CELL-INTRINSIC AND ENVIRONMENTAL FACTORS ALTERING THE NEONATAL CD8+ T CELL RESPONSE TO MCMV IN THE BRAIN

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ABSTRACT

Congenital Cytomegalovirus (CMV) infections are a major public health problem and one of the leading causes of developmental problems in children. While adults rarely experience clinical manifestations of disease, children with congenital CMV infection can develop permanent disabilities if they manifested symptoms of the disease at birth. The causes leading to this increased vulnerability to CMV in neonates is unclear. Some have speculated that it may be due to the immaturity of the neonatal immune system, which has been shown to have a decreased capacity to generate memory CD8+ T cells. An open question is whether this impaired development of memory CD8+ T cells is due to an immature priming environment or lymphocyte-intrinsic defects. One possibility is that the priming environment is affected by the presence of the vitamin A derivative retinoic acid (RA), which plays a crucial role in the development of the neonatal immune and central nervous system (CNS) as well as physiological activities like cellular proliferation, differentiation, and apoptosis. But how the RA microenvironment and the CD8+ T cell response in the neonatal brain are impacted by viral infection is poorly understood. To address this critical gap in our knowledge, we performed in-depth analysis of the CD8+T-cell response in the brains of

neonates, and our studies uncovered some surprising features: i) Neonatal CD8+ T cells are intrinsically different than adults; ii) Gut-trophic markers (CCR9, α4β7 and CD103) selectively expressed on CD8+ T cells are localized in the brain following MCMV infection; and iii) Aberrant levels of RA production were observed in MCMV-infected neonates. Together these data suggest the importance of better understanding the brain microenvironment of neonates and how it may be involved in the delayed response to MCMV infection.

BIOGRAPHICAL SKETCH

Wisler Charles is a first generation American with a Haitian heritage, and all his life he has been a tinkerer with an innate sense of curiosity. As a child of immigrants, and due to his impoverished upbringing, he was left to devise other ways to entertain himself. By the time he was six years old, he was dismantling what he found broken or in junk piles. By looking at each component, screw, washer, wire, and points of interaction, he began to understand their importance. He did not fully comprehend electricity, for example, but through determination and trial and error, he was eventually able to look at any type of electronics and determine whether it was salvageable with the limited tools he had. By the time he was eleven, he had already repaired his first television.

When he graduated from high school, due to his family's poor social status and a need to serve his country, Mr. Charles joined the U.S. Marine Corps. After the military, he worked as a part time phlebotomist for the Camp Pendleton Naval Hospital, which sparked his interest in immunology and led him to attend Revelle College at the University of California, San Diego. After admission to UCSD in the fall of 2008, he received a fellowship from the Initiative to Maximize Student Diversity (IMSD) program, which allowed him to select a lab and a mentor. He chose the lab of Dr. Antonio De Maio's at the UCSD Surgery Department working under the direct supervision of Assistant Adjunct Professor, Virginia Vega. During this period, he was trained in cellular and molecular biology techniques. The experiences he received in Dr. De Maio's lab led to an internship opportunity with the biotech company BD Biosciences, Pharmingen. The time he spent at these labs increased his fascination for the cellular and molecular

pathways associated with cancer biology, as well as the immune response. These interests later lead to his application to and enrollment at Cornell University.

DEDICATION

I would like to dedicate this dissertation to my family, who have been a critical support system for me not only during my time as a graduate student, but throughout all of my various endeavors. First, to my extraordinary wife, who has always supported my career decisions, and believed that I could succeed even when I had begun to have doubts. To my boys (Magne, and Mirai) for keeping me grounded and focused, because your educations won't be free, lol. My parents, Marie and Bazile, strongly believed in me and pushed the value of observing the world around me and being curious. To my second mom, Ada Wright, thank you for helping me see the path towards my dreams. Thank you all for helping me get here!

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
APCs	Antigen presenting cells
ATRA	All-trans retinoic acid
ROL	Retinol (vitamin A)
RE	Retinyl esters
RAL	Retinal or retinaldehyde
CFSE	Carboxyfluorescein succinimidyl ester
CRBP	Cellular retinol binding protein
CrbpI	Cellular retinol-binding protein, type 1 (protein name)
CrbpII	Cellular retinol-binding protein, type 2 (protein name)
CrbpIII	Cellular retinol-binding protein, type 3 (protein name for murine homolog of *Rbp7*)
CrbpIV	Cellular retinol-binding protein, type 4 (protein name for human homolog of Rbp7)
CRABP	Cellular retinoic acid binding protein (types I and II)
CTL	Cytotoxic effector T lymphocytes
CYP	Cytochrome P450
DC	Dendritic cell
DEAB	Diethylaminobenzaldehyde
Dhrs	Retinol dehydrogenase (Dhrs9)
dnRARα	Dominant negative retinoic acid receptor alpha
ECM	Extracellular matrix
FITC	Fluorescein Isothiocyanate
FoxP3	Forkhead box P3
GALT	Gut-associated lymphoid tissues
GM-CSF	Granulocyte macrophage-colony stimulating factor

GranB	Granzyme B
ID	Infectious diseases
IFN	Interferon
IL	Interleukin
KO	Knock-out
LBD	Ligand binding domain
LN	Lymph node
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PE	Phycoerythrin
PPAR	Peroxisome proliferator activated receptor
RA	Retinoic acid
RALDH	Retinal dehydrogenase (RALDH1, 2 and 3)
RAR	Retinoic acid receptor (isomers alpha, beta and gamma)
RARE	Retinoic acid response element
RBP	Retinol binding protein
Rbp1	Retinol-binding protein, type 1 (gene name for CrbpI)
Rbp2	Retinol-binding protein, type 2 (gene name for CrbpII)
Rbp4	Circulating retinol binding protein, type 4 (gene name for Rbp4)
Rbp5	Retinol-binding protein, type 5 (gene name for human CrpbIII)
Rbp7	Retinol-binding protein, type 7 (gene name for murine CrpbIII or human CrbpIV)
Rdh	Retinol dehydrogenase (Rdh10/12)
Reh	Retinyl ester hydrolase
Rrd	Retinal reductase (Dhrs3)

ROR	RA receptor-related orphan receptor
RXR	Retinoid x receptor (isomers alpha, beta and gamma)
SDR	Short chain dehydrogenase/reductase (Rdh/Rrd)
SNHL	Sensorineural hearing loss
Stra6	Stimulated by retinoic acid receptor-6
Tg	Transgenic
Th	T helper-type
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
VA	Vitamin A
VAD	Vitamin A deficiency
VDR	Vitamin D receptor
WT	C57BL/6J wild type mouse strain

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Cytomegalovirus (CMV)

Viral Replication of CMV

CMV has one of the largest genomes of the Herpesviridae family. The CMV genome is a linear double-stranded DNA molecule with two regions of unique sequences flanked by inverted repeats. It is approximately 240 kb and encodes over 200 genes (3-6). The CMV genome has long and short sequences and consists of 180 to 200 predicted ORFs (7). It is surrounded by a 110 nm icosahedral capsid, which is enclosed by a tegument and enveloped in a lipid bilayer with many virus-encoded glycoproteins necessary for viral attachment and rapid and efficient fusion (3-5).

The expression of cytomegalovirus proteins occurs in a tightly regulated cascade of events and is divided into several main temporal classes: immediate early, or IE; early, or E; and late, or L (41, 42, 43). Each temporal class regulates different aspects of the infectious cycle. In the IE phase of the CMV lifecycle, transcription of the immediate-early or α- genes auto-regulate their own expression and are critical because they also control the expression of both the early and late genes (44). During this period in the viral life cycle, there is an absence of *de novo* protein synthesis or replication of viral DNA (11-13). The IE phase is then followed by the E or β phase. The α-genes are required for the expression of β-genes, which encode for the viral DNA polymerase, replicative enzymes, and tegument proteins. The L phase of the CMV life cycle occurs after viral DNA replication, typically within 36 to 48 hours of infection. During this phase,

the structural proteins that are necessary for encapsidation of the virus genome and release of infectious virus progeny are produced. Encapsidation of virus genomes occurs in the nucleus of infected cells (14). The final envelopment of the immature virions occurs in the cytoplasm and mature virions are released by cell lysis.

Human Cytomegalovirus (HCMV)

The species of CMV that infects humans is known as HCMV. HCMV infects over 50% of people in the United States, making it one of the most ubiquitous viral infections in the United States (15). In developing nations, the rate of seropositivity can be as high 100% (15). The virus is shed in saliva, urine, semen, cervical secretions, and breast milk (16, 19, 20). HCMV can also be transmitted by blood transfusion, organ transplant, and sexual contact. The vast majority of these infections are asymptomatic and result in minimal overt pathology in the infected individual. Adolescents have a higher risk of infection than the general population, with a rate of approximately 13%. HCMV is so prevalent in early life that by one year of age, 40% of children are infected with the virus (15).

In healthy adults, CMV quickly becomes latent and infections are typically asymptomatic. However, the virus can occasionally reactivate (if an individual becomes immune compromised) and lead to significant CMV disease (11). This is evidenced by the fact that transplant patients and AIDS patients are highly susceptible to serious infections of the liver, kidneys, lungs, and brain (17). In fact, before its isolation, HCMV was characterized by the cytopathological abnormalities observed in tissue from

patients with fatal infection. In these cases, the cells were typically enlarged (cytomegalic) with intranuclear and cytoplasmic inclusion, which was termed cytomegalic inclusion disease (CID). Reactivation of HCMV during pregnancy can also lead to in utero infection, which can result in devastating consequences for the fetus. Understanding the pathogenesis of congenital CMV infections is the major focus of this dissertation.

Murine Cytomegalovirus (MCMV)

To better understand the pathogenesis of HCMV, a number of groups, including ours, infect mice with MCMV to model HCMV disease (19-25). MCMV is a natural mouse pathogen and homolog of HCMV. Similar to HCMV, MCMV is well adapted to its host, causes only mild symptoms in immunocompetent adults, and results in the lifelong persistence of the virus (31). Additionally, MCMV shares significant genomic homology with HCMV in terms of genome organization, the presence of gene families, and similarities in GC content (32).

Although MCMV has been used to study congenital HCMV infections, one obstacle in developing a mouse model of congenital HCMV infection is that MCMV does not cross the placenta in immunocompetent mice (4,18, 53). However, the immune system of newborn mice retains many features of the fetal immune system until day 7. Therefore, a number of groups have used a model whereby newborn mice are peripherally infected with MCMV (4,18, 53). Importantly, dissemination to the brain only occurs when mice are infected near birth, and the subsequent pathological

abnormalities observed in the brain are indistinguishable from those seen in human infants (4,18, 53). Together, these data demonstrate that inoculation of newborn mice with MCMV faithfully mimics congenital HCMV infections and can be reliably used to better understand the developmental-related susceptibility of the CNS.

Congenital HCMV Disease

Congenital HCMV infection affects about 44,000 newborns (~1% of all live births) in the US each year. This results in approximately 200 deaths yearly and up to 8,000 annual cases of permanent neurologic disability. Congenital HCMV infection of the brain is the leading viral cause of mental retardation (17-18). While the vast majority (80-90%) of these neonates are asymptomatic at birth, about 10 to 15% will succumb to a wide range of issues, including hearing loss, blindness, neurological problems, and death. In fact, HCMV infection is the number one cause of childhood hearing loss (18).

In-utero infection of the developing fetus is the most common route of infection in infants and also the most damaging; postnatal infection is not associated with CNS disease (33). However, the mechanism of vertical transmission from mother to fetus is poorly understood. It has been theorized that maternal latent virus potentially reactivates during fetal gestation or is transmitted to the fetus from newly acquired virus through intra-uterine infection. What is known is that symptomatic newborns are more likely to display severe neurological symptoms like seizures (4,18, 46, 53), and radiological tests may find brain calcification and cortical atrophy (46, 53). The most severe symptomatic infants develop cytomegalic inclusion disease (CID), which affects

multiple organs. CID can develop into intra-uterine growth retardation, hepatitis-associated jaundice, hepatosplenomegaly, microcephaly, thrombocytopenia, hemolytic anemia, and petechial rash. Approximately 50% of children with CID will develop neurologic disability (34, 35).

CID occurs more frequently when a woman develops a primary CMV infection during pregnancy. One major risk factor that increases the likelihood of congenital CMV infection is the presence of children who attend daycare in the household. Additional risk factors include maternal age under 25 years and short spacing between pregnancies (16). Timing of primary CMV infection during pregnancy also influences transmission to the fetus. For example, neonatal disease is more severe when transmission occurs in the first trimester. The relative transmission rates based on the trimester when primary CMV infection occurs are 50% in the first trimester, 40% in the second trimester, and 71% in the third trimester (36, 37).

Virus excretion from urine and saliva is more abundant and persists for longer periods of time in congenitally-infected infants with clinically apparent infection compared to those with asymptomatic infection. This data indicates that prolonged periods of active infection in infants with clinically apparent infection could contribute to increased damage to affected tissues and organs (26). Among symptomatic congenitally-infected infants, mortality may reach as high as 30% (26). The causes of these deaths in newborns are typically multi-organ disease with hepatic dysfunction and disseminated intravascular coagulation. In severe cases of neurologically-damaged children, death can occur even after the first year (26).

HCMV-associated sequelae

Interestingly, the majority of disease incidence comes from children who are asymptomatic at birth. Assuming 1000 infants are born with congenital CMV infection, 127 (12.7%) will show symptoms of cytomegalic inclusion disease (CID), while 873 (87.3%) will be asymptomatic. Of the 127 symptomatic neonates, five will die (0.5%) while 122 will survive. No asymptomatic children will die. 50-70 (40-58%) of symptomatic neonates will develop permanent neurologic sequelae, such as SNHL and cognitive difficulties. In contrast, 118 (13.5%) asymptomatic neonates will develop sequalae, mostly SNHL. Due to the large size of the asymptomatic group (873 neonates vs 127 neonates), 2/3 of children born with congenital CMV infection that develop permanent neurologic sequelae are asymptomatic at birth (49, 50).

The increased susceptibility of the CNS in utero has been attributed to various neural and extra-neural factors, which lead to increased virus dissemination and growth in the CNS. In the developing brain, the relative immaturity of the blood brain barrier (BBB), the presence of proliferating neural precursor cells that are more permissive for virus growth, and the continued formation of axonal pathways by immature neurons have all been shown to result in increased virus replication and spread in the neonatal brain. Extra-neural factors, such as the immaturity of the fetal and neonatal immune system and increased virus growth in non-neural tissues, have also been linked to the elevated incidence of encephalitis in fetuses and newborns (51, 52, 53).

Another group of individuals who are highly susceptible to infection are immunocompromised patients. Due to their impaired immune systems, organ transplant

and AIDS patients are susceptible to both primary infection and more episodes of viral reactivation. In fact, CMV is one of the most significant causes of mortality in a transplant patient. Because T-cells are an important component of controlling CMV infection, immunosuppressive drugs for organ transplant and AIDS patients place them at greater risk for developing severe manifestations of the disease. For example, CMV infection in these patients can vary from mildly symptomatic mononucleosis to full-blown organ disease with viremia. Though organ-specific disease is rare, disease can be found in multiple sites, including the lung, liver, and colon. This type of disease is typically self-limiting, but it can be life threatening in a minority of patients (54, 55).

Pathology

Acquisition of CMV in utero can lead to infection of the central nervous system (CNS). A variety of cell types in the CNS support CMV infection, including astrocytes, microvascular endothelial cells, microglia, macrophages, oligodendrocytes, and neurons. Common pathological manifestations of CNS disease include lissencephaly, microgyri, periventricular calcification, and cerebellar hypoplasia (38). Histopathological findings indicate signs of disordered migration, meningoencephalitis, mononuclear infiltrates, micronodular gliosis and viral cytopathic effect (CPE) (38).

Organ damage or malformation is potentially due to the stress caused by viral replication, which can increase programmed cell death, or apoptosis of infected cells (46). Another potential mechanism leading to tissue damage is the lysis of infected cells following release of virus progeny (47). During later stages of infection, some

groups of cells appear to fuse together and have the appearance of giant multinucleated cells. These giant cells have been shown to lyse, liberating viral progeny into the periphery (47, 48).

Infants with intra-uterine HCMV infection have been shown to have similar levels of damage to the brain parenchyma as observed in CNS infection in immune-compromised adults (45-48). But it is important to mention that progenitor cells in the developing CNS of the fetus are more susceptible to CMV infection than the differentiated CNS of the adult (46). HCMV CNS infections can present with either acute ventriculoencephalitis or focal micronodular gliosis with significant mononuclear infiltrate and gliosis in both cases. Previous studies of congenital HCMV infection of the CNS correlated focal encephalitis with inflammation. The observed difference between neonatal and adult infection could potentially be due to (i) an immature immune system in neonates; (ii) a more "leaky" blood brain barrier present in early life; or (iii) a greater number of permissive cells during early stages of development (46).

Another manifestation of congenital HCMV infection is sensorineural hearing loss (SNHL), which is the most common neurologic sequalae of this infection (39,40). HCMV infection of fetal CNS alters the development of inner ear structures and vestibular organs. Progressive hearing loss seen in CID is most likely due to chronic CMV infection of the CNS, since children with progressive SNHL shed CMV in their urine for at least four years and CNS damage can be correlated to viral load in their urine (39, 40).

Lastly, vasculitis in various tissues, including the CNS, could also contribute to the increased pathogenicity of HCMV in infants. HCMV productively infects the vascular

endothelia, which could result in increased damage to the surrounding tissue due to loss of blood supply (27, 28). Immune mediated damage could potentially add to HCMV disease by promoting cell death of infected cells as well as by collateral damage of uninfected cells (29).

Use of MCMV to model congenital CMV infection of the CNS.

Many studies of MCMV spread in the brain have employed direct intracranial inoculations of the virus (97-101). In those studies, it was determined that neural stem cells in the subventricular zone are the most susceptible to MCMV infection in the CNS (103). However, using direct intracranial inoculation not only modifies the kinetics of viral spread in the diseased host, but it also alters the host response to infection in the CNS (102). Intraventricular inoculation of MCMV potentially limits virus accessibility to other sites in the CNS, thereby masking the susceptibility of other cell types deep in the CNS parenchyma (102).

When studying the kinetics of the CMV infection, modes of infection are extremely important when choosing an infection model. Direct inoculation of MCMV into the brain can be misleading when studying infectivity of neuronal stem cells versus mature cells. A previous study by the Vanden Pol group showed that most neurons were permissive for MCMV infection when using recombinant MCMV-GFP virus to infect mixed brain cultures with pure neuronal cultures (104). But transgenic mouse cell lines expressing beta-galactosidase under an MCMV ie1 promoter showed reporter gene expression only in glial and neural progenitor cells (105). In later studies using brain slice cultures, neonates showed more MCMV infection when compared to adult

brains (103, 106). Additional studies of intracranial infection in SCID mice revealed that susceptibility to CNS infections is not dependent upon the maturity of the adaptive immune response; rather, it is primarily a function of age (101).

Another, and possibly better, mouse model for understanding host-pathogen interactions was developed by the Britt Lab (4, 10, 18, 53, 92, 123). In this model, neonatal mice are intraperitoneally infected with a small amount of MCMV within 24 hours post-partum. The attractive feature of this model is that dissemination of the virus mimics what is typically observed in humans. Using this model, Britt & colleagues observed deficits in neural maturation in the cerebellum. They also observed cerebellar hypoplasia in MCMV-infected mice, with a 10% difference in size at post-natal (PN) day 21 compared to control animals. Impairments in granule neuron migration from the external granular layer (EGL) to the internal granular layer (IGL) was observed, as characterized by thicker EGLs in MCMV-infected animals and by decreased rates of granule neuron migration. Decreased granule neuron progenitor proliferation was also observed in MCMV-infected animals, as were Purkinje neuron ectopia and delayed dendritic arborization. Cerebellar morphogenesis, fissure depth, and lobule formation were also significantly affected during infection. Due to the lack of viral cytopathic effect (CPE), it was suggested that the effects on cerebellar morphogenesis were potentially caused by the host immune response to MCMV infection and not secondary to direct cytopathic of MCMV infection (10, 53, 92, 106, 123).

Host response to CMV infection

The innate and adaptive immune responses to CMV infection have both been shown to be critical for effective control of virus infection. Early response to viral infection involves macrophage and NK cell activation, which lead to the production of inflammatory cytokines such as IL-1, IL-6, TNF-α, and IFN-γ (88-93). Whereas IFN-α/β has been shown to stimulate NK cell blastogenesis, as well as enhance IFN-γ synthesis (87), TNF-α and IL-12 both enhance Th1 polarization of activated T-cells (87-89). Overall, the innate immune response serves to curtail early infection by limiting viral load and facilitating the activation of the adaptive immune response.

The most critical adaptive immune cell for resolving acute CMV infection is the CD8+ T cell. CD8+ T cells are lymphocytes that provide immune protection against intracellular pathogens by directly killing infected cells via cytotoxic mediator or by activating other immune cell types via cytokines. Activation of CD8+ T cells requires three specific signaling events. First, the T cell receptor (TCR) must recognize peptide bound to class I major histocompatibility complexes (MHC I) on the surface of APC (14). Binding of the MHC and CD3 molecules to the TCR initiates downstream signaling cascades (15). Second, CD28 on the T cell must be ligated by CD80 (B7.I) and/or CD86 (B7.2) (16). Third, cytokines, such as IL-12 or type I IFN from APCs, are required to fully activate CD8+ T cells. After activation, CD8+ cytotoxic cells produce IL-2, which promotes the proliferation and differentiation of naïve CD8+ T cells into effector CD8+ T cells equipped to combat pathogens such as CMV (17).

Infection with CMV results in a robust CD8+ T cell response. Studies using SCID mice (94, 95), T-cell deficient mice (96, 97), and adoptive transfer of T-lymphocytes into

immune incompetent hosts (98-101) all point to a crucial role for CD8+ T cells in the host response to CMV infection (96, 98, 101, 78-80). However, the mechanism by which CD8+ T-lymphocytes mediate virus clearance remains controversial (53). One study suggested that CD8+ T cells limit viral replication in infected cells via production of cytokines. Cytokines contribute to the reduction of infectious virion synthesis and to the elimination of infected host cells via the initiation of cell death pathways (86, 87). IFNγ and TNFα have been shown to be necessary for control of virus replication and eventual viral clearance in peripheral organs (81, 82). Interestingly, the authors found that cytolytic effector molecules (granzymes and perforin) were dispensable for control of virus replication in most organs, except in salivary glands (85). However, a separate investigation showed the necessity of perforin in efficient control of MCMV infection in other organs as well (84).

Although CD8+ T-cells have been regarded as an essential mediator of virus clearance in most peripheral organs, it is important to mention that CD4+ T cells have been shown to mediate virus clearance from salivary glands and are believed to operate via an IFN-γ- dependent mechanism (83). However, other studies involving adoptive transfers of T cells into recipient mice demonstrated that CD8+ T cells are required for control of viral growth in most peripheral compartments (lung, liver, spleen), whereas CD4+ T cells alone result in lethal infection in recipient mice (73, 76). Moreover, CD4+ T cells are not required for the generation of a protective CD8+ T cell response (73). As a result, there is a belief in the field that increased susceptibility of fetuses and infants to HCMV is due to impaired cell-mediated responses to CMV (53, 60-63).

Neonatal immunity

The differences in T cell-mediated immunity between neonates and adults are not well understood. Neonates experience increased vulnerability to infectious agents, especially viral pathogens, which has been attributed to quantitative and qualitative differences between adult and neonatal immune systems (1-3). For example, neonates have 1 to 2 Log_{10} fewer numbers of T cells in both the spleen and lymph nodes when compared to adults (1-2). However, neonatal T cells are also qualitatively different due to developmental-related differences in the diversification of the TCR repertoire. In particular, TCR chains have a lower frequency of N-nucleotide additions due to a deficiency of terminal deoxynucleotidyl transferase (16). This in turn leads to shorter TCRs and fewer distinct clonotypes in neonates (17,18).

The neonatal T cell response to infection may also be altered because of developmental-related differences in the host environment. For example, there is evidence that neonatal antigen-presenting cells (APCs) are phenotypically and functionally immature, with decreased levels of MHC and co- stimulatory molecule expression (4). Together, these differences may prevent neonates from priming long lasting protective CD8+ T cells. However, it is important to mention that more robust ‘‘adult-like’’ CD8+ T cell responses can be generated in certain circumstances. This is evidenced by the fact that immunization of neonates with hepatitis B surface antigen combined with alum and CpG resulted in vigorous CTL responses (56). Neonates immunized with a DNA vaccine encoding measles-hemagglutinin (MV-HA) also produced strong CTL responses (57). Thus, the nature of the stimulus may be a key determinant in how neonatal CD8+ T cells respond to vaccination and infection.

In contrast to CD8+ T cells, more is known about developmental-related differences in CD4+ T cells. In neonates, the CD4+ T cell response is more Th2-biased to a variety of antigens (36) and is characterized by high levels of IL-4 and low levels of IFNg production during T cell memory responses. Some have speculated that this may be due to reduced production of IL-12 by neonatal APCs or hypomethylation of Th2 cytokines in neonatal CD4+ T cells (155-159). Others have suggested that neonatal memory CD4+ T cells are biased towards the Th2 lineage because neonatal Th1 cells undergo apoptosis following stimulation (39). Previous studies have determined that this effect appears to be mediated by IL-13Ra1, which is present on neonatal Th1 memory cells, but not on adult cells. IL-13Ra1 can associate with IL-4Ra and form a receptor that can bind to both IL-4 and IL-13 (40). Importantly, IL-4Ra was also highly expressed on neonatal Th1 memory cells (39). Using neutralizing antibodies against IL-4Ra, IL-13Ral, or IL4 clearly showed that neonatal Th2 memory cells induce the apoptosis of Th1 memory cells upon re-stimulation in an IL-4-dependent manner (10, 41, 42, 43-47).

In terms of B cells, antibody production is poorer in neonates, with production limited to mostly IgG. Additionally, the follicles and germinal centers (GC) of the lymphoid tissue are not fully developed at birth (139). Another study showed that differentiation of neonatal follicular DCs in response to signals from neonatal B cells is defective, leading to a delayed maturation of the follicular DC network (140). Further, there seems to be a bias for the differentiation pathway to memory- instead of plasma B cells, which limits the production of antibodies after neonatal infection (141). Maternal antibodies can confer some protection to neonates in the first few months of life;

however, passive transfer of maternal antibodies to infected infants does not afford protection against HCMV infection. Additional studies showed that i) defects in affinity maturation are observed in virus-infected neonates (11, 53, 65) and ii) HCMV virion-antibody complexes remain infectious even when high titers of anti-HCMV antibodies are present (53, 66).

