A MONOCLONAL ANTIBODY-BASED THERAPY AGAINST CNS-RESIDENT LYSSAVIRUS INFECTION

by

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DEDICATION

For my husband Frank and my family in New York.

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November 19, 2018

ABSTRACT

A monoclonal antibody-based therapy against CNS-resident lyssavirus infection:

Kate E. Mastraccio, Doctor of Philosophy, 2018

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Australian bat lyssavirus (ABLV) is a recently identified rabies-like virus that causes fatal disease in humans and horses. Clinical disease in humans is identical to that of rabies with individuals experiencing symptoms such as ataxia, muscle spasms, difficulty swallowing, paralysis, and encephalitis. Lyssavirus infection is thought to be universally fatal following central nervous system (CNS) involvement, and there are no effective therapies for rabies infections beyond clinical disease onset. In this study, we evaluated the therapeutic efficacy of F11, an anti-lyssavirus human monoclonal antibody (mAb), on established lyssavirus infection in mice. Via longitudinal tracing of lyssavirus infection in a mouse model of lethal disease, we found that a single dose of F11 mAb clears ABLV or rabies infections, even after onset of symptoms and demonstrable CNS involvement. Clearance depends on the host adaptive immune response, with CD4 T cells serving as the primary effector population. These preclinical data thus offer compelling evidence that human infections with rabies and related lyssaviruses may be treatable during the post-symptomatic stage of infection.

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CHAPTER 1: Introduction

LYSSAVIRUS OVERVIEW

Rabies is a fatal viral disease that infects wild and domestic animals and is transmissible to humans. The causative agent of this disease is Rabies virus of the genus *Lyssavirus* in the family *Rhabdoviridae*. Lyssaviruses specifically target the CNS of mammals, causing acute progressive encephalitis (307). It is estimated that 40,000-70,000 people die of rabies annually, with the majority of deaths occurring in Africa and Asia (96). While Rabies virus is regarded as the most prominent member of the *Lyssavirus* genus, there are several other lyssaviruses capable of causing fatal rabies-like disease including ABLV, Mokola virus (MOKV), and European bat lyssavirus 1 and 2 (EBLV-1, EBLV-2). Despite existing vaccines and post-exposure prophylaxis, rabies remains a neglected disease that is poorly controlled in developing countries and is still considered a serious threat to public health.

LYSSAVIRUS TAXONOMY

The *Rhabdoviridae* family is included in the order *Mononegavirales* along with the *Paramyxoviridae*, *Bornaviridae*, and *Filoviridae* families. Viruses belonging to this order are enveloped and contain a non-segmented, linear, negative-sense, single-stranded RNA genome (103). Viruses of the *Rhabdoviridae*, *Filoviridae*, and *Paramyxoviridae* families share a similar genome organization comprised of a 3' untranslated region, genes encoding core proteins, envelope protein, and an RNA-dependent RNA polymerase. Also in common are their complementary 3' and 5' termini and the presence of a single 3' promoter to initiate transcription (103). The *Rhabdoviridae* family contains six genera, designated by their serological cross-reactivity, pathobiology, and evolutionary

relatedness. The *Lyssavirus* genus, whose name derives from *Lyssa*, the Greek goddess of raging madness, contains viruses that are genetically and antigenically related. Demarcation of species within the *Lyssavirus* genus is based on serological cross-reactivity, genetic distances, topology, host and geographic range, and ecological and pathological characteristics (102; 103). The *Lyssavirus* genus contains 12 genotypes: Rabies virus (RABV) (genotype 1), Lagos bat virus (LBV) (genotype 2), MOKV (genotype 3), Duvenhage virus (DUVV) (genotype 4), EBLV-1 (genotype 5), EBLV-2 (genotype 6), ABLV (genotype 7), Aravan virus (ARAV) (genotype 8), Khujand virus (KHUV) (genotype 9), Irkut virus (IRKV) (genotype 10), West Caucasian bat virus (WCBV) (genotype 11), and Shimoni bat virus (SHIBV) (genotype 12). Bokeloh bat lyssavirus (BBLV), Ikoma lyssavirus (IKOV), Lleida bat lyssavirus (LLEBV), and Gannoruwa bat lyssavirus (GBLV) have not been classified as distinct genotypes (103; 153; 173). Lyssaviruses are also divided into phylogroups based on serological cross-reactivity and genetic distances with RABV, ABLV, EBLV-1, EBLV-2, DUVV, ARAV, IRKV, KHUV, BBLV, and GBLV belonging to Phylogroup I, LBV and MOKV belonging to Phylogroup II, and WCBV, SHIBV, IKOV, and LLEBV belonging to Phylogroup III (158; 371) (Table 1). According to phylogenetic analysis, all lyssaviruses have been isolated from bats and have bat origins, not including MOKV and IKOV (25; 32; 219-221). Indeed, the greatest diversity of lyssaviruses occurs in bats in Africa and Eurasia (126; 171). Notably, human cases have been documented in RABV, MOKV, EBLV-1, EBLV-2, ABLV, and IRKV.

GENOME ORGANIZATION OF LYSSAVIRUSES

The lyssavirus genome is comprised of a negative-sense single-stranded RNA

Table 1. Lyssavirus classification by genotype and phylogroup.

