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REVIEW

Immunobiology of congenital cytomegalovirus infection of the central nervous system—the murine cytomegalovirus model

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Congenital human cytomegalovirus infection is a leading infectious cause of long-term neurodevelopmental sequelae, including mental retardation and hearing defects. Strict species specificity of cytomegaloviruses has restricted the scope of studies of cytomegalovirus infection in animal models. To investigate the pathogenesis of congenital human cytomegalovirus infection, we developed a mouse cytomegalovirus model that recapitulates the major characteristics of central nervous system infection in human infants, including the route of neuroinvasion and neuropathological findings. Following intraperitoneal inoculation of newborn animals with mouse cytomegalovirus, the virus disseminates to the central nervous system during high-level viremia and replicates in the brain parenchyma, resulting in a focal but widespread, non-necrotizing encephalitis. Central nervous system infection is coupled with the recruitment of resident and peripheral immune cells as well as the expression of a large number of pro-inflammatory cytokines. Although infiltration of cellular constituents of the innate immune response characterizes the early immune response in the central nervous system, resolution of productive infection requires virus-specific CD8⁺ T cells. Perinatal mouse cytomegalovirus infection results in profoundly altered postnatal development of the mouse central nervous system and long-term motor and sensory disabilities. Based on an enhanced understanding of the pathogenesis of this infection, prospects for novel intervention strategies aimed to improve the outcome of congenital human cytomegalovirus infection are proposed.

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Keywords: central nervous system; congenital infection; cytomegalovirus; immune response

THE MAGNITUDE OF THE PROBLEM

Human cytomegalovirus (HCMV) is the most common cause of congenital viral infection, occurring in 0.4%–1.2% of pregnancies in the developed world.^{1,2} During maternal primary infection, and, to a lesser extent, non-primary infection, HCMV can pass the placental barrier and infect the developing fetus.^{3,4} Infection acquired *in utero* may have no clinical manifestation during the newborn period or may manifest with clinically evident disease, most commonly growth retardation, jaundice, hepatosplenomegaly, microcephaly, hearing impairment and thrombocytopenia.^{5–8} In addition to being at risk for severe, occasionally life-threatening disease,⁹ most symptomatic infants develop one or more long-term sequelae. Among them, the most devastating are those of the central nervous system (CNS)

because, in contrast to other end-organ injuries that are transitory, CNS injury is irreversible and persists for life. Common CNS morbidity includes microcephaly, cerebral palsy, mental retardation, seizures, hearing loss, ocular abnormalities and cognitive impairment.^{10–13} Even congenitally infected infants who are asymptomatic at birth have an increased risk for long-term neurodevelopmental sequelae, in particular, sensorineural hearing loss (SNHL).^{5,14} Additionally, recent investigations suggest that more subtle disorders of brain development, such as autism, may be related to congenital HCMV infection.^{10,15,16}

MOUSE MODEL OF CONGENITAL CMV INFECTION

The species specificity of cytomegaloviruses (CMVs) has limited the study of CMV infection in animal models. These

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models have been successfully used to study many aspects of congenital CMV infection due to comparable genetics between CMVs, with many genes having sequence and functional homologs, and generally similar pathogenic mechanisms in their respective hosts.^{17–19} Among the several described animal models, only guinea pig CMV, rhesus macaque CMV and porcine CMV are known to cross the placenta and cause fetal infection.^{17,18,20,21} Another animal model that has provided considerable insight into the correlation between CMV neuro-pathogenesis and immune protection is the murine CMV (MCMV) model.²² To overcome the shortfall of the inability of MCMV to cross the placenta, investigators have directly injected the virus into either the placenta or embryo.^{23,24} Alternative routes for establishing MCMV infection of the CNS include direct intracranial inoculation of the virus^{25,26} or infection of mice during the neonatal period using milk from MCMV-infected dams²⁷ or intraperitoneal (i.p.) inoculation of the virus.²⁸ Following i.p. MCMV infection of newborn mice, the virus disseminates to the CNS during hematogenous spread and replicates in the brain parenchyma.^{28,29} The latter model, when compared to models that utilize direct intracranial inoculation of the virus, more closely recapitulates the presumed route of HCMV dissemination into the CNS during systemic viremia. However, the exact mechanism that the virus utilizes to cross the blood–brain barrier is unknown. In fact, following i.p. inoculation, MCMV can be detected in both plasma and blood cells of newborn mice, indicating that MCMV may enter the CNS either as a cell-free or cell-associated virus.²⁹ CNS infection of newborn mice consistently results in widespread, focal, non-necrotizing encephalitis and defects in brain development.²⁸ It should be stated that the neonatal mouse CNS is embryologically equivalent to the human fetus at 15 weeks of gestation and that HCMV infection in humans is most frequently acquired during the early periods of the second trimester of pregnancy;³⁰ therefore, CNS disease in newborn mice closely mimics that associated with vertically transmitted HCMV infection in humans.²² Finally, mice infected with MCMV during the neonatal period exhibit neurobehavioral sequelae, such as impaired performance on a stationary balance beam and sensorineural hearing loss, as adult animals (Britt WJ, 2014, unpubl. data).

CMV SHOWS NO CELL TROPISM IN THE CNS

Following i.p. inoculation of newborn mice with MCMV, the virus initially replicates in peripheral organs and then disseminates to the CNS, where it is readily detected on postnatal day 7 (Figure 1a). Virus titers peak in the CNS between postnatal days 10 and 14, and infectious virus becomes undetectable by day 21.^{28,29} After the resolution of productive infection, clearance of the viral genome is not achieved, and MCMV establishes life-long latent infection with potential periodic reactivations. The hallmark of CNS infection is focal involvement in different regions of the brain that is consistent with hematogenous virus spread and is nearly indistinguishable from histological findings in infants with congenital HCMV infection.²⁸ Discrete foci containing small numbers of MCMV-infected cells are distributed

