Evaluation of the protective roles of complement activation and T cells during Venezuelan equine encephalitis virus infection

Christopher Byron Brooke

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Approved by:

Ralph Baric, PhD

Jeffrey Frelinger, PhD

Mark Heise, PhD

Glenn Matsushima, PhD

ABSTRACT

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Venezuelan Equine Encephalitis Virus Infection

(Under the direction of Robert E. Johnston)

Venezuelan equine encephalitis virus (VEEV) is a mosquito-borne alphavirus that can cause a potentially lethal encephalomyelitis in humans and equids. There is currently no licensed vaccine available for use in humans. Efforts to understand the host requirements for successfully controlling VEEV infection have been limited by the extreme lethality of the virus in small animal models. Here we describe the use of the V3533 mutant of VEEV as a model for successful control of VEEV infection. Following peripheral inoculation of a mouse, V3533 behaves similarly to lethal strains of VEEV. Rapid replication of V3533 in secondary lymphoid organs results in the development of a serum viremia, followed by viral invasion of the central nervous system (CNS). The infection is short-lived, however, as the development of an adaptive immune response results in clearance of infectious virus from all tissues by day 8 post-infection. Using this model we identified previously unappreciated roles for complement activation and T cells in promoting recovery from VEEV infection.

Complement activation within the first 24 hours of infection enhanced serum clearance and delayed viral invasion of the CNS, preventing the development of overt encephalomyelitis.   This effect was independent of anti-VEEV antibody induction or inflammatory cell recruitment. In addition, we showed that T cells, particularly CD4$^+$ T cells, were capable of controlling V3533 infection in the CNS and facilitating recovery from severe encephalomyelitis in the absence of antibody. Together, these results provide a starting point for future efforts to identify the requirements of a protective host response to VEEV infection.

ACKNOWLEDGMENTS

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LIST OF COMMONLY USED ABBREVIATIONS

AF – Alexa-fluor

APC – Antigen presenting cell, or allophycocyanin

AST – Average survival time

BBB – Blood brain barrier

cDNA – Complementary DNA

CNS – Central nervous system

DNA – Deoxyribonucleic acid

eGFP – Enhanced GFP

FITC – Fluorescein isothiocyanate

GFP – Green fluorescent protein

H&E – Haematoxylin and eosin

HSV – Herpes simplex virus

i.c. – Intra-cranial

IFN - Interferon

i.p. – Intra-peritoneal

LCMV – Lymphocytic choriomeningitis virus

MAb – Monoclonal antibody

MAC – Membrane attack complex

MHC – Major histocompatibility complex

MHV – Mouse hepatitis virus

nsP – Nonstructural protein

PE – Phycoerythrin

PFU – Plaque forming unit

P.I. – Post-infection

PMA – Phorbol 12-myristate 13-acetate

PRR – Pattern recognition receptor

RLR – Rig-I-like receptor

RNA – Ribonucleic acid

s.c. – Sub-cutaneous

SEM – Standard error of the mean

SF – Semliki Forest virus

SIN – Sindbis virus

TD – Trinidad donkey (VEEV strain)

TLR – Toll-like receptor

TMEV – Theiler's murine encephalomyelitic virus

VEEV – Venezuelan equine encephalitis virus

VRP – VEEV replicon particle

CHAPTER 1:

INTRODUCTION

Venezuelan equine encephalitis virus (VEEV) is a mosquito-borne RNA virus of the genus *alphavirus* that is responsible for a significant disease burden in Central and South America through sporadic outbreaks into human and equid populations (256). VEEV infection in humans causes a spectrum of disease that ranges form asymptomatic to mild, flu-like illness to overt encephalomyelitis, with an overall case mortality rate of about 0.5-1%. In equid populations, the development of overt encephalomyelitis is more common, and the overall mortality rate often exceeds 50% (252). Much of our current understanding of VEEV pathogenesis and immunity comes from studies carried out in a well-characterized mouse model of infection. Typically, VEEV infection of mice results in the development of a lethal encephalomyelitis within 6-8 days of infection, however, one unique VEEV mutant called V3533 invades and replicates within the central nervous system (CNS) of mice without inducing severe morbidity or mortality. This virus has provided an excellent model for understanding the viral and host factors that determine whether infection progresses to death or successful immune control and recovery. Specifically, the studies described here reveal previously unappreciated roles for T cells and the complement system in controlling VEEV infection and influencing disease outcome. In order to provide the pertinent background, the following sections review the current literature concerning VEEV biology, the host complement system, and the regulation of immune responses within the CNS.

VENEZUELAN EQUINE ENCEPHALITIS VIRUS BIOLOGY

GENOMIC ORGANIZATION

Venezuelan equine encephalitis virus (VEEV), a member of the genus *alphavirus*, is encoded by a single positive-sense RNA of approximately 11,400 nucleotides that is capped with a 5' terminal 7-methylguanosine as well as polyadenylated at its 3' terminus (119). The 5' two-thirds of the viral genome encodes the four non-structural proteins (nsp1-4) in a single open reading frame. The 3' third of the genome contains a second open reading frame that encodes a subgenomic, 26S RNA that yields the three major structural proteins, capsid and the E1 and E2 glycoproteins, as well as the 6K polyprotein (232). In between these two regions lies a ~38 nucleotide junction region that contains the transcriptional promoter, start site, and non-translated leader sequence for the 26S subgenomic mRNA encoding the structural polyprotein. Four conserved sequence elements (CSE) that are important for replication have been identified, two near the 5' end of the genome, one in between the nonstructural and structural gene cassettes, and one immediately upstream of the poly(A) tail (125-127). Finally, the VEEV genome is flanked by short nontranslated regions (NTR) at the 5' and 3' termini, 44 and 121 nucleotides in length respectively, both of which are required for replication of the genomic RNA (232).

REPLICATION STRATEGY

While the molecular mechanisms underlying VEEV replication have not been extensively examined, a great deal of research has been performed using related alphaviruses, including Sindbis virus (SIN) and Semliki Forest virus (SF), and it is thought that findings obtained in those systems apply generally across the genus

Alphavirus. Therefore, findings from various alphaviruses will be discussed below with the assumption that they apply to VEEV as well.

ENTRY AND UNCOATING

Alphavirus attachment and entry are mediated primarily by the viral E1 and E2 glycoproteins. These two proteins form stable heterodimers on the surface of the virion particle that are further organized into trimeric spikes by E1-E1 interactions (198, 232). The E2 protein, which protrudes from the virion surface, is thought to mediate target cell recognition via interaction with an unidentified host protein receptor (43, 198). The exact mechanism by which alphaviruses transmit their genomic RNA into the cytoplasm remains unclear. A series of studies have suggested that entry occurs through virus-cell membrane fusion in the early endosome. Following E2-mediated attachment, viral particles rapidly enter the cell via endocytosis, demonstrated by the disappearance of viral glycoproteins from the cell surface following SIN infection (47). The acidification of the early endosome to pH ≤ 5.5 causes the reduction of glycoprotein disulfide bridges, leading to a conformation change in E1 that exposes the fusion domain (1, 57, 115, 150). With SF, this process has been shown to occur within five minutes of attachment (89). Exposure of the hydrophobic, 17-amino acid fusion domain then triggers fusion of viral and host lipid bilayers by an as-yet undescribed mechanism, releasing the viral nucleocapsid into the cytoplasm (232). Alternatively, Brown and others have suggested that SIN infection can occur through a membrane fusion-independent mechanism (191). They showed that, following a low pH-induced conformational

change in the viral glycoprotein coat, SIN could bind the cell surface and release the viral genomic RNA into the cytoplasm without inducing membrane fusion or disassembling the viral protein shell. Other studies have demonstrated SIN infection in the presence of weak bases, calling into question the low pH requirement for alphaviral entry (23, 30).

The process of nucleocapsid uncoating has also best been described in the Sindbis virus and Semliki Forest virus systems. Following release into the cytoplasm, incoming nucleocapsids rapidly associate with the large 60S subunit of host ribosomes (222, 258). Binding of viral capsid protein to the 60s subunit was shown to require exposed rRNA sequence, as RNase pretreatment abolished binding, potentially a 400 nucleotide stretch of the 28S rRNA molecule that shares 54% sequence homology with the SIN encapsidation sequence (222, 257). The association with ribosomes has been demonstrated to be both necessary and sufficient for the uncoating of SF virus capsid protein from the viral RNA *in vitro* (222). Singh and Helenius hypothesized that uncoating resulted from capsid molecules being stripped away from the viral RNA by higher affinity binding sites on the 60S subunit. This hypothesis was supported by the findings that the uncoating process required no energy input, occurred in a stoichiometric rather than catalytic fashion, and resulted in the long-term association of un-degraded capsid with the 60S subunit (222, 232). One problem that has kept this hypothesis from being universally accepted is that it does not explain how newly synthesized capsid

proteins are able to stably associate with viral RNA during assembly in the continued presence of ribosomes.

TRANSLATION AND REPLICATION

Much of what is known about alphavirus protein translation and RNA replication was described in the SIN system (232). Once released from the nucleocapsid structure, the incoming viral genomic RNA acts as a messenger RNA for the translation of the four nonstructural proteins (230). In the case of VEEV, as well as many other alphaviruses including Sindbis virus, the nonstructural proteins are translated as two distinct polyproteins (119, 232). The predominant species produced is the P123 polyprotein that eventually gives rise to nsP1, nsP2, and nsP3. Translation of the P123 polyprotein is terminated by an opal codon at the nsP3-nsP4 junction. Readthrough of the opal codon, which is estimated to occur with a frequency of about 10-20% in SIN, results in the production of the second polyprotein, P1234 (139). Upon translation of the P1234 polyprotein, *cis* cleavage at the nsP3/4 junction by the protease domain of nsP2 occurs rapidly, yielding P123 and nsP4 as products (132, 133). Subsequent cleavage of P123 at the nsP1/2 and nsP2/3 junctions to yield mature nsP1, nsP2, and nsP3 is dependent upon the viral protease acting in *trans* and thus occurs with slower kinetics than the nsP3/4 cleavage (219).

The four mature nsPs are multifunctional proteins that are all required for viral replication (232). The nsP1 protein contains the viral guanine-7-methyltransferase and guanylyltransferase activities necessary for capping genomic and 26S RNA

(128, 164). In addition, nsP1 is the only alphavirus nonstructural protein that has

been shown to directly associate with cellular membranes (194). This association,

which is thought to occur through the combined effects of a palmitoylated cysteine

residue at residue 420 (position in SIN) and an amphipathic α-helix between

residues 245 and 264 (position in SF), is probably responsible for anchoring

replication complexes to cellular membranes (127, 130). As mentioned earlier, the

viral papain-like serine protease is contained within the C terminus of nsP2 (229). At

its N-terminus, nsP2 contains helicase, NTPase, and RNA triphosphatase activities

that are required for viral RNA replication (67, 205, 246). The exact function of the

nsP3 protein in viral replication remains unclear, though mutational studies have

made it clear that it does play a role (131). Finally, the nsP4 protein provides the

viral RNA-dependent RNA polymerase activity (76).

Productive infection of a cell by an alphavirus requires the highly orchestrated

production of three distinct RNA species: plus-strand genomic RNA, the

complementary minus-strand RNA, and the plus-strand subgenomic 26S RNA. This

process, which takes place on the cytoplasmic side of endosomal and lysosomal

membranes, is initiated by cleavage of the P1234 polyprotein into P123 and nsP4 by

nsP2 (55, 132). The resultant P123+nsP4 complex catalyzes minus-strand RNA

synthesis beginning from the 3' CSE of the parental genome, possibly with

unidentified host proteins serving as co-factors (62, 79, 132, 133). Due to the

initially small pool of both positive-sense template RNA (ostensibly a single molecule

at first) and nsP4 (due to inefficient readthrough of the opal codon at the nsP3/4

junction), minus strand RNA is not produced to great abundance during infection (254). Meanwhile, as P123 and P1234 continue to be translated from the input genomic RNA and their local concentration increases, the reaction velocity of nsP2-mediated *trans* cleavage of nsP1/2 and nsP2/3 also increases, giving rise to nsP1+nsP2+nsP3+nsP4 complexes (232). The transition from a P123+nsP4 conformation to an nsP1+nsP2+nsP3+nsP4 conformation apparently alters the initiation template specificity of the replicase complex, converting it irreversibly to an efficient producer of plus-strand, but not minus strand viral RNAs (134). Thus, the alphaviral replicase complex acts as a feedback switch that allows for tight temporal control of both plus-strand and minus-strand RNA synthesis, regulated by the concentration of nsPs.

Once the replicase complex has converted from minus-strand to plus-strand synthesis, two distinct RNA species are produced: the full-length genomic 49S RNA and the subgenomic 26S RNA. The genomic and subgenomic RNAs utilize different promoters, located in the minus-strand 3' NTR and the junction between the nonstructural and structural gene cassettes, respectively (232). Roughly ten times as much subgenomic RNA is produced, compared with genomic RNA, during infection, though the mechanism underlying this disparity is not fully understood (203). A panel of mutations in nsP2 of SIN result in less subgenomic RNA being produced relative to genomic RNA, suggesting that nsP2 might play some role in determining promoter choice during plus-strand synthesis (232). Additionally, differential binding of host factors to the two promoters may also play a role (44).

PACKAGING AND EGRESS

Translation of the subgenomic 26S RNA results in production of the capsid-PE2-6K-E1 structural polyprotein. The capsid precursor is located at the N-terminus of the polyprotein and is thus translated first. Once the ribosome has cleared the junction between capsid and PE2, a serinelike protease domain within the C-terminus of capsid triggers the release of mature capsid from the polyprotein by *cis*-cleavage (75). Upon cleavage from the polyprotein, capsid remains associated with the ribosome and appears to rapidly assemble into core complexes around genomic RNA (223, 244). This assembly process is dependent upon capsid binding of a specific structural element within the genomic RNA molecule called the encapsidation signal (141, 248). This process has been demonstrated to occur very rapidly *in vitro*, with nothing but purified genomic RNA and capsid protein, yielding nucleocapsid core-like particles with T=4 icosahedral symmetry similar to cores purified from mature virion particles (170).

Following release of the capsid protein by self-cleavage, the resultant N-terminus of the remaining nascent structural polyprotein acts as a signal sequence that directs translocation of the PE2 sequence into the ER lumen (60). Additional downstream stop-transfer and signal sequences result in the structural polyprotein being stitched through the ER membrane such that long stretches of PE2 and E1, as well as the N-terminus of the 6K polyprotein, are translocated into the ER lumen as well (232). Immediately upon translocation into the ER lumen, the envelope proteins begin a

complex sequence of folding and conformational changes that is dependent upon host chaperones and disulfide bonding (18, 19, 41, 123, 172-174). In parallel with this process, the VEEV envelope proteins contain 4 N-linked glycosylation sites, one on E1 and three on E2, that are glycosylated within the ER lumen (118). In the case of SIN, N-linked glycosylation sites on the viral envelope proteins initially receive high mannose chains that are then trimmed and further processed into complex sugars based on their physical availability to the host glycosylation machinery (218, 255). The glycosylation of PE2 and E1 is thought to be essential for their proper folding, possibly by increasing their solubility, as inhibition of the host glycosylation machinery prevents transport to the cell surface (155). At an undefined stage in the glycoprotein folding process, PE2 begins to oligomerize with a folding intermediate of E1, triggering egress from the ER into the Golgi network (144). As the viral glycoproteins pass into the trans-Golgi network, host furin cleaves the N-terminus of PE2, yielding the mature E2 glycoprotein (38).

Budding of virus particles requires direct interaction between the glycoproteins and the nucleocapsid core (235). Studies using cryo-EM as well as viral genetics have suggested that the association of the viral glycoprotein and newly formed nucleocapsids is mediated by interactions between the cytoplasmic tail of E2 and a hydrophobic pocket located within capsid (171, 234, 235, 264). Nucleocapsids are thought to diffuse freely through the cytoplasm until they encounter and bind the E2-E1 complex at the plasma membrane, at which point the actual process of membrane extrusion required to produce viral particles is driven by lateral

interactions between the glycoproteins (249). The interactions between the nucleocapsid and the glycoproteins are thought to induce a further maturation of the nucleocapsid, as nucleocapsids isolated from mature particles sediment more rapidly and are more sensitive to RNase treatment, compared with nucleocapsids isolated fom infected cells (29). The 6K protein, of which about five to ten molecules are incorporated into virions, also plays some undefined role in the budding process, as deletion of 6K or substitution of a heterologous 6K from another alphavirus results in budding defects with SF or SIN, respectively (140, 261).

HOST CELL EFFECTS

TRANSCRIPTION/TRANSLATION SHUTOFF

One of the most studied effects of alphavirus infection is the virus-induced shutoff of host protein synthesis that can occur as early as three hours after infection (232). This process, which does not affect the production of viral protein, is primarily considered as a means to limit the induction of a type I interferon response by the infected cell (69). Viral mutants that lack the ability to induce shutoff of host protein synthesis are less cytopathic, and are often capable of establishing a persistent infection *in vitro* (54). During SIN infection, shutoff of host protein synthesis results from viral inhibition of host transcription and translation by discrete mechanisms (69). Mechanisms of host protein synthesis shutoff differ between Old World and New World alphaviruses, so findings obtained with SIN or SF may not apply to VEEV (59). Most of the work on VEEV-mediated host protein synthesis shutoff has focused on inhibition of host transcription by the capsid protein. Residues 33-68 of

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the VEEV capsid protein have been demonstrated to be sufficient to induce

transcriptional shutoff, and full-length virus lacking this sequence was shown to be

non-cytopathic, suggesting that it was incapable of mediating shutoff (58). Both the

full-length VEEV capsid protein, as well as the 33-68 domain by itself, associate with

nuclear pore complexes and inhibit importin-mediated nuclear import, though no

direct connection between this function and shutoff of host transcription has been

demonstrated (6). Finally, full-length VEEV virus with mutations in the 33-68 domain

of capsid were shown to be less virulent in neonatal mice than wild-type VEEV (58).

INHIBITION OF IFN SIGNALING

Like many viruses, VEEV is sensitive to the effects of type I interferon (IFN)

signaling (102, 260). The sensitivity of VEEV to type II IFN has not been reported,

however SIN is quite sensitive to type II IFN treatment (12, 15, 16). While the

inhibition of host protein synthesis can serve as a potent means of preventing IFN

induction in response to infection, Simmons et al. have described a separate,

shutoff-independent mechanism by which VEEV is able to antagonize the host IFN

response (221). Expression of the viral nonstructural genes by non-propagating

VEEV replicon particles (VRP) prevented the induction of IFN-stimulated gene (ISG)

expression in response to exogenous IFN-$\alpha\beta$ and IFN-γ by preventing the activation

and nuclear trafficking of STAT1. The existence of multiple viral mechanisms for

antagonizing the induction of the host IFN response underscores how critical this

system is in restricting VEEV infection.

APOPTOSIS

Alphavirus infection of mammalian cells frequently results in apoptosis *in vitro*, and it is thought that the induction of apoptosis in infected cells *in vivo* plays an important role in determining infection outcome (136). Much of what is known about apoptosis induction by alphavirus infection comes from studies of SIN infection of mouse neurons. *In vitro* induction of apoptosis by SIN has been demonstrated to be dependent upon caspase activity and Ras signaling, and can be inhibited in some cell types by overexpression of the host anti-apoptotic protein Bcl-2 (105, 114, 178). Additional studies have demonstrated that the susceptibility of neurons to SIN-induced apoptosis depends on both the strain of virus used and the age of the animal, with neurons from neonatal mice (≤14 days of age) being much more susceptible than those from weanling or adult mice (72, 73, 243). The ability to induce apoptosis in adult neurons is associated with increased neurovirulence in mice (243). This pattern might help explain the extreme neurovirulence of VEEV, as it can induce apoptosis in adult neurons *in vivo* as well (99). Whether VEEV induces apoptosis in other *in vivo* cell targets, such as dendritic cells, remains unknown.

The exact mechanism(s) by which VEEV is able to induce apoptosis in neurons, or other cell types for that matter, is not known. SIN has been demonstrated to induce apoptosis in a neuronal cell line by a fusion-dependent, but replication-independent mechanism, suggesting that the viral glycoproteins are involved (103, 104). Entry of SIN rapidly activated acidic sphingomyelinase, which in turn hydrolyzed spingomyelin in the plasma membrane to release ceramide, a potent inducer of

apoptosis (103, 183). In fact, overexpression of acid ceramidase was sufficient to prevent SIN-induced apoptosis (103). In addition to the clear effects of the viral glycoproteins, there is almost certainly a role for the nonstructural proteins as well, as mutations in nsP2 result in a virus that is incapable of inducing apoptosis *in vitro* (54).

VEEV MOUSE MODEL

Efforts to understand the pathogenesis of VEEV infection are aided by the fact that experimental infection of rodents closely mirrors many aspects of disease observed in humans and equids (66, 100). Infection of mice with molecularly cloned VEEV viruses, as well as the use of non-propagating VEEV replicon particles (VRP), have allowed the elucidation of multiple stages in the progression of VEEV infection and the development of disease (4, 28, 34, 35, 148).

Sub-cutaneous (s.c.) inoculation into the footpad of a mouse is used in the lab to mimic the bite of an infected mosquito. Following s.c. injection, non-propagating, GFP-expressing VRP (GFP-VRP) were used to identify the initial targets of VEEV infection *in vivo*. By immune-fluorescence, the first cells to be infected by VEEV, as determined by GFP expression, stained positive for both DEC-205 and MHC class II, leading Macdonald et al. to conclude that they were Langerhans cells (148). Langerhans cells are a skin-resident dendritic cell population that is thought to play a key role in surveilling the epidermis for microbial insult (13, 87, 162). This result is complicated somewhat by the absence of detectable CD11c, a universal dendritic

cell marker, on these cells (61). Given recent studies defining at least three distinct skin-resident dendritic cell populations, further work is needed to more rigorously define the initial targets of VEEV infection in the skin (45, 87). Regardless, the MacDonald et al. study clearly showed that GFP-positive cells with a dendritic morphology rapidly traffic to the draining popliteal lymph node following infection, with infected cells appearing in the draining lymph node by 30 minutes post-infection (148). This observation is in line with multiple studies describing the rapid migration of Langerhans and other tissue resident dendritic cell populations to draining lymph nodes upon encounter of microbial stimulus (85-87, 162).

Following migration of infected skin-resident cells to the draining lymph node, subsequent rounds of viral replication rapidly lead to extremely high viral titers (up to 10^7 pfu/g) being detectable by 6 hours post-infection (4, 70, 148). The pool of lymph node-resident cells that are susceptible to VEEV infection remains poorly defined, though a fraction have been shown to be DEC-205$^+$, suggesting that they are dendritic cells (148). Efficient replication within the draining lymph node then feeds into a serum viremia that is detectable by 12 hours post-infection (4, 70).

The development of a sufficiently high concentration ($\geq 10^4$ pfu/mL) of infectious virus within the serum is required for viral invasion of the central nervous system (CNS) (K. Bernard, unpublished results). The concentration-dependence of this process supports the hypothesis that neuroinvasion occurs when the diffusion of infectious virus through the permeable tight junctions of fenestrated capillary endothelial cells

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facilitates infection of nearby cells of the peripheral nervous system (28). Following infection of peripheral nerves, VEEV is then thought to disseminate to the CNS via centripetal spread (28). The anatomical site where this process is thought to occur most efficiently is the olfactory neuro-epithelium, a unique mucosal surface that is densely innervated with the dendritic cilia of olfactory sensory neurons (28, 262). Using *in situ* hybridization, Charles et al. were able to demonstrate that olfactory sensory neurons within the neuro-epithelium were susceptible to VEEV infection (28). The axons of these cells directly synapse with CNS neurons within the olfactory bulb, thus providing a potential route for the virus to circumvent the blood brain barrier and gain entry to the CNS (262). Chemical ablation of this tissue delayed entry of the virus into the CNS, but did not prevent it, indicating that CNS entry via olfactory nerves is merely the most efficient of multiple possible entry routes (28). One of the implications of this particular mechanism is that the efficiency of neuroinvasion is a function of the ability of the host to control the amount of infectious virus present in the serum in the first 24 to 48 hours following infection.

Once VEEV has crossed from the serum into the CNS, it replicates predominately within neurons (28, 70, 217). Consistent with neuroinvasion occurring via olfactory sensory neurons, virus is first detectable in the CNS by *in situ* hybridization within the glomerular layer of the olfactory bulb within 30 hours of peripheral infection (28). The glomerular layer of the olfactory bulb is where the axons of olfactory sensory neurons connect with post-synaptic cells of the CNS and thus would serve as the

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entry point for virus entering the CNS via the olfactory neuro-epithelium (195). Subsequent spread throughout the olfactory bulb is followed by gradual dissemination to other areas of the brain connected through the olfactory network, including the pyriform cortex, entorhinal cortex, hypothalamus, amgdala, hippocampus, supraoptic nucleus, cerebellum, and brain stem, by day 6 post-infection (28, 70).

