Receptor Utilization and Antiviral CD8 T Cell Responses during Central Nervous System Infection with a Murine Coronavirus

Susan J. Bender
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Abstract
Murine coronavirus (mouse hepatitis virus, MHV) infection of the CNS provides a model system for studying viral and host factors affecting pathogenic outcome. CNS infection with the highly neurovirulent rJHM.SD is characterized by extensive viral antigen distribution throughout the brain and few antiviral CD8 T cells at the site of infection, and infected mice succumb to disease by approximately seven days post-infection. In contrast, the less neurovirulent rA59 strain establishes acute infection in the CNS and liver; a robust antiviral CD8 T cell response peaks in the brain at day seven post-infection and coincides with clearance of infectious virus. Mice surviving acute rA59 infection later develop immune-mediated demyelination, and viral RNA persists at low levels in the CNS for the life of the mouse. We aimed to evaluate these pathogenic differences between rJHM.SD and rA59 by assessing: 1) the involvement of known viral receptors in CNS infection and spread and 2) the induction and modulation of antiviral CD8 T cell responses during acute and chronic CNS disease. Using primary CNS-derived cells and quantitative RT-PCR, we demonstrate differential expression of MHV receptor genes in CNS cell types and virus strain-specific differences in receptor requirements in neurons, the predominant cell type infected in vivo. Using adoptive transfer methods, we demonstrate that naïve virus-specific CD8 T cells are poorly primed during CNS infection with rJHM.SD; however, a robust antiviral CD8 T cell response is elicited following peripheral inoculation of rJHM.SD, suggesting a passive mechanism specific to CNS infection. Finally, by comparing the quality of antiviral CD8 T cells during different scenarios of viral persistence, we show that rA59 persistence in the CNS modulates the differentiation of effector CD8 T cells into protective memory cells. Together, these results support the idea that extent of viral dissemination and induction/modulation of antiviral CD8 T cells collectively contribute to pathogenic outcome during MHV infection.

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RECEPTOR UTILIZATION AND ANTIVIRAL CD8 T CELL RESPONSES DURING CENTRAL NERVOUS SYSTEM INFECTION WITH A MURINE CORONAVIRUS

Susan J. Bender

A DISSERTATION

in

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DEDICATION

For Brian and Rita
ACKNOWLEDGMENTS

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ABSTRACT

RECEPTOR UTILIZATION AND ANTIVIRAL CD8 T CELL RESPONSES DURING CENTRAL NERVOUS SYSTEM INFECTION WITH A MURINE CORONAVIRUS

Susan J. Bender
Susan R. Weiss

Murine coronavirus (mouse hepatitis virus, MHV) infection of the CNS provides a model system for studying viral and host factors affecting pathogenic outcome. CNS infection with the highly neurovirulent rJHM.SD is characterized by extensive viral antigen distribution throughout the brain and few antiviral CD8 T cells at the site of infection, and infected mice succumb to disease by approximately seven days post-infection. In contrast, the less neurovirulent rA59 strain establishes acute infection in the CNS and liver; a robust antiviral CD8 T cell response peaks in the brain at day seven post-infection and coincides with clearance of infectious virus. Mice surviving acute rA59 infection later develop immune-mediated demyelination, and viral RNA persists at low levels in the CNS for the life of the mouse. We aimed to evaluate these pathogenic differences between rJHM.SD and rA59 by assessing: 1) the involvement of known viral receptors in CNS infection and spread and 2) the induction and modulation of antiviral CD8 T cell responses during acute and chronic CNS disease. Using primary CNS-derived cells and quantitative RT-PCR, we demonstrate differential expression of MHV receptor genes in CNS cell types and virus strain-specific differences in receptor requirements in neurons, the predominant cell type infected in vivo. Using adoptive transfer methods, we demonstrate that naïve virus-specific CD8 T cells are poorly primed during CNS infection with rJHM.SD; however, a robust antiviral CD8 T cell response is elicited following peripheral inoculation of rJHM.SD, suggesting a passive mechanism specific to CNS infection. Finally, by comparing the quality of antiviral CD8 T cells during different scenarios of viral persistence, we show that rA59 persistence in the CNS modulates the differentiation of effector CD8 T cells into protective memory cells. Together, these results support the idea that extent of viral dissemination and induction/modulation of antiviral CD8 T cells collectively contribute to pathogenic outcome during MHV infection.
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CHAPTER 1

General introduction

This chapter was adapted from the review article “Pathogenesis of murine coronavirus in the central nervous system” by S. J. Bender and S. R. Weiss. In press, Journal of Neuroimmune Pharmacology.
BACKGROUND AND SIGNIFICANCE

The family Coronaviridae is comprised of large, enveloped, RNA viruses that induce a variety of diseases in avian and mammalian species, including humans, poultry, livestock, and domestic animals. Coronaviruses, along with toroviruses and roniviruses, are members of the order Nidovirales ("nido" meaning "nest"), so named because of the nested set of subgenomic RNAs generated during the life cycle of these viruses (28). Coronaviruses are typically categorized into three groups based on antigenic similarity, with viruses in all groups being able to infect a range of different host species. Several human coronaviruses have been identified, including the mild respiratory pathogens HCoV-229E (31) and HCoV-OC43 (66), an etiologic agent of croup known as HCoV-NL63 (9, 102), and most notably SARS-CoV, the causative agent of severe acute respiratory syndrome (SARS) (16, 43, 75, 79). While coronaviruses are commonly regarded as being highly species-specific, the recent emergence of SARS-CoV in humans has brought renewed awareness to the potential for cross-species virus transmission from animal reservoirs.

Perhaps the best-studied member of the Coronaviridae is the murine coronavirus known as mouse hepatitis virus (MHV). Despite its name, not all strains of MHV are hepatotropic, with individual isolates inducing respiratory, enteric, or neurologic disease alone or in combination with hepatitis (108). While enteric strains are typically responsible for MHV outbreaks in housed rodent colonies (33), the most frequently studied are the neurotropic strains due to their ability to induce acute encephalomyelitis with or without chronic demyelination. These neurotropic strains differ widely in terms of cellular tropism, spread throughout the central nervous system (CNS), host immune response, and disease outcome, making them useful for analysis of viral and host determinants of neurovirulence (108).
MURINE CORONAVIRUS STRUCTURE AND LIFECYCLE

The RNA genome of MHV is single-stranded, positive-sense, and approximately 31 kb in length (Fig. 1-1) (46, 50). The 5’ two thirds of the genome (ORF1a and ORF1b) encode the viral replicase as well as an assortment of enzymes and other nonstructural proteins, while the 3’ one third of the genome (ORFs 2-7) largely encodes the structural proteins of the virion. These structural proteins include the spike (S), small envelope (E), membrane (M), nucleocapsid (N), and, in some strains, hemagglutinin-esterase (HE) (Fig. 1-1).

Figure 1-1. Genome organization and virion structure of MHV. A) Schematic of MHV genome. B) Schematic of MHV virion. L, leader; ORF1a/1b, replicase; structural genes/proteins: HE, hemagglutinin-esterase; S, spike; E, envelope; M, membrane; N, nucleocapsid; I, internal. ORFs 2a, 4, and 5a encode nonstructural proteins.
**Spike (S).** The MHV spike plays an integral role in the viral lifecycle as it binds to the cellular receptor CEACAM1a and mediates membrane fusion and subsequent entry into a target cell. The spike is a type I membrane protein that is synthesized as a 180 kDa precursor protein, co-translationally glycosylated, and processed by a furin-like enzyme into two approximately 90 kDa non-covalently-linked subunits, the amino-terminal S1 and the carboxyl-terminal S2 (Fig. 1-2) (21, 54). The spike glycoprotein is expressed on the virion membrane as a trimer in which the S1 subunits form a globular head structure and the S2 subunits form a transmembrane stalk (Fig. 1-1). Spike trimers project from the virion surface, forming the characteristic "corona" or halo-like appearance for which coronaviruses are named. Considerable evidence has accumulated over the years showing that the MHV spike is the major determinant of viral tropism and pathogenicity. There are at least three domains within the spike that have been shown to influence pathogenic outcome: 1) the receptor binding domain (RBD) contained within the N-terminal 330 amino acids, 2) the hypervariable region (HVR) within S1, and 3) the heptad repeat domains (HR1 and HR2) within S2 (Fig. 1-2). The role of spike in MHV pathogenesis will be detailed below.

*Figure 1-2. Structure of the JHM.SD spike glycoprotein.* RBD, receptor binding domain; HVR, hypervariable region; HR, heptad repeat domain; TM, transmembrane domain; S510 and S598, H-2b-restricted T cell epitopes. Large arrowhead indicates cleavage site yielding S1 and S2 subunits. Mutations/deletions found in selected neurotropic MHV strains are indicated below the structure.
Small envelope (E) and membrane (M). In addition to spike, all coronaviruses encode two additional transmembrane proteins, E and M (Fig. 1-1). The coronavirus E protein is an integral membrane protein (119) that plays an important role in viral assembly (103). Surprisingly, E is not an essential protein; however, a recombinant MHV lacking E expression replicates very inefficiently, consistent with the important role of E in production of infectious virus (45). The M protein is the most abundant viral membrane protein and spans the membrane multiple times (1). M has been shown to interact with the N protein during viral assembly (70). Both E and M are targeted to membranes of the Golgi, where they likely participate in viral assembly and budding.

Nucleocapsid (N). The MHV N protein plays important structural roles by both complexing with genomic RNA to form the capsid (96) and interacting with the viral membrane protein (M) during virion assembly (Fig. 1-1) (35). N increases efficiency of transcription (11), significantly enhances recovery of infectious virus from cells transfected with genome-length synthetic RNA (30), and has been implicated to play a role in translation of viral mRNA (99). Furthermore, MHV N has been reported to associate with microtubules in a neuronal cell line in vitro (77), suggesting a possible role for N in virus trafficking and axonal transport. Recent evidence from our laboratory further demonstrates that the N protein is a determinant of MHV neurovirulence (13), though the mechanism remains unclear. Also encoded within the N gene is the internal (I) protein, a structural protein of unknown significance (20).

Hemagglutinin-esterase (HE). The nonessential HE glycoprotein forms a second, smaller spike on the envelope of some coronaviruses, including some MHV strains (Fig. 1-1) (40, 92, 117, 118). The HE protein has sialic acid-binding and acetyl esterase (or receptor destroying) activities, both of which could potentially contribute to viral entry and/or release from the cell surface via interaction with sialic acid-containing moieties. While the viral HE glycoprotein is not essential for virulence in the CNS, isogenic viruses expressing full-length HE polypeptides or HE proteins with nonfunctional esterase activity are more virulent when inoculated intracranially into mice and spread more extensively than viruses expressing a truncated HE polypeptide (39).

Virus lifecycle. MHV binds to a target cell via interaction of the spike glycoprotein with its cellular receptor CEACAM1a (112). Receptor binding triggers a conformational change in the
spike that is believed to expose a fusion peptide that inserts into the host cell membrane and mediates fusion (4). MHV virions fuse either at the cell surface or from within endosomes, likely depending on target cell type and MHV strain (24, 41, 71). Genome replication and viral protein synthesis occur in the cytoplasm, and N proteins complex with copies of the viral genome; these capsids acquire their lipid envelopes and surface proteins by budding through internal membranes of the ER/Golgi, and the newly formed virions are released at the cell surface (15).

**MURINE CORONAVIRUS PATHOGENESIS**

Two MHV strains commonly used to study coronavirus-induced CNS disease are the highly neurovirulent JHM strain and the more neuroattenuated but demyelinating A59 strain (Table 1-1). Neuroattenuated variants of JHM are also common. While highly neurovirulent strains, such as JHM.SD, cause severe and uniformly lethal encephalitis in naïve mice, more neuroattenuated strains, such as A59 and some JHM variants, induce a less severe encephalomyelitis followed by chronic demyelination (Fig. 1-3) (108). For this reason, MHV infection is commonly studied as a model for the human demyelinating disease multiple sclerosis. The JHM strain, named for Professor John Howard Mueller, was initially isolated by Cheever, Bailey, and colleagues in 1949 from the brain of a paralyzed mouse and shown to induce encephalitis with extensive destruction of myelin (2, 6). Dr. Leslie Weiner later serially passaged this virus multiple times through mouse brains (106, 107). Most JHM isolates used since, including those described below, were derived from this mouse-passaged virus. The A59 strain was isolated independently in 1961 from a mouse with leukemia (56).

CNS disease induced by neurotropic MHV strains can be loosely divided into two phases, acute encephalitis and chronic demyelinating disease (Fig. 1-3). Following intracranial or intranasal inoculation, mice develop a mild to severe encephalomyelitis, characterized by infiltration of a variety of inflammatory cells. Viral titers typically peak at day 5 post-infection and then begin to decline (52), with infectious virus becoming undetectable by approximately 2 weeks post-infection (63). Innate immune responses are apparent within the first few days of infection and then give way to adaptive immunity. CD8 T cells, which play a dominant role in controlling
virus replication, are most numerous in the brain at day 7 post-infection, coinciding with viral clearance (115). However, despite clearance of infectious virus, viral RNA persists long-term in the CNS and demyelination, largely immune-mediated, develops and peaks around 4 weeks post-infection (48, 49). A notable exception to this disease course is CNS infection with the highly neurovirulent JHM isolates, particularly JHM.SD, which grow to increasing titers and induce severe encephalitis that is lethal within the first week of infection (Fig. 1-3) (74). The degree of viral spread throughout the brain and spinal cord, tropism of virus for individual CNS cell types, and dissemination of virus to other organs is largely dependent on viral strain (Table 1-1).

Figure 1-3. Kinetics of MHV-induced CNS disease. Kinetics of CNS disease after intracranial inoculation of A) demyelinating MHV strains like A59 or B) the highly neurovirulent JHM.SD strain. JHM.SD-infected mice succumb to acute CNS disease by 1 week post-infection.
Table 1-1. Neurotropic MHV strains and variants.

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<td>JHM.SD (MHV-4)</td>
<td>Highly lethal; severe encephalitis</td>
<td>Neurons, glial cells</td>
<td>Gly310; Leu1114; CEACAM1-independent spread</td>
<td>(Dalziel et al., 1986)</td>
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<td>V5A13.1 (mAb escape mutant of JHM.SD)</td>
<td>Neuroattenuated; spreads more slowly in CNS</td>
<td>Neurons, glial cells</td>
<td>HVR deletion (142 aa)</td>
<td>(Fazakerley et al., 1992)</td>
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<td>OBLV60 (variant of JHM.SD isolated from persistently-infected OBL21A cells)</td>
<td>Neuroattenuated</td>
<td>Olfactory bulb neurons</td>
<td>L1114R; CEACAM1-dependent spread</td>
<td>(Gallagher et al., 1991; Pearce et al., 1994)</td>
</tr>
<tr>
<td>JHM-DL</td>
<td>Highly lethal</td>
<td>Neurons, glial cells</td>
<td>Leu1114</td>
<td>(Stohlman et al., 1982; Wang et al., 1992)</td>
</tr>
<tr>
<td>2.2-V-1 (mAb escape mutant of JHM-DL)</td>
<td>Neuroattenuated; subacute demyelination</td>
<td>Glial cells, primarily oligodendrocytes</td>
<td>L1114F; CEACAM1-dependent spread</td>
<td>(Fleming et al., 1986; Wang et al., 1992)</td>
</tr>
<tr>
<td>JHM cl-2</td>
<td>Highly lethal</td>
<td>Neurons, glial cells</td>
<td>Gly310; Leu1114; CEACAM1-independent spread</td>
<td>(Taguchi et al., 1985)</td>
</tr>
<tr>
<td>sr7 (soluble receptor-resistant mutant of JHM cl-2)</td>
<td>Neuroattenuated</td>
<td>Macrophages/ microglia (in vitro)</td>
<td>L1114F; CEACAM1-dependent spread</td>
<td>(Matsuyama et al., 2001; Nakagaki and Taguchi, 2005)</td>
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<td>JHM.IA</td>
<td>Highly lethal, but less than JHM.SD</td>
<td>Neurons, glial cells</td>
<td>Ser310; Leu1114; CEACAM1-dependent spread</td>
<td>(Ontiveros et al., 2003)</td>
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<tr>
<td>rJIA.S310G (mutant of JHM.IA)</td>
<td>Highly lethal; more than JHM.IA</td>
<td>Neurons, glial cells</td>
<td>S310G; CEACAM1-independent spread</td>
<td>(Ontiveros et al., 2003)</td>
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<td>A59</td>
<td>Neuroattenuated; mild encephalitis; subacute demyelination; hepatitis</td>
<td>Neurons, glial cells</td>
<td>HVR deletion (52 aa); CEACAM1-dependent spread</td>
<td>(Lavi et al., 1984)</td>
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RECEPTOR AND TROPISM

The primary cellular receptor for MHV has been identified as CEACAM1a (also referred to as mmCGM1, BGP1a, and CD66a) belonging to the carcinoembryonic antigen family of cell adhesion molecules within the immunoglobulin (Ig) superfamily (112, 113). CEACAM1a is a multifunctional protein shown to play diverse roles in a variety of cellular processes, including intercellular adhesion, tumor suppression, angiogenesis, and immune cell signaling (29, 44). The ceacam1 gene is highly conserved among mammalian species, and human CEACAM1 proteins serve as receptors for a variety of pathogens, including Neisseria species and Haemophilus influenzae. In the mouse, ceacam1 exists in two allelic forms, ceacam1a and ceacam1b, and the particular ceacam1 allele expressed largely determines susceptibility of individual mouse strains to MHV; mouse strains expressing ceacam1a, including C57BL/6 and BALB/c, are highly susceptible to MHV infection whereas strains homozygous for ceacam1b, such as SJL, are relatively resistant (17).

Ceacam1a transcripts typically undergo alternative splicing, giving rise to four distinct splice variants and protein isoforms in the mouse (Fig. 1-4). These murine CEACAM1a isoforms contain either 2 or 4 extracellular Ig-like domains linked by a transmembrane domain to either a short (10 amino acids) or long (73 amino acids) cytoplasmic tail (64, 65). Using recombinant CEACAM1a constructs with deletions within the extracellular domains, the site of MHV binding was shown to be within the N-terminal Ig-like domain, D1 (18). This N-terminal domain is present in all four murine CEACAM1a isoforms, thus all serve as functional MHV receptors. Interestingly, long-tailed CEACAM1a isoforms contain phosphorylatable tyrosine residues within immunoreceptor tyrosine-based inhibitory motifs (ITIM) that have been shown to participate in protein-protein interactions and downstream signaling cascades in a variety of cell types, including T cells (8) and dendritic cells (38).
Figure 1-4. Structural isoforms of the MHV receptor CEACAM1a. D, extracellular immunoglobulin-like domain; TM, transmembrane domain; L, long cytoplasmic tail; S, short cytoplasmic tail.
While CEACAM1a is commonly regarded as the sole in vivo receptor for MHV, several lines of evidence suggest the presence of an alternative receptor or mechanism of viral infection. Despite the high predilection of some MHV strains for cells of the CNS, expression of CEACAM1a is relatively low in neural tissue compared to other MHV targets, such as liver and intestine (27). CEACAM1a is highly expressed on epithelial cells, endothelial cells, and cells of hematopoietic origin, including macrophages, B cells, and activated T cells (12, 27, 69). However, in the brain, only endothelial cells and microglia have been shown to express CEACAM1a protein (26, 84). Yet, perhaps paradoxically, many neurotropic MHV strains are able to infect a wide range of CNS cell types in addition to endothelial cells and microglia, including neurons, astrocytes, and oligodendrocytes. It has been suggested for the highly neurovirulent JHM cl-2 strain that CEACAM1a-positive microglia serve as the initial target of infection and that virus subsequently spreads to other CNS cell types in a CEACAM1a-independent manner; a soluble receptor-resistant mutant of cl-2 known as srr7 (61) cannot spread without CEACAM1a and is thus restricted to microglia in mixed neural cultures (68). Curiously, strain A59, which has also been shown to depend on CEACAM1a for spread (101), infects a variety of CNS cell types in vivo in addition to microglia. These seemingly disparate results raise the question as to whether CNS cell types other than microglia express low levels of CEACAM1a that are simply not detected by routine methods or whether some neurotropic MHV strains may use an alternative mechanism to enter these cell types.

The generation of a knockout mouse deficient in ceacam1a (ceacam1a<sup>−/−</sup>) by targeted deletion of the exon encoding the N-terminal domain has made it possible to evaluate MHV infection in the absence of CEACAM1a (32). Interestingly, two neurotropic MHV strains, A59 and JHM.SD, differ in their ability to cause CNS disease in ceacam1a<sup>−/−</sup> mice following intracranial inoculation. JHM.SD, a highly neurovirulent isolate previously shown to spread cell-to-cell in vitro in a CEACAM1a-independent manner, is able to cause lethal CNS disease in ceacam1a<sup>−/−</sup> mice, albeit at considerably higher doses than are required in wild-type C57BL/6 mice, whereas doses as high as 1 million PFU of A59 do not cause CNS disease; the ability of JHM.SD to cause disease in these mice was mapped to the spike gene (32, 67). While this finding is intriguing, it is
unclear whether the inability of A59 to cause disease in ceacam1a⁻/⁻ mice is due to a lack of initial virus entry or a deficiency in cell-to-cell spread in the CNS in the absence of CEACAM1a.

Several alternative receptors have been identified and shown to mediate MHV infection in non-murine cells when overexpressed in vitro. An additional ceacam gene, ceacam2 (bgp2), is uniquely expressed in the mouse and can facilitate infection with A59, JHM, and the hepatotropic MHV-3 strain when transiently transfected into hamster cells, though much less efficiently than ceacam1a; ceacam2 mRNA was shown to be expressed in brain tissue (73). The alternative ceacam1b allele expressed by MHV-resistant mice can similarly mediate infection with A59 when overexpressed in vitro (17). While the relative efficiencies of these alternative receptors is unclear, the decreased infection efficiency compared to CEACAM1a is likely attributable to sequence differences within the MHV binding site in the N-terminal domain. Yet another putative receptor, psg16 (bCEA), belonging to the more distantly related pregnancy-specific glycoprotein family, was identified in the brain due to its weak homology with ceacam1a; curiously, psg16 is reported to function in vitro as a receptor for A59 but not JHM (7). It is unclear what CNS cell types express these alternative receptors and thus whether they are positioned to mediate the MHV infection observed in ceacam1a⁻/⁻ mice. It is possible that an alternative receptor used by MHV in the brain may be completely unrelated to CEACAM1a, making its identification more difficult. Furthermore, the possibility that a traditional receptor molecule is not required to trigger MHV fusion cannot be excluded as some MHV strains, like JHM.SD, are inherently more fusogenic or may have acquired unique mechanisms to spread to cells expressing low or no CEACAM1a.

ROLE OF SPIKE IN VIRAL TROPISM AND SPREAD

It is not surprising that the spike, which interacts with the receptor CEACAM1a to mediate entry as well as cell-to-cell fusion, is crucial in determining the extent of viral spread within the CNS. Characterization of isogenic recombinant MHVs differing only in spike has definitively demonstrated the important role of spike in determining neurovirulence during infection in the mouse (36, 72, 82, 83). The replacement of the A59 spike gene with the spike of JHM.SD
(rA59/SJHM,SD) confers high neurovirulence on the resulting virus (72, 82). These studies have also demonstrated, perhaps unexpectedly, that a chimeric recombinant virus expressing the spike of the hepatotropic A59 within the background of the non-hepatotropic JHM.SD (rJHM.SD/S\textsubscript{A59}) cannot induce hepatitis (72). Thus, spike alone is unable to dictate organ tropism.

Mutations within the RBD have an influence on tropism and virulence. While two closely related JHM viruses, JHM.SD and JHM.IA, are both highly neurovirulent, the enhanced neurovirulence of JHM.SD can be mapped to a single amino acid difference within the RBD at residue 310 (Gly rather than Ser), as the introduction of a S310G substitution within the JHM.IA spike confers increased virulence on a recombinant JHM.IA. Furthermore, this Gly substitution at position 310 is associated with the ability to spread cell-to-cell in a CEACAM1a-independent manner (74). Characterization of viruses in which the RBDs of A59 and JHM.SD were exchanged further demonstrates that the ability to carry out CEACAM1a-independent spread requires both the RBD and the rest of the spike to be derived from JHM (101). Interestingly, a single amino acid substitution, Q159L, within the RBD eliminates the ability of A59 to infect the liver while having no measurable effect on neurovirulence (51, 52).

Among the many JHM isolates, high neurovirulence is correlated with the presence of a long hypervariable region (HVR) within S1 (Fig. 1-2). There are several JHM isolates with a similar long spike (Table 1-1), including MHV.SD (14, 74); JHM cl-2 (98), and JHM-DL (104). The extremely high neurovirulence of these viruses is believed to be due, at least in part, to their ability to induce cell-to-cell fusion and viral spread in the absence of the receptor CEACAM1a (14, 22, 23). This lack of requirement for CEACAM1a is associated with a less stable association of S1 and S2 such that the conformational changes that lead to fusion are more easily triggered, even in the absence of CEACAM1a (22, 42). The important role of the HVR in neurovirulence is further supported by the observation that the neuroattenuated phenotypes of a group of monoclonal antibody escape variants of JHM.SD, including V5A13.1 (19), are associated with single site mutations and/or deletions within the HVR (14, 25, 81). Consistent with the comparison of different JHM spikes, the genome of the neuroattenuated A59 strain encodes a large deletion (52 amino acids) within the HVR. However, replacement of the HVR of A59 with that of JHM.SD
does not confer a highly neurovirulent phenotype to the virus (81), suggesting that cooperation of several regions of spike, including the long HVR, is likely required for the high neurovirulence conferred by the JHM.SD spike.

Single amino acid substitutions in the heptad repeat (HR) domains within S2 have been shown to have dramatic effects on pathogenesis as well (Fig. 1-2). This region of the spike undergoes conformational changes during the fusion process, and therefore it is not surprising that it plays a role in pathogenic phenotype. Amino acid substitutions at position 1114 within the heptad repeat 1 (HR1) of the JHM spike (L1114R/F) are particularly intriguing in that they have been reported in multiple studies and in association with several mutant phenotypes (Table 1-1). The spike protein of the OBLV60 mutant of JHM.SD, which is restricted in replication to the murine olfactory bulbs, contains three amino acid substitutions within HR1 that have been associated with the requirement for low pH to induce fusion. One of these substitutions alone, L1114R, is sufficient to confer neuroattenuation and restriction to the olfactory bulbs (24, 78, 100). A L1114F substitution has also been identified in the spike of the 2.2-V-1 glial-tropic variant of JHM-DL (104) and in the spike of the highly attenuated soluble receptor-resistant mutant srr7 derived from JHM cl-2 (88, 89). These substitutions are associated with an inability to induce CEACAM1a-independent cell-to-cell fusion as well as neuroattenuation (59, 60, 97). Interestingly, viruses expressing the JHM spike with a L1114F substitution have lost their tropism for neurons while the OBLV60 mutant, expressing a spike carrying the L1114R substitution, can readily infect neurons of the olfactory bulb in vivo. Thus, small changes within the HR domains, even different substitutions of the same residue, may result in alterations in spike/receptor interaction and subsequent virus entry and pathogenesis in vivo.

