Lymphocytic choriomeningitis infection of the central nervous system

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1. ABSTRACT

Viral infection of the central nervous system (CNS) can result in a multitude of responses including pathology, persistence or immune clearance. Lymphocytic choriomeningitis virus (LCMV) is a powerful model system to explore these potential outcomes of CNS infection due to the diversity of responses that can be achieved after viral inoculation. Several factors including tropism, timing, dose and variant of LCMV in combination with the development or suppression of the corresponding immune response dictates whether lethal meningitis, chronic infection or clearance of LCMV in the CNS will occur. Importantly, the functionality and positioning of the LCMV-specific CD8^{+} T cell response are critical in directing the subsequent outcome of CNS LCMV infection. Although a basic understanding of LCMV and immune interactions in the brain exists, the molecular machinery that shapes the balance between pathogenesis and clearance in the LCMV-infected CNS remains to be elucidated. This review covers the various outcomes of LCMV infection in the CNS and what is currently known about the impact of the virus itself versus the immune response in the development of disease or clearance.

2. INTRODUCTION

The parameters that dictate pathology versus clearance after CNS viral infection are relatively undefined. The CNS contains highly integrated populations of neurons and glial cells responsible for our livelihood, and immune infiltration has the potential to severely disrupt the function of this sensitive compartment. Consequently, through evolutionary pressures the CNS has become immunologically specialized and now possesses the ability to modulate (or suppress) leukocyte behavior in a manner that fundamentally differs from that observed in most peripheral tissues. This evolutionary acquisition, which is advantageous from the standpoint of host survival, likely facilitates the entry and replication of viruses within the CNS. A multitude of viruses have the capacity to gain access to the CNS and upon doing so can establish a chronic infection, drive immunopathology, or alter the function of residents cells, making clearance of the invading pathogen essential. Therefore, a fundamental knowledge of viral-immune interactions in the CNS is essential if we ultimately intend to therapeutically modulate CNS immune responses in humans to achieve viral clearance in the absence of immunopathology. LCMV

infection of mice provides an outstanding model system to explore such interactions and extend the lessons learned to virus infections of humans.

3. GENERAL BACKGROUND ON LCMV

LCMV is a natural pathogen of both human and murine populations (1). Human transmission has been shown to occur *in utero*, upon transplantation with infected organs or upon contact with infected hamsters or laboratory animals (2-4). Congenital LCMV infection acts as an abortifacient and a fetal teratogen commonly manifested as chorioretinitis, hydrocephalus and microcephaly or macrocephaly (5-9). Adult infection with LCMV can lead to the development of fever, malaise, headaches, seizure and in some cases fatal meningitis (3, 10-13). Through the study of murine models of infection, a mechanistic understanding of LCMV induced immunopathology can be gained and applied to human disease. In addition, study of the LCMV model has served as a Rosetta stone for the field of viral immunology, resulting in the discovery of numerous generalizable findings that apply to a variety of viral and microbial infections in humans.

3.1. LCMV structure

LCMV is the prototypic member of the Arenaviridae family, which encompasses old and new world viruses including Lassa, Junin, Machupo and Tacaribe (14, 15). LCMV is a negative strand RNA virus that has two genome segments referred to as L and S. The L RNA segment is the larger portion of the genome with a size of 7.2kb compared to the 3.4kb size of the S RNA segment. A bidirectional coding strategy is used to synthesize the polymerase (L) and small RING finger protein Z from the L segment and the viral nucleoprotein (NP) and glycoprotein (GP) from the S segment (16-21). The GP protein is later post-translationally cleaved into GP1 and GP2, which are expressed on the envelope of the virus (22). GP1 mediates attachment of the virus to its cellular targets to initiate infection and thus determines tropism of LCMV. Therefore, mutations in this protein have the potential to alter viral targeting and influence the course of disease.

3.2. Advantages to the LCMV model system

LCMV has been used to make significant contributions to both the fields of virology and immunology, including seminal work detailing concepts such as immune tolerance, immunodominance, MHC restriction and the basis for viral persistence (23-29). Utilization of the LCMV model system provides some key advantages in our understanding of viral-immune interactions. One important benefit to the LCMV system is its great flexibility in the resultant outcome after infection. Depending on the strain, dose and route of LCMV infection, a wide variety of responses including viral clearance, immune suppression, viral persistence, hepatitis or fatal choriomeningitis can be induced (30-33). Another major advantage of the LCMV model system is the generation of experimental tools that can be used to trace and follow the anti-viral response. The ability to trace populations of LCMV-specific T cells gave way to

identification of immuodominant epitopes that are observed after infection. Extensive mapping of the LCMV Armstrong (Arm) specific immune response in C57BL/6 mice revealed the generation of a polyclonal T cell response with defined MHC I and II restricted epitopes against both the glycoprotein (GP) and nucleoprotein (NP) of the virus (MHC I: D^b GP_{33-41}, K^b GP_{33-43}, D^b $NP_{396-404}$, D^b $GP_{276-286}$, D^b GP_{92-101}, K^b $GP_{118-125}$, K^b $NP_{205-212}$; MHC II: I-A^b GP_{61-80}, I-A^b $NP_{309-328}$) thought to represent most of the LCMV Arm specific repertoire (34-36). Additionally, the creation of LCMV specific $CD4^+$ and $CD8^+$ T cell receptor (TCR) transgenics (referred to as SMARTA (37) and P14 (38), respectively) have advanced the field by providing traceable and transferable populations of virus specific T cells. The accumulation of such tools and their permutations allows for more detailed analyses of the immunopathogenesis observed following LCMV infection, which drives a variety of disease states.