The neuropathology observed in mice infected with VEEV results from a combination of virus-induced cell death in infected neurons as well as the detrimental effects of the host immune response (27). Neuropathology following infection with other encephalitic alphaviruses such as Sindbis virus and Semliki Forest virus is predominately driven by an immunopathic host response to the virus (48, 117, 135). In SCID mice, however, which lack functional B and T cells, VEEV infection results in extensive neuropathology, characterized by progressive vacuolation of the neuropil and spongiosis, which eventually results in the death of the animal despite the absence of inflammation (27, 149). This suggests that VEEV replication alone is sufficient to induce a lethal amount of neuronal pathology. A potential mechanism for this effect is provided by the observation that, unlike many strains of Sindbis virus or Semliki Forest virus, VEEV infection of CNS neurons in adult mice can trigger apoptotic cell death (27, 99, 226). One caveat with studies carried out in SCID mice is that microglial and other innate immune cell populations are intact and might be contributing to the observed pathology.

In immunocompetent mice, VEEV infection of the CNS results in the development of neuropathology that is distinct from that observed in SCID mice (27). By 6 days post-infection, large numbers of mono- and polynuclear cells are seen in perivascular regions as well as within the parenchyma (27). Meningitis and scattered micro-hemorrhaging are also characteristic. In studies by Charles et al., SCID mice infected with VEEV showed an average survival time (AST) of 8.9 ± 0.9 days compared with immunocompetent CB17 mice which survived an average of 6.8 ± 1.2 days, demonstrating that while the direct pathology induced by viral replication within the CNS is sufficient to induce mortality, the effects of the host inflammatory response exacerbate this process.

IMMUNITY TO VEEV

Efforts to identify the host immune mechanisms required for the successful development of immunity against VEEV have been hampered by the extreme lethality of the virus in small animal models. Our current understanding of VEEV immunity comes primarily from *in vitro* studies, mouse studies using vaccination or transfer of immune serum or cells, and extrapolation from SIN experiments. Correlates of protection in humans remain poorly understood, and no licensed vaccine exists for human usage.

The importance of the innate immune system in controlling VEEV infection has been clearly demonstrated by studies examining the interaction of the virus with the type I IFN system in mice. Following infection of a mouse with VEEV, large amounts of

IFN-α/β are detectable in the serum within six hours, though the mechanism of this induction remains unknown (27, 215, 260). In mice lacking the IFN-α/β receptor, the AST of VEEV-infected mice is shortened significantly in the absence of an intact type I IFN system, to 30 hours versus 7.7 days in control mice in one study (260). The dramatically reduced survival time of these animals appears to result from an inability to control viral replication and dissemination in the periphery, resulting in rapid (<14 hours) entry of the virus into the CNS (260). Additionally, treatment of VEEV-infected mice with pegylated IFN-α was shown to prevent mortality and facilitate viral clearance, demonstrating the *in vivo* IFN sensitivity of VEEV (147).

Antiviral antibodies can act to limit viral infection by multiple mechanisms. Antibody binding to virus particles can prevent them from binding or entering target cells either through direct steric interference with cellular receptors or indirectly by preventing conformational changes within the viral envelop proteins required for infectivity (77). Non-neutralizing antibodies can also limit viral spread by facilitating clearance of virus from the blood by Fc- or complement receptor bearing cells in the marginal/sinusoidal zones of secondary lymphoid organs (77, 184). Finally, virus-specific antibody can directly bind and trigger the destruction of infected cells (214). In the case of infection with a neuronotropic RNA virus, the production of anti-viral antibodies, particularly neutralizing antibodies, is thought to be the key determinant of immunity (71).

The advent of hybridoma technology facilitated the identification of antigenic sites within the E1 and E2 glycoproteins of VEEV (208). Analysis of a panel of neutralizing and non-neutralizing monoclonal antibodies (MAb) using a combination of competitive-binding and neutralization-escape assays allowed the identification of the major neutralization domains in E1 and E2 (208, 209). For E2, the key neutralization domain was reported as lying between residues 182 and 207, based on the location of *in vitro*-generated neutralization escape mutations. Neutralizing anti-E1 antibodies appear to also act by obscuring this domain, as an anti-E1 neutralizing MAb competed with neutralizing anti-E2 MAbs for binding (208). This finding mirrors work with SIN that showed binding competition between anti-E1 and anti-E2 neutralizing MAbs (231).

In vitro neutralizing activity has been shown to be highly predictive of *in vivo* protective effect in mice infected with VEEV, as well as a variety of other viruses (77, 209). In fact, two different neutralizing anti-E2 MAbs were shown to protect mice against death when administered up to 24 hours after infection with aerosolized VEEV, an extremely rigorous challenge (197). Non-neutralizing MAbs have been demonstrated to be protective *in vivo* as well. An MAb against epitope b of VEEV E1 exhibited no neutralizing activity *in vitro*, and yet was highly protective against extremely high doses of multiple strains of VEEV in three week old mice when given prior to infection (154). This result, as well as similar results obtained with non-neutralizing anti-SIN MAbs, underlines the fact that anti-viral antibodies can limit viral

infection *in vivo* by numerous mechanisms that are not assessed by the standard *in vitro* neutralization assay.

Studies by Levine and Griffin using SIN suggested an additional mechanism by which anti-viral antibodies may mediate protection during encephalitic alphavirus infection (135). They showed that neutralizing anti-E2 MAbs could non-cytolytically clear infectious virus from the CNS of SCID mice persistently infected with SIN. Recapitulating this effect *in vitro* using rat dorsal root ganglion cultures, they showed that MAb treatment inhibited viral protein production, though the molecular mechanism involved remains unknown. Though intriguing, the strain of SIN used was avirulent and it remains to be seen if anti-viral antibodies can exert a similar effect on neurons infected with a more pathogenic virus such as VEEV.

$\alpha\beta$ T cells are thought to play a minimal role in mediating protection or recovery from encephalitic alphavirus infection, compared with antibody (71, 189). In the case of SIN, mice deficient in $\alpha\beta$ T cells had lower mortality rates, compared with control mice, indicating that $\alpha\beta$ T cells were playing no significant role in mediating protection (212). For VEEV, the literature is contradictory. In a study by Jones, L.D. et al., no anti-VEEV CTL activity was detectable in the spleen or draining lymph node of mice infected with two different live VEEV vaccines, TC-83 and CAAR 508, suggesting that significant CTL activity was not induced during VEEV infection and was likely unimportant in mediating recovery (107). In contrast, Paessler et al. demonstrated that $\alpha\beta$ T cells were required for protection against lethal VEEV

challenge following vaccination with a live SIN/VEEV chimeric vaccine (190).

Additionally, the same group showed that $CD4^+$, but not $CD8^+$ T cells isolated from

mice vaccinated with the same SIN/VEEV chimera could protect $\alpha\beta$ T cell-deficient

mice from an otherwise lethal VEEV challenge (263). A key difference between

these two studies is that the conclusions of Jones, L.D. et al are based on the

absence of data (no detectable CTL activity), while those of Paessler et al. are

based on a positive phenotype (protection), thus making them more convincing.

ANTI-VEEV VACCINES

Despite decades of effort, there is currently no anti-VEEV vaccine licensed for use in

humans. Early attempts to vaccinate equids during the middle of the 20th century

using formalin-inactivated tissue from VEEV-infected animals resulted in short-lived

immunity, and may potentially have served as the causative agent of some

outbreaks due to live-virus contamination of vaccine stocks (256). In order to

produce a more immunogenic live attenuated anti-VEEV vaccine, the virulent

Trinidad donkey strain (TD) was serially passaged 83 times through guinea pig heart

cells (10). The resulting vaccine strain, TC-83, has since been shown to be safe and

highly immunogenic in equids and is currently in use in Central and South America

(251, 256). In humans, TC-83 has only been approved for at-risk laboratory

personnel under the supervision of the U.S. Army Special Immunizations Program

due to a high rate of adverse side effects and a relatively low seroconversion rate

(256). In an effort to produce a safer, more immunogenic vaccine for use in

humans, several attenuating mutations were engineered into a cDNA clone of the

TD strain. The vaccine produced by this method, V3526, is safe and immunogenic in mice and non-human primates (50, 81, 200, 204). Unfortunately, human clinical trials with V3526 revealed an unacceptably high rate of adverse side effects (152). Thus, the development of a safe VEEV vaccine candidate for use in humans remains an active area of research.

USE OF VEEV REVERSE GENETICS

The study of VEEV-host interaction has been greatly aided by the development of an easy to use reverse genetics system. The system was originally developed by Davis et al. by generating a full-length cDNA from viral genomic RNA isolated from the TD strain of VEEV (37). This cDNA was cloned into a plasmid under the control of the T7 promoter, for easy *in vitro* synthesis of full-length, infectious viral genomic RNA. *In vitro*-transcribed RNA can then be introduced into permissive cells, allowing the production of virus stocks. The plasmid encoding the wild-type TD sequence was named pV3000, and the virus generated from pV3000-derived RNA, which should be genetically identical to the original TD virus, was called V3000 to indicate that it is clone-derived. With the viral cDNA fixed into a stable clone, it is possible to introduce desired mutations into the viral sequence using standard molecular biology approaches, and reliably produce viral stocks that incorporate the desired genomic sequence. In effect, one can use the system to mutate any site in the viral genome, and then precisely track the effect of that mutation on any and all aspects of virus biology.

This system was used in a series of studies to dissect the early stages of VEEV infection of the mouse. These studies began with work by Johnston and Smith that took TD as a starting point, and then selected viral mutants that could rapidly penetrate baby hamster kidney (BHK) cells (106). Those mutants that were able to rapidly penetrate BHK cells were then demonstrated to be avirulent in adult mice, compared with the parental TD virus as well as other slowly penetrating mutants. The attenuated mutants were sequenced, and the sequence changes, all within E2 or E1, were then cloned either singly or in combinations into the pV3000 infectious clone, to yield a panel of genetically defined, attenuated VEEV mutants (36). When introduced into mice by s.c. injection, these mutants exhibited defects at various early stages of the infection (4, 11, 34, 70, 148). By directly linking changes in the viral glycoprotein sequence to specific defects in viral spread and pathogenicity, these mutants provided excellent tools for defining mechanisms of *in vivo* attenuation.

Characterization of several of these attenuated glycoprotein mutants revealed the importance of heparan sulfate binding affinity as a determinant of *in vivo* attenuation (4, 11). Four of the attenuating mutations were found to be substitutions of positively charged lysine for negatively charged glutamic acid (E->K) at positions 3, 4, 76, and 209 in the E2 protein, each of which resulted in a higher affinity for negatively charged heparan sulfate (11). The E76K and E209K mutations resulted in viruses that were unable to efficiently spread from the inoculation site or early lymphoid sites of replication, respectively, resulting in an absence of detectable serum viremia or

neuroinvasion (70). Heparan sulfate and other related glycosaminoglycans are ubiquitous on the surfaces of every cell type as well as the extracellular matrix, and it has been hypothesized that increased affinity for heparan sulfate might limit the ability of a virus to disseminate *in vivo* (17, 121, 216). Additionally, Bernard et al. showed that viruses with these mutations were more efficiently cleared from the blood following intravenous injection (11). Despite showing profound defects early in the infection process and inducing little to no morbidity in mice, the heparan sulfate-binding mutants replicated to a sufficient degree *in vivo* to generate protective immunity against subsequent lethal VEEV challenge (36). This suggests that mutations that confer high heparan sulfate affinity should be considered as part of an attenuation strategy when engineering future live attenuated vaccines.

Attenuated VEEV mutants have also been useful in exploring the roles of viral variation and selection in pathogenesis. One example involves the mutant, V3010, which is identical to wild-type V3000 except for the E76K mutation in E2 that conveys heparan sulfate binding. When introduced into the footpad of a mouse, V3010 is unable to spread efficiently from the site of inoculation or the draining lymph node, ostensibly due to its affinity for heparan sulfate (4, 70). In effect, the ubiquity of heparan sulfate was acting as a bottleneck that prevented further dissemination and infection. Virus was, however, sporadically detected outside of the draining lymph node, and when these viruses were sequenced, they were found to contain reversion mutations (4). Many of these were single-site revertants, containing a K76E mutation that resulted in wild-type V3000 sequence, but a

proportion of them were second-site revertants that maintained the lysine at position 76 but gained an additional K116E mutation. These revertant viruses were detectable by 24 hours post-infection, suggesting that VEEV was able to generate sufficient genetic variability within the first 24 hours of infection to overcome a potent host bottleneck.

An infectious clone containing both the E76K and K116E mutations in the TD background was generated, and the resultant virus, V3533, was shown to have low affinity for heparan sulfate, similar to the wild-type V3000 virus (11). Having lost the ability to bind heparan sulfate, V3533 was no longer subject to the early bottleneck that inhibited infection with the attenuated, heparan sulfate-binding mutants. This was clearly demonstrated when mice were infected in the footpad with V3533. V3533 spread from the inoculation site to the draining lymph node to other secondary lymphoid organs with similar kinetics to wild-type V3000 (4). In addition, V3533 infection resulted in the development of a serum viremia and viral invasion of the CNS, though to lower titers than V3000. Unlike V3000, however, V3533 was cleared from the CNS in most infected animals, resulting in a significantly lower mortality rate (4). Because of these unique properties, V3533 has been used in the studies described here as a model for successful recovery from VEEV-induced encephalomyelitis.

COMPLEMENT SYSTEM OVERVIEW

The host complement system is an ancient network of over thirty soluble and cell-associated proteins that can be activated by conserved molecular patterns of infection or injury to restrict microbial infection through multiple distinct mechanisms. These mechanisms range from opsonization and/or direct killing of pathogens to recruitment and activation of downstream cellular responses (21, 111, 210, 265). Predating the evolutionary split between vertebrates and invertebrates, and thus the development of the adaptive immune system, the complement system is considered part of the innate immune system (56). However, during the evolution of the adaptive immune system, the complement system was co-opted to mediate regulatory cross-talk between the innate and adaptive systems (21). Due to its wide range of effector and regulatory capabilities, the complement system appears to play a role in influencing every stage of the host response to viral infection. Depending on the virus, the effects of complement activation can be protective or pathogenic.

COMPLEMENT ACTIVATION PATHWAYS

There are three major pathways that mediate complement activation: the classical pathway, the lectin pathway, and the alternative pathway. Each of these pathways terminates in the formation of a C3-convertase complex that can catalyze the cleavage of C3. The classical pathway is typically initiated by the binding of soluble C1q to complement-fixing antibody-antigen complexes, though binding of C1q by the C-type lectin SIGN-R1 can also activate the classical pathway in an antibody-independent manner (109). Each C1q molecule has six globular head domains that can bind the Fc portion of complement-fixing IgG and IgM. The affinity of any single

globular head for an Fc portion is relatively low, so it takes the aggregation of

several Fc portions within an immune complex to stably bind C1q (120). Stable C1q

binding then activates two serine proteases, C1r and C1s, that in turn cleave C4 and

C2 to yield the C4b-C2a C3 convertase (167). The lectin pathway is activated by

soluble collectin proteins, primarily mannose-binding lectin (MBL) and members of

the ficolin family (213). These proteins act as pattern recognition receptors that bind

specific sugar groups displayed on the surface of invading pathogens or apoptotic

cells (53, 213). This binding results in the recruitment and activation of the MBL-

associated serine proteases MASP-1 and MASP-2, which, similar to C1r and C1s,

then cleave C4 and C2 to generate the C4b-C2a C3 convertase (238). Finally,

activation through the alternative pathway can occur independently of any

recognition event. Spontaneous hydrolysis of C3 produces the cleavage product

C3b, which can indiscriminately bind to plasma membranes. Bound C3b then

recruits factor B, and the resultant C3bB is cleaved by the soluble serine protease

factor D to produce the C3bBb C3 convertase. This cascade also provides an

amplification loop for C3 cleavage following activation through the other pathways.

While the alternative activation cascade is capable of occuring in the absence of any

specific recognition event, there is growing evidence that recognition of invading

microorganisms or apoptotic cells by properdin can significantly enhance the

efficiency of the pathway (112).

COMPLEMENT CASCADE

All of the activation pathways converge at the cleavage of C3 into C3a and C3b.

C3b contains a highly reactive thiolester that binds with nearby surfaces, resulting in

rapid opsonization. This opsonization process can occur extremely rapidly, as up to

10^7 C3b molecules can be deposited within 5 minutes under ideal conditions (186).

Further cleavage of C3b by factor I produces iC3b, C3c, C3d, and C3g (111). Newly

produced C3b can bind to either of the C3 convertases, C3bBb or C4bC2a, and

convert them into C5 convertases, C3bBbC3b and C4bC2aC3b, that can efficiently

cleave C5 into C5a and C5b. C5b production initiates the terminal complement

cascade, in which C5, C6, C7, C8, and C9 are recruited to form the membrane

attack complex (MAC) (111).

EFFECTOR MECHANISMS

One of the best studied anti-viral effects of the complement system is its ability to

enhance the ability of antibodies to reduce viral infectivity. This effect has been

demonstrated with a variety of RNA and DNA viruses, though the exact mechanisms

remain poorly understood (33, 142, 163, 233). Suggested mechanisms include

MAC-mediated lysis of viral particles, C1q-mediated enhancement of antibody

avidity, and steric hindrance of entry resulting from opsonization. In the case of

parainfluenza virus or HIV, complement can reduce infectivity of antibody-bound

virus through direct virolysis, probably due to MAC formation (225, 245). For

vaccinia virus, West Nile virus, and influenza A virus, the neutralization-

enhancement effect of complement was mediated by the coating or opsonization of

viral particles, rather than virolysis (9, 49, 160). In the case of West Nile virus, the

presence of C1q was shown to reduce the number of bound antibody molecules required to neutralize a virion particle, potentially through a steric hindrance mechanism (160). Complement-mediated enhancement of neutralizing antibody activity may play a key role in controlling infection by limiting viral dissemination and spread from early sites of replication (169, 184).

The complement system also acts to limit viral dissemination through antibody-independent mechanisms. At least seven distinct complement receptors (CR1, CR2, CR3, CR4, C1qR, CRIg, and SIGN-R1) have been identified as binding and facilitating clearance of complement component-bound pathogens from the blood (64, 90, 94, 109, 111, 122). These receptors are expressed by macrophage and dendritic cell populations at sites of blood and lymph filtration, such as the liver, splenic marginal zone, and the subcapsular sinus of lymph nodes. Collectively, these receptors can bind and trigger the phagocytosis of viruses or cell debris opsonized by MBL, C1q, C3b, iC3b, or C4b. In addition to clearing virus from circulation, complement receptor-mediated uptake can also result in efficient presentation of viral antigen to B cells. This effect has been shown to be required for maximal antibody induction during infection with both VSV and influenza A virus (68, 108).

The complement cascade produces several cleavage products that act to recruit innate immune cells to sites of complement activation. The cleavage of C3, C4, and C5 during the complement cascade results in the production of the anaphylatoxins

C3a, C4a, and C5a. These protein fragments act through the receptors C3aR and C5aR to recruit inflammatory cells such as neutrophils, macrophages, and dendritic cells to sites of complement activation (32, 74, 157, 181, 250). Signalling through C3aR and C5aR, as well as other complement receptors such as CR3, can also enhance the activation and effector function of NK cells, macrophages, neutrophils, and eosinophils (138, 165, 181, 211). The roles of these receptors in the context of viral infection have been poorly studied, though that is beginning to change. Morrison et al. recently showed that the majority of the pathology observed following Ross River virus infection was dependent upon CR3 expression and its role in regulating the activation of tissue-infiltrating inflammatory cells (168).

In addition to its role during the innate response to viral infection, host complement is an important regulator of the T cell response, both through effects on antigen presenting cells (APC) as well as directly on T cells (111). Maximal T cell responses to West Nile virus, influenza A virus, and lymphocytic choriomeningitis virus (LCMV) have all been shown to be dependent upon complement activation (125, 159). Release of C3a and C5a can induce the migration of APC populations, such as dendritic cells, to sites of infection (224, 259). APCs express a range of complement receptors and can thus respond to the presence of MBL, C1q, C3b, and C4b bound to antigen at the site of infection (145, 166). In fact, C1q and C3 were shown to be essential for optimal antigen uptake and maturation by dendritic cells (24, 101). Additionally, signaling through C3aR, C5aR, and/or CD46 (recognizes C3b and C4b) can either positively or negatively modulate IL-12 production by APCs, depending on

the model and specific cell population, thus affecting the Th1-Th2 skew of the downstream T cell response (42, 84, 110).

As is the case with different APC populations, C5a can attract T cells to sites of complement activation (177). While signaling through the primary complement receptors, CR1, CR2, CR3, and CR4, does not appear to play a significant role in directly regulating mouse T cells, signaling through the complement regulatory receptors CD46, CD55, and CD59 during priming can modulate downstream proliferation and effector phenotype (5, 88, 113, 146). CD59 signaling in particular was shown to significantly blunt the CD4$^+$ T cell response to vaccinia virus (146).

Finally, the complement system has been demonstrated to promote the induction of anti-viral antibody responses through several mechanisms (21). B cells express both CR1 and CR2, and signaling through these receptors following uptake of antigen coated with C3b, iC3b, C3d, or C4b synergizes with B cell receptor signaling, thus lowering the threshold for B cell activation (22). CR1/CR2 expression by follicular dendritic cells is thought to enhance antibody induction through retention of complement-coated antigen within the B cell follicle (8). During West Nile virus infection, CR1/CR2 signaling is absolutely required for the induction of a protective antibody response, though it remains unclear what CR1/CR2-dependent mechanisms are important (161). CR1/CR2 expression, along with myeloid cell-derived C3, was also required for the humoral response to herpes simplex virus (HSV) infection (31, 247). In contrast, during influenza A virus infection, CR1/CR2

signaling was dispensable for virus-specific IgG production, though complement-dependent priming of CD4 T cells was required, demonstrating the potential importance of indirect effects of complement on B cell function (125). Additionally, the capture of viral particles by SIGN-R1 expressed by dendritic cells within the lymph node medulla was also recently shown to be important for the humoral response to influenza A virus, though it remains to be seen whether this effect is dependent upon complement activation (68).

OVERVIEW OF CNS IMMUNITY

The CNS has long been considered an "immune-privileged" site based on the scarcity of immune cells within the brain and spinal cord parenchyma, as well as the exclusion of circulating immune cells and soluble mediators by the blood brain barrier (BBB). Studies demonstrating the rapidity of the response to infection within the brain, as well as systemic inflammatory signals, have made clear that the CNS is not immunologically inert (25, 78, 129, 202). Instead, the CNS is uniquely regulated in order to facilitate control of infection while maintaining the functional integrity of a delicate and vital tissue (71, 206, 227).

RESTING STATE

There are four major cell types present within the CNS parenchyma, though further functional subdivision is probable. Neurons are the essential cells responsible for transmitting and processing electrical signals within the CNS. For the most part, these cells are terminally differentiated and not easily replaced, thus the protection

of individual cells from infection-associated damage is important for maintaining host viability (143). Neuronal viability is sustained by three major supporting cell populations: astrocytes, oligodendrocytes, and microglia. Astrocytes help maintain neuronal health by producing various neuroprotective factors and metabolizing excess neurotransmitters and toxins. They also play a role in regulating the integrity of the BBB (237). Oligodendrocytes are responsible for producing the myelin sheathing that insulates neuronal axons and enhances electrical signal conduction. Microglia are macrophage-lineage cells that serve as the primary immune cells of the CNS (78). In the resting state, these cells remain mostly stationary, constantly scanning the surrounding environment with long, highly mobile processes (180). It is through this scanning process that microglia are able to rapidly detect and initiate the response to infection or other distress.

The generally immune-suppressive environment within the CNS is maintained through several mechanisms. Within the parenchyma, microglia are normally maintained in a resting state by constitutive interaction with healthy neurons via the ligand-receptor pairs CD200-CD200R, CX_3CL1-CX_3CR1, and SIRPα-CD47 (78, 93). Expression of major histocompatibility complex (MHC) molecules is low to undetectable, especially on neurons, in the absence of inflammation (117, 240). In addition, several other factors, such as TGF-β and various gangliosides and neurotrophins, are constitutively produced by astrocytes and neurons and help suppress inflammation (46, 71). Glucocorticoids released from the adrenal cortex can antagonize IRF-3-, TBK1-, and NF-κB-dependent pro-inflammatory signaling

through multiple mechanisms (175, 182, 185). Under normal conditions, most immune cells are excluded from entering the CNS from circulation by a combination of BBB-mediated exclusion and the low level of adhesion molecule expression by CNS endothelial cells (7, 91). The BBB mainly consists of specialized tight junctions between CNS endothelial cells, supported and regulated by the end-feet structures of perivascular astrocytes (7). These specialized tight junctions normally prevent the entry of cells, cytokines, antibodies, viruses, and other large hydrophilic bodies from the blood. This exclusion is not absolute, as activated T cells can cross the intact BBB during normal surveillance, partially though the expression of P-selectin by the CNS endothelium (20, 92). The impermeability of the BBB is not uniform, however, as permeability is much higher in certain regions of the brain involved in regulating the autonomic nervous and endocrine systems, specifically the circumventricular organs and choroid plexus (7). The enhanced permeability in these regions allows CNS-resident cells to detect and respond to some systemic inflammatory signals, and suggests that the immuno-regulatory environment may differ between different regions of the CNS (129).