**IMMUNE RESPONSES TO MHV**

**Type I interferon.** Type I interferon (IFN-α/β) signaling is an important aspect of host defense during the early phases of MHV infection. This role is supported by several reports of the high mortality following MHV infection that were carried out in the absence of IFN signaling in type I interferon receptor deficient (IFNAR^{-/-}) mice (5, 37, 87). IFNAR^{-/-} mice inoculated
intracranially with low doses of the neurotropic MHV strains A59 and JHM.SD show dramatically accelerated clinical signs and mortality compared with wild-type C57BL/6 mice. Furthermore, increased levels of infectious virus are detected in the brain and spinal cord (and in the case of A59, the liver) of infected IFNAR^{−/−} mice, as well as spread to other organs not usually affected, compared to wild-type mice (87).

**Inflammatory cell infiltrate.** A robust innate immune response involving chemokine secretion and migration of inflammatory cells into the CNS develops during the first few days following MHV inoculation. CNS infection with the highly neurovirulent JHM.SD strain, which is characterized by rapid spread of viral antigen throughout the brain, is accompanied by greater levels of infiltrating neutrophils and macrophages, as well as increased cellular destruction, compared to infection with the weakly neurovirulent A59 strain (36, 85, 86). Not surprisingly, the increased numbers of macrophages and neutrophils within the brains of JHM.SD-infected animals correlate with higher levels of macrophage- and neutrophil-recruiting chemokines, as well as prolonged expression of IFN-β, whereas A59 infection is characterized predominantly by T cell infiltration into the brain consistent with higher levels of T cell-attracting chemokines (36, 85, 86, 90). The MHV spike gene has been demonstrated to be a determinant of neurovirulence and to influence macrophage, but not T cell, infiltration into the brain (36, 82, 83, 86).

**T cells in acute disease.** Numerous studies of MHV infection in immunocompromised SCID, RAG-deficient, or sublethally irradiated wild-type mice have demonstrated that the host adaptive immune response plays a pivotal role in viral clearance (34, 62, 105, 116). Subsequent studies in mice lacking individual lymphocyte populations, either due to antibody depletion or genetic manipulation, combined with reconstitution experiments in mice lacking these lymphocytes more clearly delineated the roles of CD4 and CD8 T cells in viral control. While both CD4 and CD8 T cells are required for effective control of MHV infection in the CNS (114), CD8 T cells are most directly responsible for clearance of infectious virus. Following intracranial inoculation of a sublethal dose of neurotropic MHV, viral titers peak in the brain at day 5 post-infection and then begin to decline, coincident with the accumulation of virus-specific CD8 T cells at the site of infection (115). The importance of CD8 T cells in control of MHV is further supported
by the increased susceptibility and delayed viral clearance observed in β₂-microglobulin-deficient mice compared to wild-type control mice (47). Using a model in which C57BL/6 mice were infected with a recombinant A59 virus expressing the GP33 epitope of lymphocytic choriomeningitis virus (LCMV), it was shown that adoptive transfer of naïve, GP33-specific CD8 T cells results in lower viral titers and reduced antigen distribution in the brain (10). Further studies using this adoptive transfer system revealed that increasing the number of naïve, virus-specific CD8 T cells, either before infection or at early times post-infection, reduces viral replication and spread throughout the brain and spinal cord as well as demyelination compared to control mice; thus, enhancing the host CD8 T cell response is protective against A59-induced CNS disease (55).

To determine the kinetics of CD8 T cell activation and expansion, BALB/c mice were infected with the glial-tropic JHM variant 2.2-V-1 and virus-specific CD8 T cells were quantified at various times post-infection in the CNS and peripheral lymphoid organs. After intracranial inoculation, virus-specific CD8 T cells are first detected in the draining cervical lymph nodes (CLN) by day 3 post-infection, followed by further expansion in the spleen and ultimate accumulation in the brain, peaking at day 7 post-infection (58). These results indicate that the draining CLN are the initial site of CD8 T cell priming during neurotropic MHV infection. Interestingly, intracranial inoculation of the highly neurovirulent JHM.SD isolate has been shown to induce a weak antiviral CD8 T cell response in the brain compared to other neurotropic strains (36, 85); infectious virus is not cleared and mice typically succumb to CNS disease by day 7 post-infection. Interestingly, studies of recombinant viruses in which the spike genes of A59 and JHM.SD were exchanged reveal that this differential T cell induction is not due to spike, as replacement of the A59 spike with that of JHM.SD (rA59/SJHM.SD) results in a virus that spreads extensively in the CNS like JHM.SD but induces a robust CD8 T cell response similar to A59. Furthermore, though rA59/SJHM.SD has an intracranial LD₅₀ similar to JHM.SD, mice infected with rA59/SJHM.SD survive several days longer than those infected with JHM.SD, perhaps due, at least in part, to the effects of antiviral CD8 T cells (36, 82).
Activated CD8 T cells possess numerous effector mechanisms that serve to clear pathogens from infected cells, and the particular mechanisms required for clearance of MHV appear to be specific to the infected cell type. In studies using perforin-deficient mice, clearance of two JHM variants, the moderately virulent JHM-DM isolate (93) and the neuroattenuated 2.2-V-1 isolate, was delayed compared to wild-type control mice, suggesting that perforin-mediated cytolysis contributes to, but is not required for, clearance of MHV from the CNS; furthermore, JHM-infected perforin-deficient mice still developed encephalomyelitis and demyelination, indicating that these processes are independent of perforin activity (53). Interestingly, while CD8 T cell-mediated cytolysis appears to be important for clearance of MHV from astrocytes and microglia (94), viral clearance from oligodendrocytes instead relies on IFN-γ secretion, as indicated by persistence of the JHM variant 2.2-V-1 in oligodendrocytes of IFN-γ-deficient mice (76). Though several neurotropic MHV strains infect large numbers of neurons in addition to glial cells, mechanisms of MHV control in this unique cell type are poorly understood.

While CD8 T cells play a direct role in viral clearance, CD4 T cells are also required for efficient control. In studies using the JHM-DM variant, transfer of activated virus-specific CD8 T cells into infected wild-type mice resulted in viral clearance, whereas cells transferred into CD4-depleted mice are unable to effectively clear virus from the CNS (93). Furthermore, CD4 T cells are required for production of MHV-specific antibodies during infection with the JHM-DS variant (114). Recent studies in which IFN-γ- and perforin-deficient mice were reconstituted with memory CD4 T cells competent in just one of these functions and challenged with the JHM variant 2.2-V-1 suggest an additional, more direct role for CD4 T cells in viral clearance; while viral replication is initially controlled by both IFN-γ- and perforin-deficient CD4 T cell populations, only those cells competent to express IFN-γ are able to maintain prolonged control (95).

**T cells in chronic disease.** After clearance of an acute viral infection, most antiviral effector CD8 T cells are eliminated via apoptosis while a small subset continue to differentiate into a long-lived pool of resting memory cells; upon re-exposure to antigen, these memory CD8 T cells rapidly expand and reactive antiviral effector functions to protect the host against reinfection
(109). Using the lymphocytic choriomeningitis virus (LCMV) model of chronic infection, this pattern of differentiation from effector to memory CD8 T cell was shown to be significantly altered in the presence of ongoing viral replication; CD8 T cells present during chronic LCMV infection show a hierarchical loss of antiviral effector functions (functional exhaustion) and rely on continued viral antigen stimulation for maintenance in the host (91, 110, 111). Unlike chronic LCMV infection, MHV persists at lower levels and thus provides and interesting comparison to examine the effects of viral persistence on CD8 T cell function.

After clearance of infectious MHV, CD8 T cells in the CNS decrease in number but are not eliminated. Demyelination, largely immune-mediated, peaks at approximately four weeks post-infection and is accompanied by low levels of viral RNA that may persist in the CNS for the life of the mouse. A study comparing two glial-tropic JHM variants, one that persists and one that is cleared, showed that maintenance of CD4 and CD8 T cells within the infected CNS correlates with the continued presence of viral RNA, suggesting that viral persistence (as evidenced by detection of viral RNA) provides a signal that is necessary to maintain these lymphocyte populations within the CNS (57). Further studies using the glial-tropic JHM variant 2.2-V-1 revealed that CD8 T cells maintained in the CNS after viral clearance display a loss of ex vivo cytolytic activity while maintaining the ability to secrete IFN-γ (3). More recent studies indicate that CD8 T cells maintain expression of the inhibitory receptor programmed death 1 (PD-1) during persistence of the JHM variant 2.2-V-1 in the CNS, while expression of the PD-1 ligand B7-H1 is concurrently maintained on oligodendrocytes; the authors suggest that this PD-1/B7-H1 interaction contributes to CD8 T cell dysfunction during persistence (80). Studies by Zhao et al. further reveal, using adoptive transfer techniques and bone marrow chimeras, that antiviral CD8 T cell populations are maintained during persistence in the CNS, at least in part, by recruitment of both antigen-experienced and naïve CD8 T cells from the periphery (120). While MHV persistence in the CNS appears to compromise CD8 T cell function during chronic disease, the relative effect of low level RNA persistence compared to high levels of persisting infectious virus on CD8 T cell function is unclear.
SUMMARY AND FOCUS

High neurovirulence of MHV is routinely associated with increased viral dissemination throughout the CNS, yet it is unclear how neurotropic strains of MHV infect and spread in CNS cell types reportedly expressing low to nonexistent levels of the receptor CEACAM1a. Furthermore, while virus-specific CD8 T cells are crucial for controlling MHV in the infected host, it is unknown how the JHM.SD strain of MHV avoids the induction of an antiviral CD8 T cell response during acute disease or how persistence of MHV impacts CD8 T cell differentiation during chronic disease. The following chapters discuss studies that were undertaken to elucidate mechanisms of viral infection/spread as well as the interaction of MHV with the host immune system to improve our understanding of how these determinants contribute to neurovirulence.

VIRUS NOMENCLATURE

The nomenclature of recombinant viruses in our laboratory has evolved over the years, thus different names for the same recombinant virus are used in different chapters. RJHM and rJHM.SD refer to a recombinant version of the highly neurovirulent strain MHV-4. RA59 and rA59 refer to a recombinant version of the less neurovirulent but demyelinating strain A59. SJHM/RA59 and rA59/S_{JHM.SD} refer to a chimeric recombinant A59 virus expressing the spike gene of JHM.SD.
REFERENCES


CHAPTER 2

Murine coronavirus receptors are differentially expressed in the central nervous system and play virus strain-dependent roles in neuronal spread

This chapter has been submitted to The Journal of Virology as the article “Murine coronavirus receptors are differentially expressed in the central nervous system and play virus strain-dependent roles in neuronal spread” by S. J. Bender, J. M. Phillips, E. P. Scott, and S. R. Weiss.
ABSTRACT

Coronavirus infection of the murine CNS provides a model to study viral encephalitis and demyelinating disease. While MHV-A59 causes mild encephalomyelitis and demyelination, the highly neurovirulent JHM.SD (MHV-4) causes fatal encephalitis with extensive neuronal spread of virus. The carcinoembryonic antigen family member CEACAM1a is the primary receptor for MHV, yet CEACAM1a expression is very low in the CNS and has been demonstrated on endothelial cells and microglia only despite infection of additional cell types in vivo. Therefore, ceacam1a mRNA expression was quantified in murine tissues and primary cells. Among CNS cell types, ceacam1a was most highly expressed in microglia, with decreasing levels in oligodendrocytes, astrocytes, and neurons. Given low neuronal expression of ceacam1a, primary neurons from wild-type and ceacam1a-deficient mice were inoculated with MHV to determine the extent to which ceacam1a-independent infection might contribute to CNS infection. While both A59 and JHM.SD infected small numbers of ceacam1a-deficient neurons, only JHM.SD spread efficiently to adjacent cells in the absence of ceacam1a. Quantification of mRNA for ceacam1a-related genes ceacam2 and psg16 (bCEA), which encode proteins shown to serve inefficiently as MHV receptors when overexpressed in vitro, revealed low ceacam2 expression in microglia and oligodendrocytes and psg16 expression exclusively in neurons. Importantly, CEACAM2 mediated infection when expressed in human 293T cells while PSG16 receptor activity was not detectable. Thus, CEACAM2 on endothelial cells, microglia, and/or oligodendrocytes may facilitate initial infection in the absence of ceacam1a but is unlikely responsible for CEACAM1a-independent neuronal spread of JHM.SD.
INTRODUCTION

Murine coronavirus (mouse hepatitis virus, MHV) is a member of the Coronaviridae family of large, enveloped, RNA viruses. Central nervous system (CNS) infection with neurotropic strains of MHV provides a model for studying acute virus-induced neurological disease with or without chronic demyelination. These neurotropic strains differ widely in terms of tropism, spread, host response, and disease outcome, making them useful for identifying viral and host determinants of neurovirulence (58). Two strains commonly used to study coronavirus-induced CNS disease are the highly neurovirulent JHM.SD (formerly called MHV-4) strain and the more neuroattenuated and hepatotropic A59 strain (3, 28). Following intracranial (i.c.) or intranasal (i.n.) inoculation, JHM.SD causes severe and uniformly lethal encephalitis, whereas A59 induces a less severe encephalomyelitis followed by chronic demyelination (58). The extreme neurovirulence of JHM.SD largely maps to the spike glycoprotein, as a recombinant A59 virus expressing the JHM.SD spike (rA59/S_{JHM.SD}) shows increased virulence and viral dissemination throughout the brain compared to parental A59 (43, 44). Viral genes other than spike also contribute to neurovirulence (7, 23).

MHV binds to a target cell via interaction of the viral spike glycoprotein with a cellular receptor. This binding leads to a conformational change in spike that allows the virion membrane to fuse with the host cell membrane. Subsequent viral spread can occur via release of new virions from the infected cell and/or syncytium formation mediated by viral spikes expressed on the cell surface. The receptor for MHV is the murine carcinoembryonic antigen family member CEACAM1a (also referred to as mmCGM1, BGP1a, CD66a) (60). In the mouse, the ceacam1 gene exists in two allelic forms, ceacam1a and ceacam1b, and the ceacam1 alleles expressed largely determine mouse susceptibility to MHV. Mouse strains expressing ceacam1a (such as C57BL/6, BALB/c and C3H) are highly susceptible while strains homozygous for ceacam1b (such as SJL) are resistant to infection (9). Ceacam1a transcripts are alternatively spliced, yielding four distinct variants in the mouse. These splice variants encode either two or four extracellular immunoglobulin-like (Ig-like) domains linked by a transmembrane domain to a short (10 amino acids) or long (73 amino acids) cytoplasmic tail (33, 34). The MHV binding site resides within the
N-terminal Ig-like domain, D1 (10). This domain is present in all four isoforms of CEACAM1a and thus all serve as functional receptors for MHV (9).

While CEACAM1a is commonly regarded as the sole in vivo receptor for MHV, several lines of evidence suggest the presence of an alternative receptor and/or mechanism of viral infection/spread. Curiously, despite the high predilection of neurotropic MHV strains for the CNS, CEACAM1a expression is relatively low in neural tissue compared to other MHV targets such as the liver and intestine (17). While CEACAM1a is highly expressed on epithelial cells, endothelial cells, and cells of hematopoietic origin (6, 17, 37), CNS expression of CEACAM1a has only been demonstrated on endothelial cells by immunohistochemistry (16) and on microglia by flow cytometry (46). Yet, both A59 and JHM.SD infect multiple CNS cell types, with neurons being the predominant cell type infected (11, 35, 44). This apparent paradox raises the question of whether resident CNS cell types such as neurons, astrocytes, and oligodendrocytes express low levels of CEACAM1a that are simply not detected by routine methods or whether some MHV strains use an alternative mechanism to enter these cell types.

Additionally, in vitro studies reveal that the highly neurovirulent JHM.SD strain can spread efficiently from CEACAM1a-positive cells to cells lacking murine CEACAM1a (15, 38, 41, 54). Similar in vitro studies using primary mixed neural cultures demonstrated that the closely related JHM cl-2 variant spreads to adjacent neural cells in the presence of CEACAM1a-blocking antibodies (36). This “receptor-independent spread” phenomenon should more accurately be referred to as “CEACAM1a-independent spread” since the process may or may not require an alternative receptor. The generation of a knockout mouse deficient in ceacam1a (ceacam1a<sup>-/-</sup>) further facilitates analysis of CEACAM1a-independent spread both in vivo and in vitro.

Interestingly, both JHM.SD and the chimeric rA59/S<sub>JHM.SD</sub> induce lethal CNS disease in ceacam1a<sup>-/-</sup> mice, albeit at higher doses compared to wild-type mice, while doses of A59 as high as 1 million PFU i.c. are insufficient to cause disease (21, 35). While this finding is intriguing, it is unclear whether A59 fails to cause disease in ceacam1a<sup>-/-</sup> mice due to a lack of virus entry, deficiency in cell-to-cell spread in the absence of CEACAM1a, or inability to achieve a high enough viral dose to initiate infection.
Notably, two ceacam1a-related genes expressed in the mouse brain encode proteins that serve inefficiently as MHV receptors when overexpressed in vitro. The more closely related ceacam2 (bgp2) gene is uniquely expressed in the mouse and facilitates infection with both A59 and JHM when transiently transfected in hamster cells (40). Additionally, the more distantly related psg16 (bCEA) gene, belonging to the CEA-related pregnancy-specific glycoprotein family, is reported to function as a receptor for A59 but not JHM when transfected in monkey cells (4). It is unclear how these alternative receptors compare in terms of receptor functionality, and the cellular expression patterns of these receptor genes have not been explored. Therefore, in the current study we aimed to evaluate expression of ceacam1a and related genes in murine tissues and CNS cell types and to investigate the role of these receptor genes in neuronal infection and spread.
MATERIALS AND METHODS

Mice. Virus-free C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). *Ceacam1a*-deficient (*ceacam1a<sup>−/−</sup>*) mice on a C57BL/6 background were provided by Nicole Beauchemin (McGill University) (21). Mice were housed and bred at the University of Pennsylvania in accordance with Institutional Animal Care and Use Committee guidelines.

Viruses and inoculations. Recombinant A59 (rA59) (previously referred to as RA59, S<sub>A59</sub>R13, wtR13), recombinant JHM.SD (rJHM.SD) (previously referred to as RJHM and derived from the MHV-4 isolate of JHM), and a chimeric recombinant expressing the JHM.SD spike in the A59 background (rA59/S<sub>JHM.SD</sub>) (previously referred to as SJHM/RA59 or SrR22) have been described (39, 43, 44). Recombinant viruses of the same genotypes, only expressing enhanced green fluorescent protein (EGFP) in place of gene 4 (29, 49), were used to monitor infection in primary cells. Viruses were propagated in murine 17Cl1 fibroblasts and titrated by standard plaque assay on murine L2 fibroblasts. To remove cell fragments from the virus preparations, filtered virus stocks were prepared by passage through a 0.22 µm filter apparatus and retitrated after filtration. For intracranial (i.c.) inoculations, 4-week-old mice were anesthetized with isoflurane and 50 PFU of virus diluted in 30 µL PBS containing 0.75% BSA was injected into the left cerebral hemisphere. Mock infections were performed with lysate from 17Cl1 fibroblasts.

Immunofluorescence. For identification of infected cell types, brains were perfused with PBS, fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sagittally sectioned. Sections were deparaffinized and rehydrated, treated with Antigen Unmasking Solution (Vector Labs), blocked with 1.5% normal goat serum, and dual immunolabeled with a mouse monoclonal antibody (1.16.1) directed against the MHV nucleocapsid protein (a gift from Julian Leibowitz, Texas A&M University) and a rabbit polyclonal antibody directed against either glial fibrillary acidic protein (GFAP) for astrocytes (Dako USA), Iba1 for microglia (Wako Pure Chemical Industries), OLIG2 for oligodendrocytes (Millipore), or microtubule-associated protein 2 (MAP2) for neurons (a gift from Virginia Lee, University of Pennsylvania). Primary antibodies were detected with goat-anti-mouse and goat-anti-rabbit Alexa Flours 488 and 594 (Invitrogen).
Cultured cells were fixed in PBS containing 4% paraformaldehyde (Electron Microscopy Sciences), blocked with 1.5% normal goat serum, and immunolabeled as above with the exception of microglia, which were labeled with rat monoclonal anti-CD11b (Abcam) and goat-anti-rat Alexa Fluor 594 (Invitrogen). Fluorescence was visualized with a Nikon Eclipse TE2000-U microscope and images were acquired using SPOT Imaging Software (Diagnostic Instruments).

**Primary hepatocyte cultures.** Primary hepatocytes were prepared from Avertin-anesthetized 8-week-old mice by *in situ* perfusion and digestion with Liver Perfusion Medium and Liver Digestion Medium (Invitrogen), respectively, followed by mechanical disruption through nylon mesh and centrifugation through 45% Percoll. Hepatocytes were seeded onto BioCoat collagen I-coated plates (BD Biosciences) and cultured overnight in RPMI 1640 medium containing 10% FBS.

**Primary glial cultures.** Mixed glial cells were prepared from the cortices of P1-3 neonates by mechanical disruption through nylon mesh and cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 ng/mL streptomycin for 5-14 days. Mixed glial cultures were enriched for either astrocytes or microglia based on differential adhesion to tissue culture plastic as previously described (1). Briefly, microglia were detached from the astrocyte monolayer by brief gentle shaking of the culture flasks. Cells in the supernatant were collected by centrifugation and seeded onto tissue culture plastic. After 30 min., medium was replaced to remove non-adherent cells from microglia cultures. Microglia cultures were routinely ≥98% pure as determined by positive immunostaining for CD11b and negative staining for MAP2, GFAP, and OLIG2. Astrocyte monolayers were shaken at 200 RPM for 45 min. to remove loosely adherent cells. Remaining cells were detached with trypsin/EDTA and the resulting suspension was placed in tissue cultures flasks at room temperature for 30 min. to allow adherence of microglia to the plastic. This step was repeated, and the remaining astrocyte suspension was seeded onto tissue culture plastic. Astrocyte cultures were routinely 90-95% pure as determined by positive immunostaining for GFAP and negative staining for MAP2, CD11b, and OLIG2. Oligodendrocytes were prepared from minced forebrains of P1-3 neonates by trypsin/DNase digestion and cultured in DMEM containing 10% FBS, 1X non-essential amino acids solution (Invitrogen), 2 mM L-glutamine, 100 U/mL penicillin, and 100 ng/mL streptomycin as previously described (12). After
24 hours, culture medium was replaced with neurobasal medium containing B-27 supplement, 10 ng/mL bovine FGF basic (R&D Systems), 2 ng/mL recombinant human PDGF (R&D Systems), and 1 ng/mL recombinant human NT-3 (PeproTech Inc.). After 7 days, oligodendrocytes were removed from underlying astrocytes by gentle rinsing and seeded onto poly-D-lysine-coated tissue culture plates. Oligodendrocyte cultures were routinely 90-95% pure as determined by positive immunostaining for OLIG2 and negative immunostaining for MAP2, CD11b, and GFAP.

**Primary neuronal cultures.** Hippocampal neurons were prepared from E15-16 mouse embryos by trypsin digestion and mechanical disruption with a heat-polished Pasteur pipette, seeded onto poly-L-lysine-coated coverslips or tissue culture plates, and cultured in neurobasal medium containing B-27 supplement (Invitrogen), 100 U/mL penicillin, 100 ng/mL streptomycin, 2 mM L-glutamine, and 4 µg/mL glutamate for 4 days in the absence of an astrocyte feeder layer as previously described (2, 42, 45). Cortical neurons prepared from E18 mouse embryos were provided by Marc Dichter (University of Pennsylvania) (8, 50). Neuron cultures of both types were routinely 95-98% pure as determined by positive immunostaining for MAP2 and negative immunostaining for CD11b, GFAP, and OLIG2. After 4 days *in vitro*, neuron cultures were inoculated with virus diluted in neurobasal medium for 1 hour at 37°C, washed, and cultured an additional 24-72 hours in neurobasal medium. To quantify extracellular virus, neuron supernatants were collected at various times post-infection and stored at -80°C until titration. Fresh neurobasal medium was added to the remaining cells and intracellular virus was released by repeated freeze/thaw cycles at -80°C. Infectious virus was titrated by plaque assay on L2 fibroblast monolayers.

**Cell lines.** Murine DBT astrocytoma cells, 17Cl1 fibroblasts, and L2 fibroblasts were cultured in DMEM supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 100 U/mL penicillin, 100 ng/mL streptomycin, and 2.5 µg/mL amphotericin B. Human 293T cells were cultured in high glucose DMEM containing 10% FBS, 100 U/mL penicillin, and 100 ng/mL streptomycin.
Quantitative RT-PCR. RNA was isolated from liver, brain, and spinal cord tissue by homogenization in TRIzol Reagent (Invitrogen) followed by phenol/chloroform extraction and purification with an RNeasy Mini Kit (Qiagen) as previously described (47). RNA was isolated from cultured cells using an RNeasy Mini Kit according to manufacturer’s instructions. Tissue and cellular RNA was DNase-treated using a Turbo DNA-free Kit (Ambion) and quantitative PCR reactions without reverse transcriptase were performed to ensure adequate removal of genomic DNA. For cDNA synthesis, 350 ng RNA was combined with 0.5 mM dNTP mix (Invitrogen) and 50 ng random hexamers (Invitrogen) in a total volume of 13 µL, heated to 65°C in a PCR thermocycler for 3 minutes, and cooled to room temperature. 1X First Strand Buffer (Invitrogen), 5 mM DTT (Invitrogen), 200 U SuperScript III Reverse Transcriptase (Invitrogen), and nuclease-free water were added for a final reaction volume of 20 µL and the mixture was heated to 50°C for 60 minutes followed by 70°C for 15 minutes. cDNA was stored at -20°C until use. Quantitative PCR was performed in duplicate using 2 µL cDNA, 12.5 µL iQ SYBR Green Supermix (Bio-Rad), and 0.4 µM forward and reverse primers (listed in Table 2-1) in a total volume of 25 µL in an iQ5 iCycler (Bio-Rad). For copy number determination, cycle threshold (C_T) values were compared to a plasmid standard curve run in parallel for each target. Copy numbers are expressed as target cDNA copies per million cDNA copies of mouse beta-actin (actb). For relative expression compared to mock-infected samples, C_T values were normalized to actb levels resulting in a ΔC_T value (ΔC_T = C_T target – C_T actb). ΔΔC_T values were then calculated (ΔΔC_T = ΔC_T infected – ΔC_T mock) and results are expressed as fold change over mock-infected samples (2^{-ΔΔC_T}).
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**TABLE 2-1.** Primer sequences for quantitative RT-PCR.
**Plasmids.** pLXSN-BgpC (CEACAM1a-2S) and pLXSN-BgpD (CEACAM1a-4L) used for ceacam1a splice variant standard curves were provided by Nicole Beauchemin (McGill University). pCMV-SPORT6-CEACAM1, pCMV-SPORT6-CEACAM2, pCMV-SPORT6-PSG16, and pCMV-SPORT6-ACTB (mouse beta-actin, partial clone) were purchased from Open Biosystems (Thermo Scientific).