4. LCMV INDUCED MENINGITIS

LCMV has the capacity to induce meningitis in both human and murine hosts (12, 13, 33). In adult immunocompetent mice, intracerebral (i.c.) injection of LCMV results in the development of an acute, fatal meningitis 6-8 days post infection (33, 39). Prior to death, mice demonstrate symptoms including ruffled fur, blepharitis, hunched posture and seizure. Notably, the ultimate death of the mouse is often associated with massive seizure induction. LCMV has been shown to localize and infect the ependyma, choroid plexus and meningeal regions of the brain, with relatively little virus detected in the brain parenchyma after i.c. infection (Figure 1) (31, 40, 41). Because LCMV is a non-cytopathic virus, the damage induced in the CNS is not caused by the virus, but rather is caused by the subsequent immune response. Accordingly, massive leukocytic infiltrates are found to accumulate in the meningeal and ventricular regions of the brain, which corresponds with the viral distribution (41-43). Infiltration of the brain parenchyma is also observed and may be critical for the disease process (44).

4.1. The role of $CD8^+$ T cells and histocompatibility during meningitis

Induction of lethal LCMV induced meningitis is dependent on the generation of a productive immune response. T cells were directly implicated as key mediators of mortality when it was discovered that thymectomized mice had increased protection after i.c. infection with LCMV (45). Although the virus is administered i.c., approximately 90% of the inoculum is released into the blood and becomes available for the peripheral T cell priming that occurs over the next 3-5 days (42). Detailed T cell analyses in the cervical lymph node of LCMV infected BALB/c mice revealed significant activation marked by increased $CD8^+$ T cell numbers starting on day 3 post infection and elevated levels of both CD44 and CD25 by day 6 post infection (46). Proliferative and cytotoxic capacities also increased progressively over time, corresponding to the development of a functional T cell response that preceded immunopathology (46).
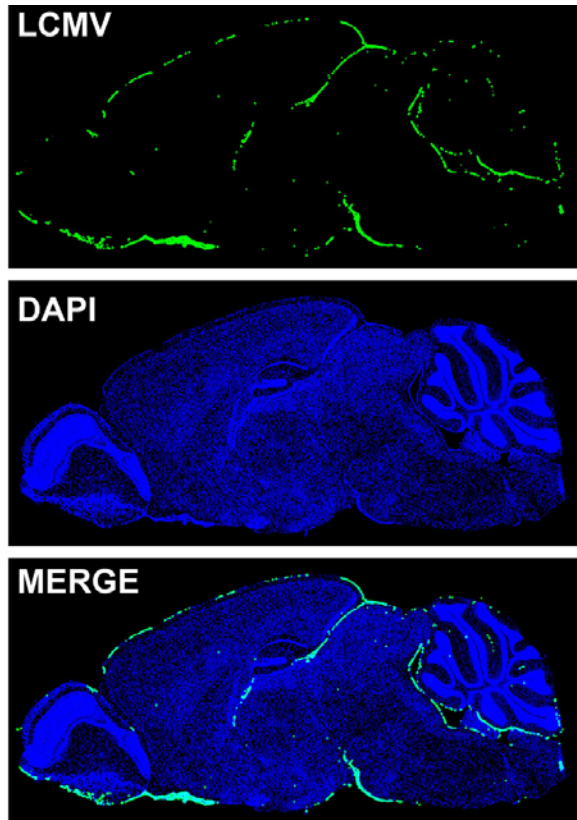
Figure 1. LCMV distribution during acute meningitis. The brain from an 8 week old C57BL/6 mouse infected with LCMV i.c. was harvested on day 6 post infection when severe seizures were apparent. Shown is the localization of LCMV (green) on a sagittal brain section. The virus was detected with a polyclonal anti-LCMV antibody and cell nuclei (blue) were stained with DAPI. Note that LCMV is distributed in the meninges with little to no infiltration into the brain parenchyma.

Following priming activated T cells are then assumed to enter the circulation, traffic to the CNS and cause inflammation and disease. Accordingly, CD8+ T cells have been identified in and appear to be recruited to the cerebral spinal fluid (CSF) after LCMV infection (47, 48). CD8+ T cells were further defined as the essential T cell population needed for LCMV-induced meningitis by studies involving CD8+ T cell depletion (49), genetic knockouts (50-52), blocking peptides (53) and cytotoxic lymphocyte (CTL) transfer (43, 54-56). CD4+ T cells, B cells and NK cells are not essential for meningitis (49, 55, 57-59); however, in the absence of CD8+ T cells, the wasting disease that subsequently develops has been attributed to CD4+ T cells (50, 52). Disease induction by CD8+ T cells necessitates LCMV specificity and therefore cannot be mediated by non-specific bystander CD8+ T cells alone (43). Importantly, meningitis can be initiated in mice harboring a T cell repertoire with a low number naïve LCMV-specific CD8+ T cells. Intracerebral inoculation of ovalbumin CD8 TCR-tg mice (OT-I mice) (60, 61) with LCMV does not result in meningitis, but adoptive transfer of as few as 1,000 monospecific, naive D^bGP_{33-41} CD8+ T

cells (P14 cells) was sufficient to reconstitute disease in these mice (43). Therefore, LCMV-specific CD8+ T cells are absolutely required for the development of meningitis.