IMMUNE INDUCTION

Despite the immuno-suppressive environment, CNS-resident cells are capable of quickly responding to viral infection. A variety of pattern recognition receptors (PRR) are expressed with the CNS, including those that recognize signs of viral replication, such as toll-like receptors (TLR) and RIG-I-like receptors (RLR) (206, 236). Viral infection causes neurons to rapidly produce IFN-β through both TLR3- and RLR-

dependent pathways (201, 236). The CNS is unique in that IFN-β, rather than IFN-α, is the predominant type I IFN produced in response to infection (71). This may be due to the fact that IFN-β appears to have neuro-protective effects *in vivo*, while IFN-α is highly toxic to neurons (2, 14, 156). Microglia can be activated in response to viral infection either by direct sensing of virus associated PAMPs or complement activation, loss of CD200R or CX$_3$CR1 signaling due to neuronal distress, or by cytokines produced by other activated microglia or astrocytes (26, 93, 176, 187). Activated microglia upregulate surface expression of MHC class I and II, and can express a number of pro-inflammatory cytokines, depending on the specific virus infection, including IL-1, IL-6, IL-12, TNFα, MIP1β, MCP1, MCP3, RANTES, and IP10 (71, 117, 199). The production of pro-inflammatory cytokines by CNS resident cells can rapidly override many of the immune-suppressive factors that protect the CNS. Inflammation within the CNS can induce an increase in permeability of the BBB, as well as increased expression of adhesion molecules such as ICAM1 and VCAM1 on the CNS endothelium, facilitating entry of immune cells from circulation (3, 196, 253). Exposure to type I and type II IFN induces neurons to express MHC class I (179).

REGULATION OF CNS IMMUNE RESPONSES

Another attribute of the CNS that has contributed to its description as immunologically inert is the lack of lymphatic drainage from the CNS parenchyma (228). This is most likely of little consequence, as all viral infections are initiated in the periphery prior to neuroinvasion, and thus the adaptive response would be

primarily initiated in the lymph nodes draining those early sites of replication. Once

the BBB has been opened by CNS inflammation, circulating innate effector cells, as

well as adaptive lymphocytes primed in the periphery, are free to enter the CNS

parenchyma and traffic to sites of infection and inflammation. Usually, the first

inflammatory cells to enter the CNS from the periphery are myeloid-lineage,

inflammatory monocytes, characterized as CD11b$^+$ Ly6c$^+$ MHC class II$^+$, typically

within a few days of infection (63, 71, 116). The recruitment of these cells is

dependent upon MCP1-CCR2 signaling, with possible additional roles for CCR5,

TNFα, and IL-23 (65, 126, 220, 239). The cells within this population most likely

exhibit some phenotypic variation, and have been described as playing either

pathogenic or protective roles depending on the specific virus infection (63, 116,

239). Within the CNS, these cells likely function as they do elsewhere in the body,

secreting pro-inflammatory cytokines, clearing cellular debris, and regulating the

activity of other infiltrating cell populations.

B and T cells appear later, following priming in the periphery. Not much is known

about CNS-specific regulation of B cells during viral infection. In the case of mouse

hepatitis virus (MHV) infection, CNS-infiltrating B cells showed a delay in the onset

of antibody secretion, compared with peripheral B cells, suggesting some CNS-

specific regulation (241). Virus-specific, antibody-secreting B cells are retained

within the CNS parenchyma long after infectious virus has been cleared (241, 242).

CNS-specific regulation of the T cell response has been more thoroughly examined.

The abundance of gangliosides within the CNS can inhibit T cell responses at the

level of antigen presentation, by inhibiting MHC class I and II expression by astrocytes (153). More importantly, exposure to gangliosides inhibits NF-κB activation and Rb phosphorylation within T cells, resulting in an inability to produce IL-2 and limited ability to survive and proliferate (97). This effect was demonstrated in the context of SIN infection where T cells isolated from the brain, but not the spleen, were incapable of producing IL-2, an effect recapitulated *in vitro* using brain-derived lipids (98). Microglia may also play a role in restricting T cell IL-2 production and proliferation, as T cells primed *ex vivo* by primary microglia, but not other APC populations, exhibit defects in IL-2 production, proliferation, and survival (52). In addition to the effects of gangliosides on T cell survival and proliferation, neurons constitutively express FasL, which can trigger apoptosis in Fas-expressing activated T cells (51, 158). Given the potential destructive effects of activated T cells, it is likely that additional mechanisms of control remain to be described.

CLEARANCE FROM NEURONS

Infection of CNS neurons with a cytopathic virus presents a unique problem to the host. These cells are essential for host function, and yet are for the most part irreplaceable (143). As a result, the immune response must curtail viral replication and spread without destroying the infected cells in the process. This requirement should preclude the widespread use of standard cytolytic methods of viral clearance, such as CTL-mediated cell killing. As a consequence of this limitation, "clearance" in many cases may result from a host-mediated shutdown of viral replication within infected cells, rather than a total eradication of all viral protein and nucleic acid.

Support for this possibility comes from numerous studies in which viral RNA or antigen was found to persist for long periods of time within the CNS following apparent clearance of infectious virus (40, 83, 151, 242).

Host strategies for mediating neuronal clearance differ somewhat between different viruses, and are probably partially dependent upon the specific biology of the virus in question (71). As described earlier, studies using SIN have suggested an important role for virus-specific antibody in mediating non-cytolytic clearance of virus from neurons (135). A similar effect has been described during rabies virus infection (95). For many neuronotropic viruses, including Borna disease virus, Theiler's murine encephalomyelitis virus, SIN, MHV, and measles virus, clearance appears to be primarily mediated by the effects of T cell-delivered IFNγ (12, 82, 192, 193, 207). In the case of vesicular stomatitis virus, inducible nitric oxide might also play a role (124). In these models, IFNγ appears to block viral replication while maintaining the viability of the infected neurons, though the exact molecular mechanisms by which IFNγ signaling exerts these effects remain unclear. Interestingly, different CNS neuron populations vary in their sensitivity to the anti-viral effects of IFNγ. Following SIN infection, spinal cord motor neurons, but not cortical neurons in the brain, were susceptible to IFNγ-mediated clearance (12).

Recent studies with SIN have suggested a role for autophagy in neuronal clearance (188). Autophagy is a cellular process in which intracellular material is packaged within double-membrane enveloped structures that are then targeted to the autolysosome for

destruction (137). This pathway has been shown to be critically involved in cellular survival, development, carcinogenesis, and host defense (39, 137). Orvedahl et al. showed that mice in which the autophagy pathway has been inactivated by an induced deletion in the essential *Atg5* gene are defective in their ability to clear SIN from neurons, resulting in a higher mortality rate (188). They went on to show that the SIN capsid protein interacted with the cellular protein p62, an adaptor protein that targets cytosolic proteins for autophagic degradation. IFNγ signaling has previously been demonstrated to induce autophagy in macrophages (80, 96). It will be interesting to see if the same is true in neurons, possibly providing a specific mechanism for the antiviral effects of IFNγ signaling in neurons.

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CHAPTER 2:

EARLY INTERACTIONS WITH THE HOST COMPLEMENT SYSTEM DETERMINE

DOWNSTREAM DISEASE OUTCOME FOLLOWING VENEZUELAN EQUINE

ENCEPHALITIS VIRUS INFECTION

ABSTRACT

Venezuelan equine encephalitis virus (VEEV) is a mosquito-borne RNA virus of the genus *Alphavirus* that is responsible for a significant disease burden in Central and South America through sporadic outbreaks in human and equid populations. In mice, the virus initiates a biphasic disease course in which initial replication within lymphoid tissue seeds a serum viremia that in turn facilitates virus invasion of the CNS. Replication within CNS neurons leads to a paralyzing, consistently lethal encephalomyelitis due to the combined detrimental effects of virus-mediated cytopathology and the host inflammatory response within the brain. The host complement system is capable of playing both protective and pathogenic roles during viral infection. In order to ascertain the role that complement plays in resolving VEEV-induced encephalomyelitis, we infected complement deficient $C3^{-/-}$ mice with a VEEV mutant (V3533) that causes mild, transient disease in immune-competent mice. In the absence of a functional complement system, peripheral inoculation with V3533 induces a much more severe encephalomyelitis, characterized by substantial weight loss, ataxia, and hind limb paresis. This enhanced pathology is associated with a delay in clearance of infectious virus from the serum and more rapid invasion of the CNS in $C3^{-/-}$ mice, despite the presence of an intact anti-VEE antibody response. If V3533 is directly inoculated into the brain, however, disease outcome in wild-type and $C3^{-/-}$ mice is identical. These results indicate that early interactions of VEEV with the host complement system in the periphery can have dramatic effects on downstream disease outcome.

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INTRODUCTION

Venezuelan equine encephalitis virus (VEEV) is a mosquito-borne alphavirus that is endemic to Central and South America (18). Though normally maintained in an enzootic transmission cycle between various rodent host species and the *Culex* mosquito vector, VEEV periodically emerges from its natural cycle to cause local epidemics in human and equid populations (57). The most recent major outbreak occurred in 1995 in Columbia and Venezuela, where 75,000 to 100,000 human cases were reported (59). VEEV infection in humans causes a spectrum of disease that ranges from asymptomatic to mild, flu-like illness to overt encephalomyelitis, with an overall case mortality rate of about 0.5-1% (58). In equid populations, the development of overt encephalomyelitis is more common, and the overall mortality rate often exceeds 50% (56). Though specific viral sequence determinants associated with epidemic emergence have been identified, outbreaks remain unpredictable (1). As a result, VEEV remains a significant public health threat in the region.

Much of our current understanding of VEEV pathogenesis and immunity comes from studies carried out in a well-characterized mouse model of infection. VEEV infection of the mouse closely replicates many aspects of infection in humans and equids (15). Subcutaneous injection of VEEV into a mouse
is followed by efficient replication in the skin-draining popliteal lymph node and rapid dissemination to other secondary lymphoid organs (2, 35). Replication at these sites leads to the development of a serum viremia within the first 12 hours of infection.

Viral invasion of the CNS first occurs through the olfactory neuro-epithelium, a mucosal surface that is densely innervated with olfactory sensory neurons (9). It is thought that these cells are infected following diffusion of virus through the permeable tight junctions of adjacent capillaries. The virus then disseminates into the olfactory bulb of the brain by centripetal spread, usually within 36 hours of infection (9). Once the virus has crossed into the CNS, it replicates predominately within neurons, triggering the death of the animal from a paralyzing encephalomyelitis within 6 to 8 days post-infection (8, 17). The mortality rate in mice is 100%, and results from a combination of virus-mediated cytolysis of infected neurons and the detrimental effects of the host immune response within the CNS (8, 56).

Engagement of both the innate and adaptive arms of the immune response are required for successful control of VEEV infection. The type I interferon (IFN) system plays a critical role in limiting early viral replication and dissemination (60). In mice with a genetic deficiency in this system, VEEV invades the CNS much earlier, resulting in a dramatically shorter time to death, compared with control mice. Anti-viral antibodies can limit viral dissemination in the periphery, as well as aid in clearance of virus from infected neurons (33, 37). The role of $\alpha\beta$ T cells during VEEV infection is unclear, as they can contribute both to control of VEEV infection within the CNS, as well as VEEV-induced immune-pathology (4, 8, 49). Further efforts to identify the components of a successful immune response to VEEV infection have been limited by the extreme lethality of the virus in mice.

The host complement system is a complex network of over 30 soluble and cell-associated factors that contribute to both innate and adaptive control of microbial infection (6). Activation of the complement system in response to infection or injury can occur through three major pathways, classical, lectin, and alternative, all of which result in the cleavage of the complement factor C3. C3 cleavage products act to limit infection through a number of mechanisms ranging from opsonization and/or direct killing of pathogens to recruitment and regulation of innate and adaptive effector cells (6, 29, 51). Complement plays a critical protective role during infection with a number of viruses, including influenza A virus, West Nile virus, ectromelia virus, and herpes simplex virus (32, 39, 45, 54). In contrast, complement activation enhances virus-induced pathology following infection with the alphaviruses Ross River virus and Sindbis virus (22, 43).

In this study, we used an established model of acute, non-lethal VEEV infection to ascertain the role of the complement system in VEEV pathogenesis and immunity. Sub-cutaneous infection of wild-type C57BL/6 mice with the V3533 strain of VEEV results in the development of a mild febrile illness with minimal signs of CNS complications. In contrast, infection of complement deficient $C3^{-/-}$ mice with V3533 resulted in the development of a severe encephalomyelitis, suggesting that the complement system plays an important role in limiting VEEV-induced pathology. No differences in disease outcome were observed following intracranial infection, however, indicating that complement was acting at a step prior to neuroinvasion.

Further studies revealed delayed serum clearance and earlier neuroinvasion in C3$^{-/-}$ mice that could not be explained by defects in inflammatory cell recruitment or anti-VEEV antibody induction. Together, these results demonstrate that early interactions with the host complement system can have profound effects on downstream disease outcome.

MATERIALS AND METHODS

Viruses

The isolation of the V3533 mutant of VEEV, as well as the generation of the pV3533 molecular clone has been described previously (2). Virus stocks of V3533 were generated by *in vitro* transcription from a linearized plasmid, pV3533, which encodes the full-length V3533 cDNA, using a T7-specific mMessage mMachine *in vitro* transcription kit (Ambion). *In vitro*-generated transcripts were then electroporated into BHK-21 cells using a Bio-Rad electroporator as described previously (2, 12). Culture supernatants were harvested 18 hours after electroporation, clarified by centrifugation at 3000 rpm for 20 min, and stored as single use aliquots at -80° C. Viral titers were determined by standard plaque assay on BHK-21 cells, as previously described (53).

Mouse studies

C3$^{-/-}$ and μMT mice (both on the C57BL/6J background) were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in house under specific pathogen-free conditions. C57BL/6J mice were purchased from The Jackson Laboratory as needed. All experimental manipulation of mice was performed in a biosafety level 3

animal facility following a 7 day acclimatization period. For infections, 6-10 week old

female mice were anesthetized via intra-peritoneal (i.p.) injection with a mixture of

ketamine (50 mg/Kg body weight) and xylazine (15 mg/Kg body weight) and then

inoculated either in the left rear footpad with 10^6 PFU of virus in diluent [phosphate

buffered saline (PBS) + 1% donor calf serum, Ca^{2+}, Mg^{2+}] for sub-cutaneous (s.c.)

infections, or directly into the brain with 10^3 PFU of virus in diluent for intracranial

(i.c.) infections. Mock-infected mice received diluent alone. Weight loss and

disease score were assessed daily in infected animals. The scale used for disease

scoring was as follows: (0) no signs; (1) hunched posture; (2) mild motor

dysfunction, altered gait; (3) moderate motor dysfunction, ataxia; (4) hind limb

paresis; (5) hind limb paralysis; (6) moribund. Mice that lost more than 35% of their

starting weight or became moribund were euthanized according to UNC Institutional

Animal Care and Use Committee guidelines.

Virus titers

To assess VEEV titers *in vivo*, infected mice were sacrificed, bled, and then

perfused through the heart with 10 mL of PBS. Spleen, draining popliteal lymph

node, brain and spinal cord were then removed, weighed, and frozen at -80°C in

diluent. Tissues were thawed, homogenized, and used to infect BHK-21 cells in a

standard plaque assay.

Antibody analysis

VEEV-specific IgG and IgM levels were assessed by standard indirect ELISA.

Purified, intact VEEV particles (2.5 μg/mL) were used to coat 96-well NUNC

Immulon 4HBX plates (Thermo Scientific) overnight at 4˚C. After washing, plates

were incubated with serial dilutions of heat inactivated mouse serum + 10% Sigma Blocking Buffer (Sigma) overnight at 4°C. Plates were washed again, incubated with HRP-conjugated goat anti-mouse IgM or IgG (Southern Biotech) for 2 hours at 4°C, and then developed using o-phenylenediamine dihydrochloride tablets (Sigma) in equal parts 0.1M citric acid and 0.1M sodium citrate. Development proceeded for 30 minutes before the reaction was terminated with 0.1M NaF. The OD_{450} values were measured using a FLUOstar Omega microplate reader with Omega software v1.02 (SPSS, Inc.). Log_{10} half-maximum ELISA titers were calculated using GraphPad Prism software v5.0 (GraphPad) and represent the log of the reciprocal dilution at which the half-maximum absorbance values were achieved.

To assess anti-VEEV neutralizing activity, serum was collected and either left untreated or heat inactivated at 56°C for 1 hour. Serum was then diluted in diluent and coincubated with gfp-expressing VEEV viral replicon particles [gfp-VRP, described in (50)] for 1 hour at 37°C. VRP-serum mixtures were then used to infect BHK-21 cells. 18 hours post-infection, infected cells were harvested by trypsinization, washed, and fixed with 2% paraformaldehyde in PBS and analyzed on a CyAn flow cytometer using Summit 5.2 software (Dako). IC50 titers were calculated using GraphPad Prism software v5.0 (GraphPad) and represent the log of the reciprocal dilution at which 50% inhibition of infectivity was achieved.

Flow Cytometry

Mock- and V3533-infected mice were sacrificed by exsanguination and perfused with PBS. Draining popliteal lymph nodes were harvested, minced, and then

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incubated for 30 minutes with shaking at 37°C in digestion media [RPMI, 1% fetal calf serum, 25mM HEPES, 1 mg/mL Collagenase A (Roche)]. Homogenates were then passed through a 40 μm strainer, washed with media, and the absolute number of live cells in each sample was then determined by trypan blue exclusion. Cells were washed in flow cytometry wash buffer (1 x Hank's balanced salt solution, 1% fetal calf serum, 0.1% sodium azide) and then stained with the following antibodies: anti-CD11c-PE-Texas red (Invitrogen), anti-Ly6G-FITC (clone 1A8), anti-B220-FITC, anti-CD3ε-PE, anti-Gr1-PE, anti-CD49b-APC, anti-MHC class II-APC, anti-CD11b-PE-Cy7 (all eBioscience). Following staining, samples were washed, fixed in 2% paraformaldehyde in PBS and analyzed on a CyAn flow cytometer using Summit 5.2 software (Dako). Absolute numbers of each specific cell type were calculated by determining the number of total live cells within a sample by trypan blue exclusion and then multiplying that number by the percentage of live cells within the sample bearing the appropriate surface staining profile.

IFN Bioassay

Total amounts of biologically active type I IFN were determined by bioassay as described previously (11). Briefly, murine L929 cells were plated in 96-well plates. Experimental samples, as well as IFN standards of a known IU/mL concentration (Chemicon), were acidified to pH=2 overnight, neutralized to pH=7.4, and then UV-inactivated for 10 minutes. Samples were then added to L929 cells in serial 2 fold dilutions. After 24 hours of incubation, cells were infected with 2 x 10^5 pfu of encephalomyocarditis virus (EMCV). 24 hours later, the sample dilution that

protected 50% of L929 cell culture from EMCV-induced cytopathic effect was determined and compared to the IFN standard to determine the IU/mL value.

RESULTS

C3$^{-/-}$ mice develop more severe encephalomyelitis following V3533 infection

Previous work has demonstrated that sub-cutaneous infection of C57BL/6 mice with V3533 results in the development of mild disease, associated with viral neuroinvasion and replication within the CNS, followed by clearance and recovery (4). Using V3533 infection of C57BL/6 mice as a model of successful control of VEEV infection, we used complement deficient C3$^{-/-}$ mice to ask what role, if any, the host complement system played during VEEV infection. C3$^{-/-}$ and C57BL/6 mice were infected with 10^6 pfu of V3533 sub-cutaneously in the footpad. Over the first 4 days, both mouse strains responded similarly, with a slight loss of weight but no other outward signs of disease. By day 6 post-infection, however, responses in C3$^{-/-}$ and C57BL/6 mice began to diverge (Fig. 1.1). Weight loss in C57BL/6 mice was minimal, and clinical signs of disease were mild, consisting of hunched posture, ruffled fur, and mild motor dysfunction in a subset of infected animals. In contrast, C3$^{-/-}$ mice uniformly lost significantly more weight, and developed more severe signs of encephalomyelitis, with all infected animals developing pronounced ataxia and in some cases, hind limb paresis or paralysis. In both C57BL/6 and C3$^{-/-}$ mice these signs of disease were transient, however, and all mice infected went on to recover. The significantly enhanced severity of encephalomyelitic disease observed in C3$^{-/-}$

mice compared with C57BL/6 clearly demonstrated that the host complement system was playing a protective role during V3533 infection.

Enhanced disease severity in C3$^{-/-}$ mice is associated with more extensive inflammation and pathology within the brain

VEEV-induced encephalomyelitis in mice is associated with extensive inflammatory cell infiltration within the brain parenchyma (8). To determine whether the differences in weight loss and clinical course of wild-type and C3$^{-/-}$ mice following V3533 infection correlated with differences in inflammation and neuropathology within the brain, we examined H&E stained brain sections from V3533-infected mice (Fig. 1.2). In both wild-type and C3$^{-/-}$ mice, comparable amounts of inflammatory cell infiltration were apparent within the olfactory bulb and meninges by day 4 post-infection. By day 6 post-infection, when weight loss and clinical signs began to diverge between wild-type and C3$^{-/-}$ mice, focal and diffuse inflammation were observed in the cerebellum and brain stems of C3$^{-/-}$, but not wild-type mice. Inflammation was associated with occasional micro-hemorrhaging. Inflammatory foci were occasionally observed within the cerebellum and brain stems of wild-type mice by day 8, though these were not nearly as extensive as those observed in C3$^{-/-}$ mice (data not shown). In all V3533-infected mice examined inflammation and observable neuropathology were limited to the olfactory bulb, meninges, cerebellum, and brain stem. Thus, the differences in outward disease signs between wild-type and C3$^{-/-}$ mice following V3533 infection correlated with the extent and severity of inflammation within the cerebellum and brain stem.

Viral burdens within the CNS of C3$^{-/-}$ mice are higher and less variable than those of wild-type mice

V3533 infection of C57BL/6 mice results in viral replication within the brain and spinal cord, followed by clearance of infectious virus by day 8 post-infection. Given the differences in disease outcome between C57BL/6 and C3$^{-/-}$ mice following V3533 infection, we next asked whether the more severe disease in C3$^{-/-}$ mice was associated with a larger viral burden within the CNS or a defect in viral clearance. To answer this question, C57BL/6 and C3$^{-/-}$ mice were infected s.c. in the footpad with 10^6 pfu of V3533, animals were sacrificed at 0.5, 1, 2, 4, 6, and 8 days P.I., and viral burdens in serum, spleen, draining popliteal lymph node, brain and spinal cord by were assessed by plaque assay (Fig. 1.3).

In C57BL/6 mice, virus was first detected in the brain at day 2, with peak titers being reached between day 4 and day 6. At day 4 titers were highly variable, ranging from below the limit of detection up to 10^6 pfu/g, but by day 6 titers were much more consistent, with a mean of about 10^4 pfu/g. In the spinal cord, virus was detectable only on days 4 and 6, with no virus being detectable in a subset of mice at each time-point (2/9 at day 4, 3/7 at day 6). In both brain and spinal cord, infectious virus was undetectable by day 8. In contrast, C3$^{-/-}$ mice had detectable virus in both the brain and spinal cord within 24 hours of infection. Viral titers in both tissues peaked at day 4, with mean titers in both brain and spinal cord significantly higher than those observed in C57BL/6 mice (p<0.05, Mann Whitney). Viral titers in the brains of C3$^{-/-}$

mice at day 4 were also much less variable than observed in C57BL/6 mice, as all

mice tested had titers of at least 10^5 pfu/g. Clearance kinetics were similar between

C57BL/6 and C3$^{-/-}$ mice, though at day 8, some C3$^{-/-}$ animals still had detectable viral

titers in the brain (4/7) and spinal cord (1/7). Thus, the more severe disease

observed in C3$^{-/-}$ mice was associated with more rapid neuroinvasion, and higher

and more consistent viral burdens within the CNS.

C3$^{-/-}$ mice exhibit delay in viral clearance from the serum relative to wild-type mice

To assess the effect of host complement on early replication of V3533 in the

periphery, we compared viral burdens within the serum of C57BL/6 and C3$^{-/-}$ mice,

as well as the draining popliteal lymph node and spleen, two anatomical sites of

replication thought to contribute to serum viremia (Fig 1.3). In the serum, peak titers

in both C57BL/6 and C3$^{-/-}$ mice occurred at 12 hours post-infection and were similar,

but clearance kinetics were quite different. Serum titers in C57BL/6 were reduced

about 10-fold between 12 and 24 hours post-infection, and by day 2 virus was

undetectable in all but one animal tested. In contrast, C3$^{-/-}$ mice sustained

significantly higher serum titers at days 1 and 2 compared with C57BL/6, and virus

was still detectable in 3 of 6 animals at day 4. In both lymph node and spleen of

both mouse strains, viral titers were statistically indistinguishable, except for the

spleen at day 1 where the mean titer was actually higher in C57BL/6 mice. Though

not achieving statistical significance, mean titers in C3$^{-/-}$ mice trended higher in the

draining lymph node at all times tested and in the spleen at days 2 and 4.

Host complement plays no significant protective role following intra-cranial introduction of V3533

Given that C3$^{-/-}$ mice were deficient in their ability to control V3533 in both the periphery and the CNS, it was possible that the effect of host complement that resulted in reduced neuropathology could be occurring prior or subsequent to viral invasion of the CNS. In order to address where in the infection process host complement was acting to limit pathology, we introduced 10^3 pfu of V3533 directly into the CNS of C57BL/6 and C3$^{-/-}$ mice by i.c. injection. By circumventing peripheral infection, we were able to directly assess the importance of host complement within the CNS. Following i.c. infection, both C57BL/6 and C3$^{-/-}$ mice behaved similarly (Fig. 1.4). Both groups began losing weight within 24 hours of infection, and rapidly began exhibiting clinical signs of ascending encephalomyelitis. Peak weight loss and disease scores were observed at days 7-8 in both groups, and were followed by protracted recovery. In both the kinetics of disease onset and recovery, as well as magnitude of peak weight loss and disease score, C57BL/6 and C3$^{-/-}$ mice were indistinguishable, indicating that once V3533 has entered the CNS, the host complement system no longer plays a significant role in influencing disease outcome.