**Transfections.** To compare MHV receptor activity, human 293T cells were seeded in a 12-well plate at 1.5 x 10^5 cells per well and transfected with 0.4 µg pCMV-SPORT6-CEACAM1, pCMV-SPORT6-CEACAM2, or pCMV-SPORT6-PSG16 plus 0.6 µg empty vector (1 µg total DNA) using 6 µL FuGENE 6 Transfection Reagent (Roche Applied Science) in 100 µL serum-free DMEM. A control plasmid encoding GFP was used in parallel to assess transfection efficiency. At 36 hours post-transfection, cells were inoculated with 3 x 10^5 PFU of virus diluted in DMEM containing 2% FBS for 1 hour at 37°C. At 8 hours post-infection, cells were fixed in 4% paraformaldehyde and immunolabeled for MHV nucleocapsid protein as described above. Cell nuclei were labeled with DAPI, and percent infection was determined as the percentage of DAPI-positive cells per field that also stained positively for MHV nucleocapsid.
RESULTS

rA59 and rJHM.SD infect multiple CNS cell types *in vivo* but predominantly infect neurons.

To determine what CNS cell types are infected at early times after intracranial (i.c.) inoculation, 4-week-old C57BL/6 mice were inoculated i.c. with 50 PFU of rA59 or rJHM.SD. At days 3 and 5 post-infection (p.i.), infected mice were euthanized and brains were fixed for immunofluorescence. By day 3, MHV antigen was readily detected in GFAP-positive astrocytes, Iba1-positive microglia, and MAP2-positive neurons (Fig. 2-1A, white arrowheads) as well as occasional OLIG2-positive oligodendrocytes (data not shown). By day 5, large patches of MHV-infected neurons were evident in both rA59- and rJHM.SD-infected brains (Fig. 2-1B), consistent with previous data that neurons are the predominant cell type infected by these strains (11, 35, 44). Importantly, while rA59 and rJHM.SD both infect large numbers of neurons, rJHM.SD is more extensively distributed throughout the brain than rA59, which remains more focal (23).
**Figure 2-1. MHV infection of CNS cell types.** C57BL/6 mice inoculated i.c. with 50 PFU of rA59 or rJHM.SD were sacrificed at days A) 3 and B) 5 post-infection. Formalin-fixed, paraffin-embedded tissues were sectioned and dual immunolabeled for GFAP, Iba1, or MAP2 (red) and MHV nucleocapsid protein (green). Arrowheads indicate double positive cells. 400X magnification. Data are representative of two independent experiments.
**Ceacam1a mRNA is expressed in murine tissues, primary cells, and cell lines.** Since rA59 and rJHM.SD infect multiple cell types not yet shown to express CEACAM1a protein by routine methods, a quantitative RT-PCR (qRT-PCR) approach was taken to assess expression of ceacam1a mRNA in murine tissues, primary CNS cells, and cell lines. Primers were designed to amplify a region of the N-terminal Ig-like domain. This domain contains the site of MHV spike binding and is present in all four known ceacam1a splice variants, thus allowing amplification of total ceacam1a mRNA. Results are expressed as cDNA copies of ceacam1a per million cDNA copies of actb. To determine if mRNA expression data parallels known CEACAM1a protein expression data, RNA isolated from liver, brain, and spinal cord of 4-week-old C57BL/6 mice was subjected to qRT-PCR analysis for total ceacam1a mRNA using the primer set listed in Table 2-1. Consistent with published data that the liver expresses more CEACAM1a protein than the brain (17, 60), ceacam1a mRNA levels in the liver were approximately 10- to 100-fold higher than both brain and spinal cord in wild-type (WT) mice (Fig. 2-2A). To ensure specificity of these primers, qRT-PCR analysis was performed on RNA from ceacam1a<sup>−/−</sup> (KO) mouse tissues. As expected, no product was amplified from ceacam1a<sup>−/−</sup> RNA (Fig. 2-2A), confirming the specificity of the assay.

To evaluate which CNS cell types express ceacam1a, enriched primary cell cultures were generated from WT mice as described in Materials and Methods. RNA isolated from these cell cultures was then subjected to qRT-PCR analysis for total ceacam1a expression. Primary hepatocytes and microglia, two cell types known to express CEACAM1a protein (17, 46), were used as positive controls. As expected, both hepatocytes and microglia were positive for ceacam1a expression, with hepatocytes expressing approximately 10-fold higher levels than microglia (Fig. 2-2B). Interestingly, ceacam1a expression was readily detected in both astrocyte and oligodendrocyte cultures, albeit to lower levels than microglia (Fig. 2-2B). While ceacam1a expression was also detected in cortical and hippocampal neuron cultures, these expression levels were extremely low (Fig. 2-2B). Importantly, since primary cell cultures are enriched but not entirely pure, immunofluorescent labeling was performed as described in Materials and Methods to identify contaminating cell types in the individual cultures. Microglia cultures were routinely
≥98% pure, with rare astrocyte and oligodendrocyte contamination (data not shown). Astrocyte cultures were routinely 90-95% pure, with contaminating microglia and rare oligodendrocytes (data not shown). Oligodendrocyte cultures were routinely 90-95% pure, with contaminating astrocytes and rare microglia (data not shown). Neuron cultures were routinely 95-98% pure, with contaminating astrocytes, oligodendrocytes, and rare microglia (data not shown). It is important to consider these low levels of contaminating cells when interpreting the qRT-PCR results obtained from primary cell cultures.

Murine DBT, 17Cl1, and L2 cell lines are routinely used for propagation and/or titration of MHV. DBT cells are derived from astrocytoma tissue in CDF1 mice (22), whereas 17Cl1 and L2 fibroblasts are derived from the BALB/c 3T3 cell line and C3H/An L929 cell line, respectively (51). Given the different mouse strains of origin as well as ongoing propagation in tissue culture, it was important to characterize the levels of ceacam1a expression in these cell lines. To this end, RNA isolated from DBT, 17Cl1, and L2 cells was subjected to qRT-PCR analysis for total ceacam1a expression. As expected, ceacam1a mRNA was readily detected in all three cell lines (Fig. 2-2C), consistent with their permissiveness to MHV infection in vitro. Furthermore, all cell lines examined showed similar levels of ceacam1a expression (Fig. 2-2C).

WT mice exhibit age-dependent differences in susceptibility to CNS infection with some neurotropic strains of MHV, including rA59. Since ceacam1a expression is developmentally regulated (20), it was of interest to determine if ceacam1a mRNA levels in the CNS changed during adulthood. To this end, RNA was isolated from liver and brain tissue of WT mice aged 2 to 14 weeks. This age range encompasses the different subsets of weanling and adult mice typically used for MHV studies. Interestingly, ceacam1a expression levels were relatively stable in both the liver and brain at all ages observed (Fig. 2-2D).

Previous flow cytometric studies demonstrated that CEACAM1a expression is downregulated on microglia during acute infection of BALB/c mice with the J2.2-V-1 variant of JHM; this downregulation, largely mediated by CD4 T cells, is restored following viral control (46). To determine if ceacam1a expression was altered in the whole brain (including brain-resident cells not easily observed by flow cytometry), C57BL/6 mice were inoculated i.c. with 50 PFU of
rA59 or rJHM.SD and RNA was isolated on days 1-5 post-infection. Within this period of acute infection, ceacam1a expression levels were unchanged compared to mock-infected brains (Fig. 2-2E). Importantly, these ceacam1a expression levels remained constant despite robust levels of virus replication, as demonstrated by qRT-PCR for nucleocapsid messenger RNA (mRNA7) (Fig. 2-2F).

Figure 2-2. Ceacam1a mRNA expression in murine tissues, primary cells, and cell lines.

RNA isolated from A) 4-week-old C57BL/6 mouse tissues, B) primary cell cultures, C) cell lines, D) 2-14-week-old C57BL/6 mouse tissues, and E-F) C57BL/6 mouse brains inoculated i.c. with 50 PFU of rA59 or rJHM.SD were analyzed by qRT-PCR for expression of total ceacam1a mRNA (A-E) or MHV mRNA7 (F). Results are expressed as cDNA copies of ceacam1a per million cDNA copies of actb (A-D) or fold change over mock (E-F). Error bars represent standard error of the mean (n=3). Data are representative of two or more independent experiments. LIV, liver; BR, brain; SC, spinal cord; WT, wild-type; KO, ceacam1a−/−; HEP, hepatocyte; MIC, microglia; AST, astrocyte; OLI, oligodendrocyte; CN, cortical neuron; HN, hippocampal neuron.
*Ceacam1a* splice variant mRNAs are differentially expressed in murine tissues and primary cells. To more fully characterize the expression patterns of *ceacam1a* in CNS tissues and cell types, primer sets were designed to detect *ceacam1a* splice variants expressing 2 versus 4 Ig-like domains (2D vs. 4D) and short versus long cytoplasmic tails (S vs. L). Primer sets are listed in Table 2-1, and a schematic depicting primer amplification sites is shown in Fig. 2-3A. While traditional RT-PCR has been used to distinguish all four splice variants based on product size alone, these assays are complicated by differences in amplification efficiency for shorter versus longer splice variants. While the qRT-PCR assay described here does not allow for distinction of each individual splice variant (2S, 2L, 4S, 4L), it does allow for more careful quantification of 2D versus 4D species and S versus L species. As shown in Fig. 2-3A, 2D primers amplify a region at the splice junction between D1 and D4 whereas 4D primers amplify a region at the junction of D3 and D4. Similarly, S primers amplify the region where exon 7 has been removed by splicing whereas L primers encompass a portion of exon 7 (Fig. 2-3A). Plasmids encoding *ceacam1a-2S* (BgpC) and *ceacam1a-4L* (BgpD) were used to construct standard curves for copy number determination by qRT-PCR.

Using these primer sets, the tissue and cellular RNA described for Fig. 2-2 was analyzed by qRT-PCR to compare splice variant expression. As shown in Fig. 2-3B, 4D variants predominated over 2D in the liver whereas 2D and 4D variants were more uniformly expressed in CNS tissues. Interestingly, while primary hepatocytes mirror the 4D>2D expression observed in the liver, most primary CNS cell types also expressed 4D variants to greater levels than 2D (Fig. 2-3C). This difference in brain expression versus primary cells may reflect the contribution of an additional cell type in vivo, such as endothelial cells, not analyzed in the primary cell panel. A notable exception in the primary cell panel was the oligodendrocyte cultures, which expressed slightly more 2D than 4D species (Fig. 2-3C). This unique ratio of 2D to 4D variants supports the idea that oligodendrocytes themselves express *ceacam1a* and that the total *ceacam1a* expression shown in Fig. 2-2B is not entirely due to contaminating cells. Cell line expression of *ceacam1a* isoforms also differed slightly, with DBT cells expressing relatively equal levels of 2D
and 4D variants while 17Cl1 and L2 fibroblasts expressed higher levels of 4D species compared to 2D (Fig. 2-3D).

Since most functional differences in terms of cell signaling and tumor suppression that have been described for CEACAM1a are due to the length of the cytoplasmic tail (18, 27), tissue and cellular RNA was analyzed for expression of ceacam1a mRNA species encoding S versus L cytoplasmic tails. Interestingly, liver exhibited relatively even expression of S and L variants while L predominated over S in the CNS (Fig. 2-3E). In terms of S versus L tails, the primary cells analyzed mirrored the expression seen in the tissues from which they were derived (Fig. 2-3F). Expression in all three cell lines showed a similar pattern as well, with L species predominating slightly over S (Fig. 2-3G).
Figure 2-3. *Ceacam1a* splice variant expression in murine tissues, primary cells, and cell lines. A) Schematic representing *ceacam1a* splice variants. B-G) RNA isolated from 4-week-old C57BL/6 mouse tissues (B and E), primary cell cultures (C and F), and cell lines (D and G) were analyzed by qRT-PCR for *ceacam1a* expression of 2 versus 4 Ig-like domains (B-D) or short versus long cytoplasmic tails (E-G). Results are expressed as cDNA copies of *ceacam1a* per million cDNA copies of *actb*. Error bars represent standard error of the mean (n=3). *, P ≤ 0.05; **, P ≤ 0.005 as determined by paired t-tests. Data are representative of two independent experiments. 2D, two extracellular Ig-like domains; 4D, four extracellular Ig-like domains; S, short cytoplasmic tail due to exclusion of exon 7; L, long cytoplasmic tail due to inclusion of exon 7.
**rJHM.SD spreads in wild-type neuron cultures despite low levels of infectious virus.** To assess the ability of rA59 and rJHM.SD to infect and spread among neurons, hippocampal neuron cultures were generated from embryonic C57BL/6 mice and inoculated with approximately 1 PFU/cell of filtered rA59, rJHM.SD, or a chimeric recombinant A59 virus expressing the JHM.SD spike (rA59/S\textsubscript{JHM.SD}); virus stocks were filtered prior to neuronal inoculations to remove residual cell debris. Recombinant versions of these viruses expressing EGFP were used in parallel to monitor infection over time and revealed widespread infection with all virus strains by 72 hours post-infection (data not shown). Therefore, cells were fixed at 72 hours post-infection and immunolabeled for MAP2 and MHV nucleocapsid protein as described in Materials and Methods. As expected, all three viruses were able to infect and spread in cultured neurons, as evidenced by co-localization of MAP2 and MHV antigen (Fig. 2-4A); however, foci of rJHM.SD- and rA59/S\textsubscript{JHM.SD}-infected neurons were typically larger than foci of rA59-infected neurons, consistent with previous results (44). To investigate whether spread in neuron cultures was occurring cell-to-cell or via virus release, cell lysates and culture supernatants were collected at 24, 48, and 72 hours post-infection and infectious virus was titrated by standard plaque assay on L2 cell monolayers. Interestingly, while rA59 replicated to relatively high titers within cells, rJHM.SD and the chimeric rA59/S\textsubscript{JHM.SD} titers were approximately 10- to 100-fold lower than rA59 titers at all three timepoints (Fig. 2-4B). Virus release from infected neuron cultures paralleled the intracellular differences, with extracellular rA59 titers exceeding rJHM.SD titers by up to 1000-fold by 72 hours post-infection (Fig. 2-4C). Thus, both rJHM.SD and rA59/S\textsubscript{JHM.SD} spread well in wild-type neurons despite relatively low levels of virus replication and release, suggesting that cell-to-cell spread is likely to be the major route of rJHM.SD dissemination in neurons.
Figure 2-4. MHV infection and replication in wild-type neuron cultures. Hippocampal neurons generated from C57BL/6 mice were inoculated with 1 PFU/cell of rA59, rA59/S_{JHM,SD}, or rJHM.SD. A) Cells were fixed at 72 hours post-infection and dual immunolabeled for MHV nucleocapsid protein (green) and MAP2 (red). 200X magnification. B) Cell lysates and C) culture supernatants were collected at 24, 48, and 72 hours post-infection and infectious virus was titrated by standard plaque assay on L2 cell monolayers. Error bars represent standard error of the mean (n=3), dotted line indicates limit of detection. Data are representative of two or more independent experiments.
**rJHM.SD spreads extensively in ceacam1a<sup>−/−</sup> neurons.** To determine if MHV entry and spread in neurons was dependent on the receptor CEACAM1a, hippocampal neurons were generated from embryonic ceacam1a<sup>−/−</sup> mice and inoculated with approximately 1 PFU/cell of filtered rA59, rJHM.SD, or the chimeric rA59/S<sub>JHM.SD</sub>. Recombinant versions of these viruses expressing EGFP were again used in parallel to monitor the spread of infection (data not shown). Cells were fixed at 72 hours post-infection and immunolabeled for MAP2 and MHV nucleocapsid protein as in Fig. 2-4. Surprisingly, all three viruses were able to infect ceacam1a<sup>−/−</sup> neurons, as evidenced by co-localization of MAP2 and MHV antigen (Fig. 2-5). However, foci of infected cells were much more rare (typically 1-3 foci per coverslip) for all virus strains in ceacam1a<sup>−/−</sup> neuron cultures compared to the numerous coalescing foci of infected cells observed in wild-type neuron cultures (data not shown). Thus, virus infection of neuron cultures was much less efficient in the absence of ceacam1a. Strikingly, though all viruses were able to enter individual cells in the ceacam1a<sup>−/−</sup> neuron cultures, only rJHM.SD and rA59/S<sub>JHM.SD</sub> spread efficiently to adjacent cells (Fig. 2-5). By 72 hours post-infection, rA59 remained localized to one or two ceacam1a<sup>−/−</sup> neurons while viruses expressing the JHM.SD spike spread similarly in ceacam1a<sup>−/−</sup> and wild-type cultures (Fig. 2-4A and 5). Notably, similar differences in spread were observed in blocking experiments in which wild-type neurons were first infected with rA59 or rJHM.SD and then treated with CEACAM1a-blocking antibody (data not shown). Thus, while MHV entry in neural cells appears largely dependent on ceacam1a expression, only rA59 appears to require ceacam1a expression for efficient cell-to-cell neuronal spread. This finding is consistent with previous studies showing that ceacam1a<sup>−/−</sup> mice are susceptible to infection with viruses expressing the JHM.SD spike but resistant to rA59 infection (35).
**Figure 2-5. MHV infection in ceacam1a−/− neuron cultures.** Hippocampal neurons generated from ceacam1a−/− mice were inoculated with 1 PFU/cell of rA59, rA59/SJHM.SD, or rJHM.SD. Cells were fixed at 72 hours post-infection and dual immunolabeled for MHV nucleocapsid protein (green) and MAP2 (red). 200X magnification. Data are representative of two or more independent experiments.
**Ceacam2 mRNA is expressed at low levels in the murine liver and CNS.** To determine if an alternative CEACAM1a-related receptor might be positioned to mediate viral infection/spread in ceacam1a−/− mice and neuron cultures, primers were designed to amplify the N-terminal Ig-like or equivalent domain of ceacam2 and psg16 (bCEA), two ceacam1a-related genes that encode proteins that serve inefficiently as MHV receptors when overexpressed in vitro (4, 40). Primer sequences are shown in Table 2-1. Using the tissue and cellular RNA described for Fig. 2-2, qRT-PCR was performed to detect expression of ceacam2, the most closely related gene to ceacam1a in the mouse. Results are expressed as cDNA copies of ceacam2 per million cDNA copies of actb. As shown in Fig. 2-6A, ceacam2 mRNA is expressed to similarly low levels in both liver and CNS tissue of wild-type (WT) mice. Notably, CNS expression of ceacam2 was relatively unchanged in the absence of ceacam1a (KO) while liver expression was slightly increased compared to WT (Fig. 2-6A); however, only one set of KO tissues (n=3) was available for qRT-PCR analysis. Interestingly, despite detectable levels of ceacam2 mRNA in both liver and CNS tissue, low to no expression of ceacam2 was observed in the primary cell types studied, with only microglia and oligodendrocyte cultures showing consistent low-level expression (Fig. 2-6B). Thus, other resident cell types, such as endothelial cells, likely contribute to the ceacam2 expression observed in liver and CNS tissue. Interestingly, when cell lines were observed, ceacam2 expression was detectable in L2 and to a lesser extent DBT but not 17Cl1 cells (Fig. 2-6C). As with ceacam1a, ceacam2 expression remained relatively constant from 2-14 weeks of age (Fig. 2-6D) and was not markedly affected by MHV infection (Fig. 2-6E).
Figure 2-6. *Ceacam2* mRNA expression in murine tissues, primary cells, and cell lines.

RNA isolated from A) 4-week-old C57BL/6 mouse tissues, B) primary cell cultures, C) cell lines, D) 2-14-week-old C57BL/6 mouse tissues, and E) C57BL/6 mouse brains inoculated i.c. with 50 PFU of rA59 or rJHM.SD were analyzed by qRT-PCR for expression of *cecam2* mRNA. Results are expressed as cDNA copies of *cecam2* per million cDNA copies of *actb* (A-D) or fold change over mock (E). Error bars represent standard error of the mean, n=3. Data are representative of two or more independent experiments.
**Psg16 (bCEA) mRNA is expressed in the murine CNS and primary neurons.** Using the tissue and cellular RNA described for Fig. 2-2, qRT-PCR was performed to detect expression of psg16 (bCEA), a more distantly related gene to ceacam1a described solely in the murine CNS (4). Results are expressed as cDNA copies of psg16 per million cDNA copies of actb. Consistent with the previously published data (4), psg16 expression was readily detected in the brain and spinal cord of both WT and KO mice while expression was negligible in the liver (Fig. 2-7A). Interestingly, when RNA from primary cells was examined, psg16 expression was detected exclusively in neurons (Fig. 2-7B). DBT, 17Cl1, and L2 cell lines were all negative for psg16 expression (Fig. 2-7C). As with ceacam1a and ceacam2, psg16 expression remained constant in the murine CNS from 2-14 weeks of age (Fig. 2-7D) and was not significantly affected by MHV infection (Fig. 2-7E). The neuronal localization of psg16 expression suggested PSG16 might substitute for CEACAM1a to facilitate MHV infection in neurons. Therefore, functionality of this receptor was investigated.
Figure 2-7. *Psg16* (bCEA) mRNA expression in murine tissues, primary cells, and cell lines.

RNA isolated from A) 4-week-old C57BL/6 mouse tissues, B) primary cell cultures, C) cell lines, D) 2-14-week-old C57BL/6 mouse tissues, and E) C57BL/6 mouse brains inoculated i.c. with 50 PFU rA59 or rJHM.SD were analyzed by qRT-PCR for expression of *psg16* mRNA. Results are expressed as cDNA copies of *psg16* per million cDNA copies of *actb* (A-D) or fold change over mock (E). Error bars represent standard error of the mean (n=3). Data are representative of two or more independent experiments.
**CEACAM2 mediates MHV entry in human 293T cells.** While CEACAM2 and PSG16 are both known to mediate inefficient MHV infection when overexpressed *in vitro* (4, 40), their functionality as MHV receptors has not been directly compared. Furthermore, PSG16 reportedly serves as a receptor for A59 but not JHM (4). To evaluate receptor activity, human 293T cells were transfected with equal amounts of plasmid DNA encoding CEACAM1a, CEACAM2, or PSG16 using FuGENE 6 Transfection Reagent as described in Materials and Methods. At 36 hours post-transfection, cells were inoculated with approximately 1 PFU/cell of rA59 or rJHM.SD and fixed at 8 hours post-infection for immunofluorescent detection of MHV antigen; this timepoint was selected to allow approximately one round of MHV infection. Parallel transfection with a plasmid encoding GFP was performed to assess transfection efficiency (Fig. 2-8C). Quantification of MHV infection is shown in Fig. 2-8B. As expected, CEACAM1a mediated robust infection with both rA59 and rJHM.SD, whereas CEACAM2 was much less efficient at mediating entry (Fig. 2-8A and 2-8B). Importantly, no MHV antigen staining was observed in mock-infected cells or in cells transfected with vector alone (Fig. 2-8A). Furthermore, transfection of higher amounts of receptor cDNA did not increase infection rates but did, particularly in the case of CEACAM1a, cause cells to cluster together (data not shown). Interestingly, PSG16 did not mediate infection with either rA59 or rJHM.SD in this assay (Fig. 2-8A), suggesting inefficient expression levels or a complete lack of cell surface expression and/or lack of receptor functionality.
Figure 2-8. MHV infection of 293T cells transfected with receptor. Human 293T cells were transfected with expression plasmids encoding CEACAM1a, CEACAM2, PSG16, or empty vector using FuGENE 6 Transfection Reagent. A) Cells were infected at 36 hours post-transfection with approximately 1 PFU/cell of rA59 or rJHM.SD, fixed at 8 hours post-infection, and immunolabeled for MHV nucleocapsid protein. B) Quantification of infected cells. Error bars represent standard error of the mean (n=3). C) GFP transfection control indicating transfection efficiency. 200X magnification. Data are representative of two or more independent experiments.
DISCUSSION

MHV utilizes murine CEACAM1a proteins as entry receptors, yet CEACAM1a expression in the CNS is remarkably low compared to expression levels in other MHV targets such as the liver (17, 60). Furthermore, in the brain, only endothelial cells and microglia have been shown to express CEACAM1a (17, 46). Thus, it was unclear how neurotropic strains of MHV infect additional CNS cell types in vivo, such as astrocytes, oligodendrocytes, and particularly neurons (Fig. 2-1). One possibility is that these cell types express low levels of CEACAM1a that are not detectable by routine methods and/or that CEACAM1a is sublocalized, such as at the neuronal synapse, such that antibodies cannot access it. Using immunohistochemical amplification techniques such as Tyramide Signal Amplification (PerkinElmer) designed for detection of low-level protein targets, we were unable to demonstrate CEACAM1a expression on neurons or glia, including microglia (data not shown). Thus, to avoid the potential caveats of antibody-based techniques, we instead used qRT-PCR to assess expression of ceacam1a mRNA in enriched CNS cell cultures. Importantly, our qRT-PCR data for ceacam1a expression corresponded with published protein data for the liver and brain (17, 60), with liver expressing significantly higher levels of ceacam1a than the brain in our assays (Fig. 2-2A). Furthermore, expression levels in the brain and liver remained stable in mice 2-14 weeks of age (Fig. 2-2D), suggesting that age-related differences in susceptibility to MHV are not due to differences in ceacam1a expression. As expected, ceacam1a mRNA expression was highest in primary hepatocytes and microglia (Fig. 2-2B), the two cell types in this panel known to express CEACAM1a protein (17, 46). Interestingly, decreasing levels of ceacam1a expression were also detected in oligodendrocyte, astrocyte, and neuron cultures (Fig. 2-2B). However, when interpreting this data, it is important to consider the level of cellular contaminants in these enriched cultures. Astrocyte cultures were 90-95% pure, with microglia being the primary contaminant; thus, it is difficult to distinguish low-level ceacam1a expression in astrocytes from higher-level expression by contaminating microglia. Expression of ceacam1a in neuron cultures was quite low and may also be due to low-level microglia contamination. However, the striking difference in neuronal spread observed in WT and KO cultures infected with rA59 suggests to us that neurons express CEACAM1a, though possibly
at very low levels. Interestingly, ceacam1a expression was readily detectable in oligodendrocyte cultures; these cultures were 90-95% pure, with astrocytes being the primary contaminant. Furthermore, the ratio of 2D to 4D splice variants in oligodendrocyte cultures was inverted compared to the ratio in microglia (Fig. 2-3C). Thus, the ceacam1a expression detected in these cultures is unlikely to be due entirely to contaminating microglia. Future studies aim to purify glial cells from the adult CNS and isolate RNA from these purified cell populations as described by Malone et al. (30) in order to reduce the complication of contaminating cells and to investigate ceacam1a expression in cells directly ex vivo.