Lyssaviruses	*Genotype*	*Phylogroup*
RABV	1	I
LBV	2	II
MOKV	3	II
DUVV	4	I
EBLV-1	5	I
EBLV-2	6	I
ABLV	7	I
ARAV	8	I
KHUV	9	I
IRKV	10	I
WCBV	11	III
SHIBV	12	III
BBLV	*	I
IKOV	*	III
LLEBV	*	III
GBLV	*	I

*According to the International Committee on the Taxonomy of Viruses (ICTV), these viruses are not yet classified as distinct lyssavirus genotypes.

genome that is approximately 12kb in length. The genome contains a 3' leader sequence of ~50 nucleotides followed by genes encoding structural proteins: nucleoprotein, phosphoprotein, matrix protein, glycoprotein, and RNA-dependent RNA polymerase, and a 5' trailer sequence of ~70 nucleotides. (30; 222; 350) (Figure 1). Inverted complementary sequences located at the 3' and 5' ends encode initiation signals for transcription and replication.

Nucleoprotein

The nucleoprotein (N) (450 amino acids) associates with the full-length negative-sense RNA genome as well as the full-length positive-sense RNA intermediate, protecting each from ribonuclease activity. Each N protein will bind to ~9-10 nucleotides, enwrapping the genomic RNA into a helical nucleocapsid (N-RNA complex).

Phosphoprotein

The phosphoprotein (297 amino acids) (P) serves as a non-catalytic cofactor of the viral polymerase complex and mediates the appropriate positioning of RNA-dependent RNA polymerase on the nucleocapsid, facilitating its access to the template. During synthesis of nascent nucleoprotein, the phosphoprotein complexes with nucleoprotein to prevent self-aggregation and binding to cellular RNA (11).

Matrix Protein

The matrix protein (202 amino acids) (M) is located under the viral envelope and bridges the nucleocapsid and the cytoplasmic domain of the glycoprotein. M binds and condenses the nucleocapsid into a tightly coiled complex, initiating viral budding from the host cell (253).

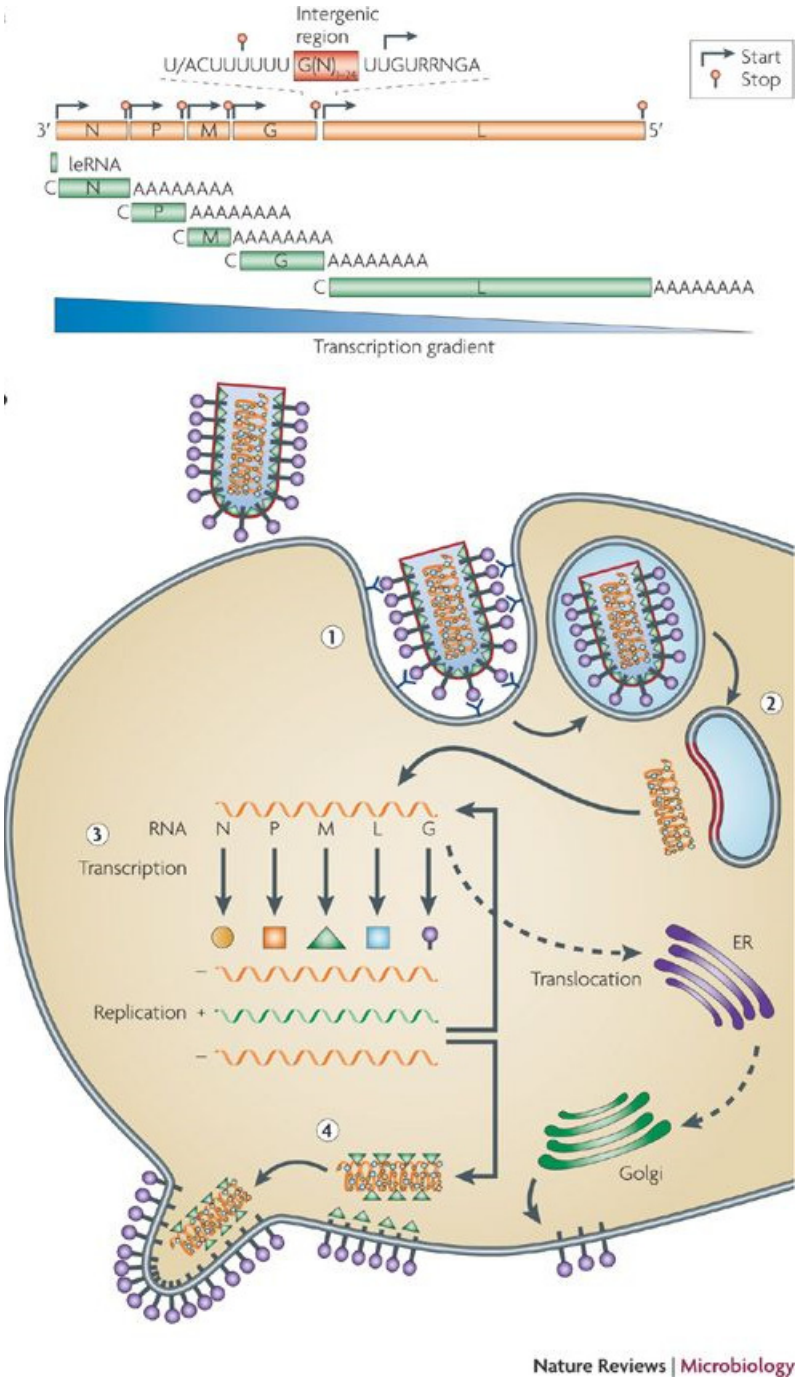


Figure 1. Genome organization, transcription and replication of lyssaviruses. Adapted from (316). Figure reprinted with permission from Springer Nature Publishing Group.