During pregnancy, the transmission rate in seronegative women is about 50%. Though the presence of maternal antibody due to pre-exposure helps manage infection, it does not guarantee prevention of fetal infection. The transmission rate in a woman whom was seropositive prior to pregnancy is 0.5% to 2% (16). These statics translate to a three-fold higher risk of CMV transmission to the fetus in CMV seronegative women. Pre-conceptional maternal immunity to CMV reduces the incidence and severity of congenital CMV disease. In most cases, CMV transmission to the fetus is limited by neutralizing antibody suppressing viral replication in the placenta of women with a history of CMV immunity before pregnancy.

Immune modulation by MCMV

Like many viruses, CMV has evolved with humans and mice for millions of years and developed strategies to evade the immune response. Transition The encoded MCMV m152, m6, and m4 gene products downregulate cell surface expression of MHC I (88-90). Another viral protein, m144, is an MHC 1 homolog that has been shown to inhibit NK activity (91). All members of the β-herpesvirus, including MCMV, encode G-protein coupled receptors (GPCR) in their genome, namely M33 and M78 (92, 93).

Interestingly, chemokine homologs are also present in the MCMV genome. For example, m131 is a murine chemokine homolog 1 (MCK1) that is similar to CC chemokines (94). mRNA splicing with another virus gene product, m129, forms MCK-2, a secreted glycoprotein shown to have monocyte-macrophage chemoattracting capabilities (95). MCK-2 is thought to play a specific role in virus dissemination in the host (96).

There are also multiple distinct strains of CMV due to interstrain recombination (11). Individuals who are infected with one strain of CMV remain susceptible to infections by other strains. These strains can be acquired simultaneously or at different times. CMV's large genome and its various strains allow it to evade immune detection, optimize vertical transmission, and efficiently establish concurrent or successive active infections in individuals.

Overview of retinoic acid (RA)

We have become particularly interested in determining the relationship among RA, CNS development, and the immune response to CMV. RA is a Vitamin A metabolite, and dietary vitamin A is one of the most essential lipid soluble vitamins for growth and central nervous system development in vertebrates. The retinoid structures of the vitamin A metabolite consist of an ionone ring and an isoprenoid tail with a polar end group. The main forms of Vitamin A are retinol (ROL), retinal (RAL), retinoic acid (RA), and carotenoid. Vitamin A is mostly derived from plants in the pro-vitamin form of

beta-carotene or from animals in the storage form of retinyl esters, with retinyl palmitate being the most abundant. Each of these active forms of vitamin A are responsible for mediating a multitude of important functions.

Studies have shown that the various vitamin A metabolites are essential for embryonic development, reproduction, vision, immune function, and tissue homeostasis. Two examples are all-*trans*-retinoic acid (ATRA) and 9-*cis*-retinoic acid (9cRA), which are involved in regulating the expression of a wide range of target genes via the activation of retinoic acid receptors (RARs), which are extremely important for embryonic development and adult tissue maintenance (3).

Furthermore, proper management of vitamin A metabolism is so vital that any deficiency or excess of retinoids are detrimental to the cells, tissues, and body as a whole. Vitamin A deficiency (VAD), for example, is defined as a whole body deficiency; symptoms include night blindness, immune deficiencies, and respiratory pathologies (6). Vitamin A toxicity (VAT), on the other hand, can be due to multi-vitamin and medication overuse/overdose and may include symptoms of headaches, nausea, altered vision, and skin rash (7). VAT during pregnancy can lead to irreversible birth defects.

During critical stages of development, VAD and VAT have significant consequences, the outcome of which could be growth defects and death (8). The severity of symptoms is impacted by the extent of acute or chronic VAD/VAT. Retinoid deficiency and excess can also be localized in a tissue or cell, or it can be related specifically to retinoid absorption, transport, storage and metabolism (6-8).

RA and embryonic development

Retinoic acid is essential for embryonic development. Abnormalities in vitamin A-deficient embryos were first described in 1933 (49), and many other malformations affecting the heart, bones, eyes, limbs, brain, and nervous system have been described since (50). Both excess and insufficient levels of RA during embryogenesis have teratogenic effects. While these reports provide insight into RA's importance during embryonic development, such studies are not ideally suited for studying its physiological roles, since the tightly-regulated homeostasis of retinoids makes it extremely difficult to achieve total depletion. More recent studies involving loss-of- function of key RA-synthesizing enzymes and receptors now provide extended information on the roles of RA throughout embryonic development. These roles are numerous and dependent upon the regulation of specific sets of target genes (16).

Treatment of mouse embryonic stem cells or embryonal carcinoma cells with high concentrations of RA can induce neural differentiation (51). In normal mouse development, however, RA is not produced until well after induction of neuroectoderm, and it has been demonstrated that neural induction does not require RA (52, 53). The role of RA in neural differentiation is to act on the neuroectoderm to induce its further differentiation. Data from knockout studies with *Raldh1/2/3* show that RA signaling in early neural development is only required in posterior structures such as the hindbrain, the spinal cord, and the eye (54, 55).

An important feature in the developing hindbrain is the differential expression along the anteroposterior axis of a cluster of genes called the Hox genes. The Hox genes are a subset of homeotic genes that are regulators of an embryo's body plans

along the cranio-caudal (head-tail) axis. The regulation of several of these genes by RA has been well documented and appears to be one of the major roles of RA in hindbrain patterning (56-58). For example, RA has been shown to directly regulate the expression of the Hox gene *Hoxb1* by both induction and repression, thereby tightly controlling the spatial expression of this gene (59, 60). Boundaries of RA signaling in the developing brain are created by the expression in the mid- and forebrain of the RA degrading enzymes *Cyp26a1* and *Cyp26c1*. Knockout models of these enzymes in developing mouse or zebrafish embryos lead to posteriorization of the expression pattern of *Hoxb1* and other RA target genes and subsequent posteriorization of the developing brain structures at the expense of the anterior structures (61, 62).

RA regulation of T cell tolerance

Numerous studies have focused on the role of RA in the regulation of T cell biology. It has been proposed that constitutive RA presence in the gut is essential for tolerance to commensal microbes and dietary antigens by inducing Tregs to dampen the potential pro-inflammatory responses that otherwise lead to pathogenesis. To maintain both efficient immunity against foreign pathogens and tolerance against food antigens, gut-associated DCs or other APCs have to distinguish, process, and present various antigens to induce an inflammatory T cell response or Treg differentiation. Numerous studies have demonstrated that in combination with TGFβ, RA can induce naïve $CD4^+$ T cell differentiation into very stable $FoxP3^+$ Tregs both *in vitro* and *in vivo* (82-85). Mucida et al. showed that TGFβ induced Th17 differentiation in the presence of

IL6, whereas RA inhibited Th17 differentiation but induced Treg differentiation (82).

Another group reported that T cell expansion induced by orally administered antigen, or homeostatic expansion of T cells *in vivo*, induced conversion of naïve $CD4^+$ T cells into Tregs in the small intestine and was dependent on RA produced by LP-DC. Dependency on RA was suggested by the fact that the RAR-specific antagonists LE540 and LE135 diminished the RA-induced FoxP3 expression (83). Benson et al. demonstrated that RA promoted TGFβ-induced Treg generation even in the presence of high-level co-stimulation. Furthermore, the induced Tregs in the presence of RA maintained their phenotype even under overt inflammation *in vivo* (84).

In an oral antigen administration-induced tolerance study, it was reported that $CD103^+$ MLN DCs induced FoxP3 expression on naïve $CD4^+$ T cells in TGFβ and RA-dependent manner (85). Studies using human peripheral blood cells proved that all-trans RA (ATRA) and TGFβ converted naïve but not memory $CD4^+$ T cells into stable and suppressive $FoxP3^+$ Tregs (86). Furthermore, ATRA treatment enhanced the suppressive capability of natural Tregs, reinforcing the key role RA plays in tolerance mediated by both natural and induced Tregs (86). Lu et al. further found that RA enhanced and stabilized FoxP3 expression in $CD4^+CD45RA^+$ naïve human T cells activated with suboptimal αCD3 and αCD28 in the presence of IL2 and TGFβ *in vitro*. These induced Tregs showed suppressive activity *in vitro* and prevented immunodeficient mice from human anti-mouse graft-versus-host-diseases (GVHD) (87).

In addition to the well-recognized role of RA in adaptive Treg induction, RA was also found to induce FoxP3 expression in $CD8^+$ T cells in models of autoimmune

diabetes and allograft transplantation. The first observation of RA-induced FoxP3 expression in $CD8^+$ T cells was reported by Kishi et al. in an autoimmune diabetes model (88). The authors generated $CD8^+FoxP3^+CD103^+$ Tregs by stimulating naïve glucose-6-phosphatase catalytic subunit-related protein (IGRP)-specific $CD8^+$ T cells with splenic DCs in the presence of IGRP peptide, TGFβ, and RA *in vitro*.

These $CD8^+$ Tregs generated *in vitro* inhibited diabetogenic $CD8^+$ T cell proliferation *in vitro* and completely prevented diabetes onset when transferred together with diabetogenic splenocytes from non-obese diabetic (NOD) mice. Using a transgenic mouse model to express hemagglutinin (HA) antigen on intestinal epithelial cells, Fleissner et al. found that $CD8^+FoxP3^+$ suppressive cells can be induced in mediastinal lymph nodes (MLN). These $CD8^+FoxP3^+$ cells suppressed both $CD4^+$ and $CD8^+$ T cell proliferation (89).

Further studies by this group illustrated that RA, in combination with TGFβ, can induce FoxP3 expression on HA-specific $CD8^+$ T cells when stimulated by MLN DCs in the presence of HA peptide. In the presence of IL2, TGFβ, and RA, donor DCs induced and expanded alloantigen-specific $CD8^+FoxP3^+$ suppressive cells with enhanced expression of CCR4, CTLA4, and CD103. The induced $CD8^+FoxP3^+$ cells protected skin allografts *in vivo* by suppressing both the co-stimulatory molecules' up-regulation on DCs and the proliferation and pro-inflammatory cytokine production by $CD4^+$ and $CD8^+$ T cells (90). All of the above studies illustrated that RA induction of FoxP3 expression was observed in both $CD4^+$ and $CD8^+$ T cells, highlighting its role in

maintaining immune tolerance.

RA regulation of T cell homing potential

The essential role of RA in gut immunity has led researchers to investigate the role of RA in the gut-tropism for T cells, an essential component in gut adaptive immunity. The interaction between integrin α4β7 and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is essential for T cell homing to intestine endothelium (91), and CCR9, which is a gut-associated chemokine receptor, mediates T cell migration to intestinal epithelial cells (92). T cells gain α4β7 and CCR9 expression during antigen stimulation by MLN-DC or PP-DC, or α-CD3 and α-CD28 stimulation in the presence of RA, while down-regulating E- and P-selectin ligands simultaneously (46).

The essential role of RA in regulating T cell gut-tropism was further demonstrated in VAD mice bearing a significantly lower number of $\alpha4\beta7^{+}$ memory/activated T cells in lymphoid organs depleted of intestinal LP (46), indicating the requirement of VA supplementation in fighting against intestinal inflammation within these hosts. Other detailed studies on the mechanism of RA-regulating α4β7 and CCR9 expression at the molecular level were performed by Kang et al., who found that RA treatment enhanced transcription of both integrin α4 on T cells, whereas both integrin βE and the constitutive expression of β7 can be enhanced by TGFβ (93). CCR9 induction depends on the delicate interaction between RAR-RXR and the nuclear factor of activated T cells (NFAT) (94).

RA regulation of $CD4^+$ T cell immunity

Despite the critical role that RA plays in maintaining tolerance, emerging data suggests that RA is critical for the development of T cell-mediated immunity. (112, 168). have discovered that RA is essential for TCR signaling in early $CD4^+$ T cell activation, thus controlling $CD4^+$ T cell proliferation and differentiation into Th1 and Th17 cells *in vitro* (95). In their study, the authors used RARα knock-out (KO) mice and showed that RARα-deficient $CD4^+$ T cells are defective in calcium signaling and ERK phosphorylation, and display insufficient activation. They further demonstrated diminished Th1 and Th17 responses against *Toxoplasma gondi* infection and failure to control the infection in the absence of RARα.

Studies using an alternative transgenic mouse model by selectively over-expressing dominant negative RARα (dnRARα) in T cells have demonstrated that $CD4^+$ T cells can proliferate as well as their control counterparts but fail to differentiate into Th1 and Th17 cells *in vivo* (51), accounting for the delay in allogeneic skin graft rejection. The discrepancy between these two studies may be due to the molecular difference in two models. This indeed calls for parallel comparison between two different transgenic mouse models to better recapitulate how VA controls immunity under different pathogenic circumstances through regulation of different immune components.

RA regulation of T cell survival

In addition to T cell activation and effector function, RA controls the survival of T

cells differently in humans and mice. In 12-o-tetradecanoyl phorbol-13- acetate-activated human T cells (96), supplementation of ATRA can increase the viability of these T cells in long-term culture through up-regulating IL2 expression. However, the same research group (97) found that, in IL2-reporter mice, ATRA treatment does not induce or up-regulate IL2 in either naïve or αCD3-activated T cells. Instead, VAD mice showed significantly higher basal levels of IL2 reporting compared to control mice, indicating a possible physiological role of ATRA in inhibiting IL2 expression *in vivo*.

The conflicting observations in these two studies suggest that either the RAR-RXR binding site in the human and murine IL2 promoter region is different, or that the IL2 requirement in T cells *in vitro* and *in vivo* is different. Of note, in both studies, $CD4^+$ and $CD8^+$ T cells were not separated and analyzed individually. It is possible that IL2 regulation by intrinsic RA signaling in $CD4^+$ and $CD8^+$ T cells may be different, and that mixed T cell analysis may make it hard to conclude the real effect of ATRA treatment on either $CD4^+$ or $CD8^+$ T cells. It remains to be elucidated whether intrinsic RA signaling may control $CD4^+$ or $CD8^+$ T cell survival via IL2 regulation.

Genetically engineered mouse models have also shed light on the function of RA in $CD8^+$ T cell biology. It has been shown that over-expression of human RARα under Lck promoter elevated the number of $CD4^-CD8^+$ single positive T cells in the thymus. These data suggest that RARα contributes to $CD8^+$ T cell development in the thymus (98). However, deletion of RARα from the hematopoietic compartment had no effect on the ontogeny of CD8+ T cells (99). Thus, it is still unclear whether RA signaling or

RARα in particular influences $CD8^+$ T cell survival during steady-state thymic development or upon activation.

RA Regulation of anti-infection T cell immunity

VAD (vitamin A deficiency) in children leads to increased susceptibility to ID (infectious diseases) and higher morbidity and mortality (115), establishing the essential role of RA for host resistance to infection. T cells are an essential component in fighting against primary infections and maintaining the long-term memory response against infectious pathogens (116). VA supplementation is still used in many countries to reduce childhood mortality (19-21). The essential role of both RA and T cells in immune responses against ID suggest that RA signaling may play a key role in the development of T cell immunity against infection. However, while VAD is associated with increased susceptibility to ID, the effect of VA supplementation on infectious disease control has been variously beneficial (117), neutral (118), and even deleterious (119). Thus, further studies are clearly warranted to fully understand how VA supplementation may help to control infectious diseases.

RA regulation of $CD8^+$ T cell responses in ID

Both pharmacological and genetic approaches have been used to elucidate the role played by RA in controlling $CD8^+$ T cell responses to infectious diseases. The gut-

homing tropism, the FoxP3 induction, and the survival effects that RA exerts on $CD8^+$ T cells may all influence the outcome of $CD8^+$ T cell immune responses. Kaufman et al. found that a VAD diet diminished the mucosal $CD8^+$ T cell response elicited by intramuscular immunization of recombinant adenovirus vaccine vector expressing OVA (rAd5-OVA), and thus abolished the protection against oral challenge of LM overexpressing ovalbumin (LmOVA) (27). Accordingly, retinyl palmitate or ATRA administration can fully restore gastrointestinal (GI) $CD8^+$ T cell responses and control the bacterial load in vaccinated VAD mice used as VA-sufficient control mice.

During murine infection of Sendai virus, a candidate human parainfluenza virus type 1 vaccine and a candidate vaccine vector for other respiratory viruses showed significantly reduced immunodominant $CD103^+CD8^+$ T cells in the lower respiratory tract (LRT) in VAD mice compared to control mice. The dominant expression of E-cadherin (ligand of CD103) on epithelial cells in the upper respiratory tract (URT) indicated that these T cells were induced to migrate to the URT but not the LRT, suggesting that VA (RA) controls $CD8^+$ T cell response in mucosa other than the gut (122). Therefore, depending on specific infectious pathogens, RA signaling may impose quite different effects on systemic $CD8^+$ T cell responses. It has been proposed that the gut-tropism of T cells imposed by ATRA has proven beneficial in eliciting more robust T cell immunity in the mucosa and better control of both *Salmonella* (123) and lymphocytic choriomeningitis virus (LCMV) infection (124). In both studies, ATRA treatment enhanced both gut-residential effector and memory $CD8^+$ T cells, which are essential for killing bacteria and viruses.

Studies on RA signaling in the manipulation of $CD8^+$ T cell immunity during viral infections in humans have also been focused on the migration properties in the mucosa. In HIV patients, the colocalization of gut-associated lymphoid tissues (GALT), HIV-specific $\beta7^+CCR6^+CXCR3^+$ $CD4^+$ target cells, and $\beta7^+CXCR3^+$ $CD8^+$ T cells may be regulated by the RA pathway (125). Peripheral blood mononuclear cells (PBMC) from slow disease progression subjects treated with ATRA and RA antagonist (LE540) showed higher and lower $\beta7$ expression levels, respectively, but unaltered CCR5 or CCR6 expression levels. The co-expression of $\beta7$ and CCR6 was up- regulated when PBMCs from slow disease progression subjects were stimulated with HIV peptide and ATRA, indicating that RA induced colocalization of HIV-specific $CD8^+$ effector and $CD4^+$ target cells in the GALT for more efficient killing of virus- infected target cells. All of the above studies illustrated that RA may promote $CD8^+$ T cell immunity during infection via regulation of $CD8^+$ T cell migration or effector function development.

With the use of genetic models that allow conditional silencing of RA signaling, precise questions can be asked as to the role of RA on specific leukocyte lineages. In one study using transgenic mice, RARα was deleted from the hematopoietic compartment and defective primary and memory $CD8^+$ T cell response against LM was observed. It was proposed that this was due to a deficiency in macrophages secreting pro-inflammatory cytokines such as TNFα and IL-6 (99). However, this study failed to establish the role of $CD8^+$ T cell intrinsic RA signaling and specifically RARα in direct control of $CD8^+$ T cell immunity.

CHAPTER 2 - CELL-INTRINSIC FACTORS

Abstract

Cytomegalovirus (CMV) is the most common cause of congenital infection in the United States and is the leading cause of developmental problems in children. While adults rarely experience clinical manifestations of disease, children infected in utero often develop permanent disabilities. The increased vulnerability to CMV in early life is poorly understood but may be due to developmental-related differences in CD8+ T cells. Previous studies have shown that CD8+ T cells are required to control viral replication, and significant numbers of CMV-specific CD8+ T cells persist in the brain even after the initial infection has been cleared. Earlier work from our lab demonstrated that neonatal CD8+ T cells have an inherent capacity to differentiate into short-lived effectors at the expense of forming memory. However, these studies were performed in the context of an acute viral infection (vaccinia virus). An important question is whether, and to what extent, cell-intrinsic differences in neonatal CD8+ T cells impair the ability to control CMV infection in the neonatal brain.

Introduction

Cytomegalovirus is the leading cause of brain damage in the U.S. (2). The severity of disease largely depends upon the gestational age of the individual at the time of infection. For example, neurological outcomes tend to be most devastating when transmission to the fetus occurs in the first trimester, but they become less severe with progressing age and development (3-6). Viral shedding can persist for years in congenitally-infected infants, whereas most adults can limit CMV replication in a matter of months (7, 8). While both lines of evidence demonstrate the importance of immune maturation to the control of infection, the primary developmental-related immune defect remains undefined. A key question is whether cell-intrinsic differences between neonatal and adult CD8+ T cells contribute to age-related differences in the ability to control CMV.

To begin answering this question, it is important to understand that neonatal CD8+ T cells are inherently different than adults for a variety of reasons. First, the neonatal CD8+ T cell pool is predominantly comprised of recent thymic emigrants (RTEs), or cells that have just left the thymus (107,108). Previous work demonstrated that RTEs in adult mice have a propensity to adopt a short-lived effector phenotype (107), similar to neonatal CD8+ T cells. However, when compared to adult RTEs, neonatal CD8+ T cells demonstrate a defective secondary recall response. Follow-up studies revealed that neonatal RTEs are phenotypically and functionally different than adult RTEs (107,108), indicating that unknown cell-intrinsic factors may underlie these differences (107,108).

Second, neonatal CD8+ T cells may behave differently than their adult counterparts because they have undergone more extensive homeostatic proliferation. Given that there are fewer T cells in neonatal mice, naïve CD8+ T cells are exposed to more homeostatic cytokines. As a consequence, they divide numerous times and become functionally more reactive than naïve cells. Whether increased homeostatic proliferation by neonatal CD8+ T cells alters their ability to respond to infection is not currently known. It is possible that neonatal CD8+ T cells rapidly become terminally differentiated after infection because they have already divided numerous times before encountering any foreign antigen (107,108).

Lastly, neonatal CD8+ T cells are created differently than adults because they are generated from a different hematopoietic stem cell population. During early stages of development, the thymus is seeded with more metabolically active hematopoietic stem cells (HSC) from the liver, which develop into the highly proliferative neonatal CD8+ T cells (107,108). The fetal HSCs are later replaced by a more quiescent wave of adults HSCs that originate in the bone marrow (107,108). Importantly, the fetal and adult HSCs express different genes (107,108), undergo different amounts of asymmetric cell division (107,108), and utilize unique programs of self-renewal (107,108). Thus, it is possible that neonatal CD8+ T cells respond differently to infections because they have an alternative origin.

This project seeks to better understand why a differential capacity to clear viral infection exists between neonates and adults. In particular, we asked whether cell-intrinsic differences between neonatal and adult CD8+ T cells contribute to increased susceptibility to MCMV brain infections. To answer this question, we conducted several

infection experiments and compared how neonatal and adult CD8+ T cells respond to CMV in the same host environment.

Materials/Methods

Animal Models

Pathogen-free pregnant C57BL/6NCr female mice were purchased from Charles River Laboratory. All experiments were conducted at Cornell University College of Veterinary Medicine, under the accordance and approval of the Cornell Institutional Animal Care and Use Committee and The Cornell Center for Animal Resources and Education. All animals were housed under standard conditions. gBT-I TCR transgenic mice (mice transgenic for TCRαβ specific for the HSV-1 glycoprotein $B_{498–505}$ peptide SSIEFARL [gBT-I in the text]) were provided by Dr. Janko Nikolich-Zugich (University of Arizona, Tucson, AZ). The *Ai9* reporter line (B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)) was purchased from Jackson Laboratories [18] and crossed with the *CD2-cre* line (B6.Cg-Tg(cre)). The *CD2-cre* × *Ai9* line was generated in-house by cross breeding and showed no adverse phenotype; the line specifically labels CD2-lineage with a fluorescent tdTomato reporter. Mice were genotyped from tail snips using real time PCR with specific probes designed for each gene. All strains were on the C57BL6/J background.

MCMV Strains

A recombinant MCMV expressing green fluorescent protein (GFP), referred to as MCMV-GFP, was constructed by inserting the GFP gene into the MCMV genome downstream of the MCMV major early immediate promoter (MIEP), such that the GFP reporter protein was expressed under control of the MIEP. Therefore, only infected cells undergoing active viral replication expressed GFP. A recombinant MCMV expressing

MCMV-specific glycoprotein B (gB), referred to as MCMV-gB, was constructed by replacing the m157 gene open reading frame with the gB epitope coding sequence. Strains courtesy of Dr. Joel Baines (Cornell University).

Infection Method and Study Design

A 1.0 mL syringe (Becton Dickinson) fitted with a 23 gauge needle (Becton Dickinson) was used to inject newborn mice (6-18 hours postpartum) intraperitoneally (IP) with 200 PFU in 50 µl of MCMV-gB. Neonatal brains and spleens from MCMV-infected and uninfected control mice were harvested by standard sterile procedure at 7, 14, 17, 21, and 28 DPI for flow cytometry to describe the CD8+ response temporality and phenotype.

Isolation of Brain Mononuclear Cells

At 7, 14, 17, 21, and 28 DPI, mice were briefly sacrificed by isoflurane inhalation to preserve intact circulation, then perfused with 30 mL ice-cold PBS. Brains were harvested in 13 mL of RPMI 1640 supplemented with 10% FBS (10% RPMI) in a 15 mL conical tube. Mechanical processing was completed through 70 µm cell strainers (Fisher Scientific) assisted by the plunger of a 3 mL syringe (Becton Dickinson). The suspension was centrifuged at 1500 RPM for 5 minutes at 4ºC. The tissue was homogenized in a 30% Percoll suspension, overlaid on a 70% Percoll solution, and centrifuged at 2600 RPM for 25 minutes at 4ºC. Cells at the solution interface layer were collected, washed in 10% RPMI, and re-suspended in ice-cold FACS buffer.

Isolation of Splenocytes

Neonatal spleens were harvested using the previously described method at the same time-points of interest, then mechanically processed using 40 µm cell strainers. Dissociated splenic tissue was centrifuged for 5 minutes at 1500 RPM and re-suspended in 3 mL of ice-cold FACS buffer.

