen in Optimal Cutting Temperature (OCT) medium (Thermo Scientific, Rochester, NY), cut into 8-um sections using a Shandon Special Motorized Electronic Cryotome (Thermo Scientific), and sections were collected onto positively charged microscope slides (Thermo Scientific). Hematoxylin and eosin staining was performed as previously described (Dix et al., 1994) with minor modifications. Ocular sections were scored for frequency and severity of retinitis using a scoring system established by us previously (Dix et al., 1994).

2.11 Immunohistochemical staining of ocular tissues

Formalin-fixed eyes were then frozen in O.C.T. medium (Thermo Scientific, Rochester, NY) and cut using a Shandon Special Motorized Electronic Cryotome (Thermo Scientific) into 8-μm sections that were placed onto positively charged microscope slides (Thermo Scientific). Immunohistochemical staining was performed by incubating slides with eye sections in 10 mM sodium citrate (Sigma Aldrich) for 10 min at room temperature followed by a 5-min rinse with PBS (Cellgro) at room temperature. Immunohistochemical staining for IL-17 in retinal tissues was accomplished by reacting slides with rehydrated eye sections with polyclonal rabbit anti-hIL-17 IgG (1:50) (Santa Cruz Biotechnology, Santa Cruz, CA) or with polyclonal normal rabbit IgG (1:400) (control, Santa Cruz Biotechnology), processed using the rabbit ABC Staining System (Santa Cruz Biotechnology), and stained with Vector Red alkaline phosphatase substrate (Vec-

tastain ABC-AP Kit, Vector Laboratories, Burlingame, CA). Slides were counterstained with DAPI (Vector Laboratories).

Double immunohistochemical staining for the identification of IL-17 producing retinal cells was accomplished by incubating rehydrated eye sections with rabbit-anti-hIL-17 IgG (1:50) (Santa Cruz Biotechnology) and goat anti-hRhodopsin IgG (1:200) for identification of rod cells (Santa Cruz Biotechnology) or goat anti-hOpsinSW IgG for identification of cone cells (1:200) (Santa Cruz Biotechnology) at 4 °C overnight in a humidified atmosphere. Following three washes for 5 min with PBS, retinal sections were reacted with secondary antibodies, donkey anti-goat DyLight488 (1:300) (Jackson ImmunoResearch Laboratories INC, West Grove, PA) and chicken anti-rabbit DyLight594 (Jackson ImmunoResearch Laboratories INC) and incubated in the dark at room temperature for 1 hr in a humidified atmosphere. Sections were washed three times with PBS, mounted with medium containing DAPI (Vector Laboratories). All stained sections were viewed at 200x or 400x magnification.

2.12 Recovery and quantification of infectious MCMV

Whole MCMV-infected eyes were collected from wild-type MAIDS-8 mice, IL-4 -/- MAIDS-8 mice, and IL-10 -/- MAIDS mice at 10 days after subretinal injection and individually stored in liquid nitrogen prior to processing. At time of quantitative plaque assay, individual eyes were thawed, individually homogenized on ice in 1.0 ml of cold Delbecco's Minimal Essential Medium (DMEM) (Cellgro, Manassas, VA), and clarified by centrifugation. Ten-fold dilutions of the resulting supernatants were titered onto monolayers of mouse embryo fibroblast (MEF) cells in 6-well plates, allowed to adsorb for 1-hr at 37C in a humidified atmosphere of 5% CO_2,

overlaid with 1.0 ml DMEM, and incubated for 5 days at 37C in a humidified atmosphere of 5% CO_2. Individual plaques were counted using an inverted light microscope, and results were expressed as the number of PFU per ml per whole eye (PFU/ml/eye).

2.13 Statistical analysis

All quantitative data obtained from quantitative real-time RT-PCR assay and ELISA were expressed as means $\pm$ standard error of mean (SEM) or standard deviation (SD), respectively. At least two independent experiments per performed for each study. Statistical analysis was performed using the Wilcoxon-rank sum or Student T-test. A p value of < 0.05 was considered significant.

3 AIM 1: TEST THE HYPOTHESIS THAT THE INCREASE IN CD4+ T CELL CYTOKINES IL-4 OR IL-10 DOWNREGULATE CELLULAR IMMUNITY TO INCREASE SUSCEPTIBILITY TO EXPERIMENTAL MCMV RETINITIS IN MICE WITH MAIDS

The data in this aim were published in Ophthalmology and Eye Diseases, 2012.

3.1 Introduction

IL-4 and IL-10 are key Th2 cytokines whose production is stimulated systemically during AIDS (and therefore during AIDS-related HCMV retinitis) as a result of HIV-induced Th2 dominance. IL-4 is an immunomodulatory Th2 cytokine that promotes a number of diverse immunological functions through binding to its two-chain receptor (IL4R) that is expressed on both immune and non-immune cells (Brown and Hural, 1997; Keegan and Zamorano, 1998). Although the immunological outcome(s) of IL-4 secretion varies remarkably depending on effector cell, target cell, and the microenvironment in which IL-4 is secreted (Brown and Hural, 1997; Chomarat and Banchereau, 1997, 1998), IL-4 promotes a number of diverse immunological functions that impact macrophage differentiation, the differentiation of CD4+ T cells into Th2 cells, and the inhibition of secretion of various inflammatory cytokines (Paul, 2008). In comparison, IL-10 is an anti-inflammatory Th2 cytokine that inhibits the production of INF-g as well as other Th1 cytokines (Ouyang et al., 2011; Paul, 2008), an accomplishment achieved through binding of IL-10 to its two-chain receptor composed of an alpha and beta subunit (Moore et al., 2001; Ouyang et al., 2011).

Of greater significance, however, are observations that CD8+ T-cell-mediated cytotoxicity is remarkably diminished at times of increased IL-4 and/or IL-10 production. For example, in-

creased IL-4 production during HIV infection results in conversion of cytotoxic CD8+ T cells to CD8- T cells that also produce more IL-4, further suppressing the Th1 response (Brown and Hural, 1997; Erard et al., 1994; Erard et al., 1993). HIV infection also results in IL-10-producing CD8+ T cells that exhibit reduced cytolytic activity to HIV as well as other viruses including HCMV (Elrefaei et al., 2007). Overexpression of IL-4 in mice also results in increased Fas-ligand (FasL) expression on T cells and a concomitant decrease in perforin production, an important observation that suggests that IL-4 favors Fas/FasL-mediated cytotoxicity over perforin-mediated cytotoxicity (Aung and Graham, 2000). Similar results have also been observed during overexpression of IL-10 (Dace et al., 2009; Furukawa et al., 2008). Taken together, these findings suggest a pivotal association between increased IL-4 and/or IL-10 production during retrovirus-induced immunosuppression and suppressed cellular immunity, especially cellular immunity involving perforin-mediated cytotoxicity.

Using our MAIDS model of MCMV retinitis, we demonstrated previously that loss of the perforin cytotoxic pathway is responsible for increased susceptibility to MCMV retinitis during MAIDS (Dix et al., 2003a), and this increased susceptibility can be reversed by immunotherapy with the Th1 cytokine IL-2 (Dix et al., 1997b). Moreover, MCMV-infected eyes of MAIDS animals susceptible to MCMV retinitis contain high amounts of IL-4 (Dix and Cousins, 2003a). These observations, coupled with those from other laboratories showing that either IL-4 or IL-10 favor the Fas/FasL cytotoxic pathway over the perforin cytotoxic pathway (Aung and Graham, 2000; Baschuk et al., 2007; Dace et al., 2009; Furukawa et al., 2008; Kienzle et al., 2002; Kienzle et al., 2005; Oshima et al., 2007; Saito et al., 1999), lead to the attractive hypothesis that an increase in systemic production of IL-4 or IL-10 during retrovirus-induced immunosuppression is respon-

sible for increased susceptibility to MCMV retinitis during MAIDS. To our surprise, however, this hypothesis proved to be incorrect since mice with MAIDS deficient in either IL-4 or IL-10 exhibited a frequency of retinitis, a severity of retinitis, and intraocular amounts of infectious virus equivalent to those found in wild-type mice with MAIDS.

3.2 Results

3.2.1 Quantification of splenic IL-4 and IL-10 mRNA levels in wildtype C57BL/6 mice during progression of MAIDS

Early work by Gazzinelli and colleagues (Gazzinelli et al., 1992; Morse et al., 1995) demonstrated that progression of MAIDS is associated with a shift in cytokine production by splenic CD4+ T cells from a Th1 profile to a Th2 profile as seen in HIV-infected patients with AIDS (Clerici and Shearer, 1993), and this shift commences at ~3 weeks after retrovirus infection (Dix and Cousins, 2004a). Although we have shown previously that IL-4 mRNA levels increase significantly within the ocular compartment of MCMV-infected eyes of mice with MAIDS (Dix and Cousins, 2003a), we sought to confirm that our animals in the absence of MCMV infection also exhibit a systemic increase in Th2 cytokines during the progression of MAIDS, especially for the Th2 cytokines IL-4 and IL-10 that have been associated with dampening of cellular immunity (Aung and Graham, 2000; Baschuk et al., 2007; Dix et al., 2003a; Kienzle et al., 2002; Kienzle et al., 2005). Initial experiments using quantitative RT-PCR assay were therefore performed to measure IL-4 and IL-10 mRNA levels within splenic cells collected from wild-type C57BL/6 mice with MAIDS of 4-weeks duration (MAIDS-4), 8-weeks duration (MAIDS-8), and 10-weeks duration (MAIDS-10), but without ocular MCMV infection. Results are shown in Fig. 3.1. Whereas splenic IL-4 mRNA levels increased nearly 2-fold in MAIDS-4 and MAIDS-8 animals, MAIDS-10 animals showed a significant 4-fold increase in splenic IL-4 mRNA levels (Fig 3.1A). In comparison, splenic IL-10 mRNA levels also increased during progression of MAIDS, but this increase was evident later in the course of MAIDS and far greater than that seen for splenic IL-4 mRNA. Whereas no significant increase in IL-10 mRNA was observed in splenic cells collected from

MAIDS-4 animals, splenic IL-10 mRNA levels were ~4-fold and ~17-fold higher in MAIDS-8 and MAIDS-10 animals, respectively (Fig 3.1B). Thus, as expected (Dix and Cousins, 2003a; Dix et al., 1994; Morse et al., 1995) , our mice with MAIDS did indeed exhibit increased systemic production of IL-4 and IL-10 mRNAs during progression of retrovirus-induced immunosuppression as measured using splenic cells, although the increase in splenic IL-10 mRNA levels was far greater than that of splenic IL-4 mRNA levels. Nonetheless, a significant increase in systemic mRNA levels to both Th2 cytokines was observed during MAIDS-8 and MAIDS-10, times during the course of retrovirus-induced immunosuppression when mice become susceptible to MCMV retinitis following subretinal infection (Dix and Cousins, 2003a; Dix et al., 1994).

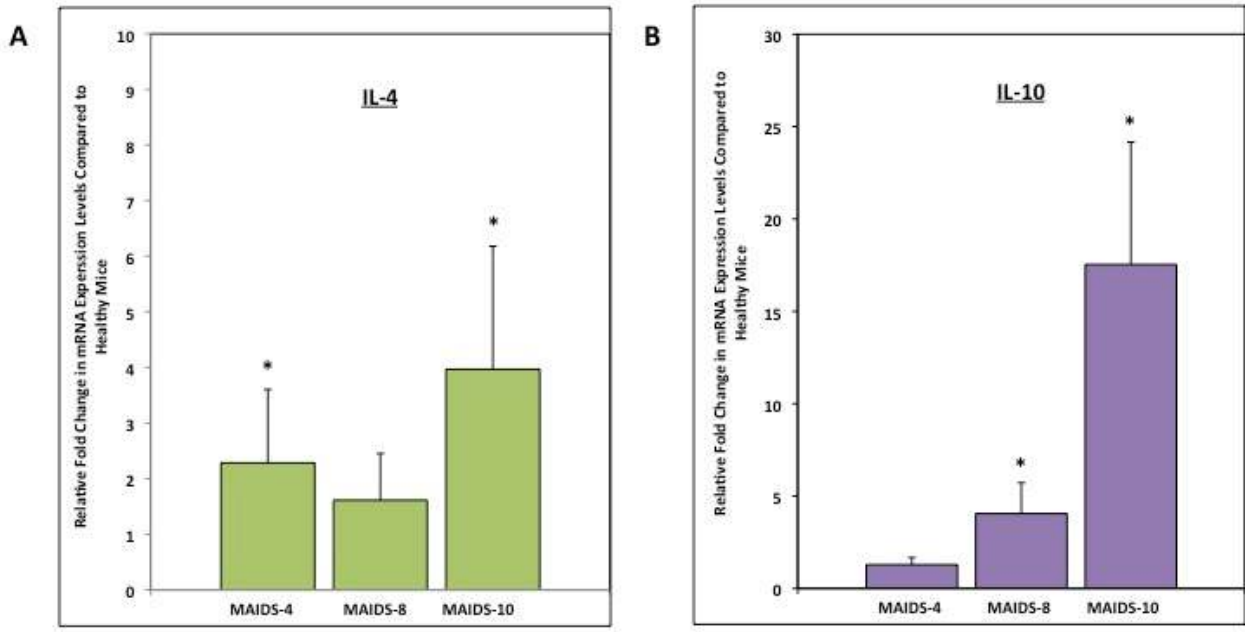

Figure 3.1. IL-4 and IL-10 mRNA levels in whole splenic cells during progression of MAIDS. A. IL-4 mRNA levels in whole splenic cells during MAIDS progression versus healthy controls, $p < 0.05$ ($n = 5$) Error bars = Standard Error of Mean (SEM) of three independent experiments. Asterisks indicate statistical significance. B. IL-10 mRNA levels in whole splenic cells during MAIDS progression versus. healthy controls, $p \leq 0.03$ ($n = 5$) Error bars = SEM of two independent experiments. Asterisks indicate statistical significance.

3.2.2 Measurement of IL-4 mRNA and protein levels in anti-IL-4 antibody treated MAIDS mice

We first sought to determine if a reduction of IL-4 during MAIDS would result in increased resistance to MCMV retinitis. Therefore, mice with MAIDS of 8-weeks duration were treated with either mouse anti-IL-4 IgG1 or matched isotype control IgG (i.p.) on days -3, 0, +3, and +6 relative to subretinal injection of left eyes with MCMV (Atherton et al., 1991; Dix et al., 1994) or subretinal mock-injection of right eyes with maintenance media (control). On day 10 after subretinal injection, whole splenic cells were collected and quantified for IL-4 mRNA or protein levels using real-time RT-PCR assay or ELISA, respectively. Results are shown in the Figure 3.2 A and B respectively. While anti-IL-4 antibody treatment protocol failed to reduce splenic IL-4 mRNA production in MAIDS animals, splenic IL-4 protein levels were reduced by ~50%.

3.2.3 Measurement of IL-10 mRNA levels in anti-IL-4 antibody treated MAIDS mice

We then wanted to confirm that reduction of IL-4 through the use of anti-IL-4 antibody would not affect the transcription of other Th2 cytokines. Thus, we quantified the mRNA expression levels of the Th2 cytokine IL-10 in whole splenic cells of anti-IL-4 antibody treated MAIDS mice and matched MAIDS controls using real-time RT-PCR assay. Anti-IL-4 antibody treatment of MAIDS mice did not alter IL-10 mRNA levels when compared to control MAIDS animals (Fig. 3.2C).

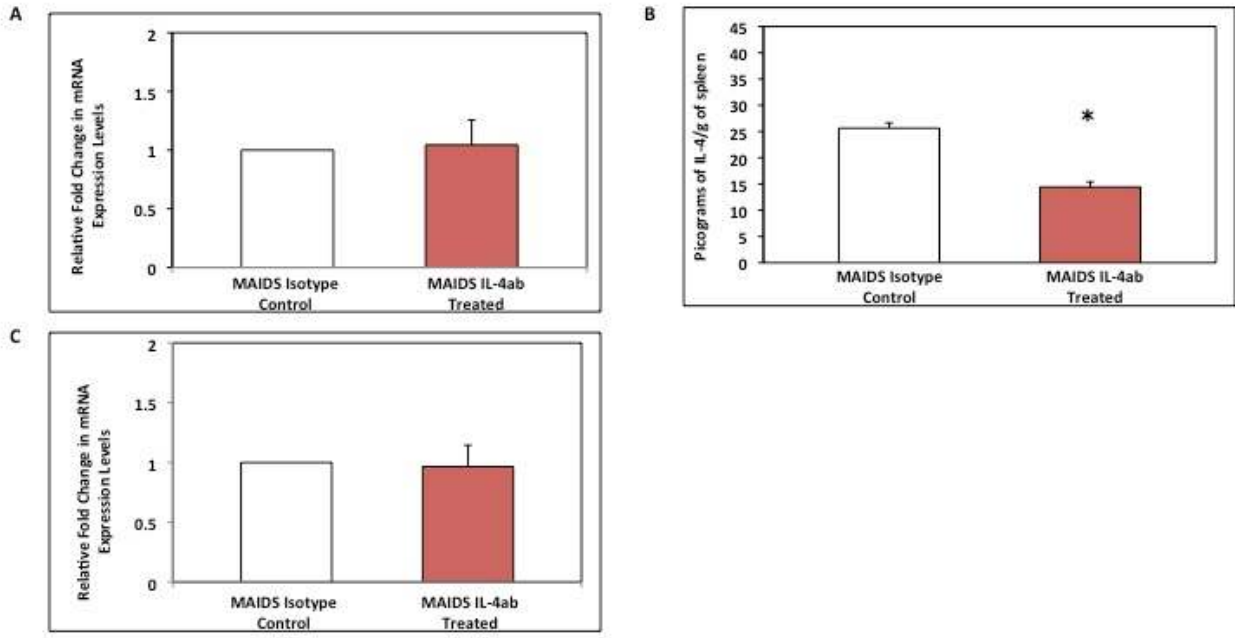

Figure 3.2. IL-4 mRNA, IL-4 protein levels, and IL-10 mRNA in whole splenic cells of anti-IL-4 antibody (Ab)-treated MAIDS mice versus respective controls. A. IL-4 mRNA levels in isotype-treated control vs. Ab-treated $p = 0.8053$ (no statistical difference) (Wilcoxon Rank Sum test) ($n = 5$ mice/group) Error bars = Standard Error of Mean (SEM) of two experiments. B. IL-4 protein levels of Ab-treated vs. isotype-treated control. $p \leq 0.05$ (Student T-test) ($n = 5$ mice/group) Error bar = Standard Deviation (SD) of two experiments. C. IL-10 mRNA levels in Isotype-treated control vs. Ab-treated p = 0.864 (no statistical difference) (Wilcoxon Rank Sum test) (n = 5 mice/group) Error bar = SEM of two independent experiments. Asterisks indicate statistical significance.