throughout the cerebrum, including the cortex, hippocampus, periventricular region as well as cerebellum and meninges overlaying different regions of the brain with a variety of resident cells being infected²⁸ (Figure 1b). Mononuclear cell infiltrates, mainly composed of T cells and F4/80⁺ macrophages/microglia, surround the foci of infection (Figure 1b). Not infrequently, F4/80⁺ macrophages/microglial cells in these inflammatory foci stain positive for viral antigens.²⁵ Characteristic histological features of the inflammatory foci include reactive astrogliosis, a finding that is similar to the glial nodules described in AIDS patients with HCMV encephalitis and fetuses infected *in utero* with HCMV.^{28,31} Remarkably, there is no evidence of necrosis or tissue damage in the CNS (Figure 1b).²⁸ In sharp contrast, MCMV infection of newborn mice induces significant tissue damage in peripheral organs, most commonly the liver with ballooning degeneration and necrosis of hepatocytes (Figure 1c).³² This observation suggests that the CNS, which is essentially a nonrenewable tissue, has unique strategies that favor clearance of the virus without marked tissue damage.

Practically all resident cells of the CNS have some degree of susceptibility to CMV infection.^{33,34} Astrocytes, microglia, oligodendroglial cells, neurons, neural stem/progenitor cells (NSPCs) and brain microvascular endothelial cells can all be infected with CMV; however, these different cell populations vary in their ability to support a complete viral replication cycle. Astrocytes, the most abundant cell type in the CNS, are fully permissive for CMV replication, which ultimately results in cell death.^{35,36} Treatment of human astrocytes with selected proinflammatory cytokines (IL-1 β , tumor necrosis factor (TNF)- α and interferon (IFN)- γ) induces an antiviral state by blocking viral replication at the level of transcription from the major immediate early promoter (MIEP).^{37,38} Endothelial cells of the brain microvasculature also support lytic infection,^{39–41} and CMV infection of endothelial cells may be a potential means the virus uses to enter the CNS. Interestingly, HCMV infection of endothelial cells also promotes monocyte recruitment, transendothelial migration and infection, which may be another mechanism of viral dissemination into the brain.⁴² Astrocytes and brain microvascular endothelial cells form the blood–brain barrier, a structure that maintains the highly regulated microenvironment in the CNS.⁴³ Infection with other neurotropic viruses has been shown to disrupt the integrity of the blood–brain barrier;⁴⁴ however, following i.p. MCMV infection of newborn mice, the integrity of the blood–brain barrier is unaffected, as determined by Evans blue dye.²⁸ Microglia, which are resident brain cells that share some functions with tissue macrophages and oligodendroglial cells, do not support productive CMV infection.^{35,45}

In the case of neural cells, it appears that the state of cell differentiation and functional status modulate permissiveness to CMV infection.³³ Differentiated neurons are generally considered refractory to CMV replication, although the published data supporting this contention are controversial.^{35,46,47} The block in viral replication occurs at the level of transcription from the MIEP³⁸ and may be, at least in part, explained by

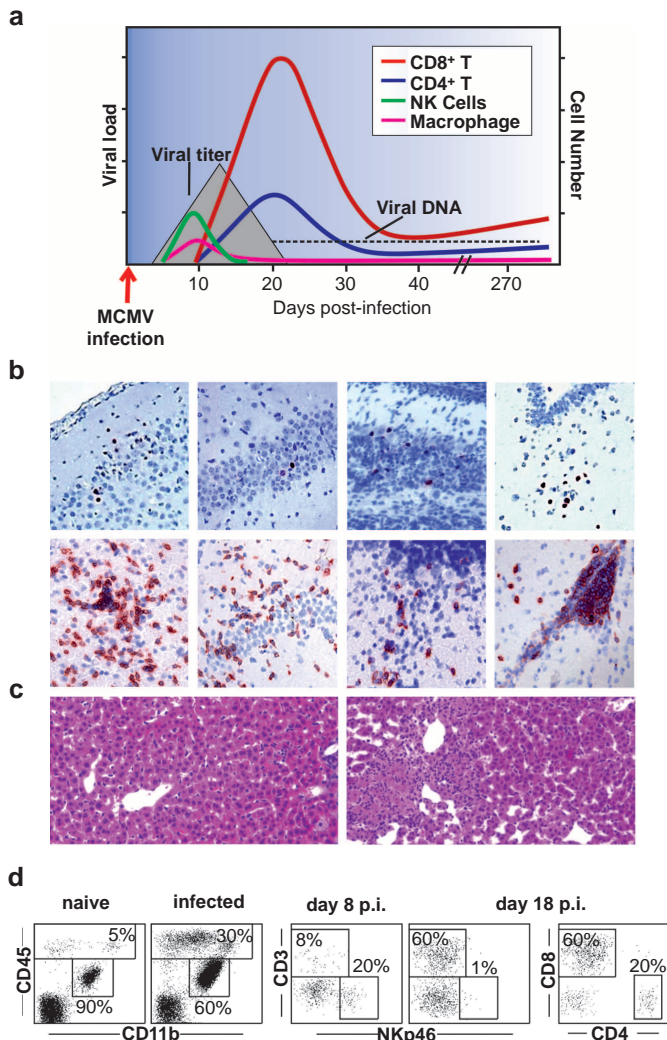


Figure 1 Kinetics of MCMV infection and immune cell influx in the CNS of newborn mice. **(a)** Following i.p. inoculation of newborn mice with MCMV, the virus reaches the CNS by postnatal day 7, and the virus titers peak between days 10–14 and become undetectable by day 21.^{24,25} After the resolution of productive infection, clearance of the viral genome is not achieved. MCMV infection induces an influx of systemic immune cells into the brain. NK cells and macrophages constitute the majority of the infiltrating cells early during infection, whereas T cells are most abundant during peak inflammation and thereafter.^{25,74,82} The accumulation of CD8⁺ T cells correlates with a rapid decline in viral titers, indicating their critical role in resolution of the MCMV infection.⁷⁴ As virus replication is controlled, the number of inflammatory cells decreases; however, CD8⁺ T cells persist in the brain after productive infection is resolved.^{74,82} The magnitude of the CD4⁺ T-cell response remains relatively constant throughout the infection and is outnumbered by CD8⁺ T cells by approximately three to one.^{74,82} **(b)** Brain paraffin sections were stained with anti-MCMV IE1/pp89 mAb (upper row), anti-CD8 mAb (bottom row, first three images) or anti-F40/80 mAb (bottom row, last image) and counterstained with hematoxylin. From left to right, virus-infected cells located in the cerebellar cortex, hippocampus, cerebellum and periventricular region are shown (PN day 11; original magnification, $\times 40$). From left to right, CD8⁺ cell infiltrates in the cerebellar cortex (PN day 42), hippocampus (PN day 42), cerebellum (PN day 42), and vascular infiltration macrophages (PN day 21) are shown (original magnification, $\times 40$). Note the focal nature of