Glycoprotein

The glycoprotein (505 amino acids) (G) is anchored in the lipid bilayer of the viral envelope by a 20 amino acid hydrophobic transmembrane domain followed by a 44 amino acid cytoplasmic domain. The glycoprotein is organized into trimers on the surface of the virion, forming individual spike-like projections that mediate attachment to host cell receptors. The glycoprotein induces cell-mediated immunity and the production of neutralizing antibodies (64; 66; 92). Several studies have suggested a role for the glycoprotein in transport of the virus to the CNS (101; 115; 248).

RNA-dependent RNA polymerase

The RNA-dependent RNA polymerase (L) (2142 amino acids) is the catalytic component of the viral polymerase complex. Along with the non-catalytic cofactor P, it mediates transcription and replication of the viral genome. L also contains a cap methyltransferase domain, mRNA 5' capping enzyme, a 3' poly A polymerase, and protein kinase domains.

LYSSAVIRUS LIFE CYCLE

Host cell receptors

The first step in the lyssavirus life cycle is entry into a host cell, which is mediated by attachment of the viral glycoprotein to a host cell receptor. Indeed, several studies have demonstrated the essentiality of the glycoprotein for host cell entry. For instance, a major determinant of pathogenicity of a tissue culture-adapted RABV strain (ERA) is located in antigenic site III of the G protein (108). A single amino acid substitution (arginine to glutamine) at position 333 in this antigenic site of the ERA G protein resulted in delayed internalization compared to WT ERA. However, an

asparagine$_{194}$ to lysine$_{194}$ mutation in ERA G resulted in significant decrease in internalization time and re-emergence of a pathogenic phenotype (117). While it is unclear which receptors mediate RABV entry, several putative receptors have been identified. The nicotinic acetylcholine receptor (nAchR) was identified as a possible receptor for RABV in 1982 (232). Given that nAchRs are expressed on muscle cells and that RABV can replicate within muscle cells before entering peripheral nerves, nAchRs are thought to be used by RABV to infect muscle cells (264; 368). Furthermore, nAchRs are also expressed on the postsynaptic side of the neuromuscular junction (NMJ) and are thought to enrich RABV at the NMJ, enabling more efficient infection of the axon terminal across the synaptic cleft (366). Proposed receptors for entry into axon terminals include neuronal cell adhesion molecule (NCAM) and nerve growth factor receptor (p75NTR) (228; 346; 352). Indeed, several studies have demonstrated that RABV-resistant cell lines are permissive to infection following induction of NCAM or p75NTR expression (346; 353). However, although RABV infection is delayed in NCAM-deficient mice, mice ultimately succumb to infection, suggesting that NCAM is not essential for viral infection *in vivo* (346). Similarly, p75NTR-deficient mice exhibit comparable disease progression to that of wild-type mice (353). Lipids, carbohydrates, and gangliosides have also been suggested as RABV receptors but their function is not clear (89; 337; 388).

Internalization

To gain access to the cytoplasm of the host cell, RABV utilizes clathrin-mediated endocytosis (286; 287). Following attachment to the host cell receptor, clathrin-coated pits (CCPs) are formed *de novo* (93; 111). Additionally, several studies have

demonstrated that actin is required for completion of virus internalization (93; 286). Following invagination, the CCPs are released from the host cell plasma membrane and sheds its clathrin coat. The uncoated vesicle is then transported to and fuses with early endosomes. The acidic environment of the endosome induces conformational changes in the glycoprotein, catalyzing the fusion of the viral and endosomal membranes, thereby releasing the nucleocapsid into the cytoplasm of the host cell (11; 350) with membrane fusion occurring in early (pH 6.5-6.0) or late (pH 6.0-5.0) endosomes (258). St. Pierre et al. demonstrated that RABV G co-localizes with Rab5a, a small GTPase that acts as a key regulator of early endocytosis. Furthermore, functional Rab5a was found to be essential for internalization of an anti-RABV G Ab/RABV G complex, suggesting that the glycoprotein fuses with early endosomes (333).