Complement is required for maximum anti-VEEV neutralizing activity of normal serum

One possible explanation for the defect in serum clearance and more rapid spread to the CNS that was observed in the C3$^{-/-}$ mice is that the anti-VEEV antibody response was diminished in the absence of complement. During an acute, cytopathic viral infection, the induction of a virus-specific antibody response occurs 2-4 days post-infection (20). During V3533 infection, significant differences in viral titers in the serum and CNS between C57BL/6 and C3$^{-/-}$ mice were apparent by 24 hours post-infection, suggesting that complement was acting prior to anti-VEEV antibody induction. This led us to examine the effect of complement on the anti-VEEV activity of natural antibody. Serum was collected from uninfected C57BL/6, C3$^{-/-}$, or B cell-deficient μMT mice, left untreated or heated to 56°C for one hour to inactivate complement, and then assayed for *in vitro* anti-VEEV neutralizing activity on BHK-21 cells.

Normal serum from C57BL/6 mice exhibited a surprising amount of anti-VEEV neutralizing activity (Fig. 1.5). Wild-type serum at a concentration of only 2.5% was sufficient to neutralize nearly 70% of VEEV infectivity, with an IC50 of 1:49. In the absence of functional complement, either due to heat inactivation or genetic deficiency in C3, neutralizing activity was reduced ~45-50%, demonstrating that the anti-VEEV neutralizing activity of normal serum was partially complement-dependent.

Anti-VEEV antibody responses are intact in C3$^{-/-}$ mice

Complement activation is a requirement for the induction of anti-viral antibody responses following infection with several different viruses. While the similar results following i.c. infection of C57BL/6 and C3$^{-/-}$ mice suggested that the protective effect of complement was not related to antibody induction, we wanted to directly confirm this. C57BL/6 and C3$^{-/-}$ mice were infected sub-cutaneously in the footpad with 10^6 pfu of V3533, and serum was collected at days 2, 4, 8, and 12 post-infection to assay anti-VEEV binding and neutralization activity.

In both C57BL/6 and C3$^{-/-}$ mice, VEEV-specific IgM was induced between days 2 and 4 post-infection, while VEEV-specific IgG appeared between days 4 and 8 post-infection (Fig. 1.6A). At all time points examined, VEEV-specific IgM titers were similar between C57BL/6 and C3$^{-/-}$ mice, with the exception of day 12 post-infection where titers were higher in the C3$^{-/-}$ mice. IgG induction in C3$^{-/-}$ mice appeared to be slightly delayed, as titers were slightly lower at day 8, compared with C57BL/6. While this difference was statistically significant (p=0.0159, Mann Whitney), titers in both groups were quite high. By day 12, IgG levels in both groups were equivalent. In addition to ELISA, which measures the amount of anti-VEEV antibody but not its biological activity, we assessed the anti-VEEV neutralizing activity of serum collected at day 4 post-infection, the timepoint when viral burdens within the CNS were most divergent (Fig. 1.6B). Similar to the ELISA results, no difference was seen in neutralizing activity between C57BL/6 and C3$^{-/-}$. Together, these results demonstrated that complement was not required for the development of an anti-VEEV antibody response.

Recruitment of inflammatory cells to early sites of V3533 replication is similar between wild-type and C3$^{-/-}$ mice

Complement activation can play a role in the recruitment and activation of inflammatory cells to sites of infection. It was possible that the sustained viremia observed in the C3$^{-/-}$ mice resulted from an inability to restrict the production of virus at early sites of replication, rather than a defect in removing virus from circulation. Thus regulation of the inflammatory response at early sites of replication might influence the duration of viremia. To assess whether the absence of complement affected the recruitment of inflammatory cells to early sites of viral replication, we examined the draining popliteal lymph node at 48 hours post-infection as a representative early site of VEEV replication. Single cell suspensions were generated from the lymph nodes, and inflammatory cells were identified based on their surface staining profile.

As would be expected, B and T lymphocytes were by far the most numerous cell populations present, and were found in similar numbers in both groups (data not shown). Looking at cell populations more associated with an inflammatory response, we observed no major differences in the numbers of NK cells (CD3-, CD49b+), NKT cells (CD3+, CD49b+), neutrophils (Ly6G+, CD11b+, MHC class II-), macrophages (CD11b+, CD11c-, MHC class II+), or conventional dendritic cells (CD11c+, B220-) between C57BL/6 and C3$^{-/-}$ mice (Fig. 1.7A). We did observe ~50% fewer plasmacytoid dendritic cells (CD11c+, B220+) in the C3$^{-/-}$ mice, though

our group sizes were too small to determine whether the difference was significant.

As the primary function of plasmacytoid dendritic cells during viral infection is

thought to be the production of type I IFN, we used a bioassay to compare the

amount of IFN present in the draining lymph nodes of C57BL/6 and C3$^{-/-}$ mice at 24

hours post-infection, the first time point at which serum titers were divergent. Using

this approach, we saw no difference in the amount of IFN present in the draining

lymph nodes at 24 hours post-infection, suggesting that there was no significant

difference in plasmacytoid dendritic cell activity between the two groups (Fig. 1.7B).

DISCUSSION

The complement system has been reported to play a key protective role in the host

response to a number of viral pathogens, though the specific mechanisms involved

differ between viruses. For many viruses, including West Nile virus, influenza A

virus, and vesicular stomatitis virus, complement acts by enhancing B and T cell

responses, thus facilitating adaptive control of the infection and clearance (32, 41,

45, 47). In the cases of Sindbis virus and ectromelia virus, complement appears to

act within the first hours of infection, limiting viral dissemination and thus

downstream pathology (23, 45). In this study, we assessed the role of the host

complement system in a model of recovery from acute VEEV infection. Together,

our results demonstrate that the complement system plays an important role in

limiting neuropathology following VEEV infection. This protective effect resulted

from complement function in the periphery during the first 24 hours of infection, and

appeared to be independent of inflammatory cell recruitment or anti-VEEV antibody

induction. While the exact mechanism responsible for this effect remains unclear, our results suggest that complement-mediated enhancement of natural antibody activity might be involved.

The results of this study clearly demonstrated that complement activity in the periphery, but not the CNS, had a profound effect on the extent of virus-induced neuropathology. In the absence of complement, clearance of VEEV from the serum was less efficient, and virus appeared within the CNS within 24 hours of infection, compared with 48 hours in the presence of complement. The earlier arrival of VEEV within the CNS of C3$^{-/-}$ mice, compared with wild-type mice, would allow the virus to replicate and spread to a greater degree prior to the generation of VEEV-specific B and T cell responses. A larger viral burden would most likely trigger a more robust T cell response within the CNS, potentially resulting in more severe immune-pathology. While we feel that this is a likely explanation for the more severe encephalomyelitis and neuropathogy observed in C3$^{-/-}$ mice relative to C57BL/6, more work is clearly needed to examine the relationship between complement, viral spread within the CNS, and the magnitude of the T cell response.

VEEV invasion of the CNS is thought to occur via diffusion of virus from the blood through fenestrated capillary endothelial tight junctions to nearby peripheral nerve endings, followed by centripetal spread to the brain (9). One consequence of this route of neuroinvasion is that the efficiency of invasion is a direct function of infectious virus concentration within the serum. This hypothesis is supported by the

observation that there was a minimum virus concentration in the serum of VEEV infected mice ($\geq 10^4$ pfu/mL) that was required for invasion of the brain (Bernard K., unpublished results). Thus any host function that limits either the magnitude or duration of serum viremia would also reduce the efficiency of neuroinvasion. While this connection has not been directly tested, a number of studies, with VEEV as well as Sindbis virus and western and eastern equine encephalitis viruses, have correlated the duration of serum viremia with virulence (5, 25-27, 36).

In C57BL/6 mice, viral titers in the serum were significantly reduced from their peak by 24 hours post-infection, and were nearly absent by 48 hours post-infection, prior to the development of a VEEV-specific antibody response. Thus, it appears likely that natural antibodies play a significant role in clearing VEEV from circulation. One possible explanation for the delay in serum clearance observed in the C3$^{-/-}$ mice is that complement is required for the maximal anti-viral activity of natural antibodies. Natural antibodies are produced constitutively, independently of internal or external stimuli, and have a fairly wide range of binding avidities, depending on the antigen and host genetic background (48). Despite being produced in a non-specific manner, the anti-viral neutralizing activity of natural antibodies can be high, ranging from titers of 1:8 to 1:32 for vesicular stomatitis virus between different mouse strains (16). When we examined the anti-VEEV neutralizing activity of normal serum, we found that it was quite high, with an IC50 of about 1:50. This could potentially explain why serum clearance occurs so rapidly in wild-type mice. In the absence of complement, the neutralizing activity of normal serum was reduced

substantially, indicating that much of its activity was complement-dependent. Surprisingly, heat-inactivated μMT serum retained a significant amount of neutralizing activity, despite lacking both antibody and functional complement. Heat inactivation at 56°C prevents the subsequent activation of the complement system, but it is possible that some complement components, the collectins for instance, may retain the ability to bind viral particles and hinder attachment or entry. Alternatively, serum may contain elements in addition to antibody and complement that exert some anti-VEEV neutralizing activity.

While these results suggest a role for complement in enhancing the anti-VEEV neutralizing activity of normal serum, further studies are needed to pinpoint the exact mechanism. The C1q protein has been shown to enhance antibody-mediated neutralization of West Nile virus by reducing the number of bound antibody molecules required to neutralize a single particle (40). We feel that is it unlikely that a similar C1q-dependent mechanism is contributing to the effect that we observed with VEEV for two reasons. First, the effect of C1q on West Nile virus neutralization was purely a function of soluble C1q protein, independent of complement activation or C3, whereas natural antibody-mediated neutralization of VEEV is C3 dependent. Second, infection of C1q$^{-/-}$ mice with V3533 did not result in the development of the overt encephalomyelitis that was observed in C3$^{-/-}$ mice, suggesting that C1q was not required for the protective effect of complement activation in our system (data not shown). Studies with ectromelia virus using normal sera from a panel of mice with deficiencies in various complement components demonstrated that both the

classical and alternative pathways of complement activation were required for maximal *in vitro* neutralization (45). Based on the absence of neutralization in the absence of C3 or C4, Moulton et al. concluded that neutralization was primarily the result of opsonization of viral particles by C3b and C4b. A slight reduction in neutralizing activity in the absence of C5 suggested an additional role for the membrane attack complex. Further work is underway to assess the relative contributions of the opsonins and the membrane attack complex to VEEV neutralization.

In addition to enhancing natural antibody-mediated neutralization of circulating virus particles, complement activation might be facilitating serum clearance of VEEV by antibody-independent mechanisms. Seven distinct complement receptors (CR1, CR2, CR3, CR4, SIGN-R1, CRIg, and C1qR) have been identified as binding and facilitating clearance of complement-bound pathogens from circulation (14, 21, 24, 28-30). These receptors are expressed by phagocytic cells such as marginal zone macrophages in the spleen and Kupffer cells in the liver, and could potentially act to eliminate complement-bound VEEV particles from the serum. This mechanism could potentially explain the higher viral burdens observed in the spleens of wild-type mice over the first 24 hours of infection, compared with C3$^{-/-}$ mice. Further evaluation of serum clearance in mice lacking the various complement receptors is needed to assess the importance of this mechanism in controlling early VEEV infection.

The finding that anti-VEEV IgM and IgG responses were intact in the absence of complement was surprising given the complement-dependence of antibody responses to a variety of other viral infections. Complement activation can enhance virus-specific antibody induction through multiple mechanisms. Recognition of complement-coated antigen by CR1 and/or CR2 expressed on B cells can lower the signaling threshold required for activation, while CR1 and CR2 expressed by follicular dendritic cells can act to retain complement-coated antigen within B cell follicles, enhancing presentation to B cells (3, 7). CR1 and CR2 have been demonstrated to be essential for the humoral response to both West Nile virus and herpes simplex virus, though it is not known which CR1/CR2 functions are involved (41, 54). It is possible that the replication of VEEV to such high titers (10^6-10^7 pfu/draining popliteal lymph node) in secondary lymphoid organs may provide enough concentrated viral antigen and inflammatory stimulus to render CR1/CR2 function unnecessary.   Type I interferon, which is produced in large amounts during VEEV infection, can also act directly on B cells to promote antibody production and may thus compensate for the lack of CR1/CR2 signaling in C3$^{-/-}$ mice (10, 13, 60).

Complement activation can play a major role in the recruitment and activation of NK cells, neutrophils, monocytes/macrophages, dendritic cells, and other inflammatory cell populations. Thus, we felt that it was possible that at least some component of the early inflammatory cell response to VEEV would be complement dependent (19, 34, 38, 42, 46, 52, 55). While more thorough analysis is still needed, it appeared that inflammatory cell recruitment was comparable between V3533-infected wild-

type and C3$^{-/-}$ mice.   This mirrors what has been described in mice infected with the arthritogenic alphavirus Ross River virus, where inflammatory cell recruitment was independent of complement activation (43, 44). The robust recruitment of inflammatory cells in the absence of complement activation might be explained by the robust inflammatory cytokine and chemokine response that is induced early during VEEV infection (31). Further work is still needed to rule out a role for complement activation in regulating the activation status and/or antiviral effectiveness of these inflammatory cell populations following recruitment to sites of infection.

Together, the results described here demonstrate that interactions between VEEV and the host complement system during the first hours of peripheral infection can have profound effects on downstream disease outcome. Furthermore, they suggest important roles for natural antibody and complement activation in limiting VEEV invasion of the CNS.

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Figure 2.1
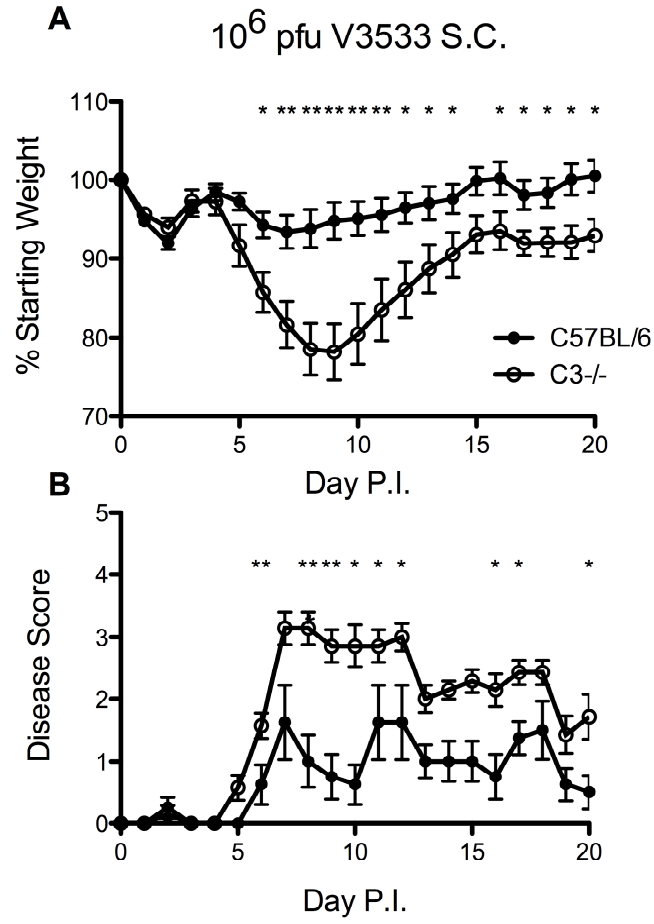
**Figure 2.1: Complement activation limits disease severity following V3533 infection.** 6-10 week old C57BL/6J or C3$^{-/-}$ mice (7-8 mice per group) were infected with 10^6 PFU of V3533 in the left rear footpad. (A) Weight loss and (B) disease score following infection. Mice were scored according to the following scale: 0 – no signs; 1 – hunched posture, ruffled fur; 2 – mild motor dysfunction, altered gait; 3 – moderate motor dysfunction, ataxia; 4 – severe motor dysfunction, hind limb paresis/paralysis; 5 – moribund. Each data point represents the arithmetic mean ± standard error of the mean (SEM). These results are representative of 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$ by Mann-Whitney testing.

Figure 2.2

	Cerebellum	Brain Stem	Meninges	Olfactory Bulb
C57Bl/6 Mock				
C57Bl/6 V3533 Day 6				
C3$^{-/-}$ V3533 Day 6				
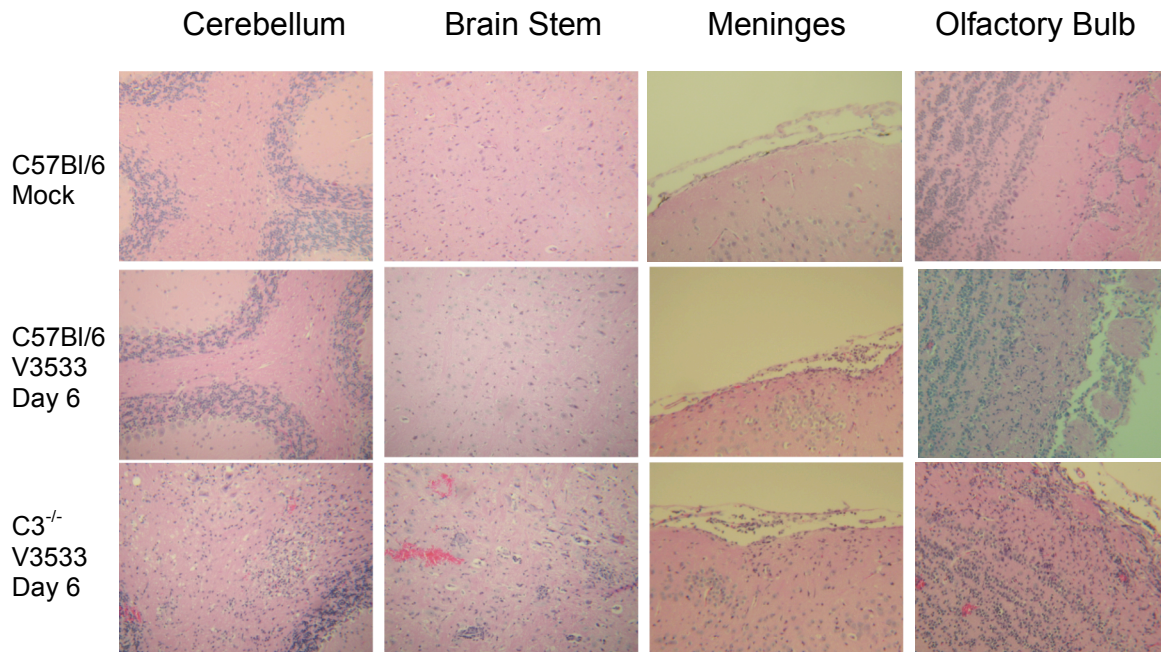
Figure 2.2: V3533-induced brain inflammation in C57BL/6 and C3$^{-/-}$ mice. 6-10 week old C57BL/6J or C3$^{-/-}$ mice were injected with either 10^6 PFU of V3533 or diluent alone in the left rear footpad. At day 6 post-infection, mice were sacrificed and perfused with 4% paraformaldehyde. 5 μM sections paraffin-embedded sagittal sections were generated and stained with H&E. Sections are representative of 2 mice per group.
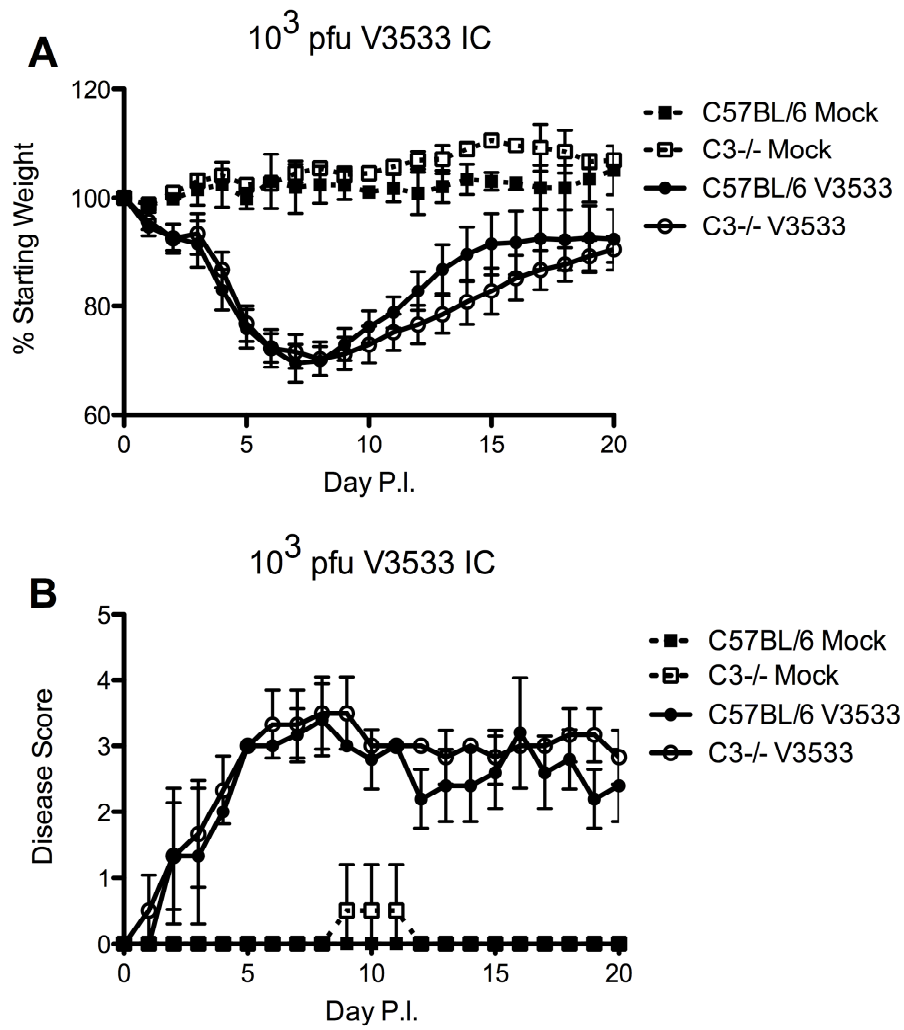
Figure 2.3
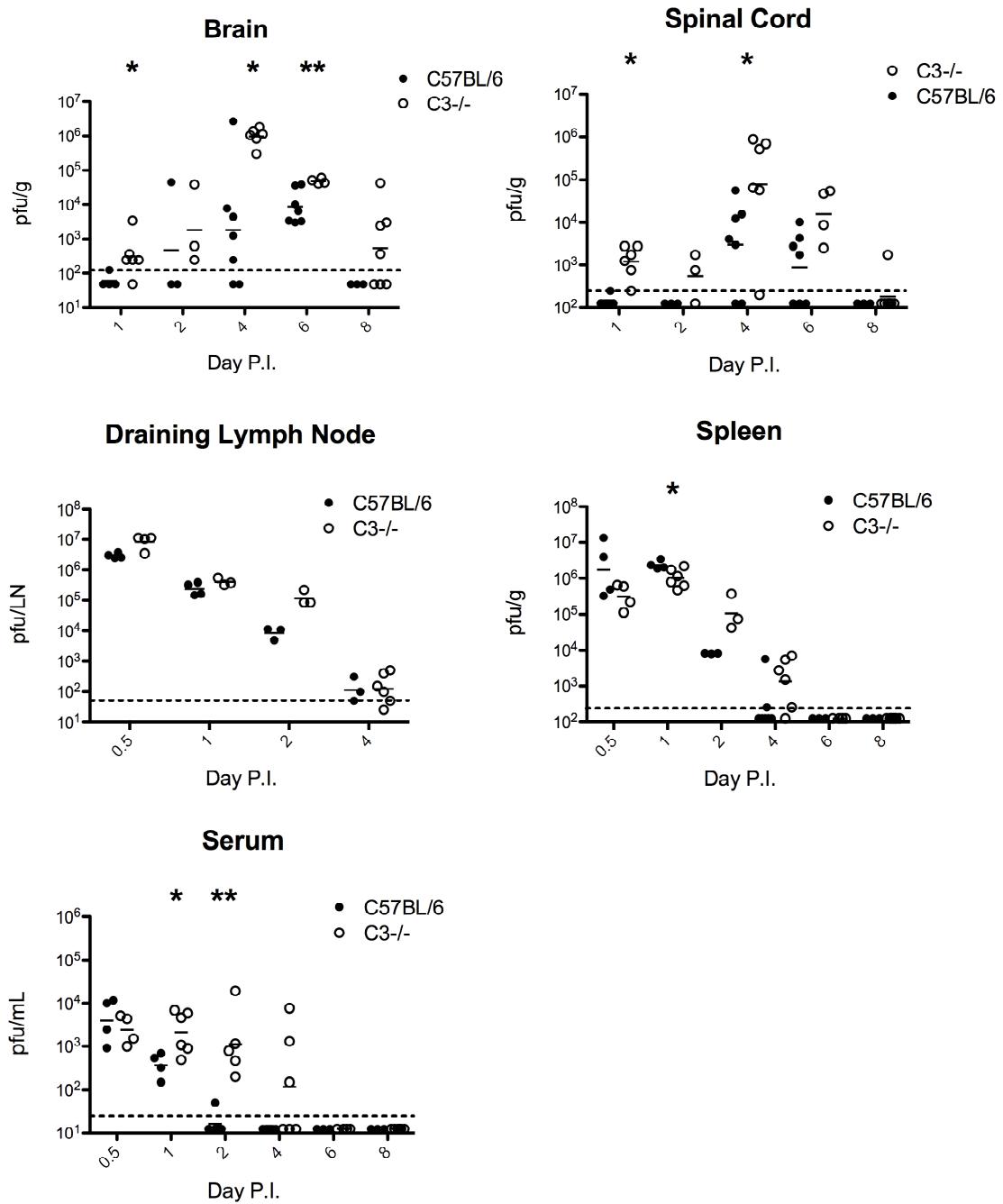
Figure 2.3: V3533 tissue titers in C57BL/6 and C3$^{-/-}$ mice. 6-10 week old

C57BL/6J or C3$^{-/-}$ mice were infected with 10^6 PFU of V3533 in the left rear footpad.