infection and mononuclear infiltrates without any evidence of tissue damage. **(c)** Liver paraffin sections from naive control (left) and MCMV-infected (right) newborn mice were stained with hematoxylin and eosin (PN day 14; original magnification, $\times 40$). Note the degeneration and coagulation necrosis of hepatocytes in infected animals. **(d)** Newborn mice (6–18 h postpartum) were inoculated i.p. with 200 PFU of tissue culture-derived MCMV. Single-cell suspensions of brain tissue obtained from naive control or MCMV-infected animals was separated on a two-layer Percoll gradient to isolate mononuclear cells that were subsequently labeled with the indicated Abs and analyzed using flow cytometry. The dot plots are representative of at least three replicates, with 2–3 mice pooled per replicate. MCMV infection induces the accumulation of CD45^{hi} cells, which represent brain-infiltrating mononuclear cells. In naive animals, the predominant population is CD45^{int}CD11b^{hi} cells, which are microglia with minimal CD45^{hi} peripheral cell infiltration. The percentage of NK and T cells or CD8⁺ and CD4⁺ T cells within the CD45^{hi} cell population are compared at the indicated times points. At day 8 p.i., NK cells represent $\sim 20\%$ of the CD45^{hi} brain-infiltrating mononuclear cells, while, at day 18 p.i., the percentage decreases to $\sim 1\%$. The predominant population becomes CD3⁺ cells, representing $\sim 60\%$ of the CD45^{hi} cells at day 18 p.i. with a ratio between CD8⁺ and CD4⁺ T cells of $\sim 3:1$. CNS, central nervous system; i.p., intraperitoneal; mAb, monoclonal antibody; MCMV, murine cytomegalovirus; NK, natural killer; PN, postnatal.

the presence of the transcription factor C/EBP and its dominant-negative isoform.^{38,48} The dominant-negative isoform of C/EBP, which has retained its DNA binding domain but has lost its transcriptional activation domain,⁴⁹ binds to and inhibits transcription from the CMV MIEP. However, inhibition of MIEP-mediated transcription can be reversed by neuron membrane depolarization dependent on activation of the cyclic AMP response element binding protein.⁵⁰ Furthermore, recent findings have demonstrated that the MIEP block in neurons can be synergistically reversed by activating the cyclic AMP signaling pathway and inhibiting histone deacetylase-mediated viral gene silencing.^{51,52} In contrast to neurons, NSPCs are fully permissive to CMV infection, suggesting a particularly vulnerable stage in neural development. CMV infection inhibits the proliferation, alters the differentiation and induces the apoptosis of infected NSPCs.^{46,53,54} It is thus likely that disruption of regular cellular processes in NSPCs accounts for a portion of the structural and migratory abnormalities in congenital CMV infection. The susceptibility of NSPCs to CMV diverges along glial and neuronal differentiation pathways; it is retained concomitantly with differentiation into glial cells but is repressed following differentiation into neurons.³⁸ More recent data revealed a preserved susceptibility to HCMV infection in neurons differentiated from infected NSPCs.⁴⁷ Some infected neurons survived and expressed viral antigens for a prolonged period of time, which argues that neuronal cells are a potential site of CMV latency.⁴⁷

CMV-INDUCED DEVELOPMENTAL BRAIN ABNORMALITIES

Perinatal MCMV infection results in profoundly altered brain development in newborn mice, which is analogous to the

finding described in brain imaging of infants with congenital HCMV infection (Figure 2).^{10,28,55} Morphological defects are most evident in the cerebella, a region of brain that undergoes extensive development in the postnatal period. By contrast, the cerebrum of infected newborn mice resembles that of uninfected animals. Thus, the impact of MCMV infection is limited to the brain regions that actively develop during the postnatal period.²⁸ Although disruptions in the orderly lamination of the cerebellar cortex can be observed in areas of the cerebellum immediately adjacent to infectious foci, the global abnormalities in cerebellar cortical development that are not spatially associated with foci of infection or infiltration of inflammatory cells are more striking.^{28,56} Granule neuron cells in the external granular layer of the cerebellar cortex show proliferation and differentiation impairments in terms of a low proliferation index and impaired expression of the differentiation-specific protein TAG-1.²⁸ As a consequence, their migration into deeper parts of the cerebellar cortex is delayed, resulting in a reduction in cerebellar foliation, decreased area of the cerebellar cortex and increased thickness of the external granular layer (Figure 2b and c).^{28,56} The Purkinje neurons of MCMV-infected newborn mice also demonstrate impairments including diminished dendrite arborization leading to decreased thickness of the cerebellar molecular layer and inadequate Purkinje neuron alignment within the stratum gangliosum (Figure 2a).²⁸ In a model of MCMV infection of mouse embryos, a similar, impeded migration of neurons is observed. MCMV inoculation into lateral ventricles at embryonic days 14.5 and 15.5 causes a disturbance in neuronal migration and a marked loss of neurons in postnatal brains.⁵⁷