Transcription and Replication

Once RABV has migrated to the cytoplasm of the neuronal cell body, the next stage of the virus life cycle can commence. Here, the nucleocapsid is recognized by L as the template for transcription. Transcription generates monocistronic positive-sense mRNAs encoding the leader RNA and the five structural proteins (11). All of the mRNAs produce a single protein from a single open reading frame (ORF) at the first AUG codon with the exception of P, which produces 3-4 proteins from downstream AUG codons (81). During transcription, RNA-dependent RNA polymerase recognizes conserved initiation and termination signals along the negative sense-RNA genome corresponding to 5' capping and 3' polyadenylation of each mRNA. Each cistron is separated by intergenic regions of 2-100 nucleotides that remain untranscribed. Following polyadenylation of each mRNA, L reinitiates transcription at the start signal of the

subsequent mRNA. Since the length of the intergenic region influences the rate of re-initiation, the amount of mRNA transcribed decreases from the 3' end, resulting in a transcriptional gradient (N>P>M>G>L). In the initial stages of transcription, the amount of N is only sufficient to encapsidate the leader RNA. The mRNAs encoding the five structural proteins do not encode the encapsidation signal and are directed to translational machinery. Viral mRNAs are translated on polysomes within the cytoplasm, with the exception of glycoprotein mRNA which is translated by ribosomes bound to the rough endoplasmic reticulum (ER). Matrix protein is synthesized as a soluble protein that associates with the cytoplasmic side of the host plasma membrane while G is first transported from the ER to the Golgi apparatus for post-translational modifications before being transported to the plasma membrane. When sufficient amounts of N have been synthesized to encapsidate nascent RNA, L ignores initiation and termination signals and synthesizes full-length positive-sense antigenome, thereby switching from transcription to replication. The full-length antigenome then serves as a template for synthesis of progeny negative-sense genome (11; 387). Genomes and antigenomes are encapsidated as they are synthesized and progeny nucleocapsids are used as templates for additional rounds of replication, secondary transcription, and for assembly into progeny virions.

The pathogenicity of RABV is inversely correlated with the rate of viral RNA synthesis and the production of infectious virions. For example, the pathogenicity of an attenuated strain of RABV (SN) was significantly increased after replacing G or M or both with those of a highly pathogenic strain (SB). This increase in pathogenicity was associated with a significant decrease in the rate of viral of RNA synthesis as well as in the rate of production of virus particles (291).

Assembly and budding

In the final stage of the RABV life cycle, viral components are transported to the host cell plasma membrane. The helical nucleocapsid associates with L and P, forming the ribonucleoprotein (RNP), also known as the internal core or capsid of the virion. M covers the RNP and maintains its condensed form and interacts with and recruits G into the assembling virion. As the virus buds from the host cell, it acquires its envelope consisting of the host cell-derived lipid bilayer.

LYSSAVIRUS PATHOGENESIS

Spread to the CNS

A major determinant of lyssavirus pathogenesis is the neurotropic nature of the virus. However, lyssaviruses typically replicate at the site of inoculation before invading the nerves that innervate muscle tissue (Figure 2). Indeed, experimental intramuscular inoculation of skunks and hamsters with street RABV revealed viral replication within striated muscle before entering axons of motor neurons (70; 264). In some cases, RABV could be detected within muscle up to 2 months post-inoculation (74). Conversely, intramuscular infection of mice with the challenge-virus standard-24 (CVS-24) strain of RABV revealed that the virus directly enters the CNS without prior replication at the site of inoculation (320).

From the infected muscles, the virus enters axon terminals of motor neurons (see Host cell receptors) and migrates to the cell body of the neuron by retrograde axonal transport (196; 342). Transport through the axon to the cell body of the neuron (soma) is required for transcription and replication within the neuron (210) (Figure 2). By hitchhiking axonal vesicle transport, RABV is able to migrate at a rapid pace of 3mm/h