Flow Cytometry

Isolated brain mononuclear cells and splenocytes were stained with anti-mouse cell surface markers and the ALDEFLUOR assay at 4ºC for 30 minutes. 300 µL of each tissue sample was transferred onto a 96-well round bottom plate (Corning), then centrifuged at 1500 RPM for 5 minutes at 4ºC. Supernatant was discarded and pellets were re-suspended in 200 µL ice-cold FACS buffer. 100 µl of each MCMV-infected sample and each control sample was transferred to another well for a fluorescence control reaction for the ALDEFLUOR buffer using the ALDH1-specific inhibitor diethylaminobenzaldehyde (DEAB), referred to as DEAB controls. ALDEFLUOR-specific flow cytometry gating strategy is shown in Figure 2. Samples were centrifuged at 1500 RPM for 5 minutes at 4ºC. Supernatant was discarded, and 100 µL of diluted Fc block was what? (Fc block stock from manufacturer diluted in FACS buffer at a 1:150 ratio). Samples were incubated on ice for 20 minutes, centrifuged at 1500 RPM for 5 minutes at 4ºC, and supernatant was discarded. The antibody panel consisted of CD8-e450, CD11b-PE, NK1.1-PeCy7, CD19-APC, CD3e-APC-e780, CD11c-PerCPe 710, and F4/80-PEe610 (all antibodies manufactured by EBioscience, Inc.) A cocktail was prepared using 50 µL ice-cold FACS buffer per sample. ALDEFLUOR reagents were

prepared according to manufacturer protocol. ALDEFLUOR reagent was activated by diluting 5 µL reagent with 1 mL ALDEFLUOR buffer, provided by the manufacturer. 50 µL activated ALDEFLUOR reagent and 50 µl antibody cocktail was added to each infected sample, each uninfected sample, and the DEAB controls, with a total volume of 100 µL in each well. Samples were stained for 30 minutes at 4ºC in the dark. Three washes were performed with FACS buffer, using volumes of 150 µL, 200 µL, and 150 µl, respectively. Cells were resuspended in 50 µL of ice-cold FACS buffer, and data was immediately collected using an LSRII flow cytometer. Lymphocytes were gated using forward and side scatter protocols. FlowJo software (TreeStar) was used for analysis. Repeat data collection and analysis was performed on 7, 14, 17, 21, and 28 DPI.

Organotypic Section Imaging Preparation

MCMV-GFP infected neonate brains were harvested intact at 7, 14, 17, 21, 28, and 60 DPI. A cryotome blade (VWR International) was used to manually dissect brains into approximately 100 µm coronal sections containing the choroid plexus of the lateral ventricle and third ventricle. Sections were placed in a 35 mm optical quality glass bottom cell culture dish (MatTek). Sections were immersed in a 1:200 dilution of ALDEFLUOR reagent at room temperature for 15 minutes. 300 µL of 300 nM DAPI solution (prepared courtesy of the Schaffer-Nishimura Lab) was added to section immersions.

Confocal Imaging Methodology

Imaging was performed on a Leica TCS SP2 confocal microscope, using a 20x or 40x

objective lens. Z-stacks with a minimum depth of 50 µm were acquired 25 minutes after the beginning of the ALDEFLUOR immersion reaction. The same stereotactic position within the brain was estimated by visually confirming locations of the lateral and third ventricles along the central dorsoventral axis of the coronal sections. Confocal imaging acquisition used a 200 Hz scanning speed, line average of 2, and frame average of 1. Objectives used included HCX PL APO 40x 0.85 Dry and HCX PL APO CS 20x 0.7 Dry. PMTs used were Leica/DAPI (emission at 455), Leica/EGFP (emission at 507), and Leica/DSRED (emission at 582). Overall laser power was 10%; UV used 22% of 405 nm laser line power; EGFP used 13% of 488 nm laser line power; and DSRED used 45% of 561 nm laser line power. Images were deconvolved using the Volocity software (PerkinElmer).

Statistical Analysis

Statistics were performed using Graph Pad Prism (GraphPad Software). For all analyses, a significance level of 0.05 was accepted.

Results

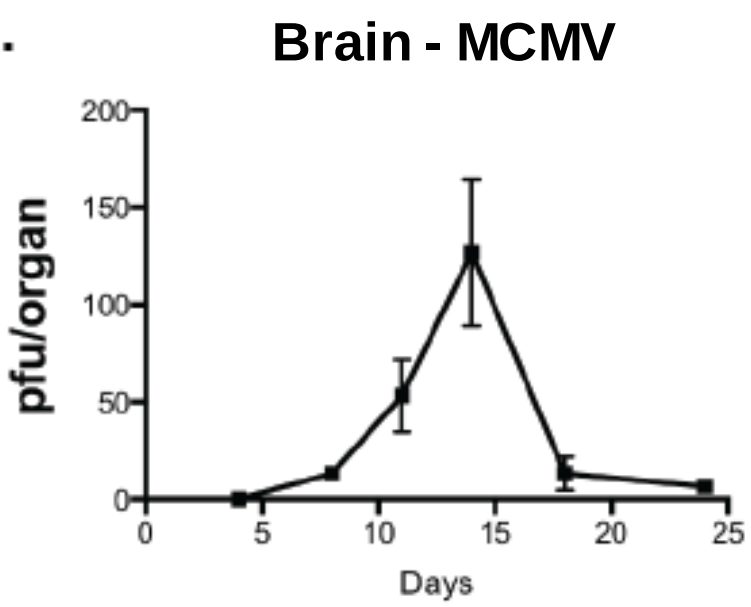
Neonatal CD8+ T cells exhibit a reduced capacity to clear MCMV

For these studies, we employed a widely accepted mouse model of congenital CMV infection that has been previously used by other labs. To verify that we could observe viral infection in the brains of neonatal mice, we first started by systemically infecting pups with 200 PFU MCMV-gB (i.p.) at birth. Brains were harvested from infected neonates on various days after infection, and the viral load was assessed using a plaque assay. Viral replication was indeed detected in the brain, and the peak in viral titer was 14 DPI (Fig. 2.1A). This is consistent with earlier studies demonstrating that significant levels of viral replication occur between 10 and 17 DPI (4,18, 46, 53).

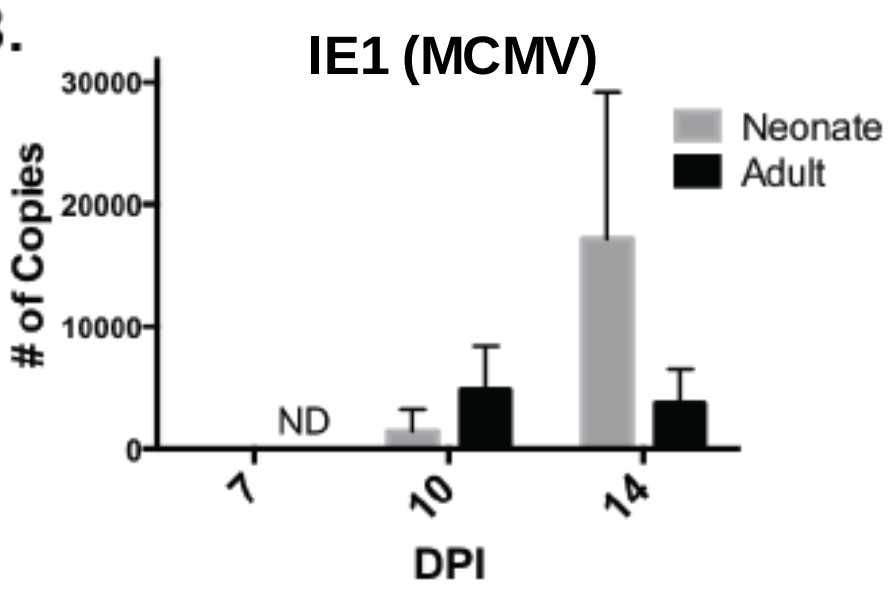
To determine whether increased viral replication in early life is due to cell-intrinsic differences between neonatal and adult CD8+ T cells, we adoptively transferred neonate or adult CD8+ T cells from gBT-I TCR transgenic mice into newborn (PND 0) TCRα $^{-/-}$ mice, which were then intraperitoneally inoculated with 200 PFU MCMV-gB at birth. By comparing equal numbers of monoclonal neonatal and adult CD8+ T cells in the same environment, we were able to focus specifically on cell-intrinsic differences. The brains of the TCRα$^{-/-}$ were then harvested on 7, 10, & 14 DPI, and levels of viral replication of the IE1 gene was measured by qPCR (Fig. 2.1B). Interestingly, there was significantly more viral DNA detected in the brains of TCRα$^{-/-}$ mice that received neonatal CD8+ T cells when compared to those that received adult cells (Fig. 2.1B). This data suggests that adult CD8+ T cells are inherently better at providing immune protection compared to neonates.

Figure 2.1 Replication of MCMV observed in the brains of neonates. (A) Virus yield per gram of homogenized liver and brain was determined by plaque assay. B6 neonates were infected on PND 0 with MCMV-gB and brains were harvested 4, 7, 14, 17, and 24 DPI for determination viral titer. (B) Naïve CD8+ T cells were isolated and purified from spleens harvested from gB-I specific TCR transgenic neonate CD90.1 (Thy1.1) and adult Ly5.1 (CD45.1) mice. The isolated neonate or adult cells were then adoptively transferred i.p. into TCRa-/- newborn (6–18 h postpartum) mice. The recipient pups were then infected with 200 PFU MCMV-gB. Brains were harvested at specified timepoints postinfection. The tissues were weighed and DNA was extracted using the Qiagen DNeasy isolation kit. Viral (MCMV IE1 genes) copies was assessed via RT-qPCR.

A.


Brain - MCMV
pfu/organ
200
150
100
50
0
0
5
10
15
20
25
Days

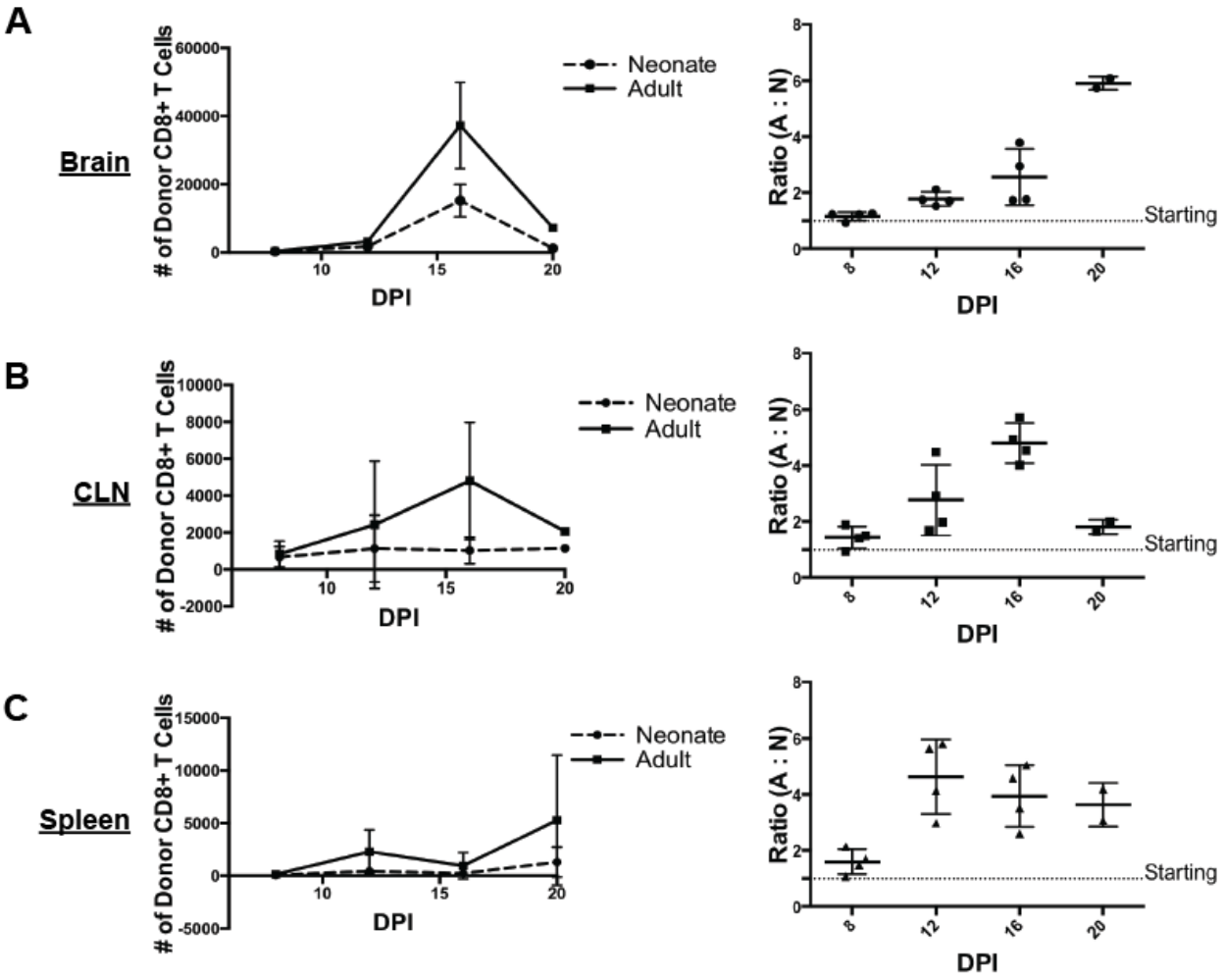
B.


IE1 (MCMV)
# of Copies
30000
20000
10000
0
ND
Neonate
Adult
7
10
14
DPI

Neonatal CD8+ T cells fail to accumulate in the brain after MCMV infection

To determine the underlying basis for why adult CD8+ T cells exhibit a greater protective capacity than adults, we co-transferred neonatal and adult gBT-I donor cells at a 1:1 ratio into newborn recipient mice and compared their numbers and phenotype after infection with MCMV-gB. Following donor cell transfer, recipient mice were systemically infected with murine cytomegalovirus expressing gB-peptide (MCMV-gB), and the ratio of adult and neonatal donor cells was tracked in the spleen, LN, and brain throughout the course of infection (Fig. 2.2). First, we analyzed the proportion of neonate and adult CD8+ T cells that responded to the infection in the brain over time. Interestingly, the data showed similar levels of neonatal and adult donor cells in various organs at early stages of infection (8DPI), suggesting that both groups of cells are recruited into the response. However, at later stages of infection (20DPI), we only observed an accumulation of adult donor CD8+ T cells. This data indicates that adult donor CD8+ T cells have a proliferative or survival advantage over neonatal donor CD8+ T cells during infection (Fig. 2.2)

Figure 2.2 When compared to neonates, more adult CD8+ T cells efficiently accumulate in the brains post-infection. Kinetics of neonate and adult gBI cells at various times post-infection. Naïve CD8+ T cells were isolated and purified from spleens harvested from gB-I specific TCR transgenic neonate CD90.1 (Thy1.1) and adult Ly5.1 (CD45.1) mice. The neonate and adult cells were then mixed together in a 1:1 ratio and adoptively transferred i.p. to congenic newborn Ly5.1 (CD45.2) pups. The recipient pups were then infected with 200 PFU MCMV-gB. Brains, spleens, and MLN were harvested at specified timepoints postinfection. The number and ratio of congenically marked donor cells from the neonate and adults harvested from the tissues were assessed using flow cytometer (LSR) and FlowJo.


A
Brain
# of Donor CD8+ T Cells
60000
40000
20000
0
10
15
20
DPI
Neonate
Adult
Ratio (A : N)
8
6
4
2
0
Starting
8
12
16
20
DPI
B
CLN
# of Donor CD8+ T Cells
10000
8000
6000
4000
2000
0
-2000
10
15
20
DPI
Neonate
Adult
Ratio (A : N)
Starting
DPI
C
Spleen
# of Donor CD8+ T Cells
15000
10000
5000
0
-5000
10
15
20
DPI
Neonate
Adult
Ratio (A : N)
Starting
DPI

Neonatal CD8+ T cells are readily activated more terminally differentiated post infection.

To gain insight into why neonatal CD8+ T cells fail to accumulate during infection, we compared important phenotypic markers associated with activation and differentiation in the donor CD8+ T cells. To compare differences in activation, we examined expression of CD69, which is known to be upregulated in T cells after stimulation through the TCR. We reasoned that reduced accumulation of neonatal donor cells may be due to reduced activation after stimulation. Interestingly, we found that neonatal donor cells in the brain actually express higher levels of CD69 shortly after infection (8 DPI) (Fig. 2.3A). However, as the infection progresses, nearly all donor CD8+ T cells from both age groups upregulate CD69, suggesting that most donor cells are coming in contact with their cognate antigen (Fig. 2.3). Additionally, we found that in the infected neonates CD8+ T cells began proliferating much sooner than the adult cells (Fig 2.4). These data suggests that neonatal CD8+ T cells become activated sooner than adults.

Given that neonatal CD8+ T cells appear to be activated earlier than their adult counterparts, we next asked whether they more quickly become terminally differentiated. To test this, we monitored expression of KLRG1, which has previously been shown to serve as a marker of terminal differentiation in CD8+ T cells. When we specifically focused on CD8+ T cells in the brain, we found that neonatal and adult CD8+ T cells upregulate KLRG1 at similar rates. However, neonatal CD8+ T cells maintain high levels of KLRG1 expression for a longer amount of time (Fig. 2.3B, D, F). Collectively, this data does not support the idea that neonatal CD8+ T cells fail to accumulate because of a failure to become activated and undergo effector cell

differentiation. Instead, it supports that idea that neonatal CD8+ T cells undergo more rapid activation and sustained effector cell differentiation after infection.

Figure 2.3 When compared to adults, neonatal CD8+ T cells are readily activated and are more terminally differentiated in the brain post infection. Naïve CD8+ T cells were isolated and purified from spleens harvested from gB-I specific TCR transgenic neonate CD90.1 (Thy1.1) and adult Ly5.1 (CD45.1) mice. The neonate and adult cells were then mixed together in a 1:1 ratio and adoptively transferred i.p. to congenic newborn Ly5.1 (CD45.2) pups. The recipient pups were then infected with 200 PFU MCMV-gB. Brains, spleens, and MLN were harvested at specified timepoints postinfection. The number and ratio of congenically marked donor cells from the neonate and adults harvested from the tissues were assessed for surface expression of CD69, CD103, and KLRG1 using flow cytometer (LSR) and FlowJo.

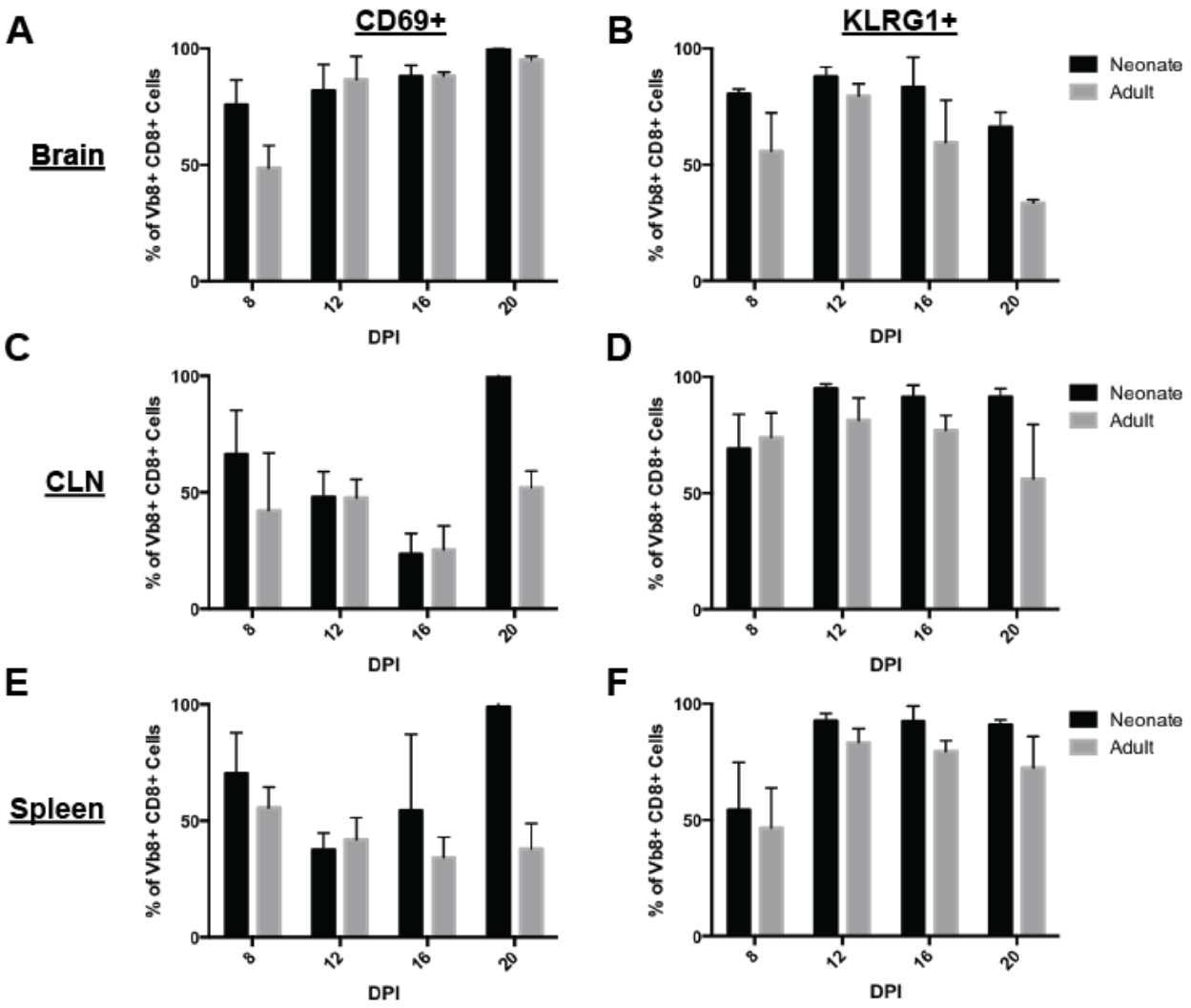
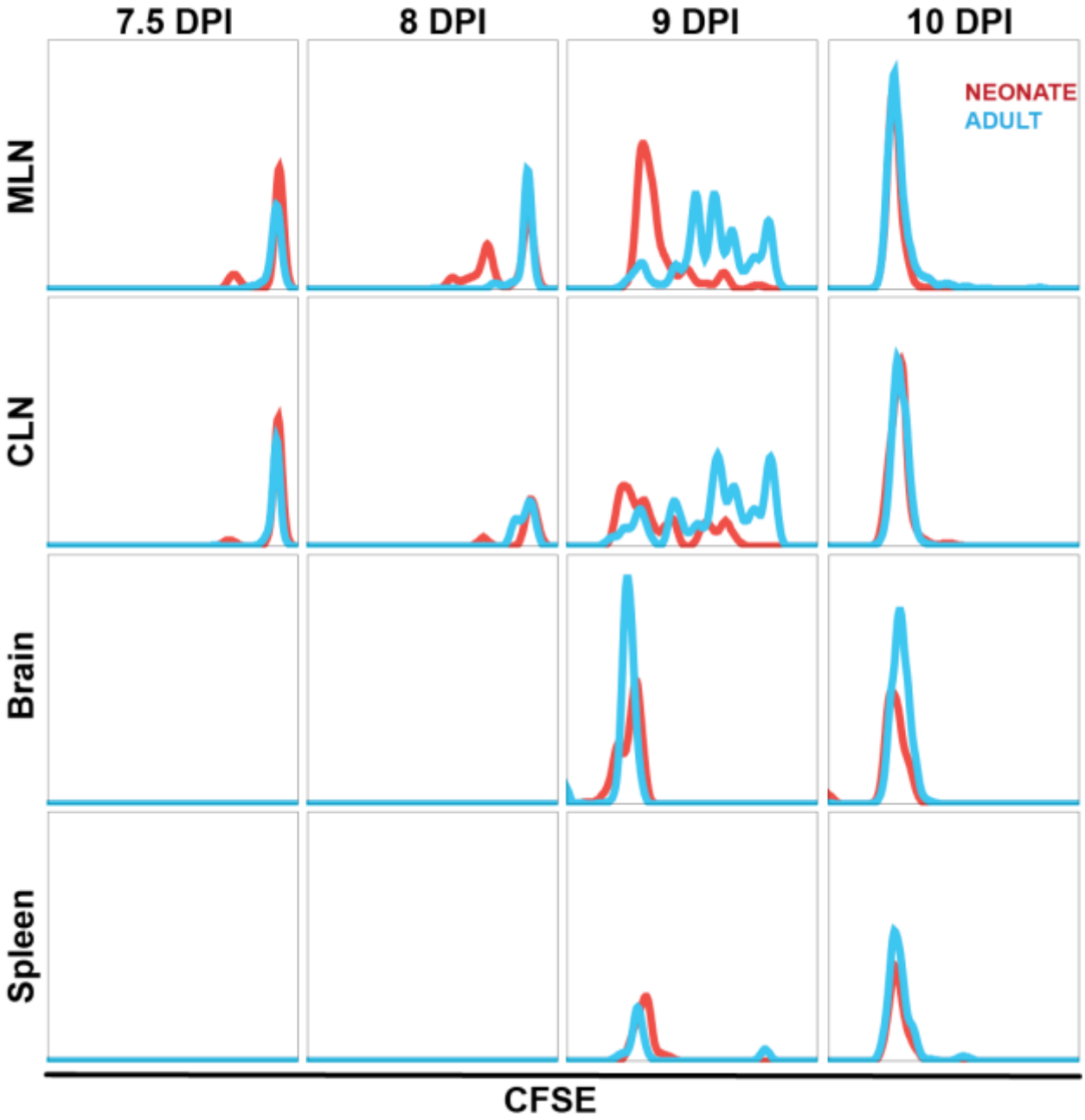

A
CD69+
B
KLRG1+
Brain
CLN
Spleen
C
D
E
F
% of Vb8+ CD8+ Cells
DPI
Neonate
Adult
0
50
100
8
12
16
20

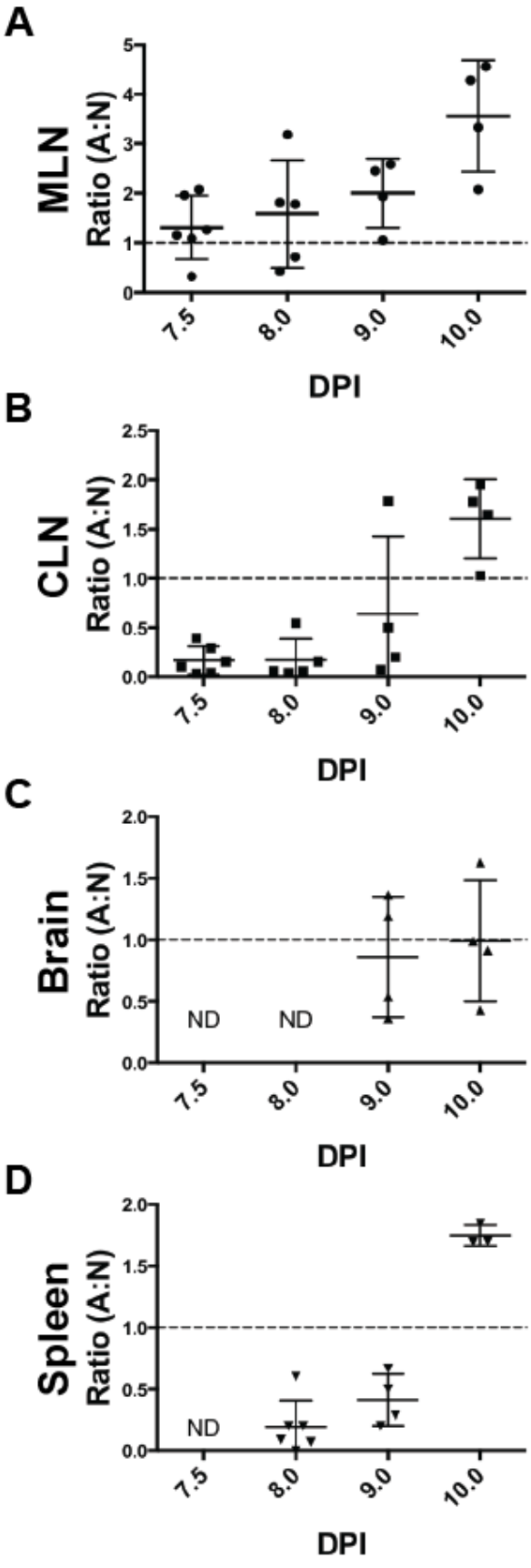
Figure 2.4 Neonatal CD8+ T cells rapidly begin proliferating before adults post infection. Newborn B6 (C57BL/6J) pups were i.p. infected with 200 PFU MCMV-gB. 7 days post infection, naïve CD8+ T cells were isolated and purified from spleens harvested from gB-I specific TCR transgenic neonate CD90.1 (Thy1.1) and adult Ly5.1 (CD45.1) mice. The purified naïve neonate and adult cells were labeled with CFSE. 5×105 neonate and 5×105 adult cells were pooled together in a 1:1 ratio and adoptively transferred i.p. to the previously infected B6 (C57BL/6J) neonates. Brains, spleens, CLN, and MLN were harvested at specified timepoints postinfection. Histograms show representative data from each time point. Cell proliferation and ration of the neonate and adults assessed using flow cytometer (LSR) and FlowJo.