What could be the mechanism(s) responsible for the altered brain development observed in CMV infection? Although the abnormalities are temporally related with MCMV replication in the CNS, there is a sharp contrast between focal distribution of the paucity of virus-infected cells and global defects in brain morphogenesis.^{28,56} These findings argue against direct virus cytopathology and suggest an indirect, virus-induced mechanism as more probable. CNS structural abnormalities and functional disorders caused by congenital CMV infection may be due to MCMV infection of radial glial cells, which play an important role in guiding neuron migration in the neonatal mouse cerebrum,^{58–60} but could also be the result of an insufficient cerebral

blood supply caused by endothelial cell infection and inflammation of the vessel wall⁶¹ (Figure 1b) or by infection of NSPCs.^{47,54,62} NSPCs, which are located predominantly in the ventricular/periventricular zone, have the ability to migrate, proliferate, and differentiate into neurons, astrocytes, and oligodendrocytes and are fully permissive to CMV infection.^{38,46,47,53,63} CMV infection causes NSPCs to alter their differentiation program, and it inhibits the proliferation and induces the apoptosis of NSPCs.^{47,62,64} Whole-genome expression analysis found rapid downregulation of the mRNA levels of several genes important for maintaining NSPC multipotency and for establishing their neural identity (nestin, doublecortin, sex-determining home box 2 and glial fibrillary acidic protein).⁶⁴ It is thus possible that HCMV infection causes *in utero* CNS defects by inducing both premature and abnormal differentiation of NSPCs.⁶⁴ More recently, our group has reported a comprehensive analysis of the host-cell transcriptional response to MCMV infection.⁶⁵ Using RNA-Seq combined with differential gene expression analysis, biological pathway analysis and gene ontology analysis, we identified 10 748 genes that were significantly altered by infection.⁶⁵ We observed upregulation and induction of many unexpected transcription factors and host genes (engrailed-2, GABA and glutamate receptor) related to nervous system development and neuron differentiation.⁶⁵ The transcription factor engrailed-2 is key to patterning cerebellar foliation during development,^{66,67} while GABA and glutamate receptors influence proliferation, migration, differentiation or survival processes during neural development.⁶⁸ As mentioned, morphological deficits within the cerebellum of MCMV-infected mice coincide with impaired transcription of developmentally regulated genes, suggesting a possible physiological link to the regulation of these genes.

A delay in cerebellar development due to infection is also associated with the inflammatory response, which transiently perturbs the developmental program. Increased expression of a number of pro-inflammatory genes is readily observed in brains of MCMV-infected, newborn mice (IFN and IFN-inducible genes, chemokine receptors genes).^{28,69} These findings suggest a host inflammatory response in the developing CNS as a possible mechanism behind impaired brain morphogenesis. Likewise, CNS disease associated with inflammatory responses in the CNS has been described in newborn mice

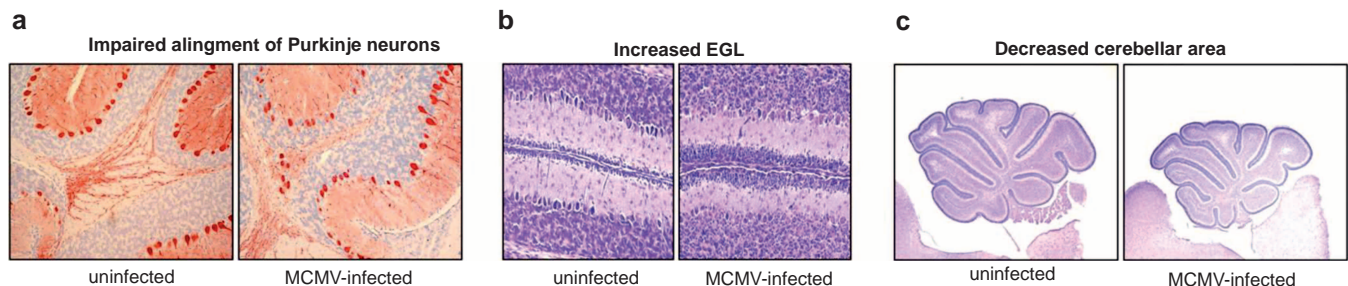


Figure 2 Altered brain development in MCMV-infected newborn mice.²² Cerebellum paraffin sections from naive control and MCMV-infected newborn mice were stained with anti-calbindin and counterstained with hematoxylin (a) or cresyl violet (b and c). Note the impaired alignment and arborization of Purkinje neurons in the stratum gangliosum (a), increased thickness of the external granular layer (b) and smaller size and delayed fissure formation in the cerebellum of infected animals. Original magnification $\times 4$ (c), $\times 10$ (a) and $\times 20$ (b). MCMV, murine cytomegalovirus.