Supporting a role for CD8+ T cells in LCMV-induced meningitis, histocompatibility at the MHC class I locus was discovered by Zinkernagel and Doherty to be an essential requirement for pathology and disease following i.c. LCMV infection (56, 62, 63) – a finding worthy of the Nobel Prize. In fact, Doherty subsequently uncovered that maximal meningeal inflammation required that histocompatible MHC I be expressed on both bone marrow derived cells and radio-resistant cells; he presumed the latter to be endothelial cells (64). *In vitro*, LCMV infection enhanced MHC I expression on brain endothelial cells (65), suggesting an increased potential for CD8+ T cell engagement *in vivo*. Recently, the CNS vascular endothelium was shown to present a model antigen (Ag) to CD8+ T cells and allow for preferential entry of Ag-specific CD8+ T cells into the brain parenchyma (66). If this mechanism holds true in the context of meningitis, then vascular endothelial MHC I expression may be important for optimal entry of CD8+ T cells into the CNS. As LCMV specific CD8+ T cells are known to produce pro-inflammatory cytokines that can affect blood brain barrier integrity (67, 68), it is interesting to further theorize that cognate peptide-MHC I interactions on the vascular endothelium with LCMV-specific CD8+ T cells could stimulate cytokine release or actual cytolysis of the endothelium. These actions could then be directly responsible for the increased blood brain barrier permeability associated with CD8+ T cells during meningitis (69).

4.2. CD8+ T cell trafficking to the CNS during acute meningitis

Normally, T cell trafficking into various tissues is a multi-step process involving selectin mediated rolling, chemokine induced activation and integrin mediated adhesion on vascular endothelium, which is followed by extravasation (70). Although these steps are not completely defined for CNS T cell entry during LCMV-induced meningitis, some insights into the process do exist in the literature. Studies in CD43, an E-selectin ligand (71), deficient mice demonstrated decreased infiltration to the CNS (72) suggesting a role for this E-selectin in the trafficking events to the CNS. However, it was previously shown that entry of CD8+ T cells into this site after infection is actually an E/P selectin independent event (73). Therefore, during LCMV infection, the selectins used for CD8+ T cell rolling on the vascular endothelium of the CNS are still unclear and remain to be elucidated.

Several candidates for chemokine induced activation of integrins have been identified. By day 6 post infection when T cells are present in the CNS, mRNA levels of chemokines such as RANTES, IP-10, MIP-1α/β and MCP-1 are increased over what is observed on day 3 post-infection – a time point prior to T cell infiltration (74). Chemokine upregulation appears to be linked to IFNγ expression (75), which is interesting because IFNγ is not detected until later time points when T cells are present in

the CNS. This suggests that the release of IFNγ by infiltrating CD8^{+} T cells may be an important step in the amplification of chemokine expression and T cell recruitment into the brain.

Chemokine receptors including CXCR3 and CCR5 are expressed on CD8^{+} T cells after LCMV infection (44, 75, 76). While CCR5 and CXCR3 are dispensable for initial T cell entry into the CSF of the LCMV infected mice (44, 77), the subsequent positioning of T cells in the brain parenchyma during LCMV-induced meningitis is controlled in part by interactions between CXCR3 and CXCL10 (44, 78). Mice genetically deficient in either the receptor (CXCR3) or the ligand (CXCL10) were found to be partially resistant to LCMV-induced meningitis, and this was attributed to a reduced number of CD8^{+} T cells found in the brain parenchyma (44, 78). The inability of researchers to identify requisite chemokine-chemokine receptor interactions for initial T cell entry into the brain of LCMV-infected mice might be due to the fact that the appropriate molecule (s) has not yet been studied or to functional redundancy in the system, which has the potential to obscure the relative importance of the previously mentioned chemokine/chemokine receptors. It is therefore of some importance to examine chemokine pathways using approaches that do not rely on genetic knockouts. Real time *in vivo* imaging of T cell extravasation in the presence of chemokine receptor blocking antibodies might provide a contemporary approach to address such an issue.

Expression of the integrins VLA-4, LFA-1 and CD11b on CD8^{+} T cells located in the secondary lymphoid organs and CSF after infection make them probable candidates for the last step of extravasation (75, 79-82). Correspondingly, the ligands for VLA-4 and LFA-1 (i.e., VCAM-1 and ICAM-1) are expressed on the vascular endothelium of the CNS during meningitis (79, 80, 83). Entry of CD8^{+} T cells into the brain is associated with massive infiltration of monocytes and non-specific CD8^{+} T cells, which is likely due to the aforementioned CD8^{+} T cell associated blood brain barrier activation and breakdown. Although many candidates for immune cell trafficking into the CNS have been identified, the multi-step process for extravasation of the initial LCMV-specific CD8^{+} T cell population, which amplifies the magnitude of CNS immune infiltrate, requires further refinement.

4.3. T cell effector functions and meningitis

Despite the identification of CD8^{+} T cells as the critical population required for disease induction, the actual mechanism that results in mortality during LCMV infection is still unknown. After entry into the CNS, LCMV-specific CD8^{+} T cells co-localize in LCMV-infected regions of the brain. Direct *in vivo* engagement of infected target cells by LCMV-specific CTL is thought be involved in the pathogenic process, as immunological synapses between D[b]GP$_{33-41}$ specific CTL and LCMV-infected targets were observed in symptomatic mice at the peak of disease (day 6 post-infection) (84). Productive CTL lysis of the LCMV infected cells is supported by the deposition of perforin onto the targets (84), the significant increase in the number

of apoptotic cells (43) and the presence of LCMV NP circulating in the CSF (85). Interestingly, in CXCR3-deficient mice delayed disease, associated with poor parenchymal CTL recruitment, was observed despite the fact that infiltration of the CSF was unimpaired (44). Though it remains possible that death in LCMV-infected mice is caused by physical occlusion of the ventricular system or by increased intracranial pressure, the aforementioned data in CXCR3-deficient mice suggests a critical link between CTL localization and mortality in the LCMV model.