3.2.4 Measurement of perforin, granzyme B and FasL mRNA levels in anti-IL-4 antibody treated MAIDS mice

Since IL-4 has been shown to inhibit perforin and granzyme B production and thereby favor the Fas/FasL cytotoxic pathway (Aung and Graham, 2000; Baschuk et al., 2007; Dix et al., 2003a; Kienzle et al., 2002; Kienzle et al., 2005), we next determined the fate of mRNAs for perforin and granzyme B as well as FasL mRNA following systemic reduction of IL-4 protein during MAIDS-related MCMV retinitis. Whole spleens were collected from anti-IL-4 antibody-treated MAIDS mice and matched control mice at day 10 after subretinal MCMV injection and quantified for these mRNAs by real-time RT-PCR assay. We did not detect a significant difference in perforin, granzyme B, or FasL mRNA production by splenic cells in anti-IL-4 antibody-treated mice with MAIDS when compared with splenic cells of control animals following subretinal MCMV injection (Fig. 3.3 A, B, and C respectively).

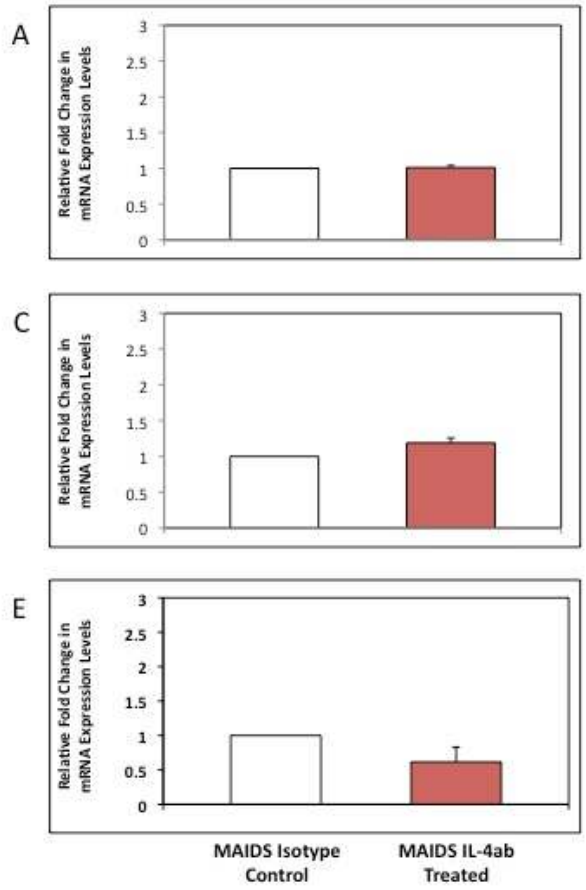

Figure 3.3. Perforin, Granzyme B, and FasL mRNA levels in whole splenic cells of anti-IL-4 antibody (Ab)-treated MAIDS mice versus respective controls. A. Perforin mRNA levels in Isotype-treated control vs. Ab-treated MAIDS splenic cells, p = 0.751 (no statistical difference) (Wilcoxon Rank Sum test) (n = 5 mice/group) Error bar = SEM of two independent experiments. B. Granzyme B mRNA levels in Isotype-treated control vs. Ab-treated MAIDS splenic cells, p = 0.105 (no statistical difference) (Wilcoxon Rank Sum test) (n = 5 mice/group) Error bar = SEM of two independent experiments. C. FasL mRNA levels in Isotype-treated control vs. Ab-treated MAIDS splenic cells, p = 0.695 (no statistical difference) (Wilcoxon Rank Sum test) (n = 5 mice/group) Error bar = SEM of two independent experiments. Asterisks indicate statistical significance.

3.2.5 Frequency and severity of MCMV Retinitis in anti-IL-4-antibody treated MAIDS mice

We then sought to determine if reduction of IL-4 levels during MAIDS resulted in increased resistance to MCMV retinitis. Ten days after subretinal MCMV infection, whole eyes of anti-IL-4 antibody treated MAIDS mice and matched control MAIDS mice were collected and analyzed for frequency and severity of retinitis. Results are shown in Table. 3.1 and Figure 3.4. As expected, MCMV-infected eyes of control mice with MAIDS exhibited a frequency of retinitis of 83% (Dix and Cousins, 2004a), and with an average severity score of 2.84. In comparison, MCMV-infected eyes of anti-IL-4 antibody-treated MAIDS animals remained susceptible to MCMV retinitis. While the frequency of retinitis in MCMV-infected eyes of mice with MAIDS treated with neutralizing antibody to IL-4 was reduced by ~30% and the severity of disease was reduced to 2.35 when compared with control animals, these reductions were not significant.

Table 3.1. Frequency and severity of MCMV necrotizing retinitis in groups of mice with MAIDS at day 10 after subretinal MCMV injection.

Group	Frequency of necrotizing retinitis (retinitis/total)	Severity Score
MAIDS Isotype Control	83% (5/6)	2.84
MAIDS Anti-IL-4 Antibody Treated	50% (3/6)	2.35

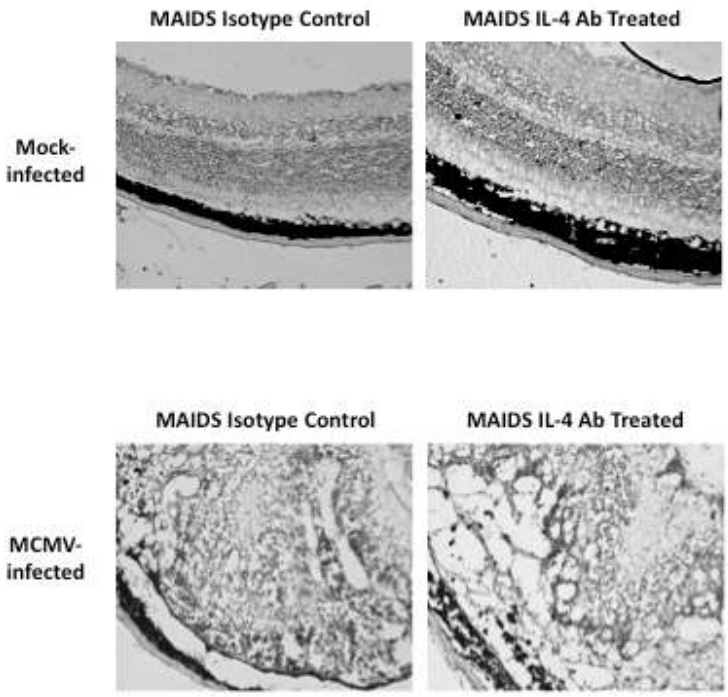

Figure 3.4. Photomicrographs of anti-IL-4 antibody (Ab)-treated MAIDS mice eyes and isotype-control mice eyes. Top panel: Photomicrographs of formalin-fixed OCT-embedded frozen sections of retina of mock-infected eyes collected from control animals at 10 days following subretinal injection of maintenance medium (right eyes) showing normal retinal architecture. Bottom panel: Photomicrographs of formalin-fixed OCT-embedded frozen sections of retina of eyes at 10 days after subretinal MCMV injection (left eyes) showing full-thickness retinal necrosis. (hematoxylin & eosin; magnification X 200)

3.2.6 Induction of MAIDS in mice deficient in IL-4 or IL-10

Due to the unexpected results obtained utilizing anti-IL-4 antibody treatment in MAIDS mice, we employed a second experimental approach utilizing IL-4 -/- and IL-10 -/- to determine if systemic reduction of IL-4 or IL-10 might lead to increased resistance to MCMV retinitis during MAIDS as hypothesized. We first attempted to induce MAIDS in IL-4 -/- mice and IL-10 -/- mice. Since IL-4 and IL-10 gene-deficient mice are not available, we elected to use for these studies IL-4 -/- and IL-10 -/- mice that possess a targeted mutation of the IL-4 gene or IL-10 gene that results in the production of truncated, non-functional IL-4 (Kuhn et al., 1991) or IL-10 (Kuhn et al., 1993) protein products. Groups of IL-4 -/- and IL-10 -/- mice were therefore infected with the immunosuppressive retrovirus mixture, LP-BM5, housed for 8 weeks, and assessed for development of MAIDS using criteria established by us previously (Dix et al., 1994). All retrovirus-infected IL-4 -/- and IL-10 -/- mice exhibited physical and immunological features consistent with development of MAIDS, and were designated as IL-4 -/- MAIDS-8 mice and IL-10 -/- MAIDS-8 mice.

Additional studies were performed to confirm that splenic IL-10 mRNA levels were not affected in IL-4 -/- mice MAIDS-8 following subretinal MCMV infection, and, conversely, splenic IL-4 mRNA levels were not affected in IL-10 -/- MAIDS-8 mice following subretinal MCMV infection. This was of interest since Green and coworkers (Green et al., 2008) noted that IL-10 -/- mice infected with LP-BM5 exhibited exaggerated disease development when compared with wild-type LP-BM5-infected C57BL/6 mice, an outcome that might affect splenic IL-4 mRNA production. Results are shown in Fig. 3.4. While equivalent amounts of IL-10 mRNA levels were observed in splenic cells collected from wild-type MAIDS-8 mice and IL-4 -/- MAIDS-8 mice follow-

ing intraocular MCMV infection, IL-10 protein levels were significantly increased in IL-4-/- MAIDS-8 mice. In comparison, while splenic IL-4 mRNA levels were reduced by ~50% in IL-10 -/- MAIDS-8 mice when compared with wild-type MAIDS-8 mice following intraocular MCMV infection, nearly equivalent amounts of IL-4 protein were found within splenic cells of both animal groups.

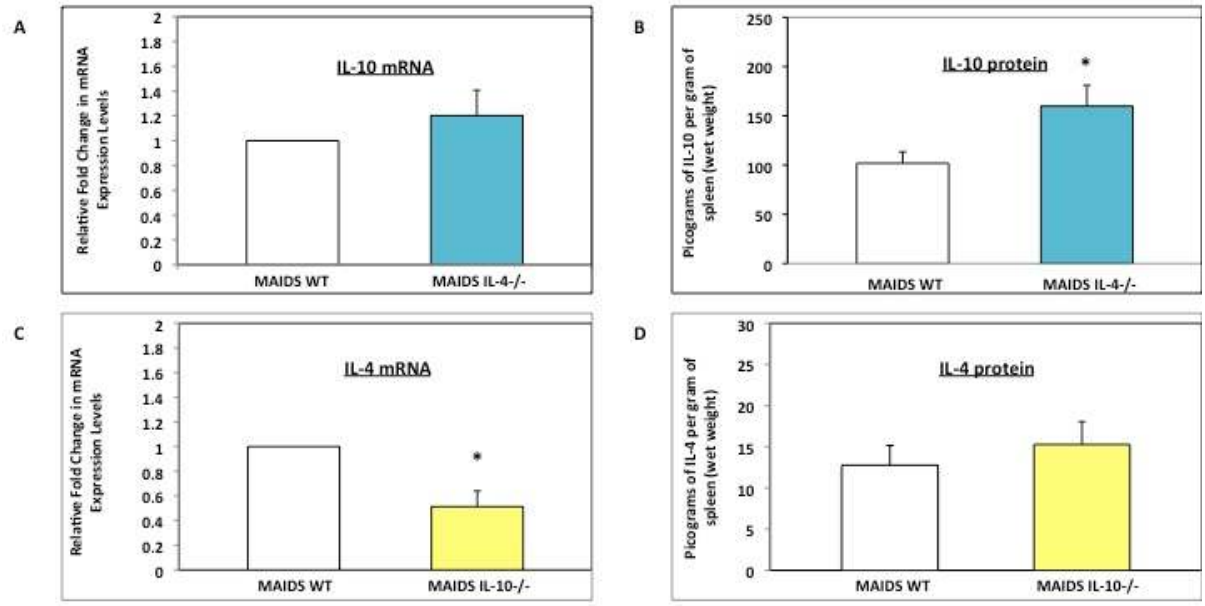

Figure 3.5. IL-10 mRNA and IL-10 protein levels in whole splenic cells of IL-4 -/- MAIDS-8 mice versus respective controls and IL-4 mRNA and IL-4 protein levels in whole splenic cells of IL-10 -/- MAIDS-8 mice versus respective controls. A. IL-10 mRNA levels in whole splenic cells wild-type MAIDS versus IL-4-/- MAIDS-8 mice, $p = 0.432$ ($n = 10$) Error bars = SEM of two independent experiments. B. IL-10 protein levels in whole splenic cells of wild-type MAIDS vs. IL-4-/- MAIDS-8 mice, $p \leq 0.02$ ($n = 10$) Error bars = Standard Deviation (SD) of two independent experiments. Asterisks indicate statistical significance. C. IL-4 mRNA in whole splenic cells of wild-type MAIDS versus IL-10-/- MAIDS-8 mice, $p \leq 0.04$ ($n = 5$) Error bars = SEM of one experiment. Asterisks indicate statistical significance. D. IL-4 protein in whole splenic cells of wild-type MAIDS versus IL-10-/- MAIDS-8 mice $p = 0.939$ ($n = 8$, wild-type MAIDS-8 mice; $n = 10$, IL-10-/- MAIDS-8 mice) Error bars = SD of one experiment.

3.2.7 Quantification of amounts of infectious MCMV within eyes of wildtype MAIDS-8 mice, IL-4 -/- MAIDS-8 mice, and IL-10 -/- MAIDS-8 mice following subretinal MCMV infection

We have shown previously that the amounts of infectious MCMV within the ocular compartments of mice with MAIDS increase remarkably during development of retinitis following subretinal MCMV injection (Dix and Cousins, 2003a; Dix and Cousins, 2004a; Dix et al., 1994). Moreover, since increased IL-4 and IL-10 production would tend to delay virus clearance by dampening of CD8+ T-cell cytotoxicity, we sought to determine if loss of IL-4 or IL-10 would affect virus replication during MAIDS. Eyes of IL-4 -/- MAIDS-8 mice, IL-10 -/- mice, and wild-type MAIDS mice were therefore infected with MCMV by subretinal injection, collected 10 days later, and subjected to standard plaque assay for quantification and comparison of amounts of infectious virus. As shown in Fig. 3.5, equivalent amounts of infectious virus were found in MCMV-infected eyes of IL-4 -/- MAIDS-8 mice, IL-10 -/- MAIDS-8 mice, and their respective wild-type MAIDS-8 controls. Thus, systemic loss of IL-4 or IL-10 during MAIDS did not appear to impact virus replication significantly within the eye, either positively or negatively.

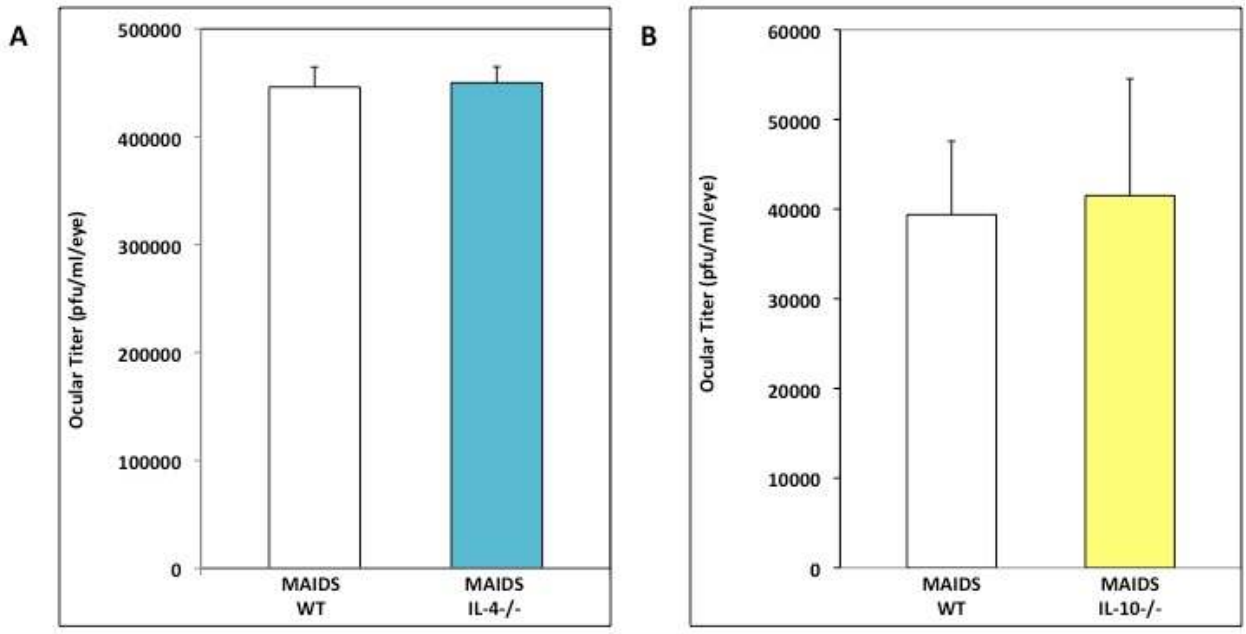

Figure 3.6. Ocular MCMV titer in wild-type MAIDS-8 mice, IL4 -/- MAIDS-8 mice, and IL-10 -/- MAIDS-8 mice. A. Average virus titer (expressed as PFU/eye/ml) in whole eyes of wild-type MAIDS-8 mice versus IL-4-/- MAIDS-8 mice at 10 days after subretinal MCMV infection $p = 0.658$ ($n = 5$) Error bars = SEM of one experiment. B. Average virus titer (expressed as PFU/eye/ml) in whole eyes of wild-type MAIDS-8 mice versus IL-10 -/- MAIDS-8 mice at 10 days after subretinal MCMV infection $p = 0.800$ ($n = 4$) Error bars = SEM of one experiment.