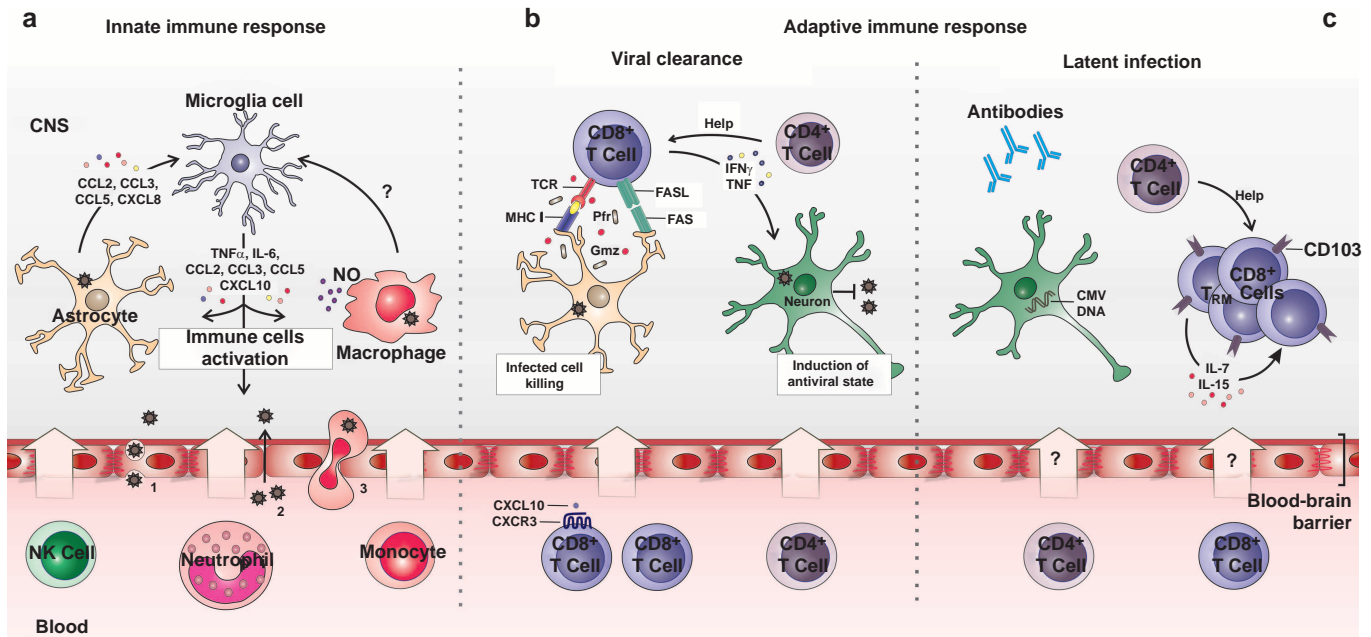


Figure 3 Immunobiology of perinatal MCMV infection in the CNS. **(a)** The possible mechanisms by which MCMV enters the CNS include infection of brain microvascular endothelial cells with basolateral spread of the virus (1), loss of integrity of the BBB with disruption of tight junctions (2) and the 'Trojan Horse' model of intracellular transport within infected monocytes (3). Once the virus is in the CNS, it infects different target cells, with astrocytes being the major, productively infected cell population.^{35,36} Infection initiates the production of chemokines and pro-inflammatory cytokines, resulting in glial cell activation and recruitment of inflammatory cells to the CNS.^{76,77} NK cells, neutrophils and monocytes are the first cells to be recruited into the brain and initiate clearance of the virus before the adaptive immune response takes place.^{25,74,82} Recruited monocytes are precursors of macrophages and possibly microglia. **(b)** Within a few days of CNS infection, infiltration of CD8⁺ and CD4⁺ T cells, which are activated in secondary lymphoid organs, begins.^{74,82} The chemoattractant CXCL10 promotes trafficking of CD8⁺ T cells *via* binding to its receptor, CXCR3.^{78,79} CD8⁺ T cells are crucial for eliminating replicating virus from the CNS,^{74,82} which may be cell type-specific. Cytolytic mechanisms (perforin, FAS–FASL) may be important for viral clearance from non-neuronal cells, whereas non-cytolytic clearance without concomitant cell loss may predominate in neurons. IFN- γ secreted by activated CD8⁺ T cells effectively inhibits virus replication but does not necessarily eliminate virus DNAs from the cell. The direct antiviral role of CD4⁺ T cells is unclear; however, they may enhance CD8⁺ T-cell survival and function.^{74,82} **(c)** Virus-specific CD8⁺ T cells persist in the brain after resolution of productive infection and play a dominant role in the maintenance of virus latency.^{74,82} A proportion of these cells may be T_{RM} cells, which are characterized by high expression of the integrin CD103 and a long-lasting resident nature. These cells most likely survive without replenishment from the circulation. In addition to IL-7 and IL-15, local environmental cues and CD4⁺ T-cell help may be required for T_{RM} cell maintenance in the brain. Antiviral antibodies limit dissemination of recurrent virus.^{112,113} CNS, central nervous system; IFN, interferon; MCMV, murine cytomegalovirus.

infected with non-lytic viruses such as murine leukemia virus or Borna disease virus.^{70–73} Furthermore, corticosteroid treatment, which modulates inflammatory responses, corrected the MCMV-induced morphological deficits within the cerebellum of infected mice.⁵⁶ Prednisolone treatment also corrected abnormalities in the transcription of developmentally regulated genes (decreased GABRA6 and CDK5 and increased *gli1* and N-myc expression) and decreased the expression of inflammatory cytokines (TNF- α , IFN- β and IFN- γ).⁵⁶

IMMUNE RESPONSE TO CMV INFECTION OF THE CNS

CMV infection of the CNS initiates a robust inflammatory response characterized by the recruitment of activated resident and peripheral immune cells as well as expression of a large number of pro-inflammatory cytokines and IFN-regulated genes.^{25,56,74,75}

MCMV infection of newborn mice brain following intracranial injection upregulates the expression of IFN-stimulated genes by 10- to 100-fold.⁶⁹ Induced IFNs substantially reduce

the number of MCMV-infected cells in the CNS and prevent virus-mediated cell death.⁶⁹ Glial cells respond to CMV infections through a highly regulated network of cytokines and chemokines that orchestrate the subsequent immune response.⁷⁵ MCMV-infected astrocytes primarily produce the chemokine CCL2, and less so, CXCL8, CCL3 and CCL5, which recruit microglial cells to the sites of infection.^{76,77} Activation of microglia leads to the production of the pro-inflammatory cytokines TNF- α and IL-6 as well as chemokines CXCL10, CCL2, CCL3 and CCL5.^{76,77} CXCL10 is a potent T lymphocyte chemoattractant that is critical for defense against CMV infection of the CNS.^{78,79} Microglial cell-derived TNF- α and T cell-derived IFN- γ inhibit CMV replication in astrocytes by suppressing transcription from the CMV MIEP.³⁷ This acute cytokine response is regulated by the anti-inflammatory cytokine IL-10.⁸⁰ Although lack of IL-10 has little effect on viral load, it leads to a severely dysregulated IFN- γ response and renders CMV brain infection lethal.⁸⁰ Interestingly, HCMV carries its own IL-10 homolog that inhibits CXCL10 production in

microglial cells, which consequently inhibits lymphocyte migration.^{77,81} An analogous IL-10 homolog has not been identified in MCMV, although MCMV infection of the brain induces a strong increase in IL-10 expression (Britt WJ, 2014, unpubl. data). Cytokine and chemokine production within the CNS precedes the infiltration of systemic immune cells.