CTL have multiple pathways that can be used for target cell killing which include perforin-granzyme delivery, Fas/FasL interactions and cytokine production (e.g., TNF-α); however, elimination of individual pathways through genetic deficiency has minimally impacted disease progression and mortality in the LCMV model. It was originally thought that pathology and death in this model involved perforin-mediated lysis of target cells by CD8^{+} T cells. This was based upon the observation that perforin-deficient mice survived i.c. challenge with a viscerotropic strain of LCMV (86). Subsequent analyses of perforin-deficient mice infected with a neurotropic LCMV variant (Armstrong) revealed a delay in disease kinetics, but not protection from death, thus disproving that perforin was the sole effector molecule responsible for disease (87). Perforin-independent, granzyme B-mediated cytolysis and Fas/FasL interactions (88) have also been eliminated as being required for disease, as both granzyme B-deficient and Fas-deficient mice succumb to LCMV-induced meningitis ((89), unpublished results). Therefore, no evidence presently exists to support a direct link between a single contact-dependent CTL effector mechanism and the highly reproducible mortality observed in the model.

CTL also use cytokine release as an alternate means to lyse target cells. TNFα and IFNγ were potential candidates for disease progression based on their increased cerebral expression in symptomatic animals and their colocalization with infected regions (90-92). Depending on the time of administration, TNFα was shown to either exacerbate or alleviate meningitis in an adoptive transfer model of disease (93). However, three observations support that TNFα is not involved in disease pathogenesis: 1.) TNFα was not found in the CSF (94), 2.) mortality was observed despite intracranial neutralization of TNFα (95), and 3.) i.c. inoculation of TNFα deficient mice results in normal disease kinetics (unpublished results). IFNγ (96) are also dispensable for LCMV-induced meningitis, as deficient mice succumb to disease with kinetics similar to wild-type mice following i.c. challenge with neurotropic LCMV Arm. However, IFNγ deficiency does improve survival when mice are infected with a viscerotropic LCMV variant (96-98). This pattern of protection versus susceptibility based on the usage of a viscerotropic versus a neurotropic LCMV variant is likely due to the peripheral organs acting as an antigen sink that divert the CD8^{+} T cell away from the CNS (99). In conclusion, although CD8^{+} T cells are known to be crucial for disease, the mechanism (s) that mediate mortality during meningitis remain elusive.
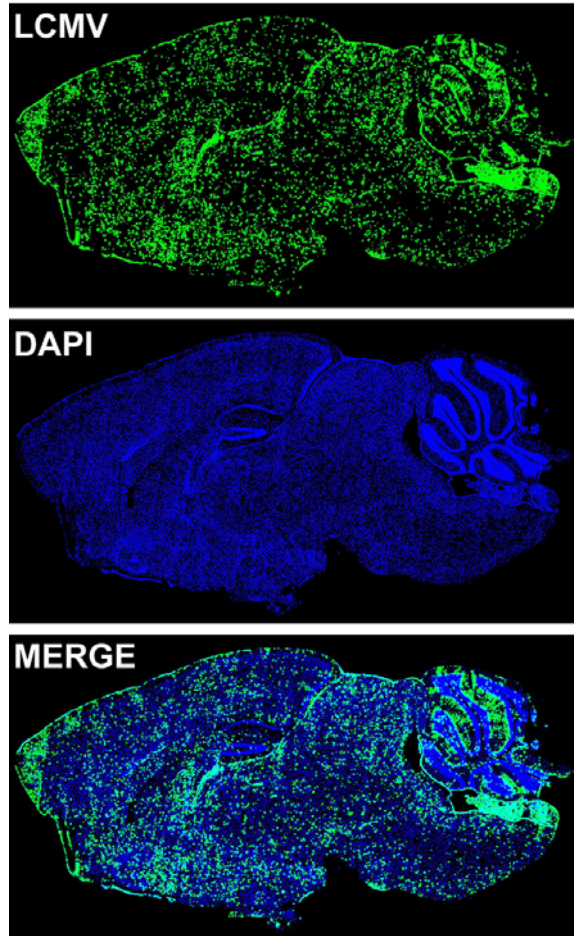
Figure 2. LCMV distribution in carrier mice infected *in utero* with LCMV. A brain from a C57BL/6 mouse infected with LCMV *in utero* via maternal vertical transmission was harvested at >8 weeks of age. Note that LCMV (LCMV) is localized throughout the brain parenchyma in a pattern reflecting neuronal infection. The meninges are also infected during this carrier state. Cell nuclei are shown in blue for anatomical purposes.

5. LCMV AND NEONATAL INFECTION

In 1936 Traub made the seminal observation that a naturally occurring state of persistent LCMV infection could arise in most tissues without causing mortality (100). Later it was determined that LCMV infection either *in utero* or within 24 hours of birth leads to a life long carrier state marked by persistent viral infection rather than the clearance or death that is normally observed in adult mice (101-103). After inoculation, virus is disseminated globally throughout the body and is detected in major organs including the lung, liver, kidney, thymus, lymph nodes, pancreas and brain (104, 105). Within the brain parenchyma of the adult carrier mouse, the virus replicates almost exclusively in neurons (106, 107) (Figure 2). Chronic infection is established in part because LCMV replicates in the thymus, which leads to clonal deletion of LCMV-specific thymocytes (38). This was directly

demonstrated by neonatal infection of LCMV-specific TCR tg mice, which resulted in the loss of cells in the $CD4^+CD8^+$ stage of thymic development and a diminished number of splenic $CD8^+$ T cells (38). As a result of clonal deletion, the repertoire of T cells in the periphery of carrier mice has little to no cytolytic activity against LCMV and is unable to effectively purge the virus (108, 109). Notably, vaccination of carrier mice with an immunodominant CTL epitope for LCMV leads to the generation of a low affinity CTL response, albeit at an exceedingly low frequency, suggesting that thymic deletion of LCMV-specific $CD8^+$ T cells is incomplete and that high affinity $CD8^+$ T cell clones are eliminated in the thymus (110). Additionally, "cured" carrier mice after immune cell transfer (discussed below) can mount a functional endogenous CTL response that is able to clear virus upon re-challenge, suggesting a reversibility of tolerance once persistent antigen is removed (111).