7.5 DPI
8 DPI
9 DPI
10 DPI
NEONATE
ADULT
MLN
CLN
Brain
Spleen
CFSE

Higher numbers of neonatal CD8+ T cells were observed in various tissues post MCMV infection

We also asked if the more rapid proliferation of neonatal cells resulted in greater numbers of cells in specific organs at early times after infection. To this end, we tracked the ratio of adult and neonatal donor cells in the MLN, CLN, brain and spleen throughout the course of infection. Indeed, more neonatal donor CD8+ T cells were found in the CLN and spleen after infection; however, the ratio tipped in favor of adults by day 20 (Fig 2.5A, B). The failure of neonatal cells to accumulate during later stages of infection could be a result of reduced proliferation or increased cell death. (Fig 2.5)

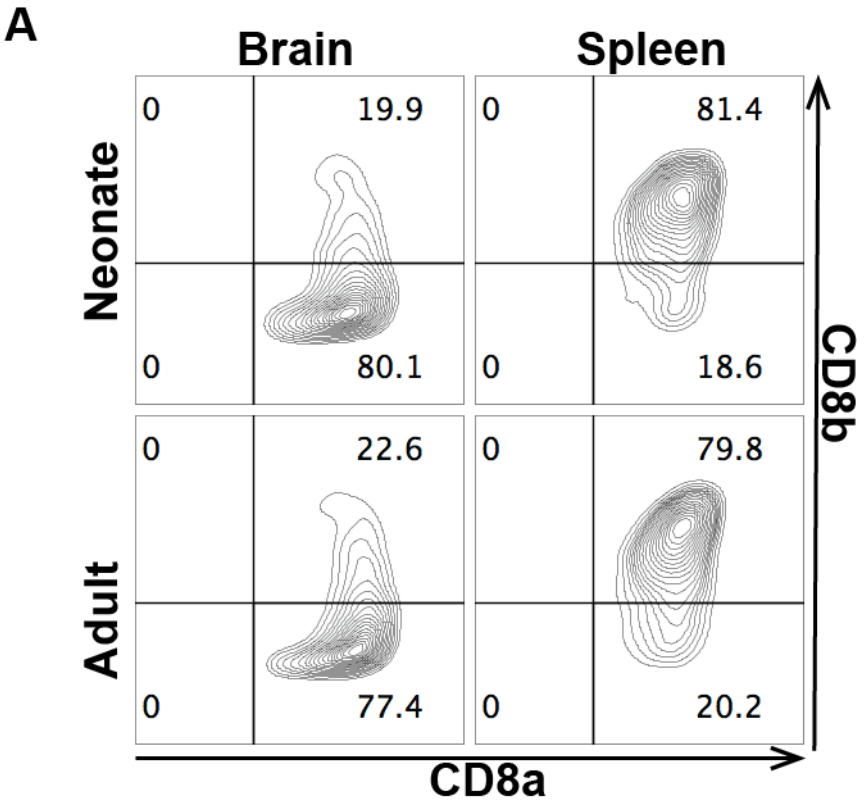
Figure 2.5 Higher numbers of neonatal CD8+ T cells migrated into various post infection. Newborn B6 (C57BL/6J) pups were i.p. infected with 200 PFU MCMV-gB. 7 DPI, naïve CD8+ T cells were isolated and purified from spleens harvested from gB-I specific TCR transgenic neonate CD90.1 (Thy1.1) and adult Ly5.1 (CD45.1) mice. The purified naïve neonate and adult cells were labeled with CFSE. 5×105 neonate and 5×105 adult cells were pooled together in a 1:1 ratio and adoptively transferred i.p. to the previously infected B6 (C57BL/6J) neonates. Brains, spleens, CLN, and MLN were harvested at specified timepoints postinfection. Histograms show representative data from each time point. Cell proliferation and ration of the neonate and adults assessed using flow cytometer (LSR) and FlowJo.

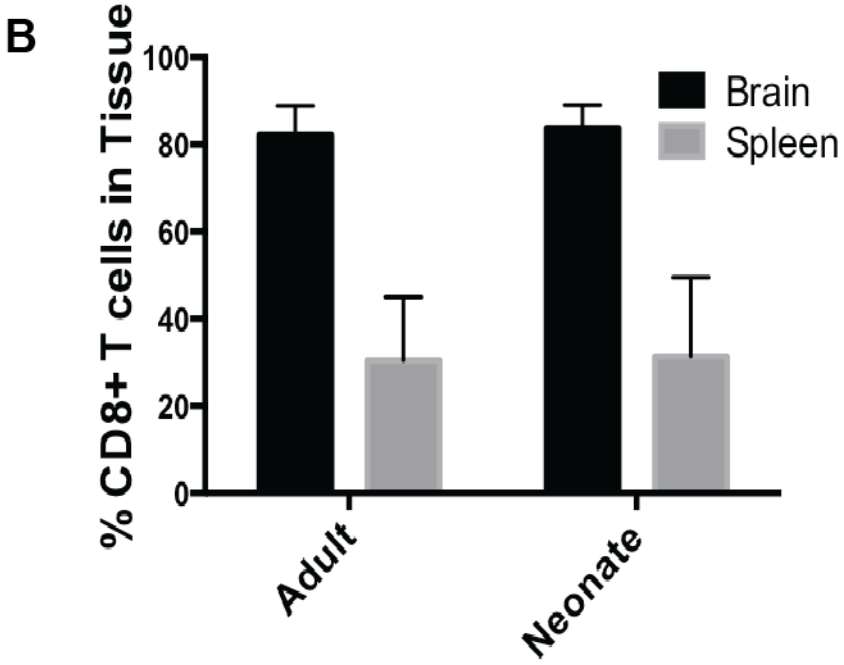

A
MLN
Ratio (A:N)
5
4
3
2
1
0
7.5
8.0
9.0
10.0
DPI
B
CLN
Ratio (A:N)
2.5
2.0
1.5
1.0
0.5
0.0
7.5
8.0
9.0
10.0
DPI
C
Brain
Ratio (A:N)
2.0
1.5
1.0
0.5
0.0
ND
ND
7.5
8.0
9.0
10.0
DPI
D
Spleen
Ratio (A:N)
2.0
1.5
1.0
0.5
0.0
ND
7.5
8.0
9.0
10.0
DPI

Neonate and adult CD8+ T cells localize in the brain with similarly efficiencies.

We also sought to determine why neonatal CD8+ T cells remain terminally differentiated in the brain after infection. One possibility may be that neonatal CD8+ T cells more efficiently leave the vasculature and infiltrate the parenchyma tissue, allowing them to remain in close contact with virally infected cells. To test this, we examined differences in trafficking between neonate and adult CD8+ T cells. Neonatal and adult donor CD8+ T cells were again co-transferred into newborn pups prior to infection with MCMV-gB. At 16 DPI, we injected α-CD8b (i.v.) in order to label all the CD8+ T cells in the vasculature, then harvested the brains and spleens 3 minutes later. Cells from these organs were then resuspended and stained with α-CD8α. In this way, we could delineate the cells in the vasculature (CD8b+) from those in the parenchyma tissue (CD8α+). The frequency of parenchyma- and vasculature-associated cells were examined by analyzing the expression of CD8α and CD8b on different-aged donor CD8+ T cells using flow cytometry. As shown in Fig. 2.6, we observed the same percentage of CD8b+ cells in neonatal and adult CD8+ T cells localized in the brain. This data suggests that neonatal and adult CD8+ T cells undergo similar patterns of homing and trafficking to the brain after MCMV infection (Fig. 2.6).

Figure 2.6 Neonate and adult CD8+ T cells both equally able to migrate into the brain post MCMV infection. Naïve CD8+ T cells were isolated and purified from spleens harvested from gB-I specific TCR transgenic neonate CD90.1 (Thy1.1) and adult Ly5.1 (CD45.1) mice. The neonate and adult cells were then mixed together in a 1:1 ratio and adoptively transferred i.p. to newborn (6–18 h postpartum) B6 pups. The recipient pups were then infected with 200 PFU MCMV-gB. 16 DPI α-CD8b was injected i.v. three minutes later, tissues were harvested. Cells were isolated from brains and spleens at specified timepoints postinfection and then stained ex vivo with α-CD8α. The frequency of parenchyma and vasculature-associated adult or neonate CD8+ T cells of brains and spleens were assessed for surface staining for of CD8α and CD8b using flow cytometer (LSR) and FlowJo.

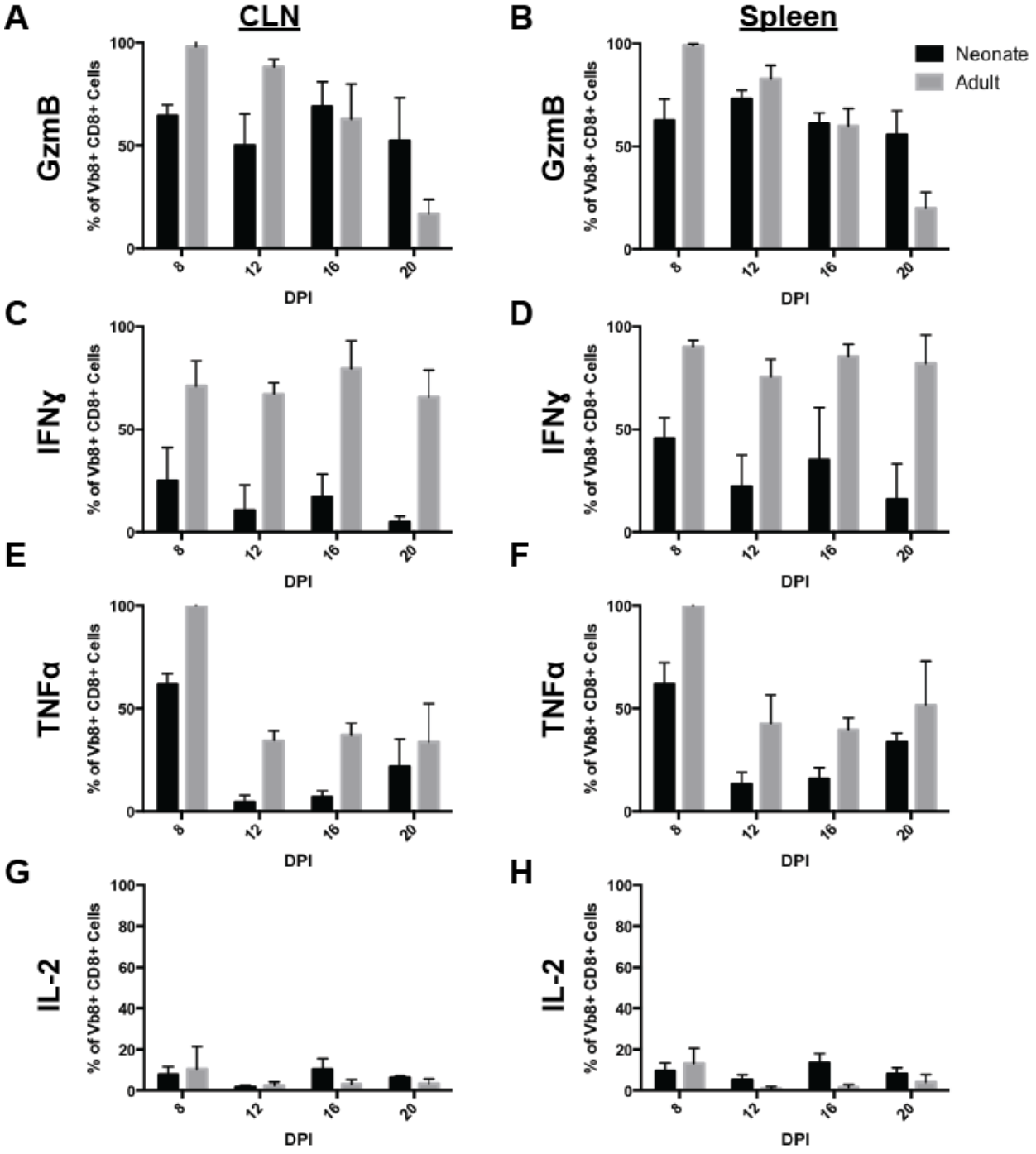

A
Brain
Spleen
Neonate
Adult
0
19.9
80.1
81.4
18.6
22.6
77.4
79.8
20.2
CD8b
CD8a


B
% CD8+ T cells in Tissue
100
80
60
40
20
0
Brain
Spleen
Adult
Neonate

Neonates exhibit impaired effector functions throughout the course of MCMV infection

Given that neonatal CD8+ T cells do not appear to be impaired in their ability to become activated, proliferate, and migrate to sites of infection in the brain, we next asked whether they were able to acquire similar amounts and types of effector functions. Once again, we performed the co-transfer experiment, but this time we measured their ability to produce cytolytic molecules and cytokines at various days after infection. This data showed dramatically lower levels of GzmB, INFγ, and TNFα expression by neonatal CD8+ T cells. In contrast, the adult and neonatal CD8+ T cells made similar amounts of the effector cytokine IL-2, though the frequency of cells producing it was fairly modest (Fig. 2.7). These data suggest that neonatal cells exhibit a reduced capacity to eliminate CMV because they fail to mobilize robust effector functions after infection.

Figure 2.7 Neonatal CD8+ T cells show signs of impaired effector function when compared to adults post-infection with MCMV-gB Neonatal CD8+ T cells show signs of impaired effector function post-infection with MCMV-gB. Naïve CD8+ T cells were isolated and purified from spleens harvested from gB-I specific TCR transgenic neonate CD90.1 (Thy1.1) and adult Ly5.1 (CD45.1) mice. The neonate and adult cells were then mixed together in a 1:1 ratio and adoptively transferred i.p. to congenic newborn Ly5.1 (CD45.2) pups. The recipient pups were then infected with 200 PFU MCMV-gB. CLN and spleen were harvested at specified timepoints postinfection. The number and ratio of congenically marked donor cells from the neonate and adults harvested from the tissues were assessed effector function with expression of GzmB, TNFa, INFγ and IL-2 using flow cytometer (LSR) and FlowJo.


A
CLN
B
Spleen
Neonate
Adult
GzmB
IFNγ
TNFα
IL-2
% of Vb8+ CD8+ Cells
DPI
8
12
16
20
C
D
E
F
G
H

Discussion

Numerous reports have demonstrated a crucial role for the $CD8^+$ T cell response to MCMV in the CNS of neonatal mice. Other studies have reported the importance of CD8+ T cells for resistance to systemic MCMV infection in neonatal mice (10, 106). Following virus spread to the neonatal brain, activated virus-specific $CD8^+$ T cells are recruited into the CNS and are the most abundant lymphocyte population in the brains during infection (52, 106, 152). While it is well known that neonatal mice exhibit a higher susceptibility to MCMV infection in the brain compared to adults, the reasons for these age-related differences are poorly understood. The studies described in this report suggest that cell-intrinsic differences between neonatal and adult CD8+ T cells may be a key factor. This conclusion is based on the fact that neonatal CD8+ T cells are not as effective at conferring immune protection as adults in the same host environment.

To better understand why neonatal CD8+ T cells are less protective, we co-transferred an equivalent number of monoclonal neonatal and adult CD8+ T cells into the same recipient mouse and tracked their response to MCMV infection in the brain. The results showed a failure of neonatal cells to accumulate in the brain after infection. Indeed, at the peak of the CD8+ T cell response, nearly 2 times more adult donor cells were present compared to neonatal donor cells. Interestingly, these differences could not be attributed to reduced activation or proliferation of neonatal CD8+ T cells because additional studies showed neonatal cells actually become activated and proliferate sooner than their adult counterparts. Importantly, this data is consistent with previous reports (104, 108, 155, 156) and suggests that reduced numbers of neonatal CD8+T cells during infection is likely due to increased apoptosis. Although future studies are

warranted, our conclusion aligns well with earlier reports demonstrating that neonatal CD8+ T cells undergo more apoptosis after activation (Adkins, 1999), and it highlights the need to consider the potential role of apoptosis in the neonatal CD8+ T cell response to CMV.

An important caveat to our study is that we used CD8+ T cells from TCR transgenic mice (gBT-I mice). This was necessary to control for developmental differences in the TCR repertoire and focus specifically on cell-intrinsic differences. We also compared the same number of neonatal and adult CD8+ T cells because previous reports have shown that the precursory frequency can influence critical aspects of the CD8+ T cell response to infection. However, it is important to point out that neonatal mice typically contain much fewer CD8+ T cells than adults. This is due to the fact that fewer CD8+ T cells have been produced and that the neonatal CD8+ T cell pool is less diverse and contains fewer distinct CD8+ T cells (Rudd repertoire papers). Thus, it would be interesting to determine whether age-related differences in the CD8+ T cell response to MCMV would be even more dramatic if we directly compared polyclonal CD8+ T cells from neonatal and adult mice.

In addition to the lower magnitude of neonatal CD8+ T cells, the numbers of IFNγ^{+} producing CD8+ T cells were also significantly lower than their adult counterparts after MCMV infection. Previous studies have shown that IFN-γ plays an important role in recovery from MCMV infection by helping to eliminate the virus (53, 121). Moreover, adoptive transfer of Tc1 cells (IFNγ^{high} CTLs) promotes clearance of viral infection, while transfer of Tc2 cells (IFNγ^{low} CTLs) does not affect viral clearance (Wiley). These published studies, along with our data, indicate that IFNγ produced by CD8^{+} T cells may

be particularly important to effectively clearing MCMV from the neonatal brain as well as explain why TCRα knockout mice receiving WT neonatal $CD8^+$ T cells showed higher viral loads than the mice receiving WT adult $CD8^+$ T cells.

In sum, this study has demonstrated several quantitative differences in how neonatal and adult CD8+ T cells respond to MCMV infections even when placed in similar environments. Our adoptive transfer data suggests that the developmental stage of the CD8+ T cell population is an important factor in determining disease outcome. Future studies to elucidate the molecular mechanisms responsible for phenotypic and functional differences of CD8+ T cells should include studies of the microenvironment, which can give us an impression of what may be causing these variances in response. Our observation that $CD8^+$ T cell responses in neonates are functionally different from those of adults have significant implications and may help the development of future strategies to enhance cellular immunity during early stages of development.

CHAPTER 3 - ENVIRONMENTAL FACTORS – RA EFFECTS ON THE CD8+ T CELL RESPONSE

Abstract

In early life, the active vitamin A derivative retinoic acid (RA) plays a crucial role in the development of the immune and central nervous system (CNS). Throughout early development, RA's important physiological activities include cellular proliferation, differentiation, and apoptosis. The impact of viral infection on the RA microenvironment of the neonatal brain, as well as its propensity to affect immune function and CNS development, has not been previously explored. Congenital infection of the developing central nervous system (CNS) with cytomegalovirus (CMV) has been shown to result in permanent brain damage and neurological impairment, including congenital sensorineural deafness, vision impairment, and cognitive impairment. The goals of this study were to observe the patterns of RA production in MCMV-infected mice and identify the cell types and brain regions involved in RA production. Collectively, these results suggest that aberrant retinoic acid production in the brain after neonatal MCMV infection may contribute to the delayed CD8+ T cell response and resulting neurological sequelae observed in congenitally infected neonates. These studies give insight into possible pathological mechanisms of brain malformations and other clinical manifestations associated with the disease.

Introduction

Congenital cytomegalovirus (CMV) infection of the developing central nervous system (CNS) has been shown to be one of the most significant sources of neurological disease in newborns and young children that inevitably leads to physical and cognitive deficits (124, 125). In the United States, an estimated 3,000 newborns suffer from developmental problems associated with CMV-induced CNS damage (18, 126). The neurodevelopmental abnormalities observed in infected children range from hearing loss to cortical and cerebellar hypoplasia (18, 118, 122-123). In severe cases, lissencephaly, ventriculomegaly, and periventricular calcifications have been observed in congenitally infected infants.

The mechanism(s) leading to neurodevelopmental disorders in infants with HCMV infection and inflammation within the CNS are poorly understood. Previous studies have alluded to various neurological abnormalities that might underlie these disorders, including disruption of vascular supply in the developing brain, loss of neural progenitors in the subventricular zone, and disordered cellular positioning secondary to altered cellular migration and positioning (116-121). CNS damage that leads to lifelong neurological disease can also be caused by the host inflammatory response (118, 121). Earlier studies on the host response to CMV have demonstrated that CD8+ T cells are required to control CMV through cytokine production and the killing of infected target cells (4, 6, 53). Whether CD8+ T cells contribute to impaired development of the CNS remains an open question.

Previous studies have suggested that RA signaling in CD8+ T cells during infection is biphasic, capable of driving T cell activation/differentiation during the early

stages of an immune response, and able to regulate the amplitude of effector responses at later stages (112-114). RA is a vitamin A-derived, non-peptidic, small lipophilic molecule that acts as a ligand for nuclear RA receptors (RARs), converting them from transcriptional repressors to activators. RA is synthesized in specific cellular locations and regulates transcription by interacting with nuclear RARs bound to RA response elements (RAREs) near target genes. The distribution and levels of RA in embryonic tissues are tightly controlled by regulated synthesis through the action of specific retinol and retinaldehyde dehydrogenases and by degradation via specific cytochrome P450s (CYP26s).

Retinoic acid (RA) has also been shown to have a central role in CNS development in vertebrates (109-111). Recent studies demonstrate that RA is involved in the creation of diffusion (morphogen-like) gradients and the establishment of signaling boundaries due to RA metabolism, thereby allowing RA to finely control the differentiation and patterning of various neural stem/progenitor cell populations (115). However, how viral infections in the brains of neonates impact these RA gradients and microenvironments is unknown. Also, how changes in RA levels affect CD8+ T cell activation and differentiation in neonatal brains is poorly understood. In this study, we examined how MCMV infection impacts RA production in the CNS. We also investigated the role of RA signaling in CD8+ T cells during infection.

Materials/Methods

Animal Models

Pathogen-free pregnant C57BL/6NCr female mice were purchased from Charles River Laboratory. All experiments were conducted at Cornell University's College of Veterinary Medicine, in accordance with and under the approval of the Cornell Institutional Animal Care and Use Committee and the Cornell Center for Animal Resources and Education. All animals were housed under standard conditions.

MCMV Strains

A recombinant MCMV expressing green fluorescent protein (GFP), referred to as MCMV-GFP, was constructed by inserting the GFP gene into the MCMV genome downstream of the MCMV major immediate-early promoter (MIEP). Therefore, only infected cells undergoing active viral replication expressed GFP. A recombinant MCMV expressing MCMV-specific glycoprotein B (gB), referred to as MCMV-gB, was constructed by replacing the m157 gene open reading frame with the gB epitope coding sequence. Strains courtesy of Dr. Joel Baines (Cornell University).

Infection Method and Study Design

A 1.0 mL syringe (Becton Dickinson) fitted with a 23-gauge needle (Becton Dickinson) was used to inject newborn mice (6-18 hours postpartum) intraperitoneally (IP) with 200 PFU in 50 µl of MCMV-gB. Neonatal brains and spleens from MCMV-infected and uninfected control mice were harvested by standard sterile procedure at 7, 14, 17, 21,

and 28 DPI for flow cytometry to describe the CD8+ T cell response temporality and phenotype.