The early cellular immune response is characterized by the efflux of innate immune constituents such as macrophages, neutrophils and natural killer (NK) cells (Figure 3a).^{25,74,82} Macrophages are target cells for CMV infection, contribute to systemic dissemination of the virus and serve as a reservoir of latent infection.⁸³ In response to MCMV infection, brain-infiltrating macrophages (CD45^{hi}CD11b⁺ cells) are activated, as assessed by the upregulated expression of MHC class II molecules and activation markers CD40 and CD80.⁷⁴ Nitric oxide derived from brain macrophages may contribute to the elimination of MCMV from the brain,²⁵ as has been shown for other organs. Neutrophils are considered significant participants for combating extracellular, bacterial pathogens as well as viral infections.⁸⁴ Although they play an important role in the innate immune response to MCMV, the absence of anti-inflammatory cytokine IL-10 neutrophils could contribute to tissue damage. MCMV brain infection of IL-10-deficient mice induces exaggerated chemoattractant CXCL2 production by microglial cells and pathological neutrophil recruitment into the brain.⁸⁵ Despite the vital roles that NK cells play during MCMV infection in peripheral organs,^{86–88} their role in the control of CNS infection has been inadequately studied.²⁵ In our model of i.p. MCMV infection, the initial neuroimmune responses are dominated by the influx of NK cells, whose appearance coincides with detection of the virus in the CNS (Figure 1d). At day 8 p.i., NK cells constitute the prominent cellular component and comprise ~20% of the total recovered brain leukocytes (CD45^{hi} cells). However, the percentage of infiltrating NK cells decreases rapidly, as they are replaced with other immune cells, and, after day 10 p.i., the percentage settles at approximately 1% (Figure 1d) (Jonjic S, 2014, unpubl. data). The roles that NK cells play in MCMV infection of the developing CNS remain to be defined.

The innate immune response provides the first line of defense against MCMV infection; however, subsequent adaptive immunity is required to clear the virus from a newborn mouse brain.^{74,79} CD8⁺ T cells play an essential role in resolving acute MCMV infection in the CNS (Figure 3b).^{74,79} Moreover, they provide resistance to the otherwise lethal infection in newborn animals.⁷⁴ Following MCMV spread to the newborn mouse brain, CD8⁺ T lymphocytes are recruited into the CNS, where they emerge as a dominant mononuclear cell population (Figure 1a, b and d). Peak infiltration is observed at postnatal day 18, when more than 70% of the brain-infiltrating peripheral mononuclear cells (CD45^{hi}) comprise the CD8⁺ T lymphocytes (Figure 1d).⁷⁴ The increasing magnitude of the CD8⁺ T-cell response correlates with a rapid decline in viral titers, indicating a critical role of CD8⁺ T cells in resolving MCMV infection in the CNS (Figure 1a).⁷⁴ Subsequently,

lymphocyte infiltration wanes; however, CD8⁺ T cells persist in the brain long after resolution of productive MCMV infection^{74,82} and can be detected for up to a year (Jonjic S, 2014, unpubl. data) (Figure 3c). Similar observations have been reported during other viral brain infections, where tissue memory T cells expressing integrin CD103 persist in the CNS long after the virus has been cleared.⁸⁹ CD8⁺ T lymphocytes that infiltrate the CNS following MCMV infection display an effector memory phenotype that is characterized by upregulated expression of CD44, a marker of antigen experienced cells, and the lack of expression of CD62L (L-selectin), which is downregulated following extravasation into tissue.^{74,82} A substantial proportion of brain-infiltrating CD8⁺ T cells expresses the activation markers CD69 and CD49, implying they are highly activated.^{74,82}

CMV infection elicits a broad CD8⁺ T-cell response that is specific for an array of viral antigens.^{90–92} However, in the brain, the MCMV-directed immune response seems to be quite focused. In newborn BALB/c mice, approximately 10% of brain-infiltrating CD8⁺ T cells are directed to a single, immunodominant IE1 epitope at the peak of the response.⁷⁴ Similar to the peripheral, IE1-specific T-cell response after the initial expansion and contraction phases, there is a slow accumulation of virus-specific cells in the CNS over time (Jonjic S, 2014, unpubl. data). CD8⁺ T lymphocytes specific for two other immunodominant viral epitopes in BALB/C mice, m164 and m04, can also be detected in the brain, albeit at lower frequencies.⁷⁴ The relevance of the immune responses to specific viral epitopes and their roles in protection against CMV brain infection is still unclear.

T lymphocytes use distinct antiviral effector functions to control infection in particular organs, and it is becoming increasingly clear that target cell-specific mechanisms within particular tissues also exist (Figure 3b).^{93–95} It has been reported that CD8⁺ T cells control MCMV brain infection through a perforin-mediated mechanism.⁷⁹ Similarly, clearance of West Nile virus from infected neurons is dependent on perforin.⁹⁶ Other viral models of CNS infections have suggested non-lytic, cytokine-dependent mechanisms that predominate in viral clearance from the CNS without concomitant neuronal loss.^{97–99} CD8⁺ T cells isolated from the brain of MCMV-infected animal produce IFN- γ and TNF- α upon stimulation with viral peptides. However, a considerable fraction of CD8⁺ T cells that secrete IFN- γ is deficient in degranulation, as assessed by CD107 staining, and potentially lack the capacity to mediate cytotoxicity.⁷⁴ These results suggest a potential role of non-cytolytic mechanisms in viral clearance from this vital, generally nonregenerating tissue. Because various types of brain cells can be infected by CMV, e.g., neurons, stem/progenitor cells, astrocytes, microglia, it is possible that CD8⁺ T cell-mediated viral clearance from the CNS depends on several antiviral effector functions and is cell type-dependent. It should be stated that CD8⁺ T lymphocytes that are recruited into the CNS following newborn mouse infection are functional and limit virus replication upon adoptive transfer into immunodepleted, MCMV-infected, syngeneic mice.⁷⁴