3.2.8 Frequency and severity of MCMV retinitis in IL-4 -/- MAIDS-8 mice and IL-10 -/- MAIDS-8 mice following subretinal MCMV infection

Since systemic loss of IL-4 or IL-10 had no effect of the amount of virus within the eyes of MAIDS-8 mice that have been shown previously by us to be susceptible to retinitis (Dix and Cousins, 2004a; Dix et al., 1994), we sought to determine whether systemic loss of IL-4 or IL-10 would result in increased resistance to retinitis as hypothesized. In separate experiments, IL-4 -/- MAIDS-8 mice, IL-10 -/- MAIDS-8 mice, and their respective wild-type MAIDS-8 controls were infected with MCMV by subretinal inoculation. Ten days later, all eyes were collected, analyzed histopathologically, and scored for frequency and severity of necrotizing retinitis using a scoring system described previously (Dix et al., 1994). Results are shown in Table 3.2, Figure 3.6, and Figure 3.7. As expected, MCMV-infected eyes of wild-type MAIDS-8 control mice were indeed susceptible to retinitis as indicated by frequencies of retinitis of 78% and 100% in separate experiments (average = 89%). In sharp opposition to our hypothesis, however, MCMV-infected eyes collected from IL-4 -/- MAIDS-8 mice and IL-10 -/- MAIDS-8 exhibited a frequency of retinitis equivalent to that observed in control animals. Whereas 89% of MCMV-infected eyes of IL-4 -/- MAIDS-8 mice showed retinitis, 85% of IL-10 -/- MAIDS-8 mice also showed retinitis. Although frequency of retinitis was unaffected in MAIDS animals with a systemic loss of IL-4 or IL-10, it was possible that loss of these Th2 cytokines would result in a decrease in severity of retinal disease. This was not the case (Table 3.2). When scored for severity of retinitis (Dix et al., 1994), a statistical difference was not observed when MCMV-infected eyes of IL-4 -/- MAIDS-8 mice or MCMV-infected eyes of IL-10 -/- MAIDS-8 mice were compared with MCMV-infected eyes of their respective controls.

Table 3.2. **Frequency and severity of MCMV necrotizing retinitis in wild-type MAIDS-8 mice, IL-4-/- MAIDS-8 mice, and IL-10-/- MAIDS-8 mice at day 10 after subretinal MCMV infection.**

Group	Frequency of Necrotizing retinitis (retinitis/total)	Severity Score
Wildtype MAIDS-8	78% (7/9)	2.94 (n = 7)
IL-4 -/- MAIDS-8	89% (8/9)	3.24 (n = 8)
Wildtype MAIDS-8	100% (4/4)	3.72 (n = 4)
IL-10 -/- MAIDS-8	85% (6/7)	3.14 (n = 6)

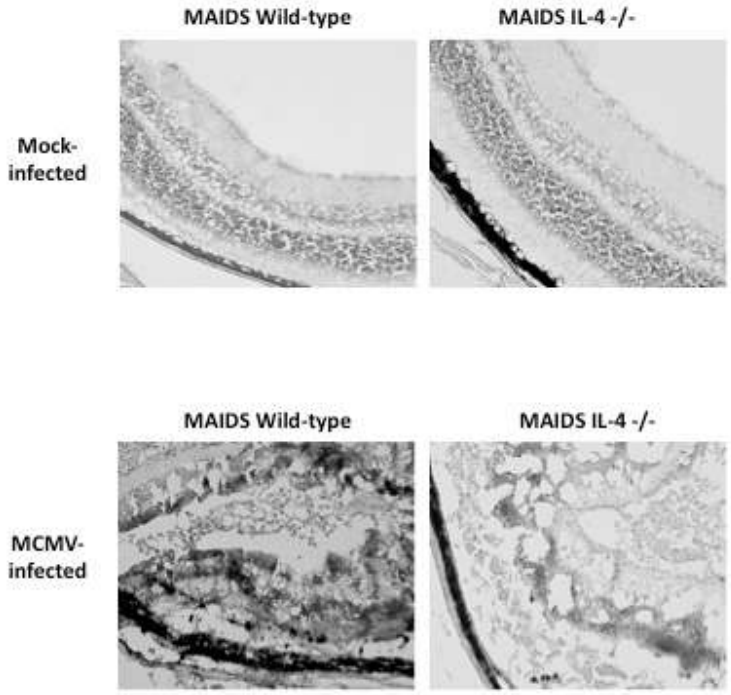

Figure 3.7. Photomicrographs of IL-4 -/- MAIDS mice eyes and wild-type MAIDS mice eyes. Top panel: Photomicrographs of formalin-fixed OCT-embedded frozen sections of retina of mock-infected eyes collected from control animals at 10 days following subretinal injection of maintenance medium (right eyes) showing normal retinal architecture. Bottom panel: Photomicrographs of formalin-fixed OCT-embedded frozen sections of retina of eyes at 10 days after subretinal MCMV injection (left eyes) showing full-thickness retinal necrosis. (hematoxylin & eosin; magnification X 200)

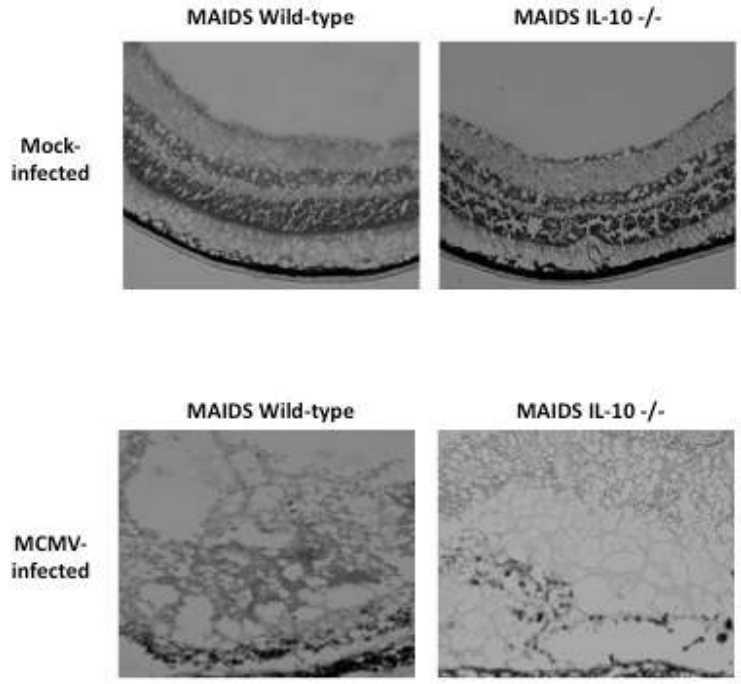

Figure 3.8. Photomicrographs of IL-10 -/- MAIDS mice eyes and wild-type MAIDS mice eyes. Top panel: Photomicrographs of formalin-fixed OCT-embedded frozen sections of retina of mock-infected eyes collected from control animals at 10 days following subretinal injection of maintenance medium (right eyes) showing normal retinal architecture. Bottom panel: Photomicrographs of formalin-fixed OCT-embedded frozen sections of retina of eyes at 10 days after subretinal MCMV injection (left eyes) showing full-thickness retinal necrosis. (hematoxylin & eosin; magnification X 200)

3.2.9 Quantification of splenic perforin, granzyme B, and FasL mRNA levels in IL-4 -/- MAIDS-8 mice and IL-10 -/- MAIDS-8 mice following subretinal MCMV infection

Since MCMV-infected eyes of mice with MAIDS deficient in systemic IL-4 or IL-10 failed to show a decrease in frequency or severity of retinitis as predicted, we were interested in knowing the fate of mRNAs of key molecules involved in CD8+ T-cell cytotoxicity in animals unable to produce functional systemic IL-4 or IL-10. These included perforin and granzyme B mRNAs associated with the perforin cytotoxic pathway (Paul, 2008) and FasL mRNA associated with the Fas/FasL cytotoxic pathway (Paul, 2008). This was accomplished by measurement of perforin, granzyme B, and FasL mRNAs originating from splenic cells collected from IL-4 -/- MAIDS-8 mice and IL-10 -/- MAIDS-8 mice following subretinal MCMV infection and comparing these amounts by quantitative RT-PCR assay with those obtained from wild-type MAIDS-8 mice following subretinal MCMV infection. As shown in Fig. 3.8, amounts of splenic perforin and granzyme B mRNAs remained relatively unchanged in mice with MAIDS deficient in systemic IL-4 or IL-10 production when compared with wild-type MAIDS animals. Moreover, MAIDS mice deficient in systemic IL-4 or IL-10 production showed a significant increase in splenic FasL mRNA production, an unexpected finding.

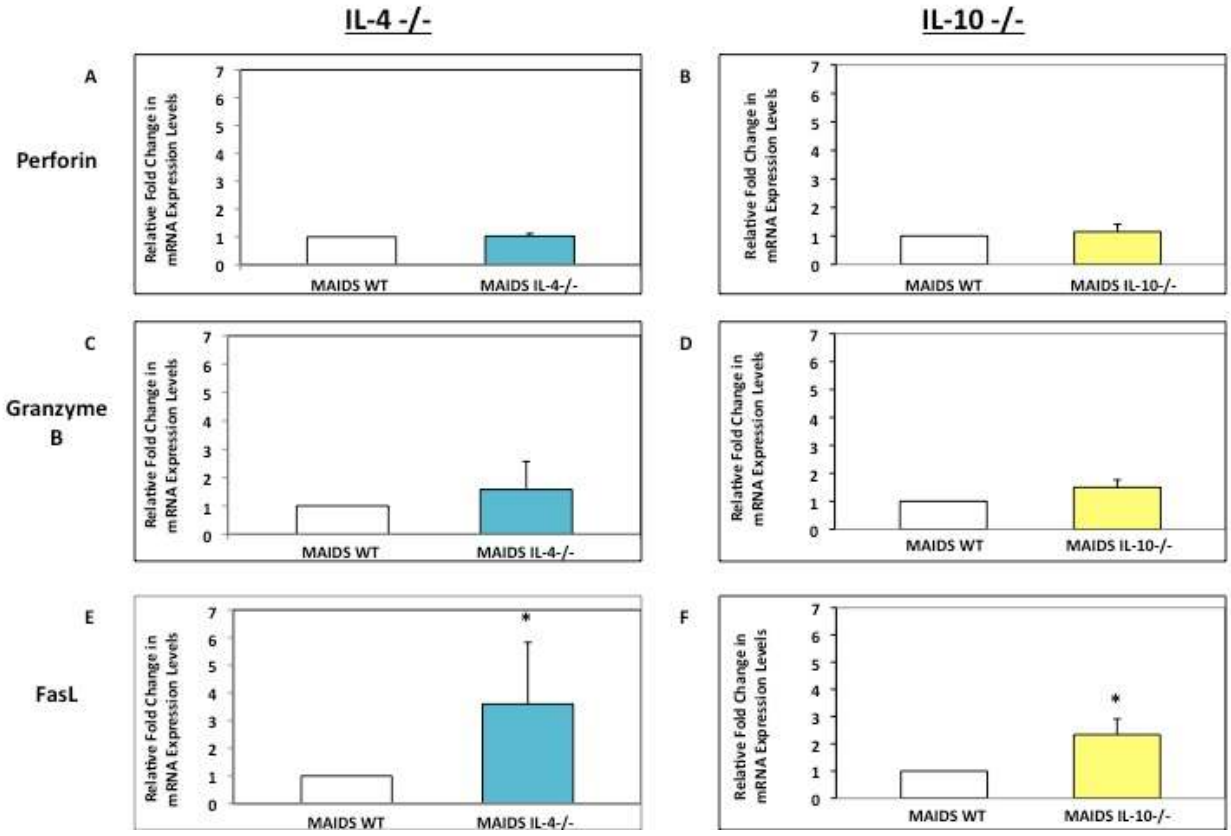

Figure 3.9. Perforin, granzyme B, and FasL mRNA levels in whole splenic cells of IL-4 -/- MAIDS-8 mice and IL-10 -/- MAIDS-8 mice versus respective controls. A. Perforin mRNA levels in splenic cells of wild-type MAIDS-8 mice versus IL-4-/- MAIDS-8 mice; $p = 0.780$ ($n = 5$) Error bars = SEM of two independent experiments. B. Perforin mRNA levels in splenic cells of wild-type MAIDS-8 mice versus IL-10-/- MAIDS-8 mice; $p = 0.49$ ($n = 5$) Error bars = SEM on one experiment. C. Granzyme B mRNA levels in splenic cells of wild-type MAIDS-8 mice versus IL-4-/- MAIDS-8 mice; $p = 0.691$ (n = 5) Error bars = SEM of two independent experiments. D. Granzyme B mRNA levels in splenic cells of wild-type MAIDS-8 versus IL-10 -/- MAIDS-8 mice; $p = 0.22$ ($n = 5$) Error bars = SEM of one experiment. E. FasL mRNA in splenic cells of wild-type MAIDS-8 mice versus IL-4 -/- MAIDS-8 mice; $p \leq 0.01$ ($n = 5$) Error bars = SEM of two independent experiments. F. FasL mRNA in splenic cells of wild-type MAIDS-8 mice versus IL-10 -/- MAIDS-8 mice; $p \leq 0.007$ (n = 5) Error bars = SEM of one experiment. Asterisks indicate statistical significance.

4 AIM 2: TEST THE HYPOTHESIS THAT INCREASED PRODUCTION OF IL-17 FROM CD4+ TH17 CELLS CONTRIBUTES TO THE PATHOGENESIS OF EXPERIMENTAL MCMV RETINITIS IN MICE WITH MAIDS

4.1 Introduction

A more recently recognized CD4+ Th cell subset, Th17 cells, secretes a unique set of cytokines that includes IL-17A (IL-17) as well as TNF-α, IL-22, and IL-6 (Annunziato et al., 2010; Bettelli et al., 2007; Furuzawa-Carballeda et al., 2007; Schmidt-Weber et al., 2007). Differentiation of the Th17 linage is dependent upon the secretion of IL-6 and TFG-ß from APCs (Bettelli et al., 2007; Furuzawa-Carballeda et al., 2007; Gaffen, 2009; Schmidt-Weber et al., 2007); the Th17 lineage is stabilized by IL-23 secretion from APCs (Bettelli et al., 2007; Furuzawa-Carballeda et al., 2007; Gaffen, 2009; Schmidt-Weber et al., 2007). A pro-inflammatory cytokine, IL-17 functions by binding to its receptor, IL-17RA, that is expressed on a wide range of cells (Annunziato et al., 2010; Weaver et al., 2007) and thereby stimulates a number of immune responses including the recruitment of macrophages and neutrophils (Annunziato et al., 2010; Stockinger et al., 2007; Weaver et al., 2007). Of significance, however, is that IL-17 has been associated with cellular damage that occurs during autoimmune diseases including rheumatoid arthritis and multiple sclerosis (Annunziato et al., 2010; Furuzawa-Carballeda et al., 2007; Korn et al., 2009; Lovett-Racke et al., 2011). Recent work by Luger and colleagues (Luger and Caspi, 2008; Luger et al., 2008) and others (Amadi-Obi et al., 2007; Caspi, 2008; Peng et al., 2007) has also linked IL-17 to uveitis, a leading cause of blindness in the United States (Caspi, 2010).

Although Th17 cells have been implicated in the pathogenesis of various autoimmune diseases, the role of IL-17 during HIV-induced immunosuppression remains controversial.

Brenchley and co-workers (Brenchley et al., 2008) found that Th17 cells were lost in mucosa of the gastrointestinal tract of HIV-infected patients, but remained intact in simian immunodeficiency virus-infected sooty mangabey monkeys. In contrast, other studies have found that IL-17 secretion from Th17 cells was increased during HIV infection (Maek et al., 2007; Yue et al., 2008). To more clearly elucidate the role of Th17 cells in HIV infection as well as the potential contribution(s) of IL-17 to the pathogenesis of AIDS-related HCMV retinitis, we performed a series of studies to test the hypotheses that systemic IL-17 is increased during retrovirus-induced immunosuppression, and this systemic increase in IL-17 production plays a role in the pathogenesis of cytomegalovirus retinitis within the ocular compartment.

These hypotheses were tested using a mouse model of MCMV retinitis that develops in mice with MAIDS, a retrovirus-induced immunodeficiency syndrome with features that closely mimic HIV/AIDS in humans (Dix and Cousins). Importantly, mice with MAIDS exhibit lymphadenopathy, polyclonal B-cell activation, hypergammaglobulinemia, and a retrovirus-induced shift in cytokine production from a dominant Th1 response to a dominant Th2 response prior to susceptibility to experimental MCMV retinitis (Dix and Cousins, 2004a; Dix et al., 1994; Morse et al., 1995), thereby making this an attractive animal model to investigate IL-17 in the context of MCMV retinal disease development.

We report herein that while IL-17 mRNA and protein levels increased in mice with MAIDS, CD4+ T cells were not the sole source of IL-17 during retrovirus-induced immunosuppression progression. Surprisingly, however, IL-17 mRNA and protein levels were dampened in MCMV-infected eyes of mice with MAIDS that were susceptible to retinitis, a result that was also observed following systemic MCMV infection of both healthy mice and MAIDS mice. Decreased

levels of IL-17 correlated with increased mRNA levels of IL-10 and suppressor of cytokine signaling (SOCS)-3 during MCMV infection. While systemic MCMV infection of healthy IL-10 -/- mice resulted in a partial recovery of IL-17 protein levels, IL-10 -/- MAIDS animals remained susceptible to MCMV retinitis at levels equivalent to wild-type MAIDS mice. Taken together, our results suggest that IL-17 plays no direct role in the pathogenesis of MAIDS-related MCMV retinitis, but that MCMV downregulates IL-17 from CD4+ T cells through the upregulation of IL-10 and potentially SOCS-3.

4.2 Results

4.2.1 IL-17 protein production in whole splenic cells and whole eyes of healthy C57BL/6 mice

We first sought to determine baseline levels of expression of IL-17 protein in the spleens and eyes of C57BL/6 mice during health. Results are shown in Figure 1. Detectable amounts of IL-17 protein were found in homogenates of whole splenic cells and whole eyes of healthy animals by ELISA (Fig. 4.1A and Fig. 4.1B) as well as by Western blot analysis (data not shown). ELISA showed 4.73 $\pm$ 1.84 pg of IL-17 per gram of spleen (wet weight) and 0.87 $\pm$ 0.31 pg of IL-17 per whole eye. Moreover, visualization of IL-17 protein by immunohistochemical staining of the posterior segment of healthy whole eyes revealed constitutive expression of IL-17 with an expression pattern confined to the photoreceptor and inner plexiform layers of the neurosensory retina (Fig. 4.1C). Thus, IL-17 was not only produced within the spleen of healthy mice, but was also found to be expressed constitutively by cells of the neurosensory retina of healthy mice. Further analysis of the photoreceptor cells revealed that both rods and cones constitutively express IL-17 during health (Fig 4.2).