At the indicated times post-infection (Day P.I.), serum, spleen, draining popliteal

lymph node, brain, and spinal cord were collected and homogenized. Viral burden in

each tissue was determined by standard plaque assay on BHK-21 cells. Data points

represent individual titers pooled from three independent experiments, and bars

indicate the geometric mean. *, $P < 0.05$; **, $P < 0.01$ by Mann-Whitney testing.

Figure 2.4

A

10^3 pfu V3533 IC

C57BL/6 Mock
C3-/- Mock
C57BL/6 V3533
C3-/- V3533

% Starting Weight

Day P.I.

B

10^3 pfu V3533 IC

C57BL/6 Mock
C3-/- Mock
C57BL/6 V3533
C3-/- V3533

Disease Score

Day P.I.

Figure 2.4: Disease outcome following intracranial inoculation of V3533. 6-10 week old C57BL/6J or C3$^{-/-}$ mice (8 mice per group) were infected intracranially with 10^3 PFU of V3533. (A) Weight loss and (B) disease score following infection. Mice were scored according to the following scale: 0 – no signs; 1 – hunched posture, ruffled fur; 2 – mild motor dysfunction, altered gait; 3 – moderate motor dysfunction, ataxia; 4 – severe motor dysfunction, hind limb paresis/paralysis; 5 – moribund. Each data point represents the arithmetic mean ± standard error of the mean (SEM). No statistically significant differences between C57BL/6J and C3$^{-/-}$ mice were detected at any time by Mann-Whitney testing.
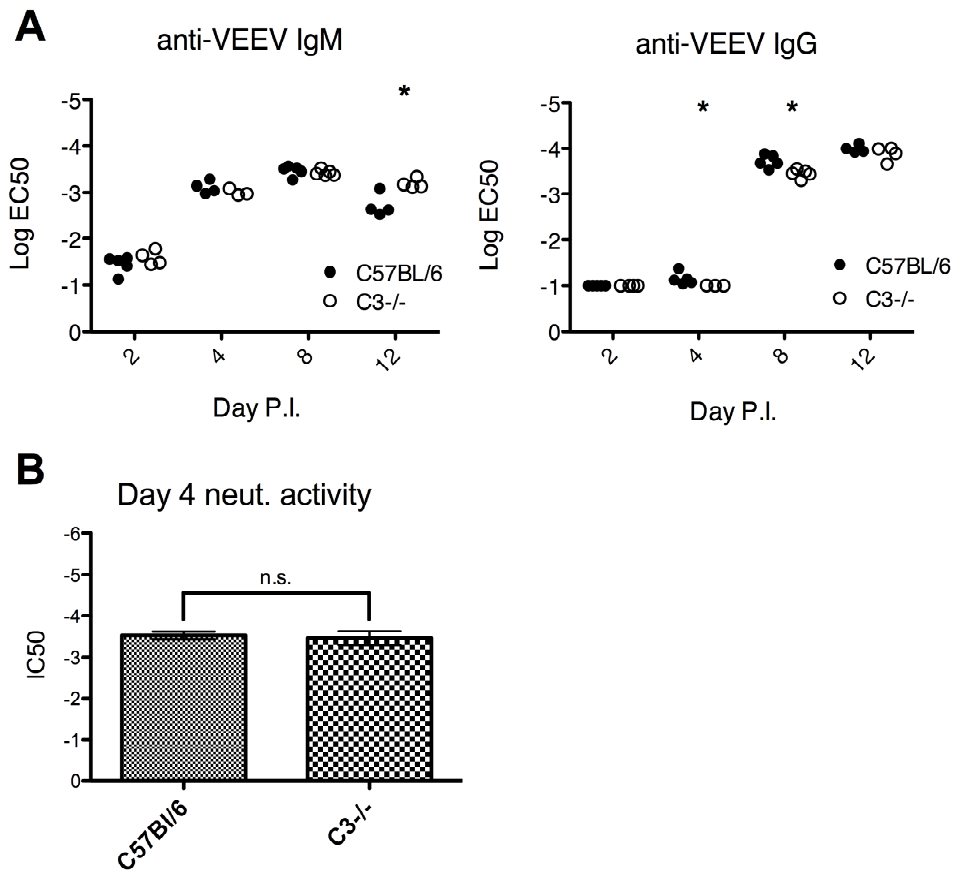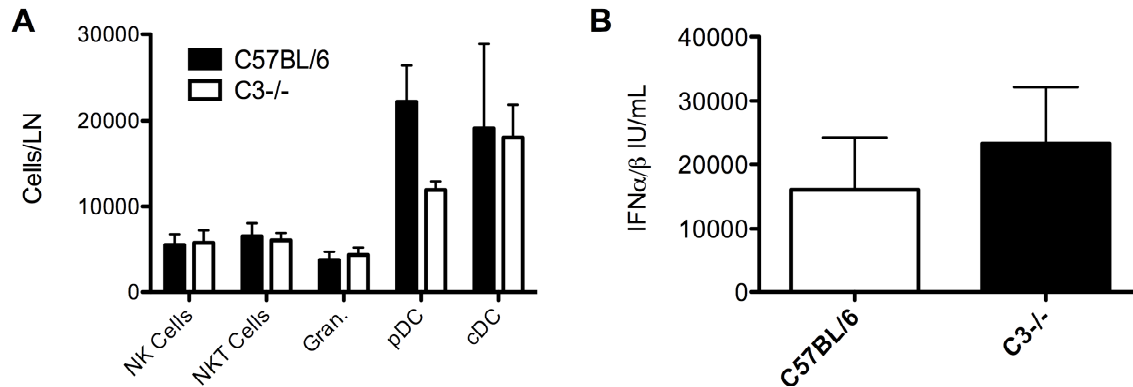
Figure 2.5
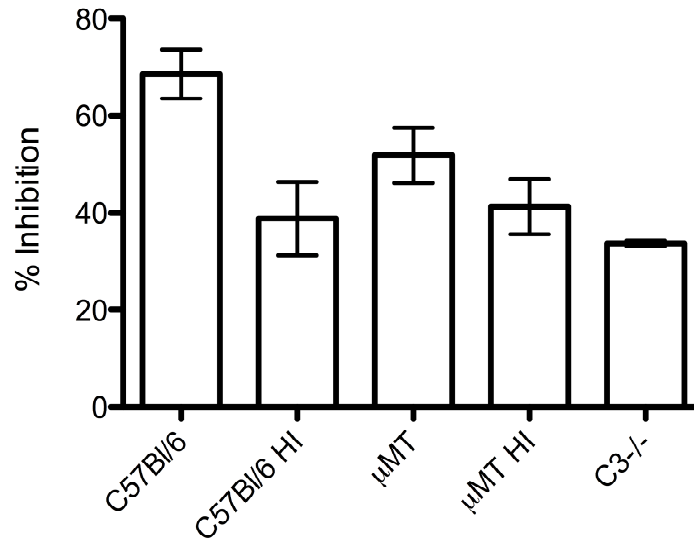
Figure 2.5: Anti-VEEV neutralizing activity of normal serum is partially dependent on complement activation. Anti-VEEV neutralizing activity of normal serum from C57BL/6J, μMT, and C3$^{-/-}$ mice, untreated or heat-inactivated for 1 hour at 56°C, was assessed *in vitro* on BHK-21 cells. Data represents the percentage of viral infectivity lost following 1 hour incubation with the indicated serum at a final concentration of 2.5%, compared with virus incubated in the absence of serum. Each bar represents the arithmetic mean of two replicates.

Figure 2.6

A

anti-VEEV IgM

anti-VEEV IgG

B

Day 4 neut. activity

Figure 2.6: Anti-VEEV antibody response is intact in C3$^{-/-}$ mice. 6-10 week old

C57BL/6J or C3$^{-/-}$ mice were infected with 10^6 PFU of V3533 in the left rear footpad.

(A) At the indicated times post-infection (Day P.I.), mice were bled, and the amount

of VEEV-specific IgM and IgG was determined by anti-VEEV ELISA. Data is

presented as the $\log_{10}$ reciprocal dilution at which 50% of the maximal absorbance

value was reached. Individual data point represent single animals, and are pooled

from two independent experiments. (B) The anti-VEEV neutralizing activity of serum

collected from C57BL/6J and C3$^{-/-}$ mice at day 4 post-infection. Data is presented as

the $\log_{10}$ reciprocal dilution at which 50% of infectivity was inhibited, and horizontal

bars represent the arithmetic means of 3-5 animals per group. *, $P < 0.05$ by Mann-

Whitney testing.

Figure 2.7

Figure 2.7: Inflammatory cell recruitment to early sites of V3533 replication is complement-independent. 6-10 week old C57BL/6J or C3$^{-/-}$ mice were infected with 10^6 PFU of V3533 in the left rear footpad. (A) At day 2 post-infection, mice were perfused with PBS and single cell suspensions were generated from the draining popliteal lymph node, stained for various surface markers, and analyzed by flow cytometry. Total numbers of NK cells (CD3$^-$/CD49b$^+$), NKT cells (CD3$^+$/CD449b$^+$), granulocytes (Ly6G$^+$/CD11b$^+$/MHC class II$^-$), plasmacytoid dendritic cells (pDC) (CD11c$^+$/B220$^+$), and conventional dendritic cells (cDC) (CD11c$^+$/B220$^-$) isolated from lymph nodes. Bars represent the arithmetic mean ± SEM of 2 mice per group. (B) At 24 hours post-infection, mice were perfused with PBS and draining popliteal lymph nodes were isolated and homogenized. Amounts of type I IFN were determined by bioassay. Bars represent the arithmetic mean of results form 3-4 mice per group. Results were statistically indistinguishable by Mann-Whitney testing.

CHAPTER 3:

T CELLS FACILITATE RECOVERY FROM VENEZUELAN EQUINE

ENCEPHALITIS VIRUS-INDUCED ENCEPHALOMYELITIS IN THE ABSENCE OF

ANTIBODY

Abstract

Venezuelan equine encephalitis virus (VEEV) is a mosquito-borne RNA virus of the genus *Alphavirus* that is responsible for a significant disease burden in Central and South America through sporadic outbreaks into human and equid populations. In humans, 2-4% of cases are associated with encephalitis, and there is an overall case mortality rate of approximately 1%. In mice, replication of the virus within neurons of the central nervous system (CNS) leads to a paralyzing, invariably lethal encephalomyelitis. However, mice infected with certain attenuated mutants of the virus are able to control the infection within the CNS and recover. To better define what role T cells responses might be playing in this process, we infected B cell-deficient μMT mice with a VEEV mutant that induces a mild, sub-lethal illness in immune competent mice.   Infected μMT mice rapidly developed the clinical signs of severe paralyzing encephalomyelitis, but were eventually able to control the infection and recover fully from clinical illness. Recovery in this system was T cell-dependent, and associated with a dramatic reduction in viral titers within the CNS, followed by viral persistence in the brain. Further comparison of the relative roles of T cell subpopulations within this system revealed that CD4$^+$ T cells were better producers of IFNγ expression and were more effective at controlling VEEV within the CNS, compared with CD8$^+$ T cells. Overall, these studies suggest that T cells, especially CD4$^+$ T cells, can successfully control VEEV infection within the CNS and facilitate recovery from a severe viral encephalomyelitis.

INTRODUCTION

Venezuelan equine encephalitis virus (VEEV) is a mosquito-borne RNA virus of the

genus *Alphavirus* that is responsible for a significant disease burden in Central and

South America through sporadic outbreaks into human and equid populations (20,

57). The most recent major outbreak occurred in 1995 with 75,000 to 100,000

human cases spread between Columbia and Venezuela (59). In humans, only 1-2%

of cases progress to full-blown encephalitis, though roughly 50% of those cases are

fatal (58). In equid populations, however, the mortality rate is much higher, often

over 50% (56). Because of the high probability of future natural outbreaks, as well

as its potential use as a bioterrorism agent, VEEV remains a significant public health

concern (43). Currently, there are no therapeutics or licensed vaccines available for

human use.

Work with multiple infection models has shown that both the innate and adaptive

arms of the host immune response are involved in successful control of viruses that

target CNS neurons (21). Disruption of the type I interferon system dramatically

decreases the average survival time of mice infected with VEEV, as well as Sindbis

and West Nile viruses (45, 46, 60). Studies with a variety of neuronotropic viruses,

including Sindbis and West Nile, have clearly demonstrated that the development of

a virus-specific antibody response is a critical step in both limiting viral spread and

facilitating non-cytolytic clearance of infectious virus from neurons within the brain

(14, 32). $\alpha\beta$-T cell responses also help limit lethality in many of these models by

directly killing infected cells, producing antiviral cytokines, and/or by enhancing the

production and quality of virus-specific antibody (4, 38, 52, 54). In the case of

Sindbis virus, the T cell compartment was able to dramatically restrict viral

replication in the CNS in the absence of anti-viral antibodies, partially through an

interferon-γ-dependent mechanism (5). While numerous components of the host

immune system play a role in mediating protection or recovery from neuronotropic

virus infection, the specific mechanisms by which the host is able to eliminate virus

from CNS neurons, while leaving these critical, irreplaceable cells intact, remain

unknown.

Our current understanding of VEEV pathogenesis comes primarily from work in a

well-established mouse model of infection and disease that closely mirrors many

aspects of disease in humans and horses (18). Following peripheral inoculation into

the footpad of a mouse, a delivery method that mimics the natural route of infection

by mosquito bite, the virus initiates a biphasic course of infection in which initial

replication within the skin-draining lymph node as well as other secondary lymphoid

tissue seeds a high-titer serum viremia (35). The viremia facilitates virus invasion of

the CNS, initially through non-myelinated olfactory neurons within the nasal neuro-

epithelium (11, 35). This leads to a second phase of infection characterized by rapid

replication and spread though CNS neurons and the eventual development of

paralyzing encephalitis (10, 19). Infection of inbred mice with most strains of VEEV

results in 100% mortality (56). Due to the extreme lethality of the virus, efforts to

understand the host mechanisms involved in mediating recovery from VEEV-

induced encephalomyelitis have been hampered by the lack of a relevant model system in which such a recovery could be reliably observed.

Using a fixed cDNA clone (pVR3000) of the Trinidad Donkey strain of VEEV as a starting point, our lab has generated a panel of genetically defined VEEV mutants that are attenuated *in vivo*, compared to virus derived from the parental pVR3000 clone (1, 3, 12, 19, 60). The use of these mutants, which are attenuated at various definable stages of *in vivo* infection, has facilitated the dissection of the sequence of host-virus interactions that give rise to pathogenesis and/or immunity during VEEV infection (1, 3, 35). One of these lab-generated mutants, labeled V3533, differs from the parental V3000 virus at only two residues, both within the E2 glycoprotein (E76K, K116E), yet these changes are sufficient to convert an invariably lethal virus into one that is non-lethal in immune-competent C57BL/6 mice (1). Sub-cutaneous infection of C57BL/6 mice with V3533 resulted in neuroinvasion followed by rapid clearance. This reduction in pathogenicity did not result from an alteration of CNS cellular tropism, as both V3000 and V3533 exclusively infected neurons within the brain (Data not shown). Thus, infection with V3533 provided a model system to study successful control of VEEV infection within the CNS.

In order to identify the components of a successful immune response to VEEV infection within the CNS, we infected a number of immunodeficient mouse strains with V3533 and assessed disease outcome. Infection of Rag1$^{-/-}$ mice with V3533 resulted in near total lethality while infection of B cell-deficient μMT mice resulted in

near total recovery, demonstrating that recovery from V3533 was dependent upon an adaptive immune response and that while antibody production contributed to recovery, it was not required. Further studies demonstrated that both CD4$^+$ and CD8$^+$ T cells had direct anti-viral effects within the CNS, but that both were required for maximal control of V3533 infection.

Materials and Methods

Viruses

The isolation of wild-type V3000 and the V3533 mutant of VEEV, as well as the generation of the pVR3000 and pVR3533 molecular clones have been described previously (1, 13). Virus stocks of V3533 were generated by in vitro transcription from a linearized plasmid, pVR3533, which encodes the full-length V3533 cDNA, using a T7-specific mMessage mMachine in vitro transcription kit (Ambion). In vitro-generated transcripts were then electroporated into BHK-21 cells using a Bio-Rad electroporator as described previously (1). Culture supernatants were harvested 18 hours after electroporation, clarified by centrifugation at 3000 rpm for 20 min, and stored as single use aliquots at -80° C. Viral titers were determined by standard plaque assay on BHK-21 cells, as previously described (53).

Mouse studies

Rag1$^{-/-}$ and μMT mice (both on the C57BL/6 background) were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in house under specific pathogen-free conditions. C57BL/6 mice were purchased from The Jackson Labs as needed. All experimental manipulation of mice was performed in a biosafety level 3 animal

facility following a 7 day acclimatization period. For infections, 6-10 week old female mice were anesthetized via intra-peritoneal (i.p.) injection with a mixture of ketamine (50 mg/Kg body weight) and xylazine (15 mg/Kg body weight) and then inoculated in the left rear footpad with 10^3 PFU of virus in diluent [phosphate buffered saline (PBS) + 1% donor calf serum, Ca^{2+}, Mg^{2+}]. Mock-infected mice received diluent alone. Weight loss and disease score were assessed daily in infected animals. The scale used for disease scoring was as follows: (0) no signs; (1) hunching; (2) ruffled fur; (3) ataxia, imbalance; (4) conjunctivitis; (5) paralysis of one or both hind limbs; (6) moribund. This scale was based on the temporal order of ascending symptoms in μMT mice following V3533 infection. Mice that lost more than 20% (following V3000 infection) or 35% (following V3533 infection) of their starting weight or became moribund were euthanized according to UNC Institutional Animal Care and Use Committee guidelines.

Virus titers

To assess VEEV titers *in vivo*, infected mice were sacrificed, bled, and then perfused through the heart with 10 mL of PBS. Spleen, draining popliteal lymph node, brain and spinal cord were then removed, weighed, and frozen at -80°C in diluent. Tissues were then thawed, homogenized, and used to infect BHK-21 cells in a standard plaque assay.

***In vivo* depletions**

μMT mice received i.p. injections of 0.5 mg of depletion antibody in 0.1 mL of PBS 24 hours prior to V3533 infection, 24 hours following infection, and every 72 hours subsequently until the termination of the experiment at day 25 post-infection.

Depleting antibodies used were 17A2 (αCD3), GK1.5 (αCD4), 2.43 (αCD8), and LTF2 (isotype control) (all Bio X Cell). At day 25 post-infection, mice were bled and then sacrificed by exsanguination. Brains and spinal cords were collected for titering as described above, and spleens were collected to assess depletion efficacy by flow cytometry.

Quantification of CNS leukocytes and flow cytometry

Mock and V3533-infected mice were sacrificed by exsanguination and perfused with PBS. Brains and spinal cords were harvested, minced, and then incubated for 1.5 hours with vigorous shaking at 37°C in digestion media [RPMI, 1% fetal calf serum, 25mM HEPES, 1.25 mg/mL Collagenase A (Roche)]. Homogenates were then passed through a 40 μm strainer and pelleted through 25% Percoll (GE Healthcare) in media (RPMI, 1% fetal celf serum, 25mM HEPES) for 20 min at 800g. Resulting pellets were then resuspended in 30% percoll, overlayed above 70% Percoll, and centrifuged for 20 min at 800g. The interface was collected and washed with media, and the absolute number of live cells in each sample was then determined by trypan blue exclusion. Cells were washed in flow cytometry wash buffer (1 x Hank's balanced salt solution, 1% fetal calf serum, 0.1% sodium azide) and then stained with the following antibodies: anti-CD45-biotin coupled with streptavidin-conjugated PerCP, anti-CD11b-PE-Cy7, anti-MHC Class II-APC, anti-Ly6G-FITC (clone 1A8), anti-CD3ϵ-PE (all eBioscience); anti-CD4-Pacific Blue, and anti-CD8-Pacific Orange (both Caltag). All staining was done in the presence of anti-mouse FcγRII/III (clone 2.4G2; BD Pharmingen) to prevent non-specific antibody binding. Following staining, samples were fixed in 2% paraformaldehyde in PBS and analyzed on a

CyAn flow cytometer using Summit 5.2 software (Dako). Absolute numbers of each

specific cell type were calculated by determining the number of total live cells within

a sample by trypan blue exclusion and then multiplying that number by the

percentage of live cells within the sample bearing the appropriate surface staining

profile.

Ex vivo analysis of CNS leukocytes

Leukocytes were isolated as described above. For samples receiving no *ex vivo*

stimulus, cells were cultured in T cell culture media (RPMI + 10% fetal calf serum, 50

μM β-mercaptoethanol) with 3 μg/mL brefeldin A (eBioscience) for 4 hours at 37° C.

For samples receiving stimulus, cells were cultured in T cell culture media with 50

ng/mL PMA and 500 ng/mL ionomycin for 6 hours at 37° C, with brefeldin A added

for the last 4 hours. CD107a staining was performed during brefeldin A treatment, in

the presence of 2 μM monensin and 1:100 anti-CD107a-AF488 or isotype control

(eBioscience). Cells were then washed in flow cytometry wash buffer and stained

with the following antibodies: anti-CD69-PE-Cy5, anti-CD3ϵ-PE (both eBioscience);

anti-CD4-Pacific Blue, and anti-CD8-Pacific Orange (both Caltag). Following

surface staining, cells were washed twice and then simultaneously fixed and

permeabilized in Cytofix/Cytoperm (BD Biosciences) for 30 min. Permeabilized

samples were stained for intracellular expression using the following antibodies: anti-

Ki-67-FITC, anti-IFNγ-PE-Cy7, anti-TNFα-FITC, anti-IL-2-APC, anti-IL-10-APC, anti-

IL-17-APC, or the appropriate isotype controls (all eBioscience). All staining was

done in the presence of anti-mouse FcγRII/III (2.4G2; BD Pharmingen) to prevent

non-specific antibody binding. Samples were then washed twice in

Cytoperm/Cytowash (BD Biosciences) and then analyzed on a CyAn flow cytometer.

RESULTS

V3533 provides a relevant model of viral clearance and recovery

Following sub-cutaneous, footpad injection of C57Bl/6 mice with 10^6 pfu of V3533,

the early stages of viral replication and spread were similar to those observed

previously with the more virulent parental virus, V3000 (Fig 1A, data not shown)

(19). Virus was first observed in the draining popliteal lymph node, spleen, and

serum by 24 hours post-infection. The virus first became detectable in the brain by

plaque assay at day 2 post-infection, while titers in secondary lymphoid organs and

serum were reduced considerably by this timepoint. Viral titers peaked within the

CNS around day 4, but unlike wild-type virus that maintained high titers within the

CNS until the inevitable death of the host (data not shown), titers of V3533 began to

decline by day 6, and by day 8, infectious virus was no longer detectable by plaque

assay. Replication of V3533 within the CNS was associated with a small, but

significant loss of weight relative to mock in multiple independent experiments (Figs

1B, 2). Upon clearance, infected mice began regaining weight and eventually

stopped exhibiting outward signs of disease. Thus, V3533 provides a model of

VEEV infection in which the host is able to successfully clear the virus from the CNS

and recover from clinical illness.

Antibody production is not required for recovery from V3533-induced encephalomyelitis

The role of the adaptive component of the immune system in mediating protection or recovery during VEEV infection has been tested primarily in the context of vaccinated animals, or animals receiving passive transfer of immune sera. While these studies have been instructive, we were interested in what role the adaptive response could play during the infection of a naïve animal. To answer this question, we determined whether the observed control of V3533 by C57Bl/6 mice was dependent on the adaptive arm of the immune system.