CEACAM1a is a multifunctional protein involved in a variety of cellular processes including intercellular adhesion, tumor suppression, angiogenesis, and immune cell signaling (18, 27). Many of these signaling and regulatory functions are specific to long-tailed CEACAM1a isoforms, which contain phosphorylatable tyrosine residues within an immunoreceptor tyrosine-based inhibitory motif (ITIM). These residues have been shown to participate in protein-protein interactions and downstream signaling cascades in a variety of cell types, including T cells (5) and dendritic cells (24). Furthermore, the balance of short (S) to long (L) isoforms in a particular cell likely contributes to signaling outcome (18). Thus, from the standpoint of host cell signaling, it was of interest to evaluate expression patterns of individual ceacam1a splice forms, particularly those encoding S versus L tails. In our studies, L splice variants predominated over S in the brain while the two were more balanced in liver, and this trend was recapitulated in primary CNS cell types (Fig. 2-3E and 2-3F). Thus, it is tempting to speculate that MHV binding to long-tailed CEACAM1a isoforms may trigger or modulate intracellular signaling pathways in neural cells in ways that are not yet appreciated, and CEACAM1a-dependent versus CEACAM1a-independent mechanisms of infection/spread could lead to different signaling outcomes in an infected cell. Future studies aim to investigate CEACAM1a signaling during MHV infection. While expression of 2D versus 4D splice variants in the CNS was more balanced than liver (Fig. 2-3B), both microglia and astrocyte cultures expressed significantly higher levels of the 4D variants (Fig. 2-3C). However, since 2D and 4D isoforms both mediate MHV entry, the functional significance of this difference, if any, in unknown.
If neurons, astrocytes, and/or oligodendrocytes are indeed CEACAM1a-negative, MHV might use an alternative receptor to enter these cells; such a receptor would likely be less functional than CEACAM1a and/or more limited in its availability given the reduced susceptibility of ceacam1a−/− mice to CNS infection with MHV. Two ceacam1a-related genes, ceacam2 and psg16 (bCEA), are expressed in the brain and encode proteins reported to act as MHV entry receptors when overexpressed in vitro (4, 40). However, it was unclear what cell types express these alternative receptors in the CNS. To address this question, qRT-PCR was again used to quantify expression of ceacam2 and psg16 in primary CNS-derived cells. Interestingly, ceacam2 mRNA levels in these cell types were quite low despite more readily detectable levels in whole brain (Fig. 2-6A and 2-6B). While microglia and oligodendrocyte cultures were the most highly positive, it is difficult to know if such expression levels are sufficient to encode functional levels of receptor on the cell surface. Furthermore, other cell types not examined, such as endothelial cells, likely contribute to the overall expression levels observed in CNS tissue (Fig. 2-6A). Consistent with previous reports (4), psg16 expression was indeed observed in the CNS; strikingly, this expression was detected in cortical and hippocampal neuron cultures only (Fig. 2-7B). To our knowledge, this is the first report of a pregnancy-specific glycoprotein family member expressed in cultured neurons, though the role of PSG16 in these cells remains unclear.

To directly compare the ability of these alternative receptors to mediate MHV entry, we transfected cDNA clones of CEACAM1a, CEACAM2, and PSG16 into human 293T cells (not normally infected by MHV), allowed time for protein expression to occur, and then inoculated with rA59 or rJHM.SD. Consistent with previous reports, CEACAM2 mediated infection with both strains but was considerably less efficient than CEACAM1a (Fig. 2-8A and 2-8B). The lower efficiency of CEACAM2 compared to CEACAM1a is likely due to structural differences at the site of MHV binding, though differences in receptor density and surface distribution could also contribute. While PSG16 is reported to be a receptor for A59 but not JHM (4), no infection of PSG16-transfected cells was observed for either rA59 or rJHM.SD in our assay (Fig. 2-8A and 2-8B). However, we could not confirm that PSG16 was actually expressed on the 293T cell surface. Computer algorithms were also unable to identify a transmembrane region or membrane anchor
in the sequence, nor could they identify a traditional secretory signal. To address the issue of surface expression, PSG16 was artificially linked to either a transmembrane domain or a GPI anchor, expressed in 293T cells, and inoculated with virus. Curiously, even when PSG16 was successfully targeted to the cell surface, no infection was observed with rA59 or rJHM.SD (Judith Phillips, unpublished data). Notably, when CEACAM1a was attached to the membrane via a GPI anchor MHV infection was indeed observed (Judith Phillips, unpublished data), indicating that the extracellular Ig-like domains of CEACAM1a are sufficient to mediate MHV entry when attached to the cell membrane in the absence of a transmembrane domain and cytoplasmic tail. It is possible that PSG16 is normally expressed in conjunction with another protein or membrane entity in the mouse and that this entity is lacking in human 293T cells. Interestingly, the placental members of the PSG family are largely secretory proteins, several of which have immunomodulatory functions (19, 59). Thus, it is intriguing to imagine that PSG16 expressed by neurons might be released under certain conditions to provide a signal to neighboring cells. Since primary neurons did not express ceacam2 (Fig. 2-6) and the product of psg16 appears to be nonfunctional (Fig. 2-8), it remains unclear how MHV initially enters ceacam1a<sup>−/−</sup> neurons (Fig. 2-5). It is possible that an alternative PSG16 isoform or PSG16 in association with another surface protein specific to neurons may mediate infection in this cell type, though previous studies suggest this mechanism would only function for A59 entry (4). It is also possible that neurons express another, thus far unidentified, receptor that can mediate inefficient MHV entry in this cell type. Furthermore, rJHM.SD could enter a non-neuronal cell type first (perhaps via CEACAM2) and subsequently spread to neurons via a CEACAM1a-independent mechanism, as has been described in vitro.

Finally, neurotropic strains of MHV may have evolved a novel mechanism to spread to and between specialized CNS cell types, particularly neurons. Using mixed neural cultures, Nakagaki et al. (36) demonstrated that the highly neurovirulent JHM cl-2 isolate spreads to non-microglial cells in the presence of CEACAM1a antibody blockade; in contrast, a soluble receptor resistant mutant of cl-2 known as srr7 remains localized to microglia when CEACAM1a is blocked. From these experiments, it was proposed that initial CNS infection with MHV occurs via entry into CEACAM1a-expressing microglia and that spread to additional CNS cell types requires
the capacity for CEACAM1a-independent spread. While microglia may indeed represent an early
site of MHV replication in the CNS, this model is insufficient to explain the diverse cellular tropism
of rA59 (Fig. 2-1), particularly since rA59 requires CEACAM1a for efficient cell-to-cell spread in
vitro (56). Further, while in vitro studies indicate that both rA59 and rJHM.SD require CEACAM1a
for initial entry (15, 54), this does not seem to be the case in vivo as rJHM.SD can infect cells in
the CNS of ceacam1a−/− mice (35). These results suggest that more than one mechanism may be
involved during infection of ceacam1a−/− animals: 1) initial entry in the absence of CEACAM1a and
2) cell-to-cell spread in the absence of CEACAM1a (which may or may not be the same as
CEACAM1a-independent spread observed in vitro). Thus, the inability of rA59 to cause CNS
disease in ceacam1a−/− mice could be the result of its dependence on CEACAM1a for cell entry
and/or cell-to-cell spread. These possibilities are difficult to distinguish in whole brain, thus we
devised an in vitro system using primary cells derived from wild-type C57BL/6 and ceacam1a−/−
mice to address these questions.

While most CNS cell types can be infected by MHV in vivo, neurons are the predominant
cell type infected by rA59 and rJHM.SD (Fig. 2-1) (11, 44). For this reason, primary neurons were
the cell type of choice to model infection in vivo. We first aimed to compare infection and
replication of rA59, rJHM.SD, and the chimeric rA59/SJHM.SD in wildtype (WT) neurons versus
ceacam1a−/− (KO) cells. When WT neurons were inoculated with MHV, foci of infected cells were
readily detected throughout the coverslip (Fig. 2-4A). Parallel infections using EGFP-expressing
viruses revealed that these foci increased in size from 24-72 hours postinfection (data not
shown), presumably due to cell-to-cell spread. Viral titration of culture supernatants and cell
lysates at 24, 48, and 72 hours post-infection revealed that rA59 infection resulted in higher levels
of infectious virus, both inside and outside the cell, compared to both rJHM.SD and rA59/SJHM.SD
(Fig. 2-4B and 2-4C). Thus, as in the brain (23, 43), widespread neuronal infection with viruses
expressing the rJHM.SD spike occurs in the absence of high levels of infectious virus. It should
be noted, however, that the JHM.SD spike is inherently less stable than that of A59 and may
inactivate more rapidly following release from an infected cell (13, 26, 52). Thus, it is possible that
some extracellular virus was no longer infectious at the time of sampling due to spontaneous

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inactivation. However, this is less likely to be the case intracellularly, where rJHM.SD titers were also lower than rA59 (Fig. 2-4B).

Given that rA59 and rJHM.SD both infect and spread well in WT neurons (Fig. 2-4A), we next asked whether these strains could infect neuron cultures generated from ceacam1a<sup>−/−</sup> (KO) mice. Surprisingly, occasional foci of infected cells (typically 1-3 per coverslip) were observed for both strains as well as with the chimeric rA59/S<sub>JHM.SD</sub> virus (Fig. 2-5). This result was initially surprising given previous reports that CEACAM1a antibody blockade prevents MHV infection in mixed neural cultures (36). However, given the low incidence of infection seen in ceacam1a<sup>−/−</sup> neurons, it is possible that the phenomenon was not appreciated in these blocking experiments or attributed to incomplete antibody blockade. Importantly, the results in Fig. 2-5 are consistent with previous data that JHM.SD successfully infects the CNS of ceacam1a<sup>−/−</sup> mice (35). Notably, as infection proceeded from 24-72 hours post-infection, differences in spread became apparent, with rA59 remaining localized to individual cells while rJHM.SD and rA59/S<sub>JHM.SD</sub> spread extensively, generating foci of infected cell that were indistinguishable from infected foci in WT neurons (Fig. 2-5). Further, while efficient cell-to-cell spread in KO neuron cultures largely maps to the JHM.SD spike (Fig. 2-5), variability in the extent of spread by rA59/S<sub>JHM.SD</sub> in these cultures suggests viral genes other than spike may also contribute to the neuronal spread of rJHM.SD (data not shown). Interestingly, when microglia cultures derived from ceacam1a<sup>−/−</sup> mice were infected by rJHM.SD, large foci of infected cells were not observed and cell-to-cell spread was limited to just a few cells (data not shown). Thus, the ability of rJHM.SD to spread in the absence of CEACAM1a may be specifically enhanced in neurons compared to other CNS cell types. Additional viral surface proteins, such as hemagglutinin-esterase (HE), or the internal viral nucleocapsid (N) protein, which shares homology with the microtubule binding protein tau (42), may contribute in unique ways to enhance neuronal spread of MHV, though such activity may not be limited to the rJHM.SD virus (7, 25).

Based on these data, we propose a model for ceacam1a<sup>−/−</sup> mouse infection in which both rA59 and rJHM.SD can enter occasional CNS cells lacking ceacam1a expression; this initial entry may occur via CEACAM2 on endothelial cells, microglia, and/or oligodendrocytes, via an
alternative form of PSG16 that has not yet been described, and/or via another as-yet-unidentified alternative receptor that can facilitate virus entry, albeit inefficiently. Once initial entry has occurred, a CEACAM1a-independent mechanism allows viruses expressing the JHM.SD spike to spread cell-to-cell. Thus, rA59 remains restricted to individual cells and does not produce detectable CNS infection while rJHM.SD and rA59/SJHM.SD spread efficiently to adjacent cells and ultimately lead to fatal neurological disease. While the precise mechanism of CEACAM1a-independent spread remains unclear, one possibility is that an alternative receptor can interact with rJHM.SD but not rA59; differences between the JHM.SD and A59 spikes could likely allow for differences in receptor utilization by these strains. Another possibility is that the less stable association of the S1 and S2 subunits of the JHM.SD spike allows conformational changes leading to fusion to be more easily triggered even in the absence of a traditional receptor, as has been proposed for spread among tissue culture cells lacking CEACAM1a expression (14, 26). Finally, JHM.SD may have developed a way to spread across the neuronal synapse; such mechanisms of transynaptic spread have been described for other neurotropic viruses. In support of this idea, low levels of infectious rJHM.SD are detected extracellularly despite widespread distribution of viral antigen in neurons in vitro.

Several amino acid residues within the JHM.SD spike have been identified in relation to CEACAM1a-independent spread or lack thereof. For example, introduction of a S310G substitution within the spike of JHM.IA (a CEACAM1a-dependent virus) confers increased virulence and the ability to spread cell-to-cell in the absence of CEACAM1a in vitro (41). Such a mutation could contribute to any of the aforementioned mechanisms of spread. Additionally, a L1114F substitution within the spike of the J2.2-V-1 glial-tropic variant of JHM-DL (57) and in the spike of the highly attenuated srr7 mutant of JHM cl-2 (48) is associated with an inability to induce CEACAM1a-independent fusion in mixed glial cultures (31, 32, 53); these viruses are neuroattenuated compared to their parental strains, further indicating the contribution of CEACAM1a-independent spread to enhanced neurovirulence. Viruses such as J2.2-V-1 with the L1114F substitution in spike are primarily glial-tropic and rarely infect neurons in vivo. However, a similar variant with a L1114R substitution, despite being CEACAM1a-dependent, infects neurons
of the olfactory bulb (55). Thus, different amino acid residues at the same position in spike have unique effects on neurotropism.

It remains unclear how neurotropic strains of MHV spread to CNS cell types expressing low to nonexistent levels of CEACAM1a, particularly neurons, and how viruses that use the same entry receptor can exhibit such varied cellular tropisms. However, the ability of rJHM.SD to spread in the absence of CEACAM1a likely contributes to neurovirulence by increasing viral dissemination throughout the CNS. Future studies using the ceacam1a<sup>−/−</sup> mouse and primary cell models combined with the variety of neurotropic MHV strains differing in tropism and CEACAM1a dependence will aim to address the mechanism of CEACAM1a-independent spread in neural cells and the contribution of this process to neurovirulence.

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REFERENCES


CHAPTER 3

Priming of CD8 T cells during central nervous system infection with a murine coronavirus is strain dependent.

This chapter appeared as the published article “Priming of CD8+ T cells during central nervous system infection with a murine coronavirus is strain dependent” by K. C. MacNamara,* S. J. Bender,* M. M. Chua, R. Watson, and S. R. Weiss. Journal of Virology, 82: 6150-6160 (2008).

*KCM and SJB contributed equally to this work.
ABSTRACT

Virus-specific CD8+ T cells are critical for protection against neurotropic coronaviruses; however, CNS infection with the recombinant JHM (RJHM) strain of mouse hepatitis virus (MHV) elicits a weak CD8+ T cell response in the brain and causes lethal encephalomyelitis. An adoptive transfer model was used to elucidate the kinetics of CD8+ T cell priming during CNS infection with RJHM as well as two MHV strains that induce a robust CD8+ T cell response (RA59 and SJHM/RA59, a recombinant A59 virus expressing the JHM spike). While RA59 and SJHM/RA59 infections resulted in CD8+ T cell priming within the first 2 days post-infection, RJHM infection did not lead to proliferation of naïve CD8+ T cells. While all three viruses replicated efficiently in the brain, only RA59 and SJHM/RA59 replicated to appreciable levels in the cervical lymph nodes (CLN), the site of T cell priming during acute CNS infection. RJHM was unable to suppress the CD8+ T cell response elicited by RA59 in mice simultaneously infected with both strains, suggesting RJHM does not cause generalized immunosuppression. RJHM was also unable to elicit a secondary CD8+ T cell response in the brain following peripheral immunization against a viral epitope. Notably, the weak CD8+ T cell response elicited by RJHM was unique to CNS infection, as peripheral inoculation induced a robust CD8+ T cell response in the spleen. These findings suggest that RJHM fails to prime a robust CD8+ T cell response during CNS infection due to inaccessibility of viral antigen in the brain and/or limited availability of viral antigen in the CLN.
INTRODUCTION

Members of the *Coronaviridae* family infect a wide range of mammalian species including humans and induce mild to severe disease of the respiratory tract, gastrointestinal tract, and central nervous system (CNS). Mouse hepatitis virus (MHV) infection provides a useful model to study acute and chronic CNS disease and specifically the process of demyelination, the hallmark of the human disease multiple sclerosis. Different strains of MHV induce disease with varying degrees of severity. For example, CNS infection with the recombinant wild-type A59 (RA59) strain causes acute encephalitis during the first week of infection; a strong CD8+ T cell response is observed in the brain coinciding with viral clearance. However, despite clearance of infectious RA59 virus, demyelination develops, peaking at approximately four weeks post-infection (p.i.) (17, 20). In contrast, infection with the recombinant wild-type JHM (RJHM) strain (derived from the JHM isolate referred to as MHV-4 or JHM.SD (7, 28)) causes severe encephalomyelitis; virus is not cleared and mice typically succumb to disease by the end of the first week of infection. Furthermore, RJHM infection of the CNS elicits a very weak virus-specific CD8+ T cell response in the brain (7, 20, 34). However, we have only examined the most virulent strain of JHM. It should be noted that there are other strains of JHM that have deletions and mutations within the spike glycoprotein, rendering them less virulent and sometimes resulting in a change in cell tropism. The ability of neurotropic strains of MHV to replicate within cells of the CNS and cause disease of varying degrees is ideal for allowing the dissection of both viral and host determinants of neuropathogenesis.

The spike glycoprotein of MHV is a major determinant of neurovirulence (32). It controls virus tropism and spread as it both binds the cellular receptor and induces fusion with target cells. In addition, it encodes neutralizing antibody epitopes and the H-2b-restricted CD8+ T cell epitopes recognized in C57BL/6 (B6) mice. The A59 spike differs from the JHM spike in that it contains a deletion of 52 amino acids within the hypervariable region (HVR). The HVR has been well documented to tolerate mutation but with attenuating effects on virulence (5, 7). RA59 and RJHM both encode an H-2Kb epitope at position S598-605 (S598); however, due to the deletion, the A59 spike lacks the immunodominant H-2Db epitope at position S510-519 (S510). We previously
selected isogenic recombinant viruses that express the JHM spike with all other genes derived from the A59 strain of MHV (SJHM/RA59). The isogenic SJHM/RA59 has a similar fifty percent lethal dose (LD_{50}) to RJHM, demonstrating that the JHM spike is sufficient to generate a highly neurovirulent phenotype and increased ability to spread within the CNS (32, 33). However, SJHM/RA59-infected mice exhibit slower kinetics of death compared to RJHM-infected mice, and notably, unlike RJHM, the chimeric SJHM/RA59 virus induces a strong CD8+ T cell response in the brain (14, 34).

In addition to spike, there is increasing evidence that other viral genes play an important role in pathogenesis. We (14, 21) and others (34, 35) have noted that the low CD8+ T cell response observed during RJHM infection is not dependent on spike since the SJHM/RA59 recombinant induces a robust virus-specific CD8+ T cell response. The difference in CD8+ T cell response elicited by SJHM/RA59 and RJHM may explain why SJHM/RA59 kills mice more slowly than RJHM. Furthermore, the reverse chimeric recombinant virus expressing the A59 spike in the JHM background (SA59/RJHM) is unable to replicate in the liver despite the fact that it expresses the spike from the hepatotropic RA59 strain (27), suggesting that background genes play a significant role in viral tropism.

It is well established that virus-specific CD8+ T cells play a protective role against MHV and are essential for clearance of infectious virus from the CNS (6, 20, 40, 41). The effector mechanisms exerted by activated, virus-specific CD8+ T cells include the ability to secrete cytokines as well as the ability to lyse target cells. Interferon gamma (IFN-γ) expression is essential for clearance of MHV from the brain (3, 22, 29), and perforin-mediated lysis of infected cells also appears to play a role in viral clearance (6, 31). In contrast to infection with RA59 or the relatively neuroattenuated glial-tropic strains of JHM, CNS infection with the highly neurovirulent RJHM strain results in very low numbers of activated, virus-specific CD8+ T cells in the spleen and brain (14, 34). Furthermore, RJHM infection induces a different expression profile of cytokines and chemokines in the brains of infected mice compared to infection with RA59 (34, 35, 38). One dramatic difference is that RA59 infection results in a robust IFN-γ response whereas
RJHM infection results in higher, sustained levels of IFN-β (34). These observations prompted us to address the following questions: 1) Does RJHM elicit a CD8+ T cell response in the brain following intranasal inoculation, a route that requires more virus and results in slower infection compared to intracranial inoculation? 2) What are the kinetics of CD8+ T cell priming during CNS infections with RA59, SJHM/RA59, and RJHM? 3) Is CNS infection with RJHM generally immunosuppressive? 4) Do RA59, SJHM/RA59, and RJHM replicate efficiently in the draining cervical lymph nodes? 5) Can RJHM elicit a secondary CD8+ T cell response in the brain following peripheral immunization against a viral epitope? 6) Is the low CD8+ T cell response elicited during RJHM infection an inherent characteristic of the viral strain or specific to RJHM infection of the CNS? Our results suggest that RJHM fails to prime a CD8+ T cell response specifically during infection of the CNS without causing generalized immunosuppression, and this lack of priming correlates with a low level of RJHM replication in the draining cervical lymph nodes, the site of CD8+ T cell priming during acute CNS infection.
MATERIALS AND METHODS

Mice and viruses. Four- to five-week-old male mice were used in all experiments. C57BL/6 (B6) and B6-LY5.2/Cr (CD45.1+) mice were obtained from the National Cancer Institute (Frederick, MD). P14 mice (CD45.2+) (4) were bred at the University of Pennsylvania. Recombinant MHV strains A59 (RA59), JHM (RJHM), and a chimeric virus expressing the JHM spike in the A59 background (SJHM/RA59, originally referred to as S4R22) were selected by targeted recombination as described elsewhere (20, 32). Recombinant A59 and SJHM/RA59 expressing enhanced green fluorescent protein (EGFP) in the place of nonessential gene 4 (referred to as RA59-gfp and SJHM/RA59-gfp, respectively) were selected by targeted recombination as previously described (37). RJHM-gfp was selected using similar techniques. Selection of RA59-gfp expressing the H-2^b^-restricted gp33 epitope of lymphocytic choriomeningitis virus (LCMV) as an N-terminal fusion protein to EGFP in place of gene 4 (referred to as RA59-gfp/gp33) is described in detail elsewhere (6). RJHM and SJHM/RA59 expressing the gfp/gp33 fusion were selected similarly via targeted recombination.

Inoculation of mice. For intracranial (i.c.) inoculations, mice were anesthetized with isoflurane and virus was injected into the left cerebral hemisphere in a total volume of 30 μL phosphate-buffered saline (PBS) containing 0.75% bovine serum albumin (BSA). For intranasal (i.n.) inoculations, virus was applied directly to the nostrils of a slightly anesthetized mouse in a total volume of 20 μL PBS containing 0.75% BSA. For intraperitoneal (i.p.) inoculations, virus was injected in a total volume of 100 μL PBS containing 0.75% BSA. Doses for individual experiments are indicated in the figure legends.

Isolation of mononuclear cells. Mononuclear cells were prepared from the brain as previously described (6, 30). Brains from 4-6 animals were pooled per group. Briefly, animals were perfused with 10 mL PBS. Brains were placed in ice-cold RPMI 1640 medium containing 10% fetal bovine serum (FBS) and homogenized through a nylon mesh bag (pore diameter 64 μm) using a syringe plunger. Cells were passed through a 30% Percoll gradient and then through a cell strainer (pore diameter 70 μm). The cell suspension was layered atop a 2 mL cushion of Lympholyte-M.
(Cedarlane Laboratories) and viable cells were removed from the interface, washed, and counted. Mononuclear cells were prepared from the spleen as previously described (6); this method was also used to isolate cells from the cervical lymph nodes (CLN). Briefly, tissues were homogenized through a nylon mesh bag (pore diameter 64 μm) in RPMI 1640 medium containing 1% FBS. Red blood cells were lysed with 0.83% NH₂Cl, and the remaining cells were washed and counted.

**Intracellular cytokine staining and flow cytometry.** Intracellular IFN-γ production was assayed in response to specific peptide stimulation as previously described (26, 33). Briefly, 1 × 10⁶ brain- or spleen-derived mononuclear cells were cultured with 10 units human recombinant IL-2 and 1 μL/mL Brefeldin A (Golgiplug; BD Biosciences) in the presence or absence of 1 μg/mL peptide in a total volume of 200 μL RPMI 1640 medium supplemented with 5% FBS for 5 hours at 37°C. Cells were stained for surface expression of CD4, CD8, and/or CD45.2 using fluorescently conjugated monoclonal antibodies (BD Pharmingen). Cells were then fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) and stained with a FITC-conjugated monoclonal rat anti-mouse IFN-γ antibody (BD Pharmingen). Cells were fixed in 2% paraformaldehyde and analyzed using a FACScan or FACSCalibur flow cytometer (Becton Dickinson). Total cell numbers per mouse were determined by multiplying the fraction of live cells positive for a given marker by the total number of live cells isolated per organ.