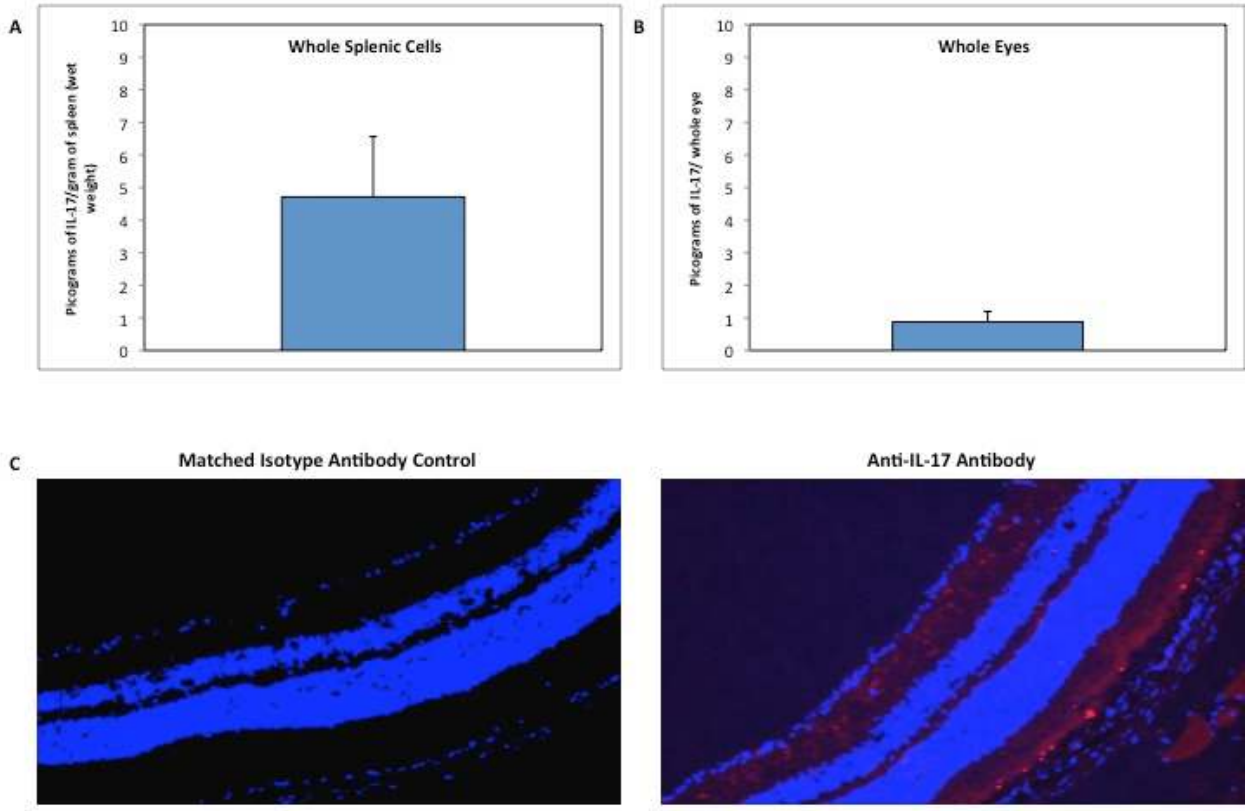

Figure 4.1. Detection and quantification of IL-17 protein in whole splenic cells and whole eyes of healthy C57Bl/6 mice. (A) IL-17 protein in whole splenic cells of adult healthy C57BL/6 mice as determined by ELISA [pg of IL-17 per gram of spleen (wet weight] (*n* = 10). Bars = Standard Deviation of 1 experiment. (B) IL-17 protein in whole eyes of adult healthy C57BL/6 mice as determined by ELISA [pg of IL-17 per whole eyes] (*n* = 10). Bars = Standard Deviation of 1 experiment. (C) Detection of IL-17 in cells of a representative retinal tissue section collected from an adult healthy mouse. Formalin-fixed cytosections were reacted with anti-IL-17 antibody (red) or an isotype-matched normal antibody (control). Nuclei were counterstained with DAPI (blue) (200X).

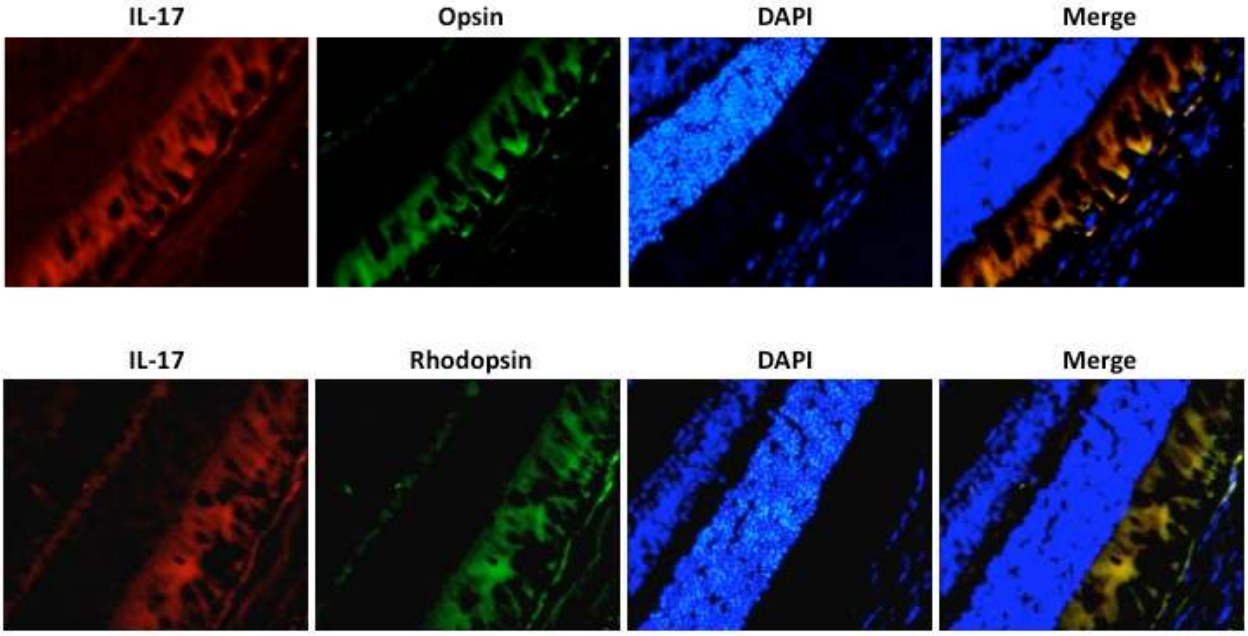

Figure 4.2. Detection of IL-17 protein in photoreceptor cells of healthy C57BL/6 mice. Formalin-fixed cytosections were reacted with anti-IL-17 antibody (red) and anti-OpsinSW antibody (green) or anti-Rhodopsin antibody (green). Nuclei were counterstained with DAPI (blue) (400X).

4.2.2 IL-17 mRNA and protein production in whole splenic cells and whole eyes of C57BL/6 mice during progression of MAIDS

After establishing baseline levels of expression of IL-17 in the spleen and eyes of healthy C57BL/6 mice, we next investigated possible changes in IL-17 mRNA and protein production during the progression of MAIDS in order to clarify the fate of IL-17 during retrovirus-immunosuppression. Whole splenic cells and whole eyes were collected from MAIDS-4, MAIDS-8, and MAIDS-10 mice and quantified for IL-17 mRNA and protein levels by real-time RT-PCR assay and ELISA, respectively. Progression of MAIDS was associated with a significant ($p \leq 0.03$) increase in IL-17 mRNA levels in whole splenic cells, with the levels peaking in MAIDS-10 animals (Fig. 4.3A). This increase in IL-17 transcript production was reflected in a significant ($p \leq 0.001$) increase in IL-17 protein production in whole splenic cells during MAIDS progression, with protein levels being highest at 41.5 ± 6.1 and 23.5 ± 5.13 pg per gram of spleen (wet weight) in MAIDS-8 and MAIDS-10 animals, respectively (Fig. 4.3B).

MAIDS progression was also associated with a significant ($p \leq 0.0006$) increase in IL-17 mRNA levels in whole eyes of MAIDS-8 and MAIDS-10 animals (Fig. 4.3C), but ocular IL-17 protein levels in these animals did not differ from IL-17 levels in the eyes of healthy mice (Figure 4.3D). We postulate that this is due to the degradation of IL-17 mRNA shortly after transcription or the storage of IL-17 mRNA in cytoplasmic vesicles such that mRNA is ultimately not being translated into protein. It is noteworthy that previous work has shown MAIDS-8 and MAIDS-10 animals are susceptible to MCMV retinitis (Dix and Cousins, 2004a; Dix et al., 1994). The elevated levels of IL-17 mRNA and protein in whole splenic cells during the progression of MAIDS suggested that numbers of IL-17-producing Th17 cells may be increased during progression of

retrovirus-induced immunosuppression, but this increase may not necessarily be found within the ocular compartment.

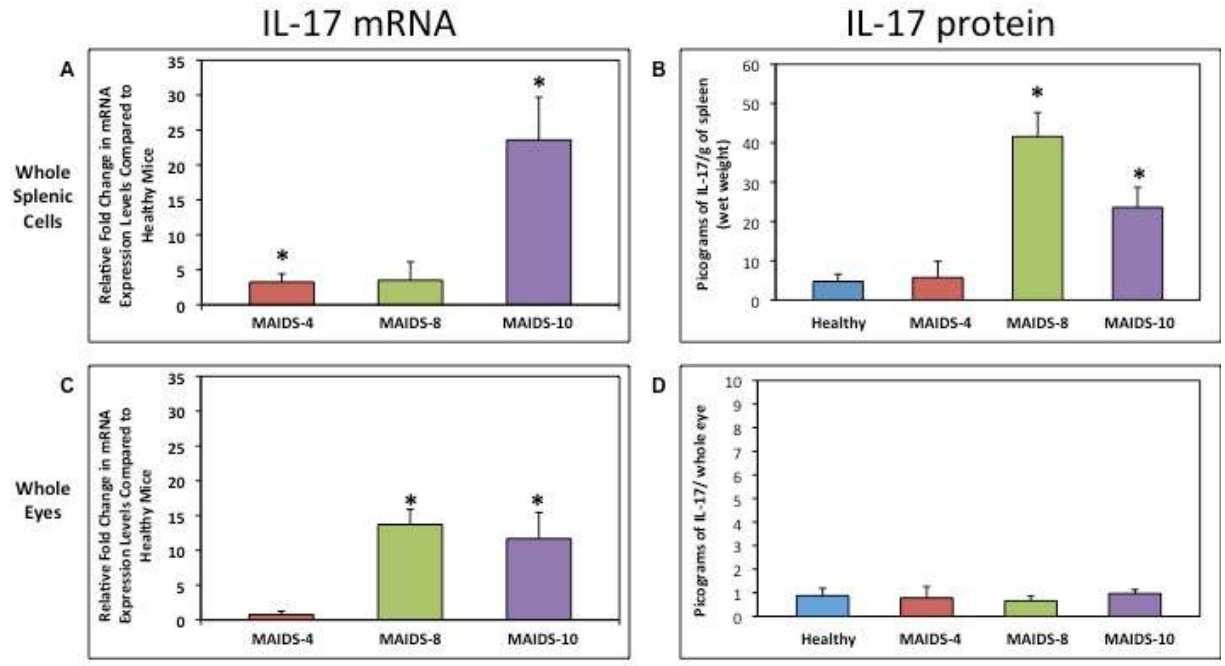

Figure 4.3. IL-17 mRNA and protein levels in whole splenic cells and whole eyes of C57BL/6 mice during the progression of MAIDS. Comparison of (A) IL-17 mRNA levels of whole splenic cells collected from groups of MAIDS-4, MAIDS-8, and MAIDS-10 mice (*n* = 5), and (C) IL-17 mRNA levels of whole eyes collected from groups of MAIDS-4, MAIDS-8, and MAIDS-10 mice (*n* = 5). Levels (fold-change) of IL-17 mRNA were determined by quantitative RT-PCR assay. Bars = Standard Error of the Mean of 3 independent experiments. Asterisks indicate statistical significance. Comparison of (B) IL-17 protein levels of whole splenic cells collected from groups of MAIDS-4, MAIDS-8, and MAIDS-10 mice (*n* = 5), and (D) IL-17 protein levels of whole eyes collected from groups of MAIDS-4, MAIDS-8, and MAIDS-10 mice (*n* = 5). Levels (fold-change) of IL-17 mRNA were determined by quantitative RT-PCR assay. Protein levels were determined by ELISA. Bars = Standard Deviation of 1 experiment.

4.2.3 IL-17 mRNA levels in enriched populations of splenic CD4+ T cells, splenic macrophages, and splenic Gr-1-expressing cells (neutrophils) during progression of MAIDS

We next sought to determine the cellular source of splenic IL-17 production in MAIDS-8 and MAIDS-10 animals. Known cellular sources of IL-17 that included populations of splenic CD4+ T cells, splenic macrophages, and splenic Gr-1-expressing cells (neutrophils) (Gu et al., 2008; Korn et al., 2009; Weaver et al., 2007) were enriched by flow cytometry (purity > 90%) from the whole spleens of MAIDS-4, MAIDS-8, and MAIDS-10 mice and quantified for IL-17 mRNA levels by real-time RT-PCR assay. Unlike whole splenic cells, the progression of MAIDS was associated with a decrease in IL-17 mRNA levels in splenic cells enriched for CD4+ T cells (Fig. 4.4). Purified splenic macrophages and splenic Gr-1-expressing cells (neutrophils) also exhibited similar yet significant ($p \leq 0.05$) decreases in IL-17 mRNA levels, and therefore contributed little to overall expression of IL-17 mRNA within the whole spleen (Fig. 4.4). Our finding of decreased IL-17 mRNA from CD4+ T cells during MAIDS progression suggested that retrovirus-induced immunosuppression might alter essential Th17 cell differentiation factors including IL-23 and IL-6.

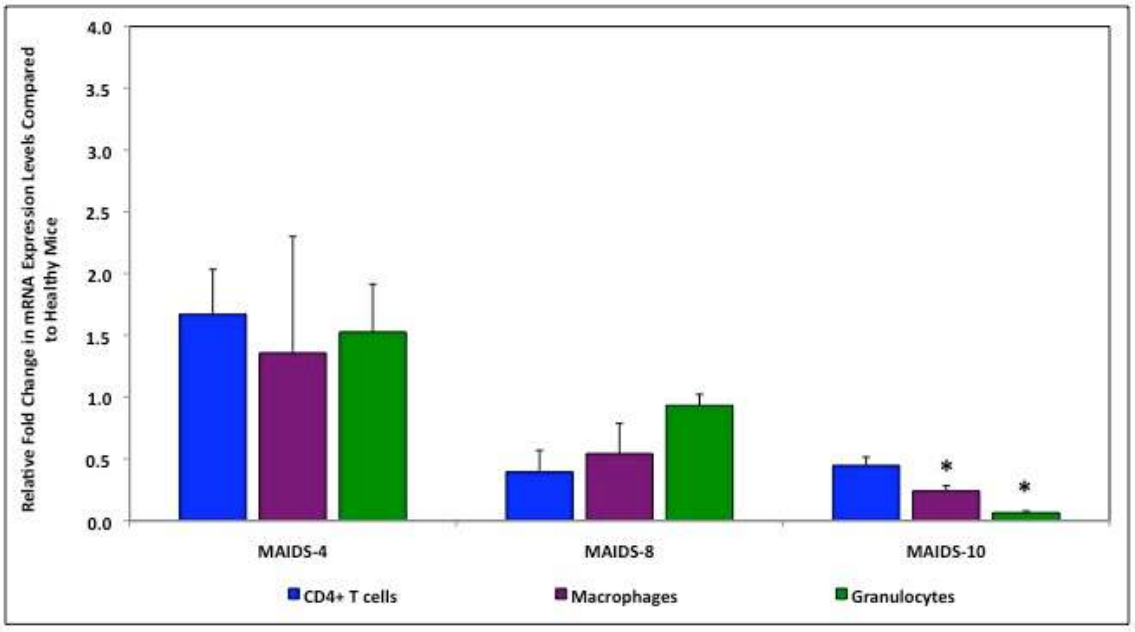

Figure 4.4. IL-17 mRNA levels of enriched populations of splenic CD4+ T cells, splenic macrophages, and splenic Gr-1+ cells (neutrophils) from C57BL/6 mice during the progression of MAIDS. IL-17 mRNA levels for enriched populations (> 90% purity) of splenic CD4+ T cells (gray bars), splenic macrophages (black bars), and splenic Gr-1+ cells (neutrophils) (white bars) of whole splenic cells collected from groups of MAIDS-4, MAIDS-8, and MAIDS-10 mice (*n* = 5). Levels (fold-change) of IL-17 mRNA were determined by quantitative RT-PCR assay. Bars = Standard Error of the Mean of 3 independent experiments. Asterisks indicate statistical significance.

4.2.4 IL-6 and IL-23 mRNA production in whole splenic cells during progression of MAIDS

IL-6 and IL-23 from APCs are required for the differentiation of the mouse Th17 lineage (Bettelli et al., 2007; Furuzawa-Carballeda et al., 2007; Gaffen, 2009; Schmidt-Weber et al., 2007). In order to address the discrepancy that was seen in IL-17 mRNA expression levels between whole splenic cells and purified populations of splenic CD4+ T cells during MAIDS progression, we measured IL-6 and IL-23 mRNA levels in order to determine whether these cytokines were reduced at various times during retrovirus-induced immunosuppression. Whole splenic cells collected from groups of MAIDS-4, MAIDS-8, and MAIDS-10 mice exhibited IL-6 and IL-23 mRNA levels equivalent to mRNA levels seen in healthy age-matched control mice (Fig. 4.5). Thus, the decrease in IL-17 mRNA production seen in CD4+ T cells during MAIDS progression was not due to the unavailability of the essential linage factors IL-6 or IL-23. Our findings suggest that in addition to splenic CD4+ T cells, other cellular sources like CD8+ T cells or NKT cells contribute to the overall increase in IL-17 production in whole splenic cells during MAIDS progression (Kondo et al., 2009; Rachitskaya et al., 2008).