5.1. Immunotherapy to treat LCMV carrier mice

Induction of a carrier state in mice has served as a useful platform to study the potential of immunotherapy as an effective means for viral clearance. Initial work conducted by Mogens Volkert established that transfer of LCMV immune cells from donor mice into carrier recipients resulted in elimination of the virus (112). $CD8^+$ T cells, H-2 compatibility and viral specificity, but not virus specific antibodies and $CD4^+$ T cells, were all later defined as necessary parameters for successful immunotherapy (113-118). However, a role for B cells and $CD4^+$ T cells may have been underestimated. In a system where high antibody production was observed, peripheral clearance of carrier mice persistently infected with LCMV WE was dependent on $CD4^+$ T cells and B cells, but not $CD8^+$ T cells (119). In the same study, immunotherapy of LCMV Arm carriers, which have lower antibody production after adoptive transfer compared to LCMV WE carriers, required $CD8^+$ T cells, $CD4^+$ T cells and B cells for viral elimination from tissues (119). Since B cells are critical for proper splenic architecture and resulting T cell responses (120), it is possible that this structural function is more important than antibody production in the context of successful clearance of carrier mice. This idea was substantiated in a study that used B cell deficient μMT mice to generate immune cells for immunotherapy (121). Adoptive transfer of LCMV immune cells from μMT-deficient mice resulted in decreased viral clearance in carrier mice infected with LCMV Arm. This defect was attributed to the impaired LCMV-specific $CD4^+$ T cell responses observed in μMT donor mice (121). Further evaluation of $CD4^+$ T cells following adoptive immunotherapy revealed that they are important for the long term maintenance of $CD8^+$ T cell function (122-124). In fact, the extent of $CD8^+$ T cell IFNγ and TNFα production directly correlated with the number of CD4 T cells co-transferred into recipient LCMV carrier mice (124). The numbers of $CD4^+$ T cells, relative to $CD8^+$ T cells, needed to provide sufficient help is surprisingly low. 350,000 $CD8^+$ T cells and 7,000 $CD4^+$ T cells (a ratio of 50:1) was defined as the minimum number of virus-specific memory cells required for successful immunotherapeutic clearance of LCMV Arm infected carrier mice (124).

While CD8^{+} T cells are likely the predominant effector cell necessary for viral clearance in persistently infected mice, CD4^{+} T cells and B cells play an important accessory role by maximizing and sustaining the activities of the transferred CTL response.

One of the more striking features of carrier immunotherapy is the varied kinetics of viral clearance based on the location of viral sequestration (116, 118, 125, 126). Oldstone and colleagues demonstrated that LCMV was purged from peripheral tissues (e.g., serum, liver, lungs and spleen) by day 15 post-immunotherapy but persisted in the brain and kidney of carrier mice until around day 120 (116). Although initial studies of brain tissues after immunotherapy demonstrated a restriction of infiltrates to the leptomeninges with little to no inundation of the brain parenchyma (116, 126), a more sensitive assay revealed that GFP-tagged LCMV-specific CTL distribute evenly throughout the brain parenchyma within 8 days following immunotherapy (107). Interestingly, LCMV clearance from neurons in carrier mice occurs with minimal neuronal drop out, suggesting a noncytopathic mechanism of clearance (116, 126).

Because neurons express low to undetectable levels of MHC I, this is thought to be one mechanism that protects them from direct CTL-mediated lysis. Consequently, this protective mechanism might also give rise to the predilection of viruses to establish persistence in neurons. Reduced MHC I expression on neurons was first demonstrated *in vitro* using a neuronal cell line (127, 128). These cells also had a reduced ability to load MHC I with peptides (128). However, stimulation of the neuronal cell line by IFNγ resulted in upregulation of MHC I and subsequent lysis by CTL (127). Based on the hypothesis that neurons had a deficiency in MHC I expression, transgenic mice were generated to express H-2D^b protein under the neuron-specific enolase promoter (NSE-D^b mice). Adoptive transfer of LCMV-specific CTL in NSE-D^b mice persistently infected with LCMV Arm resulted in CTL infiltration of the CNS, profound blood brain barrier breakdown, increased illness / death of carrier mice and faster CNS viral clearance in those that survived (129). These data suggest that neuronal MHC I expression is one bottleneck that restricts the speed with which CTL purge virus from neurons in LCMV carrier mice. This bottleneck is likely in place to minimize the amount of irreparable immune-mediated damage that can occur within the CNS.

In the absence of direct interactions between CTL and neurons, production of cytokines such as IFNα/β, IFNγ and TNFα may be important for viral clearance of the CNS. Each of these cytokines were shown be required for viral clearance following immunotherapy (107, 119, 122). A recent study examining mice 8 days post-immunotherapy revealed that a considerable number of antigen presenting cells (APCs) were recruited into the CNS of carrier mice as a direct consequence of the immunotherapy, and this APC recruitment correlated perfectly with the arrival of LCMV-specific CTL (107). Importantly, of the APC subsets only dendritic cells (not infiltrating macrophages or resident microglia) were able to stimulate LCMV-specific CTL to produce IFNγ and TNFα production, and immunological synapses between CTL and APCs were observed *in situ* (84). These data suggest that CNS-infiltrating DCs might serve as important accessory cells to support the activities of anti-viral CTL operating within the persistently infected CNS. These DCs could function either by promoting CTL survival / division or by fostering the production of anti-viral cytokines to purge adjacently infected neurons. Thus, it is conceivable that direct engagement of neurons by CTL is not an absolute requirement for clearance.