Rag1$^{-/-}$ mice, which lack functional B and T cells, as well as μMT mice, which lack functional B cells, were infected with 10^3 pfu of V3533 sub-cutaneously in the footpad. Rag1$^{-/-}$ mice initially behaved similarly to wild-type mice, losing little or no weight, and showing no outward signs of disease for the first 4 days post-infection (Fig 2). At day 4, infected Rag1$^{-/-}$ mice began losing weight, and by day 8 post-infection had lost roughly 20% of their starting weight. They also developed clinical signs of disease including hunched posture, ruffled fur, ataxia, and a marked reduction in cage exploration. Weight loss and clinical signs of disease in infected Rag1$^{-/-}$ mice progressed rapidly between days 4 and 8 post-infection, but the clinical condition of the animals then stabilized, and over the next 10 days both weight and clinical score remained virtually unchanged. Starting around day 18 post-infection however, infected Rag1$^{-/-}$ mice suffered further weight loss, culminating in death. V3533 infection of Rag1$^{-/-}$ mice resulted in 93% lethality (n=15) over 40 days of

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observation, with an AST of 30+/-5 days. The difference in disease outcome observed between C57BL/6 and Rag1$^{-/-}$ mice clearly demonstrated that successful control of V3533 infection is dependent upon an intact adaptive immune response.

The importance of an intact antibody response was demonstrated by following V3533 infection of μMT mice. Between days 4 and 9 post-infection, μMT mice lost about 30% of their starting weight and developed the outward signs of severe encephalomyelitis, including convulsions, conjunctivitis, and hind-limb paresis or paralysis. This dramatically enhanced pathology, compared with the much milder disease observed in wild-type mice, illustrated the critical role that antibody production played in mounting a successful response to VEEV infection. Surprisingly, despite the extremely severe morbidity observed, the vast majority of these animals (92.7%, 64/69) went on to recover. Recovery of μMT mice was somewhat protracted, with mice not regaining their full starting weights until weeks later. Most outward signs of encephalomyelitis or febrile illness disappeared in recovered μMT mice, though these animals retained a slight tentativeness in their movements that was still observable 15 weeks after infection. The stark contrast in disease outcome following V3533 infection in μMT mice versus Rag1$^{-/-}$ mice clearly demonstrated that other adaptive mechanisms in addition to antibody production could play a major protective role during VEEV infection.

Recovery in μMT mice is associated with dramatic reduction of viral titers in the brain and clearance of infectious virus in the spinal cord

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Persistent CNS infection by wild-type VEEV in mice has not been reported. The unexpected recovery observed in V3533-infected μMT mice lead us to ask whether the observed recovery was associated with clearance of virus from the CNS or the establishment of viral persistence. To answer this question, we infected μMT mice sub-cutaneously in the footpad with 10^3 pfu of V3533, and then sacrificed animals at various times post-infection and assessed viral burden in relevant tissues by plaque assay.

The pattern of early V3533 replication and dissemination observed in μMT mice differed somewhat from the classic biphasic course of infection that has previously been seen in VEEV-infected immune-competent mice (1). Virus was first seen in the draining popliteal lymph node within 12 hours of inoculation, and the spleen and serum by 24 hours. Rather than being rapidly cleared from these compartments, as it is in C57BL/6 mice, infectious V3533 remained detectable in both serum and spleen for weeks following inoculation (Fig 3A). Of 6 mice examined at 15 weeks post-infection, virus was still detectable in serum (4/6) and spleen (2/6) by plaque assay (Fig 3B).

V3533 first appeared in the brains of μMT mice around 24 hours post-infection, and rapidly reached peak titers of around 10^6 pfu/gram by day 4. The appearance of virus in the spinal cord was slightly delayed, with infectious virus first detectable at 48 hours post-infection and peak titers being reached at day 6 (Fig 3A). Thus, the kinetics of neuroinvasion and initial replication of the virus within the CNS were

similar to that observed in C57BL/6 mice. Between days 6 and 8 however, titers in both brain and spinal cord began to fall, and by day 15 post-infection had been reduced to within a log of the limit of detection. Following this initial reduction of viral titers within the CNS, infectious virus was cleared from the spinal cord between days 15 and 30 post-infection, but continued to persist in the brains of the majority of animals tested through week 15 (day 105), indicating the development of a chronic infection (Fig 3B). This divergence in the ability of the brain and spinal cord to clear virus in the absence of antibody mirrors what has been reported previously with Sindbis virus in these mice (5).

Given that μMT mice were able to control V3533 infection within the CNS and recover from infection, we next asked whether the mortality observed in V3533-infected Rag1$^{-/-}$ mice was due to an inability to control viral replication. To answer this question, we directly compared both systemic and CNS titers between μMT and Rag1$^{-/-}$ mice at days 6 and 25 following sub-cutaneous infection with 10^3 pfu of V3533. While viral titers were high at day 6 post-infection in both μMT and Rag1$^{-/-}$ mice, by day 25 μMT mice were able to reduce titers in both serum and brain substantially, while Rag1$^{-/-}$ mice continued to maintain extremely high titers in both tissues, potentially explaining the enhanced mortality rate in these mice (Fig 3C).

Reduction in viral CNS titers is concurrent with influx of inflammatory monocytes and T cells

The dramatic reductions in CNS titers in the absence of anti-viral antibody led us to ask which immune cell populations were responsible. We first identified leukocyte populations present in the brains and spinal cords of V3533-infected mice over the course of infection. μMT mice were infected sub-cutaneously in the footpad with 10^3 pfu of V3533. At different times post-infection, infected animals were sacrificed and CNS-infiltrating leukocytes were isolated. Different leukocyte populations were then identified by surface phenotype and quantified by flow cytometry (Fig 4A).

The vast majority of leukocytes in the uninfected CNS were identified as resting microglia, defined as CD11b$^+$ CD45^{lo}MHC class II^{lo}, with minimal numbers of other populations being detectable (17, 49). Following invasion of the CNS by V3533, however, this picture changed dramatically. Between days 4 and 10 post-infection, a massive expansion in a CD11b$^+$ CD45^{hi} Ly-6G$^-$ MHC class II^{hi} population, defined as inflammatory monocytes, was observed (16, 29, 49). This population increased in both number and activation state, assessed by MHC class II expression, over this time period (Fig 4B, data not shown) (55). Between days 6 and 10 post-infection, the number of CD4$^+$ and CD8$^+$ T cells within the CNS increased from barely detectable to 10^6 cells/brain and 10^5 cells/spinal cord. After peaking at day 10, these inflammatory cell populations began rapidly contracting, with inflammatory monocytes and T cell populations exhibiting a 5-10 fold decrease in numbers by day 15 post-infection (Fig 4B). Interestingly, the period during which infected μMT mice exhibited increasing severity of disease signs and weight loss corresponded in time precisely with the period during which inflammatory monocytes and T cell numbers

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within the CNS expanded, while the onset of recovery in those animals corresponded with the contraction of those cell populations (Fig 2).

Due to the persistence of low levels of infectious virus in the brains of recovered μMT mice, we asked whether different inflammatory cell populations were retained in the CNS during the chronic phase of the infection. Comparing total cell numbers in the brains of μMT mice 70 days after infection with V3533 with those from mock infected μMT mice, we found significantly elevated numbers of inflammatory monocytes, CD4$^+$, and CD8$^+$ T cells, but not microglia, in the infected group. In the spinal cord, all cell populations tested were significantly elevated above mock (Fig 4C; p<0.05, Mann Whitney). Additionally, microglia and inflammatory monocytes from the persistently infected mice showed significantly higher levels of MHC class II staining relative to mock, indicating that they retained an activated phenotype (p=0.0286, Mann Whitney, Data not shown).

T cells are required for control of V3533 infection and recovery in μMT mice
Numerous studies demonstrating the importance of T cells in mediating anti-viral immunity in other viral systems, as well as the dramatic difference that we observed in survival rates between μMT and Rag1$^{-/-}$ mice, suggested that T cells are required for the recovery observed in V3533-infected μMT mice. It was also possible that the relative contributions of CD4$^+$ and CD8$^+$ populations to control of the infection and recovery differed. To address these questions, we treated μMT mice with depleting antibodies against CD3, CD4, or CD8, or with an isotype control antibody. Weight

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loss and disease score were observed for 25 days following infection, at which point, surviving mice were sacrificed and assayed for viral burden in the CNS as well as serum. The efficacy of the depletion treatments also was assessed at the termination of the experiment by flow cytometric analysis of splenocytes (Fig 5A).

In terms of disease outcome, the antibody isotype control group was indistinguishable from untreated μMT mice following V3533 infection, with animals developing the same overt signs of severe encephalomyelitis and then recovering (Figs 2, 5B). The CD3-depleted group immediately began exhibiting signs of illness upon infection, including weight loss, hunching, ruffling, and ataxia. These disease signs closely mirrored what was observed in V3533-infected Rag1$^{-/-}$ mice, without the convulsions, conjunctivitis, and hind limb paresis observed in infected μMT mice. Also similar to the disease observed in Rag1$^{-/-}$ animals, illness in the CD3-depleted group was prolonged but generally sub-lethal, with 3 of 4 animals still alive at the termination of the experiment at day 25 post-infection. Those surviving animals appeared to be nearing death, however, suggesting that lethality would have been 100% had the experiment been allowed to continue. The one significant difference observed between the CD3-depleted group and Rag1$^{-/-}$ was that the onset of disease was much more rapid in the CD3-depleted group, with signs of fever and weight loss easily observable by 24 hours post-infection (Figs 2, 5B, data not shown). This is most likely not an artifact of the depletion as both the CD4 and CD8-depleted groups exhibited the same delay between infection and disease onset as untreated μMT mice.

In order to more directly examine the role of T cells in controlling V3533 infection in μMT mice, we compared viral burdens at 25 days post-infection both within the CNS and systemically between the different depleted groups with those in the control group (Fig 5C). As expected, based on infection of untreated μMT mice, the antibody isotype control treated group showed low to undetectable viral burdens in the brain and serum, and no detectable infectious virus in the spinal cord. The CD3-depleted mice however, demonstrated a complete inability to control the infection, with titers in all three compartments tested ranging from 10^5 pfu/mL in the serum to 10^7 pfu/g in the brain. These results clearly demonstrated a requirement for the T cell compartment in controlling V3533 infection in the absence of antibody.

Both CD4+ and CD8+ T cells are required for effective control of V3533 infection in μMT mice

Initial disease severity in both CD4 and CD8-depleted groups appeared to be somewhat intermediate between the control and CD3-depleted groups. During the time of peak disease severity (days 9-10), both CD4 and CD8-depleted groups lost significantly less weight than the antibody isotype control treated group ($p<0.05$; Mann-Whitney), and neither group developed the conjunctivitis and/or hind limb paresis that were characteristic of V3533 infection in untreated μMT mice (Fig 5B, data not shown). This indicated that both CD4$^+$ and CD8$^+$ T cells together were required for the most extreme pathology observed in μMT mice following V3533 infection.

While the CD4 and CD8 depletion treatments reduced the severity of V3533-induced disease during the early stages of infection, they also appeared to reduce the ability of those animals to recover compared with the control group (Fig 5B). At the termination of the experiment, 25 days post-infection, one of five CD4-depleted mice had already succumbed to infection, and the survivors were steadily losing weight, indicating that they were not successfully controlling the infection. The CD8-depleted group, while appearing to have recovered more fully than the CD4-depleted group at first, also had begun to deteriorate again by the end of the experiment, resulting in significantly lower weights and significantly higher disease scores compared with the antibody isotype control group at day 25 (both $p<0.05$; Mann-Whitney). Although the experiment was not extended long enough to determine final survival rates for the different groups, the status of the CD4 and CD8-depleted groups at the termination of the experiment strongly suggested that both CD4$^+$ and CD8$^+$ T cell populations were required for complete recovery from V3533 infection.

Having established the necessity of T cells for effective control of V3533 and recovery, the relative contributions of the CD4$^+$ and CD8$^+$ compartments to the control of the virus both systemically and within the CNS were determined (Fig 5C). In the serum at day 25 post-infection, mean virus titers in both CD4 and CD8 depleted groups were roughly three logs lower than the CD3-depleted group, though still elevated above the antibody isotype control group, indicating that each population was able to exert substantial control over systemic replication of V3533,

but that both populations were required for maximal control. In the brain, mean virus titers in mice depleted of CD8$^+$ cells were statistically indistinguishable from those of the antibody isotype control group (p=0.1761; Mann-Whitney). Brain titers in the CD8-depleted group were significantly lower than in the CD3-depleted group, however (p=0.0357; Mann Whitney). In the CD4-depleted group, titers were significantly elevated above both the control and the CD8-depleted groups (p=0.0294, 0.0159 respectively; Mann Whitney), but were indistinguishable from the CD3-depleted group (p=0.0571; Mann Whitney). In the spinal cord, we observed a similar trend where titers in CD8-depleted mice were significantly lower than those of the CD3-depleted group, but titers in CD4-depleted mice were not statistically distinguishable from the CD3-depleted group (p=0.4, p=0.0357 respectively; Mann Whitney) (Fig 5C). Together, these results suggest that within the CNS, the CD4$^+$ compartment contributes the majority of the T cell-associated anti-viral activity, but that both CD4$^+$ and CD8$^+$ cells are required for maximal control.

CD4$^+$ T cells are the primary source of T cell-associated IFNγ within the brain

The apparent difference in antiviral activity observed between CD4$^+$ and CD8$^+$ cells within the CNS might be explained by differences in expression of cytokines or other indicators of effector function. To address this possibility, we isolated T cells from the brains of V3533-infected μMT mice at days 8, 15, and 70 post-infection and assessed expression of IFNγ, TNFα, IL-2, IL-10, and IL-17 by intracellular cytokine staining. We also looked at surface expression of CD69 and CD107a as markers of recent activation and degranulation, respectively. Day 8 was chosen to provide a

snapshot of T cell behavior during the peak of antiviral activity, as T cell numbers in the CNS were increasing and viral titers were decreasing at this time. Day 15 represented a point after which both viral titers and T cell numbers within the CNS had decreased substantially and recovery had begun. Day 70 was chosen to represent a point well into the chronic phase of infection.

T cell behavior within the brains of V3533-infected mice was described using two complementary approaches. The first was to non-specifically stimulate brain-infiltrating T cells with PMA and ionomycin for 6 hours *ex vivo* prior to flow cytometric analysis to determine how these cells were capable of responding (Fig 6A). Using this approach, it appeared that both $CD4^+$ and $CD8^+$ cells were programmed to respond similarly to V3533 infection within the brain. Large numbers of $IFN\gamma$ and $TNF\alpha$-producing $CD4^+$ and $CD8^+$ cells were detected, while IL-17 and IL-10-producing cells were much less prevalent, indicating that both $CD4^+$ and $CD8^+$ cell populations appeared to be participating in a predominantly Th1-skewed response. IL-2 production was minimal, in accordance with previous findings during Sindbis infection (26). The numbers of $IFN\gamma$ and $TNF\alpha$-producing $CD4^+$ and $CD8^+$ cells were roughly equivalent at day 8, but by day 15 the number of $CD8^+$ cells producing both cytokines was approximately two-fold higher compared with $CD4^+$ cells, reflecting an overall increase in the ratio of total $CD8^+$ cells to $CD4^+$ cells at this time-point. At all time-points, a higher percentage of $CD8^+$ cells stained positive for surface expression of the recent degranulation marker CD107a, compared with $CD4^+$ cells. These results suggest that brain-infiltrating $CD4^+$ and $CD8^+$ T cells were similar in

their abilities to produce IFNγ and TNFα *ex vivo* during the acute phase response to V3533, and that the degranulation activity of CD8$^+$ T cells was superior to that of CD4$^+$ cells. Both CD4$^+$ and CD8$^+$ T cells retained multiple effector capabilities well into the chronic phase of VEEV infection, indicating that the phenomenon of T cell exhaustion was not occurring in this system (Table 1). The caveat with results obtained by PMA/ionomycin treatment however, is that they represent only how T cells are programmed to respond, not how they are actually behaving *in vivo*.

A second approach gave a more direct measurement of how T cells were actually behaving *in vivo*. Rather than treating with PMA and ionomycin following isolation, brain-infiltrating T cells were treated only with brefeldin A, with or without monensin, prior to flow cytometric analysis (Fig 6B). No external stimulus was provided. While this treatment probably resulted in artificially low levels of detectable expression due to the time lag between sacrifice of the animal and cell fixation, we feel that any results observed using this method are the direct result of the stimuli and regulatory signals present within the brains of V3533-infected mice. Brain-infiltrating T cells analyzed in the absence of *ex vivo* stimulus exhibited a dramatically different pattern of IFNγ and CD107a expression compared to that observed following PMA/ionomycin treatment. At days 8, 15, and 70 post-infection, significant IFNγ expression was detectable only within the CD4$^+$ population. IFNγ$^+$ CD8$^+$ T cells were undetectable at all timepoints. In addition, a larger percentage of CD4$^+$ cells expressing surface CD107a were detected, compared with CD8$^+$ cells during the acute phase. Finally, IL-2, IL-17, and IL-10 expressing cells were sparse in both

CD4$^+$ and CD8$^+$ T cells. Together, these results indicate that during the acute phase of the response to V3533 infection within the brain, CD4$^+$ T cells produced more IFNγ and had higher levels of degranulation activity compared with CD8$^+$ T cells, potentially explaining why these cells exhibited more potent anti-VEEV activity.

While the lack of known T cell epitopes prevented us from directly examining VEEV-specific T cells, we felt that the use of CD69 staining provided a reasonable alternative. CD69 expression on T cells serves as a marker of recent encounter with cognate antigen, and should not be expressed on bystander T cells (47). At each time point examined, CD69$^+$ cells constituted roughly 50-80% of all CD4$^+$ and CD8$^+$ T cells within the brain (Fig 6C). This is consistent with the majority of brain-infiltrating T cells being VEEV-specific during both the acute and chronic phases of infection.

Discussion

While the important protective role of B cells and virus-specific antibodies during neuronotropic alphavirus infection has been well established, the role of T cells remains relatively ill-defined. Work with avirulent Sindbis virus has suggested that T cells might directly act to limit viral infection within the CNS by non-cytolytic mechanisms; however, studies using virulent strains of Sindbis or VEEV were contradictory, suggesting instead that T cells might either act to enhance pathology or play no significant role whatsoever (5, 28, 44). Previous studies evaluating the immune response to VEEV infection have been hampered by the lack of a model

system in which successful control of infection could be reliably observed. To better understand the role of T cells during a successful immune response to acute VEEV infection, we utilized a mutant of VEEV, V3533, which is capable of invading the CNS from the periphery yet only induces a mild, transient disease in immune-competent mice. We feel that this provides a more relevant model of VEEV-induced disease in humans, compared with the universal lethality observed in mice infected with wild-type VEEV strains, as natural infections of humans very rarely progress to overt encephalomyelitis and death (58). Using V3533 infection of mice as a model of successful recovery from VEEV-induced encephalomyelitis, we asked whether T cells played a significant role during recovery from VEEV infection, independent from any effect on antibody production. We observed that B cell-deficient μMT mice were able to recover from V3533 infection while T cell-depleted μMT mice were not, clearly demonstrating that T cells could facilitate clinical recovery from VEEV-induced encephalomyelitis in the absence of antibody.

We chose to carry out these studies in μMT mice, rather than B-cell depleted immune-competent mice, in order to ensure that antibody production was completely absent. Given the highly protective effects that antibodies demonstrated in other alphavirus infection models, we were concerned that even a small percentage of endogenous B cells that survived a depletion treatment might exert enough of an effect to confound the potentially subtle T cell-associated effects in which we were interested (32). The use of μMT mice as a "B cell deficient mouse" is complicated by the observation that these mice exhibit various T cell deficiencies relative to wild-

type mice (2). We cannot rule out that these defects, which affect both expansion and function in CD4$^+$ and CD8$^+$ T cells, might contribute to the viral persistence that we observed. However, the fact that T cells are able to control V3533 infection and facilitate recovery in μMT mice despite these strain-specific deficiencies strengthens our conclusion that T cells contribute a significant anti-viral effect during V3533 infection.

Viral infection of CNS neurons presents a unique problem for the immune system in that they are absolutely essential for host function, yet are not easily replaced (34). As a result, widespread immune-mediated cytolysis of infected cells can present a greater threat to host viability than the virus itself (10). In the case of infection with a highly virulent virus, the benefits of cytolytic clearance mechanisms might be worth the cost in enhanced pathology; however, it appears that the mammalian immune system has also evolved non-cytolytic mechanisms of T cell-mediated clearance. In particular, studies with Sindbis virus, Theiler's murine encephalomyelitis virus (TMEV), and measles virus have all implicated T cell-associated IFNγ production in mediating noncytolytic clearance of virus from infected neurons (7, 38, 42). The studies described here clearly demonstrated that, during V3533 infection, T cells were able to significantly restrict viral replication within the brain and clear infectious virus from the spinal cord, thus facilitating recovery from a severe viral encephalomyelitis. While this reduction in CNS titers was clearly associated with more severe disease signs, compared with what was observed in Rag1$^{-/-}$ mice, the rapid onset of recovery and the absence of long-lived sequelae within the brain

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suggest that control of the infection was not achieved simply by destruction of infected neurons.

The failure of μMT mice to fully clear infectious virus from the CNS mirrors what has been previously observed in μMT mice infected with Sindbis virus (5). The ability of these mice to recover from V3533-induced encephalomyelitis despite the continuing presence of infectious virus both within the CNS as well as systemically raises a number of questions. The most obvious is, what are the cellular reservoirs of virus that prevent total clearance? Studies with Sindbis virus have indicated that neuronal sub-populations within the CNS are differentially susceptible to IFNγ-mediated clearance (8). Whether these differences are due to intrinsic differences in the IFNγ-signaling network within these cells or some other mechanism remains to be determined. If there is a stable reservoir of clearance-refractory neurons that are responsible for the observed persistence, how are those cells able to survive prolonged infection with a cytolytic virus? Despite the presence of robust anti-apoptotic programming within mature neurons, virulent strains of VEEV are able to induce widespread neuronal death (10, 22, 27). It may be that V3533 is less efficient at overcoming these mechanisms, thus allowing long-term survival of infected neurons. If this is the case, understanding the molecular basis of this difference will be of great interest. Another issue that remains unresolved is the extent to which host neurological function is compromised by the ongoing persistent infection and accompanying low-level inflammatory response (40). While μMT mice appear to recover fully from V3533-induced clinical disease, it is possible that a

more in-depth evaluation of cognitive function in the recovered animals might reveal subtle defects.

Given the continuing presence of infectious virus within the CNS of recovered μMT mice, it is not surprising that activated T cells were retained within the brain and spinal cord. What was somewhat surprising was that both CD4+ and CD8+ T cells retained functionality, as determined by IFNγ expression, as long as 70 days following infection. The loss of T cell function, specifically IFNγ expression, has been well documented in other models of persistent viral infection and is thought to result from prolonged antigen exposure (6, 36, 51). In this model of persistent VEEV infection however, long-term antigen exposure, as indicated by sustained CD69 expression, was not sufficient to induce exhaustion. A recent study that examined T cell responses to chronic mouse hepatitis virus infection of the CNS also observed long-term maintenance of T cell function despite ongoing antigenic stimulation, suggesting that some aspect of the CNS regulatory environment might prevent the development of the exhaustion phenotype observed in other systems (61).

Another notable aspect of the T cell response that we observed during the sub-clinical chronic phase of the infection was a shift in $CD8^+$:$CD4^+$ T cell ratios. While this ratio was roughly 1:1 during the acute phase of the response within the CNS, by day 20 post-infection it had risen to about 3:1, and by day 70 had reached nearly 10:1. One possible explanation for this finding could be increased susceptibility to apoptosis among cells of the $CD4^+$ population. Studies performed in the context of

acute LCMV infection showed that CD4$^+$ memory cells have lower levels of Bcl-2

expression compared with CD8$^+$ memory cells and that this corresponded with a

more rapid decline in CD4$^+$ T cells over time (24). Another possible explanation is

that MHC class I and MHC class II expression within the CNS might be differentially

regulated during the development of viral persistence (39). Since viral antigen

presentation is likely to be required for maintenance of T cell populations during

chronic infection, it may be that a reduction in MHC class II-restricted presentation

could result in the gradually diminishing CD4$^+$ T cell numbers that we observed (50).

More work is needed to describe the dynamics of antigen presentation within the

CNS during chronic viral infection.

It has been firmly established that the CNS presents an especially unique

microenvironment in which extremely tight regulation of host immune responses is

essential for continued host viability (9, 37). Numerous mechanisms, including

restricted expression of MHC class I and class II by neurons, and the constitutive

production of TGFβ and immune-suppressive gangliosides by glial cells, act to limit

both the magnitude and duration of inflammatory responses, thereby protecting

against excessive immune-pathology (15, 25, 30, 37, 48). The complex regulatory

environment within the CNS might explain our observation that CD4$^+$ T cells appear

to be providing the majority of T cell-associated antiviral activity in our system.

Despite both T cell populations having been clearly primed to produce IFNγ, as

evidenced by the response to PMA/ionomycin treatment, the absence of detectable

IFNγ expression by unstimulated brain-infiltrating CD8$^+$ T cells during the acute

response to V3533 suggests that CD4$^+$ and CD8$^+$ cells are subject to differential regulation within the brain. This observation could be explained by a failure of infected neurons to upregulate MHC class I, preventing antigen encounter, but this is unlikely as the vast majority of CD8$^+$ T cells retain a CD69$^+$ phenotype both during the acute and chronic phases of the response to V3533, indicating recent encounter with cognate antigen. It seems more likely that some environmental factor is specifically limiting IFNγ production by CD8$^+$ cells within the brain. There is precedent for this, as CNS-infiltrating CD4$^+$ T cells responding to Sindbis virus infection appear deficient in IL-2 expression, compared with CD4$^+$ T cells in the periphery (26). Other studies have shown that T cells primed by brain-resident microglia take on a phenotype that is distinct from T cells primed by other APC populations (16). Clearly, further work is needed to better define the regulatory elements within the CNS that dictate T cell function during VEEV infection.