Isolation of Brain Mononuclear Cells

Mice were briefly sacrificed by isoflurane inhalation to preserve intact circulation, then perfused with 30 mL ice-cold PBS. Brains were harvested in 13 mL of RPMI 1640 supplemented with 10% FBS (10% RPMI) in a 15 mL conical tube. Mechanical processing was completed through 70 µm cell strainers (Fisher Scientific), assisted by the plunger of a 3 mL syringe (Becton Dickinson). The suspension was centrifuged at 1500 RPM for 5 minutes at 4ºC. The tissue was homogenized in a 30% Percoll suspension, overlaid on a 70% Percoll solution, and centrifuged at 2600 RPM for 25 minutes at 4ºC. Cells at the solution interface layer were collected, washed in 10% RPMI, and re-suspended in ice-cold FACS buffer.

Isolation of Splenocytes

Neonatal spleens were harvested using the previously described method at the same time-points of interest, then mechanically processed using 40 µm cell strainers. Dissociated splenic tissue was centrifuged for 5 minutes at 1500 RPM and re-suspended in 3 mL of ice-cold FACS buffer.

Flow Cytometry

Isolated brain mononuclear cells and splenocytes were stained with anti-mouse cell surface markers and the ALDEFLUOR assay at 4ºC for 30 minutes. 300 µL of each

tissue sample was transferred onto a 96-well round bottom plate (Corning), then centrifuged at 1500 RPM for 5 minutes at 4ºC. Supernatant was discarded and pellets were re-suspended in 200 µL ice-cold FACS buffer. 100 µl of each MCMV-infected sample and each control sample were transferred to another well for a fluorescence control reaction to? the ALDEFLUOR buffer using the ALDH1-specific inhibitor diethylaminobenzaldehyde (DEAB), referred to as DEAB controls. ALDEFLUOR-specific flow cytometry gating strategy is shown in Figure 2. Samples were centrifuged at 1500 RPM for 5 minutes at 4ºC. Supernatant was discarded, and 100 µL of diluted Fc block was what? (Fc block stock from manufacturer diluted in FACS buffer at a 1:150 ratio). Samples were incubated on ice for 20 minutes, centrifuged at 1500 RPM for 5 minutes at 4ºC, and supernatant was discarded. The antibody panel consisted of CD8-e450, CD11b-PE, NK1.1-PeCy7, CD19-APC, CD3e-APC-e780, CD11c-PerCPe 710, and F4/80-PEe610 (all antibodies manufactured by EBioscience, Inc.) A cocktail was prepared using 50 µL ice-cold FACS buffer per sample. ALDEFLUOR reagents were prepared according to manufacturer protocol. ALDEFLUOR reagent was activated by diluting 5 µL reagent with 1 mL ALDEFLUOR buffer, provided by the manufacturer. 50 µL activated ALDEFLUOR reagent and 50 µl antibody cocktail was added to each infected sample, uninfected sample, and DEAB controls, with a total volume of 100 µL in each well. Samples were stained for 30 minutes at 4ºC in the dark. Three washes were performed with FACS buffer, using volumes of 150 µL, 200 µL, and 150 µl, respectively. Cells were resuspended in 50 µL of ice-cold FACS buffer, and data was immediately collected using an LSRII flow cytometer. Lymphocytes were gated using forward and side scatter protocols. FlowJo software (TreeStar) was used for analysis.

Repeat data collection and analysis was performed on 7, 14, 17, 21, and 28 DPI.

Organotypic Section Imaging Preparation

MCMV-GFP infected neonate brains were harvested intact at 7, 14, 17, 21, 28, and 60 DPI. A cryotome blade (VWR International) was used to manually dissect brains into approximately 100 µm coronal sections containing the choroid plexus of the lateral ventricle and third ventricle. Sections were placed in a 35 mm optical quality glass bottom cell culture dish (MatTek). Sections were immersed in a 1:200 dilution of ALDEFLUOR reagent at room temperature for 15 minutes. 300 µL of 300 nM DAPI solution (prepared courtesy of the Schaffer-Nishimura Lab) was added to section immersions.

Confocal Imaging Methodology

Imaging was performed on a Leica TCS SP2 confocal microscope, using a 20x or 40x objective lens. Z-stacks with a minimum depth of 50 µm were acquired 25 minutes after the beginning of the ALDEFLUOR immersion reaction. The same stereotactic position within the brain was estimated by visually confirming locations of the lateral and third ventricles along the central dorsoventral axis of the coronal sections. Confocal imaging acquisition used a 200 Hz scanning speed, line average of 2, and frame average of 1. Objectives used included HCX PL APO 40x 0.85 Dry and HCX PL APO CS 20x 0.7 Dry. PMTs used were Leica/DAPI (emission at 455), Leica/EGFP (emission at 507), and Leica/DSRED (emission at 582). Overall laser power was 1%; or: UV used 22% of 405 nm laser line power; EGFP used 13% of 488 nm laser line power; and DSRED used

45% of 561 nm laser line power. Images were deconvolved using the Volocity software (PerkinElmer).

Statistical Analysis

Statistics were performed using Graph Pad Prism (GraphPad Software). For all analyses, a significance level of 0.05 was accepted.

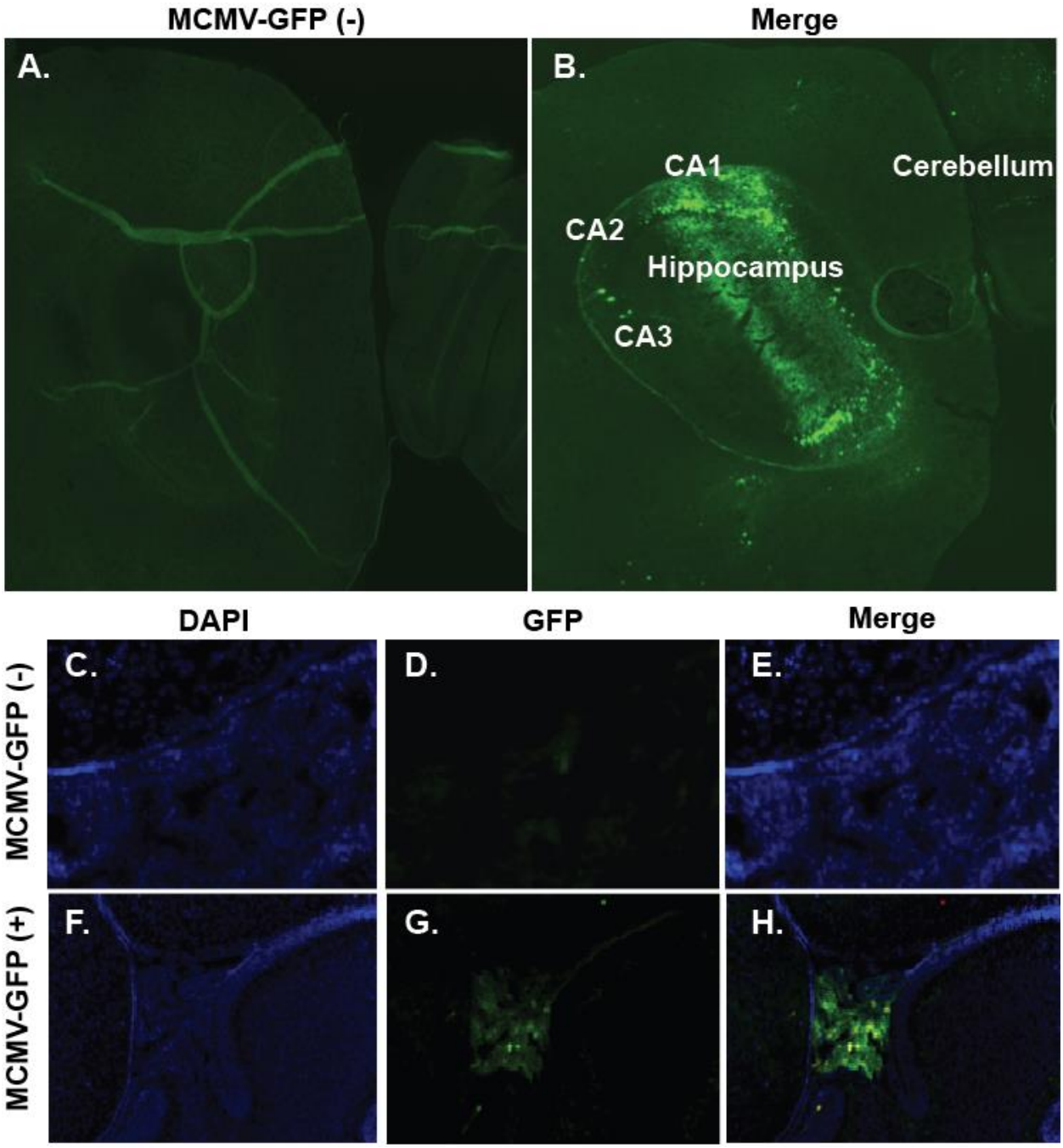
Results

MCMV preferentially localizes to the hippocampus, cerebellum, and choroid plexus after systemic infection

Many studies of the hippocampus have shown its distinct importance and role in learning and declarative memory. The cerebellum, on the other hand, plays a key role in motor and cognitive functions, including attention and language (175, 176). The choroid plexus consists of modified ependymal cells that help to produce cerebrospinal fluid (CSF) in the ventricles of the brain and act as the blood-CSF barrier, facilitating transport of different substances in and out of the brain (177).

When we first sought to determine which regions of the brain were infected with MCMV-GFP, we found that each of the above regions were affected. Following intraperitoneal inoculation of newborn mice with MCMV-GFP, active replication of virus was detected in the hippocampus (CA1, CA2, & CA3 regions) and cerebellum, which peaked around 10 to 14 DPI (Fig. 3.1A-B). MCMV-GFP infection was also observed throughout the choroid plexus of the lateral, third, and fourth ventricle of developing brains in early stages of the infection (~7 DPI) (Fig. 3.1C-H).

Figure 3.18 Active replication of MCMV observed in the brains of neonatal mice. Confocal image of murine cytomegalovirus (MCMV) replication observed in the brains of neonatal mice. C57BL/6 neonates (PND 0) were i.p. infected with MCMV-GFP (200 PFU). Active viral infection and replication was determined by expression of EGFP. At the peak of infection (10 DPI), EGFP positive cells are mainly observed in the Hippocampal and cerebellar regions of the brain. (A-B) Infection was also observed in the choroid plexus (C-H)


MCMV-GFP (-)
Merge
A.
B.
CA1
Cerebellum
CA2
Hippocampus
CA3
DAPI
GFP
Merge
MCMV-GFP (-)
MCMV-GFP (+)
C.
D.
E.
F.
G.
H.

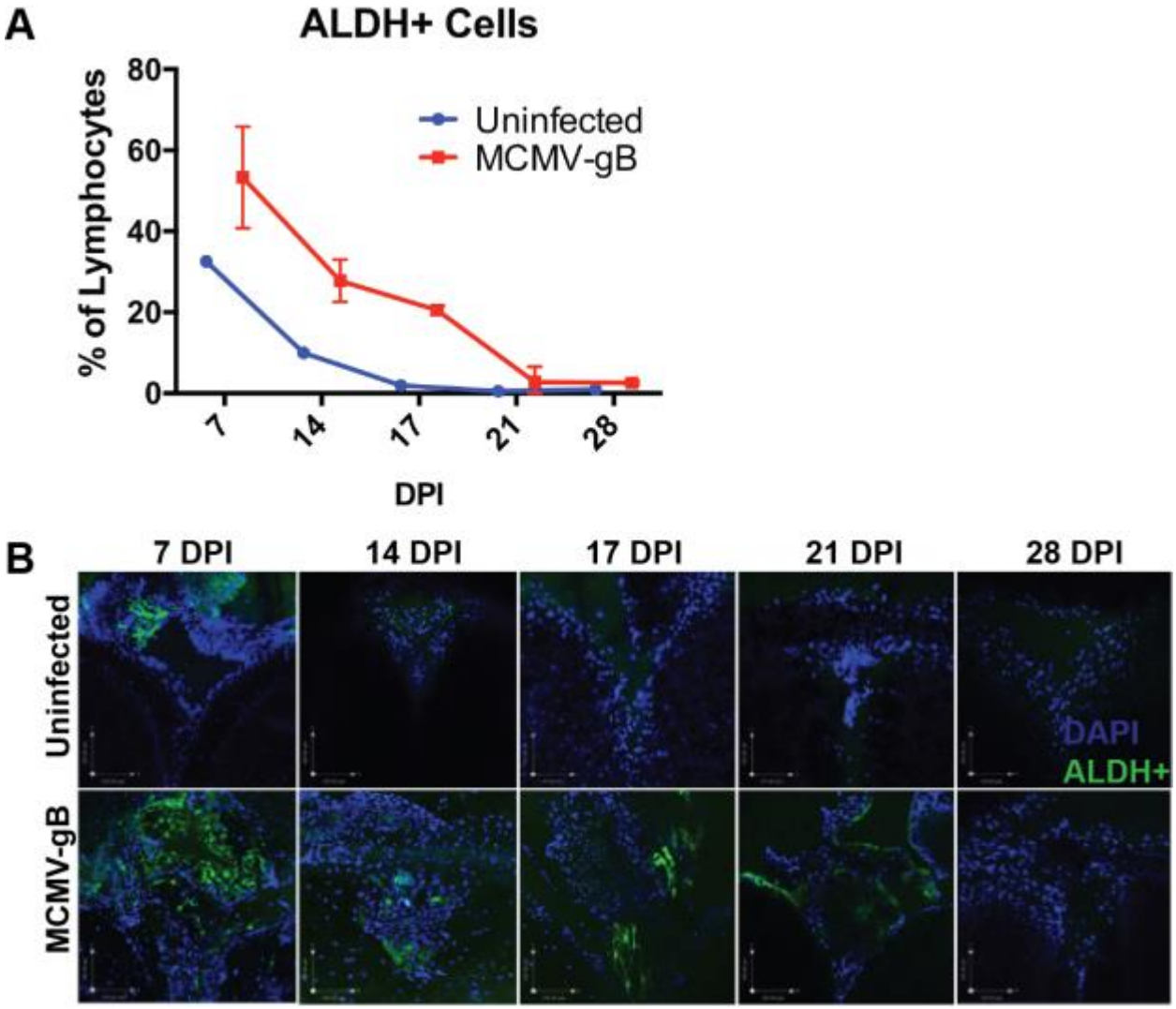
MCMV-infected brains show aberrant levels of RA production.

During the early stages of brain development, RA is an established signalling molecule involved in neuronal patterning, neural differentiation, and axon outgrowth. To determine whether MCMV alters the pattern of RA production during infection, we next assessed cytosolic levels of RA. This was accomplished by measuring retinaldehyde dehydrogenase (RALDH) activity in cells, the enzyme family responsible for metabolism of retinal to RA. For these studies, we employed the ALDEFLUOR assay, allowing us to examine RALDH activity and determine which regions of the brain were involved in RA production. The ALDEFLUOR assay has been optimized to detect activity of the ALDH1 isoforms in various cell types. Activated ALDEFLUOR reagent is a green fluorescent substrate of ALDH1 that freely diffuses into live cells. ALDH1 catalyzes the binding of aminoacetate to ALDEFLUOR reagent, which is then retained within cells. Concentration of intracellular fluorescent aminoacetate-bound reagent is proportional to ALDH1 activity and can be measured using flow cytometry. Background fluorescence is controlled for by diethylaminobenzaldehyde (DEAB), a specific inhibitor of ALDH1 activity.

Live brain sections stained with ALDEFLUOR assay showed increased levels of ALDH1+ activity (FITC) in MCMV-infected mice in the choroid plexus (Fig. 3.2B). The highest levels were observed in the choroid plexus of the lateral (data not shown) and third ventricles at 7 DPI (Fig. 3.2B). ALDH1+ activity was also observed in ventricular regions dorsal to the hippocampus, and in dorsal surfaces of the hippocampus itself, most notably at 7 and 14 DPI (Fig. 3.2B). Visible levels of ALDH1+ activity decreased over the course of infection, with minimal fluorescence by 21 DPI (Fig. 3.2B). The same

decreasing trend in fluorescence was observed in uninfected control mice (Fig. 3.2B). However, infected mice showed dramatically more fluorescence at all time points under identical imaging conditions. We also used flow cytometry to examine the kinetics of RA production in lymphocytes throughout the response to infection (Fig. 3.2A). Strikingly, the results showed that nearly twice as many lymphocytes produce RA in mice infected with MCMV compared to controls (Fig. 3.2A). Collectively, this data indicates that MCMV infection in the brain results in an aberrant increase in RA levels for extended periods of time during development.

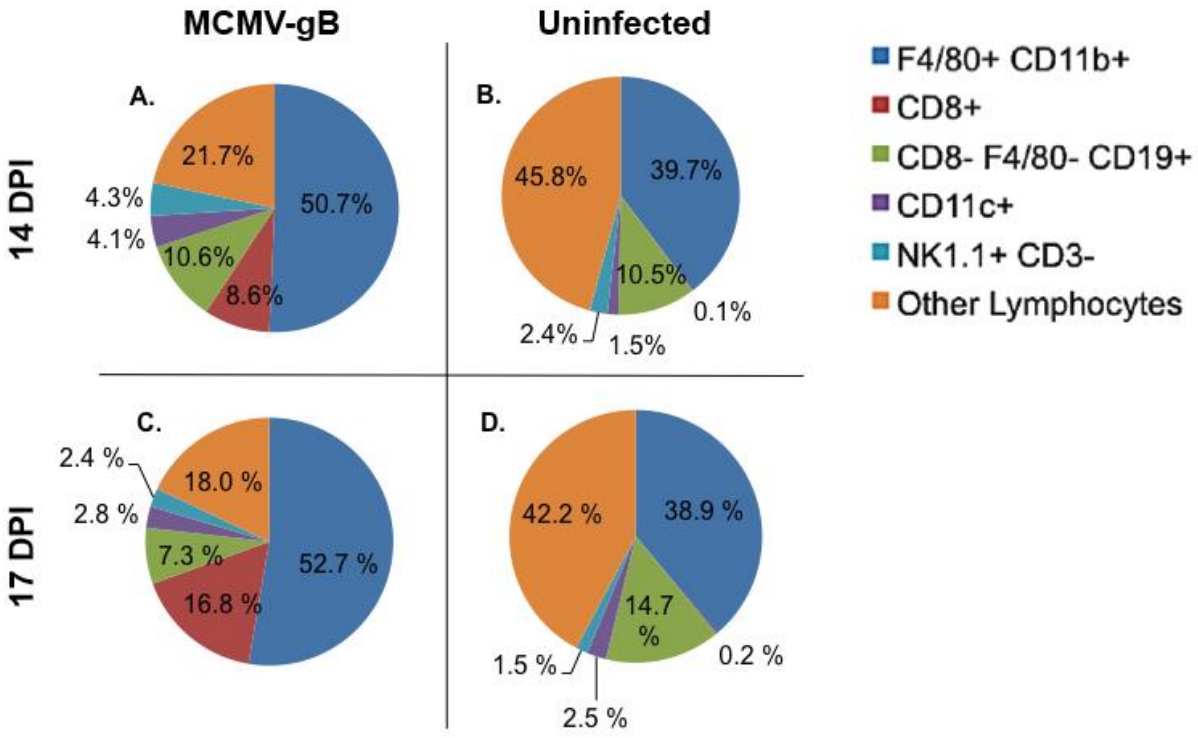
Figure 3.29Prolonged ALDH1 enzyme activity observed in choroid plexus of infected neonates. ALDEFLUOR substrate is oxidized by ALDH1 enzymes and the charged fluorescent product accumulates in cells with active ALDH1 enzymes. DEAB, a general inhibitor of ALDH1 enzymes, was used as a control for background fluorescence. (A) Quantification and kinetic of ALDEFLUOR fluorescence in mononuclear cells from the brains of infected and uninfected pups. The data were made relative to DEAB control. Neonates were infected on PND 0 with MCMV-gB and brains were harvest 7, 14, 17, 21, 28DPI. Mononuclear cells were isolated from brain tissue using percoll gradient and cells were treated with ALDEFLUOR assay. ALDH1 activity was accessed via flow cytometric analysis. (B) Confocal image of choroid plexus of the third ventricle in the brain. Imaging was performed on a Leica TCS SP2 confocal microscope, using a 20x objective lens. Image is made up of Z-stacks at a depth of 50 µm which were then merged. The image were acquired 25 minutes after the beginning of the ALDEFLUOR immersion reaction. ALDH1+ cells are visualized in green fluorescence. Neonates were infected on PND 0 with MCMV-gB and brains were harvest 7, 14, 17, 21, 28DPI. Organotypic brain slices were then visually accessed for ALDH1+ activity.


A
ALDH+ Cells
% of Lymphocytes
80
60
40
20
0
Uninfected
MCMV-gB
7
14
17
21
28
DPI
B
7 DPI
14 DPI
17 DPI
21 DPI
28 DPI
Uninfected
MCMV-gB
DAPI
ALDH+

Increased macrophage and CD8+ T cell numbers observed in the neonatal brain following MCMV infection.

We next set out to determine the immune cell types that were producing RA after infection. To start, we first characterized the composition of immune cells in the brain at the peak of viral replication (14 dpi), as well as at the peak of the CD8+ T cell response (17 dpi). This was accomplished by staining mononuclear brain cells from control and infected mice with an array of antibodies that identify various immune cells. At both 14 and 17 DPI, we observed a large percentage of F4/80+ CD11b+ macrophages in the brains of MCMV-infected mice compared to controls (50% vs 39%) (Fig. 3.3A, B). We also observed significant numbers of CD8+ T cells at both timepoints, but differences between infected and control mice were most dramatic at 17 DPI (17% vs 0.2%) (Fig. 3.3C, D). Other cell types, such as B-cells, NK cells, and DCs appeared to make up a small fraction of the inflammatory response (Fig. 3.3).

Figure 10 Increased number in macrophages and CD8+ T cells observed in infected neonates. Graph of cell composition post infection with MCMV. (A) F4/80+ CD11b+ macrophages compose the majority of the total lymphocyte population observed at 14 DPI in the brain. (B) F4/80+ CD11b+ macrophages compose a significantly lesser portion total lymphocytes in the uninfected brain at 14 DPI. CD8+ T-cells are not notably present. (C) F4/80+ CD11b+ macrophages remain the dominant lymphocyte in the infected brain at 17 DPI. CD8+ T-cells reach peak levels. (D) F4/80+CD11b+ macrophages are the dominant lymphocyte in uninfected neonatal brains, but to a notably lesser extent than MCMV-infected mice.


MCMV-gB
Uninfected
14 DPI
17 DPI
A.
B.
C.
D.
21.7%
4.3%
50.7%
4.1%
10.6%
8.6%
45.8%
39.7%
10.5%
0.1%
2.4%
1.5%
2.4 %
18.0 %
2.8 %
7.3 %
52.7 %
16.8 %
42.2 %
38.9 %
14.7 %
0.2 %
1.5 %
2.5 %
F4/80+ CD11b+
CD8+
CD8- F4/80- CD19+
CD11c+
NK1.1+ CD3-
Other Lymphocytes

F4/80+ CD11b+ Microglia and CD8+ T cells are the predominant source of elevated ALDH1 activity in infected neonatal mice.

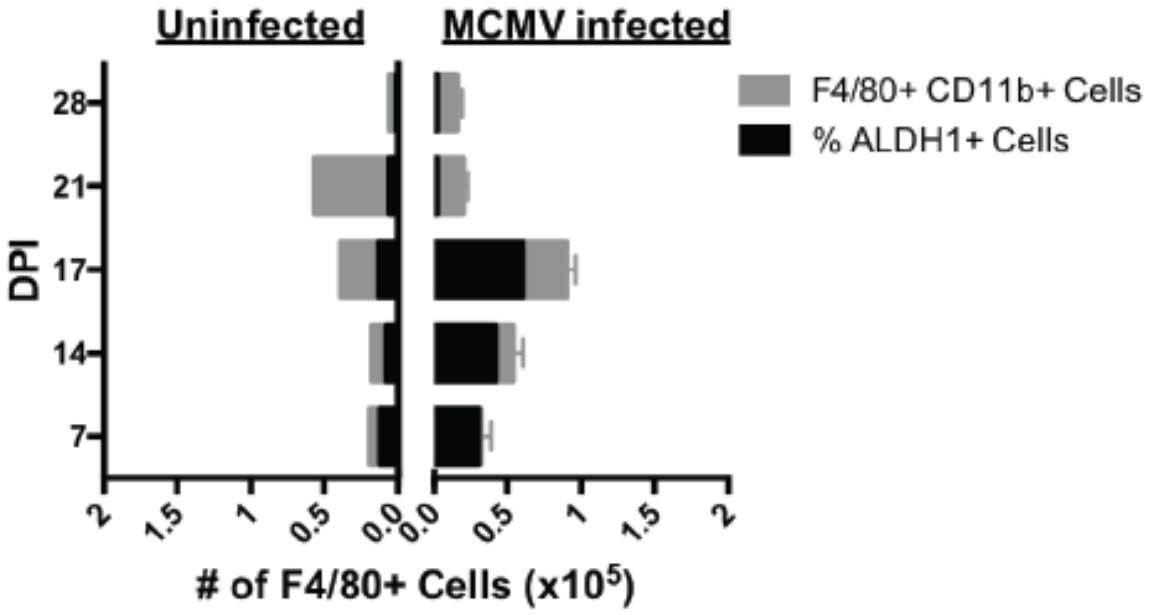
Since large numbers of macrophages and CD8+ T cells are present in the brain at 14 and 17 DPI, we hypothesized that these cell types may be responsible for the increased amount of RA in the brain after MCMV infection. Our approach here was to examine the frequency of RA-producing macrophages and CD8+ T cells from the brains of control and infected mice throughout the response. For the macrophages, we observed the highest levelsALDH1activity in the MCMV-infected brain at 7-17 DPI, after which levels sharply decreased and were no longer significantly different from age-matched control mice (Fig. 3.4A). At 17 DPI time point, macrophages were still the predominant leukocyte in MCMV-infected mice. (Fig. 3.4A). They also accounted for the majority of the ALDH1+ activity; 90.2% showed ALDH1+ activity (Fig. 3.4A). This is a significant increase from macrophage ALDH1+ activity at 14 DPI, where 77.4% of macrophages showed ALDH1+ activity (Fig. 3.4A). Control mice showed an inverse trend, with macrophage ALDH1 activity decreasing from 51.8% at 14 DPI to 41.4% at 17 DPI (Figure 8B, Figure 8D).