5.2. Behavioral abnormalities in carrier mice

Although carrier mice do not display signs of neuronal loss during infection, they do exhibit signs of neuronal dysfunction manifested as abnormalities in behavior and learning (130-132). Specifically, persistently infected mice show reductions in spatial-temporal learning, assessed in a Y maze discriminated avoidance learning task, and decreases in explorative behavior in the context of novel environments (130-132). Expression of viral antigen is found in neurons localized in numerous regions of the brain including the neocortex, limbic system, hypothalamus, brain stem, thalamus, basal ganglia and the hippocampus - a region that is implicated in the development of memory and learning (104, 106). Recently, it was shown that persistent viral infection is associated with alterations in the host gene profile of the brain (132). Most of the genetic perturbations were downstream of type 1 interferon (132) - a cytokine known to be systemically elevated in LCMV carrier mice (133, 134). Because the carrier state is maintained in the absence of any major inflammation or necrosis in the brain parenchyma, it has been theorized that alterations in the expression of neuronal proteins are a direct result of the LCMV infection itself. Neurochemical abnormalities, manifested as hypersensitivity to cholinergic antagonists during learning and motor tests, and decreased choline acetyltransferase in neuroblastoma cells have been linked to LCMV infection (130, 131, 135). These data suggest that dysfunctional cholinergic responses might be responsible for the neurobehavioral defects observed in the carrier mice. In addition to neurochemical alterations, viral persistence was also associated with decreased neuronal plasticity. *In vitro* studies showed that LCMV prevented nerve growth factor (NGF) induced upregulation of growth-associated protein-43 (GAP-43) in PC12 cells (136). Importantly, these data were confirmed *in vivo*, as decreased expression of GAP-43 was observed in the hippocampus of LCMV carrier mice (136). Because GAP-43 has been implicated in neuronal plasticity (137), decreased expression in the hippocampus of carrier mice might also contribute to the virus-induced defects in learning capacity. These studies clearly indicate that LCMV has the capacity to significantly alter neuronal function in the absence of actual cytolysis.

5.3. LCMV and growth hormone deficiency

Neonatal infection of C3H/St mice results in the development of a growth hormone deficiency syndrome. This is characterized by growth retardation manifested as a decrease in body length and weight within weeks of birth (138). Disease induction is dependent on the strain of

mouse and virus used. C3H/St mice were susceptible to disease, whereas both BALB/c and SWR/J mice were resistant (139, 140). The ability of LCMV variants to differentially infect growth hormone-producing cells localized in the anterior pituitary gland directly correlated with growth hormone deficiency during persistence (138, 141). LCMV Armstrong but not LCMV WE or Traub was able to induce disease (139). Growth hormone deficiency occurred in the absence of necrosis or inflammation in the pituitary gland, suggesting that LCMV altered cellular function rather than induce cell death (142). In support of this statement, LCMV was subsequently shown to selectively disrupt growth hormone production by interfering with the growth hormone transactivator factor GHF1 (Pit1) that binds to the growth hormone promoter (143). This interference resulted in decreased production of growth hormone mRNA and subsequent development of the growth hormone deficiency syndrome (144, 145). Therefore, the ability of LCMV to cause selective cellular dysfunction without lysis is not restricted to neurons and can be extended to other persistently infected cell populations.

6. LCMV AND ADULT INFECTION

In general, chronic LCMV infection in adult mice is favored under conditions where there is rapid viral replication and/or an impaired immune response. For example, mechanisms that allow for increased viral titers, including viral interferon insensitivity, higher replicative rates, increased inoculation dosage and suppression of the immune system can facilitate the transition from an acute to more chronic state of persistence (26, 146-149). Therefore, under permissive conditions LCMV can establish a state of persistence in adult mice.

6.1. LCMV clone 13 and chronic infection

The prototypic LCMV strain associated with chronic infection of immunocompetent adult mice is referred to as LCMV clone 13. This variant was isolated from mice persistently infected with Armstrong from birth (150). Genetic analysis of LCMV clone 13 compared to the parental Armstrong virus revealed only 2 genomic changes that led to alterations in the resultant amino acid sequence: a change from K to Q at position 1079 of the L segment encoding the polymerase and a change from F to L at position 260 of the S segment encoding the viral glycoprotein (28, 29, 151). Notably, the glycoprotein (F to L at position 260) mutation has been associated with both viral persistence and decreased CTL activity in addition to conferring an increased affinity for the LCMV receptor, α-dystroglycan (α-DG) (29, 152). Initial studies in carrier mice infected with LCMV Armstrong revealed that the F to L mutation at position 260 of the glycoprotein, which is observed in the clone 13 variant, appeared to occur preferentially in macrophages and peripheral tissues including the spleen, liver, lymph node, thymus, heart and lung within 2 months post infection (151, 153, 154). However, in the brain conversion from LCMV Armstrong to clone 13 occurred at lower levels (151), and in general, LCMV Armstrong was observed to out compete clone 13 for infection of neurons *in vitro* and brain tissue upon co-infection *in vivo* (154, 155). These data suggested that

LCMV clone 13 was a more viscerotropic variant, whereas Armstrong was a more neurotropic variant.