Our observation that, within this highly reductionist model, CD4$^+$ cells played a significantly more potent anti-viral role in response to V3533 compared with CD8$^+$ cells correlated with substantially higher levels of IFNγ expression and signs of recent degranulation. This differs from results obtained in Sindbis virus-infected mice, where CD4$^+$ and CD8$^+$ T cells appeared to have equivalent effects on viral titers within the CNS (5). Based on our CD8 depletion studies in μMT mice, CD4$^+$ T cells appear to exert considerable anti-viral effect, independent of any role in helping B cell or CD8$^+$ T cell responses. This effect is most likely mediated primarily by IFNγ signaling within infected neurons, similar to what has been described with Sindbis

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virus, Borna virus, TMEV, and measles virus (7, 23, 38, 42). However, we cannot rule out additional mechanisms. One possible alternative mechanism could involve the release of lytic granules as CD4$^+$ T cells and, to a lesser extent, CD8$^+$ T cell both exhibited signs of recent degranulation both during and after the acute phase response. Lytic granule release could act to clear V3533 from infected neurons either by cytotoxic mechanisms (i.e. perforin release), as has been described during West Nile virus infection, or by non-lethal mechanisms, as has been described during HSV-1 infection (31, 52). A second possible alternative mechanism is that CD4$^+$ T cells, possibly through IFNγ expression, might act to enhance monocyte/microglia responses to the virus, as has been observed following LCMV infection of the CNS (33). Obviously, these different mechanisms are not mutually exclusive. Given that neurons do not express MHC class II, the mechanism by which CD4$^+$ T cells recognize V3533-infected neurons also remains unclear (41).

In summary, our results confirm the established importance of anti-viral antibodies in limiting disease following neuronotropic alphavirus infection, but also clearly demonstrate that T cells can facilitate recovery from severe viral encephalomyelitis in the absence of antibodies. The recovery that we observed in V3533-infected μMT mice was associated with a dramatic reduction in viral titers within the CNS, followed by the establishment of a persistent sub-clinical infection. These results demonstrate for the first time that T cells are able to directly control infection by a neuronotropic virus that causes encephalomyelitis in humans. We also showed that, in the context of VEEV infection, the majority of T cell-associated antiviral activity

resides within the CD4$^+$ population, possibly due to significantly enhanced IFNγ expression in these cells compared with CD8$^+$ cells. Taken together, these studies suggest that the promotion of T cell effector function, within both CD4$^+$ and CD8$^+$ populations, should be an important consideration when designing and evaluating new vaccines against encephalitic alphaviruses.

ACKNOWLEDGEMENTS

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Figure 3.1 (Figure 1)

Figure 1

A

B
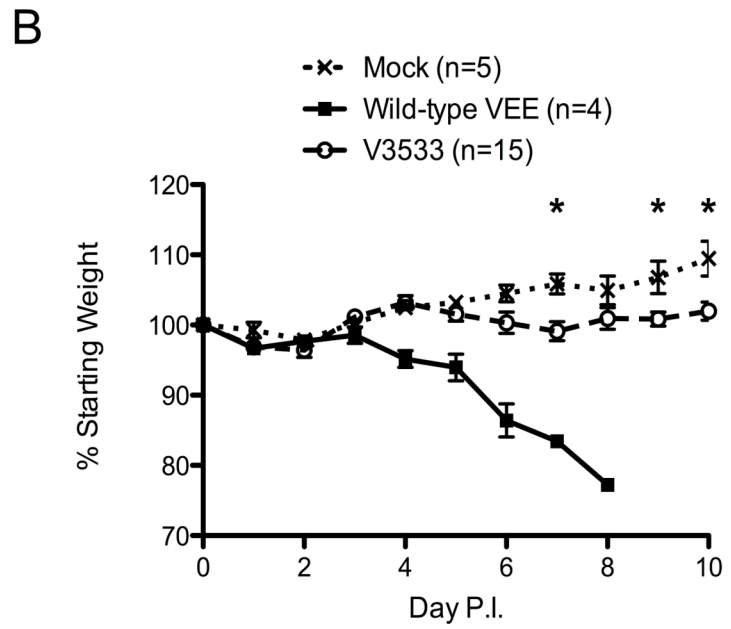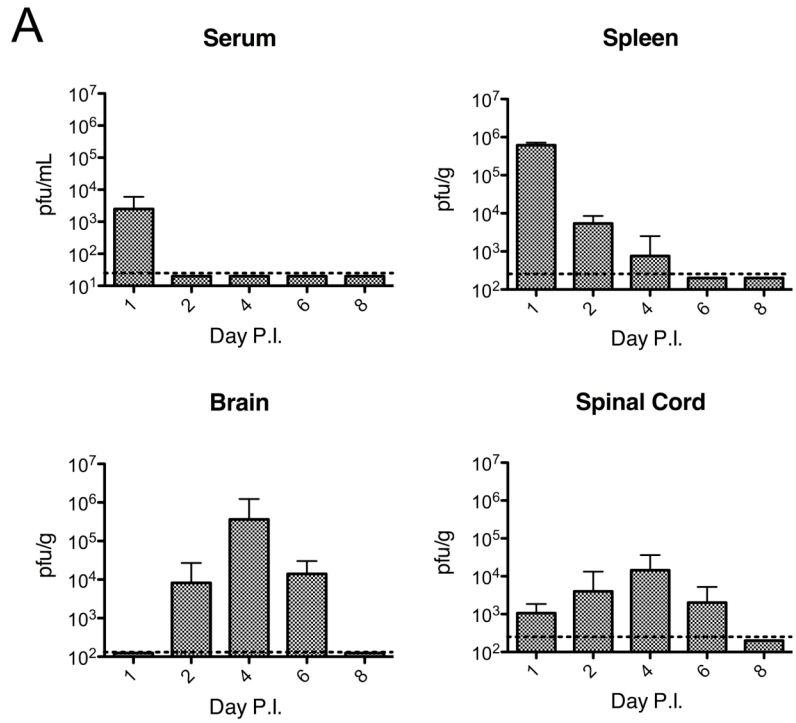
Figure 1: V3533 induces mild, transient disease followed by clearance in C57BL/6. (A) 7-10 week old female C57BL/6 mice were inoculated with 10^6 pfu of V3533 by infection in the left rear footpad. At the indicated days post-infection (Day P.I.) serum, spleen, brain, and spinal cord were collected from V3533-infected mice and homogenized. The amount of infectious virus present in serum, spleen, brain, and spinal cord was then quantified by plaque assay on BHK-21 cells. Data is presented as the mean +/- standard deviation of results pooled from two independent experiments with 3-10 animals per timepoint. Dotted line represents the limit of detection. (B) 7-10 week old female C57BL/6 mice were inoculated with 10^3 pfu of wild-type VEEV (V3000) or 10^6 pfu of V3533 by infection in the left rear footpad. Mock-infected mice were infected with diluent alone. Mice were weighed daily, with those losing more than 20% of their starting weight being euthanized as required by UNC IACUC regulations. *, $p < 0.05$ by Mann-Whitney.
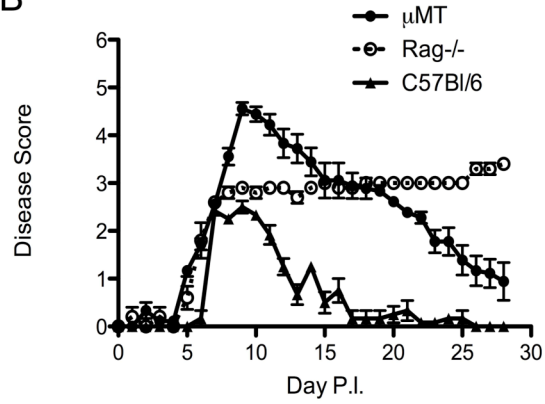
Figure 3.2 (Figure 2)

Figure 2

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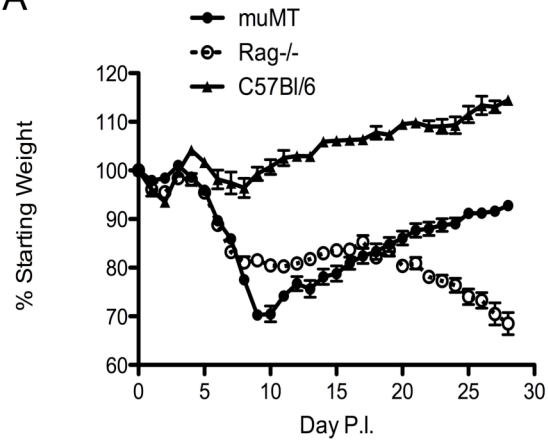
B

Figure 2: Rag1$^{-/-}$ mice succumb to V3533 infection while μMT mice recover. 7-10 week old female μMT or Rag1$^{-/-}$ mice were inoculated with 10^3 pfu of V3533 by injection in the left rear footpad. C57BL/6 mice received 10^6 pfu of V3533. (A) Mice were weighed daily, with those losing more than 35% of their starting weight being euthanized as required by UNC IACUC regulations. (B) Mice were scored for the development of encephalomyelitis based on the following scale: 1 – hunched posture; 2 – ruffled fur; 3 – ataxia, imbalance; 4 – conjunctivitis; 5 – hind limb paresis/paralysis; 6 – moribund. Each data point represents the mean +/- SEM of 6 animals per group from a single, representative experiment.

Figure 3.3 (Figure 3)

Figure 3
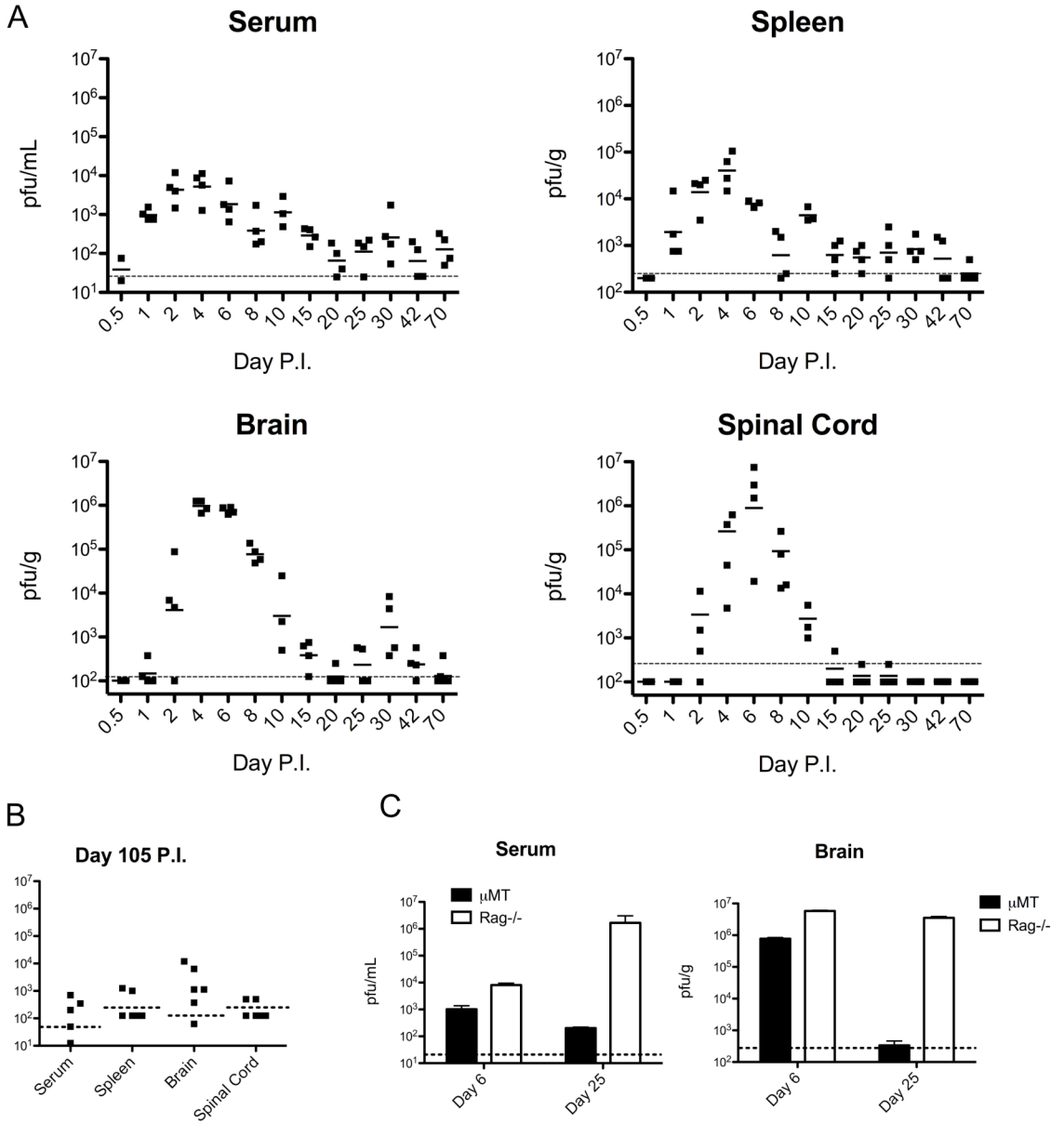
Figure 3: Recovery in μMT mice is associated with control of viral replication in the brain, clearance in the spinal cord. 7-10 week old female μMT or Rag1$^{-/-}$ mice were inoculated with 10^3 pfu of V3533 by injection in the left rear footpad. (A) At the timepoints indicated serum, spleen, brain, and spinal cord were collected from infected μMT mice and homogenized. The amount of infectious virus present in serum, spleen, brain, and spinal cord was then quantified by plaque assay on BHK-21 cells. Data points represent individual tissue titers pooled from two independent experiments. (B) Tissue titers from infected μMT mice at day 105 post-infection. (C) Comparison of tissue titers between μMT and Rag1$^{-/-}$ mice.   Data is presented as the mean +/- SEM of titer values from 3-4 animals per group. In all cases, dotted line represents the limit of detection.
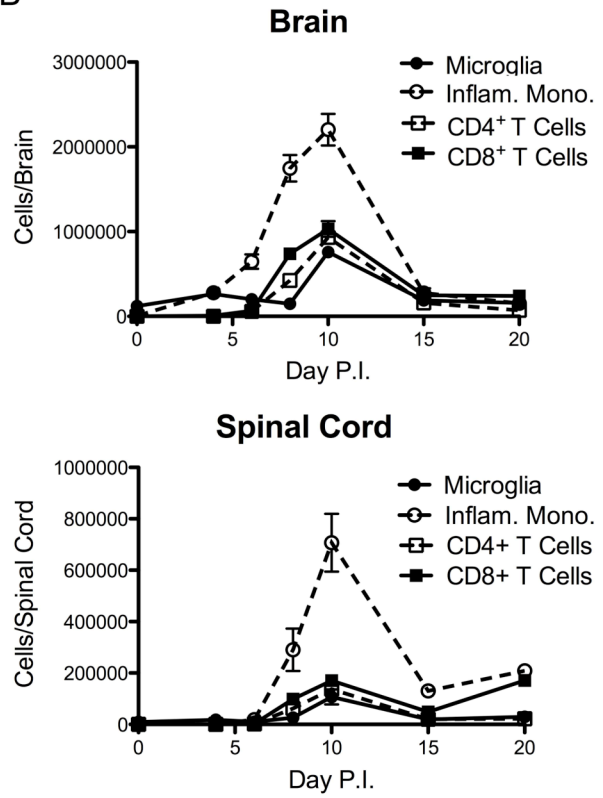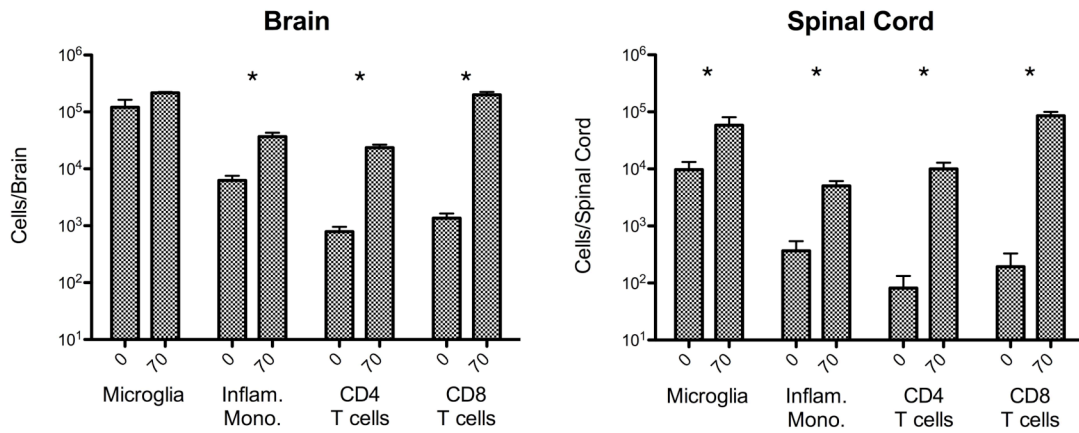
Figure 3.4 (Figure 4)

Figure 4

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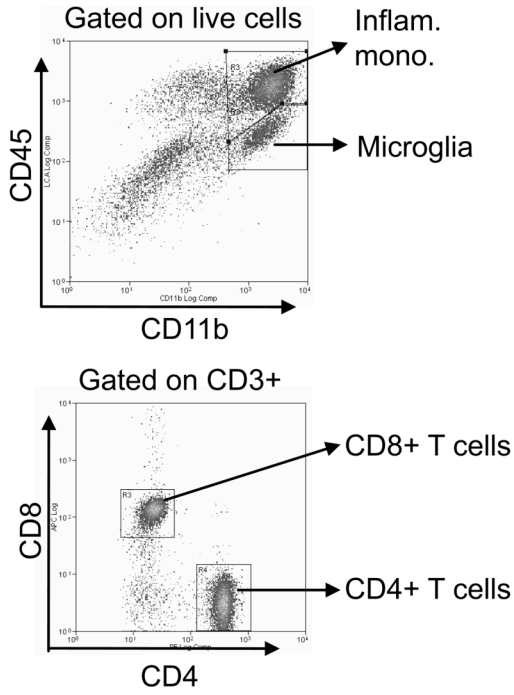
B

C

Figure 4: Reduction of viral titers in the CNS coincides with influx of T cells, inflammatory monocytes. 7-10 week old female μMT mice were inoculated with 10^3 pfu of V3533 or diluent alone by injection in the left rear footpad. At various timepoints post-infection, mice were perfused with PBS and CNS-infiltrating leukocytes were isolated. Infiltrating cells were stained for various surface markers and analyzed by flow cytometry. (A) Representative dot plots illustrating the gating scheme used to define cell populations. (B) Total numbers of microglia ($CD11b^+/CD45^{lo}$), inflammatory monocytes ($CD11b^+/CD45^{hi}$), CD4+ T cells ($CD3^+/CD4^+$), and CD8+ T cells ($CD3^+/CD8^+$) isolated from brain and spinal cord. For each timepoint, data is presented as the mean +/- the standard error of 3-4 mice and is representative of 2 independent experiments. (C) Comparison of total cell numbers of indicated infiltrating leukocyte populations between mock and V3533-infected mice 70 days post-infection. Data is presented as means +/- standard error of four animals per group. *, $p < 0.05$ by Mann-Whitney.

Figure 3.5 (Figure 5)

Figure 5
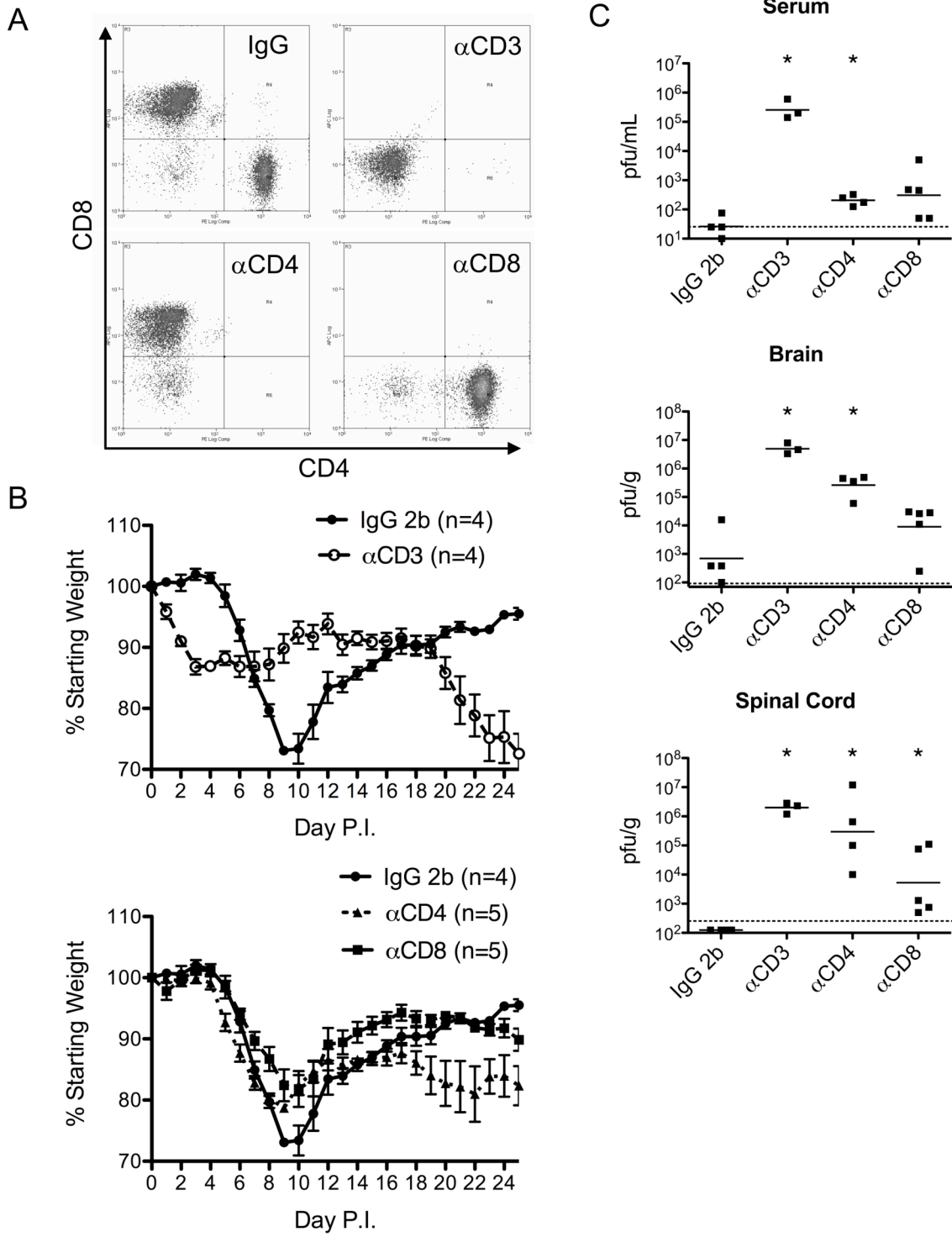
Figure 5: T cells are required for control of infection, recovery in μMT mice. 7-10 week old female μMT mice were treated with depleting antibodies against CD3, CD4, or CD8, or with an isotype control antibody, and then inoculated with 10^3 pfu of V3533 by injection in the left rear footpad. Depletion treatments were continued for 25 days post-infection, at which point the experiment was terminated. (A) Representative dot plots of CD3+ splenocytes from each group, taken at day 25 post-infection. (B) Effect of T cell depletions on weight loss following V3533 infection. Data presented as mean +/- standard error of 4-5 animals per group. (C) Infectious virus from tissues harvested 25 days post-infection, assessed by plaque assay on BHK-21 cells. Each data point represents a single animal, with bars indicating the geometric mean. *, $p < 0.05$ compared to control by Mann Whitney.
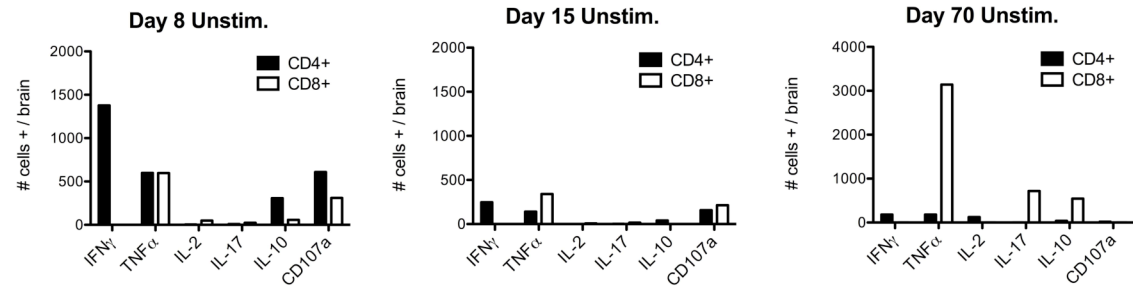
Figure 3.6 (Figure 6)

Figure 6
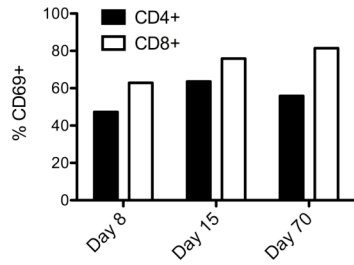
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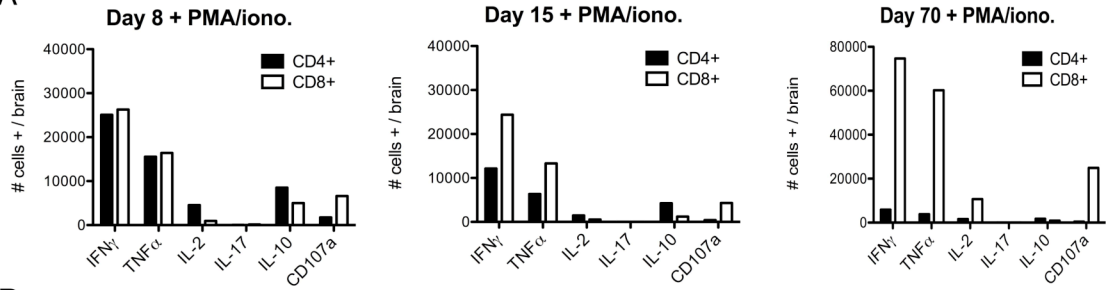
B

C

Figure 6: CD4+ T cells are the main producers of T cell-associated IFNγ within the brains of V3533-infected μMT mice. 7-10 week old female μMT mice were inoculated with 10^3 pfu of V3533 by injection in the left rear footpad. At the times indicated, mice were perfused with PBS and brain-infiltrating leukocytes were isolated. Harvested cells were then pooled and either (A) cultured in the presence of PMA/ionomycin for 6 hours with brefeldin A +/- monensin added for the final 4 hours, or (B) cultured in the presence of brefeldin A +/- monensin with no additional stimulus for 4 hours. Following treatment, cells were surface stained for CD3α, CD4, and CD8, and then stained for the intracellular presence of multiple cytokines. Each bar depicts the number of cells of a given cell type that stained positive for the indicated cytokine/surface marker per brain (days 8 & 15) or the percentage of pooled cells that stained positive (day 70). (C) Percentage of T cells positive for CD69 surface expression in the absence of *ex vivo* stimulation. Data shown was generated in a single experiment but is representative of 2-3 independent experiments.