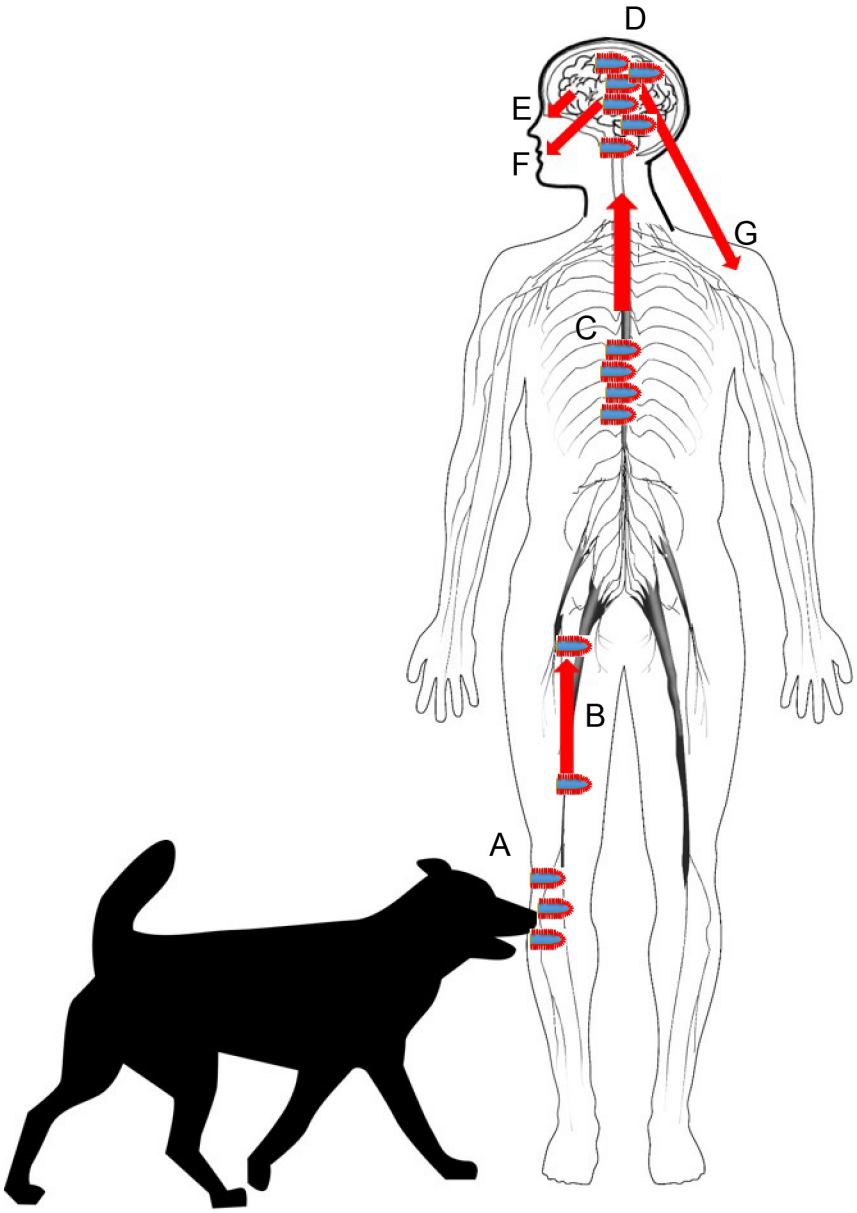


Figure 2. Lyssavirus pathogenesis. Following the bite or scratch from an infected animal, the virus replicates at the site of inoculation before entering nerve endings at neuromuscular junctions (A). The virus then travels to the spinal cord via retrograde transport in axons (B). Once in the spinal cord, replication takes place in the dorsal root ganglia and spinal interneurons (C). The virus ascends to the brain to replicate mainly within the brain stem, hippocampus, and cerebellum (D). Centrifugal spread of the virus to extraneural organs leads to infection of the eye (E), salivary glands (F), skin (G), and skeletal and cardiac muscle.

(351). Several groups have suggested that RABV utilizes the cytoplasmic dynein motor complex for transport based on findings that the viral phosphoprotein interacts with dynein light chain 8 (LC8) (186; 294). Under this hypothesis, direct attachment of rabies P to the dynein LC8 would enable axonal transport of viral proteins or the ribonucleoprotein complex (N, P, L, and genomic RNA). However, other studies have demonstrated that interaction of phosphoprotein and LC8 is not required for RABV transport to the neuronal cell body (252; 341). Others have suggested that the viral glycoprotein is a ligand for vesicular transport of whole virions through the p75NTR (248; 384). Indeed, Klingen et al. demonstrated that RABV virions remain within the endosome during transport to the neuronal cell body but the mechanism remains unclear (202).

Extensive study of RABV infection in animal models has provided insight into the early events of lyssavirus infection of the CNS. In one study, striped skunks were inoculated intramuscularly in the right hindlimb with a pathogenic strain of RABV. Immunohistochemical analysis revealed virus entry into the lumbar region of the spinal cord with replication at the L2 and L3 levels of the spinal column (72; 124). Similarly, in mice infected with pathogenic strains of RABV (isolate from bat and CVS-11), virus could be detected in the thoracic region of the spinal cord 4 days post-infection with ascension to the cervical region and brain on days 5 and 6 post-infection, respectively (207; 240; 278).

Interneuronal spread

Within days of infection of motor neurons, local spread of virus in the spinal cord can result in infection of connected spinal interneurons and dorsal root ganglia, which are

clusters of neurons in a dorsal root of a spinal nerve (72; 265; 335). The transneuronal transfer of virus allows ascension from the spinal cord to the brain. Immunohistochemical studies of RABV distribution in the brain of several domestic and wildlife animal species found that the virus predominantly infects the brainstem, cerebellum, hippocampus, and cerebrum (334).

RABV infection spreads between neurons that are connected by synaptic junctions but the mechanism facilitating interneuronal spread remains unclear. It is thought that infectious naked nucleocapsids cross synapses based on the observation that budding of virus is rarely seen at synapses via electron microscopy (143). However, recent studies have identified that the RABV glycoprotein plays a predominant role in the transneuronal spread of the virus. For instance, intracranial inoculation of mice with a G-deficient recombinant RABV revealed that virus was restricted to neurons at the inoculation site without spreading to secondary neurons (115). Furthermore, a single amino acid substitution (arginine to glutamine) at position 333 of ERA G protein results in a loss of pH-dependent cell fusion *in vitro* as well as a reduction in viral spread from cell-to-cell, suggesting that the viral glycoprotein plays a key role in cell-to-cell spread and thus viral transport (91; 107; 214; 262).

Infection of extraneural organs

In the final stages of infection, centrifugal spread of RABV from the CNS to extraneural organs via their sensory innervations is essential for transmission (Figure 2) and has been shown to involve central, peripheral, and autonomic nervous systems (266). For instance, following migration to salivary glands, RABV replicates in mucogenic acinar cells and is shed into the saliva, facilitating transmission to the next host (71). In

addition to salivary glands, RABV can also infect the retina, cornea, skin, gastrointestinal tract, liver, pancreas, and skeletal and cardiac muscle (28; 99; 185; 204). In addition, immunoperoxidase staining for RABV antigen in postmortem tissue sections of human rabies cases revealed virus within salivary glands of the tongue, muscle fibers of the heart, tongue, and larynx, as well as the gastrointestinal tract (185).