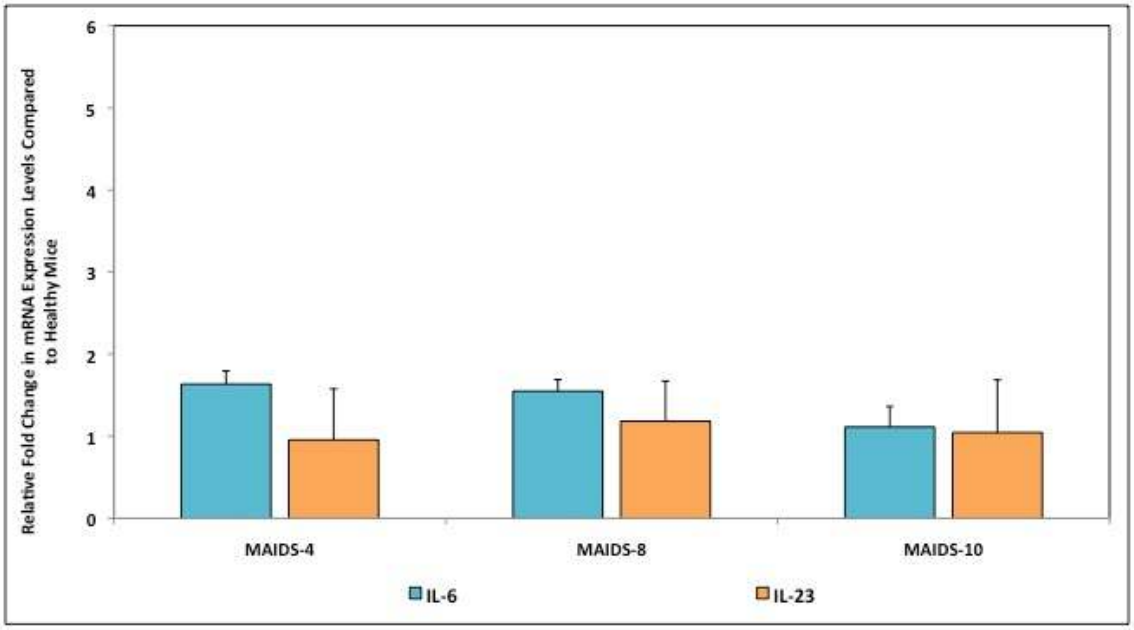

Figure 4.5. IL-6 and IL-23 mRNA levels of whole splenic cells from C57BL/6 mice during the progression of MAIDS. IL-6 and IL-23 mRNA levels of whole splenic cells collected from groups of MAIDS-4, MAIDS-8, and MAIDS-10 mice (*n* = 5). Levels (fold-change) of IL-6 and IL-23 mRNA were determined by quantitative RT-PCR assay. Bars = Standard Error of the Mean of 3 independent experiments. Asterisks indicate statistical significance.

4.2.5 IL-17 mRNA levels in MCMV-infected eyes of MAIDS-4 mice resistant to retinitis and MCMV-infected eyes of MAIDS-10 mice susceptible to retinitis

We next sought to determine whether the increased systemic levels of IL-17 from whole splenic cells of MAIDS mice contributed to the pathogenesis of MCMV-related retinitis. We have previously reported that while MCMV-infected eyes of MAIDS-4 and MCMV-infected eyes of MAIDS-10 mice have equivalent amounts of infectious virus at 10 days after subretinal MCMV infection, MAIDS-4 animals are resistant to MCMV retinitis whereas MAIDS-10 animals are susceptible to MCMV retinitis (Chien and Dix, 2012; Dix and Cousins, 2004b). Because MAIDS-4 and MAIDS-10 mice exhibit such divergent pathogenic outcomes following subretinal MCMV infection, we compared MCMV-infected eyes of MAIDS-4 and MCMV-infected eyes of MAIDS-10 mice for amounts of IL-17 mRNA. Whole eyes were collected from parallel groups of MAIDS-4 and MAIDS-10 animals inoculated subretinally with MCMV or maintenance medium only (control), and analyzed on days 3, 6, and 10 post-infection for amounts of IL-17 mRNA. Subretinal MCMV infection of eyes of MAIDS-4 mice did not result in increased IL-17 mRNA levels when compared to mock-infected eyes of MAIDS-4 mice (Fig. 4.6A). In sharp contrast, MCMV-infected eyes of MAIDS-10 mice exhibited a significant ($p \leq 0.05$) decrease in IL-17 mRNA levels on day 10 when compared to mock-infected eyes of MAIDS-10 mice (Fig. 4.6A).

Due to the significant decrease in IL-17 mRNA levels in MCMV-infected eyes of MAIDS-10 mice, ELISA and immunohistochemical staining were also utilized to detect and quantify intraocular IL-17 protein in these animals. IL-17 protein levels were decreased, although not significantly, when compared to contralateral mock-infected eyes (data not shown). Immunohistochemical staining also revealed that IL-17 protein was detectable in cells of the neurosensory

retina and RPE of the eyes of both MCMV-infected and mock-infected MAIDS-10 mice (Fig 4.6B), but IL-17 production in the MCMV-infected eyes of MAIDS-10 mice was dampened when compared to mock-infected eyes. Taken together (Fig 4.6A and 4.6B), these unexpected results suggested that MCMV infection may downregulate IL-17 production within the ocular compartment during MAIDS.

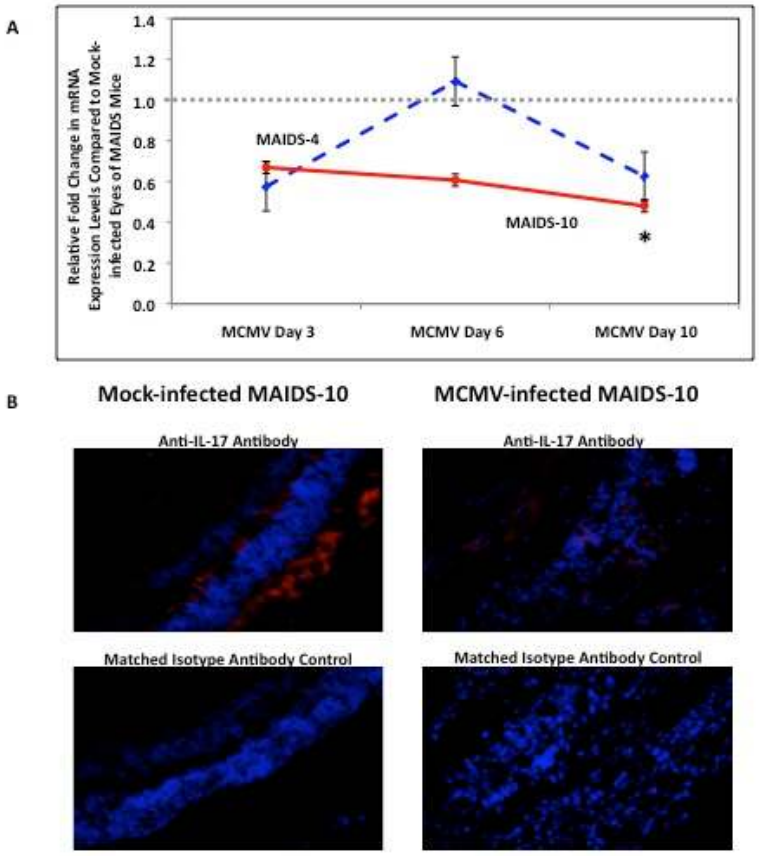

Figure 4.6. IL-17 mRNA levels in MCMV-infected eyes of MAIDS-4 versus MAIDS-10 mice and detection of IL-17 protein in MCMV-infected eyes of MAIDS-10 mice. A) Comparison of IL-17 mRNA levels in MCMV-infected eyes of MAIDS-4 mice (blue dashed line) versus MCMV-infected eyes of MAIDS-10 mice (red solid line) collected at 3, 6, or 10 days after subretinal MCMV infection (*n* = 5). Levels (fold-change) of IL-17 mRNA were determined by quantitative RT-PCR assay. Bars = Standard Error of the Mean of 2 independent experiments. Asterisks indicate statistical significance. (B) Detection of IL-17 in cells of representative retinal tissue sections collected from eyes of MAIDS-10 mice at 10 days after subretinal MCMV infection or mock infection. Formalin-fixed cytosections were reacted with anti-IL-17 antibody (red) or an isotype-matched normal antibody (control). Nuclei were counterstained with DAPI (blue) (200X).

4.2.6 Effect of systemic MCMV infection on IL-17 mRNA levels in splenic CD4+ T cells during health and MAIDS

The observation of significantly decreased IL-17 mRNA levels and dampened protein levels in MCMV-infected eyes of MAIDS-10 mice led us to investigate the remarkable possibility that MCMV infection might lead to a preferential downregulation of IL-17 from CD4+ Th17 cells systemically. To investigate this possibility, IL-17 mRNA levels were quantified by real-time RT-PCR assay in populations of splenic CD4+ T cells enriched by flow cytometry (purity > 90%) from groups of healthy, MAIDS-4, or and MAIDS-10 mice infected systemically by i.p. injection with a sublethal dose of MCMV for 6 days. Systemic MCMV infection of all animals resulted in a significant ($p \leq 0.01$) decrease in IL-17 mRNA levels in splenic CD4+ T cells when compared to mock-infected controls (Figs. 4.7A and 4.7B). Whole splenic cell populations from all animal groups also exhibited a significant ($p \leq 0.008$) decrease in IL-17 mRNA levels (data not shown). Thus, systemic MCMV infection did indeed result in a significant downregulation of IL-17 mRNA production from CD4+ T cells.

In order to determine whether productive MCMV infection was required to downregulate IL-17 mRNA production in CD4+ T cells, groups of healthy, MAIDS-4, or MAIDS-10 mice were systemically inoculated with either UV-inactivated MCMV or infectious MCMV for 6 days, and splenic CD4+ T cells enriched by flow cytometry (purity >90%) were analyzed for IL-17 mRNA expression via real-time RT-PCR assay. IL-17 mRNA levels were significantly ($p \leq 0.05$) reduced in all animals inoculated with UV-inactivated MCMV (Fig. 4.7C), but IL-17 mRNA levels were further reduced when animals were inoculated with infectious MCMV (Fig. 4.7C). These results suggest that MCMV structural protein(s) (tegument proteins and/or glycoproteins) as

well as one or more virus-induced proteins produced during active virus replication alter the host cell response to decrease IL-17 production during systemic MCMV infection.

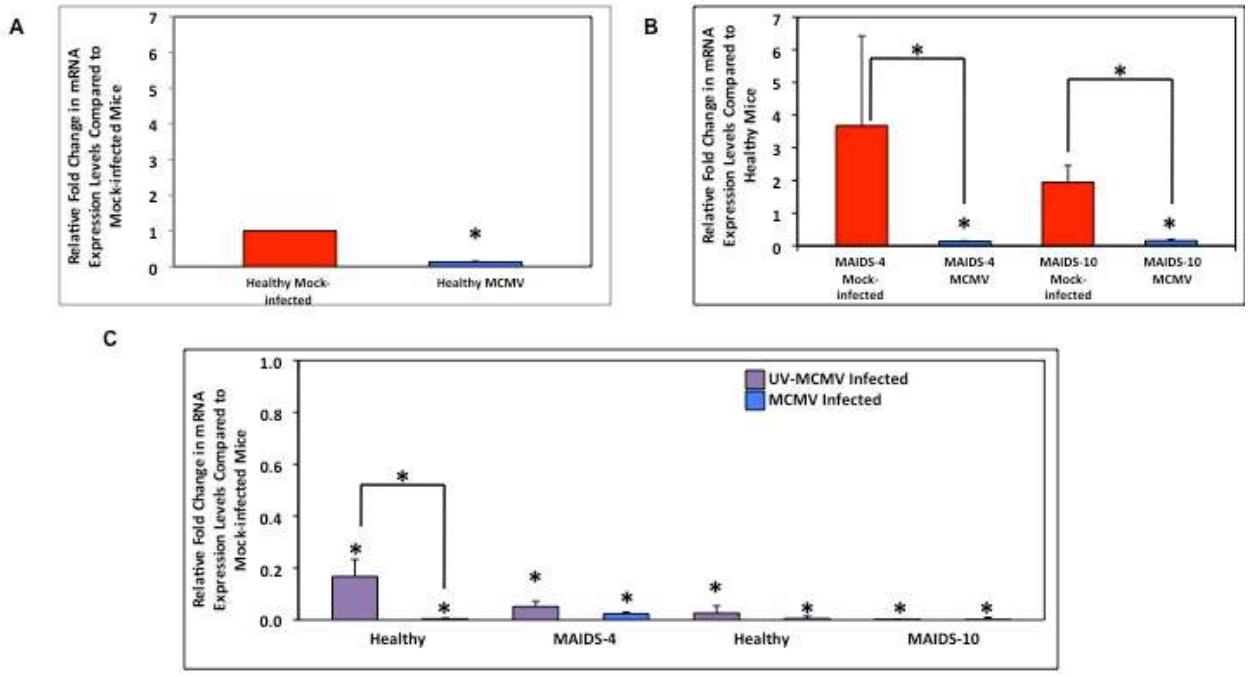

Figure 4.7. IL-17 mRNA levels in splenic CD4+ T cells of healthy C57BL/6 mice, MAIDS-4 mice, and MAIDS-10 mice following systemic MCMV infection. IL-17 mRNA levels of splenic CD4+ T cells collected from (A) groups of healthy C57BL/6 mice (*n* = 5) and (B) groups of MAIDS-4 and MAIDS-10 mice (*n* = 5) at 6 days after i.p. inoculation with MCMV or mock-infection. Levels (fold-change) of IL-17 mRNA were determined by quantitative RT-PCR assay. Bars = Standard Error of the Mean of 3 independent experiments. Asterisks indicate statistical significance. (C) IL-17 mRNA levels of splenic CD4+ T cells collected from MAIDS-4 mice, MAIDS-10 mice, and their age-matched healthy controls inoculated with either UV-inactivated MCMV or infectious MCMV. Levels (fold-change) of IL-17 mRNA were determined by quantitative RT-PCR assay. Bars = Standard Error of the Mean of 1 experiment. Asterisks indicate statistical significance.

4.2.7 SOCS-3, SOCS-1, and IL-10 mRNA expression levels in whole splenic cells of mice with MAIDS.

We then sought to investigate the possible mechanism(s) by which IL-17 mRNA was being downregulated by CD4+ T cells during MAIDS progression and ultimately during MCMV infection. Previous studies conducted by Chen and colleagues (Chen et al., 2006) identified the SOCS-3 protein as a negative regulator of IL-17 in CD4+ T cells. Additionally, loss of the SOCS-1 protein leads to defective Th17 cell differentiation (Tanaka et al., 2008; Yoshimura et al., 2007). Ongoing work in our laboratory by Dr. Hsin Chien has also tied SOCS-1 and SOCS-3 to MCMV-related disease. Although both SOCS-1 and SOCS-3 are increased during HIV infection (Akhtar and Benveniste, 2011), we investigated whether SOCS-3 mRNA levels were increased in mice with retrovirus-induced immunosuppression and/or whether SOCS-1 mRNA levels were decreased, thereby leading to a decrease in IL-17 expression from CD4+ T cells during MAIDS. Whole splenic cells collected from groups of MAIDS-4, MAIDS-8, or MAIDS-10 mice were quantified for SOCS-1 and SOCS-3 mRNA levels by real-time RT-PCR assay. Whereas splenic mRNA levels of SOCS-1 in MAIDS animals remained equivalent to mRNA levels of healthy mice (Fig. 4.8A), SOCS-3 mRNA levels were significantly ($p \leq 0.05$) increased during MAIDS progression (Fig. 4.8B). Importantly, increased SOCS-3 mRNA levels correlated with decreased IL-17 mRNA levels in splenic CD4+ T cells seen during MAIDS progression (Fig. 4.4).

Recent work has also led to the identification of the IL-10R on the surface of Th17 cells (Huber et al., 2011); IL-10 is able to bind its receptor and negatively regulate the secretion of IL-17 from Th17 cells (Chaudhry et al., 2011). We therefore sought to determine if IL-10 mRNA expression during MAIDS progression was also increased. Whole splenic cells collected from

groups of MAIDS-4, MAIDS-8, and MAIDS-10 mice exhibited a significant ($p \leq 0.03$) increase in IL-10 mRNA production (Fig. 4.8C). In particular, levels of IL-10 mRNA were high in whole splenic cells of MCMV retinitis-susceptible MAIDS-8 and MAIDS-10 animals (Dix and Cousins, 2004a; Dix et al., 1994). The increase in IL-10 mRNA levels in whole splenic cells of MAIDS-8 mice and MAIDS-10 mice correlated with the decrease in IL-17 mRNA levels seen in CD4+ T cells during MAIDS progression (Fig. 4.4).

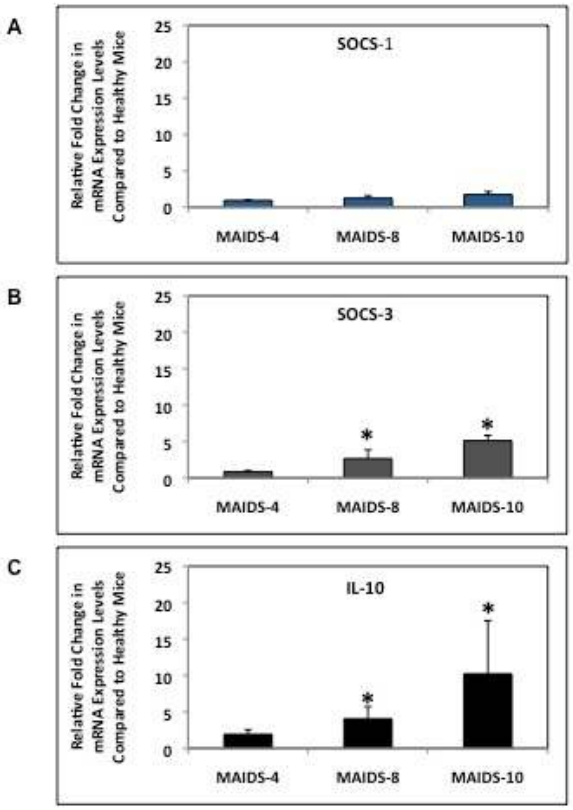

Figure 4.8. SOCS-1, SOCS-3, and IL-10 mRNA levels in whole splenic cells of C57BL/6 mice during the progression of MAIDS. (A) SOCS-1 mRNA levels, (B) SOCS-3 mRNA levels, and (C) IL-10 mRNA levels of whole splenic cells collected from groups of MAIDS-4, MAIDS-8, and MAIDS-10 mice (*n* = 5). Levels (fold-change) of SOCS-1 mRNA, SOCS-3 mRNA, and IL-10 mRNA were determined by quantitative RT-PCR assay. Bars = Standard Error of the Mean of 2 independent experiments. Asterisks indicate statistical significance.