After inoculation of adult mice with LCMV clone 13, virus is distributed throughout the body in areas including the serum, blood, spleen, liver, kidney and brain (28, 153, 154, 156, 157). In contrast to LCMV Armstrong infection, which is cleared within 2 weeks, LCMV clone 13 challenge results in persistent infection and diminished CTL responses (29, 152, 158, 159) in adult mice. Whereas clone 13 infection of neonates results in suppression of LCMV-specific responses, challenge of adult mice generates a generalized immune suppression that also diminishes cytotoxic responses to other viruses (158). This global inhibition is partially attributed to alterations in splenic architecture that occurs after clone 13 infection of adult but not neonatal mice (158). Further studies in adult mice demonstrated that enhanced viral infiltration into the white pulp of the spleen was associated with increased binding to α-DG (152) and that clone 13 also more effectively targeted fibroblastic reticular cells resulting in decreased conduit function of the spleen (160). The disruption in splenic function (158, 160), physical destruction of dendritic cells (161), and inhibition of dendritic cell differentiation and maturation (162, 163) are likely contributors to the immunosuppression observed during clone 13 infection. More recently, it has also been revealed that inhibitory molecule such as PD-1 (164) and IL-10 (165) are upregulated early following clone 13 infection and also contribute to the immunosuppressive milieu. In terms of CTL responses, clone 13 infection was shown to disrupt their dominance hierarchies and tissue distribution (157), induce deletion of immunodominant populations (159), and promote profound functional exhaustion, evidenced by a diminished capacity to produce IFNγ, TNFα, and IL-2 (157). Similar functional disruptions were also noted in the CD4 compartment (166, 167). However, despite this suppression in virus-specific immunity, systemic clone 13 clearance is eventually achieved in chronically infected mice.

6.2. LCMV clone 13 infection and clearance from the CNS

Intravenous inoculation of adult mice with LCMV clone 13 results in state of protracted viral clearance. In contrast to Armstrong, which is cleared acutely in 8-10 days, clone 13 distributes systemically and is not purged from most peripheral tissues (e.g. serum, spleen, lymph nodes, lung, and liver) until 40-80 days post-infection (156, 157). The time period preceding viral clearance is associated with profound immune exhaustion, during which LCMV-specific T cells show a diminished capacity to produce anti-viral cytokines and engage in cytolytic activities (157). Within the CNS clearance of LCMV clone 13 is delayed considerably and does not occur until day 200 post infection (156).

To gain novel insights into clone 13 infection of the CNS, our group recently studied clone 13 tropism and the responding CNS immune response to this variant following intravenous inoculation (156). At early time points post-infection, viral antigen was detected in the
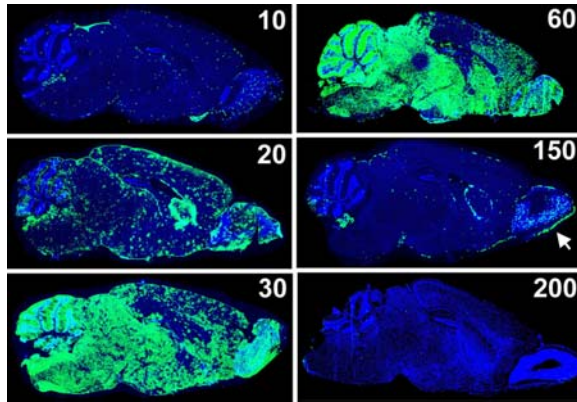
Figure 3. Localization of LCMV in the brain of clone 13 infected mice**.** The brains of C57BL/6 mice infected intravenously with LCMV clone 13 were harvested at time points spanning 10 to 200 days post infection. The distribution of LCMV (green) is shown on representative sagittal brain sections, and cell nuclei are shown in blue. On day 10 post-infection clone 13 is detected in the meninges, choroid plexus, ependyma as well as throughout the parenchyma in areas around blood vessels (note the punctuate pattern observed in the parenchyma). From days 20-60 post-infection, the virus inundates the brain parenchyma, spreading almost exclusively through the astrocytic network. Concurrent with reanimation of the peripheral T cell response clone 13 is purged from parenchymal astrocytes, and by day 150 the virus is found in its last refuge - olfactory bulb neurons as well as the meninges, ependyma and choroid plexus. Clone 13 is finally cleared from the brain by day 200 post infection.

choroid plexus, meninges, ependymal cells and in regions surrounding the blood vasculature of the brain (Figure 3). Within a month, viral entry and dissemination throughout the brain parenchyma was achieved through astrocyte infection. Because astrocytes are a critical element of the blood brain barrier (BBB) (168), it is possible that the virus gains access from the blood stream into these cells through astrocytic foot processes that comprise the second layer of the BBB. Additionally, astrocytic expression of the LCMV receptor, α-dystroglycan, may increase the ability of clone 13 to readily gain access to this cell type (169, 170). Over time the virus moved throughout the astrocytic network, and complete inundation of the brain parenchyma was observed between days 30-60 post infection. In contrast, peak viral loads were achieved in the periphery by day 10 post infection. By 150 days post-infection, a remarkable shift in the distribution of parenchymal virus was observed. Viral antigen in astrocytes was reduced to an almost undetectable level and olfactory bulb neurons emerged as the last bastion supporting viral persistence. When LCMV clones were isolated from the brains of clone 13 infected mice at early and late stages of persistence, it was revealed that the leucine at position 260 of the viral glycoprotein, indicative of the clone 13 phenotype, was maintained throughout infection. Therefore, reversion or selection of a more neurotropic LCMV Armstrong virus was not a likely cause for the altered tropism observed between these two stages of chronic infection.