The magnitude of the CD4⁺ T-cell response in the CNS remains relatively constant throughout the infection and is outnumbered by CD8⁺ T cells by approximately three to one (Figure 1a).^{74,82} CD4⁺ T lymphocytes are necessary for the resolution of productive MCMV infection in salivary glands,¹⁰⁰ but adoptive transfer of CD4⁺ T cells is not protective against systemic nor CNS diseases.^{79,101,102} Interestingly, in salivary glands, the ratio between CD8⁺ and CD4⁺ T cells is also disproportionately skewed in favor of CD8⁺ T cells.^{103–105} Thus, although there is no direct correlation between the CD4⁺ T lymphocyte response and the infectious virus titer in MCMV-infected newborn mouse brains,⁷⁴ CD4⁺ T lymphocytes may contribute to viral control in the CNS by providing crucial accessory functions for virus-specific CD8⁺ T cells, as shown for other infections (Figure 3b).^{106,107}

The accumulation of virus-specific B-lineage cells within the CNS is a hallmark of many neurotropic viral infections.^{108–110} A model of intracranial MCMV infection of mice demonstrated B-cell recruitment into the CNS with a steady increase throughout the time-course of infection.¹¹¹ The recruitment of B-cells was dependent upon the cytokine and chemokine responses generated by infiltrating T cells, including IL-21. Unlike the above-mentioned model of direct intracranial inoculation of the virus, which bypasses the peripheral immune response, in our model of i.p. MCMV infection, no B-lineage cell infiltration into newborn mice brains was detected (Jonjic S, 2014, unpubl. data).

ANTIBODY-MEDIATED IMMUNITY TO CMV INFECTION

Antiviral antibodies, although dispensable for the resolution of acute CMV infection, play an essential role in limiting virus dissemination following recurrent infection (Figure 3c).^{112,113} The presence of maternal antiviral antibodies has been claimed to be associated with a decreased incidence of viral transmission and improved neurological outcome in congenital HCMV infection.^{114–117} In addition, a beneficial effect of CMV-specific, hyperimmune globulin against symptomatic, congenital HCMV infection has been suggested,^{118,119} but has not been confirmed by recent studies.¹²⁰ This proposed protective activity is difficult to reconcile with the finding of a significant number of infants born to mothers undergoing non-primary infections during pregnancy.¹²¹ Lastly, it should be noted that a recently reported, well controlled trial of human hyperimmune globuline to prevent intrauterine HCMV transmission demonstrate no efficacy of this approach.¹²² The proposed mechanisms of protection are virus neutralization, with proteins in the gH/gL/UL128/UL130/UL131 complex being the major targets of neutralizing antibodies, reduction of placental inflammation and perhaps reduction in the cytokine-mediated cellular immune response.^{123,124} Although antibodies play an important role in protection against CMV infection and disease, the level of protection is incomplete.^{125,126} Notably, maternal preconceptional immunity fails to provide absolute protection against congenital HCMV infection, which is related to reinfection with a new HCMV serotype or reactivation of an endogenous virus.³ In either case, infection is caused by a virus variant that is not recognized by preexisting antibodies. However, low avidity

maternal CMV antibodies, paradoxically, have been suggested to promote transmission of virus across the syncytiotrophoblast *via* the neonatal Fc receptor.¹²⁷

Antibodies that are effective in limiting virus-induced neuropathology has been documented in a model of perinatal MCMV infection.²⁹ Treatment of MCMV-infected newborn mice with either MCMV-immune serum or MCMV-specific monoclonal antibodies reduced viral titers and the severity of inflammatory lesions in the CNS. More specifically, administration of antiviral antibodies during the early post-infection period completely prevented virus replication in brain parenchyma, indicating antibodies may prohibit virus dissemination to the CNS either by neutralizing cell-free virus or limiting the spread of cell-associated virus by mechanisms such as antibody-dependent cellular cytotoxicity. Administration of antiviral antibodies at the peak of MCMV replication in the CNS promoted viral clearance, suggesting antibodies may limit virus dissemination and/or replication within the CNS.²⁹ Finally, antibodies diminished the developmental alterations associated with MCMV infection of the CNS by limiting virus replication and presumably the host inflammatory response.²⁹ The protective capacity of antiviral antibodies in perinatal MCMV infection is further outlined by a study using recombinant MCMV expressing RAE-1 γ , a high-affinity ligand for activating the NKG2D receptor, which is present on both NK cells and CD8⁺ T lymphocytes.¹²⁸ This recombinant virus, by eliciting tight innate immune control, is highly attenuated in adult and neonatal mice but induces strong and long-lasting protective immunity. Moreover, infection of female mice with MCMV expressing RAE-1 γ elicited an antibody response that, upon placental transfer, limited virus dissemination and protected offspring from MCMV disease.¹²⁸

SENSORINEURAL HEARING LOSS DUE TO CONGENITAL CMV INFECTION

Congenital CMV infection is the leading infectious cause of hearing deficits in children, contributing to approximately 14%–25% of all congenital hearing-loss cases.^{5,14} Remarkably, hearing defects due to congenital CMV infection can progress through early childhood, even when they are clinically unapparent at birth. It is assumed that hearing defects are caused by CMV-induced labyrinthitis; however, the mechanisms surrounding its pathogenesis and the role of the inflammatory response remain unclear.¹²⁹

Sparse temporal bone autopsy specimens from infants with congenital HCMV infection have shown damage to vestibular structures with only minor involvement of the cochlea, mainly as hydrops at the basal turn.^{130–132} The infection is coupled with cytomegalic inclusion cells that are positive for CMV antigens and mononuclear cell infiltrates in the cochlea. Two recent studies on terminated fetuses have extended our knowledge about congenital HCMV infection of the inner ear.^{133,134} Inflammatory cells mainly composed of activated CD8⁺ T lymphocytes were more abundant in the inner ear structures, where the virus was detected within stria vascularis followed by Reissner's membrane. Inflammatory cells were also found

along cochlear nerve fibers and in the spiral ganglion.¹³³ The authors presumed that inflammation altered the delicate ion circulation throughout the stria vascularis, damaging the endocochlear potential formation.¹³³ Without the endocochlear potential, the hair cells of the organ of Corti might become inactive or degenerate.¹³⁵ In addition, the vestibular labyrinth shows CMV infection of sensory cells in the utricle as well as crista ampullaris.¹³³ The clinical consequences of these findings are still unknown, although some authors have reported abnormal vestibular tests in congenitally infected babies.^{136,137}