4.2.8 SOCS-1, SOCS-3, and IL-10 mRNA levels in MCMV-infected eyes of MAIDS-4 mice resistant to retinitis and MCMV-infected eyes of MAIDS-10 mice susceptible to retinitis

To determine whether SOCS-3 and IL-10 production were also negatively influencing the expression of IL-17 during the onset and progression of retinal necrosis in the eyes of MAIDS mice, MCMV-infected and mock-infected eyes from MAIDS-4 and MAIDS-10 mice were collected on days 3, 6, and 10 after MCMV subretinal infection and analyzed for SOCS-3 and IL-10 mRNA expression. Additionally, mRNA levels of the required Th17 lineage differentiation factors SOCS-1 and IL-6 (Bettelli et al., 2007; Yoshimura et al., 2007) were also examined in MCMV-infected and mock-infected eyes of MAIDS-4 and MAIDS-10 mice. While SOCS-3 mRNA levels were indeed significantly ($p \leq 0.03$) increased in MCMV-infected eyes of MAIDS-4 mice on day 6 prior to the appearance of retinal necrosis on day 10 (Chien and Dix, 2012; Dix et al., 1994), MCMV-infected eyes of MAIDS-10 mice exhibited a far greater increase ($p \leq 0.007$) in SOCS-3 mRNA levels when compared with mRNA levels of MCMV-infected eyes of MAIDS-4 mice on day 6 post-infection (Fig. 4.9B). Similarly, IL-10 mRNA levels in MCMV-infected eyes of MAIDS-10 mice exhibited a dramatic 12-fold and ~60-fold ($p \leq 0.01$) increase in IL-10 mRNA on days 3 and 6 post-infection, respectively, when compared with mRNA levels in MCMV-infected eyes of MAIDS-4 mice (Fig. 4.9C). In comparison, SOCS-1 (Fig. 4.9A) and IL-6 (data not shown) mRNA levels mirrored those seen for SOCS-3 and IL-10, with mRNA levels of both molecules being significantly ($p \leq 0.03$) increased in MCMV-infected eyes of MAIDS-10 mice when compared to MCMV-infected eyes of MAIDS-4 mice. It is noteworthy that increased mRNA levels of SOCS-3 and IL-10 in the MCMV-infected eyes of MAIDS-10 mice preceded the decrease in IL-17 mRNA

levels seen in the eyes of these animals during retinitis development (Fig 4.6A), suggesting that SOCS-3 and/or IL-10 contribute to the downregulation of IL-17 during ocular MCMV infection.

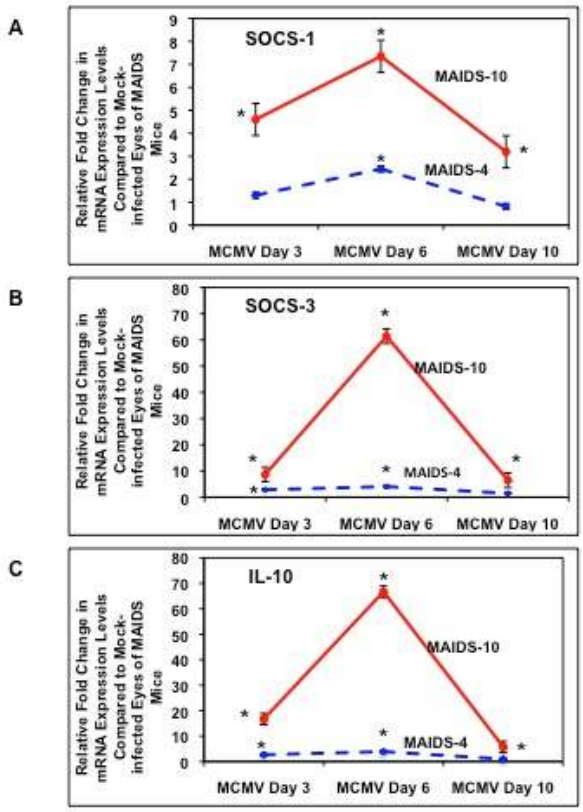

Figure 4.9. SOCS-1, SOCS-3, and IL-10 mRNA levels in MCMV-infected eyes of MAIDS-4 versus MCMV-infected eyes of MAIDS-10 mice. (A) SOCS-1 mRNA levels, (B) SOCS-3 mRNA levels, and (C) IL-10 mRNA levels of MCMV-infected eyes of MAIDS-4 mice (dashed line) versus MCMV-infected eyes of MAIDS-10 mice (solid line) collected at 3, 6, or 10 days after subretinal MCMV infection (*n* = 5). Levels (fold-change) of SOCS-1 mRNA, SOCS-3 mRNA, and IL-10 mRNA were determined by quantitative RT-PCR assay. Bars = Standard Error of the Mean of 2 independent experiments. Asterisks indicate statistical significance.

4.2.9 Effect of systemic MCMV infection on IL-17 mRNA and protein levels in whole splenic cells and splenic CD4+ T cells of wildtype mice and IL-10 -/- mice without MAIDS

Because we demonstrated that IL-10 mRNA levels were upregulated in whole splenic cells of MAIDS animals during the evolution of immunosuppression (Fig. 4.8C) as well as in the eyes of retinitis-susceptible MAIDS animals following subretinal MCMV infection (Fig. 4.9C) and that the increase in IL-10 mRNA is correlated with the decrease in IL-17 mRNA observed in CD4+ T cells, we investigated whether knockout of IL-10 would restore IL-17 levels. Wild-type mice without MAIDS and IL-10 -/- mice without MAIDS were systemically infected with a sublethal dose of MCMV. Six days p.i., whole splenic cells and splenic CD4+ T cells were collected and analyzed for IL-17 mRNA and protein levels by real-time RT-PCR assay and ELISA, respectively. Systemic MCMV infection of wild-type mice resulted in a significant ($p \leq 0.03$) decrease in IL-17 mRNA levels (Fig. 4.10A) confirming our previous findings (Fig 4.7A). However, while IL-17 mRNA levels were dampened in splenic cells of systemically infected IL-10 -/- mice when compared to mock-infected controls (Fig. 4.10A), IL-17 protein levels were significantly ($p \leq 0.05$) increased in whole splenic cells of these mice [12.28± 7.7 pg of IL-17 per gram of spleen (wet weight)] when compared to systemically infected wild-type mice [4.47± 3.5 pg of IL-17 per gram of spleen (wet weight)] (Fig. 4.10B). The production of IL-17 mRNA in splenic CD4+ T cells was significantly ($p \leq 0.03$) reduced in both animal groups (Fig. 4.10C). However, IL-17 mRNA levels were moderately increased, showing ~2-fold higher levels of production in systemically infected IL-10 -/- animals (Fig. 4.10D). These results suggest that IL-10 knockout partially restored IL-17 levels during systemic MCMV infection.

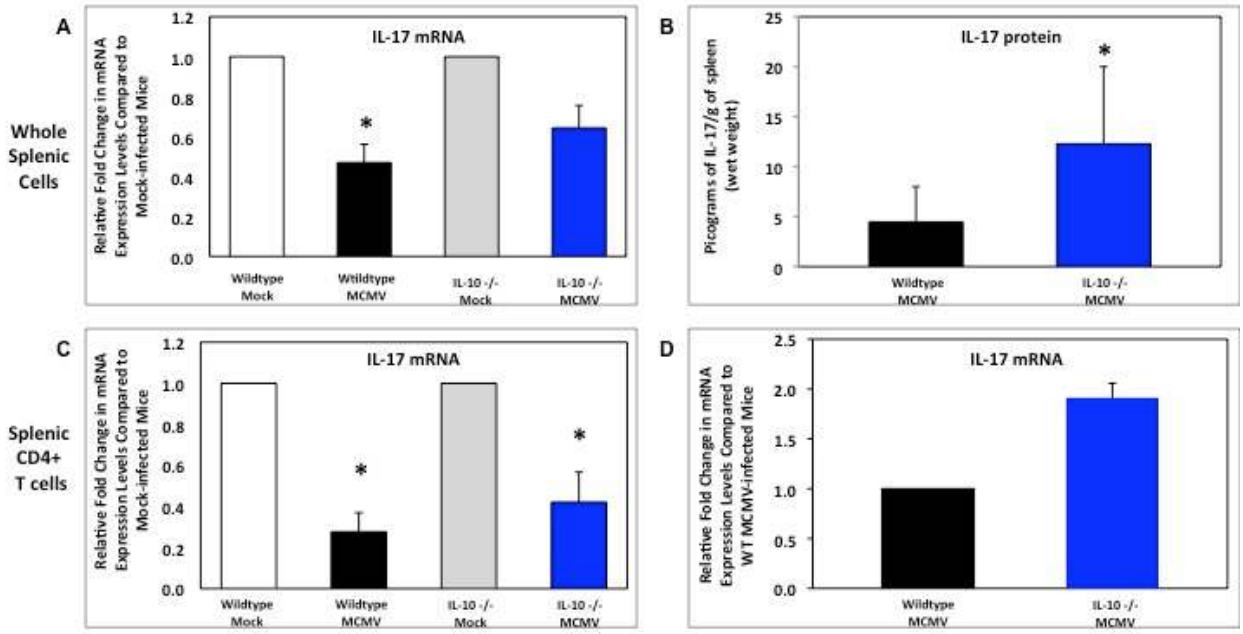

Figure 4.10. IL-17 mRNA and protein levels in whole splenic cells and splenic CD4+ T cells of wild-type mice and IL-10 -/- mice following systemic MCMV infection. Comparison of IL-17 mRNA levels of (A) whole splenic cells, (C) and (D) splenic CD4+ T cells collected from wild-type mice (*n* = 5) and IL-10 -/- mice (*n* = 5) at 6 days following i.p. MCMV infection. Levels (fold-change) of IL-17 mRNA were determined by quantitative RT-PCR assay. Bars = Standard Error of the Mean of 2 independent experiments. Comparison of IL-17 protein levels of (B) whole splenic cells collected from wild-type mice (*n* = 5) and IL-10 -/- mice (*n* = 5) at 10 days following i.p. MCMV infection. Protein levels were determined by ELISA. Bars = Standard Error of the Mean of 2 independent experiments. Asterisks indicate statistical significance.

4.2.10 L-17 mRNA and protein levels in whole splenic cells and eyes of wildtype MAIDS-8 mice and IL-10 -/- MAIDS-8 mice following subretinal MCMV inoculation

Since we demonstrated that systemic MCMV infection of IL-10 -/- mice without MAIDS resulted in a partial restoration of IL-17 mRNA and protein levels, we next wanted to determine whether subretinal MCMV infection of retinitis-susceptible IL-10 -/- MAIDS-8 mice also resulted in partial recovery of IL-17 mRNA and protein levels. Whole spleens and eyes were collected from wild-type MAIDS-8 mice and IL-10 -/- MAIDS-8 mice on day 10 after subretinal MCMV inoculation and analyzed for IL-17 mRNA and protein levels by real-time RT-PCR assay and ELISA, respectively. Results are shown in Figure 4.11. While IL-17 mRNA levels did not increase in either whole splenic cells or whole eyes of IL-10 -/- MAIDS-8 mice following subretinal MCMV inoculation (Fig. 4.11A and 4.11B), IL-17 protein levels were consistently elevated, although not significantly, in both splenic cells and eyes of the IL-10 -/- MAIDS-8 animals (Fig. 4.11C and 4.11D). Ocular tissues collected from IL-10 -/- MAIDS-8 mice and analyzed for IL-17 protein via immunohistochemistry exhibited an enhancement in IL-17 protein production when compared to MCMV-infected eyes of wild-type MAIDS-8 mice (Fig. 4.12), although both groups remained equally susceptible to MCMV retinitis (85% frequency of retinitis, n = 7 mice/group). As observed during systemic MCMV infection, these results suggest that partial restoration of IL-17 was not sufficient to protect MAIDS mice against MCMV retinitis.

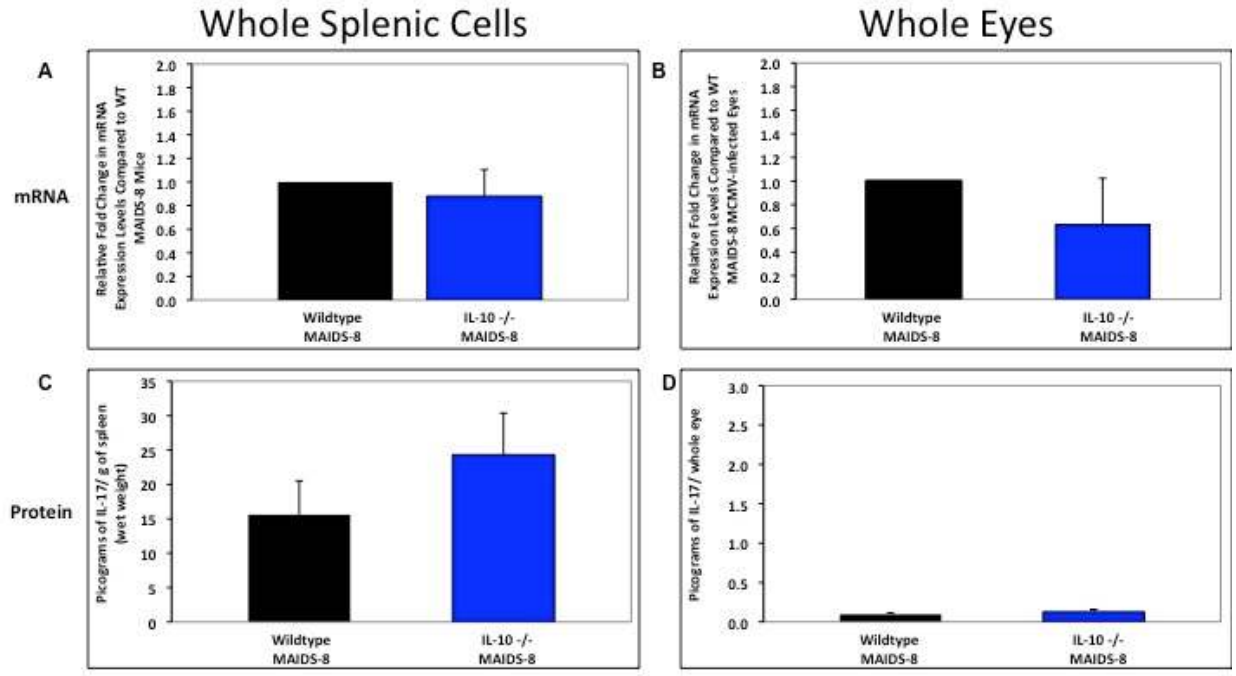

Figure 4.11. IL-17 mRNA and protein levels in whole splenic cells and MCMV-infected eyes of wild-type MAIDS-8 and IL-10 -/- MAIDS-8 mice following subretinal MCMV infection. Comparison of IL-17 mRNA levels in (A) whole splenic cells and (B) MCMV-infected eyes of groups of wild-type MAIDS-8 mice (*n* = 10) and IL-10 -/- MAIDS-8 mice (*n* = 10) at 10 days after subretinal MCMV infection. Levels (fold-change) of IL-17 mRNA were determined by quantitative RT-PCR assay. Bars = Standard Error of the Mean of 1 experiment. Comparison of IL-17 protein levels in (C) whole splenic cells and (D) MCMV-infected eyes of groups of wild-type MAIDS-8 mice (*n* = 10) and IL-10 -/- MAIDS-8 mice (*n* = 10) at 10 days after subretinal MCMV infection. Protein levels were determined by ELISA. Bars = Standard Error of the Mean of 1 experiment. All comparisons showed no statistical significance.

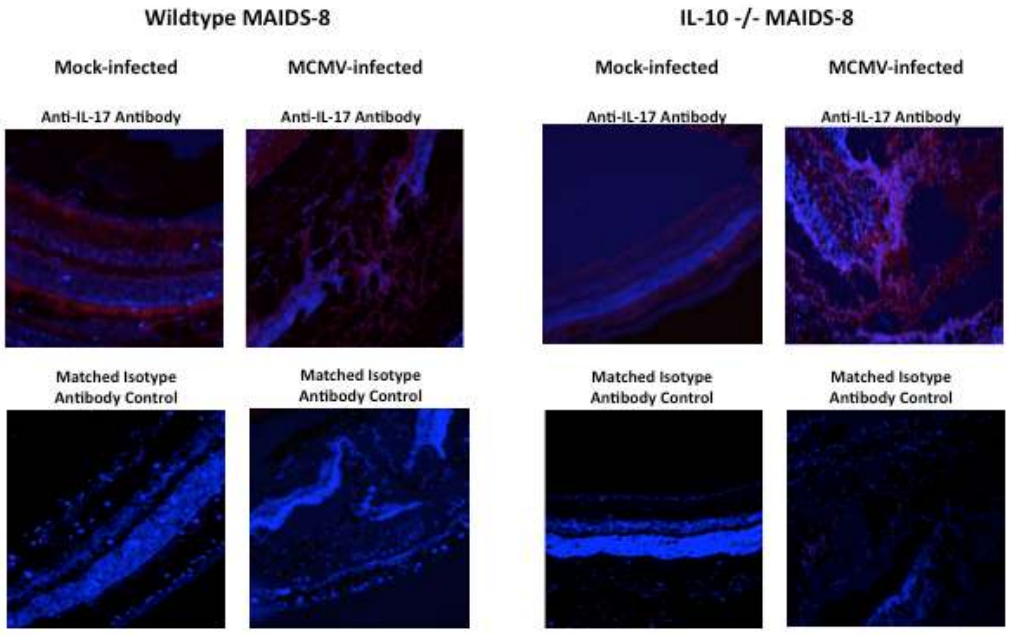

Figure 4.12. Detection of IL-17 protein in MCMV-infected eyes of wild-type MAIDS-8 mice and IL-10 -/- MAIDS-8 mice. Detection of IL-17 in cells of representative retinal tissue sections collected from eyes of wild-type MAIDS-8 mice and IL-10 -/- MAIDS-8 mice at 10 days after subretinal MCMV infection or mock infection. Formalin-fixed cytosections were reacted with anti-IL-17 antibody (red) or an isotype-matched normal antibody (control). Nuclei were counterstained with DAPI (blue) (200X).