Interestingly, we observed a different trend in RA production for CD8+ T cells. While RA production increased in macrophages between 14 and 17 DPI (Fig. 3.4B), ALDH1 activity decreased in CD8+ T cells during the same time interval. For example, at 14 DPI, 46.9% of CD8+ T cells showed ALDH1+ activity (Figure 9A). This was nearly double the proportion of ALDH1+ CD8+ T cells observed in uninfected controls, which was 28.8% (Fig. 3.4B). However, by 17 DPI, the proportion of CD8+ T cells showing ALDH1 activity dropped to 5.4% (Fig. 3.4B). Collectively, this data indicates that a

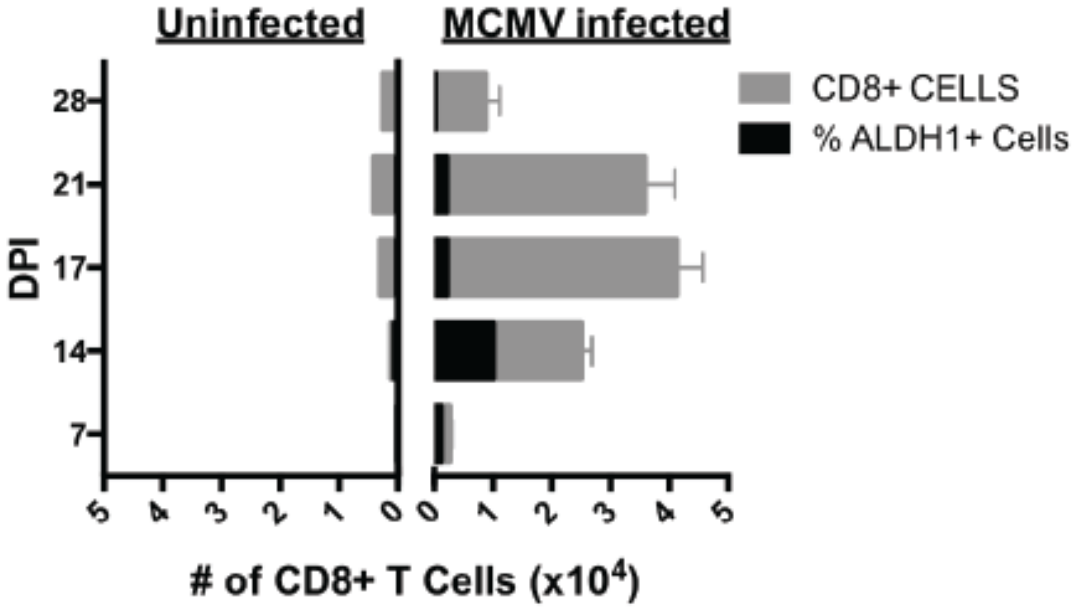
greater number and percentage of CD8+ T cells and macrophages produce RA after MCMV infection in the brain, though macrophages appear to be the dominant source at both early and late timepoints.

Figure 11 F4/80+ CD11b+ Microglia and CD8+ T cells show elevated ALDH1 activity in infected neonatal mice. Newborns were infected within 18 hours of birth with 200 PFU of MCMV-gB-Δ157 i.p. and brains were harvested on 7, 14, 17, 21, 28 DPI. Cells were isolated from infected and uninfected brains using a percoll gradient and washed with complete medium. Isolated F4/80+ and CD8+ T cells were then washed with ALDEFLUOR buffer for measurement of ALDH1 activity. ALDH1 Activity of F4/80+ CD11b+, (A) and CD8+ T cells (B) were analyzed using flow cytometer. ALDH1 activity was analyzed for ALDEFLUOR fluorescence in the presence and absence of the ALDH1 inhibitor DEAB. ALDEFLUOR fluorescence was measures by using the DEAB-treated samples as reference point for the corresponding non-DEAB-treated samples.

A. **F4/80+ CD11b+ cell Population - Brain**



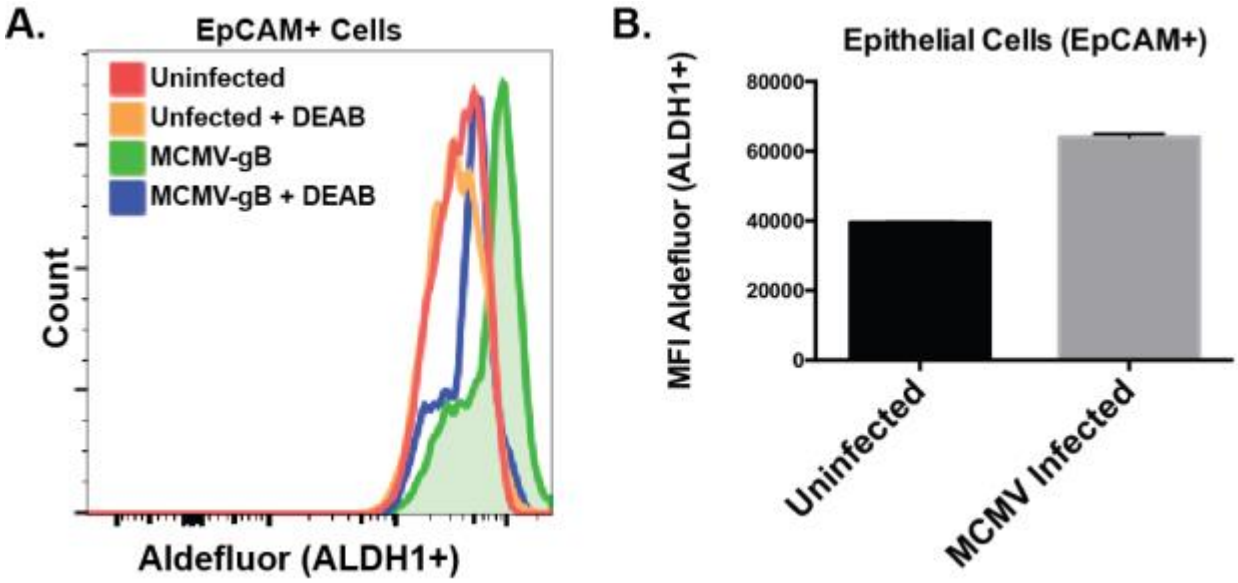
B. **CD8+ T cell Population - Brain**



Elevated RA production observed in epithelial cells of infected neonates

We also considered that structural cells in the brain are capable of producing RA. Indeed, epithelial cells in the choroid plexus are known to be dominant producers of RA in the developing CNS (Engberg et al. 2010, Mizee et al. 2007). To determine whether MCMV upregulates RA production in epithelial cells after infection, we isolated cells from MCMV-gB infected and control mice and stained them for EpCAM (a marker for epithelial cells). Interestingly, MCMV-gB-infected neonates showed a distinct increase in the percentage of EpCAM+ ALDH+ when compared to uninfected brains. (Fig. 3.5A-B).

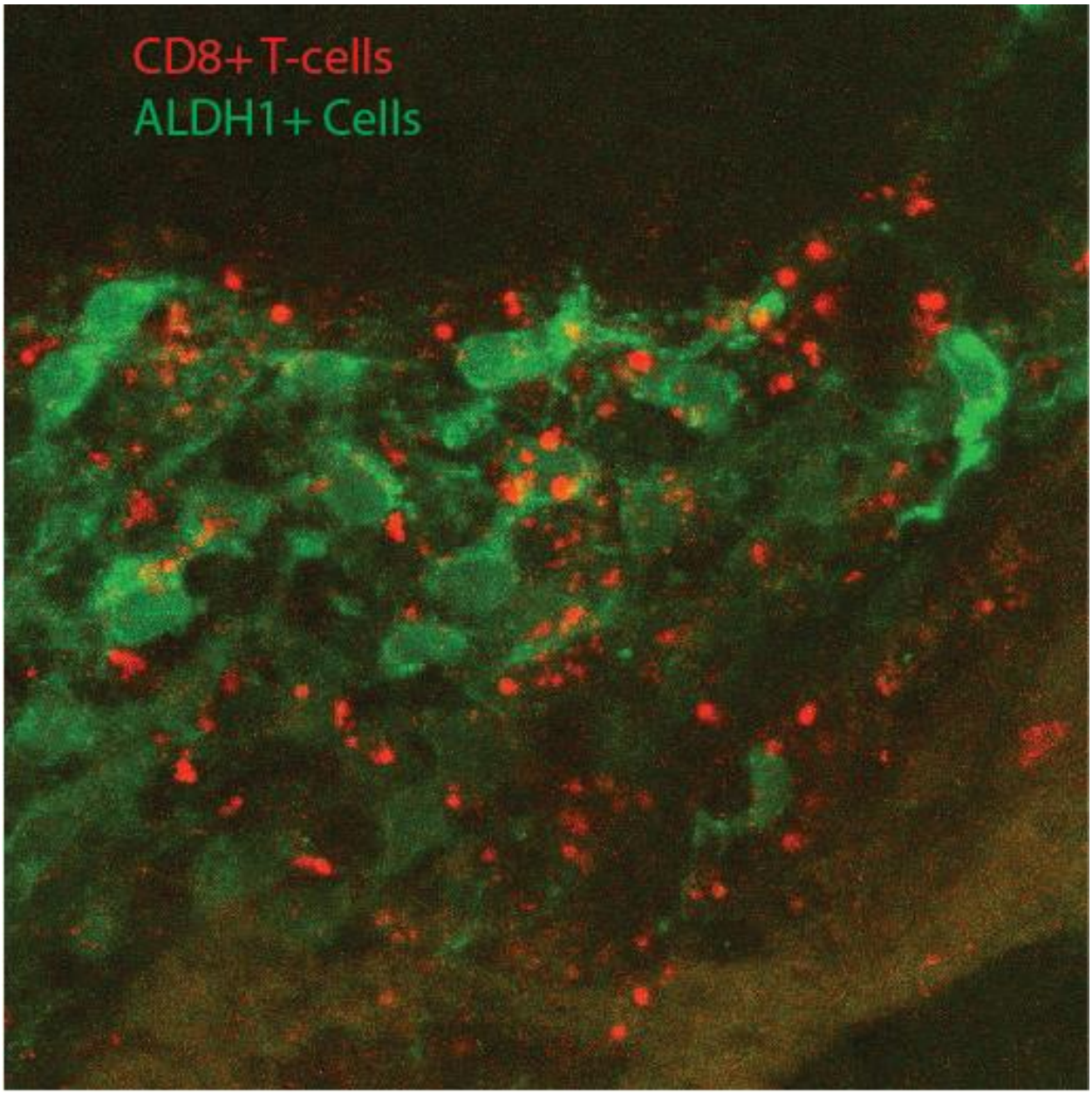
Figure 12 Elevated ALDH1 ezyme activity observed in epithelial cells of infected neonates. ALDEFLUOR substrate is oxidized by ALDH1 enzymes and the charged fluorescent product accumulates in cells with active ALDH1 enzymes. DEAB, a general inhibitor of ALDH1 enzymes, was used as a control for background fluorescence. Neonates were infected on PND 0 with MCMV-gB and brains were harvest 14 DPI. Mononuclear cells were isolated from brain tissue using percoll gradient and cells were treated with ALDEFLUOR assay and then stained for EpCAM+ cells. (A, B) Histograms from flow cytometric analysis of cells from infected and uninfected brains for populations of Aldh+ cells treated with the ALDEFLUOR reagent with and without DEAB. Quantification of ALDEFLUOR fluorescence in mononuclear cells from the brains of infected and uninfected pups. The data were made relative to DEAB control.


A.
EpCAM+ Cells
Uninfected
Unfected + DEAB
MCMV-gB
MCMV-gB + DEAB
Count
Aldefluor (ALDH1+)
B.
Epithelial Cells (EpCAM+)
MFI Aldefluor (ALDH1+)
80000
60000
40000
20000
0
Uninfected
MCMV Infected

CD8+ T cells migrate to regions of the brain containing elevated levels of RA.

Given that MCMV appears to increase RA production at sites of infection, we sought to determine whether CD8+ T cells localize to these RA-rich microenvironments. For these experiments, we generated a T cell reporter mouse by crossing CD2cre mice with a mouse that contain a floxed stop-cassette upstream of an RFP (TdTomato) in the Rosa26 locus. This mouse allows us to visualize CD8+ T cells because the cells express TdTomato from the time they are initially created in the thymus. We infected these mice at birth with MCMV-gfp and then examined whether CD8+ T cells (red) were co-localized in the region of the brain that was ALDH+ (green). Indeed, we found extensive overlap between CD8+ T cells and RA-producing cells in the choroid plexus on day 14 (Fig. 3.6). This data raises the possibility that RA production at sites of infection influence the function or maintenance of CD8+ T cells during MCMV infection in the brain.

Figure 13.6 Virus specific CD8+ T cells localize to regions of elevated RA production in infected neonates. Isolated naïve gB-specific dsRed CD8+ T-cells from 7 day old pups using negative selection MACS purification system. Then adoptively transferred the CD8+ T-cells into newborn pups and infected them with MCMV-gB within 18 hours. At 10 DPI, estimated peak of viral titer, we harvested the brains and stained the tissue using ALDEFLUOR buffer to show RA production. The brains were manually sectioned and then imaged using confocal microscopy. Confocal image of choroid plexus of the lateral ventricle in the brain. Imaging was performed on a Leica TCS SP2 confocal microscope, using a 20x objective lens. Image is made up of Z-stacks at a depth of 50 µm. The stacks were rotated 30 degrees and then merged to create this image. The image were acquired 25 minutes after the beginning of the ALDEFLUOR immersion reaction. ALDH1+ cells are visualized in green fluorescence.


CD8+ T-cells
ALDH1+ Cells

Discussion

In this study, we investigated how the chemical environment of the brain is altered after CMV infection. Tissue microenvironments define the properties and necessary functions of the tissues themselves, including cell trafficking capabilities, permeability, and growth. We were particularly interested in studying changes in RA because of its important role in neurodevelopment. Our interest in RA also stems from ongoing studies in the lab that point toward RA as a key factor regulating the expression of specific chemokine receptors in CMV-specific CD8+ T cells found in the brain, which is the focus of chapter 4. The experiments in this chapter are important because they describe how RA production in the CNS change during CMV infection and identify the key cell types contributing to these changes.

In this study, we used a measure of ALDH1 activity as a surrogate measure of RA synthesis. The ALDEFLUOR assay quantifies intracellular ALDH1 activity, not direct RA levels, providing confidence that the ALDH1 levels we observed are indicative of RA production. While MCMV-infected neonates demonstrated RA metabolism levels significantly greater than uninfected control neonates, both groups showed highly elevated levels of RA metabolism at 7 dpi followed by a decreasing trend over time. This trend may reflect the critical role of RA in neurodevelopment.

RA-regulated genes are associated with cell differentiation, cell proliferation, apoptosis, and embryonic development (180-184). In brain morphogenesis, RA has been identified as a key endogenous signaling molecule. RA gradients are found in the microenvironment of the developing CNS and specify the CNS anterior-posterior and dorsoventral axes (180). While both infected and uninfected mice showed upregulated

RA metabolism at early neonatal stages, it is notable that MCMV-infected neonate lymphocytes performed nearly double the RA metabolism of uninfected neonates at 7 DPI, which was the earliest and most developmentally active time point included in this experiment. The elevated levels of RA metabolism observed at early time points in MCMV-infected mice may be due to a combination of typically elevated RA levels for developmental functions, in conjunction with an altered brain microenvironment due to the immune response. It is also notable that previous studies have concluded that the availability of RA increases production of RA itself, creating a positive feedback loop (179).

In addition to its involvement in the patterning and morphogenesis of the developing brain, RA is important in the CNS because it plays a key role in neuronal differentiation. RA acts as a transcription factor for translation of several proteins involved in the differentiation of neural stem cells (NSCs) 109-111) and increases rates of NSC differentiation (178). ALDH1 expression has also been recently identified as a characteristic of non-neoplastic NSCs (185-189). NSCs have been identified as a preferentially targeted cell type in MCMV infection of the developing brain (46), which may lead to the structural damage and loss of function associated with congenital MCMV infection of the brain. In light of the data present in our report, it is also interesting to speculate that aberrant RA production may lead to a dysregulation in NSC development and differentiation.

Our flow cytometry analysis of RA-producing cells point toward the choroid plexus as a major source of RA production after MCMV infection. ALDH1 activity, represented by increased fluorescence, was observed in the choroid plexus during the

first 3 weeks of life in both infected and uninfected mice. While RA is metabolized in the developing brain in nearly all vertebrate animals, viral challenge appears to elevate the level of RA metabolism above the control baseline. The choroid plexus is a key structure in neurodevelopment, and previous literature supports its role as an important region of RA metabolism (194-198). The choroid plexus is a network of capillaries, ventricles, and ependymocytes, the glial cells responsible for production of cerebrospinal fluid (CSF). As a vasculature system, much of the choroid plexus is composed of epithelial cells that are noted to be a dominant cell type in RA synthesis (192, 193). There are two major regions of choroid plexus in the brain: one contained within the lateral and third ventricles and a second located ventral to the cerebellum within the fourth ventricle. Both regions have been observed to express high levels of ALDH1 during pre- and post-natal periods of brain morphogenesis (188).

Each of the four brain ventricles has a periventricular area, a thin layer of tissue lining the ventricle. The largest ventricles are the lateral ventricles, which serve as reservoirs for the CSF produced by the choroid plexus. CSF circulates throughout the brain, clearing cellular waste products and supplying nutrients to tissues throughout the brain. CSF is produced actively and passively. Through active processes, cells in the periventricular areas secrete salts into the lateral ventricles to create a salt gradient that causes diffusion of blood from blood vessels, across the blood-brain-barrier, and into the ventricles. As such, the blood-brain barrier (BBB) is critical in the maintenance of the microenvironment of the brain, especially the highly regulated solute concentrations (191).

Our results may shed light on the biology and function of the blood-brain barrier, since the endothelial cells of the BBB control the entry of cells and metabolites into the CNS. Studies using human tissue have hypothesized that RA plays a role in the development of the BBB because glial cells produce RA during development and are closely associated with vasculature development. RAR ß, the member of the human RAR family important for RA ligand-mediated cell signaling, is highly expressed in developing brain vasculature (190). In mice, RA signaling has been shown to be essential for development of the BBB (190). Thus, it is possible that changes in RA levels also influence the recruitment of immune cells after CMV infection. While this remains to be formally tested, our study provides crucial insights into how neonatal CMV infection in the CNS changes the chemical environment, which may pave the way for therapeutic strategies to reduce the burden of CMV-associated neurological disorders in humans.

CHAPTER 4 - ROLE OF CCL25/CCR9 DURING MCMV BRAIN INFECTIONS

Abstract

Congenital Cytomegalovirus (CMV) is a major public health problem. Previous work has shown that CD8+ T cells are the main cells involved in the clearance of CMV infection in the brains. However, the mechanism by which CD8+ T cells gain entry into the brain is presently unclear. To address this critical gap in our knowledge, we performed in-depth analysis of the CD8+T cell response. Our initial studies uncovered the following surprising features of the CD8+ T cell response in the brains of neonates: (i) CCR9 is selectively expressed on CD8+ T cells localized in the brain following MCMV infection; (ii) the ligand for CCR9 (CCL25) is expressed in the choroid plexus at the peak of the CD8+ T cell response; iii) the gut homing markers, α4β7 and CD103, are co-expressed on CCR9+ CD8+ T-cells in the brains of the neonates; (iv) CD8+ T cells in CCR9 KO mice fail to accumulate in the brain after neonatal CMV infection. These observations are significant because CCR9 has primarily been associated with T-cell homing to the gut epithelium and has not been shown to be expressed on lymphocytes in the brain.

Introduction

Previous studies have shown that CD8+ T cells play an integral role in the clearance of CMV in the brain, (18, 53) but how these cells are recruited and maintained in the CNS remains poorly understood. During viral infection, chemokines have been shown to play a pivotal role in the recruitment of CD8+ T cells to various tissues and organs. Chemokines are a family of small (8–14 kDa) proteins that induce migration and accumulation of cells to a certain location using a concentration gradient. Some chemokines are homeostatic in nature and are constitutively produced and secreted. Its structurally related molecule is the chemokine receptor, which is part of a subset of seven transmembrane-, G protein-coupled receptors (132). Chemokines are essential in the process of extravasation of leukocytes, which includes multiple steps involving interactions of adhesion molecules and the chemoattractant function of these proteins (133, 134). Both B- and T-cell maturation involve several chemokines at different stages of development (135-137). Chemokine agonists and receptors that have been observed to regulate T-cell maturation include CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), and the receptors CCR2 and CCR5 (132, 135).

Our lab has recently become interested in one chemokine in particular, known as TECK (thymus-expressed chemokine) or CCL25. Expression of CCL25 was initially detected in medullary dendritic cells of the thymus (136). However, later studies reported its expression in thymic epithelial cells in both the cortex and medulla (137,138). CCL25 is also upregulated in the thymus (139) and mediates chemotaxis and maturation of thymocytes via the C-C chemokine receptor type 9 (CCR9) (16, 17, 18, 19). CCR9 is expressed on the majority of immature $CD4^{+}CD8^{+}$ (double-

positive (DP)) thymocytes, is down-regulated during transition to the $CD4^+$ or $CD8^+$ (single-positive (SP)) stage, and is expressed again on a minor subset of $CD8^+$ lymph node T cells (20, 21). $CD69^+$ thymocytes demonstrate enhanced CCL25-induced migration compared with $CD69^-$ thymocytes (21, 22), and thymocyte migration in response to CCL25 is augmented by TCR signaling (21). Thus, the CCL25/CCR9 axis may be important for the development, homeostasis, and function of T lymphocytes.

An additional site of CCL25 production has been found in the epithelial layer of the small intestine (138, 140-142), implicating its involvement in the recruitment of CCR9+ lymphocytes to gut epithelium (144). Indeed, there is a large body of work that demonstrates a critical role for CCR9 in regulating inflammatory immune responses in the large intestinal mucosa. These studies have clearly shown that CCL25 production by surrounding epithelial cells lining the small intestinal villi result in the recruitment, proliferation, and maintenance of T cells in the gut.

In this study, we investigated the role of CCR9 during the CD8+ T cell response to murine cytomegalovirus (MCMV) in the brains of neonatal mice. Our studies suggest that CCR9 may play a key role in the recruitment of CD8+ T cells to infected cells in the brain. Collectively, these results suggest that, in addition to the gut, the CCL25/CCR9 axis is employed by the brain for the recruitment of lymphocytes after infection.

Materials/Methods

Animal Models

Pathogen-free pregnant C57BL/6NCr female mice were purchased from Charles River Laboratory. All experiments were conducted at Cornell University's College of Veterinary Medicine, in accordance with and under the approval of the Cornell Institutional Animal Care and Use Committee and the Cornell Center for Animal Resources and Education. All animals were housed under standard conditions.

MCMV Strains

A recombinant MCMV expressing green fluorescent protein (GFP), referred to as MCMV-GFP, was constructed by inserting the GFP gene into the MCMV genome downstream of the MCMV major immediate-early promoter (MIEP). Therefore, only infected cells undergoing active viral replication expressed GFP. A recombinant MCMV expressing MCMV-specific glycoprotein B (gB), referred to as MCMV-gB, was constructed by replacing the m157 gene open reading frame with the gB epitope coding sequence. Strains courtesy of Dr. Joel Baines (Cornell University).

Infection Method and Study Design

A 1.0 mL syringe (Becton Dickinson) fitted with a 23-gauge needle (Becton Dickinson) was used to inject newborn mice (6-18 hours postpartum) intraperitoneally (IP) with 200 PFU in 50 µl of MCMV-gB. Neonatal brains and spleens from MCMV-infected and uninfected control mice were harvested by standard sterile procedure at 7, 14, 17, 21,

and 28 DPI for flow cytometry to describe the CD8+ T cell response temporality and phenotype.

Isolation of Brain Mononuclear Cells

Mice were briefly sacrificed by isoflurane inhalation to preserve intact circulation, then perfused with 30 mL ice-cold PBS. Brains were harvested in 13 mL of RPMI 1640 supplemented with 10% FBS (10% RPMI) in a 15 mL conical tube. Mechanical processing was completed through 70 µm cell strainers (Fisher Scientific), assisted by the plunger of a 3 mL syringe (Becton Dickinson). The suspension was centrifuged at 1500 RPM for 5 minutes at 4ºC. The tissue was homogenized in a 30% Percoll suspension, overlaid on a 70% Percoll solution, and centrifuged at 2600 RPM for 25 minutes at 4ºC. Cells at the solution interface layer were collected, washed in 10% RPMI, and re-suspended in ice-cold FACS buffer.

Isolation of Splenocytes

Neonatal spleens were harvested using the previously described method at the same time-points of interest, then mechanically processed using 40 µm cell strainers. Dissociated splenic tissue was centrifuged for 5 minutes at 1500 RPM and re-suspended in 3 mL of ice-cold FACS buffer.

Flow Cytometry

Isolated brain mononuclear cells and splenocytes were stained with anti-mouse cell surface markers and the ALDEFLUOR assay at 4ºC for 30 minutes. 300 µL of each

tissue sample was transferred onto a 96-well round bottom plate (Corning), then centrifuged at 1500 RPM for 5 minutes at 4ºC. Supernatant was discarded and pellets were re-suspended in 200 µL ice-cold FACS buffer. 100 µl of each MCMV-infected sample and each control sample were transferred to another well for a fluorescence control reaction to? the ALDEFLUOR buffer using the ALDH1-specific inhibitor diethylaminobenzaldehyde (DEAB), referred to as DEAB controls. ALDEFLUOR-specific flow cytometry gating strategy is shown in Figure 2. Samples were centrifuged at 1500 RPM for 5 minutes at 4ºC. Supernatant was discarded, and 100 µL of diluted Fc block was what? (Fc block stock from manufacturer diluted in FACS buffer at a 1:150 ratio). Samples were incubated on ice for 20 minutes, centrifuged at 1500 RPM for 5 minutes at 4ºC, and supernatant was discarded. The antibody panel consisted of CD8-e450, CD11b-PE, NK1.1-PeCy7, CD19-APC, CD3e-APC-e780, CD11c-PerCPe 710, and F4/80-PEe610 (all antibodies manufactured by EBioscience, Inc.) A cocktail was prepared using 50 µL ice-cold FACS buffer per sample. ALDEFLUOR reagents were prepared according to manufacturer protocol. ALDEFLUOR reagent was activated by diluting 5 µL reagent with 1 mL ALDEFLUOR buffer, provided by the manufacturer. 50 µL activated ALDEFLUOR reagent and 50 µl antibody cocktail was added to each infected sample, uninfected sample, and DEAB controls, with a total volume of 100 µL in each well. Samples were stained for 30 minutes at 4ºC in the dark. Three washes were performed with FACS buffer, using volumes of 150 µL, 200 µL, and 150 µl, respectively. Cells were resuspended in 50 µL of ice-cold FACS buffer, and data was immediately collected using an LSRII flow cytometer. Lymphocytes were gated using forward and side scatter protocols. FlowJo software (TreeStar) was used for analysis.