To better understand the damage caused by CMV infection of the inner ear, experimental animal models have been extensively used. In a model of systemic infection of newborn mice with MCMV combined with intracranial inoculation of bacterial lipopolysaccharide to stimulate labyrinthitis, virus-infected cells were detected in spiral ganglia and the perilymphatic area, including the scala tympani and scala vestibuli. Interestingly, the organ of Corti was completely void of MCMV-infected cells.¹³⁸ Infection induces the infiltration of immune cells largely composed of macrophages and T lymphocytes that populate MCMV-positive regions and persist after viral clearance from the cochlea. Lymphocytic infiltration is concomitant with the production of pro-inflammatory cytokines and chemokines, including TNF- α and IL-6, whose levels in the scala tympani are increased;¹³⁹ however, the inner ear injury possibly being mediated by the host inflammatory response was suggested by a study using intracranial MCMV inoculation. High levels of pro-inflammatory cytokines and macrophage-produced reactive oxygen species induce the progressive loss of cochlear hair cells, despite the lack of their direct viral infection.¹³⁹ Analogously, guinea pig CMV lacking a CC chemokine gene displays reduced propensity for inducing SNHL.¹⁴⁰ Other documented pathological changes are located in the spiral ganglia and include reduced numbers of spiral ganglia neurons and changes in their ultrastructures (e.g., swelling of the endoplasmic reticula, ribosomes and mitochondria).¹⁴¹ Moreover, with increasing age, the number of neurons decreases, and ultrastructural lesions become more severe. Later findings are in agreement with the progressive nature of SNHL in children, suggesting that there may be ongoing inflammation of the inner ear that continues to be active throughout early childhood.³³

FUTURE PROSPECTS FOR THE PREVENTION AND THERAPY OF CONGENITAL CMV INFECTION

Antiviral therapy is recommended for infants with congenital CMV infection involving CNS or serious end-organ disease. Ganciclovir, a synthetic nucleoside analog that is structurally similar to guanine, is the keystone for treatment of CMV infections.¹⁴² Six-week ganciclovir therapy reduces the risk of long-term disabilities in children with symptomatic CNS congenital infection by improving hearing and possibly neurodevelopmental outcomes.^{143–145} However, antiviral treatment cannot reverse already established CNS injury and, due to its teratogenic effect, ganciclovir is not suitable for pregnant women with documented CMV infection. Therefore, prophylactic

strategies aimed at preventing fetal infection and subsequent neurological sequelae are under intensive investigation.

CMV immune globulin is indicated, either alone or in combination with nucleoside analogs, for the prophylaxis of CMV disease in solid organ transplant patients.¹⁴⁶ Its potential benefit in protection against congenital CMV infection has been evaluated. Former, non-randomized studies have shown decreased numbers of congenitally infected infants born to mothers treated with hyperimmune globulin and improved outcomes in CMV-infected infants.^{114–117} However, a recently published, controlled, randomized study failed to confirm any significant differences in either the rate of transmission or in the secondary clinical and biologic outcomes between infants born to women receiving hyperimmune globulin compared with those receiving placebo.¹²⁰ The authors argue that, due to the small effect of immune globulin administration, more patients would have to be enrolled in the study to have the power to detect differences in the outcome. Currently, two other randomized, phase-3 studies are under way that will hopefully further our understanding of the CMV immune globulin efficacy in the prevention of congenital CMV infection and its sequelae.^{147,148}

Development of a CMV vaccine is a major public health priority, as identified by the US National Vaccine Program Office and the Institute of Medicine.¹⁴⁹ A number of CMV vaccine candidates have been evaluated in preclinical and clinical trials.^{150–152} Diverse expression strategies have been used that can conceptually be divided into live, attenuated vaccines and subunit vaccines that target individual CMV proteins. In the setting of congenital CMV infection, it is not clear what would constitute an optimal vaccine. Due to the complexity of CMV immunobiology, a simple vaccine approach such as induction of an adaptive immune response that mirrors the one following natural infection would be of limited value in the prevention of congenital CMV infection. Namely, not only CMV-naïve but also CMV-immune women can acquire CMV infection during pregnancy with subsequent transmission to the fetus.^{3,4} Although primary maternal infection carries a greater risk of CMV transmission and severe sequelae for the neonate than do recurrent infections, the burden associated with non-primary infection is nonetheless substantial. Development of a vaccine able to induce immunity that is more effective than the natural infection or able to ameliorate the preexisting immune response is a highly challenging concept. Thus, a vaccine that is competent to prevent severe CNS sequelae and other disabilities in congenitally infected infants seems a more feasible goal than a vaccine that is able to avoid CMV transmission and fetal infection. However, as suggested by some authorities, the force of CMV infection is low, indicating that even a vaccine of only modest effectiveness could, by conferring herd immunity, substantially reduce the incidence of congenital CMV infection.¹⁵³

The potential role that the inflammatory response plays in neuropathogenesis of congenital CMV infection, including SNHL, is becoming increasingly clear.^{28,56,139} In a mouse model, administration of corticosteroids dramatically decreased the inflammatory response in the brain of CMV-infected newborn

mice and normalized deficits in CNS development.⁵⁶ If a similar mechanism could account for the neurodevelopmental deficits found in infants with congenital HCMV infection, interventions that modulate host inflammatory responses could provide a promising new therapeutic strategy for congenital CMV infection.

In conclusion, the public health and economic burdens of congenital CMV infection are tremendous which underscores the need for future research to improve our understanding of the pathogenesis of CNS injury and immune protection correlates. It is conceivable that these observations could provide novel strategies for the development of effective interventions to help address the problem of congenital CMV infection.

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