5 DISCUSSION, FUTURE DIRECTIONS, AND SIGNIFICANCE

5.1 Aim 1: Increased CD4+ T cell cytokines IL-4 or IL-10 dowregulate cellular immunity to increase susceptibility to experimental MCMV retinitis in mice with MAIDS

Since its appearance as a major cause of vision loss and blindness within the United States nearly 30 years ago, AIDS-related HCMV retinitis has become well characterized clinically and histologically (Dix and Cousins, 2004a; Holland, 1996). Despite many years of extensive clinical and laboratory investigation, however, a number of basic issues related to the virology, immunology, and pathogenesis of this sight-threatening disease within the unique immunosuppressive environment of HIV infection and disease remain unresolved. Among these is a crisp and comprehensive understanding of the basic immunological changes that take place during retrovirus-induced immunosuppression, especially those involved during loss of cellular immunity. Toward this end, we previously used our MAIDS model of MCMV retinitis to show that the perforin-mediated cytotoxic pathway is more important than the Fas/FasL-mediated cytotoxic pathway in protection against MAIDS-related MCMV retinitis (Dix et al., 2003a). We (Dix et al., 2003a), and others (Loh et al., 2005), showed that loss of the perforin cytotoxic pathway results in an increased susceptibility to MCMV-induced disease, including retinitis. Resistance to MCMV-related disease, however, could be restored by immunotherapy with the Th1 cytokine IL-2 (Dix et al., 1997b), a cytokine that has robust effects on cytotoxic T cell and natural killer (NK) cell functions (Smith, 1988). Unanswered in these investigations, however, was an understanding of the precise mechanism(s) by which the perforin cytotoxic pathway is diminished during MAIDS in favor of the Fas/FasL cytotoxic pathway. These findings, coupled with the fact that progression of MAIDS is also associated with a prominent shift in cytokine production by

CD4+ T cells from a Th1 profile to a Th2 profile (Dix and Cousins, 2004a) (Fig. 3.1) as seen during AIDS (Clerici and Shearer, 1993), lead to the attractive hypothesis that IL-4 or IL-10, both Th2 cytokines that have been shown to be involved in downregulation of cellular immunity (Aung and Graham, 2000; Baschuk et al., 2007; Dix et al., 2003a; Kienzle et al., 2002; Kienzle et al., 2005), play key roles in the pathogenesis of MAIDS-related MCMV retinitis.

Results reported herein suggest that this is not the case, and our hypothesis is incorrect. In fact, MCMV-infected eyes of mice with MAIDS deficient in systemic IL-4 or IL-10 displayed a frequency of retinitis, a severity of retinitis, and intraocular amounts of virus equivalent to those found in MCMV-infected eyes of wild-type mice with MAIDS.

That IL-4 or IL-10 should play a pivotal role in loss of cellular immunity during the pathogenesis of MAIDS-related MCMV retinitis was a reasonable prediction. In addition to studies that have shown that increased IL-4 or IL-10 production during HIV infection results in decreased cytotoxic CD8+ T-cell activity against many viruses including HCMV (Elrefaei et al., 2006; Elrefaei et al., 2007; Erard et al., 1994; Erard et al., 1993), numerous in vitro and in vivo studies have also noted an association between elevated levels of the Th2 cytokine IL-4 and decreased CD8+ T-cell-mediated cytotoxicity, all associated with an increase in FasL expression and a concomitant decrease in perforin and granzyme B expression. For example, Kienzle and colleagues (Kienzle et al., 2002; Kienzle et al., 2005) demonstrated that incubation of naïve mouse CD8+ T cells to high concentrations of IL-4 resulted in a population of T cells with significantly lower expression of CD8 and reduced cytotoxic ability. Aung and coworkers (Aung and Graham, 2000) infected mice with an IL-4-expressing respiratory RSV recombinant and observed a subsequent increase in expression of FasL on CD4+ and CD8+ T cells coupled with al-

teration of the mechanism of CD8+ T-cell cytotoxicity from a perforin-mediated pathway to a favored Fas/FasL-mediated pathway. Moreover, systemic treatment of mice with anti-IL-4 antibody resulted in decreased morbidity following challenge with wild-type RSV due to an enhanced CD8+ cytotoxic response that lead to more effective virus clearance (Aung and Graham, 2000). Similar findings were reported by Jackson and colleagues (Jackson et al., 2001) for mousepox virus infection. These workers observed suppressed CD8+ T-cell cytotoxicity as well as suppressed NK-cell cytotoxicity in mice infected with an IL-4-expressing mousepox virus, outcomes that resulted in increased virus-induced mortality.

Despite this convincing body of evidence, we did not observe increased resistance to MCMV retinitis in mice with MAIDS in the absence of functional IL-4 or IL-10 production as hypothesized. Without functional IL-4 or IL-10, two Th2 cytokines thought to dampen cellular immunity during retrovirus-induced immunosuppression (Elrefaei et al., 2006; Elrefaei et al., 2007; Erard et al., 1994; Erard et al., 1993; Furukawa et al., 2008), we predicted a resurgence in CD8+ T-cell cytotoxicity that would provide protection against onset and progression of MCMV-induced retinal disease, possibly through stimulation of the perforin cytotoxic pathway associated with a concomitant decline in the Fas/FasL cytotoxic pathway. Surprisingly, this was not the case. In fact, loss of IL-4 or IL-10 during MAIDS resulted in no significant change in splenic mRNA levels for perforin or granzyme B, two key molecules involved in the perforin cytotoxic pathway, when compared with wild-type mice with MAIDS. More importantly, an increase in splenic mRNA levels for FasL was observed following loss of either IL-4 or IL-10 during MAIDS when compared with wild-type mice with MAIDS. One explanation for these unexpected results is that while IL-4 or IL-10 may work individually to suppress cellular immunity during MAIDS,

they also function collectively toward this end. Our findings suggest that loss of IL-4 or IL-10 during MAIDS is an independent event that fails to affect production of the other Th2 cytokine. In fact, whole splenic cells from IL-4 -/- MAIDS mice exhibited increased IL-10 protein levels and whole splenic cells from IL-10 -/- MAIDS mice exhibited equivalent IL-4 protein levels to wild-type MAIDS mice, which potentially explains why these individual knockout MAIDS animals remained susceptible to retinitis. Thus, loss of one of these Th2 cytokines is not sufficient to reduce the frequency or severity of MAIDS-related MCMV retinitis in these animals.

Another possible explanation for our unexpected findings may be that neither IL-4 nor IL-10 play a role in governing susceptibility to MCMV retinitis. It is noteworthy that more recent findings from other laboratories have suggested that IL-4 can indeed support development of cytotoxic T cells when incubated with highly purified primary T cells collected from lymph nodes of BALB/c mice (Miller et al., 1990; Trenn et al., 1988). Studies conducted by Bachmann and coworkers (Bachmann et al., 1995) also demonstrated that IL-4 -/- C57BL/6 mice, when compared with wild-type C57BL/6 mice, failed to exhibit changes in cytotoxic T-cell responses when infected with lymphocytic choriomeningitis virus (LCMV) or vaccinia virus. These animals were also able to clear virus as a result of an effective cytotoxic T-cell response. Additional studies conducted by Mo and colleagues (Mo et al., 1997) noted that CD8+ T cells collected from IL-4 -/- 129/J mice and wild-type 129/J mice exhibited equivalent cytotoxic activity against Sendai virus infection. Similarly, IL-10 has also been found to promote T cell development as well as CD8+ T cell cytotoxicity (Chen and Zlotnik, 1991; MacNeil et al., 1990; Santin et al., 2000). In fact, human pappillomavirus-specific CD8+ T cells isolated from cervical cancer patients that were incubated with IL-10 + IL-2 exhibited increased cytotoxicity over that observed in CD8+ T

cells incubated with IL-2 alone (Santin et al., 2000). This increase CD8+ T cell cytotoxicity was correlated with increased perforin levels in the IL-10 + IL-2-treated CD8+ T cells (Santin et al., 2000). While it is important to recognize that none of these studies investigated IL-4 or IL-10 and cellular immunity in the context of retrovirus-induced immunosuppression, these data indicate that perhaps neither IL-4 nor IL-10 decrease CD8+ T cell cytotoxicity to increase susceptibility to MCMV retinitis.

In summary, we show herein that mice with MAIDS deficient in either IL-4 or IL-10 do not develop resistance to MCMV retinitis as measured by amounts of intraocular infectious virus, frequency of retinitis, and severity of retinitis. Our findings therefore disprove our initial working hypothesis that IL-4 or IL-10 are key Th2 cytokines that promote increased susceptibility to MAIDS-related MCMV retinitis by dampening of cellular immunity, possibly by favoring the Fas/FasL cytotoxic pathway over the perforin cytotoxic pathway. However, IL-4 and IL-10 may work collectively with each other as well as with other host factors to create the unique intraocular environment that allows for pathogenesis of MCMV retinitis.

5.1.1 Future directions

Future studies should address whether IL-4 and IL-10 work in a synergistic manner to govern susceptibility to MCMV-related retinitis during MAIDS. One experimental approach to test this hypothesis would be to perform parallel studies using mice with MAIDS that suffer systemic loss in both IL-4 and IL-10 production. Unfortunately, double knockout mice exist only on a BALB/c background (Jackson Laboratory, Strain 004291), and MAIDS cannot be induced in BALB/c mice (Hartley et al., 1989). Alternatively, we could reduce systemic levels of both IL-4 and IL-10 in mice with MAIDS using an antibody approach to neutralize their functions. How-

ever, studies to reduce systemic levels of only IL-4 in mice with MAIDS using anti-IL-4 antibody proved to be ineffective, resulting in only ~50% reduction in splenic levels of IL-4 mRNA and protein (Fig. 3.2). Lastly, future studies of CD8+ T cell cytotoxicity utilizing flow cytometry-based killing assays would be a nice addition to this study in order to show that CD8+ T cell-mediated cytotoxic activities in the IL-4 -/- MAIDS mice and the IL-10 -/- MAIDS mice are equivalent to the CD8+ T cell-mediated cytotoxic activities seen in wild-type MAIDS mice and that this loss in cell-mediated cytotoxicity is correlated with increased FasL mRNA expression in whole splenic cells of the knockout MAIDS animals.

5.2 Aim 2: Increased production of IL-17 from CD4+ Th17 cell contributes to the pathogenesis of experimental MCMV retinitis in mice with MAIDS

The aims of the present study were to determine the fate of IL-17 during HIV infection, and to determine whether IL-17 contributes to the pathogenesis of AIDS-related HCMV retinitis through the use of a clinically relevant mouse model of MAIDS-related MCMV retinitis. Whereas IL-17 expression was detected within the spleen and eyes of healthy mice, IL-17 mRNA and protein were increased systemically during MAIDS progression when compared to healthy mice. However, MCMV-infected eyes of retinitis-susceptible MAIDS-10 mice, but not MCMV-infected eyes of retinitis-resistant MAIDS-4 mice, exhibited a significant decrease in IL-17 mRNA expression and dampened IL-17 protein levels. IL-17 mRNA was also decreased in whole splenic cells as well as in splenic CD4+ T cells of healthy mice and MAIDS animals upon systemic MCMV infection. This decrease was associated with increased levels of SOCS-3 and IL-10 mRNAs during

MCMV infection. However, knockout of IL-10 in MAIDS mice only partially restored IL-17 levels and failed to protect mice against MCMV retinitis.

5.2.1 IL-17 production in splenic cells and eyes of C57BL/6 mice during health

IL-17 was detected at low levels in the spleens of healthy mice, an observation consistent with that of Brenchley and coworkers (Brenchley et al., 2008) who detected low levels of IL-17-expressing CD4+ T cells in the peripheral blood of healthy human patients. IL-17 was also detected in cells of the neurosensory retina and the photoreceptor layer of healthy mice, but IL-17 production in the eye was lower than that in the spleen. Further investigation determined that both cell types that comprise the photoreceptor layer, rod cells and cone cells, constitutively express IL-17 during health. To our knowledge, ours is the first study to identify resident IL-17-expressing cells within the ocular compartment of healthy mice. We postulate that in addition to retinal photoreceptors cells, retinal pericytes and/or microglia also produce IL-17 in the healthy mouse eye due to their distribution within the neurosensory retina and their ability to secrete inflammatory cytokines (Chen et al., 2002; Crane and Liversidge, 2008; Guillemin and Brew, 2004). Future experiments should be focused on determining the cellular source of IL-17 production in the neurosensory retina of healthy mice.

5.2.2 IL-17 production in splenic cells and eyes of C57BL/6 mice during progression of MAIDS

Previous studies on IL-17 production during HIV/AIDS remain discordant (Brenchley et al., 2008; Maek et al., 2007; Yue et al., 2008). Our findings using mice with MAIDS to determine

the fate of IL-17 during the progression of retrovirus-induced immunosuppression agreed with previous studies (Maek et al., 2007; Yue et al., 2008) showing IL-17 production was increased in patients during HIV infection. We observed a significant increase in IL-17 mRNA and protein levels in whole splenic cell populations as well as in mRNA in whole eyes when compared to healthy age-matched controls. However, unlike whole splenic cells, MAIDS progression was also associated with a concomitant decrease in IL-17 mRNA levels in splenic CD4+ T cells, a finding that was in agreement with Brenchley and coworkers (Brenchley et al., 2008) observations in HIV-infected individuals. Other known cellular sources of IL-17, splenic macrophages and splenic Gr-1-expressing cells (neutrophils), contributed little to the overall IL-17 mRNA levels observed during MAIDS progression suggesting that there is not one sole source of IL-17 production by these animals. Thus, CD4+ T cells, macrophages, and Gr-1-expressing cells (neutrophils) in combination with other known sources of IL-17 including natural killer T cells (NKTs), CD8+ T cells, and gamma-delta ($\gamma\delta$) T cells (Gaffen, 2009; Iwakura et al., 2011; Rachitskaya et al., 2008) might all collectively contribute to the overall increase of IL-17 observed during MAIDS. The contributions of each of the other known cellular sources of IL-17, including CD8+ T cells, NKT cells, and $\gamma\delta$ T cells, to the increased levels of IL-17 observed in whole splenic cells of MAIDS mice during retrovirus-induced immunosuppression progression should be assessed in future studies.

5.2.3 IL-17 production and susceptibility to MCMV retinitis during MAIDS

Intraocular IL-17 production has been associated with uveitis, an inflammatory disease of the retina and uvea. Disease etiology is autoimmune in nature, with patients exhibiting im-

mune responses to ocular antigens including retinal arrestin and interphotoreceptor retinoid-binding protein (Caspi, 2010). EAU in mice has defined with some precision the effector cells involved in retinal and uveal tissue destruction during disease pathogenesis (Caspi, 2010; Luger and Caspi, 2008; Luger et al., 2008). Originally thought to be solely a Th1-mediated disease, uveitis is also induced through the actions of Th17 cells and IL-17 secretion (Caspi, 2010; Luger and Caspi, 2008; Luger et al., 2008). Neutralization of IL-17 through passive transfer of anti-IL-17 antibody has been shown to mitigate EAU severity significantly (Amadi-Obi et al., 2007; Luger et al., 2008; Peng et al., 2007). Conversely, adoptive transfer of retinal antigen-specific Th17 cells has been shown to induce EAU development in naïve mice (Luger et al., 2008; Peng et al., 2007).

Because IL-17 is involved in uveitis pathogenesis, we sought to determine whether the increase in IL-17 levels seen in mice with MAIDS played a role in susceptibility to MCMV retinitis. Whereas MAIDS-4 mice resistant to retinitis did not exhibit changes in IL-17 mRNA levels following subretinal MCMV inoculation when compared to mock-infected controls, MCMV-infected eyes of MAIDS-10 mice susceptible to retinitis exhibited a significant decrease in IL-17 mRNA levels. Additionally, ocular IL-17 protein levels and patterns of IL-17 immunohistochemical staining were dampened in MCMV-infected eyes of these retinitis-susceptible animals when compared to mock-infected eyes. We therefore conclude that IL-17 plays no direct role in increased susceptibility to MCMV retinitis during MAIDS. It is possible, however, that IL-17 may act indirectly to increase susceptibility to MCMV retinitis or may play a protective role in MCMV retinitis development in MAIDS mice.

5.2.4 IL-17 production in splenic CD4+ T cells of healthy mice and MAIDS mice following systemic MCMV infection

The surprising observation of significantly reduced IL-17 mRNA and dampened protein levels during ocular MCMV infection of MAIDS-10 mice led us to investigate whether MCMV actually downregulated IL-17 production from CD4+ T cells systemically. In agreement with this hypothesis was our finding that systemic MCMV infection resulted in a significant reduction of IL-17 mRNA levels in whole splenic cells and splenic CD4+ T cells from groups of healthy mice, MAIDS-4 mice, and MAIDS-10 mice. Moreover, experiments using UV-inactivated virus suggested that active MCMV infection was not an absolute requirement for reduction of IL-17 mRNA in CD4+ T cells, although productive MCMV replication did result in a more profound decrease in IL-17 mRNA levels. Taken together, these results suggest that a structural MCMV protein(s) as well as one or more virus-induced proteins during virus replication might work in concert to contribute to the downregulation of IL-17 through the upregulation of IL-10 and SOCS-3 (Fig. 5.1).