Table 1. Percentage of brain-infiltrating T cells expressing indicated cytokines at day 70 post-infection

	CD4+ PMA/iono.	CD8+ PMA/iono.	CD4+ Unstim.	CD8+ Unstim.
IFNγ	62.03	77.96	1.92	0.0
TNFα	40.51	62.84	1.92	3.25
IL-2	16.91	11.19	1.32	0.0
IL-17	0.0	0.0	0.0	0.75
IL-10	18.75	0.93	0.4	0.57
CD107a	4.81	26.01	0.2	0.0

CHAPTER 4:

COMPARISON OF V3000 AND V3533 CELLULAR TROPISM WITHIN THE BRAIN

INTRODUCTION

Once VEEV has invaded the CNS, it predominantly replicates within neurons (2, 4, 6). Viral replication within these critical, difficult to replace cells results in cell death, usually through the induction of apoptosis (4). VEEV-induced killing of infected neurons is sufficient to induce mortality in mice (1). Viral infection of neurons presents a unique problem for the immune system in that indiscriminate cytolysis of infected neurons would most likely present a greater threat to the host than allowing the virus to replicate unchecked. Thus, non-cytolytic mechanisms of controlling viral replication and spread are thought to be favored within the CNS (3).

The extreme lethality of VEEV in mice is thought to result from its ability to efficiently infect and kill neurons within the CNS. One possible explanation for the outcome of V3533 infection, where viral replication within the CNS does not result in severe morbidity or mortality, is that the two mutations that differentiate V3533 from its parental virus V3000 alter the *in vivo* cellular tropism of the virus such that it no longer replicates in neurons. This is quite plausible, given that the two mutations, E76K and K116E, occur in the protein responsible for host cell binding, E2 (7). Preferentially replication of V3533 in some other, less critical CNS resident cell population, such as astrocytes, could explain why the immune-competent mice are able to rapidly clear the virus in the absence of severe morbidity.

Therefore, we asked whether the V3533 mutations altered the cellular tropism of VEEV within the mouse brain. To answer the question we used eGFP-expressing

VEEV replicon particles (VRP) that were packaged with the glycoproteins from either

V3000 or V3533 (5). These VRP should exhibit the exact same cellular tropism as

full-length virus bearing the same glycoproteins, but should be unable to propagate

beyond the initially infected cells. Following intracranial injection, we showed that

cells infected with either V3000- or V3533-packaged VRP stained positively for the

neuronal marker NeuN. Thus, the V3533 mutations do not alter the neuronal

tropism of VEEV *in vivo*.

MATERIALS AND METHODS

VRP production

VEEV replicon particles (VRP) expressing the enhanced green fluorescence protein

(eGFP) under the control of the 26S subgenomic reporter were packaged with either

the V3000 or the V3533 mutant envelope proteins, as described previously (5). For

assembly, the appropriate replicon and packaging RNAs were electroporated into

BHK-21 cells using a Bio-Rad electroporator. After confirming the absence of

propagating virus by passage in BHK-21 cells, VRP were concentrated by

ultracentrifugation and titers were determined by immunohistochemistry on BHK-21

cells.

Mouse studies

C57BL/6 mice were purchased from The Jackson Labs (Bar Harbor, ME) as needed.

For virus infections, 6-10 week old female mice were anesthetized via intra-

peritoneal (i.p.) injection with a mixture of ketamine (50 mg/Kg body weight) and

xylazine (15 mg/Kg body weight) and then injected directly in the brain with 10^6

infectious units of VRP in diluent [phosphate buffered saline (PBS) + 1% donor calf serum, Ca^{2+}, Mg^{2+}]. Mock-infected mice received diluent alone.

Immunohistochemistry

Mice were inoculated intracranially with VRP as described above. At 24 hours post-infection, mice were perfused with 4% paraformaldehyde (PFA). Brains were removed and fixed in 4% PFA overnight, and then rehydrated for 24 hours in 30% sucrose in PBS. Following rehydration, brains were frozen in Tissue-Tek OCT Compound (Sakura) using a histobath, and sectioned at 10 μm with a cryostat. Cryosections were mounted on poly-L-lysine-coated slides and air dried for 30 min. Slides were then stained with streptavidin-conjugated anti-NeuN (neuronal nuclei, clone A60; Chemicon), anti-GFAP (glial fibrillary acidic protein, clone GA5; Chemicon), and anti-CD11b (microglia, clone M1/70; eBioscience). Following incubation with the appropriate biotinylated secondary antibodies (anti-mouse IgG and anti-rat IgG; Vector), slides were incubated with a streptavidin-conjugated Alexa Fluor 546, washed and dried, and examined with a Nikon FXA microscope.

RESULTS AND DISCUSSION

The V3533 mutations do not significantly alter *in vivo* cellular tropism within the CNS

The mechanism underlying the attenuation of V3533 relative to the V3000 parental virus is not yet known. One potential explanation for how V3533 is able to replicate within the CNS without triggering death or even severe morbidity in immune-competent mice is that the mutations in E2 have altered the *in vivo* cellular tropism

of the virus such that it no longer replicates predominantly in neurons. If V3533

instead replicated in non-neuronal cells, cytolytic clearance of the virus could occur

without the widespread destruction of neurons. To test whether the *in vivo* cellular

tropism within the CNS of V3533 differed from that of V3000, we infected C57BL/6

mice i.c. with 10^6 IU of eGFP-expressing VRP, packaged with either the V3000 or

V3533 glycoproteins. Animals were sacrificed at 24 hours post-infection, and the

brains prepared as frozen sections. Analysis of V3000-eGFP-VRP-infected sections

by fluorescence microscopy revealed numerous eGFP+ cells within the brain

parenchyma. Staining of these sections with NeuN, a neuronal marker, GFAP, an

astrocyte marker, and CD11b, a microglial marker, revealed that eGFP+ cells

exclusively costained with the NeuN marker, indicating that the infected cells were

neurons (Fig. 3.1). Evaluation of V3533-eGFP-VRP-infected sections revealed a

similar pattern, with eGFP+ cells exclusively costaining with NeuN. Thus, V3533

appears to predominantly target neurons within the CNS, similar to V3000.

The fact that the V3533 mutations did not alter the neuronal tropism of VEEV *in vivo*

rules out one potential explanation for the unique attenuation pattern of V3533. By

confirming the neuronal tropism of V3533, these results confirm the relevance of

V3533 infection as a model for VEEV-induced disease and recovery. Further work

is needed to determine whether V3533 affects infected neurons differently than

V3000. The observation that V3533 is able to establish a persistent infection within

CNS neurons while V3000 is not, suggests that such differences may exist (1).

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Figure 4.1

	Anti-NeuN	GFP	Merge
V3000-eGFP-VRP			
V3533-eGFP-VRP			
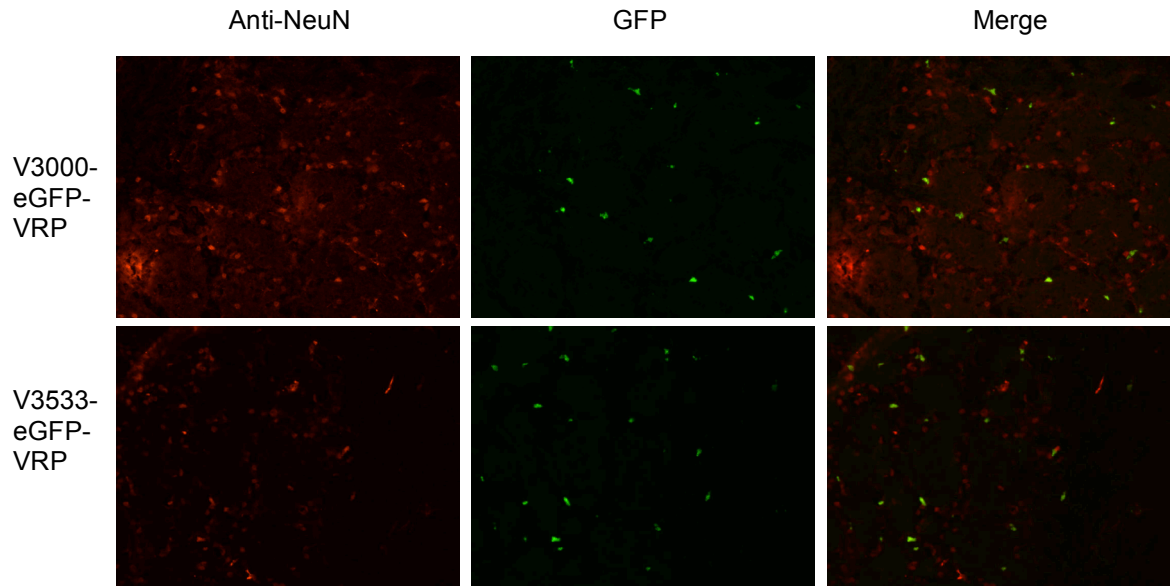
Figure 4.1: V3533 mutations do not alter the cellular tropism of VEEV within the CNS. Representative sections from C57BL/6 mice infected intracranially with 10^6 infectious units of either V3000-eGFP-VRP or V3533-eGFP-VRP in diluent. At 24 hours post-infection, brains were harvested and frozen sections generated. Sections were stained for NeuN (neuronal marker), and then evaluated on a Nikon FXA microscope. Images were merged using ImageJ software.

CHAPTER 5:

DISCUSSION AND FUTURE DIRECTIONS

All of the studies described here have taken advantage of the unique *in vivo* virulence pattern of the VEEV mutant V3533. Similar to its lethal parental virus, V3000, V3533 replicates and spreads efficiently in the periphery, replicating to high titers in multiple lymphoid organs and producing a serum viremia. This serum viremia is sufficient to facilitate viral invasion of the CNS, where V3533 replicates predominantly in neurons. Unlike lethal strains of VEEV, however, V3533 infection is halted within the CNS and cleared, resulting in the development of a mild, transient febrile illness rather than the uniform lethality that occurs when other strains of VEEV make it into the CNS. In many ways, this better replicates VEEV-induced disease in humans, where most symptomatic cases present with generic "flu-like" symptoms such as fever and headache, and very few immune-competent patients progress to overt encephalitis (15). In addition, as a model of a successful recovery from VEEV infection, V3533 infection of C57BL/6 mice allows the identification of host factors required for a successful immune response to VEEV using knock-out mice, *in vivo* depletions, and other standard immunological tools. Here, we have described studies that used this model to identify previously unappreciated protective roles for T cells and the complement system during VEEV infection. A great deal of further work is needed to better understand the mechanisms of attenuation for V3533, the actions of complement and T cells during VEEV infection, and the requirements for a successful host response to VEEV infection.

DETERMINATION OF THE MECHANISMS OF V3533 ATTENUATION

The course of infection and disease outcome following V3533 infection of C57BL/6

mice is unlike that of any other strain of VEEV. Attenuated VEEV mutants are

usually unable to spread beyond early sites of replication or invade the CNS (1, 2, 4,

16). Those strains of VEEV that do make it into the CNS are typically lethal. V3533

is unique in its ability to replicate within the CNS without inducing the death of the

animal. Determining the mechanisms underlying this attenuation pattern would both

expand our understanding of alphavirus pathogenesis and potentially provide new

attenuation strategies for use in live attenuated vaccines.

Efforts to determine where in the infection process V3533 differs from its lethal

parental virus V3000 would have to start in the periphery. Previous work has

suggested that V3533 and V3000 targeted different cells within the draining popliteal

lymph node (11). Using a combination of GFP-expressing VRP and double

promoter viruses, the *in vivo* cellular tropism of V3000 and V3533 at early sites of

replication should be more rigorously compared. Differences in the specific cell

populations that VEEV infects, and potentially modulates the behavior of, could

significantly affect the quality of the downstream immune response. To further

characterize the effects of the V3533 mutations on early interactions with the host, a

luminex-based comparison of V3000 and V3533 induced cytokine/chemokine

production at early sites of infection would also be useful. An additional approach,

that would also complement the targeting analysis, would be to compare the gene

expression profiles of V3000- and V3533-infected cells both within the draining

lymph node, as well as within cultured dendritic cells, using poly-A binding protein

(PABP)-expressing VRP (9). This would illuminate any differences in the responses to these two viruses at the level of the infected cell.

Of greater importance, most likely, would be an analysis of the interactions between V3533 and neurons. The fact that V3533 is able to replicate to high titers within CNS neurons without inducing severe morbidity or mortality suggests that there are important differences in the ways that V3000 and V3533 interact with these cells. The primary function of the E2 glycoprotein is to facilitate cellular attachment, however studies with Sindbis virus have suggested an additional role for E2 as a determinant of apoptosis induction following infection (6, 10). Thus, the E2 mutations present in V3533 could influence replication and spread within CNS, or the eventual fate of infected neurons. Preliminary studies could be carried out in primary cortical neuron cultures. These cells could be used to compare the relative neuronal infectivities and replication kinetics of V3533 and V3000. The use of PABP-expressing VRP would be useful for determining whether different patterns of neuronal gene expression are induced by the two viruses. Another key experiment would be to compare the kinetics and magnitude of neuronal cell death following V3533 and V3000 infection. One possible explanation for the attenuation of V3533 is that it is less efficient at killing infected neurons *in vivo*.

The limited anatomical distribution of inflammation within the brains of V3533-infected C57BL/6 mice suggests that V3533 is restricted in its ability to disseminate within the brain. Though the exact mechanism of cell-cell spread of VEEV between

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neurons is not known, there is evidence that the virus follows established neural networks *in vivo*, suggesting a predominant role for trans-synaptic transmission (3). *In vitro* studies using compartmentalized neuronal cultures could shed more light on the exact mechanisms of neuron-neuron spread (13). Infecting mice intra-nasally with V3533 and V3000, and then tracking dissemination within the CNS by in situ hybridization over short time intervals would indicate whether V3533 was defective in spread *in vivo*.

Finally, the V3533 system remains a virtually untapped resource for dissecting the host response to VEEV infection. Further screening of different knock-out mouse strains could identify further unappreciated components of a successful immune response to VEEV.

FURTHER EXPLORATION OF VEEV-COMPLEMENT INTERACTIONS

The infection of C57BL/6 and C3$^{-/-}$ mice with V3533 revealed a role for the complement system in limiting the severity of VEEV-induced disease. More specifically, we showed that complement activation within the first 24 hours of infection limited the efficiency of neuroinvasion and the extent of downstream viral replication and inflammation within the brain. These results demonstrated that early virus-immune interactions can significantly influence the outcome of infection days later at distal anatomical sites. In addition, these studies suggested an important role for natural antibody in restricting VEEV-induced pathology. Finally, in contrast

with what has been observed during infection with many other viruses, the induction of an anti-VEEV antibody response was not dependent upon complement activation.

Our efforts to understand the complement-dependent mechanisms that are important in determining disease outcome following V3533 infection are still underway. As a result, some of the data presented here is still preliminary. The histopathology analysis shown in Figure 2 of the complement chapter grossly illustrates that there are differences in the extent of inflammation within the brains of wild-type and C3$^{-/-}$ mice, however blinded scoring of a more extensive time-course of sections is needed to present a more definitive picture of the effect of complement activation on CNS pathology and inflammation. Efforts to assess the complement dependence of the anti-VEEV neutralizing activity of natural antibody still need quite a bit of work. The *in vitro* anti-VEEV neutralization experiments using normal serum need to be repeated, with a tighter dilution curve to generate more robust IC50 values for comparison. In addition, the experiment comparing the inflammatory cell populations present in the draining lymph nodes of V3533-infected wild-type and C3$^{-/-}$ mice needs to be repeated with larger group sizes and earlier timepoints. Finally, the comparison of type I IFN levels at early replication sites needs to be repeated for earlier timepoints, starting just a few hours after inoculation. The completion of these experiments will allow us to draw more definitive conclusions about the effect of complement activation on inflammatory cell recruitment and activation.

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Our work to date strongly suggests a role for complement activation in facilitating the clearance of VEEV from the serum and, as a result, delaying the arrival of VEEV in the CNS. Future work should aim to uncover the molecular interactions between VEEV, natural antibody, and complement that govern this effect. Determining which complement activation pathways are being triggered by VEEV infection would be a good start. Preliminary studies suggest that the protective effect of complement *in vivo* is dependent upon C4, but not C1q, suggesting that complement is primarily activated through the lectin pathway during VEEV infection (data not shown). Further *in vitro* experiments assessing complement activation and anti-VEEV neutralization using serum deficient in each of the three activation pathways could better address this question. To determine which complement components are binding and potentially opsonizing VEEV particles, virus could be immuno-precipitated either following serum incubation *in vitro*, or directly from the serum of viremic mice, and bound complement components could then be detected by western blot. Additional *in vivo* examination of the effects of complement activation on VEEV serum clearance kinetics could either support or contradict our hypothesis that serum clearance and neuroinvasion are linked. Comparing the actual clearance rate following intravenous injection of VEEV between wild-type and different complement component knock-out mice would be a good first step in this area.

Though the recruitment of inflammatory cells to early sites of V3533 replication appears to be complement independent, we cannot yet rule out a role for complement-dependent regulation of the effector phenotypes of those cells. The

importance of complement activation in regulating the activation status, but not the recruitment, of inflammatory cells during infection with another alphavirus, Ross River virus, highlights the importance of addressing this issue (12). A good starting point would be to isolate inflammatory cells from wild-type and C3$^{-/-}$ mice within the first 24 hours of infection, and to compare gene expression levels by microarray. Candidate genes that are found to be regulated by complement activation could be further investigated for potential anti-VEEV activity.

Additional work will be focused on determining the role of C5 cleavage during V3533 infection. Some preliminary work using C5-deficient mice has suggested that C5 cleavage-dependent mechanisms play a role in limiting the development of overt encephalomyelitis following V3533 infection (data not shown). Studies are currently underway to confirm that V3533-induced disease is more severe in the absence of C5, as well as to assess the effects of C5 on serum clearance and neuro-invasion kinetics. The relevance of these studies to those performed in the C57BL/6 and C3$^{-/-}$ mice is limited, however, by the fact that the C5-deficient mice are in a different genetic background.

Finally, the complement-independent induction of a robust anti-VEEV antibody response raises the question of what other virus-induced factors enhance the anti-VEEV antibody response. This question could be addressed initially by shutting down the various signaling axes known to influence B cell activation through the use of knock-out mice and/or adoptive transfer of B cells from knock-out mice.

Answering this question could be useful in evaluating future live attenuated vaccine candidates.

FURTHER INVESTIGATION OF T CELL RESPONSE DURING VEEV INFECTION AND VEEV PERSISTENCE

Our studies of V3533 infection of μMT mice resulted in several intriguing findings. First, T cells can control VEEV replication within the CNS in the absence of antibody, facilitating recovery from infection. Second, CD4+ T cells were better able to control VEEV infection within the CNS, relative to CD8+ T cells. CD8+ T cells did not produce detectable amounts IFNγ within the brain during V3533 infection, despite being fully capable of producing large amounts in response to *ex vivo* PMA/ionomycin treatment. Finally, in the absence of antibody, V3533 was able to establish a persistent infection in the brain in the absence of apparent morbidity.

The persistent infection that we observed in the μMT mice was surprising, and raised a number of intriguing questions. It would be interesting to determine which specific cell population is maintaining the infection. This could possibly be determined through careful in situ hybridization experiments on brain sections taken late in infection. If there is a specific subset of neurons that is completely refractory to T cell-mediated clearance, it would interesting to know why. Additionally, it would be worthwhile to fully sequence virus isolates from different anatomical sites at late times post-infection. This might reveal viral sequence elements that influence interaction with neuronal cells or viral susceptibility to T cell-induced effector

mechanisms. Finally, while μMT mice persistently infected with V3533 appear outwardly normal in their behavior, more in depth examination of their cognitive function might reveal subtle defects. This would allow us to better assess whether these mice have truly recovered from infection.

One of the most surprising results of these studies was that CD4+ T cells, in the absence of B cells or CD8+ T cells, were able to significantly reduce VEEV titers within the CNS. Neurons are not thought to express MHC class II, so the mechanism by which VEEV-specific CD4+ T cells were able to recognize infected cells is unclear. Microglia and astrocytes are the only CNS-resident cells thought to express MHC class II, so one possibility is that these cells play some role in facilitating contact between VEEV-specific T cells and infected neurons (5). Identifying the mechanism by which CD4+ T cells are recognizing VEEV-infected neurons would be interesting, but quite difficult, and would probably require visualizing the interaction using labeled cells. This could possibly be performed in either *ex vivo* brain slice cultures, or through live animal imaging (8, 14).

Another interesting observation was the lack of IFNγ production by CD8+ T cells in the brains of V3533-infected μMT mice. This was not due to a generalized defect in the CD8+ response in these mice, as CD8+ T cells produced significant amounts of another Th1-associated cytokine, TNFα, and were fully capable of producing IFNγ *ex vivo* following PMA/ionomycin treatment. Instead, it suggests that IFNγ production by CD8+ but not CD4+ cells was being specifically suppressed in the

brain during V3533 infection. This finding is similar to the observation that IL-2 production was specifically repressed in the brains of Sindbis virus infected mice (7). To shed light on the mechanism underlying this phenomenon, CD8+ T cells isolated from the spleens and brains of V3533-infected μMT mice could be compared to determine whether the lack of IFNγ production was brain-specific. If IFNγ production was detectable in the spleen, microarray-based comparisons of spleen- and brain-infiltrating CD8+ T cells would indicate whether there were corresponding differences in gene expression. The lack of reagents for identifying VEEV-specific T cells would complicate this and other potential experiments. Regardless, pursuing this finding could potentially shed some light on the unique immune-regulatory environment within the CNS.

Much has been made in the alphavirus field about the role of IFNγ in clearing virus from infected CNS neurons. Our work here did not directly address this issue, but did find a correlation between IFNγ production and antiviral activity in T cells during V3533 infection. Future studies should more directly address the relationship between IFNγ, VEEV, and neurons *in vivo*. Effects seen in IFNγ- or IFNγR- knockout mice are complicated by an inability to separate the direct effects of IFNγ on infected neurons from other effects in the periphery. Additionally, teasing apart the antiviral and immune-regulatory functions of IFNγ will be difficult. A good starting point might be to use the transfer of IFNγ-knockout T cells prior to V3533 infection to ascertain the importance of T cell-derived IFNγ within the CNS.

The overall purpose of these studies was to determine whether T cells exerted any anti-VEEV effect, independent of antibodies. While we were able to demonstrate that T cells could be quite potent in restricting VEEV replication within the CNS in μMT mice, more work is needed to determine the importance of T cell-mediated clearance mechanisms in immune-competent animals. It is quite possible that the protective effects of anti-VEEV antibodies are so strong, they render T cell-mediated effects superfluous. Additionally, the potential protective effects of memory T cells need to be better studied. Efforts are currently underway to determine if memory T cells generated during V3533 infection can protect naïve mice from lethal VEEV challenge. Answering these questions will help determine how important the development of T cell effector function should be as a criterion for evaluating future encephalitic alphavirus vaccine candidates.

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