**CFSE staining of spleen-derived mononuclear cells for adoptive transfer.** Mononuclear cells were prepared from the spleens of P14 mice as described above. Cells were incubated with 5 μM CFSE for 10 minutes with periodic agitation. Staining was quenched by the addition of an equal volume of FBS and cells were washed three times with PBS. Cells were then counted and resuspended in PBS for adoptive transfer into B6 mice. 2 × 10⁷ cells were injected intravenously (i.v.) into the lateral tail vein in a total volume of 0.5 mL PBS.

**Mononuclear cell proliferation analysis.** Mononuclear cells were isolated from the spleen or CLN as described above. Since CFSE labeling was performed on different days, it is important to note that the peak fluorescence intensity is not always the same; only those transfers performed
on the same day can be compared. The proliferative index (PI), the average number of divisions undergone by the divided population, was determined using FlowJo software (Tree Star, Inc.).

**Virus replication in mice.** To measure *in vivo* virus replication, mice were sacrificed and perfused with 10 mL PBS. Brains and/or CLN were placed in 1-3 mL gel saline, an isotonic saline solution containing 0.167% gelatin, weighed, and stored frozen at -80°C. Tissues were subsequently homogenized and standard plaque assays were performed on murine L2 fibroblast monolayers (13). Neutralization assays were performed using monoclonal antibodies raised against either the JHM spike (J7.2 and J7.18) or the A59 spike (A2.1); these antibodies were a kind gift from John Fleming (University of Wisconsin, Madison).

**Listeria immunization.** Recombinant *Listeria monocytogenes* (rLm) strains expressing the H-2b-restricted gp33-41 epitope (KAVYNFATC; referred to as gp33) or the H-2d-restricted np118-126 epitope (RPQASGVYM; referred to as np118) from LCMV were engineered as previously described (36, 39). Both epitopes are expressed as fusion proteins with dihydrofolate reductase. Strain XFL703 expresses the gp33 epitope and will be referred to as rLm-gp33. Strain XFL303 expresses the np118 epitope and will be referred to as rLm-np118. Mice were inoculated i.p. with $10^4$ CFU of rLm in 0.5 mL PBS, rested for three weeks, and then challenged i.c. with gp33-expressing strains of MHV.
RESULTS

RJHM elicits a weak virus-specific CD8+ T cell response in the brain. Intracranial (i.c.) inoculation of RJHM results in severe encephalitis with high numbers of innate immune cells, including macrophages and neutrophils, being recruited into the brain parenchyma early during infection. However, the recruitment of T cells into the brain, both total and virus-specific CD8+ T cells, is extremely low (14, 34). One possibility that could account for the low CD8+ T cell response in the brain following i.c. RJHM inoculation is that RJHM infection causes extensive tissue changes (more than observed with SJHM/RA59) that prevent proper immune cell trafficking into or out of the brain. To address this issue, mice were inoculated with virus via the intranasal (i.n.) route, which results in slightly slower spread of virus within the brain and less tissue destruction during the first week of infection compared to i.c. inoculation. Mice inoculated i.n. do not succumb to the disease until after the first week of infection, thus allowing us to examine the antiviral T cell response before massive tissue destruction occurs. It is important to note that the LD$_{50}$ for RJHM when administered i.n. is approximately 2 log$_{10}$ higher than when administered i.c. In this experiment, all mice inoculated by the i.n. route survived the first seven days of infection. However, similar to i.c. inoculation, i.n. infection with RJHM elicited a very weak CD8+ T cell response in the brain (Fig. 3-1). This result was in contrast to infection with the chimeric SJHM/RA59 strain of MHV, which induced a robust CD8+ T cell response to both H-2$^b$-restricted viral epitopes, S510 and S598 (Fig. 3-1). While the total number of cells isolated per brain from RJHM- and SJHM/RA59-infected mice varied by only 2-fold ($6.4 \times 10^5$ versus $1.3 \times 10^6$, respectively), the total number of CD8+ T cells isolated from RJHM-infected brains was nearly 50-fold lower compared to SJHM/RA59-infected brains ($5.3 \times 10^3$ versus $2.6 \times 10^5$) (Fig. 3-1). Total epitope-specific cells per brain were also significantly lower following RJHM infection compared to SJHM/RA59 (Fig. 3-1). Thus, RJHM infection of the CNS elicits a weak CD8+ T cell response in the brain following either i.c. or i.n. inoculation. Furthermore, this weak response is not solely dependent upon the JHM spike. This difference in CD8+ T cell response between RJHM and SJHM/RA59 following i.n. inoculation is similar to what we have previously reported.
for i.c. inoculation (14), demonstrating that the minimal CD8+ T cell response observed following i.c. inoculation of RJHM is unlikely due to the rapid spread of virus and massive tissue destruction in the brain before a T cell response can be elicited.

![Graph showing CD8+ T cell response in the brain](image)

**Figure 3-1. Intranasal inoculation of RJHM elicits a weak CD8+ T cell response in the brain.**

Brain lymphocytes harvested on day 7 p.i. from mice inoculated i.n. with $10^4$ PFU SJHM/RA59 or $10^3$ PFU RJHM were stimulated with S510 or S598 peptides and then stained for intracellular IFN-γ to evaluate the virus-specific CD8+ T cell response. The number in the upper right quadrant of each plot represents the percentage of CD8+ T cells that are epitope-specific as determined by IFN-γ production. Data represent cells pooled from the brains of 4-6 animals per group and are representative of two independent experiments. The average numbers of live cells and CD8+ T cells harvested per brain are located above each column.
Kinetics of CD8+ T cell priming during CNS infection with MHV is strain-dependent. To assess the protective or pathogenic effects of virus-specific CD8+ T cells during the course of MHV disease, we previously developed an adoptive transfer system in which we could modulate the level of epitope-specific CD8+ T cells in vivo. This system utilized recombinant MHV strains expressing the gp33 epitope of LCMV and the transfer of gp33-specific CD8+ T cells from P14 transgenic mice. We previously observed that adoptive transfer of P14 splenocytes during the first 2 days p.i. with RA59-gfp/gp33 can protect against disease; however, transfer on days 3 or 5 p.i. neither protects nor enhances disease (20). Further analysis revealed that the P14 cells do not accumulate at the site of infection, the brain, when transferred at these later times; this result is in contrast to the dramatic accumulation of P14 cells within the brain when transferred early or prior to infection (20). We next aimed to determine the kinetics of CD8+ T cell priming and, importantly, whether CD8+ T cell priming was occurring during CNS infection with RJHM. Increasing the number of precursor cells by adoptive transfer allowed us to visualize CD8+ T cell expansion and proliferation, and cell proliferation was used as an indicator of CD8+ T cell priming. In this experiment, mice were inoculated with 10^4 PFU of RA59, SJHM/RA59, or RJHM expressing gfp/gp33. The gfp/gp33-expressing viruses are attenuated relative to their respective parental viruses, thus allowing inoculation of higher doses. On days 1, 2, or 3 p.i., P14 splenocytes were labeled with CFSE and transferred into the infected mice. At 3 days post-transfer, cells were isolated from the spleen and cervical lymph nodes (CLN) and examined for CFSE dilution as an indicator of cell proliferation. Cells expressing low amounts of CFSE (CFSE^hi) represent the divided population.

To analyze proliferation of the transferred P14 cells (CD45.2+), we used CD45.1+ mice as transfer recipients, thus allowing us to identify CD8+CD45.2+ cells as the transferred cells. The results of this experiment are shown in Fig. 3-2. As expected, the transferred cells did not proliferate and remained CFSE^hi in uninfected and RA59-gfp-infected animals (data not shown). During RA59-gfp/gp33 infection, when cells were transferred on day 1 p.i. and examined 3 days later, we observed that a significant number of the transferred CD8+CD45.2+ cells underwent several rounds of division in both the CLN and the spleen as indicated by dilution of CFSE (Fig. 87.
3-2A). However, transfers performed on day 2 p.i. or later showed significantly lower percentages of proliferated cells, indicating that CD8+ T cells are primed within the first 2 days of RA59-gfp/gp33 infection. Importantly, the absence of proliferating cells in the animals receiving transfer on day 3 p.i. was not due to their presence in another location, as we have already shown that transferred cells are not present in the brains of these recipients (20). Similarly, infection with the chimeric gp33-expressing virus SJHM/RA59-gfp/gp33 resulted in early and robust CD8+ T cell priming (Fig. 3-2B), consistent with the observation that this virus induces a strong CD8+ T cell response (Fig. 3-1).

In sharp contrast, infection with RJHM-gfp/gp33 resulted in a dramatically different proliferation profile. Interestingly, little to no proliferation was observed in the populations of cells transferred on days 1, 2, or 3 p.i. (Fig. 3-2C). Thus, priming of naive, virus-specific CD8+ T cells is greatly reduced during RJHM-gfp/gp33 infection compared to both RA59-gfp/gp33 and SJHM/RA59-gfp/gp33 infections. Importantly, this reduced response was not due to a lack of gp33 expression during infection with RJHM-gfp/gp33 as sequencing of RNA from infected cells confirmed the presence of an intact gp33 sequence. Furthermore, EGFP, which is fused to the carboxyl terminus of the gp33 epitope, continued to be expressed for at least three days p.i. in mice (data not shown).

Reports indicate that CD8+ T cells are not infected by MHV in vivo (9); however, to confirm that donor CD8+CD45.2+ cells were not infected with the EGFP-expressing recombinant viruses (and thus contributing to the population of CFSElo cells), we harvested cells from the spleen and CLN of RA59-gfp/gp33- and RA59-gfp-infected animals that either did not receive adoptive transfer or received adoptive transfer of unlabeled P14 cells. As expected, these cells were negative for fluorescence in the FL1 channel (data not shown). Additionally, the experiments in Fig. 3-2 were repeated using an alternative dye, PKH26, that fluoresces in the FL2 channel to confirm that the divided CFSElo population did not include MHV-infected CD8+CD45.2+ cells (data not shown).
Figure 3-2. Kinetics of naïve CD8+ T cell priming. CD45.1+ mice inoculated i.c. with $10^4$ PFU gfp/gp33-expressing virus received adoptive transfer of CFSE-labeled P14 cells on days 1, 2, or 3 p.i. Histograms represent transferred CD8+CD45.2+ T cells. The number in the upper left of each histogram indicates the percentage of transferred cells that have divided; in parentheses below is the proliferative index, which represents the average number of cell divisions that have occurred in the dividing population. Panels represent proliferation of transferred cells in mice infected with A) RA59-gfp/gp33, B) SJHM/RA59-gfp/gp33, or C) RJHM-gfp/gp33. The numbers to the left of each row indicate the days p.i. when cells were transferred/harvested. Each histogram represents data collected from a single mouse and is representative of the entire group (n=3). Data are representative of two independent experiments.
**RJHM is not generally immunosuppressive.** The induction of cytokines and chemokines in the brain has been shown to differ among different strains of MHV (34, 38). Based on these findings, we next investigated whether the RJHM-induced cytokine response could suppress the development of a virus-specific CD8+ T cell response to RA59. To determine which strain of MHV dominates the adaptive response, the weak CD8+ T cell inducer RJHM or the robust CD8+ T cell inducer RA59, we inoculated mice with both viral strains simultaneously and analyzed the CD8+ T cell response in the brain at day 7 p.i. (Fig. 3-3).

The RA59 strain of MHV expresses only the subdominant S598 epitope, whereas RJHM expresses both S598 and the immunodominant S510 epitope. While similar numbers of mononuclear cells were isolated per brain from mice infected with either RA59 or RJHM (9.3 x 10^5 versus 9.4 x 10^5, respectively), the total number of CD8+ T cells per brain was approximately 25-fold less in the RJHM-infected animals (1.6 x 10^5 versus 6.6 x 10^3) (Fig. 3-3). Furthermore, the S598-specific CD8+ T cells accounted for approximately 5% of the CD8+ T cells in RA59-infected mice, whereas RJHM infection resulted in minimal levels of S510- and S598-specific CD8+ T cells (Fig. 3-3). In contrast to infection with RJHM alone, co-infection with RA59 and RJHM resulted in significant recruitment of CD8+ T cells into the brain. While co-infected mice exhibited a robust response (similar to RA59 infection in both percentage and total number) to S598, the epitope expressed by both RA59 and RJHM, there was no appreciable response to the immunodominant S510 epitope expressed only by RJHM (Fig. 3-3). Thus, under these conditions, the ability of RA59 to elicit a robust, virus-specific CD8+ T cell response was dominant over the low CD8+ T cell response induced by RJHM. Importantly, the striking observation that co-infection did not result in an S510-specific CD8+ T cell response in the brain suggests that RJHM is unable to elicit a robust CD8+ T cell response even in the presence of immune mediators governing an adaptive immune response to RA59.
Figure 3-3. RJHM inoculation does not suppress the CD8+ T cell response elicited by RA59. Mice were inoculated i.c. with either 500 PFU RA59, 10 PFU RJHM, or both 500 PFU RA59 and 10 PFU RJHM; these doses represent approximately one LD₅₀ of RJHM and less than one LD₅₀ of RA59. Brain lymphocytes harvested on day 7 p.i. were stimulated with peptide and stained as in Fig. 3-1. The number in the upper right quadrant of each plot represents the percentage of CD8+ T cells that are epitope-specific as determined by IFN-γ production. Data represent cells pooled from the brains of 4-6 animals per group and are representative of two independent experiments. The average numbers of live cells and CD8+ T cells harvested per brain are located above each column.
In addition to analyzing the virus-specific CD8+ T cell response in the brain, we also monitored survival in the co-infected mice. As shown in Fig. 3-4A, the majority of mice co-infected with RA59 and RJHM survived the infection, and those that died did so with slower kinetics compared to mice infected with RJHM alone, suggesting that some component of the host response elicited by RA59 is protective even in the context of infection with the destructive and highly lethal RJHM strain. To ensure that both viruses were indeed present within the brain and that preferential replication and spread of RA59 was not responsible for the RA59-like immune response, tissue homogenates were titrated for the presence of both RA59 and RJHM (Fig. 3-4B). At day 4 p.i., which is close to the peak of virus replication in vivo, brains and CLN were harvested from co-infected mice. The CLN were evaluated to determine if both RA59- and RJHM-infected cells were present at the site where CD8+ T cell priming is thought to occur during acute MHV infection of the CNS.

At day 4 p.i., viral titers in the brains of RA59-infected mice were significantly higher than titers in RJHM-infected brains (Fig. 3-4B). Interestingly, RA59 also replicated to relatively high titers in the CLN whereas RJHM titers were below the limit of detection (Fig. 3-4B). Titers observed in co-infected mice appeared to mirror the titers observed in mice infected with RA59 or RJHM alone (Fig. 3-4B). However, to confirm that both RA59 and RJHM were indeed replicating in the brains of co-infected animals, a neutralization assay was performed in which brain homogenates from co-infected animals were incubated with monoclonal antibodies specific for either the A59 spike (A2.1) or the JHM spike (J7.2 and J7.18) prior to performing standard plaque assays. This allowed selective detection of either RA59 or RJHM. While both RA59 and RJHM were present in the brains of co-infected animals, only RA59 replicated to measurable titers within the CLN (Fig. 3-4C). This difference in RA59 and RJHM replication in the draining CLN correlates with the difference in CD8+ T cell priming observed during these infections.
Figure 3-4. Survival and virus replication in co-infected mice. Mice were inoculated i.c. as described in Fig. 3-3. A) The survival of infected mice was monitored in 10 mice per group. Mice co-infected with RA59 and RJHM (open circles) displayed an intermediate survival phenotype compared to RJHM-infected mice (black squares) and RA59-infected mice (black diamonds). B) Virus replication in the brains and CLN of mice infected with RA59 (A; white bar), RJHM (J; black bar), or co-infected with RA59 and RJHM (C; gray bar) was examined in tissues harvested on day 4 p.i. Tissue homogenates were titrated on L2 fibroblasts. Bars represent mean viral titers (3 mice per group), and error bars represent the standard error of the mean. C) Brain and CLN homogenates from co-infected animals were used in a neutralization assay using mAb specific for either the A59 spike (A2.1) or the JHM spike (J7.2 and J7.18). Tissue homogenates were incubated with a 1:10 dilution of anti-JHM Ab (α-J; white bar), anti-A59 Ab (α-A; black bar), or no Ab (N; gray bar) for one hour prior to performing standard plaque assays. (The CLN from all RJHM-infected mice had titers below the limit of detection.)
RJHM replicates poorly in the CLN compared to RA59 and SJHM/RA59. The difference in viral titers in the CLN of RA59- and RJHM-infected mice at day 4 p.i. (Fig. 3-4C) suggested that the poor CD8+ T cell priming during RJHM infection could be due, at least in part, to the absence of infectious virus in the CLN, the site of priming during acute MHV infection of the CNS. Thus, we further compared the replication of RA59, SJHM/RA59, and RJHM in the draining CLN to determine if RJHM replicates in the CLN at early times post-infection when priming is thought to occur. Mice were infected with 50 PFU of RA59, SJHM/RA59, or RJHM, and infectious virus was titrated from brain and CLN homogenates on days 1-5 p.i. While all three viruses replicated efficiently in the brain during the course of five days p.i. (Fig. 3-5A), only RA59 and SJHM/RA59 replicated to appreciable titers in the CLN during this time while RJHM replicated to only a minimal extent (Fig. 3-5B). Thus, the ability of these strains to prime an effective CD8+ T response correlates with the presence of infectious virus in the draining CLN. Interestingly, RJHM replication in the brain was lower than RA59 and SJHM/RA59 during the first two days p.i. (Fig. 3-5A), the time during which priming occurs (Fig. 3-2). These data suggest that the minimal CD8+ T cell priming observed during RJHM infection of the CNS may be due to a lack of RJHM antigen in the CLN, the site of T cell priming during acute CNS infection.
Figure 3-5. RJHM replicates inefficiently in the draining cervical lymph nodes (CLN). Mice were inoculated with 50 PFU RA59 (white bars), SJHM/RA59 (gray bars), or RJHM (black bars). A) Brains and B) CLN were removed on days 1-5 p.i. and tissue homogenates were titrated on L2 fibroblasts to assess viral replication. Bars represent mean viral titers (5 mice per group), and error bars represent the standard error of the mean. (Individual tissues with no measurable titer were assigned a log$_{10}$ value of zero.) Data are representative of two independent experiments.
RJHM elicits a weak secondary CD8+ T cell response in the brain after immunization against a viral epitope. To further investigate the mechanism by which RJHM fails to elicit a robust CD8+ T cell response in the brain, we next asked whether RJHM infection could elicit a strong secondary response in the brain following peripheral immunization against a single viral epitope. Since the S510- and S598-specific CD8+ T cell responses differ for RA59 and RJHM, we instead utilized the gp33 epitope and the gfp/gp33-expressing MHV strains for this experiment. Immunization was performed using two recombinant *Listeria monocytogenes* (rLm) strains engineered to express either the gp33 epitope (expressed by recombinant gfp/gp33-expressing MHV strains) or the non-specific np118 epitope of LCMV (not expressed by these MHV strains). Mice were inoculated i.p. with $10^4$ CFU of rLm-gp33 or rLm-np118, rested for three weeks, and then challenged i.c. with approximately one LD$_{50}$ of either RA59-gfp/gp33 or RJHM-gfp/gp33. Since a secondary immune response typically occurs more rapidly than a primary response, mice were sacrificed at day 5 p.i. and brain lymphocytes were analyzed to assess the magnitude of the CD8+ T cell response. While the average number of live cells per brain varied less than 2-fold among all groups, striking differences were observed in the number of CD8+ T cells per brain (Fig. 3-6A and 3-6B). While RA59-gfp/gp33-infected mice immunized with non-specific *Listeria* yielded approximately $9.66 \times 10^4$ CD8+ T cells per brain at 5 days p.i. (representing approximately 8.09% of the live cells recovered), those mice immunized with rLm-gp33 showed a significantly greater response ($3.51 \times 10^5$ CD8+ T cells per brain, representing approximately 29.61% of live cells) (Fig. 3-6A). In sharp contrast, mice infected with RJHM-gfp/gp33 showed little to no difference in CD8+ T cell response following immunization with rLm-gp33 compared to non-specific *Listeria* (approximately 2.87% vs. 2.25% CD8+ T cells, respectively) (Fig. 3-6B). Importantly, the secondary CD8+ T cell response elicited by RJHM-gfp/gp33 following rLm-gp33 immunization was significantly reduced compared to the secondary response elicited by RA59-gfp/gp33, both in total number and percentage (2.87% vs. 29.61% CD8+ T cells, respectively) (Fig. 3-6A and 3-6B). Since the percentage of CD8+ T cells specific for gp33 was similar in all cases, the absolute number of gp33-specific CD8+ T cells elicited during the secondary response
to RJHM-gfp/gp33 was no greater than the response in mice immunized with non-specific *Listeria* and was significantly lower than the secondary response to RA59-gfp/gp33 (Fig. 3-6A and 3-6B). Thus, while RA59-gfp/gp33 induced a robust secondary response in the brain following peripheral immunization against a viral epitope, RJHM-gfp/gp33 was deficient in this ability.

To verify that the lack of CD8+ T cell response in RJHM-gfp/gp33 infected mice following peripheral immunization was not due to poor replication of RJHM-gfp/gp33 in the brain, tissue homogenates from infected mice were titrated at day 5 p.i. As shown in Fig. 3-6C, RJHM-gfp/gp33 replicated to higher titers than RA59-gfp/gp33 in the brains of infected mice, demonstrating that the poor secondary CD8+ T cell response to RJHM-gfp/gp33 is not due to poor viral replication in the brain. Furthermore, immunization with rLm-gp33 did not reduce the amount of replication in the brains of RJHM-gfp/gp33-infected mice compared to rLm-np118-immunized mice (Fig. 3-6C); this is not surprising given the very small numbers of gp33-specific CD8+ T cells infiltrating the brain (Fig. 3-6B). There was, however, an approximately ten-fold reduction in RA59-gfp/gp33 titers (*P = 0.04*) when mice were immunized with rLm-gp33 compared to rLm-np118, similar to what we have previously observed (6).
Figure 3-6. RJHM fails to elicit a robust secondary response in the brain following peripheral immunization against a viral epitope. Mice were inoculated with $10^4$ CFU rLm-np118 or rLm-gp33 i.p., rested for 3 weeks, and then challenged i.c. with A) $10^5$ PFU RA59-gfp/gp33 or B) $10^3$ PFU RJHM-gfp/gp33. Brain lymphocytes were harvested on day 5 p.i., stimulated with gp33 peptide, and stained as in Fig. 3-1. The number in the upper right quadrant of each plot represents the percentage of CD8+ T cells that are epitope-specific as determined by IFN-γ production. Data represent cells pooled from the brains of 4-6 animals per group and are representative of three similar experiments. The average numbers of live cells and CD8+ T cells harvested per brain are located above each column. C) Virus replication in the brain was examined on day 5 p.i. Tissue homogenates were titrated on L2 fibroblasts. Bars represent mean viral titers (3 mice per group), and error bars represent the standard error of the mean (*$P = 0.04$).
RJHM elicits a robust CD8+ T cell response in the spleen following peripheral inoculation.

Since CNS infection with RJHM elicits a weak virus-specific CD8+ T cell response in the brain, we next aimed to determine if this feature was inherent to the virus or a unique characteristic of CNS infection. To address this issue, mice were inoculated with RA59, SJHM/RA59, or RJHM via the intraperitoneal (i.p.) route. Spleen lymphocytes were analyzed on day 8 p.i. (the peak of the CD8+ T cell response following this route of inoculation) to assess the magnitude of the response. As expected, i.p. inoculation with RA59 elicited a robust CD8+ T cell response to S598 in the spleen (Fig. 3-7A). Interestingly, both SJHM/RA59 and RJHM elicited a robust response to epitopes S510 and S598 following i.p. inoculation (Fig. 3-7A). Total numbers of viable splenocytes per mouse as well as CD8+ T cells per spleen were not statistically different for any of the viruses examined (Fig. 3-7B). Similarly, no statistical difference was observed in total numbers of S510-specific (SJHM/RA59 and RJHM only) and S598-specific CD8+ T cells harvested per spleen (Fig. 3-7B). Thus, the RJHM strain of MHV induces a robust virus-specific CD8+ T cell response when inoculated i.p. This result is in sharp contrast to the response observed following CNS infection.
Figure 3-7. Intraperitoneal inoculation of RJHM elicits a robust CD8+ T cell response in the spleen. A) Spleen lymphocytes harvested on day 8 p.i. from mice inoculated i.p. with 10^4 PFU RA59, SJHM/RA59, or RJHM were stimulated with peptide and stained as in Fig. 3-1. The number in the upper right quadrant of each plot represents the percentage of CD8+ T cells that are epitope-specific as determined by IFN-γ production. Each column represents cells harvested from one individual mouse and is representative of the entire group (n=4). B) Total numbers of live cells, CD8+ T cells, and S510- and S598-specific CD8+ T cells per spleen were compared for each virus. Bars indicate the mean value for each group (n=4), and error bars represent the standard error of the mean. All P values were > 0.10, as determined by one-way analysis of variance. Data are representative of two independent experiments.
DISCUSSION

The presence of CD8+ T cells in the CNS during infection with neurotropic strains of MHV has been implicated in both protection and pathogenesis. While virus-specific CD8+ T cells likely contribute to the pathogenesis of demyelination through the secretion of macrophage-recruiting cytokines (34), their role in protection and clearance of infectious virus appear to outweigh their detrimental effects. This protective role is illustrated by the remarkable susceptibility of β2-microglobulin-deficient mice to infection with RA59 (10). Furthermore, we speculate that the lethality of the highly neurovirulent RJHM strain of MHV may be due, at least in part, to the poor CD8+ T cell response elicited by this virus after i.c. (14) or i.n. inoculation (Fig. 3-1). A similarly low CD8+ T cell response has been reported with MHV-4, a closely related JHM isolate (34). The data presented here suggest that during CNS infection RJHM is largely able to avoid CD8+ T cell priming and that this ability leads to the poor CD8+ T cell response observed in the brains of RJHM-infected mice. Importantly, this finding appears to be unique to CNS infection with RJHM, as i.p. inoculation resulted in a robust virus-specific CD8+ T cell response in the spleen (Fig. 3-7). Furthermore, the poor CD8+ T cell response during CNS infection with RJHM does not appear to be a function of disease severity as illustrated by i.n. infection (Fig. 3-1) and co-infection (Fig. 3-3), both of which were less severe than i.c. infection with RJHM alone. In addition, a chimeric recombinant expressing the A59 spike within the background of RJHM causes a very mild infection and still fails to induce a CD8+ T cell response (14). The poor CD8+ T cell response during CNS infection with RJHM also appears to be independent of viral dose as very high doses of RJHM-gfp/gp33 were used in the priming experiments (Fig. 3-2) and since doses as low as 20 PFU of RA59 i.c. induce a robust CD8+ T cell response in the brain (14).