Repeat data collection and analysis was performed on 7, 14, 17, 21, and 28 DPI.

Organotypic Section Imaging Preparation

MCMV-GFP infected neonate brains were harvested intact at 7, 14, 17, 21, 28, and 60 DPI. A cryotome blade (VWR International) was used to manually dissect brains into approximately 100 µm coronal sections containing the choroid plexus of the lateral ventricle and third ventricle. Sections were placed in a 35 mm optical quality glass bottom cell culture dish (MatTek). Sections were immersed in a 1:200 dilution of ALDEFLUOR reagent at room temperature for 15 minutes. 300 µL of 300 nM DAPI solution (prepared courtesy of the Schaffer-Nishimura Lab) was added to section immersions.

Confocal Imaging Methodology

Imaging was performed on a Leica TCS SP2 confocal microscope, using a 20x or 40x objective lens. Z-stacks with a minimum depth of 50 µm were acquired 25 minutes after the beginning of the ALDEFLUOR immersion reaction. The same stereotactic position within the brain was estimated by visually confirming locations of the lateral and third ventricles along the central dorsoventral axis of the coronal sections. Confocal imaging acquisition used a 200 Hz scanning speed, line average of 2, and frame average of 1. Objectives used included HCX PL APO 40x 0.85 Dry and HCX PL APO CS 20x 0.7 Dry. PMTs used were Leica/DAPI (emission at 455), Leica/EGFP (emission at 507), and Leica/DSRED (emission at 582). Overall laser power was 1%; or: UV used 22% of 405 nm laser line power; EGFP used 13% of 488 nm laser line power; and DSRED used

45% of 561 nm laser line power. Images were deconvolved using the Volocity software (PerkinElmer).

Statistical Analysis

Statistics were performed using Graph Pad Prism (GraphPad Software). For all analyses, a significance level of 0.05 was accepted.

Results

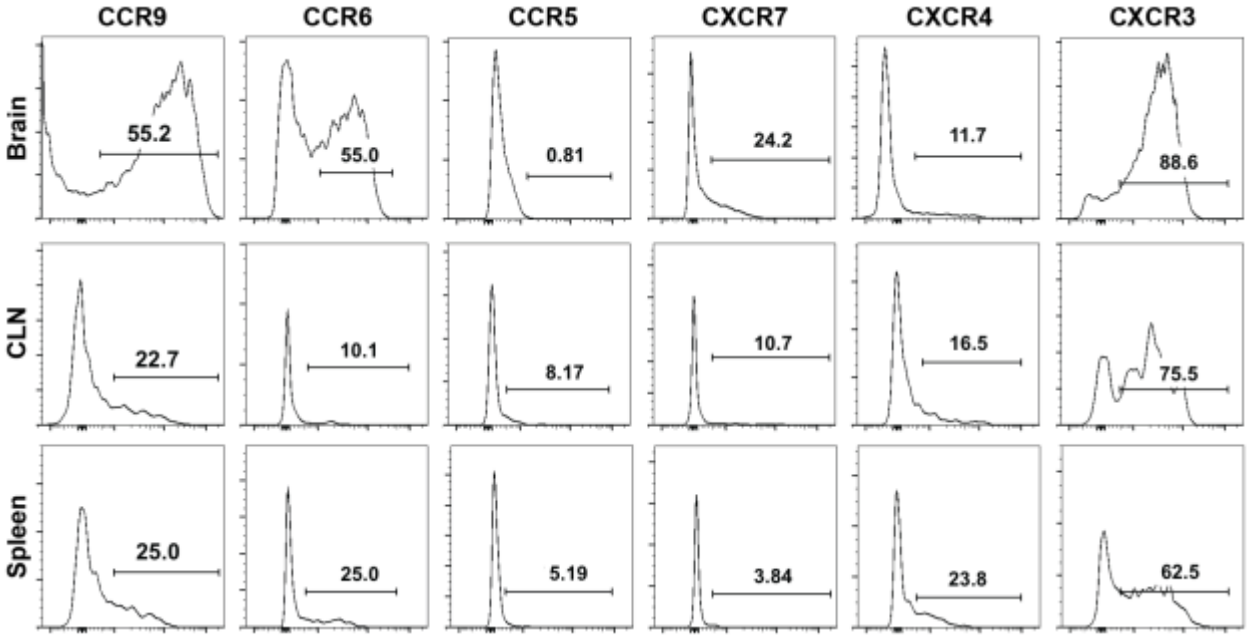
CCR9 is highly expressed on CMV-specific CD8+ T-cells in the brains of neonatal mice

In this study, we investigated the mechanism involved in lymphocyte homing to MCMV infection in the brain. To start, we first determined the kinetics of the CD8+ T cell infiltration into the brain. For these studies, we employed a recombinant strain of MCMV (denoted MCMV-gB-ΔM157), where the M157 viral epitope was replaced with the gB peptide from herpes simplex virus. By infecting newborn pups with MCMV-gB-ΔM157, the CD8+ T cell response becomes overwhelmingly dominant towards the gB peptide, allowing us to track virus-specific CD8+ T cells responding to the infection using tetramers. Following infection, we harvested the brains on days 7, 14, 17, 21, and 28 DPI and enumerated the numbers of gB-specific CD8+ T cells by flow cytometry. As shown in Fig. X, we found that the antigen-specific CD8+ T cells peaked at 17 DPI.

In an effort to identify the mechanism(s) of CD8+ T cell homing to the brain, we evaluated the expression of various chemokine receptors (CCR5, CXCR7, CXCR4, CXCR3 and CCR9) known to play a role in T cell recruitment to sites of infection. On day 17 post-infection (the peak of the CD8+ T cell response), we harvested the brains, spleen, and cervical lymph nodes (CLN) and measured the expression of various chemokine receptors by flow cytometry. Unexpectedly, we found that chemokine receptor 9 (CCR9) was preferentially expressed on CD8+ T cells in the brain compared to the spleen and cervical lymph nodes (Fig. 4.1). It is also important to note that CCR6 and CXCR3 were also highly expressed on the CD8+ T cells found in the infected brains of neonates. Previous studies in mouse models of multiple sclerosis (EAE) found that CCR6 was expressed on autoreactive IL-17-producing T helper cells (T_H-17 cells)

entering the CNS through the choroid plexus/blood-brain barrier axis. As for CXCR3, earlier work has highlighted a role for this receptor in the recruitment of CD8+ T cells to the liver during MCMV infection (199). Together, these results implicated a role for CCR6, CXCR3, and CCR9 during the CD8+ T cell response to MCMV infection in the brains of neonates.

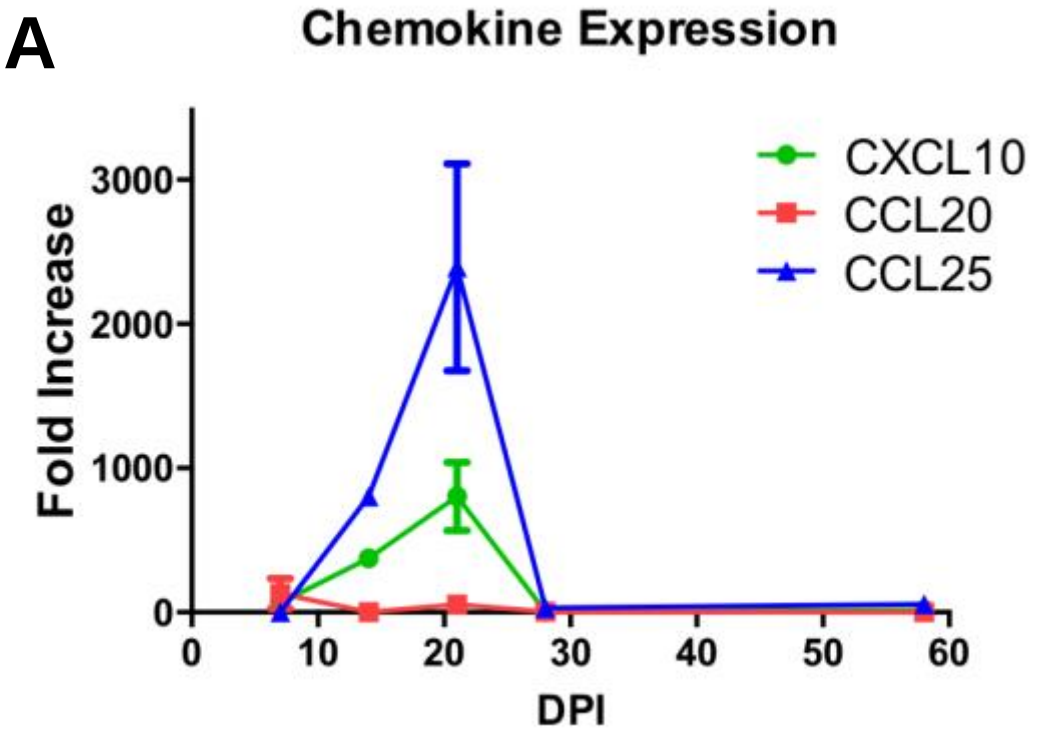
Figure 14.1 CCR9 exhibits one of the most divergent patterns of expression, with much higher levels expressed on CD8+ T cells in the brain compared to the spleen. Representative flow cytometry contour plots of gB-tet+ CD8+ T-cells in the brain, lymph node, and spleen stained for various chemokine receptors 17DPI.

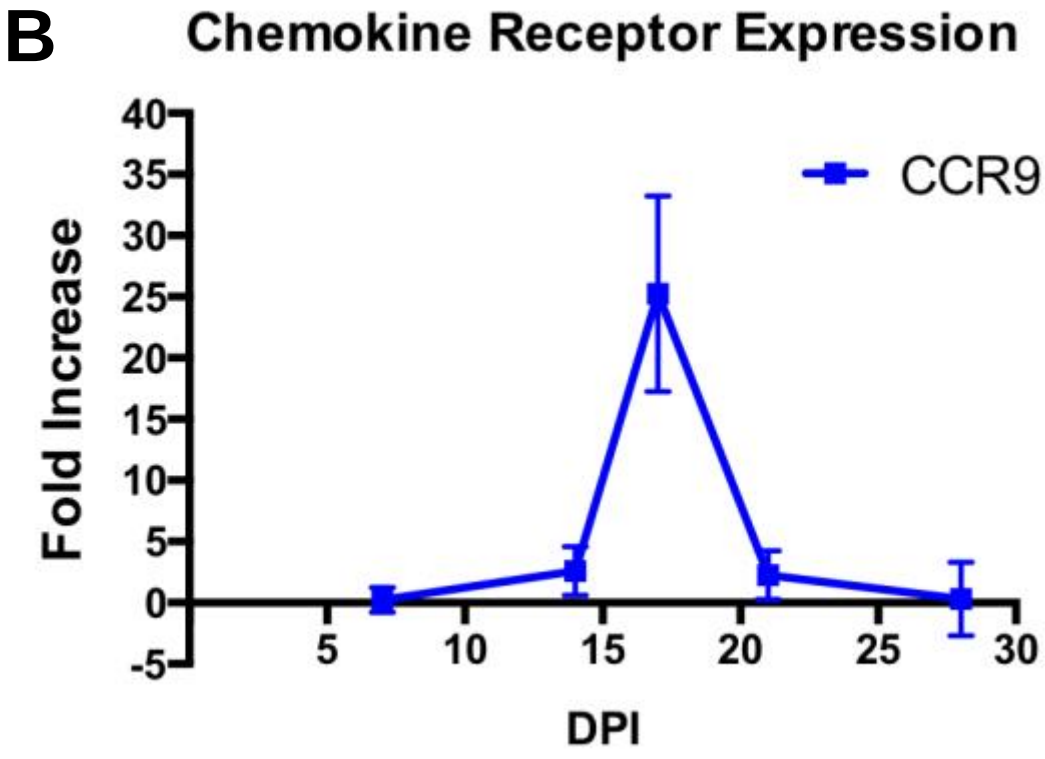

CCR9
CCR6
CCR5
CXCR7
CXCR4
CXCR3
Brain
CLN
Spleen
55.2
55.0
0.81
24.2
11.7
88.6
22.7
10.1
8.17
10.7
16.5
75.5
25.0
25.0
5.19
3.84
23.8
62.5

CCL25 is expressed in the brain after MCMV infection.

Given the expression patterns of CCR6, CXCR3, and CCR9 in CD8+ T cells after MCMV infection, we next investigated whether the ligands for these receptors were also expressed in the infected brains of neonates. Using qPCR, we examined the kinetics of their expression in the brains following infection with MCMV. While we did not observe an increase in the expression of CXCL10 (CXCR3 ligand), we did observe increased amounts of both CCL20 (CCR6 ligand) and CCL25 (CCR9 ligand) in the brains of the infected neonates. The patterns of expression of CCL20 and CCL25 are consistent with the influx of CD8+ T cells in the brain at 17-21 dpi (Fig. 4.2A, B). These data further highlight the potential importance of CCR6 and CCR9 expression in CD8+ T cells in the brains of the infected pups.

Figure 15 Differential levels of CCR9 and CCL25 expression is observed in infected brains. Quantification and kinetic of CCL25 mRNA expression in the brains of infected pups relative to uninfected pups. Neonates were infected on PND 0 with MCMV-gB and brains were harvest on 7, 14, 21, 28, and 60 DPI. For relative expression the CCL25 mRNA analyzed as a reference for fold-change in expression. Data are represented as fold-change for 8 neonates per time point in 2 independent experiments. Each experiment was performed in duplicate. Neonates were infected on PND 0 with MCMV-gB and brains were harvest on 7, 14, 17, 21, and 28 DPI. For relative expression the CCR9 mRNA analyzed as a reference for fold-change in expression. Data are represented as fold-change for 8 neonates in 2 independent experiments. Each experiment was performed in duplicate.

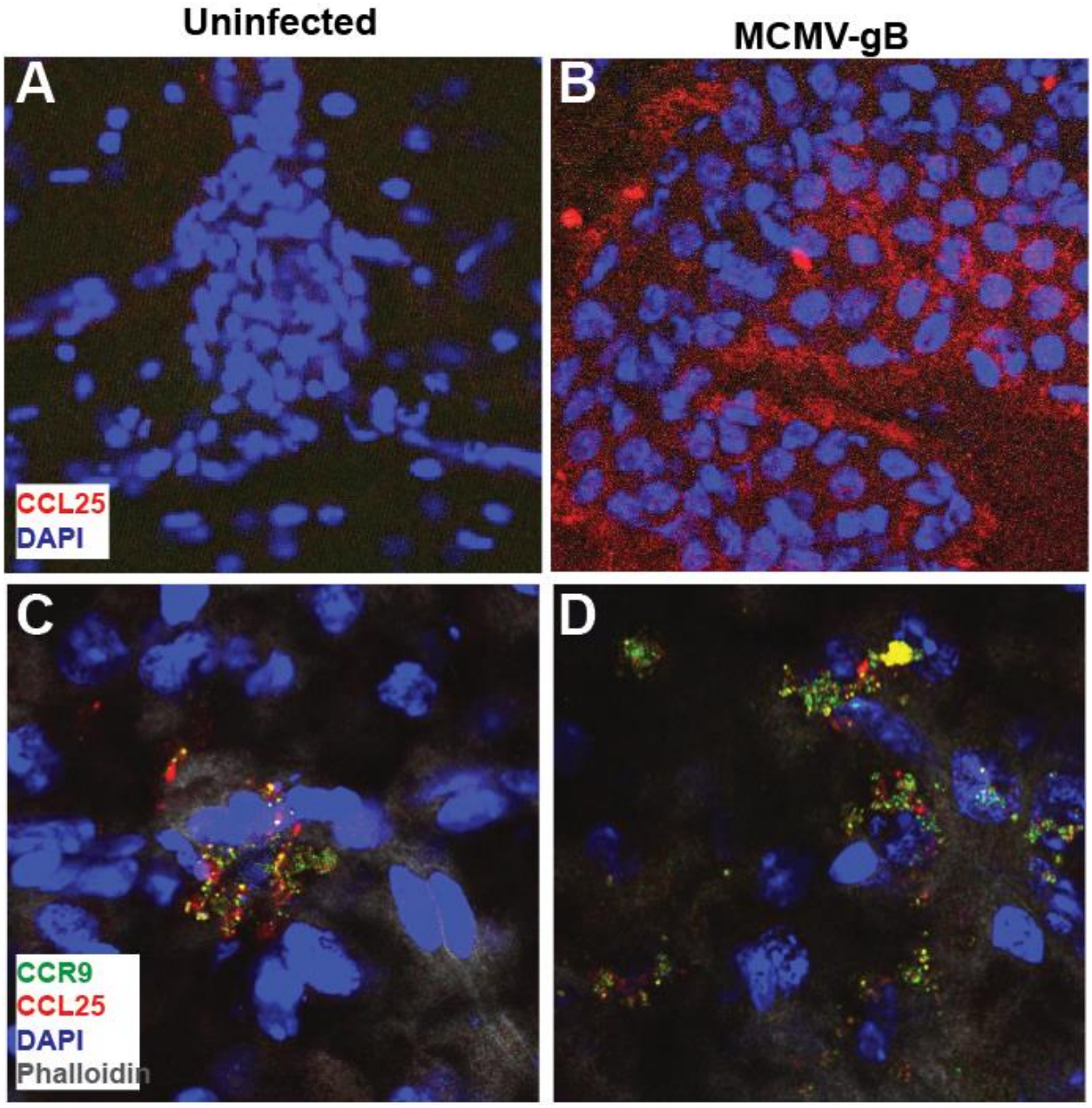

A
Chemokine Expression
Fold Increase
3000
2000
1000
0
0
10
20
30
40
50
60
DPI
CXCL10
CCL20
CCL25


B
Chemokine Receptor Expression
Fold Increase
40
35
30
25
20
15
10
5
0
-5
5
10
15
20
25
30
DPI
CCR9

Upregulation of CCL25 is linked to patterns of viral replication.

Given that CCL25 expression is more abundant than CCL20 and CCR9+ CD8+ T cells have never been detected in the brain after viral infection, we decided to focus on the role of CCR9 in the MCMV-infected brains of neonatal mice. A key objective was to determine the factors driving expression of CCL25 after infection. To this end, we examined the association of CCL25 expression in relation to viral replication. We used a recombinant MCMV that expresses GFP for these studies, allowing us to visualize regions of infection in the brain. Using single photon microscopy, we observed active replication of MCMV-GFP in the choroid plexus as early as 4 DPI in these pups (Fig. 3.1). At the peak (~10-14 dpi) of the viral infection, we also observed infection in the cortex, the hippocampus, and the cerebellum of the pups (Fig. 3.1). Then, we used immunohistochemistry (IHC) to determine whether production of CCL25 and expression of CCR9 was localized to the same regions. This data showed expression of CCL25 in ependymal cells in the lateral ventricle (Fig. 4.3C, D). We also observed expression of CCL25 in the choroid plexus, which is a major site of viral replication (Fig. 4.3A, B). These results suggest that viral replication may be driving production of CCL25 in the choroid plexus.

Figure 16 CCL25 and CCR9 expression observed in the choroid plexus and ependymal cells of infected neonates. Confocal image showing staining for CCL25 in MCMV infected brains of neonates at the 10 DPI. Dual color immunofluorescence shows colocalized staining with CCL25 antibody (Red) and Nuclei were counterstained with DAPI blue. C57BL/6 neonates (PND 0) were i.p. infected with MCMV-gB (200 PFU). CCL25 were mainly observed in the choroid plexus. Confocal image showing colocalized expression of CCR9 and CCL25 in MCMV infected brains of neonates at the 10 DPI. Dual color immunofluorescence show colocalized staining with CCL25 antibody (Red) and CCR9 antibody (green) Nuclei were counterstained with DAPI blue and f-actin stained with phalloidin. C57BL/6 neonates (PND 0) were i.p. infected with MCMV-gB (200 PFU). CCL25 and CCR9 were mainly observed in the choroid plexus.


Uninfected
MCMV-gB
A
B
CCL25
DAPI
C
D
CCR9
CCL25
DAPI
Phalloidin

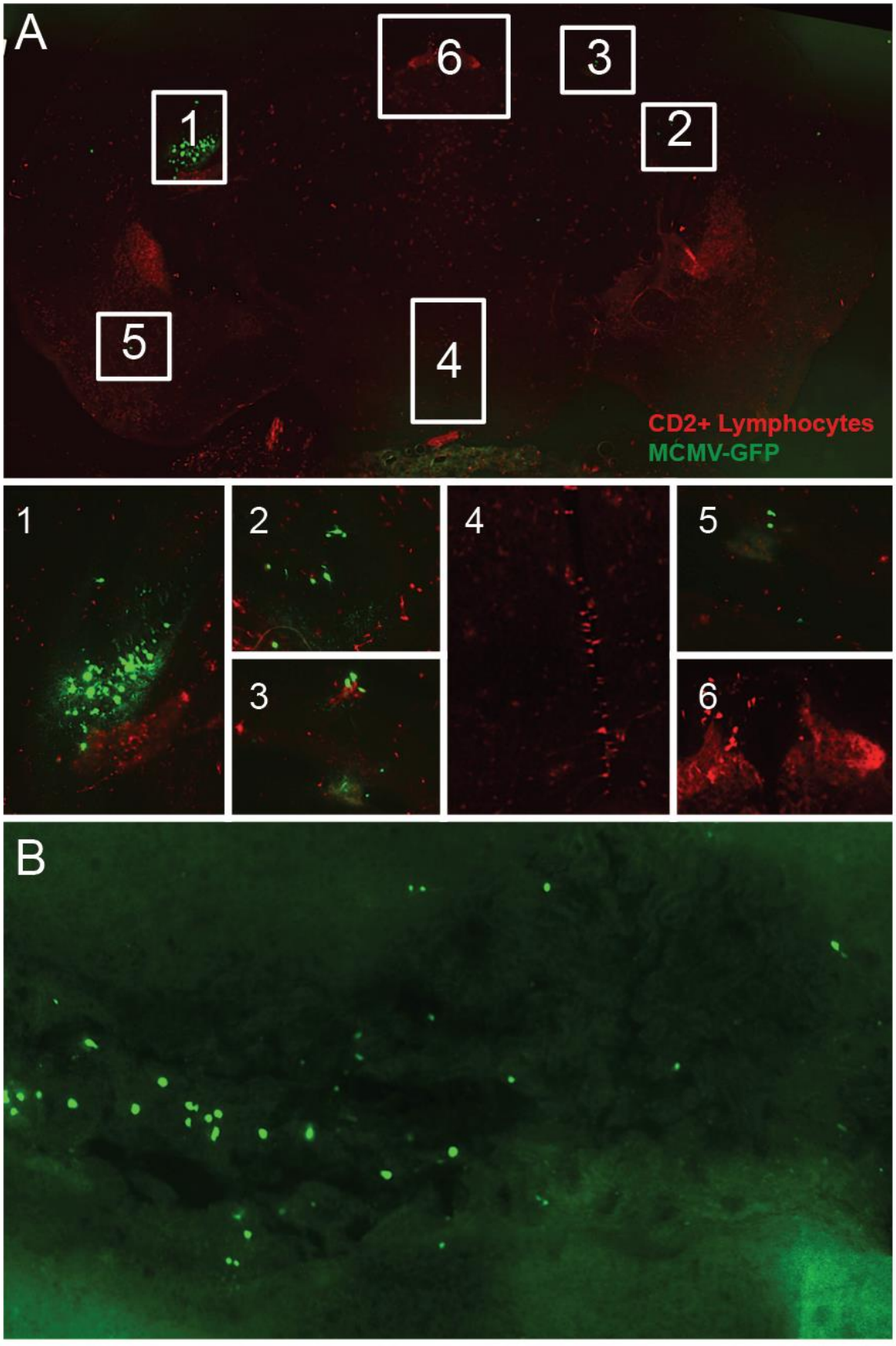
CD8 T-cells localize to sites of CCL25 expression.

To determine whether CD8+ T cells localize to sites of CCL25 expression, we mapped the regions where lymphocytes (CD2+ cells) were localized. For these experiments, we used mice in which CD2+ cells fluoresce red and infected their pups with MCMV-GFP. In this way, we could not only visualize regions of MCMV infection in the brain, we could also investigate the proximity of the CD2+ lymphocytes to infection. At various time points (10, 14, and 17 DPI), we harvested and processed the tissue for imaging using single photon microscopy. We observed CD2+ lymphocytes clustering near foci of MCMV-GFP infection in the hippocampus and the choroid plexus of these infected pups (Fig 4.4A 1-6). Lymphocytes could also be observed clustered near the lateral ventricles in close proximity to the choroid plexus (Fig 4.4 A6).

While the above experiment provides key insight into where endogenous lymphocytes are located in the MCMV-infected brain under physiological conditions, one limitation to this approach is that it is not specific to CD8+ T cells. Therefore, we employed an adoptive transfer experiment to determine whether CD8+ T cells specifically localize to sites of CCL25 expression. In this experiment, we isolated CD8+ T cells from gB-I GFP-transgenic mice, whereby all CD8+ T cells have rearranged a TCR specific for the gB-peptide and fluoresce green. We then adoptively transferred these cells into newborn mice prior to infection with MCMV-gB and harvested and processed the brain at various days after infection for imaging using single photon microscopy. As expected, we visualized the presence of the gB-I GFP CD8+ T cells in

in the choroid plexus of MCMV-infected mice at the peak of CCL25 expression (Fig 4.4B). These results demonstrate that CD8+ T cells do indeed localize to regions of CCL25 expression in the brains of infected neonates.