It is interesting to note that a structural protein of HCMV, the pp65 tegument protein, has been shown to alter the host immune response early during infection by blocking the interferon response and inhibiting the activity of NK cells through direct interaction with its receptor (Arnon et al., 2005; Kalejta, 2008; Miller-Kittrell and Sparer, 2009). Since MCMV encodes a pp65 homolog, M83/84 (Cranmer et al., 1996; Kattenhorn et al., 2004), it is possible that the M83/84 tegument protein of MCMV might also serve to downregulate IL-17 production in MCMV-infected cells. The action of these tegument proteins leads to decreased T cell activation and ultimately a decreased immune response that favors viral replication and persistence. It is pos-

sible then that during MCMV infection, the actions of tegument proteins indirectly leads to the decreased production of inflammatory cytokines including IL-17 from CD4+ T cells, macrophages, and neutrophils (Gr-1-expressing cells).

Structural HCMV glycoproteins also exhibit immune evasion properties. MCMV gp34, gp40, and gp48 are functional homologs of HCMV glycoproteins encoded by viral genes US2, US3, and US11, respectively (Kavanagh et al., 2001). These MCMV viral glycoproteins decrease MHC I protein expression on virus-infected cells (Kattenhorn et al., 2004; Kavanagh et al., 2001; Loewendorf and Benedict, 2010; Loewendorf et al., 2011; Mocarski, 2002; Wagner et al., 2002), leading to decreased CD4+ T cell and CD8+ T cell activation. In addition, the MCMV m155 protein inhibits expression of the CD4+ T cell and CD8+ T cell stimulator protein CD40 in virus-infected monocytes/macrophages as well as in virus-infected dendritic cells (Elgueta et al., 2009; Loewendorf et al., 2011; Ma and Clark, 2009). The action of these viral glycoproteins to decease the activation of CD4+ T cells might account for the reduction in IL-17 production from CD4+ T cells that was observed during MCMV infection of healthy mice and mice with MAIDS. Future experiments should be done to investigate the exact role of these viral proteins in IL-17 downregulation during systemic and ocular MCMV infection. Selective overexpression or knockdown of MCMV immunoregulatory tegument proteins m83/84 and/or MCMV immune evasion viral glycoproteins utilizing a bacterial artificial chromosome (BAC) system would reveal their direct involvement in downregulating IL-17 during systemic MCMV infection and whether the downregulation of IL-17 by MCMV is directly correlated with increased IL-10 and SOCS-3 levels.

In addition to MCMV viral tegument and glycoproteins contributing to the downregulation in IL-17 during infection, cytokine secretion from infected, activated macrophages may also contribute to the downregulation of IL-17 during systemic infection of mice with both actively replicating MCMV as well as UV-inactivated MCMV. Both HCMV and MCMV infection induce the production of immunosuppressive IL-10 (Humphreys et al., 2007; Redpath et al., 1999) in infected macrophages as well as in CD4+ T cells. We confirmed the upregulation of IL-10 in splenic CD4+ T cells of MAIDS-10 mice during MCMV infection (data not shown). In addition, IL-10 secretion from anti-inflammatory M2 macrophages promotes wound healing as well as angiogenesis, which consequently leads to the reduction of pro-inflammatory cytokines (Cassetta et al., 2011; Gordon, 2003; Mosser and Edwards, 2008; Sunderkotter et al., 1994). We believe that an upregulation of IL-10 from M2 macrophages as well as CD4+ T cells during MCMV infection as well as during UV-inactivated MCMV infection may potentially contribute to the downregulation of IL-17 we observed in both our healthy and MAIDS mice. However, MCMV and HCMV have been also noted to induce the secretion of pro-inflammatory cytokines from macrophages including TNF-α, IL-1β, and IL-6 during infection (Iwamoto et al., 1990; Pulliam et al., 1995; Smith et al., 1992; Vliegen et al., 2004). Pro-inflammatory M1 macrophage cytokines have been associated with increased IL-17 production and vise versa (Jovanovic et al., 1998; Sutton et al., 2006). Based on our results from these studies, we believe that acute MCMV infection leads to the induction of M2 macrophages as well as CD4+ T cells that secrete IL-10, which ultimately contributes to the downregulation in IL-17 that we observed in whole splenic cells as well as splenic CD4+ T cells of systemically infected healthy and MAIDS mice.

5.2.5 Proposed mechanism of IL-17 downregulation during MCMV infection

The observation that IL-17 was decreased in CD4+ T cells during MAIDS progression as well as during ocular and systemic infection of retinitis-susceptible MAIDS animals led us to investigate possible mechanisms by which retrovirus-induced immunosuppression might downregulate IL-17 production by CD4+ T cells. Recent studies have implicated both SOCS-3 and IL-10 as negative regulators of IL-17 secretion (Chaudhry et al., 2011; Gu et al., 2008; Huber et al., 2011). It is therefore possible that increases in one or both of these proteins would cause the IL-17 mRNA decrease observed by us in CD4+ T cells during MAIDS progression. SOCS-3 is induced by cytokine signaling (Yoshimura et al., 2007) and negatively regulates Th17 cell differentiation by suppressing STAT3 activation of IL-6 and IL-23 receptors on Th17 cells (Chen et al., 2006; Yoshimura et al., 2007). Additionally, SOCS-3 levels are increased during HIV infection (Akhtar and Benveniste, 2011; Akhtar et al., 2010). We have shown that SOCS-3 mRNA levels were significantly increased during retrovirus-induced immunosuppression, with mRNA levels peaking in MAIDS-10 mice when most susceptible to MCMV retinitis (Dix and Cousins, 2004a; Dix et al., 1994). Importantly, the increase in SOCS-3 mRNA levels correlated with the decrease in IL-17 mRNA levels seen in CD4+ T cells during MAIDS progression, suggesting a potential explanation for the observed downregulation in IL-17 mRNA from CD4+ T cells during MAIDS progression.

While little is currently known about the potential induction of SOCS-3 or SOCS-1 during MCMV infection, a number of viruses including influenza, respiratory syncytial virus (RSV), HIV, and HSV-1 exploit SOCS proteins, activating them in order to evade the host cell immune response (Akhtar and Benveniste, 2011; Frey et al., 2009; Yokota et al., 2005). We propose that

MCMV acts in a similar manner to upregulate SOCS-1 and SOCS-3 proteins. While we observed increases in both SOCS-1 and SOCS-3 mRNA levels in the MCMV-infected eyes of MAIDS-4 and MAIDS-10 mice, levels were far higher in the MCMV-infected eyes of MAIDS-10 mice that were susceptible to retinitis. Importantly, the increase in ocular SOCS-3 mRNA levels preceded the decrease in IL-17 mRNA seen in MCMV-infected eyes of MAIDS-10 mice. In addition, the increase in SOCS-3 mRNA on day 6 was approximately 10-fold higher than the SOCS-1 mRNA levels we observed in the MCMV-infected eyes of MAIDS-10 mice. These results indicate to us that while positive regulator of Th17 cell differentiation SOCS-1 was significantly increased during the development of MCMV retinitis in MAIDS-10 mice, these mRNA levels were not high enough to overcome the dramatic increase in mRNA that we observed in the Th17 cell negative regulator SOCS-3. Protein levels for both SOS-1 and SOCS-3 determined by Dr Hsin Chein in our laboratory (unpublished data) mirrored our mRNA results, where both SOCS-1 and SOCS-3 protein levels were significantly increased in the MCMV-infected eyes of MAIDS-10 mice when compared to MCMV-infected eyes of MAIDS-4 mice. However, protein levels in MCMV-infected eyes of both MAIDS-4 and MAIDS-10 mice peaked on day 10. The highest levels of SOCS-3 protein on day 10 correlated with the significant downregulation of IL-17 we observed the in MCMV-infected eyes of MAIDS-10 mice. This suggests that in addition to the action of its viral tegument proteins and glycoproteins, MCMV may also upregulate SOCS-3 for efficient viral replication or for evasion of the host immune response, which, in turn, results in the downregulation of IL-17.

IL-10 is an anti-inflammatory cytokine secreted by numerous immune cells, and it is able to regulate IL-17 secretion by binding to its receptor on Th17 cells (Chaudhry et al., 2011;

Couper et al., 2008; Huber et al., 2011). In our study, we demonstrated that IL-10 mRNA levels were significantly increased during MAIDS progression, and peaked at 10 weeks after retrovirus-induced immunosuppression when animals were susceptible to MCMV retinitis (Dix and Cousins, 2004a; Dix et al., 1994). Similar to SOCS-3, the increase in IL-10 mRNA levels correlated with the decrease in IL-17 mRNA levels seen in CD4+ T cells during MAIDS progression.

Systemic MCMV infection also induces expression of IL-10 in both CD4+ T cells as well as in MCMV-infected macrophages (Humphreys et al., 2007; Redpath et al., 1999). Increased production of IL-10 during MCMV infection leads to reduction of MHC class II on virus-infected cells and subsequently a decreased host response (Redpath et al., 1999). In agreement with these studies, we observed a significant increase in IL-10 mRNA levels in MCMV-infected eyes of MAIDS-10 mice that were susceptible to MCMV retinitis. As IL-10 mRNA levels increased during MCMV retinitis development, IL-17 mRNA levels decreased. It is therefore possible that MCMV infection orchestrates the downregulation of IL-17 through the induction of IL-10 along with SOCS-3.

Although systemic MCMV infection of IL-10 -/- mice without MAIDS resulted in an increase in IL-17 mRNA and protein in whole splenic cells and an increase in IL-17 mRNA expression levels in CD4+ T cells, and subretinal MCMV infection of MAIDS-8 IL-10 -/- mice led to elevated levels of IL-17 protein in whole splenic cells and MCMV-infected eyes, this partial recovery was not sufficient to protect MAIDS-8 mice against MCMV retinitis development. These results suggest that knockout of IL-10 results only in a partial restoration of IL-17 expression levels. Thus, other proteins, such as SOCS-3 and/or MCMV viral proteins, contribute to the downregulation of IL-17 during MCMV infection.

In conclusion, we have demonstrated that IL-17 plays no direct role in the pathogenesis of MAIDS-related MCMV retinitis. Remarkably, however, systemic MCMV infection of mice with MAIDS resulted in the downregulation of IL-17 production from CD4+ T cells, possibly through the actions of MCMV viral proteins and/or the upregulation of SOCS-3 and IL-10 mRNA production (Fig. 5.1) during infection. SOCS-3 and IL-10 may work in a synergistic manner to downregulate IL-17 from CD4+ T cells, although the direct role of SOCS-3 in downregulation of IL-17 during MCMV infection remains unclear. Lastly, while partial restoration of IL-17 through knockout of IL-10 did not reduce the frequency and/or severity of MCMV retinitis in MAIDS animals, it remains to be determined whether full restoration of IL-17 from Th17 cells during MAIDS plays a protective role in resistance to MCMV retinitis.

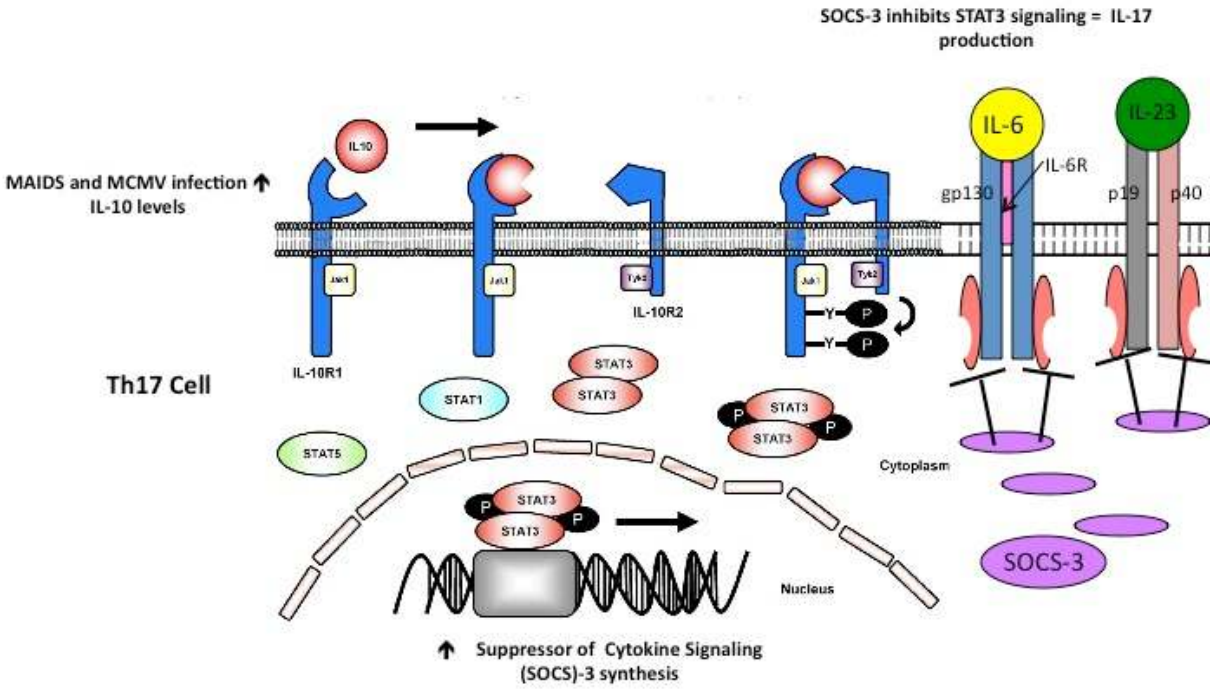

Figure 5.1. Proposed mechanism for downregulation of IL-17 in Th17 cells by MCMV. MCMV infection stimulates IL-10 production during health and retrovirus-induced immunosuppression. IL-10 binds to its receptor on Th17 cells that induces activation of Janus kinase-1 (Jak1) and tyrosine kinase-2 (Tyk2) that leads to the phosphorylation of signal transducer and activator of transcription-3 (STAT-3). Phosphorylated STAT-3 translocates to the nucleus where it upregulates SOCS-3 transcription that results in an increase in SOCS-3 protein intracellularly and ultimately inhibition of STAT-3 phosphorylation at the IL-6 and IL-23 receptors on Th17 cells. This inhibition culminates in decreased production of IL-17 by Th17 cells. [Adapted from (Sabat et al., 2010; Yoshimura et al., 2007)]

5.2.6 Future directions

While the results obtained from this study indicated that IL-17 does not play a direct role in MCMV-related retinitis pathogenesis, IL-17 may play an indirect role in governing susceptibility to MCMV retinitis development in MAIDS mice. For example, the progression of MAIDS results in increased numbers of neutrophils and macrophages systemically (Mosier et al., 1985), and it is possible that the increased systemic IL-17 we observed during the progression of retrovirus-induced immunosuppression, from multiple cellular sources, leads to the activation of these neutrophils and macrophages (Annunziato et al., 2010; Iwakura et al., 2011; Stockinger et al., 2007; Weaver et al., 2007). IL-17-activated macrophages and neutrophils in MAIDS mice may then perfuse into the tissues, including the eyes of MAIDS animals. This influx of activated macrophages and neutrophils into the eyes of MAIDS mice during subretinal MCMV infection caused by elevated IL-17 levels could thus contribute to retinitis development through the production of pro-inflammatory cytokines (i.e., TNF-α) by these cells (Chien and Dix, 2012; Dix and Cousins, 2004b). The use of IL-17A -/- mice in future experiments, through a collaboration with Dr. Chen Dong (Martin-Orozco et al., 2009), would allow us to decipher the exact role of IL-17 during the development of MAIDS as well as during MCMV-retinitis development in MAIDS animals.

On the other hand, because we observed decreased in IL-17 levels during MCMV infection of retinitis-susceptible MAIDS-10 mice, it is possible that IL-17 is important in mitigating rather than exacerbating MCMV-related retinal disease development. Future studies should investigate whether or not treatment of retinitis-susceptible MAIDS mice with recombinant IL-17 prior to subretinal MCMV infection provides protection against MCMV-related retinitis de-

velopment. However, ocular administration of recombinant IL-17 of retinitis-susceptible MAIDS mice during subretinal MCMV infection may be necessary in order to determine the potential protective properties of IL-17 during MCMV retinitis development. Additionally, if knockdown of the tegument proteins and/or the immune evasion viral glycoproteins leads to the upregulation of IL-17 during infection, this would provide an alternative approach for us to determine whether IL-17 plays a protective role in MCMV retinitis pathogenesis.

Due to the current unavailability of IL-17A -/- mice, we aimed to better decipher the role of IL-17 during MCMV retinitis development in MAIDS mice by altering an IL-17 negative regulator in this current study, IL-10, that was upregulated systemically during MAIDS progression as well as in during ocular MCMV infection of retinitis-susceptible MAIDS mice. Additionally, SOCS-3 was also upregulated in both whole splenic cells during the progression of MAIDS and in MCMV-infected eyes of MAIDS-10 mice. We believe that the loss of SOCS-3 would partially restore IL-17 levels in a similar fashion to what we observed in our IL-10 -/- mice. However, knockdown of SOCS-3 in mice is lethal (Robb et al., 2005; Takahashi et al., 2003). SOCS-3 conditional knockouts, mice devoid of SOCS-3 protein production in particular tissues, could be utilized, but mice lacking SOCS-3 protein in the ocular tissues are not commercially available. Additionally, SOCS-3 antibody treatment could be utilized; however, when we utilized antibody treatment to downregulate IL-4 in our MAIDS animals, we only obtained a 50% reduction in IL-4 protein (Aim 1, Fig. 3.2). Thus, SOCS-3 antibody treatment of MAIDS mice does not present a practical model to decipher the role of IL-17 in MCMV-related retinitis. We could also consider developing mice that overexpress either SOCS-3 or IL-10 as well as both SOCS-3 and IL-10 in ocular tissues in order to determine the degree to which each of these cytokines downregulates

IL-17 during infection. Based on our results from this study utilizing IL-10 -/- MAIDS mice, we believe that in addition to IL-10, SOCS-3 contributes to the downregulation of IL-17 during MCMV infection, and that the loss of both of SOCS-3 and IL-10 is essential for fully restoring IL-17 levels in Th17 cells during systemic and ocular MCMV infection.

5.3 Significance

Overall, the results from these studies furthers our basic understanding of the pathogenesis of AIDS-related HCMV retinitis as well as our understanding of the immunobiology of HCMV. Analysis of the host cell CD4+ T cell cytokines IL-17, IL-4, and IL-10 during retrovirus-induced immunosuppression as well as during the development of retinal disease provide evidence for their collaborative involvement in disease development (IL-4 and IL-10) as well as their potential protective capabilities (IL-17). Further study of the mechanism of action of these cytokines will help to direct the development of novel drug targets to manage existing HCMV retinitis and/or prevent its development in HIV/AIDS patients.

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