Since minimal priming of virus-specific CD8+ T cells was observed in RJHM-infected mice (Fig. 3-2), we further investigated whether activated CD8+ T cells transferred into RJHM-gfp/gp33-infected mice could traffic to the infected CNS and protect against neurological disease. To this end, P14 splenocytes were transferred into B6 mice and activated in vivo with rLm-gp33. Activated P14 cells were then transferred to naïve B6 mice, and these recipients were infected with RA59-gfp/gp33 or RJHM-gfp/gp33. Analysis of brain lymphocytes on day 7 p.i. demonstrated
that activated P14 cells trafficked efficiently to the brains of RA59-gfp/gp33-infected mice while only small numbers of activated cells were present in the brains of RJHM-gfp/gp33-infected mice (data not shown). Furthermore, those RJHM-gfp/gp33-infected mice that received adoptive transfer of activated cells showed similar clinical signs and mortality as controls not receiving transfer (data not shown). While transfer of activated virus-specific CD8+ T cells was unable to protect mice from RJHM-induced neurological disease, such experiments are complicated by the low levels of T cell recruiting cytokines expressed in the brain following RJHM infection compared to RA59 infection (34, 38). Thus, RJHM may avoid the induction of a CD8+ T cell response in the brain due to both an inefficiency of priming and an inability to recruit activated cells to the CNS.

Many studies have demonstrated that a robust adaptive immune response can be mounted to antigens expressed in the brain. While antigen presentation during some chronic infections can occur within the brain itself (24), the ability to recruit an adaptive immune response during acute CNS infection relies on antigen presentation in the draining CLN (15, 23, 25). Soluble antigen injected directly into the brains of mice reach the CLN within minutes to hours (11, 19). The development and migration of a protective CD8+ T cell response has been examined for several CNS infections and likely depends on the pathogenic organism. Mice infected with an attenuated variant of JHM (J2.2-V-1) that primarily infects glial cells were used to characterize the trafficking of CD8+ T cells into the CNS during acute infection (23). Interestingly, in contrast to the highly neurovirulent RJHM strain used in our studies, J2.2-V-1 induces a robust antiviral CD8+ T cell response. Similar to what we observe with RA59, CNS infection with J2.2-V-1 results in a high percentage of virus-specific CD8+ T cells in the brain during the acute stage of disease. Using tetramers specific for the immunodominant S510 epitope, it was determined that the initial expansion of CD8+ T cells occurs in the CLN, followed by further expansion of virus-specific CD8+ T cells in the spleen and eventual accumulation in the brain (23). In this model, only highly activated CD8+ T cells are evident in the brain. The data from our T cell priming experiments are in agreement with this finding, as only CFSE-negative P14 cells were detected in the brains of infected mice (data not shown).
In a mouse model using ocular herpes simplex virus-1 (HSV-1) infection and the adoptive transfer of epitope-specific CD8+ T cells, the site of T cell priming was determined to be the submandibular lymph node (16). Interestingly, however, when cells are transferred one day prior to HSV-1 infection, proliferation cannot be detected until day 5 p.i.; these kinetics are delayed compared to what we observed during infection with RA59-gfp/gp33 (Fig. 3-2A) and raise the question as to what factors contribute to the kinetics of T cell priming. Important factors likely include the tropism of the infectious organism, the kinetics of infection, the route of inoculation, and the potential interactions of the infectious organism with antigen presenting cells (APC). Each of these factors may influence antigen distribution and availability, the rate at which APC encounter antigen and thus the length of time it takes them to interact with naïve CD8+ T cells, and/or the capacity of APC to present antigen and provide appropriate co-stimulation. The poor CD8+ T cell priming observed during acute infection with RJHM-gfp/gp33 compared to infection with RA59-gfp/gp33 (Fig. 3-2), the lack of response to S510 in mice co-infected with RA59 and RJHM (Fig. 3-3), and the inability of RJHM to elicit a robust secondary response in the brain (Fig. 3-6) together suggest an inefficiency of RJHM antigen presentation that could potentially be due to viral effects on the APC and/or inaccessibility of RJHM antigen.

The data presented here support the hypothesis that the poor CD8+ T cell priming observed during RJHM infection of the CNS likely results from an inaccessibility of RJHM antigen to APC in the CLN rather than a direct effect on APC function. The data in Fig. 3-2 and Fig. 3-5 show that the ability to prime a CD8+ T cell response correlates with viral replication in the CLN during the first 2 days p.i. when antigen presentation is taking place. RJHM replicates very poorly in the CLN compared with RA59 and SJHM/RA59 while all three viruses replicate to high titers in the brain. Furthermore, RJHM induces a robust CD8+ T cell response when inoculated i.p. (Fig. 3-7). While little is known about the site of replication or the site of T cell priming following i.p. inoculation with MHV, dendritic cells (DC) are believed to be the relevant APC following this route of inoculation (43), thus arguing against an interaction of RJHM with DC that prevents priming. Furthermore, we compared the phenotype of DC present in the CLN of mice infected with RA59 and RJHM on days 1-3 p.i. and were unable to detect any differences in DC activation despite an
increase in CD11c+ cells in the CLN of both RA59- and RJHM-infected mice (data not shown); this increase in CD11c+ cells was similar to that reported for CNS infection with an attenuated variant of JHM (J2.2-V-1) (42). Nevertheless, we cannot exclude the possibility that there may be other phenotypic or functional differences between DC from RA59- and RJHM-infected mice that could contribute to the differences in T cell priming. Finally, the observation that co-infected mice mount a robust CD8+ T cell response to RA59 even in the presence of RJHM infection (Fig. 3-3) indicates that RJHM is not generally immunosuppressive; thus, RJHM does not appear to alter the cytokine milieu in the brain or CLN in a way that compromises antigen presentation.

We further speculate that a lack of antigen availability to APC in the CLN may be linked to the cellular tropism of RJHM. The highly neurovirulent RJHM strain of MHV disseminates widely in neurons throughout the brain (8), in contrast to RA59 and neuroattenuated JHM variants that infect a significant number of glial cells and induce a robust CD8+ T cell response. Indeed, we have observed that RJHM spreads more extensively than RA59 in primary neuronal cells in vitro (data not shown). Infected neurons are less likely to lyse and release viral antigen than other CNS cell types due to their ability to prevent apoptosis (reviewed in (2)), and cell-mediated lysis of infected neurons is limited by their low surface expression of MHC class I molecules. Thus, mechanisms that preserve neuronal integrity in the face of viral infection may limit the availability of RJHM antigen to be taken up and presented by APC via cross-presentation pathways. Furthermore, MHV may drain directly from the CNS to the CLN via lymphatics (1) where virus is then able to infect APC in the LN. It was recently shown that, following peripheral infection, both vaccinia virus and VSV rapidly reach the draining lymph nodes via lymphatics and infect DC, which then present antigen to T cells (12). While infectious RJHM is present in the brains of infected mice (Fig. 3-5 and 3-6), titers are comparatively low at early times p.i. when T cell priming takes place (Fig. 3-5). If RJHM is indeed highly neuronal, it may be highly cell associated as primary neurons infected in vitro produce less infectious virus than other CNS cell types (18, 33). In contrast, RA59 replication in CNS cell types such as microglia/macrophages and astrocytes as well as its measurable replication in the draining CLN (Fig. 3-4 and 3-5) likely yields higher levels of viral antigen that can be processed for presentation by APC. Ongoing studies aim
to quantify the relative cellular tropisms of RA59 and RJHM, particularly at early times p.i. when T
cell priming occurs.

The data presented here demonstrate that closely related neurotropic strains of MHV
have a differential ability to induce a CD8+ T cell response during CNS infection. We suggest that
the decreased ability of RJHM to induce a CD8+ T cell response in the brain leads to a lack of
viral clearance and thus contributes to the high neurovirulence of RJHM compared to RA59.
Notably, this feature appears to be unique to RJHM infection of the CNS, as i.p. inoculation of
RJHM induces a robust CD8+ T cell response in the periphery. The precise mechanism by which
RJHM avoids the induction of a protective antiviral CD8+ T cell response during CNS infection is
an area of ongoing research.

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CHAPTER 4

Severity of CD8 T cell dysfunction during chronic viral infection in the brain and liver correlates with level of viral persistence

This chapter is in preparation for submission to The Journal of Immunology as the article “Severity of CD8 T cell dysfunction during chronic viral infection in the brain and liver correlates with level of viral persistence” by S. J. Bender,* H. Shin,* S. D. Blackburn, K. C. MacNamara, S. R. Weiss, and E. J. Wherry.

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ABSTRACT

Clearance of acute viral infections typically results in the generation of highly functional memory CD8 T cells that provide long-lived protective immunity. However, during chronic viral infections, the differentiation of effector CD8 T cells into protective memory cells is altered, leading to progressive stages of T cell dysfunction. We, and others, have demonstrated in confined systems that the stage of CD8 T cell exhaustion correlates with viral load; however, this prediction has never been tested in a system comparing CD8 T cell responses to unrelated viruses expressing a common epitope. Here, we report a model system that allows direct comparison of acute LCMV Armstrong infection that is completely cleared, chronic LCMV clone 13 infection with high levels of viral replication, as well as infection with a murine coronavirus (MHV) characterized by low levels of persisting viral RNA accompanied by demyelination in the CNS. Importantly, since this recombinant MHV expresses the GP33 epitope of LCMV (MHV-GP33), we can directly compare antiviral CD8 T cell responses to these unrelated viruses via this shared epitope. Given the intermediate level of MHV persistence compared to acute versus chronic LCMV infection, we predict, and show data to support, that CD8 T cells maintained in the brain during MHV-GP33 persistence are 1) functionally intermediate compared to cells maintained in the brains of Armstrong- or clone 13-infected mice and 2) less functional than CD8 T cells present in the liver, an organ where virus is cleared and MHV-GP33 does not persist.
INTRODUCTION

During an acute viral infection, naïve virus-specific CD8 T cells become activated and expand to form an antiviral effector CD8 T cell pool that functions to clear virus from the host. This clearance is largely mediated by antiviral cytokines, including IFNγ and TNFα, as well as cytotoxic molecules, such as perforin and granzymes, that cooperate to lyse infected target cells. As viral titers decline and viral antigen (Ag) is cleared, the majority of these antiviral effector T cells die by apoptosis while a subset of cells persists and continues to differentiate, forming a population of long-lived memory CD8 T cells. Optimal memory CD8 T cells are typically characterized by long-term self-renewal in the absence of Ag, mediated by IL-7 and IL-15, as well as rapid reactivation of effector functions and robust expansion following re-exposure to Ag (14, 29, 37). Together, this set of properties allows memory CD8 T cells to confer long-term protective immunity. However, this pattern of CD8 T cell differentiation is markedly altered during viral persistence.

We have previously addressed the differentiation of CD8 T cells during viral persistence using the lymphocytic choriomeningitis virus (LCMV) model of chronic infection. Infection of mice with LCMV, an arenavirus, is commonly used to study antiviral T cell responses following acute versus chronic infection because different strains of this virus can be rapidly cleared or result in persisting infection. In adult mice, the Armstrong (Arm) strain of LCMV causes an acute infection that is successfully cleared by the host within 8-10 days post-infection (p.i.). Following viral clearance, a subset of virus-specific CD8 T cells differentiates into a population of highly functional memory cells that provide long-term protective immunity in the absence of viral Ag. In contrast, the clone 13 (Cl-13) strain of LCMV establishes a chronic infection in adult mice characterized by approximately 2 months of viremia, after which time viral replication is controlled in tissues such as the spleen and liver while high levels of infectious virus persist long-term in organs such as the kidney and brain (1, 22, 38). The virus-specific CD8 T cells present during chronic Cl-13 infection show signs of severe T cell dysfunction that lead to poor pathogen control. This loss of effector functions occurs in a hierarchical manner, with IL-2 production and cytolytic
functions disappearing first, followed by TNFα and then IFNγ production (39). This functional impairment also correlates with increased expression of PD-1 on exhausted CD8 T cells (2). Furthermore, these CD8 T cells require the presence of viral Ag for maintenance during chronic infection rather than using IL-7 and IL-15 for homeostatic proliferation (31, 38).

Until now, comparative studies of CD8 T cell dysfunction during chronic infection have been limited to cross-virus comparisons of T cell responses to dissimilar epitopes or infections with an individual virus where viral loads are varied. We now describe a model system that allows direct comparison of CD8 T cell responses to a common epitope expressed by unrelated viruses with inherently different levels of persistence. Importantly, this system can be used to test our working model about the relationship between the level of virus persistence and the stages of T cell exhaustion during chronic viral disease. Using acute Arm and chronic CI-13 infection as our standards, we have expanded our comparison to include persisting infection with a recombinant murine coronavirus (mouse hepatitis virus, MHV) expressing the GP33 epitope of LCMV (MHV-GP33). In weanling mice, intracranial (i.c.) inoculation of MHV strain A59 results in acute infection in the liver and central nervous system (CNS). Viral titers peak by day 5 p.i., after which time infectious virus is cleared (24). Despite clearance of infectious virus, MHV RNA persists in the CNS long-term and demyelination, largely immune-mediated, develops and peaks around 4 weeks p.i. (23). Previous studies assessing the protective capacity of GP33-specific CD8 T cell against MHV-GP33-induced demyelination showed that increasing the numbers of virus-specific T cells during acute infection correlates with decreased severity of demyelination; these virus-specific CD8 T cells were maintained in the brain for at least one month following clearance of infectious virus (21). However, little is known about the differentiation state of these cells, how the amount of MHV persistence in different tissues impacts the functional quality or exhaustion of CD8 T cells responding to MHV infection, or how these cells compare to CD8 T cells responding to cleared versus other persisting infections.

Given the intermediate level of MHV persistence in the brain compared to cleared Arm and chronic CI-13 infection, our model predicts an intermediate level of CD8 T cell dysfunction during persistent MHV-GP33 infection. Furthermore, the differences in MHV infection in the brain
versus liver (persisting infection versus viral clearance) would suggest that virus-specific CD8 T cells in the liver during late stages of infection will be more highly functional than cells in the brain, where viral RNA persists. Thus, using this model system, we can now begin to make direct comparisons regarding pattern and extent of virus persistence on the overall quality of antiviral CD8 T cells responding to an individual epitope that were not possible in previous systems.
MATERIALS AND METHODS

Animals and viruses. Four- to six-week-old male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or National Cancer Institute (Frederick, MD). P14 transgenic mice bearing a TCR specific for the GP33-41 (GP33) epitope of LCMV (27) were maintained on a Ly5.1* background at The Wistar Institute. To generate P14 chimeras, small numbers (5-7.5×10^4 for Arm and MHV-GP33 infections, 2-5×10^3 for Cl-13) of naïve P14 CD8 T cells (Ly5.1*) were adoptively transferred i.v. to congenic C57BL/6 mice (Ly5.2*). P14 chimeras were subsequently inoculated with 2×10^5 PFUs of LCMV Armstrong (Arm) i.p., 2×10^6 PFUs LCMV clone-13 (Cl-13) i.v., or intracranially (i.c.) with 1×10^4 PFUs of recombinant MHV strain A59 expressing the GP33 epitope fused to enhanced green fluorescent protein in place of nonessential gene 4 (MHV-GP33) (previously referred to as RA59-gfp/gp33) (4) as described (1, 20, 39). Viruses were grown and titrated by plaque assay as previously described (1, 6). All mice were used in accordance with Institutional Animal Care and Use Committee guidelines.

Quantitative RT-PCR. RNA was isolated from MHV-GP33-infected tissues by homogenization in TRIzol reagent (Invitrogen) followed by phenol/chloroform extraction and purification using an RNeasy Kit (Qiagen) then DNase treated using a Turbo DNA-free Kit (Ambion) as previously described (30). Quantitative PCR was performed in the absence of reverse transcriptase to ensure adequate removal of genomic DNA. For cDNA synthesis, 1 μg RNA was combined with 0.5 mM dNTP mix and 50 ng random hexamers (Invitrogen) in a total volume of 13 μL, heated to 65°C for 3 minutes, and cooled to room temperature. 1X First Strand Buffer, 5 mM DTT, and 200 U SuperScript III Reverse Transcriptase (Invitrogen) were added for a final reaction volume of 20 μL and the mixture was heated to 50°C for 60 minutes followed by 70°C for 15 minutes.

Quantitative PCR reactions were performed in duplicate in an iQ5 iCycler (Bio-Rad) using 2 μL cDNA, 12.5 μL iQ SYBR Green Supermix (Bio-Rad), and 0.4 μM forward and reverse primers in a total volume of 25 μL. MHV genome primers (forward 5'-ATGCGTCTACATTAACACGAC-3’, reverse 5'-TTACCTGTGGGTCCGGTA-3’) amplified a conserved region of ORF1b. MHV GP33 primers (forward 5’-GACGATGGCGTGTGTCCT-3’, reverse 5’-
GGTGGCGACCGGTGGATCCGG-3’) amplified a region of gene 4 containing the GP33 sequence. Mouse beta-actin primers (forward 5’-CAGATGGATCAGCAAGCAGGA-3’, reverse 5’-CGCAGCTCAGTAACAGTCGCCTA-3’) were used as a normalization control. Cycle threshold (C_T) values for MHV genome and GP33 were normalized to mouse beta-actin levels, resulting in ΔC_T values [ΔC_T = C_T(target) – C_T(beta-actin)]. ΔΔC_T values were then calculated [ΔΔC_T = ΔC_T(infected) – ΔC_T(mock)] and results are expressed as fold change over mock-infected tissues (2^ΔΔC_T).

Lymphocyte isolation and flow cytometry. Lymphocyte isolation from lymphoid and nonlymphoid tissues, surface staining, and intracellular cytokine staining (ICS) were performed as previously described (2, 38, 39). Briefly, lymphocytes were isolated from spleen by mechanical disruption through nylon mesh followed by lysis of RBCs. Lymphocytes were isolated from liver by mechanical disruption through nylon mesh and separation on a 44/56% Percoll gradient, followed by lysis of RBCs. Lymphocytes were isolated from brain by mechanical disruption through nylon mesh and separation on a 35/70% Percoll gradient. PBMCs were isolated from blood using Histopaque 1083 (Sigma). Data in Fig. 4-2 and 4-3 represent lymphocytes pooled from multiple animals. Data in Fig. 4-4 represent lymphocytes isolated from individual animals. All antibodies were purchased from Biolegend except for CD127, TNFα, IL-2 (eBioscience), and granzyme B (Caltag). LIVE/DEAD dead-cell stain was purchased from Invitrogen. MHC class I tetramers of H-2Db complexed with GP33 peptide were made and used as previously described (39). All flow cytometry data were acquired on an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Treestar).

Adoptive transfers and bacterial challenge. Approximately 30 days after viral infection, donor lymphocytes from LCMV- or MHV-GP33-infected brain, liver, or spleen were adoptively transferred i.v. to C57BL/6 recipient mice. In each individual experiment, identical numbers of DbGP33 tetramer+ CD8 T cells were adoptively transferred to each recipient mouse. Recipient mice were subsequently challenged i.v. with recombinant Listeria monocytogenes strain XFL203 expressing the GP33 epitope of LCMV (LM-GP33) (11) and T cells were analyzed at 5-7 days p.i.
RESULTS

LCMV CI-13 and MHV-GP33 are chronic viral infections. To investigate the impact of different types of viral persistence on the quality of antiviral CD8 T cells, we utilized two strains of LCMV, Arm and CI-13, and a recombinant MHV expressing the GP33 epitope (MHV-GP33) (4). Arm causes acute infection in adult mice that is completely cleared within 8-10 days post infection (p.i.) (1, 16, 39), whereas CI-13 establishes chronic infection with viremia lasting approximately 2 months p.i. (1, 39). MHV persistence, characterized by the maintenance of viral RNA in the brain but not in the liver (18, 19), is intermediate compared to Arm and CI-13 and thus provides an important comparison in terms of CD8 T cell quality and viral load. To facilitate our T cell analyses, adult C57BL/6 mice received adoptive transfer of 5x10^4 naïve P14 CD8 T cells prior to virus infection (referred to here as P14 chimeras). Therefore, we first aimed to confirm the persistence of MHV-GP33 in this model.

To evaluate viral persistence, P14 chimeras were inoculated i.c. with 1x10^4 PFUs of MHV-GP33 and sacrificed on days 3, 14, 21, and 28 p.i. Spleen, liver, and brain tissue were divided in half to provide paired samples for virus titration and RNA analysis. Standard plaque assays were performed to quantify infectious virus in tissue homogenates. As shown in Fig. 4-1A, infectious MHV-GP33 was recovered from both liver and brain on day 3 p.i. By day 14 p.i., infectious virus was eliminated from the liver and had declined to relatively low titers in the brain (Fig. 4-1A). As expected, no infectious virus was detected in any of the organs at days 21 and 28 p.i. (Fig. 4-1A). Though wild-type MHV has been shown to replicate at low levels in the spleen (17), no infectious virus was detected in spleens of P14 chimeras infected with MHV-GP33 at the times shown (Fig. 4-1A). Since viral RNA typically persists in the brain following clearance of infectious virus, we next performed quantitative RT-PCR (qRT-PCR) to evaluate levels of MHV genome and the genome region encoding GP33. Using primers designed to a conserved region of the MHV replicase gene, viral genome was detected in both liver and brain at day 3 p.i. (Fig. 4-1B). By day 14 p.i., viral genome levels remained elevated in the brain but were no longer detectable in the liver (Fig. 4-1B). Despite a gradual decline, genomic RNA remained detectable
in the brain by day 28 p.i. In contrast, viral genome levels in the liver were no longer detectable (Fig. 4-1B). We have previously shown that GP33 epitope immunization prior to MHV-GP33 infection provides high selective pressure that results in mutation and/or deletion of the viral GP33 epitope during the acute stage of infection (4); similar results were observed when large numbers of P14 CD8 T cells (2×10^7/mouse) were adoptively transferred prior to viral infection. To determine if the GP33 region of the genome remained intact during the course of MHV-GP33 infection in our system, we designed qRT-PCR primers to amplify the region of gene 4 surrounding the GP33 sequence. As shown in Fig. 4-1B, the pattern of GP33 expression paralleled that of MHV genome. While nucleotide sequencing of these qRT-PCR products revealed an intact consensus sequence (data not shown), we cannot exclude the possibility of point mutations in the GP33 RNA sequence. However, agarose gel electrophoresis of the qRT-PCR products revealed no significant changes in product size (data not shown), indicating that no gross deletions had occurred in the region encoding the GP33 epitope.

For comparison, we evaluated the replication of CI-13 in the spleen, liver, and brain at early and late times p.i. by plaque assay of tissue homogenates. Wild-type mice were inoculated with LCMV CI-13 and sacrificed on days 8 and 42 p.i. As expected, infectious CI-13 was detected at high levels in all three tissues both early and late after infection (Fig. 4-1C). Notably, CI-13 titers in the brain were considerably higher than titers in the liver at this late timepoint (Fig. 4-1C). In contrast, LCMV Arm is rapidly cleared from all tissues tested by day 8 p.i. (Fig. 4-1D); while viral RNA might be detectable for a short time after day 8 (25), there is no evidence for long-term persistence of LCMV Arm viral RNA (16). These data confirm that MHV-GP33 and CI-13 establish vastly different levels of persistence in the mouse. Thus, we now have a model system with which to compare virus-specific CD8 T cell responses to different viruses exhibiting high, low, or no viral persistence and to evaluate the impact of viral persistence on CD8 T cell responses in different tissues.
Figure 4-1. MHV-GP33 and LCMV CI-13 are chronic viral infections. A) 5×10^5 naïve T cells from Ly5.1^+ P14 Tg mice were adoptively transferred to congenic naïve recipients. Recipients were then infected with MHV-GP33. Viral titers were measured in multiple tissues by plaque assay of tissue homogenates at the indicated days p.i. (n=3-5). B) Quantitative RT-PCR for total viral genome and GP33 in liver and brain during MHV-GP33 infection. C-D) Naïve mice were infected with CI-13 (C) or Arm (D). Viral titers in multiple tissues were determined by plaque assay at the indicated days p.i. (n=7-8).
**CD8 T cell phenotype correlates with level of viral persistence.** To assess the effect of virus persistence on CD8 T cell differentiation, we next examined the differentiation state of virus-specific CD8 T cells at 30 days p.i. This timepoint was chosen to allow comparison of CD8 T cells in mice that had fully cleared Arm infection, mice chronically infected with CI-13, and mice that had cleared infectious MHV-GP33 but had persisting viral RNA in the brain (Fig. 4-1). As shown in Fig. 4-2A, D\(^{b}\)GP33 tetramer\(^{a}\) CD8 T cells were detected in spleen, liver, and brain of virus-infected P14 chimeras. As expected, these cells were predominantly CD44\(^{hi}\), indicative of prior activation (Fig. 4-2A). The frequency of tetramer\(^{a}\) CD8 T cells in the brain following Arm and MHV-GP33 infection was similar at approximately 40% of total CD8 T cells (Fig. 4-2A). In contrast, however, the frequency of D\(^{b}\)GP33-specific CD8 T cells in the spleen and liver of MHV-GP33-infected mice was substantially lower than for Arm-immune mice (Fig. 4-2A). The frequency of D\(^{b}\)GP33-specific CD8 T cells in the brains of CI-13-infected mice was slightly lower than in Arm- or MHV-GP33-infected mice, while the frequencies of D\(^{b}\)GP33-specific cells in the spleen and liver of CI-13 infected mice were intermediate between Arm- and MHV-GP33-infected mice. Some of the differences in virus-specific CD8 T cell frequencies between CI-13- versus Arm- or MHV-GP33-infected mice could reflect the lower number of P14 CD8 T cells initially transferred. In contrast, the MHV-GP33- and Arm-infected mice received the same number of naïve P14 cells, suggesting different dynamics and tissue distribution of the virus-specific CD8 T cell responses (for cells responding to the same epitope) following these infections. The number of virus-specific CD8 T cells in the spleens of MHV-GP33-infected mice was relatively low compared to other tissues. In all cases, however, a substantial number of virus-specific CD8 T cells were present in the brain and liver, allowing qualitative comparisons of antiviral CD8 T cells generated during these different infections.