LYSSAVIRUS-INDUCED CNS PATHOLOGY

Regarding RABV-induced pathology within the brain, experimental infections of mice with a fixed strain of RABV have proposed apoptotic cell death as a potential pathogenic mechanism (184) while infection of mice with silver-haired bat RABV does not result in apoptosis (391). Similarly, hemorrhages or tissue necrosis is not commonly observed within the brain of RABV-infected mice (184). Fu et al. found the expression of several housekeeping genes to be significantly reduced in RABV-infected neurons, resulting in a generalized inhibition of protein synthesis, suggesting that RABV infection causes CNS dysfunction through impairment of neuronal function (129). Following natural or experimental infections with RABV, several pathological features can be observed including: non-suppurative encephalitis, necrosis of neurons, destruction of nerve cells by phagocytes, lymphocytic infiltrates, and morphological changes in glial cells (69; 73). However, such features are not uniform in all rabies infections. For instance, human cases of ABLV and MOKV exhibit pronounced CNS inflammation in contrast to human RABV infections, in which extensive inflammation is a rarity (155; 263). For instance, encephalitic changes induced by RABV infection in the brain can vary in severity and distribution and appears to be strain dependent. Indeed, apoptosis, necrosis, and perivascular cuffing are evident throughout the midbrain, pons, thalamus,

and hypothalamus of mice infected with a street RABV isolate (bat isolate) while such features are absent in the brains of mice infected with silver-haired bat rabies virus (SHBRV) (336; 391).

LYSSAVIRUS RESERVOIRS

Rabies has been documented on all continents, with the exception of Australia and Antarctica. However, Australia is home to ABLV, with confirmed cases of documented in bats, horses, and humans. In fact, all Australian bat species are considered to be potential host reservoirs of ABLV (371). Variants (or strains) of RABV are maintained in several different host reservoirs throughout the world. These variants undergo genetic adaptation to each host which results in new clades of RABV (97; 156; 276; 296; 355) . In the New World, RABV reservoirs include bats and terrestrial carnivores. Bat species most commonly associated with RABV infection in the Americas are as follows: Big brown bat (*Eptesicus fuscus*), Mexican/Brazilian free-tail bat (*Tadarida brasiliensis*), Silver-haired bat (*Lasionycteris noctivagens*), Tri-coloured bat (*Perimyotis subflavus*), and the Vampire bat (*Desmodus rotundus*) (31). The small Indian mongoose (*Herpestes auropunctatus*) is the primary reservoir for rabies on the islands of Cuba, Puerto Rico, Grenada, and the Dominican Republic (270; 271). In Canada, Arctic RABV circulates amongst Arctic Foxes (*Vulpes lagopus*), red foxes (*Vulpes vulpes*), and the striped skunk (*Mephitis mephitis*) (218; 268). While dog rabies in Latin America has declined in the past decade, bats are still considered an important reservoir (114). Within the Unites States, primary reservoirs of RABV include raccoons (*Procyon lotor*), bats (multiple species), skunks (*Mephitis spp*), and foxes (*V. lagopus* and *Urocyon cinereoargenteus*) (45). Raccoon and south-central skunk RABV are phylogenetically-

linked with bat RABV, yet neither lineage is associated with a specific bat RABV lineage (25; 269). Furthermore, it is unclear whether the raccoon RABV lineage emerged from the south-central skunk or a bat RABV lineage. In North America, skunks are very permissive to infection with various RABV lineages and are often infected by the raccoon RABV whereas raccoons tend to be infected exclusively with raccoon RABV (46; 47). Several species of foxes, coyotes (*Canis latrans*), the north central-skunk, and the California skunk belong to a 'cosmopolitan' RABV lineage, which likely originated from dogs.

In the Old World, terrestrial carnivores are reservoirs for RABV but not bats, as detection of RABV in Old World bats has not been confirmed (31; 216; 217). While sporadic cases of rabies in wildlife do occur throughout Africa, maintenance of RABV in wildlife populations exists only in South Africa (3). The two main variants of RABV in South Africa are the mongoose and canid RABV variants (198; 276). In particular, the yellow mongoose (*Cynictis penicillata*) is the main reservoir and vector for mongoose rabies and maintains a distinct RABV variant from the slender mongoose (*Galarella sanguinea*) (43; 125; 199; 275; 340). The African civet (*Civettictis civetta*) and honey badger (*Mellivora capensis*) are also reservoirs of mongoose RABV (43). Reservoirs of canid RABV include the domestic dog (*Canis familiaris*), the bat-eared fox (*Otocyon megalotis*), the side-striped jackal (*Canis adustus*), and the black-backed jackal (*Canis mesomelas*) (41; 42; 276; 310; 311; 345). However, several studies suggest that domestic dogs are solely responsible for maintenance of rabies in some South African ecosystems and that jackals are nonessential to maintenance (86; 231; 299).