Reflective of what is observed in the periphery, LCMV specific CD8$^+$ T cells that reside in the CNS undergo a state of functional impairment after clone 13 infection. When analyzed *ex vivo* CNS-derived LCMV-specific CD8$^+$ T cells showed signs of decreased IFNγ, TNFα, and IL-2 production by day 8 post-infection, but the functional impairment of CD8$^+$ T cells was far more severe in peripheral tissues (e.g., spleen and liver). After day 60 post-infection, just prior to the initiation of viral clearance from astrocytes, LCMV-specific CD8$^+$ T cells underwent a reanimation phase in both the periphery and CNS characterized by a resurgence of full cytokine production capacity. At this time increased leukocyte numbers were observed in the CNS, and diversification in the composition of the infiltrate was observed. In particular, a significant increase in the number of virus-specific CD4$^+$ T cells and B cells was observed. These events coincided with the eventual clearance of clone 13 from the CNS and were initiated at a time when virus was purged almost entirely from peripheral tissues. These findings suggest that diversification of the CNS immune infiltrate is an important step in achieving viral clearance from this specialized compartment and that the timing of CNS clearance might be delayed by the peripheral "antigen sink" which spreads out the resources of the adaptive immune response (99). Once the peripheral viral burden is removed, the adaptive immune system can redirect all of its resources to CNS. Studies are underway to elucidate the exact components of CNS immune infiltrate responsible for purging clone 13 during this reanimation phase.

6.3. Chronic LCMV infection in immunocompromised mice

Chronic infection can also be established in adult immunocompromised mice after inoculation with LCMV Armstrong. Although intracranial injection of immunocompetent mice with LCMV normally results in acute lethal meningitis, a deficiency in the CD8$^+$ T cell response enables the virus to establish persistence. For example, in CD3δ deficient mice, a delayed LCMV-specific CD8$^+$ T cell response provides the virus enough time to switch tropism into neurons (51). The targeting of the virus to neurons changes the accessibility of LCMV peptide presentation because of low neuronal MHC I expression, and therefore the virus remains "hidden" even after a functional immune response is generated. Similarly, mice that mount a negligible LCMV-specific response due to forced expression of a CD8$^+$ ovalbumin specific TCR (OT-I mice) (60, 61) also survive an intracranial challenge with LCMV (43). Initially, the anatomical viral distribution is confined to the meninges, ependyma and choroid plexus, as observed in wild type mice; however, by day 55 the localization changes and LCMV persists in neurons and astrocytes in addition to the meninges and ependyma. Importantly, transfer of as few as 1000 naive D^{b}GP$_{33-41}$ CD8$^+$ T cells into OT-I mice prior to infection was sufficient to reconstitute the acute meningitis (43). These studies reflect two important points for LCMV viral persistence and disease. First, the immune response, particularly LCMV-specific CD8$^+$ T cells, is necessary for clearance / immunopathology and thus a deficiency in this compartment can prevent disease and lead to uncontrolled

infection. Second, a single monospecific CD8^{+} T cell population operating in sea of bystander T cells of an irrelevant specificity is sufficient drive an entire disease process (e.g., meningitis) with normal kinetics. These data suggest that bystander T cells are of minimal importance in mediating acute CNS diseases initiated by virus infection.

7. CONCLUSION

LCMV infection provides a powerful model system to study viral-immune cell interactions in the CNS because of the great diversity in observed outcomes. These outcomes range from severe immunopathology to chronic infection to viral clearance. Because LCMV is non-cytolytic, the outcome of infection is largely influenced by two factors: 1.) viral tropism within the CNS, and 2.) the functionality of the adaptive immune system. In some instances, LCMV alone is sufficient to alter the function of the cells it infects, which can result in modifications in behavior and hormone regulation. However, the integrity of the CNS as well as the state of LCMV persistence within this compartment is usually dictated by the immune response that is mobilized to fight the infection. The most dramatic example of this is the fatal choriomeningitis that develops in response to intracerebral inoculation of immunocompetent mice with LCMV.

CD8^{+} T cells have emerged as key mediators of both pathology and clearance following LCMV infection. While acute lethal meningitis is caused by a robust LCMV-specific CD8^{+} T cell response, neonatal and adult persistence develops when CD8^{+} T cells are suppressed. This is observed when an immunosuppressive variant of LCMV is used (e.g. clone 13) or when immunologically compromised (or immature) mice are infected with LCMV. Once LCMV establishes persistence, it is possible to therapeutically purge the pathogen by supplementing the carrier mouse with virus-specific memory T cells. This remarkable therapeutic approach is also dependent on the presence of a functional CD8^{+} T cells, further emphasizing the importance of this cell population. Moreover, factors that aid in the induction and/or maintenance of virus-specific CTL, such as MHC and CD4^{+} T cell responses, also have a profound impact on the final outcome after infection. For example, adoptive immunotherapy is not successful unless CD8^{+} and CD4^{+} T cells cooperate to achieve clearance. Given the importance of CTL in the LCMV system as well as other viral models, we propose that it is important to gain additional insights into the behavior and function of these cells within the CNS. It is also of great importance to better understand how to therapeutically modulate CTL function, especially in patients that have an established CNS infection.

While peripheral responses have been extensively studied in the LCMV model, relatively little is known about viral and leukocyte behavior within the LCMV-infected CNS. There is a paucity of knowledge as to how and why LCMV tropism within the CNS shifts after infection, which effector mechanisms are critical for T cell clearance versus pathology, which molecules direct T cell trafficking into the LCMV-infected CNS, and whether LCMV-specific T

cell priming (or restimulation) occurs within the CNS. The degree to which neurons and glia regulate virus-specific immune cells and the impact of immune-target interactions on the function the LCMV-infected CNS cells also remains to be elucidated. Addressing these unanswered questions in the LCMV model will provide important, fundamental information pertaining to CNS virus-immune interactions that can hopefully be extrapolated to states of viral infection in humans.

8. ACKNOWLEDGEMENTS

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