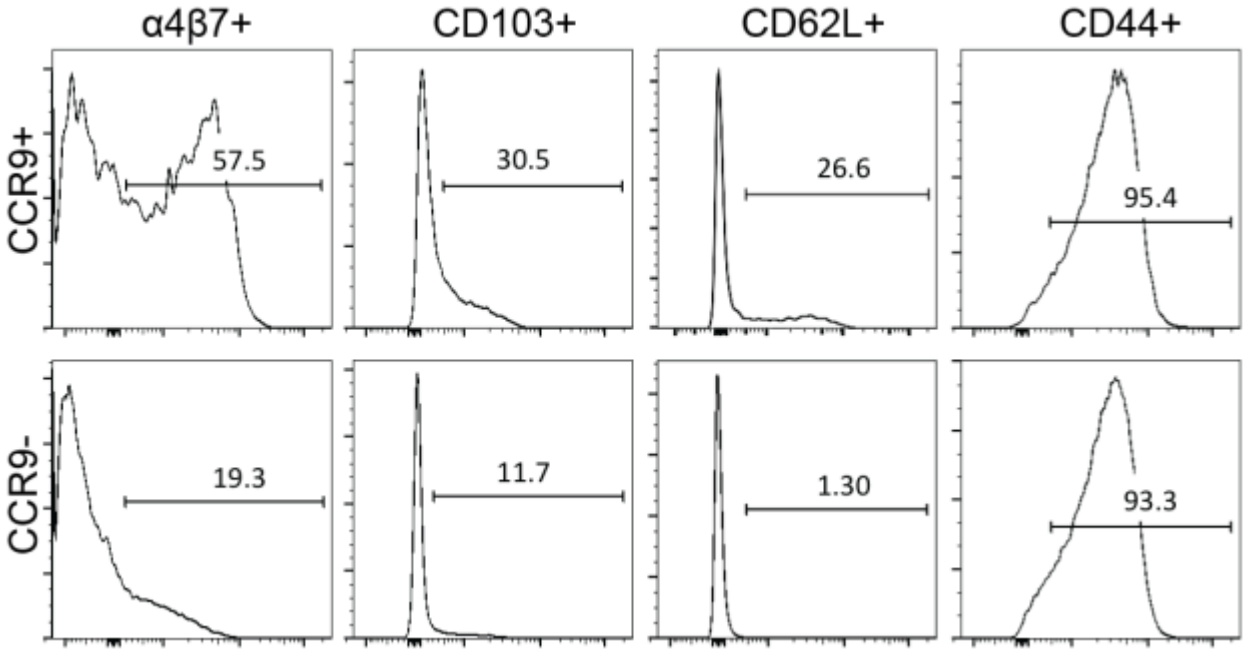
Figure 17 dsRed CD2+ Lymphocytes observed targeting MCMV-GFP infected cells in infected neonate brains. Confocal image of CD2+ lymphocytes (tdTomato) targeting murine cytomegalovirus (MCMV-GFP) infected cells in the brain. Neonatal mice (PND 0) from a CD2-cre × Ai9 cross mouse line were i.p. infected with 200 PFU MCMV-GFP. The CD2-cre × Ai9 line specifically labels cells from a CD2+ lymphocytes with a fluorescent tdTomato reporter. (A1-A6) Active viral infection and replication was determined by expression of EGFP. At the peak of infection (10 DPI), pups were euthanized brains were harvested and treated with 4% PFA. EGFP positive cells are mainly observed in the Hippocampal and cerebellar regions of the brain. (A1-A6) Infection was also observed in the hippocampus (A1-A3), Amygdaloidal region (A5) Confocal image GFP+ CD8+ T cells infiltrating of the choroid plexus in MCMV-gB infected mice. (B) CD8+ T cells were isolated from gB-specific GFP reporter mice. The GFP+ CD8+ T cells were purified from Neonatal (PND 7) splenocytes using negative selection MACS purification system and adoptively transferred i.p. at 200 GFP+ CD8+ cells per neonate (PND 0). Recipient pups are then infected. (PND 1) and at 14 DPI brains were harvested from infected animals.


A
6
3
1
2
5
4
CD2+ Lymphocytes
MCMV-GFP
1
2
4
5
3
6
B

CCR9+ CD8+ T-cells upregulate gut-associated adhesion molecules.

To determine whether other CCR9 or gut-associated molecules could be involved in homing of CD8+ T cells to the brain, we studied adhesion molecule/integrin expression (CD44, CD62L, α4β7, and CD103). Using flow cytometry, we showed that CCR9+ CD8+ T cells exhibit increased surface expression of β7 integrins (α4β7 and CD103) at the peak (17 DPI) of the response (Fig. 4.5). These results suggest that the CCR9/CCL25 axis is not only important for CD8+ T cell homing to the brain, but may also play a role in regulating the development and maintenance of tissue-resident memory T-cells.

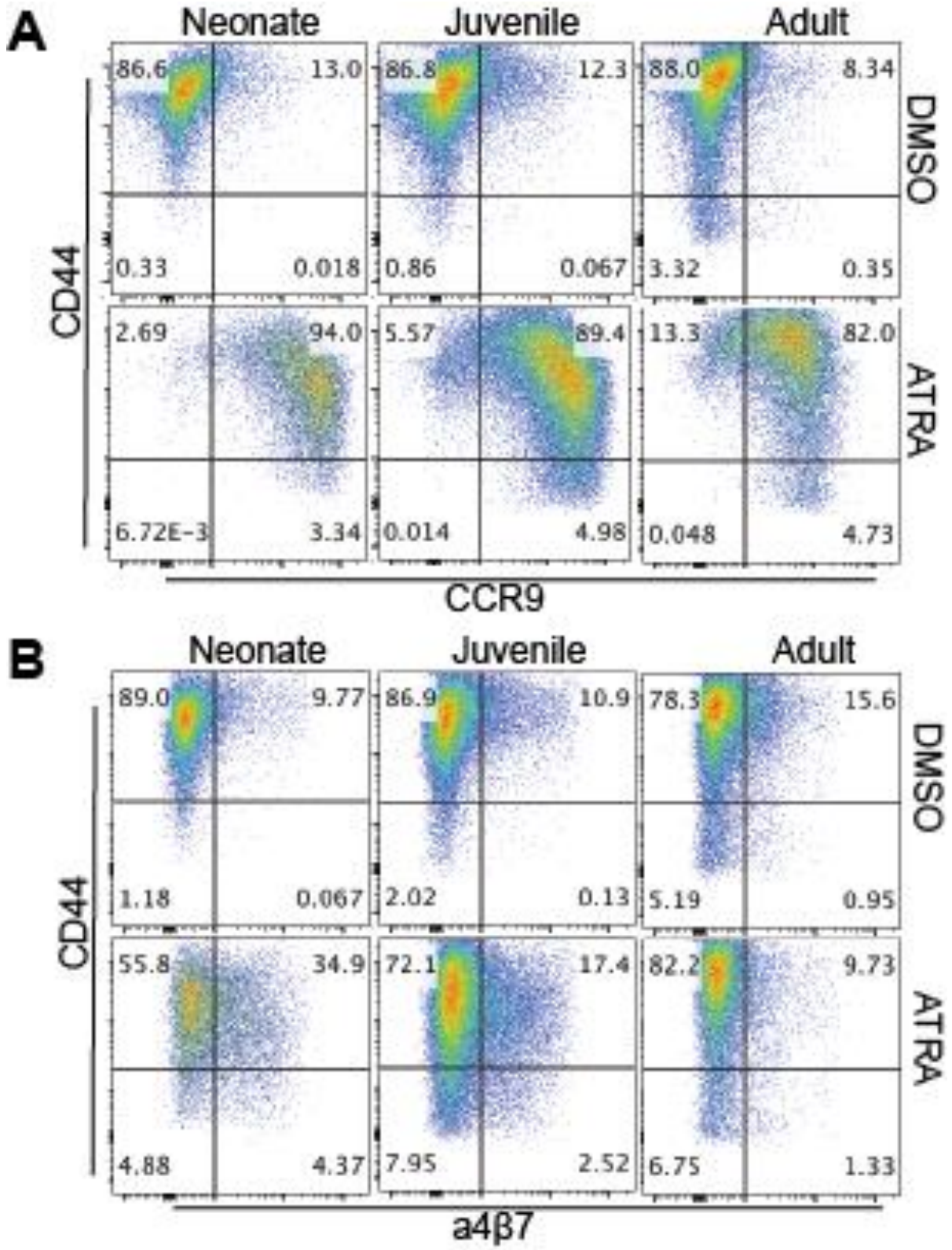
Figure 18 CCR9+ CD8+ T Cells found in infected brains of neonates show elevated expression of α4 integrins and adhesion molecules. Neonates were infected on PND 0 with MCMV-gB and brains were harvested 17 DPI. Mononuclear cells were isolated from brain tissue using percoll gradient and then stained for flow cytometric analysis. Representative flow cytometry contour plots of gB-tet+ CCR9+ CD8+ T-cells in the brain stained for a4b7, CD103, CD62L, and CD44 at 17DPI.


α4β7+
CD103+
CD62L+
CD44+
CCR9+
CCR9-
57.5
30.5
26.6
95.4
19.3
11.7
1.30
93.3

RA upregulates CCR9 and is required for efficient homing to brain during infection

In the previous chapter, we found that RA is elevated in the brain after neonatal MCMV infection. Since previous studies have shown that RA stimulation can lead to increased expression of CCR9 and integrin a4b7 on adult CD8+, we decided to test whether RA also regulates the expression of CCR9 on neonatal CD8+ T cells. To do this, we cultured neonatal and adult CD8+ T cells in vitro with various amounts of ATRA or DMSO and measured the expression of CCR9 and a4b7 by flow cytometry at various times post-stimulation (Fig 4.6A, B). Interestingly, this data indicated that neonatal CD8+ T cells are hypersensitive to RA, as evidenced by the fact that they upregulate more gut-associated molecules than adults in response to equivalent amounts of RA (Fig 4.6A, B).

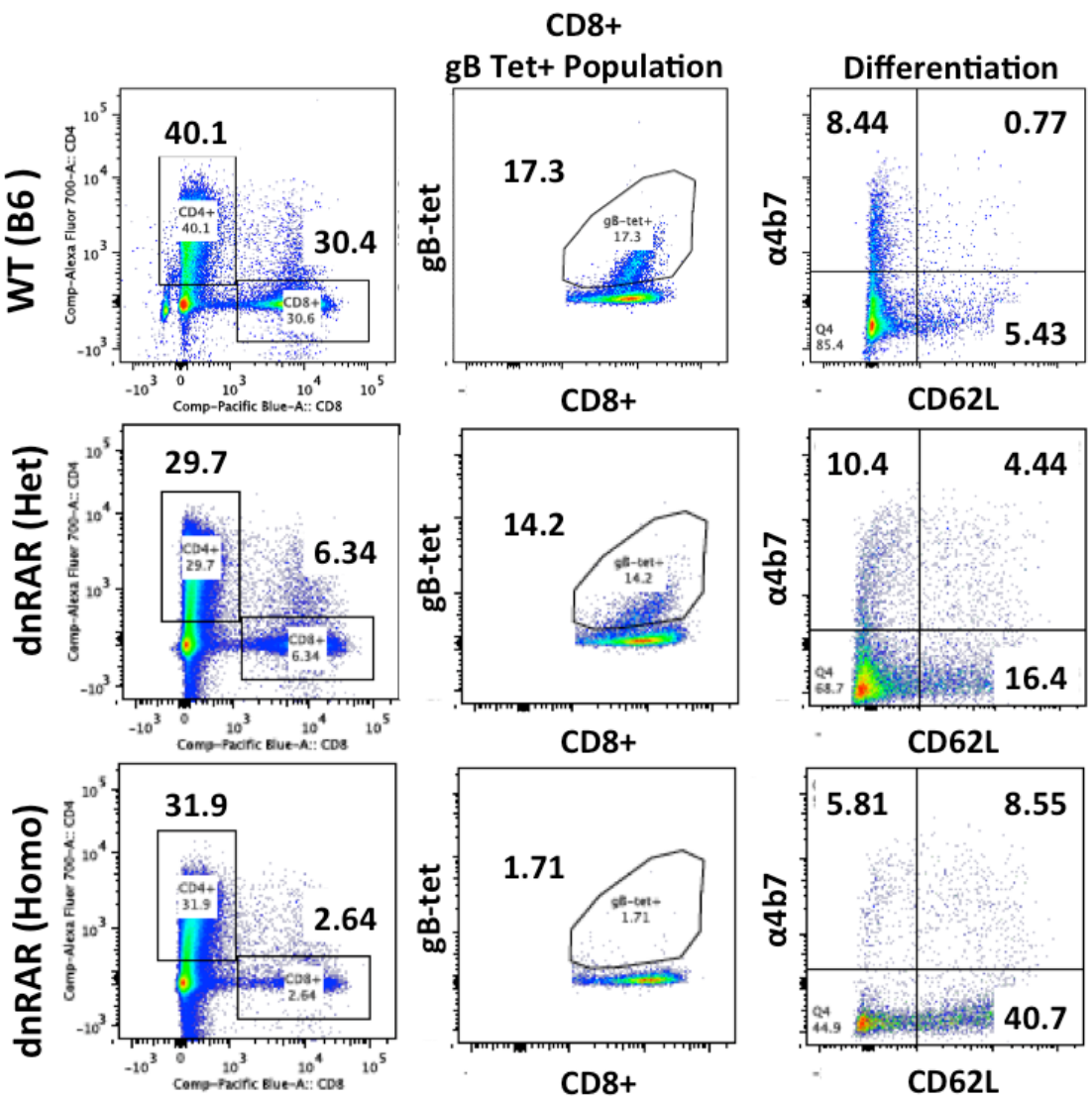
Figure 19 Neonatal CD8+ T cells in the presence of ATRA enhances expression of CCR9 and a4β7. Naïve WT (B6) CD8+ T cells were isolated from the spleens of a neonate (PN 7), juvenile (PN 14) and adult (PN 60) mice. The CD8+ T cells were treated either with or without ATRA for 3 days. Cultures were stimulated with 100 nmol/L ATRA, or DMSO alone. Murine naïve CD8 T cells were incubated with CD3/CD28 coated plates for 3 days. CCR9 and α4β7 expression were measured using LSRII flow cytometer.) Representative FACS plots are shown with percentage expression.


A
Neonate
Juvenile
Adult
86.6
13.0
86.8
12.3
88.0
8.34
DMSO
0.33
0.018
0.86
0.067
3.32
0.35
CD44
2.69
94.0
5.57
89.4
13.3
82.0
ATRA
6.72E-3
3.34
0.014
4.98
0.048
4.73
CCR9
B
Neonate
Juvenile
Adult
89.0
9.77
86.9
10.9
78.3
15.6
DMSO
1.18
0.067
2.02
0.13
5.19
0.95
CD44
55.8
34.9
72.1
17.4
82.2
9.73
ATRA
4.88
4.37
7.95
2.52
6.75
1.33
a4β7

RA is required for vigorous CD8+ T cell response

A key question is whether RA plays a role in CD8+ T cells trafficking to the brains of MCMV-infected mice. RA regulates cell differentiation/proliferation and gene transcription via binding to the RA receptor (RAR)/retinoid X receptor (RXR) heterodimer. Unfortunately, knocking out the receptor does not result in a useful mouse model because KO mice will die in utero. Thus, we obtained CD4cre x dnRAR mice that express a dominant negative (dnRARα) transgene under control of the CD4+ T cell promoter, which only blocks RA signaling in T cells. We then infected WT, het, and homozygote KO mice with MCMV at birth and compared the number and phenotype of tetramer positive CD8+ T cells in the brain at the peak (17 DPI). While large numbers of CD8+ T cells were observed in the brains of WT mice, we found relatively few CD8+ T cells in the homozygote KO mice. Moreover, the small numbers of CD8+ T cells present in the homozygote KO mice were significantly less differentiated. Collectively, this data indicates that RA signaling is important for CD8+ T cells to respond vigorously to CMV in the brain (Fig 4.7).

Figure 20 Disruption of RA signaling in CD8+ T cells impairs differentiation and virus specific immune response. WT, and dnRAR (heterozygotes or homozygotes) newborns were infected with 200 PFU of MCMV-gB-ΔM157 within 18 hours of birth. At 17 DPI, neonatal brains were harvested and processed for analysis with flow cytometer.


CD8+
gB Tet+ Population
Differentiation
WT (B6)
dnRAR (Het)
dnRAR (Homo)
40.1
30.4
17.3
8.44
0.77
5.43
29.7
6.34
14.2
10.4
4.44
16.4
31.9
2.64
1.71
5.81
8.55
40.7
gB-tet
α4b7
CD8+
CD62L
Comp-Alexa Fluor 700-A:: CD4
Comp-Pacific Blue-A:: CD8

Discussion

CD8+ T cells have been identified by numerous studies to be the dominant cell type responding to neurotropic MCMV infection. However, the exact trafficking methods of CD8+ T cells into the brain during MCMV infection are not known. In this study, we investigated the expression of several chemokine receptors primarily implicated in lymphocyte homing. Preferential expression of CCR9, a major lymphocyte gut-homing receptor, was observed. The ligand for CCR9 (CCL25) is thought to be selectively expressed in the thymus and small intestine and play an important role in the recruitment of T cells to gut mucosa. However, we made the novel observation that CCL25 is also expressed in the choroid plexus after infection with MCMV. Additionally, we found that CCR9+ CD8+ T cells are co-localized to the region of CCL25 expression, and CCR9-deficient CD8+ T cells are impaired at homing to the brain after infection. These data implicate the CCR9/CCL25 axis as a major regulator of the CD8+ T cell response to MCMV in the brain during early stages of development.

A key question is; which molecules are driving expression of CCR9 on CD8+ T cells in the brain? Other groups have shown that retinoic acid (RA) stimulation can lead to increased expression of the gut-homing specific markers CCR9 and integrin α4β7 on T-cells. GALT-derived CD8α dendritic cells have been shown to produce retinoic acid and are capable of up-regulating CCR9 and α4β7, resulting in their preferential homing to the gut. The expression of CCR9 on lymphocytes in other environments or in vitro has also been linked to retinoic acid. Indeed, we found that CCR9 is upregulated in neonatal CD8+ T cells following stimulation with RA and CD8+ T cells lacking RA signaling are impaired at trafficking (or surviving) in the brain after MCMV infection.

Whether their inability to migrate to the brain during infection is linked to reduced CCR9 expression remains an open question.

One of the more interesting findings in our study is the association of CD103 in CCR9+ CD8+ T cells. Previous work has shown that CD103 is typically expressed in tissue resident memory CD8+ T cells (173,174). This population of memory CD8+ T cells remains sequestered at sites of infection and is phenotypically distinct from circulating memory CD8+ T cells (173,174). Moreover, tissue resident memory CD8+ T cells confer immune protection during reinfection or reactivation of antigen. Thus, CCL25 may have a role in the transition of effector CD8+ T cells into long-lived tissue resident memory CD8+ T cells in the CNS.

It is also worth noting that CCR9 is preferentially expressed on recent thymic emigrants. Given that nearly all CD8+ T cells in early life are RTEs, it is likely that neonatal CD8+ T cells express more CCR9 than adults. Thus, we speculate that neonatal CD8+ T cells may have a selective advantage in migrating to the brain after infection with CMV. Nonetheless, this data provides key insight into the factors that may be involved in recruiting CD8+ T cells to the CNS during congenital CMV infection.

CHAPTER 5 – FINAL DISCUSSION AND FUTURE DIRECTIONS

In early development, the immune system and CNS of a neonate are quite different from those of adults. In this study, we wanted to improve our understanding of why neonates exhibit reduced immune protection against CMV. To investigate age-related differences in the susceptibility to CMV, we first asked whether cell-intrinsic differences between neonatal and adult CD8+ T cells play a key role. We were also interested in the microenvironment of the neonatal brain and how it may impact immune function, especially during viral infection. We specifically studied the vitamin A metabolite retinoic acid (RA) because of its dual roles in CNS development and immune function. In particular, we imaged the immune response to CMV in the brain and studied the effects of viral replication on the RA microenvironment. We also investigated the expression of chemokine receptors involved in CD8+ T cell homing and its localization to RA-producing cells in the brain following neonatal MCMV infection. Together, these data highlight the importance of the microenvironment in relation to the CD8+ T cell response to viral infection.

In our first study, we used an adoptive cell transfer approach to examine cell-intrinsic differences between different-aged CD8+ T cells and found that neonatal CD8+ T cells were less efficient at clearing MCMV compared to their adult counterparts. To understand the underlying basis for these differences, we also co-transferred neonatal and adult donor CD8+ T cells into the recipient mice and tracked their response to infection. What we found was that the failure of neonatal CD8+ T cells to clear CMV was not due to differences in activation, proliferation, or recruitment to the brain after

infection. Instead, we found that neonatal CD8+ T cells failed to acquire effector functions.

While this work clearly establishes a link between impaired immune defense against CMV and reduced effector function in neonatal CD8+ T cells, future studies may be warranted to further dissect out the precise immune lesion that is present in neonatal CD8+ T cells. CD8+ T cells control viral infections by either cytolytic molecules (gzmB) or cytokines (IFNg, TNFa). Previous reports have indicated that the specific effector function required to control viral infection in the CNS depends upon the particular pathogen. For example, elimination of measles or Sindbis virus from the CNS requires IFNg (49, 50), whereas perforin is required for the control of encephalitic West Nile virus infection (51). The effector functions that are necessary for controlling CMV in the CNS are presently not known. We found that a number of effector functions (GzmB, IFNg, TNFa) were depressed in neonatal CD8+ T cells, which is linked to their inability to clear virus during infection. However, it would be useful to know which effector function (assuming one is most important) needs to be restored in order to confer immune resistance to infection. This could be accomplished by repeating our studies with adult donor CD8+ T cells lacking key effector functions (e.g. IFNg KO). Additionally, we could administer recombinant cytokines (e.g IFNg) to recipient mice receiving WT neonatal donor CD8+ T cells to see if immune protection is enhanced.

In addition to examining how cell-intrinsic differences may alter the CD8+ T cell response to CMV in the brain, we also examined the role of a key environmental factor (RA) in the brains of neonatal mice infected with MCMV. In these studies, we observed elevated and prolonged levels of retinoic acid production in regions of the brain (choroid

plexus) that were infected with MCMV. By modifying the ALDEFLUOR assay™ for flow cytometry, we determined that the major source of elevated RA production during infection is primarily macrophages, although some contribution is made from CD8+ T cells at later stages of infection. While beyond the scope of our current study, it would be interesting to determine the relationship between aberrant RA production and abnormal brain development in animals infected with CMV in utero. Given that RA regulates the proliferation and differentiation of neural stem cells, we hypothesize that elevated amounts of RA could contribute to impaired development of neurons, resulting in microcephaly. One way to test this would be to cross floxed Raldh2 mice with Vav-cre mice to generate mice that contain immune cells with reduced ability to produce RA. Then we could determine whether there is a relationship among RA levels, brain pathology, and neural stem cell development following CMV infection.

RA may also lead to the development of abnormalities in the CNS via an indirect mechanism. For example, previous work has shown that RA limits the production of TGF-B, which plays an important role in the proliferation and differentiation of neurons and glia (175). Maternal administration of RA in group II rats was associated with congenital malformations, including microcephaly, exencephaly, hydrocephaly, gastrochisis, omphalocele, exophthalmus, mandibular hypoplasia, facial dysmorphia, limb reduction defects, and reduction of the crown-rump length (174). Another study showed that mouse embryos from mothers that had received RA on the 7.5 day of gestation showed a significant reduction of TGF-β2 expression (173). In preliminary studies, we observed a drop in TGF-beta production in the CNS following MCMV infection, which coincides with elevated levels of RA. While additional studies are

certainly warranted, it is possible that CMV-induced RA levels inhibit the production of TGF-beta and thus inhibit the normal development of the CNS during early stages of life.

Given that RA production is typically found at sites of viral replication in the CNS, it is also possible that RA influences the viral load after infection. Previous work has demonstrated that the promoter of the hCMV major immediate-early (MIE) gene, the product thought to be necessary for non-latent viral replication, is activated by physiological concentrations of RA [82]. Angulo and co-workers [84] have also demonstrated that the MIE gene of murine cytomegalovirus (MCMV) contains multiple RAREs and can be activated by RA. Others have demonstrated that RA can induce differentiation of human embryonal carcinoma cells (NT-21D2) into the neuronal lineage, converting non-permissive cells to HCMV infection into permissive ones (128). Collectively, these studies raise the possibility that MCMV sets up a positive feedback loop, whereby virus upregulates RA and in turn stimulates the production of more virus.

Perhaps one of the most interesting findings of our studies was the discovery that CCR9 is preferentially expressed on CD8+ T cells in the brain after MCMV infection. This finding is particularly exciting because CCR9+ expression has always been referred as a gut-trophic marker and has never been recognized on lymphocytes in the brain. To better understand the role CCR9 in CD8+ T cells during MCMV brain infection, we attempted to import CCR9 KO mice into our colony. Unfortunately, upon further inspection, we discovered that CCR9 was still intact in these mice, preventing us from using them in our experiments. However, previous work has shown that RA leads to the upregulation of CCR9 in adult CD8+ T cells, which we confirmed in vitro with

neonatal CD8+ T cells. This observation allowed us to use CD4cre dnRAR mice, which lack RA signaling in both CD4+ and CD8+ T cells. When we compared the numbers of CD8+ T cells in the brain after neonatal MCMV infection, we found fewer CD8+ T cells accumulate in dnRAR mice compared to their WT littermate controls.

While our studies with dnRAR mice shed light on the role of RA in the CD8+ T cell response to CMV in the CNS, it would also be of interest to expand the scope of our studies to other immune cell types. Indeed, RA has been shown to induce conversion of naive CD4+ T cells to adaptive regulatory T cells (Tregs) (166-168), as well as to drive (169) or inhibit (170) Th17 differentiation, depending on the concentration. Thus, it is possible to speculate that RA also influences the outgrowth of different CD4+ T cell subsets during MCMV infection.

In conclusion, our studies demonstrate that both cell-intrinsic and environmental factors influence susceptibility to CMV in early life. On one hand, neonatal CD8+ T cells appear to be inherently less capable at clearing infection because of reduced immune functionality. On the other hand, elevated levels of RA in the brain in early life may lead to the upregulation of key chemokine receptors (CCR9) and facilitate the recruitment of CD8+ T cells after infection. These results help broaden our fundamental understanding of the CD8+ T cell response to viral infections in the brain and have important implications for the development of therapeutic strategies to enhance immunity against CMV in early life.

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