In South Asia, mongoose (*Herpestes* spp.), golden jackals (*C. aureus*), Indian

foxes (*V. bengalensis*), and wolves (*Canis lupus*) are suggested as wildlife reservoirs of RABV (142). Interestingly, mongoose in South Asia maintain circulation of dog-related RABV (97; 275; 354). There is also evidence of 'cosmopolitan' RABV circulating amongst domestic dogs and wild canids (raccoon dogs, red foxes, and wolves) in Eurasia as well as Africa (25; 200; 269; 329). In East Asia, raccoon dogs (*Nyctereutes procyonoides*) maintain Artic "like" RABV and contribute to circulation of 'cosmopolitan' RABV in areas that are typically enzootic for fox RABV. RABV is also present in raccoon dogs in Korea (392). In India, dogs are the main reservoir of rabies and dog RABV variants are the single major variants in circulation (135; 272). The red fox is the main reservoir of RABV in western Europe, while introduced raccoon dogs are the primary reservoir in eastern Europe (3; 52; 217; 326). Corsac foxes (*V. corsac*) and golden jackals (*C. aureus*) also maintain RABV in Eurasia, namely the Former Soviet Union (FSU) (217).

LYSSAVIRUS TRANSMISSION

Rabies is a classic example of zoonosis in that it is transmitted from animals to humans. When a rabid animal bites or scratches another animal or a human, RABV is transmitted from the animal's saliva. While the most dangerous and common route of rabies exposure is from the bite of rabid mammal, contamination of open wounds, scratches, or mucous membranes with saliva or neural tissues from a rabid animal also constitutes an exposure. While rare, human-to-human transmission has occurred through transplantation of corneas, solid organs, and vascular tissue from individuals who died of rabies (57; 159; 331). Two cases of rabies have been attributed to probable aerosol exposure in laboratory workers. Additionally, two cases of potential airborne exposure of

rabies occurred in caves containing millions of free-tailed bats (*Tadarida brasiliensis*) in the southwest United States (61; 87; 88; 382; 383). All warm-blooded animals can contract rabies but some are more likely to transmit the virus than others. For example, RABV reservoirs are considered high risk for transmitting the virus. While a particular variant typically circulates among a reservoir species, spillover events, in which a species other than the reservoir species becomes infected, can occur. An example of this is depicted in Figure 3. Furthermore, the risk for transmission depends on the species of the biting animal, the anatomical bite site, and the severity of the wound (62) .

Worldwide, the dog is the main vector for transmission of rabies, accounting for 90% of human exposures and 99% of human deaths (381). Rabies causes approximately 55,000 deaths annually, the majority of which occur in countries in Africa and Asia where dog rabies is still endemic (120; 203). In addition to dogs, a sylvatic cycle of transmission also exists in which wild animals (e.g. bats, raccoons, and foxes) serve as maintenance hosts. Within the United States, the source of RABV transmission can vary from state to state. For example, raccoons are the main source of transmission along the east coast whereas in Texas, foxes, skunks, bats, coyotes, and raccoons are at high risk for transmitting rabies. However, most human rabies deaths in the US are due to exposure to rabid bats. Oftentimes, the bite wound from a bat is so small, the individual may not know they were bitten and therefore does not seek post-exposure prophylaxis (381).

CLINICAL PRESENTATION

Clinical presentation of rabies can be divided into five stages: the incubation period, the prodrome phase, the acute neurological phase, coma, and death (160; 161; 165). The incubation period is on average, 30-60 days, but can be as short as 5 days to

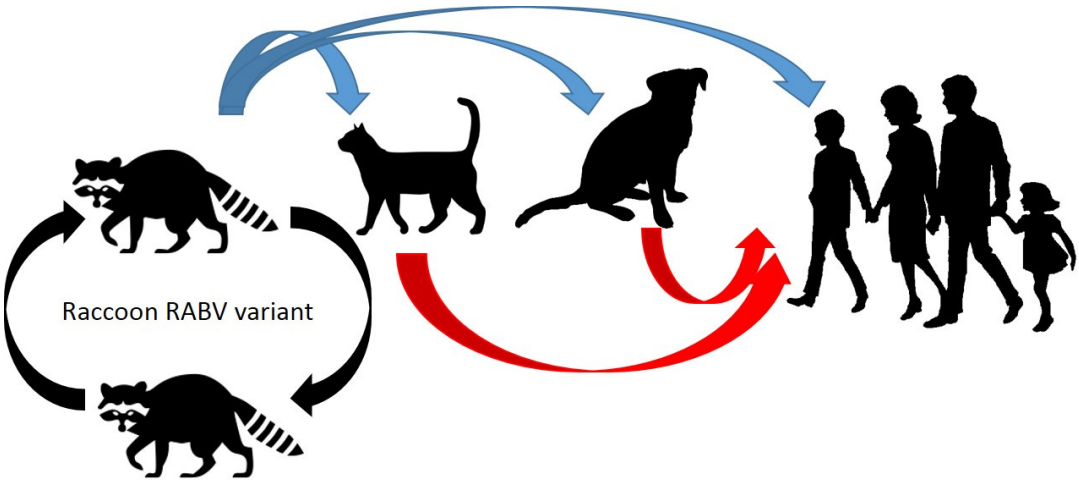


Figure 3. RABV transmission. While the raccoon rabies variant typically circulates among the raccoon reservoir species (black arrows), spillover events to domestic animals and humans can occur (blue arrows). In addition to direct transmission between the reservoir species and humans, a domestic animal infected by the reservoir species may serve as an intermediate host to subsequently infect humans (red arrows).