To begin to investigate the activation and differentiation state of virus-specific CD8 T cells following LCMV Arm, CI-13, or MHV-GP33 infection, we first examined expression of CD69, an early activation marker that is highly expressed on virus-specific CD8 T cells in the brain at late times p.i. with other neurotropic viruses such as dengue virus, influenza virus, and the MHV variant J2.2-V-1 (3, 7, 33). As shown in Fig. 4-2B, tetramer\(^{a}\) CD8 T cells isolated from the liver of...
Arm- and MHV-GP33-infected mice were predominantly negative for CD69 expression while most tetramer<sup>+</sup> cells in Cl-13 liver expressed low levels of CD69. In contrast, tetramer<sup>-</sup> CD8 T cells isolated from the MHV-GP33-infected brain more closely resembled cells isolated from Cl-13-animals rather than Arm-immune mice, with the majority of tetramer<sup>+</sup> cells in both persisting infections expressing high levels of CD69 while the Arm-immune CD8 T cells were largely CD69<sup>lo</sup> (Fig. 4-2B). These data correlate with the level of MHV-GP33 and Cl-13 persistence in these organs; in the liver where infectious MHV-GP33 and viral RNA are cleared, MHV-GP33-primed CD8 T cells more closely resemble Arm-immune cells whereas in the brain where viral RNA persists CD8 T cells appear more like cells in Cl-13-infected brain, even though the level of MHV-GP33 versus Cl-13 persistence in the brain is different. It should be noted that CD69 expression could reflect persisting antigen stimulation but also might indicate ongoing inflammation at the site of viral persistence (41). Also, the consequence of expressing CD69 on virus-specific CD8 T cells in the brain, for example inhibition of S1P<sub>1</sub>-mediated egress (32), could have important implications.

The transition of CD8 T cells from effector to memory cells is typically associated with alterations in cell surface molecule expression. We next evaluated surface expression of killer cell lectin-like receptor G1 (KLRG1), CD127, and CD62L on virus-specific CD8 T cells in the liver and brain on day 30 p.i. High expression of KLRG1 and low CD127 has been demonstrated on short-lived effector cells during resolution of LCMV Arm infection (10). Conversely, high level expression of CD127, the homeostatic cytokine IL-7 receptor alpha (IL-7Rα) chain, and low KLRG1 is associated with memory precursor CD8 T cells that have the capacity to differentiate into long-lived memory CD8 T cells (9, 13, 28). The lymphoid homing molecule L-selectin (CD62L) is also upregulated as CD8 T cells transition from effector to memory (8). During Cl-13 infection, in contrast, virus-specific CD8 T cells remain CD127<sup>lo</sup> and CD62L<sup>lo</sup> long-term, but also, despite exhibiting some characteristics of terminal differentiation, these cells do not express KLRG1 (35). As with CD69 expression, D<sup>b</sup>GP33 tetramer<sup>+</sup> CD8 T cells isolated from MHV-GP33-infected liver were phenotypically similar to cells isolated from LCMV Arm-immune liver with regards to KLRG1, CD127, and CD62L expression (Fig. 4-2C). Approximately 39% of the
tetramer^+ cells in Arm- and MHV-GP33-infected liver were KLRG1^+ compared to only 7% in CI-13-infected liver (Fig. 4-2C). Strikingly, nearly 70% of the tetramer^+ cells in Arm- and MHV-GP33-infected liver expressed CD127 compared to only 39% in CI-13-infected liver and the overall MFI was substantially lower during CI-13 infection (Fig. 4-2C). Furthermore, roughly 10% of the tetramer^+ cells in Arm- and MHV-GP33-infected liver had upregulated CD62L on the cell surface whereas only 2% were CD62L^+ in CI-13-infected liver (Fig. 4-2C).

Interestingly, a different pattern of surface expression was observed in MHV-GP33-infected brain compared to liver (Fig. 4-2C and 4-2D). While over 55% of the tetramer^+ cells in LCMV Arm-immune brain were KLRG1^+, only 1-4% of the tetramer^+ cells were positive for KLRG1 in CI-13- and MHV-GP33-infected brain (Fig. 4-2D). Similar results were observed for CD62L, with 2-4% of tetramer^+ cells being positive in CI-13- and MHV-GP33-infected brain compared to over 7% in Arm-immune brain (Fig. 4-2D). Curiously, the majority of the tetramer^+ cells in MHV-GP33-infected brain expressed high levels of CD127 similar to cells isolated from Arm-immune brain (Fig. 4-2D). This intermediate surface phenotype of cells in MHV-GP33-infected brain compared to tissues infected with Arm and CI-13 correlates with the intermediate level of viral persistence and inflammation/demyelination present during this stage of MHV-GP33 infection.
Figure 4-2. Antigen-specific CD8 T cells responding to MHV-GP33 or LCMV infection follow distinct differentiation pathways. 5x10^5 naive CD8 T cells from Ly5.1+ P14 Tg mice were adoptively transferred to congenic naïve recipients. Recipients were infected with LCMV Arm, CI-13, or MHV-GP33. Data represent pooled cells isolated from the indicated tissues at 30 days p.i. 

A) Frequency of donor antigen-specific CD8 T cells in multiple tissues during MHV-GP33 or LCMV infection. Frequencies of D^bGP33-specific CD8 T cells were measured in the indicated tissues at 30 days p.i. All plots are gated on total CD8 T cells. Numbers in plots show the percentage of total CD8 T cells that are D^bGP33^+. 

B) CD69 expression on donor antigen-specific CD8 T cells during MHV-GP33 or LCMV infection. CD69 expression on D^bGP33-specific CD8 T cells
cells isolated from the indicated tissues at 30 days p.i. was quantified. All plots are gated on total CD8 T cells. Numbers in plots show the percentage of DbGP33-specific CD8 T cells that are CD69+. C-D) Phenotype of antigen-specific CD8 T cells during MHV-GP33 or LCMV infection. Expression of indicated markers was measured on cells isolated from the liver (C) or brain (D) at 30 days p.i. Plots are gated on total CD8 T cells. Numbers in each plot indicate the percentage of DbGP33-specific CD8 T cells that are positive for each indicated marker. For all panels, plots are representative of 5 independent experiments.
**CD8 T cell functionality is slightly impaired during MHV-GP33 persistence.** A prediction from models about T cell exhaustion during chronic viral infections is that the level of viral and/or antigen persistence impacts the severity of T cell dysfunction; that is, the hierarchical stages of exhaustion get progressively worse as viral and/or antigen burden increases. However, these models are based largely on examining T cells responding to different viral loads for the same infection or by comparing responses to different epitopes during different viral infections. Using the MHV-GP33 versus LCMV Arm and Cl-13 system outlined here, we next examined whether the differences in memory CD8 T cell differentiation and phenotype also corresponded to different functional properties or stages of exhaustion. Typically, during T cell exhaustion, functional properties are lost in a hierarchical manner as viral/antigen load increases, with the ability to secrete IL-2 lost early followed successively by defects in efficient cytotoxicity, robust proliferative capacity and TNFα production, and, finally, compromised production of IFNγ and chemokines such as MIP1-α or -β (39). An increase in expression of the inhibitory receptor PD-1, and other inhibitory receptors, is also associated with increasing severity of exhaustion (5). Therefore, we evaluated expression of these cytokines, as well as granzyme B and PD-1, in CD8 T cells from the liver following GP33 peptide stimulation. As shown in Fig. 4-3A and 4-3B, the majority of IFNγ⁺ cells in liver of Arm-immune mice also expressed TNFα whereas most IFNγ⁺ cells from Cl-13-infected mice failed to produce TNFα. CD8 T cells in the MHV-GP33-infected liver, though intermediate with respect to IFNγ/TNFα co-production, more closely resembled cells from Arm-immune mice (Fig. 4-3A and 4-3B). A similar pattern was observed in the brain, with GP33-specific CD8 T cells from MHV-GP33-infected mice being intermediate between GP33-specific cells from Arm- or Cl-13-infected brain; however, cells in MHV-GP33-infected brain were more similar to cells in Arm-immune brain than to CD8 T cells present during Cl-13 infection. In terms of IL-2 expression, the percentage of IFNγ⁺ cells in MHV-GP33-infected tissues that were also positive for IL-2 was again intermediate between Arm and Cl-13 (Fig. 4-3A and 4-3B), suggesting a partial loss of function during MHV-GP33 persistence. Thus, as with cell phenotype, MHV-GP33 was again intermediate between Arm and Cl-13 in terms of cytokine production by GP33-specific
CD8 T cells, further supporting our model that viral persistence contributes to CD8 T cell dysfunction during chronic infection.

We also evaluated intracellular levels of granzyme B in D\textsuperscript{b}GP33 tetramer\textsuperscript{*} cells from liver and brain. As CD8 T cells differentiate from effector cells to resting memory cells, they downregulate expression of cytotoxic molecules including granzyme B (12). However, during chronic LCMV infection, granzyme B expression is sustained, though killing in some assays (e.g. 51Cr-release) is compromised in some settings. As expected, the lowest percentages of tetramer\textsuperscript{*} cells that were positive for granzyme B (approximately 39%) were observed in the liver and brain of Arm-immune mice (Fig. 4-3D). Consistent with ongoing viral replication, a larger percentage of tetramer\textsuperscript{*} cells in CI-13-infected liver and brain (52-57%) expressed granzyme B (Fig. 4-3D). Interestingly, while granzyme B expression in MHV-GP33-infected liver cells was similar to cells from Arm-immune liver, over 85% of the tetramer\textsuperscript{*} cells from MHV-GP33-infected brain continued to express granzyme B (Fig. 4-3D). The elevated granzyme B levels in cells from MHV-GP33-infected brain even compared to CD8 T cells in CI-13-infected brain could suggest additional defects in degranulation, resulting in retention of granzyme B within the cell. Thus, CD8 T cells retained in the brain during MHV-GP33 persistence appear more similar to the dysfunctional cells present during chronic CI-13 infection, lending additional support to the proposed link between virus persistence and T cell dysfunction.

Given the important role for inhibitory receptors during persisting viral infections (2, 5), we next assessed expression of PD-1 on the D\textsuperscript{b}GP33 tetramer\textsuperscript{*} cells. Previous studies show high levels of PD-1 on the surface of exhausted CD8 T cells during chronic LCMV CI-13 infection compared to functional memory cells present in Arm-immune mice (2). As expected, the mean fluorescence intensity (MFI) of PD-1 on tetramer\textsuperscript{*} cells from CI-13-infected liver and brain was significantly higher (>100-fold) compared to cells from Arm-immune mice (Fig. 4-3E). Interestingly, D\textsuperscript{b}GP33\textsuperscript{*} CD8 T cells from MHV-GP33-infected liver expressed a similar amount of PD-1 compared to D\textsuperscript{b}GP33\textsuperscript{*} CD8 T cells from the livers of Arm-immune mice. In contrast, the PD-1 MFI of D\textsuperscript{b}GP33\textsuperscript{*} CD8 T cells in the brains of MHV-GP33-infected mice was increased approximately 5-fold compared to the D\textsuperscript{b}GP33\textsuperscript{*} CD8 T cells from Arm-immune animals (Fig. 4-
3E). This PD-1 expression profile is consistent with the modest functional defects illustrated above. Notably, the MHV-GP33-primed D\textsuperscript{p}GP33-specific CD8 T cells are largely functional, and any modest compromise of their functional capacity has only been revealed through direct comparison to CD8 T cells responding to the same epitope following LCMV Arm infection, further emphasizing the utility of this model for identifying subtle alterations in T cell quality.
Figure 4-3. Antigen-specific CD8 T cells do not become exhausted during chronic MHV-GP33 infection. 5×10⁵ naïve splenocytes from Ly5.1⁺ P14 Tg mice were adoptively transferred to congenic naïve recipients. Recipients were infected with LCMV Arm, CI-13, or MHV-GP33. Data represent pooled cells isolated from the indicated tissues at 30 days p.i. A-B) Cytokine production by antigen-specific CD8 T cells. Cells isolated from the liver (A) or brain (B) at 30 days p.i. were stimulated with GP33 peptide for 5 hours. Plots are gated on total CD8 T cells. Numbers in plots show the percentage of IFNγ⁺ CD8 T cells that co-produce the second indicated cytokine. Data are representative of 3 independent experiments. C) Cytokine production by antigen-specific CD8
T cells. Graphs show co-production of IFNγ and TNFα (top) or IFNγ and IL-2 (bottom) after stimulation with GP33 peptide. Data are representative of 3 independent experiments. D) Cytotoxicity of antigen-specific CD8 T cells. Granzyme B expression was measured in DβGP33-specific CD8 T cells isolated from the indicated tissues at 30 days p.i. Plots are gated on total CD8 T cells. Numbers in plots show the percentage of DβGP33-specific CD8 T cells that are granzyme B	extsuperscript{hi}. E) Inhibitory receptor expression on antigen-specific CD8 T cells. Amount of PD-1 expression on DβGP33-specific CD8 T cells isolated from the indicated tissues was measured at 30 days p.i. Data are representative of 5 independent experiments.
Antigen-specific CD8 T cells isolated during chronic MHV-GP33 infection proliferate in response to pathogen rechallenge. The data in Fig. 4-2 and 4-3 suggest that GP33-specific CD8 T cells isolated from the liver and brain at day 30 p.i. with MHV-GP33 are intermediate in phenotype and function compared to cells present during Arm and Cl-13 infections, though they appear to fall toward the Arm end of the spectrum in terms of low viral persistence and high functional capacity. Furthermore, cells isolated from the brains of MHV-GP33-infected mice appear slightly more impaired than cells isolated from the liver of the same mice, consistent with viral RNA and inflammation in the brain tissue. We therefore wanted to compare GP33-specific CD8 T cells from the different infections and different tissues in terms of their ability to mount a recall response upon challenge infection. As outlined in Fig. 4-4A, P14 chimeras were infected with LCMV Arm, Cl-13, or MHV-GP33. At day 30 p.i., donor lymphocytes were harvested from spleen, liver, and brain, the number of D\textsuperscript{b}GP33-specific CD8 T cells was normalized, and \(1 \times 10^4\) Ly5.1\(^{+}\) D\textsuperscript{b}GP33 tetramer\(^{+}\) CD8 T cells were adoptively transferred i.v. to naïve Ly5.2\(^{+}\) C57BL/6 recipients. Recipient mice were subsequently challenged i.v. with Listeria monocytogenes expressing the GP33 epitope (LM-GP33) (11). At day 7 post-challenge, lymphocytes were isolated from the spleen, liver, and peripheral blood of the challenged recipients and analyzed by flow cytometry for quantification of total cells. As expected, donor GP33-specific CD8 T cells isolated from the spleen of Arm-immune mice at day 30 p.i. proliferated to high levels when challenged with LM-GP33, with donor GP33-specific cells comprising roughly 1.6% of total CD8 T cells in the recipient liver (Fig. 4-4B). In sharp contrast, donor GP33-specific CD8 T cells isolated from Cl-13-infected spleen only constituted 0.04% of total CD8 T cells in the recipient liver (Fig. 4-4B). Interestingly, comparison of MHV-GP33 donor cells again revealed intermediate results, with MHV-GP33 donor cells comprising a greater percentage of total CD8 T cells in the recipient liver than Cl-13 donor cells regardless of whether the MHV-GP33- and Cl-13-derived D\textsuperscript{b}GP33-specific CD8 T cells were from the liver or brain (Fig. 4-4B). However, the D\textsuperscript{b}GP33\(^{+}\) CD8 T cells from brain and liver of MHV-GP33-infected mice did not expand as well as the D\textsuperscript{b}GP33-specific CD8 T cells from the spleen of Arm-immune mice. It is possible that some of the differences between MHV-GP33- and Arm-derived D\textsuperscript{b}GP33-specific CD8 T cells reflect differences between CD8 T
cells from the liver versus spleen, but Arm-immune CD8 T cells from the liver and spleen have relatively modest differences in phenotype and function at day 30 p.i. (data not shown).

To more rigorously assess donor cell proliferation in response to LM-GP33 challenge, the absolute number of responding P14 cells from MHV-GP33-infected mice was quantified in the recipient spleen and liver and normalized to the number of responding P14 cells from Arm-immune spleen. As shown in Fig. 4-4C, donor GP33-specific CD8 T cells isolated from MHV-GP33-infected liver and brain proliferated to similar levels in both compartments examined. Thus, GP33-specific CD8 T cells maintained during MHV-GP33 persistence retain the ability to proliferate in response to Ag re-exposure but are slightly impaired compared to cells from Arm-immune mice. Taken together, our data fit well with our working model that the amount of viral persistence dictates the severity of CD8 T cell dysfunction during chronic infection. Thus, MHV-GP33, which persists at a low level in the brain but not in the liver, has only a modest impact on the overall functionality of antiviral CD8 T cells and would thus be positioned closer to Arm than to Cl-13 on a spectrum of persistence as well as CD8 T cell impairment.
Figure 4.4. Antigen-specific CD8 T cells isolated during chronic MHV-GP33 infection proliferate well in response to pathogen rechallenge. A) Schematic of experiment. 5 \times 10^6 naïve T cells from Ly5.1\ber P14 Tg mice were adoptively transferred to congenic naïve recipients that were subsequently infected with Arm, Cl-13, or MHV-GP33. At 30 days p.i., donor P14 T cells were isolated from tissues, pooled from multiple mice, and adoptively transferred to naïve recipients (1 \times 10^6 P14/mouse) that were subsequently challenged with LM-GP33. Donor P14 T cells were quantified in spleen, liver, and PBMC at day 7 post-challenge. B) Plots show the population of Ly5.1\ber P14 donor CD8 T cells in recipient liver. Numbers in plots represent the frequency of donor P14 T cells in recipient liver at 7 days p.i. C) Quantification of MHV-GP33 donor Ly5.1\ber P14 T cells in recipient spleen and liver. Data are representative of two independent experiments.
DISCUSSION

As mentioned throughout this report, the LCMV model of acute and chronic viral infection suggested a model in which increased levels of virus persistence parallel severity of CD8 T cell dysfunction. Acute Arm infection is completely cleared, resulting in highly functional memory CD8 T cells, while chronic CI-13 infection, characterized by high levels of replicating virus, results in the development of highly dysfunctional exhausted CD8 T cells. Given the phenotypic and functional data described, we can now place MHV-GP33, a virus characterized by low level persistence of viral RNA in the brain, much closer to the Arm end of the LCMV spectrum due to its low viral persistence and high memory functionality. The similarities between Arm and MHV-GP33 are perhaps most obvious in terms of cytokine production (Fig. 4-3); CD8 T cells isolated from MHV-GP33-infected tissues were surprisingly more polyfunctional than their phenotype suggested. The severe impairment of cytokine expression by virus-specific CD8 T cells isolated from CI-13-infected tissues, along with the decreased capacity to proliferate following LM-GP33 challenge, distances this virus considerably from MHV-GP33. Furthermore, MHV-GP33 infection in the liver, where virus is completely cleared, is slightly more Arm-like while MHV-GP33 infection in the brain during persistence is slightly more similar to CI-13. Importantly, differences in brain versus liver were also apparent during chronic CI-13 infection, particularly in terms of proliferation following LM-GP33 challenge (data not shown); less evidence of CD8 T cell exhaustion was observed in the liver consistent with lower levels of replicating CI-13 in that organ.

During chronic CI-13 infection, virus-specific CD8 T cells undergo an aberrant pathway of differentiation compared to cells from Arm-immune mice that is characterized by functional impairment, upregulation of PD-1, and failure to respond to the homeostatic cytokines IL-7 and IL-15. While virus-specific CD8 T cells in Arm-immune mice are maintained long-term via IL-7 and IL-15 signaling in the absence of Ag, maintenance of CD8 T cells during CI-13 infection instead relies on Ag stimulation provided by ongoing virus replication (31, 38). While the mechanism by which MHV-GP33 RNA persists is not known and viral Ag is not detectable by currently available techniques, viral mRNA is present in the CNS at four weeks p.i. (unpublished data), suggesting a low level of gene expression and thus low level of viral Ag. This low level expression could be
sufficient to modulate CD8 T cell differentiation in the brain during MHV-GP33 infection, resulting
in the modest defects in cytokine expression and upregulation of PD-1 observed.

While our model fits nicely with the data at hand, inherent differences in the pattern of
virus infection must be considered. In the brain, LCMV likely infects cells of the monocyte lineage
whereas MHV has a tropism for neurons and glia. Such differences in cellular tropism within a
highly specialized organ like the brain could have profound impacts on CD8 T cell responses by
differentially impacting levels of virus replication, Ag release, innate immune responses, and
interactions with antiviral CD8 T cells. Furthermore, MHV replicates primarily in the liver and brain
while LCMV strains are more widely disseminated throughout the body resulting in a more
systemic infection. In light of these differences between LCMV and MHV pathogenesis, it is
perhaps even more surprising that our model largely holds true when applied to such a dissimilar
virus. Importantly, the data presented for MHV-GP33 are largely in agreement with previous
studies evaluating the response to endogenous MHV epitopes during CNS persistence of the
MHV variant J2.2-V-1. Zhao et al. demonstrated that a proportion of antiviral CD8 T cells retained
in the CNS during MHV persistence remain multifunctional at days 7, 42, and 70 p.i., as
evidenced by IFNγ, TNFα, IL-2, and granzyme B expression (42). Additional studies using this
MHV variant show that virus persists in oligodendrocytes despite the presence of antiviral CD8 T
cells and that these CD8 T cells express PD-1 even after control of infectious virus (26). It should
be noted that this MHV variant differs significantly from MHV-GP33 in terms of tropism, with J2.2-V-1
replication being restricted to glial cells while MHV-GP33 replicates in both neurons and glia;
Furthermore, J2.2-V-1 does not replicate in the liver while MHV-GP33 does. Thus, the MHV-GP33
virus used in our studies offers several advantages for these types of studies due to 1) the ability
to increase precursor cell numbers and monitor donor cell proliferation by adoptive transfer of
congenic P14 Tg T cells, 2) the ability to directly compare the MHV-GP33-specific CD8 T cell
responses with LCMV, and 3) the ability to directly compare MHV-specific cells in the liver, where
virus is completely cleared, and brain where viral RNA persists. Nevertheless, it would be
informative to compare CD8 T cell quality during infection with these two MHV strains in order to
evaluate the contribution of acute viral tropism on subsequent antiviral CD8 T cell responses.
Furthermore, it would be of value to compare endogenous GP33-specific CD8 T cell responses to CD8 T cell responses directed against an endogenous MHV epitope like S598; expression of this epitope, which is encoded within the spike glycoprotein, may differ from that of GP33, which is expressed in place of nonessential gene 4 and thus could result in yet another comparison of CD8 T cell responses to an individual virus.

Unique aspects of tissues such as liver and brain must also be considered. The liver, which filters blood draining from the gastrointestinal tract and other abdominal organs, is continually exposed to a variety of insults whereas brain tissue is typically maintained in a more quiescent state with low levels of inflammation. Interestingly, previous studies in which mice were immunized against GP33 and then infected with MHV-GP33 revealed that the GP33/GFP region of this virus is mutated and/or deleted more rapidly in the liver than in the brain (4). Studies isolating LCMV variants from different organs of carrier mice infected at birth with Arm also reveal organ-specific differences in selection of viruses; notably, viruses isolated from the CNS remained biologically similar to the parental Arm virus whereas viruses isolated from the liver or spleen contained reproducible mutations resulting in conversion to a more persistent phenotype like Cl-13 (34). Thus, tissue-specific differences likely play significant roles with regard to virus infection and host immune responses that are only beginning to be appreciated. Furthermore, the distinct CD8 T cell phenotypes observed in liver versus brain with both Cl-13 and MHV-GP33 suggest there is little fluidity between CD8 T cell populations in the brain and liver. Zhao et al. demonstrated using the J2.2-V-1 variant of MHV that virus-specific CD8 T cells in the brain during viral persistence are maintained in part by recruitment of cells from the periphery (42); while CD8 T cells can likely enter the inflamed brain with relative ease, they may become trapped in the brain parenchyma, consistent with CD69 expression as mentioned previously.

Finally, considering the demyelinating process present in mice chronically infected with MHV-GP33, our data raise the question as to whether having antiviral CD8 T cells that are more highly functional would be an advantage or disadvantage for the host. One can imagine that increasing T cell functionality early during persistence may more effectively clear residual infectious virus, thereby limiting the severity of demyelinating disease. In support of this idea,
previous studies using adoptive transfer of P14 CD8 T cells prior to MHV-GP33 infection reduced the severity of acute CNS disease and demyelination (21). However, contrasting studies suggest that antiviral CD8 T cells may contribute to the pathogenesis of demyelination in MHV-infected mice. RAG-deficient mice infected with MHV do not develop demyelination; however, adoptive transfer of splenocytes from infected immunocompetent mice into RAG-deficient mice restores demyelination (36, 40). Furthermore, both CD4 and CD8 T cells alone can restore the development of demyelination in RAG-deficient mice (15). These somewhat conflicting studies highlight the delicate balance between virus control and immune-mediated pathology that can be particularly detrimental if thrown off balance in a delicate tissue like the brain. Thus, additional work is needed to delineate the precise mechanisms of viral control and immune-mediated pathology in the brain during persistent viral infections in order to guide therapeutic approaches for controlling neurotropic infections. As demonstrated here, comparative models of viral infection offer a significant advantage for elucidating subtle mechanisms of viral pathogenesis.

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CHAPTER 5

Discussion
GENERAL CONCLUSIONS AND DISCUSSION

The neurotropic MHV strains rA59 and rJHM.SD differ strikingly in terms of viral dissemination, induction of host immune responses, and ultimate disease outcome in the CNS, thereby providing a useful comparison for studying viral and host determinants of neurovirulence. Two hallmarks of rJHM.SD-induced CNS disease in the mouse are widespread localization of viral Ag throughout the brain parenchyma and low numbers of activated antiviral CD8 T cells at the site of infection (13, 34). We propose that these features cooperatively contribute to the high neurovirulence and uniform lethality of rJHM.SD in adult mice. However, prior to the studies described, relatively little was known about how rJHM.SD spreads so extensively in the brain while avoiding the induction of a CD8 T cell response.

One characteristic of rJHM.SD that has been documented over the years is its capacity to spread cell-to-cell in vivo in the absence of the primary MHV receptor CEACAM1a (8, 27, 28, 40); yet, it was unclear if this CEACAM1a-independent spread phenomenon plays a role in vivo during CNS infection. Addressing the role of CEACAM1a during rJHM.SD infection has been complicated by the fact that CEACAM1a expression is difficult to detect on the surface of CNS cell types and has thus far only been demonstrated on endothelial cells and microglia (9, 31). In Chapter 2, we described a series of studies aimed at identifying expression of MHV receptor genes in CNS cell types and utilized primary cell cultures derived from ceacam1a−/− animals to address the role of CEACAM1a-independent mechanisms of MHV infection and spread in neuronal cells, the predominant target of JHM.SD infection. While low to moderate expression of ceacam1a and ceacam1a-related genes was detected in a variety of CNS cell types, the cell type distribution of mRNA expression and in vitro functionality of these receptors suggest they are unlikely to account for the pattern of rJHM.SD infection observed in ceacam1a−/− mice and cells. Thus, rJHM.SD likely utilizes an additional mechanism of CNS entry/spread that is independent of previously identified ceacam1a-related receptors.

In Chapter 3, we continued to explore the enhanced neurovirulence of rJHM.SD by examining the induction of antiviral CD8 T cell responses during CNS infection. Using an adoptive transfer model, we showed that few naïve CD8 T cells are primed during acute CNS infection with
rJHM.SD, as evidenced by low levels of virus-specific CD8 T cell proliferation in the draining CLN and spleen. This lack of CD8 T cell priming is unlikely to be due to an active viral mechanism as rJHM.SD did not prevent the induction of a CD8 T cell response to rA59 in co-infected animals. Furthermore, peripheral inoculation of rJHM.SD resulted in a robust antiviral CD8 T cell response in the spleen, suggesting that the weak CD8 T cell response to rJHM.SD is specific to CNS infection. After intracranial inoculation of virus, infectious rJHM.SD was not readily detected outside the CNS while rA59 replicated to reasonably high titers in both brain and CLN at early times post-infection. Based on these data and the data presented in Chapter 2, we propose that cell-to-cell spread of rJHM.SD, predominately in neurons, and subsequent restriction of rJHM.SD replication to the CNS limit the availability of viral Ag to antigen presenting cells (APC) and thus result in poor priming of virus-specific CD8 T cells in the CLN.

In Chapter 4, we focused on the persistent demyelinating rA59 strain to study the relationship between viral persistence and CD8 T cell functionality during chronic disease. We presented a model comparing virus-specific CD8 T cell responses during acute and chronic LCMV infection as well as infection with a recombinant MHV A59 expressing the GP33 epitope (MHV-GP33). This system allowed us to directly test the model that increased viral load/persistence corresponds to increased stages of CD8 T cell exhaustion during chronic viral infections; importantly, these comparisons were made between different viruses as well as between organ systems within an individual mouse. The quality of virus-specific CD8 T cells maintained during persistence was evaluated in terms of cell phenotype, effector functionality, and proliferative capacity in the face of pathogen rechallenge. Taken together, the low level of MHV-GP33 persistence compared to chronic CI-13 infection resulted in antiviral CD8 T cells that were of high quality, similar to but slightly less polyfunctional than cells present after Arm clearance. Furthermore, virus-specific CD8 T cells maintained after MHV-GP33 clearance in the liver were more functional than cells present in the persistently infected brain. Thus, our data fit extremely well within this model of viral persistence, lending further support to the idea that persisting virus, whether in the form of RNA, antigen, or infectious virus, can have a direct impact on the quality and protective capacity of antiviral CD8 T cells during chronic disease. Together,
the studies presented in Chapters 2, 3, and 4 further enhance our understanding of how different MHV strains interact with the murine host, from receptor utilization for viral entry/spread to shaping of the antiviral immune response during CNS persistence.

RECEPTOR EXPRESSION AND UTILIZATION IN THE CNS

The MHV lifecycle typically begins with binding of the viral spike glycoprotein to its receptor CEACAM1a on the surface of a host cell (44). This binding triggers a conformational change in spike that is thought to reveal an internal fusion peptide that inserts into the host cell membrane and mediates fusion for virus entry (2). However, expression of CEACAM1a in the CNS is very low compared to other targets of MHV infection like the liver, and only a small subset of cells (microglia and endothelial cells) have thus far been demonstrated to express this receptor. Yet, we and others have shown that both rA59 and rJHM.SD infect multiple CNS cell types including astrocytes, oligodendrocytes, and especially neurons (Fig. 2-1). Using enriched primary CNS cell cultures, we assayed mRNA expression of documented MHV receptor genes. In our studies, ceacam1a expression was detected in microglia as well as oligodendrocyte, astrocyte, and to a much lesser extent neuronal cultures (Fig. 2-2). While we cannot exclude the possibility that contaminating microglia may account for the ceacam1a expression observed in neuron and astrocyte cultures, our data raise the likely possibility that some CNS cell type(s) other than microglia express CEACAM1a in vivo. Based on data from mixed neural cultures, it has been proposed that MHV infection is initiated in CEACAM1a-bearing microglia and that dissemination to additional CNS cell types occurs via a CEACAM1a-independent mechanism (26). Since we have been unable to definitively demonstrate CEACAM1a expression on cells other than microglia we cannot rule out this possibility; however, data from our lab and others argue against this hypothesis. In wild-type neuron cultures, rA59 infects clusters of neurons (Fig. 2-4) (30) despite its dependence on CEACAM1a for spread. Furthermore, the striking observation that spread of rA59 in ceacam1a−/− neurons is severely hindered compared to spread in wild-type cells (Fig. 2-4 and 2-5) indicates that CEACAM1a does indeed play a role in neuronal spread of rA59, further suggesting that neurons likely express low levels of CEACAM1a that are not
detectable by routine methods. Interestingly, the normally glial-tropic JHM variant 2.2-V-1, which is CEACAM1a-dependent, spreads more extensively among glial cells types and also infects neurons in mice deficient in type I interferon signaling (IFNAR\(^{-}\)), arguing that it is not an absence of CEACAM1a that prevents neuronal infection with J2.2-V-1 in wild-type mice (14). Given that CEACAM1a is specifically targeted to cell-cell contact areas and apical surfaces of polarized epithelial cells (39), we hypothesize that CEACAM1a may be similarly sublocalized on the surface of neurons, for example to synaptic membranes, such that receptor density is sufficient to facilitate CEACAM1a-dependent spread to adjacent neurons at these specialized locations. Moreover, MHV has previously been reported to spread transneuronally (1, 42). Sophisticated amplification techniques combined with traditional immunofluorescence and/or in situ hybridization procedures will likely allow for more accurate assessment of receptor expression in individual cells.

While CEACAM1a clearly plays a role in initial virus entry for both rA59 and rJHM.SD (as evidenced by decreased rates of infection in ceacam1a\(^{-}\) compared to wild-type mice and cells) as well as in neuron-to-neuron spread of rA59 (Chapter 2), an additional mechanism appears to be at play in the CNS that allows spread of rJHM.SD in the absence of ceacam1a; this mechanism is largely dependent on the JHM.SD spike (Fig. 2-5) (25). The capacity of rJHM.SD and other highly neurovirulent JHM isolates to spread cell-to-cell in vitro in the absence of CEACAM1a correlates with the presence of a long hypervariable region (HVR) within the spike (5, 7, 8, 28); notably, rA59 contains a 52 amino acid deletion within the HVR, correlating with its inability to spread without CEACAM1a. It remains unclear whether the phenomenon of CEACAM1a-independent spread described for rJHM.SD in vitro is the same phenomenon observed during infection of ceacam1a\(^{-}\) mice and cells; however, the availability of a recombinant JHM.IA virus with a mutation rendering it capable of CEACAM1a-independent spread in vitro (rJIA.S310G) (28) and the prospect of a recombinant JHM.SD virus expressing the reverse mutation (rJSD.G310S) should enable this question to be addressed. To begin to address the mechanism of CEACAM1a-independent entry/spread, we explored the possibility that CEACAM1a-related proteins may facilitate MHV infection in the absence of ceacam1a by
evaluating expression of ceacam2 and psg16 in primary CNS cells. While ceacam2 expression was detected at low levels in microglia and oligodendrocyte cultures (Fig. 2-6), neuron cultures were strikingly positive for psg16 expression (Fig. 2-7). Based on previous reports of receptor functionality (3) and the data presented here (Fig. 2-8), it is unlikely that PSG16 mediates rJHM.SD entry or spread. However, as we propose for CEACAM1a, selective localization to a particular region of the neuron, either by direct protein targeting to the membrane or association with another membrane moiety, may position PSG16 to mediate infection in ways that were not appreciated in transfection experiments using cultured cell lines. Furthermore, the possibility that PSG16 might be secreted from neurons and/or other cell types during infection could suggest a role in intercellular signaling as has been described for PSG17 and PSG18 (12, 43). We cannot exclude the possibility that an alternative receptor unrelated to CEACAM1a exists in the CNS and functions as a receptor for rJHM.SD; however, such a receptor would be expected to function for cell-to-cell spread only as infection rates are significantly reduced in the absence of ceacam1a.

Given the range of differences in spike glycoproteins of different MHV strains and their impact on viral tropism and virulence (Fig. 1-2, Table 1-1), it is possible that small changes in sequence could drastically affect the binding of the MHV spike to an alternate receptor and/or lower the threshold required to trigger a conformational change rendering the spike fusion competent in the absence of receptor binding. As such, spontaneous activation of the JHM.SD spike due to inherent instability of the S1/S2 interaction has been suggested as a mechanism for CEACAM1a-independent spread (7). We further postulate that the HE protein expressed on the surface of a subset of MHV strains, including rJHM.SD, may enhance viral attachment to a host cell via interaction with sialic acid-containing molecules on the surface (33); binding of these smaller HE spikes to surface moieties may bring virus and host membranes in close enough proximity such that spontaneous activation of the JHM.SD spike could trigger membrane fusion. Indeed, expression of the HE gene enhances virulence of a recombinant A59 expressing the JHM.SD spike (16). Alternatively, rJHM.SD may be passively transported from neuron to neuron within synaptic vesicles, thereby eliminating the requirement for CEACAM1a in cell-to-cell spread; virus transport within synaptic vesicles could allow transmission of either whole virions or
nucleocapsid complexes to a neighboring cell without the need for a virus receptor, similar to the process described for measles virus (22). Importantly, such a mechanism would be specific to rJHM.SD (possibly due to characteristics of the long spike, as discussed above) since rA59 requires CEACAM1a for efficient neuron-to-neuron spread.

Since ceacam1a mRNA is alternatively spliced yielding four distinct species in the mouse, we compared expression of individual ceacam1a splice variants and showed that different organs and cell types express different ratios of short-tailed to long-tailed species (Fig. 2-3). Notably, splice variants encoding a long cytoplasmic tail predominated in the CNS while short- and long-tailed species were more balanced in the liver (Fig. 2-3). Isoforms with long cytoplasmic tails contain phosphorylatable serine, threonine, and tyrosine residues that participate in protein-protein interactions and intracellular signaling cascades, with roles in insulin regulation and tumor suppression (17). While short cytoplasmic tails lack these phosphorylatable residues, they have instead been shown to bind elements of the cytoskeleton like actin, calmodulin, and tropomysin (10). Of particular interest, long-tailed CEACAM1a isoforms expressed on murine dendritic cells have been shown to stimulate cell maturation as well as secretion of the chemokines CCL3 (MIP1α) and CXCL2 (MIP2) and cytokines IL-6 and IL-12 in response to monoclonal antibody stimulation (15). Therefore, we hypothesize that binding of the MHV spike to CEACAM1a, similar to antibody binding, may trigger release of cytokines from infected CNS cells. Furthermore, MHV binding to CEACAM1a on dendritic cells may activate these APC, leading to DC migration and priming of naïve T cells in the CLN. Given the functional differences attributed to long- and short-tailed CEACAM1a isoforms, is tempting to speculate that MHV binding to long-tailed isoforms may trigger or modulate intracellular signaling pathways in ways that virus binding to short-tailed isoforms may not and that the induction of protective immune responses could be limited in situations where MHV spreads in the absence of CEACAM1a.

CD8 T CELL PRIMING DURING ACUTE DISEASE

Numerous studies have highlighted the importance of antiviral CD8 T cells in protection against coronavirus-induced CNS disease and clearance of infectious virus. During acute CNS
infection with rA59 or neuroattenuated JHM variants, activated virus-specific CD8 T cells accumulate at the site of infection, peaking in the brain at day 7 p.i. and coinciding with viral clearance (21, 45). Interestingly, CNS infection with the highly neurovirulent JHM.SD induces a weak virus-specific CD8 T cell response in the brain despite high levels of infectious virus and widespread distribution of viral Ag (Fig. 3-5)(13, 34). However, it was unclear why mice fail to mount an effective immune response to JHM.SD in the brain. In collaboration with Katherine MacNamara, we addressed this issue by comparing CD8 T cell priming during infection with rA59, rJHM.SD, and the chimeric rA59/S\textsubscript{JHM.SD}. Previous studies using an attenuated variant of JHM demonstrated that naïve virus-specific CD8 T cells are primed by APC in the draining CLN, where they begin to expand and upregulate antiviral effector functions; these activated cells increase further in number in the spleen and ultimately traffic to the site of infection, the brain (24). Using adoptive transfer of naïve CFSE-labeled P14 cells prior to or early after CNS infection with GP33-expressing viruses, we observed significant proliferation of P14 donor cells following infection with either rA59 or rA59/S\textsubscript{JHM.SD} but not with rJHM.SD (Fig. 3-2); these results correlate with the accumulation of virus-specific CD8 T cells in the brain during infection with these viruses. Interestingly, rJHM.SD did not cause a generalized immunosuppression in the brain, as mice co-infected with rJHM.SD and rA59 mounted a CD8 T cell response directed solely against rA59 (Fig. 3-3). Notably, i.p. inoculation of rJHM.SD resulted in a robust antiviral CD8 T cell response in the spleen (Fig. 3-7), indicating that the weak CD8 T cell response to rJHM.SD observed in the brain is limited to the context of CNS infection.

Prior to these studies, we hypothesized that rJHM.SD might actively evade the host immune system, as is the case for many other viruses. However, the data in Fig. 3-3 and 3-7 instead suggest that the failure of rJHM.SD to elicit a robust antiviral CD8 T cell response in the brain is more of a passive mechanism. The predominant neuronal tropism of rJHM.SD (Fig. 2-1)(6), paired with unique mechanisms of neuronal survival in the face of insult, may enable rJHM.SD to spread, at least temporarily, without being detected by APC in the brain. The low level of rJHM.SD replication in cultured neurons (Fig. 2-4) and delayed viral replication in the brain compared to rA59 (Fig. 3-5) support the hypothesis that viral Ag is sequestered away from
APC at early times post-infection. Furthermore, rJHM.SD replication was largely restricted to the CNS, as infectious rJHM.SD virus (Fig. 3-5) and viral RNA (unpublished data) were barely detectable in the draining CLN while replication of rA59 and rA59/S_{JHM.SD} was readily detected in the CLN. While virus replication in the LN is not required for priming of a CD8 T cell response, one can imagine that additional virus replication at the site of T cell priming could increase the amount of viral Ag available for presentation by APC; therefore, minimal levels of rJHM.SD replication at sites outside the CNS would further limit the accessibility of viral Ag to APC. However, sequestration of virus from APC is unlikely to be complete, and some level of infectious rJHM.SD would ultimately be expected to contact APC in the brain; in this case, viral infection could lead to lysis of APC or otherwise compromise APC function such that Ag presentation in the CLN does not successfully occur. While cytopathic effects and impairment of APC maturation have been observed in vitro in bone marrow-derived dendritic cells infected with MHV (48) (unpublished data), the data presented in Fig. 3-7 argues against rJHM.SD-mediated impairment of APC function as peripheral inoculation elicits a robust antiviral CD8 T cell response. However, we cannot exclude the possibility that APC encountering viral Ag under different conditions (CNS vs. periphery) may have disparate outcomes with regard to functional maturation and subsequent CD8 T cell priming. As such, the inherent instability of the JHM.SD spike could render virions noninfectious if they are not immediately engaged by another cell; thus, APC in the periphery could potentially encounter exogenous rJHM.SD Ag more readily than in the brain and process it via cross-presentation pathways for presentation to CD8 T cells, as opposed to processing of endogenous Ag during direct viral infection, thereby avoiding cytotoxic effects of the virus. Additional studies are needed to elucidate the mechanisms of Ag processing following different routes of viral infection.

Regardless of how rJHM.SD avoids the induction of an antiviral CD8 T cell response in the brain, the question remains as to whether CD8 T cells could actually protect mice during CNS infection with a highly neurovirulent virus like rJHM.SD. Recent studies in our lab suggest that antiviral CD8 T cells may be ineffectual against some highly neurovirulent viruses, as evidenced by a virus expressing the A59 replicase paired with the structural genes of JHM.SD; this virus is
highly virulent despite the induction of a robust virus-specific CD8 T cell response (Timothy Cowley, unpublished data). Conversely, induction of antiviral CD8 T cells correlates with delayed mortality in mice infected with rA59/S$_{jHM,SD}$ compared to rJHM.SD, despite the fact that these viruses have the same intracranial LD$_{50}$ (13). Furthermore, co-infection of rA59 and rJHM.SD was protective against rJHM.SD-induced CNS disease as fewer co-infected animals succumbed to infection by day 8 p.i. compared to mice infected with rJHM.SD alone (Fig. 3-4); however, we cannot exclude the possibility that some non-CD8 T cell aspect of the host response to rA59 contributed to this partial protection in co-infected animals. While CD8 T cell-mediated cytolysis is important for clearing MHV from astrocytes and microglia (36) and viral clearance from oligodendrocytes requires IFN-γ (29), it is not yet clear what antiviral mechanisms are required to clear infectious MHV from neurons. Given the rapid dissemination of rJHM.SD throughout the brain and the pathology caused by infiltrating neutrophils early after infection (13), it seems unlikely that a primary antiviral CD8 T cell response could completely resolve rJHM.SD infection; rather, antiviral CD8 T cells would likely slow the course of CNS disease, as is the case with rA59/S$_{jHM,SD}$. Furthermore, even if virus-specific CD8 T cells were effectively primed during rJHM.SD infection, the lower levels of T cell-recruiting chemokines, such as CXCL9 (MIG) and CXCL10 (IP-10), expressed in the brains of mice infected with rJHM.SD compared to rA59 or rA59/S$_{jHM,SD}$ infected animals (35) could lead to poor recruitment of CD8 T cells to the site of infection.

**CD8 T CELL DYSFUNCTION DURING CHRONIC INFECTION**

Persistence of demyelinating MHV strains, such as rA59, in the CNS provides a unique model system in which to assess the effect of low-level virus persistence on antiviral CD8 T cell differentiation. The LCMV models of acute (Arm) and chronic (Cl-13) infection have been well described, as have the quality of CD8 T cells responding to these viruses at late times post-infection. As outlined in Chapter 4, we utilized a recombinant A59 expressing the GP33 epitope of LCMV in place of nonessential gene 4 (MHV-GP33) (4), thereby allowing the transfer of naïve P14 (GP33-specific) CD8 T cells and comparison of the quality of these cells during subsequent
viral infection. Like parental A59, MHV-GP33 replicated in the brain and liver during the first week of infection (Fig. 4-1), after which time virus is completely cleared from the liver but persists in the brain in the form of viral RNA accompanied by immune-mediated demyelination. This intermediate strategy of viral persistence provided an important comparison to Arm, which is cleared by 8-10 days p.i., and CI-13, which continues to replicate for approximately 2 months p.i. Interestingly, GP33-specific CD8 T cells isolated from the liver of MHV-GP33-infected mice at day 30 p.i. phenotypically resembled cells from the liver of Arm-immune mice while cells isolated from MHV-GP33 infected brain looked slightly more like the dysfunctional CD8 T cells isolated from the brains of mice chronically infected with CI-13 (Fig. 4-2); these differences in T cell quality directly correlate with tissue differences in MHV-GP33 persistence (complete clearance in liver versus RNA persistence in brain). In terms of cytokine production, GP33-specific CD8 T cells from the brain and liver of MHV-GP33-infected mice were largely dual functional in terms of IFNγ and TNFα expression following peptide stimulation; furthermore, granzyme B remained high, particularly in cells isolated from the brain (Fig. 4-3). These results are in agreement with previous studies using the attenuated 2.2V-1 variant of JHM, where a proportion of antiviral CD8 T cells retained in the CNS during viral persistence remained multifunctional at days 7, 42, and 70 p.i., as assessed by IFNγ, TNFα, IL-2, and granzyme B expression (47). Similar studies showed that viral challenge following epitope immunization resulted in accumulation of virus-specific IFNγ+ cells, half of which co-produced TNFα; a high frequency of these secondary responding cells were also granzyme B+ (32).

In our final experiment, we tested to ability of GP33-specific CD8 T cells from persistently-infected mice to proliferate in response to LM-GP33 rechallenge in an otherwise naïve host. As expected from our functional data, donor P14 cells isolated from the brain and liver of MHV-GP33-infected animals proliferated well in response to Ag re-exposure (Fig. 4-4). Together, our results fit exceptionally well with our predictions from the LCMV model that low-level virus persistence, as with MHV-GP33, will have modest effects of CD8 T cell quality. Further, low levels of MHV-GP33 persistence in the brain compared to viral clearance in the liver
resulted in CD8 T cells in the brain that were slightly less functional than their liver counterparts. Whether the persistent viral RNA allows for low-level translation of viral Ag that continues to stimulate CD8 T cells or whether some other aspect of MHV persistence, such as inflammation associated with demyelination, is having a direct effect on CD8 T cell differentiation is unclear; however, we now have a working model in which to test individual aspects of MHV persistence for their effects on T cell responses. Furthermore, additional analyses will be important to more fully characterize the subtle T cell defects during MHV persistence and determine if the functionality of these cells changes over time.

Studies of J2.2V-1 infection in IL-15−/− mice suggest that activated memory-like cells undergo a low level of proliferation in the absence of IL-15 in persistently-infected mice (49). Previous studies by this group also demonstrated that persistent viral RNA is required to retain antiviral CD8 T cells in the brain during persistent J2.2V-1 infection (23). Given that CD127 is highly expressed on MHV-specific CD8 T cell in the brain and liver (Fig. 4-2), it is likely that IL-7 plays a role in driving homeostatic proliferation of GP33-specific CD8 T cells during MHV-GP33 persistence; however, future studies should address the capacity of these cells to maintain their population numbers in the absence of persistent viral RNA and inflammation. Furthermore, endogenous comparisons of CD8 T cell responses directed against the GP33 epitope versus the naturally occuring S598 epitope will be informative with regard to the integrity of the GP33-encoding region of the MHV-GP33 genome at late times post-infection. While we did not detect gross deletions of consensus mutations, it is possible that the virus-specific CD8 T cells present in the brain and/or liver exert selective pressure that could render this epitope nonfunctional; since S598 is encoded within a key structural protein, the spike, it is less likely to tolerate mutation as well as the nonessential GP33 and thus may result in a different pattern of persistence/expression. Another aspect of MHV persistence that is unclear is the role of cellular tropism in shaping the antiviral CD8 T cell response during persistent rA59 and J2.2-V-1 infection; these viruses have unique cellular tropisms during acute disease but are suggested to persist in similar cells, primarily oligodendrocytes, during the chronic phase of infection. If this is the case, it would be interesting to test whether acute neurotropism, specifically of rA59 for neurons, plays a
role in shaping the differentiation of CD8 T cells observed during chronic infection. Conversely, if these viruses persist in different cell types a comparison between the two viruses could reveal cell type-specific mechanisms of viral persistence and immune modulation. Inherent differences between viruses, even within the same organ, should not be overlooked.

Notably, while viral RNA persistence appears to correlate with the differentiation of virus-specific CD8 T cells during chronic disease, we cannot rule out effects of the demyelinating process on CD8 T cell differentiation. MHV-induced demyelination is characterized by infiltration of lymphocytes and lipid-containing macrophages (19, 20, 37, 46). Using infections with the attenuated JHM 2.2-V-1 variant, it was demonstrated that both monocyte-derived macrophages and microglia are present in regions of demyelination in contact with demyelinated axons, suggesting that both cell types participate in the demyelinating process (41). Furthermore, while infected RAG-deficient mice do not develop demyelination, adoptive transfer of either CD4 or CD8 T cells restores the development of demyelination in RAG-deficient mice (18). Chronic activation of astrocytes during persistent infection also contributes to demyelination via the secretion of macrophage- and T cell-chemoattractants, as well as TNFα, IL-1β, IL-6, and type 2 nitric oxide synthesis (iNOS) that may directly contribute to the dysregulation of oligodendrocyte function and resulting myelin loss (11, 38). While the precise role of these immune cell types in MHV-induced demyelination is unknown, it will be important to assess the quality of persisting CD8 T cells in the brain at multiple times p.i. as demyelination waxes and wanes in the continued presence of viral RNA.

FUTURE DIRECTIONS AND FINAL COMMENTS

Many questions remain regarding the precise mechanisms contributing to MHV neurovirulence, and these questions will be most effectively addressed utilizing the multitude of available MHV strains and variants that differ in tropism, receptor utilization, persistence, and host response. All MHV strains analyzed to date utilize CEACAM1a as the primary receptor. Therefore, it is unclear why rJHM.SD disseminates so widely in the CNS but is unable to establish productive infection in the liver, an organ known to express higher levels of CEACAM1a.
than the brain. It is clear that the JHM.SD spike can mediate entry into the liver, suggesting that early host responses may limit rJHM.SD infection in this organ; these processes are beginning to be explored. Furthermore, it is curious that J2.2-V-1 infects only glial cells while other JHM isolates infect neurons as well. In terms of MHV receptor expression, it remains unclear whether neurons express CEACAM1a, though several lines of evidence suggest this is the case. It is also unknown if PSG16 is expressed on the surface of neurons and whether this protein can actually function as a receptor for MHV. It is likely that CEACAM1a-independent spread of rJHM.SD in neurons also occurs independently of CEACAM1a-related proteins, though this mechanism of spread must be more carefully explored. Additionally, the impacts of virus replication on neuronal function, both direct and indirect, have yet to be examined. Overall, it will be important to more fully characterize how different MHV strains enter individual CNS cell types and how infection is subsequently recognized and combated by the host while maintaining the integrity of the delicate CNS tissue. Finally, much remains unknown regarding the establishment of viral persistence in the CNS and interactions of virus with CD8 T cells during chronic disease. The diversity of neurotropic strains and recombinants available combined with the relative ease of genetic manipulation makes MHV an important tool for studying virus-induced pathogenic processes in the CNS, dissecting the development of antiviral immune responses during acute and chronic disease, and elucidating viral and host determinants of neurovirulence.
REFERENCES