more than six years (162; 165; 306; 328). The length of the incubation period depends on several factors. For instance, the amount of virus in the inoculum, severity of the wound, and proximity of the wound to the CNS can all influence the time to symptom onset (109). For instance, an incubation period of <5 days was observed in a patient sustained a brachial-plexus injury from a dog bite which presumably facilitated direct entry of RABV into the nervous tissue (165). The prodrome phase, which lasts between 2-10 days (306), begins as the virus migrates from the periphery to the CNS (163). Symptoms during this stage are variable and nonspecific and include fever, fatigue, malaise, sore throat, anorexia, nausea, vomiting, difficulty swallowing, headache, irritability, anxiety, and vertigo. More notable symptoms include agitation, photophobia, insomnia, and pain or paresthesia at the site of virus inoculation. Oftentimes, localized pain at the bite site will spread to involve the entire limb (160).

The next stage of rabies, the acute neurological period, begins with signs of CNS dysfunction and can manifest as “furious” rabies or “dumb” rabies although nervous system dysfunction is most commonly seen in patients with the furious form (160; 161; 165). “Furious” or encephalitic rabies can involve the spinal cord, basal ganglia, thalamus, hypothalamus, brain stem, cerebrum, and limbic system and occurs in approximately 80% of human rabies cases (293). Many individuals with the furious form of rabies experience fluctuating consciousness, hyperactivity, hydrophobia, aerophobia, agitation, seizures, hypersalivation, photophobia, fixed and dilated pupils, and convulsions (339). The infection eventually progresses to coma and death from cardio-respiratory arrest. “Dumb” or paralytic rabies accounts for approximately 20% of human rabies cases and involves the spinal cord and medulla (259; 293). This form presents as

flaccid muscle weakness that is often confused with Guillain-Barré syndrome (GBS). However, a persistent fever and urinary incontinence distinguishes paralytic rabies from GBS (178). Another common feature is percussion myodema, which is a contraction of skeletal muscle at the site of a slight blow by a percussion hammer (134). Paralytic rabies progresses to paralysis, coma, and death by respiratory failure. While patients with encephalitic rabies die within 7 days of symptom onset, the average survival period in paralytic rabies is typically 2 weeks (160; 165). The pathogenesis underlying these two clinical forms of rabies remains to be determined. For instance, there is no relation between bite site and the development of encephalitic or paralytic rabies (349). Furthermore, while it has been suggested that different RABV variants influence which clinical form develops, sequence analysis of N, P, and G genes suggests this is not the case, or at least is not the sole contributor to which clinical form manifests. Rabies can also present as a non-classical form, which is typically associated with bat bites but has been described following dog bites. Non-classical rabies presents as neuropathic pain, involuntary muscle twitching, focal brainstem signs, and sensory and motor deficits (163).

TREATMENT

Post-exposure prophylaxis

Each year, approximately 40,000 people receive post-exposure prophylaxis (PEP) in the United States due to exposure to a potentially rabid animal (175). Globally, it is estimated that more than 15 million people receive PEP, mostly in China and India (174). PEP consists of wound cleansing, vaccination, and administration of human rabies immune globulin (HRIG). To properly cleanse an animal bite wound, gentle irrigation

with water or a dilute water and providone-iodine solution is recommended (62). The importance of wound cleansing has been demonstrated in several animal studies, in which thorough wound cleansing alone without vaccination reduced the likelihood of rabies (98; 193). Following wound care, vaccination and HRIG are administered. Two vaccines are licensed for use in the United States: Human diploid cell vaccine (HDCV) (Imovax® Rabies) and Purified chick embryo cell vaccine (PCECV) (RabAvert®). HDCV is prepared from the fixed RABV strain Pitman-Moore grown on a diploid human fibroblast cell line (MRC-5) and is inactivated with β-propiolactone. PCECV is prepared from the fixed RABV strain Flury LEP grown on primary cultures of chicken fibroblasts and is inactivated with β-propiolactone (62). The first dose of HDCV and PCECV should be administered IM as soon as possible after exposure, which is considered day 0 of the vaccination series. Additional doses of vaccine are administered on days 3, 7, 14, and 28.

Two HRIG products are currently available in the US: Imogam® Rabies-HT and HyperRab™ S/D. These products are made from the plasma of individuals hyperimmunized with rabies antigen. HRIG is administered once on day 0 to provide an immediate, passive, neutralizing antibody response until the individual mounts an antibody response to the vaccine. If HRIG is not administered on day 0, it should be administered no later than day 7 because an antibody response to vaccination is presumed to have occurred at that time. Ideally, HRIG should be infiltrated into and around the wound with any remaining volume administered IM at a site distant from the vaccination site. For previously vaccinated individuals, PEP consists of 2 IM doses of vaccine on day 0 and day 3. HRIG administration is not recommended, as it may impede the relative strength or quickness of an immune response to vaccination (123).

Outside of the United States, individuals exposed to rabies may receive purified vero cell rabies vaccine (Verorab™, Imovax-Rabies vero™, TRC Verorab™), purified duck embryo vaccine (Lyssavac N™), and different formulations of PCECV (Rabipur®) and HDCV (Rabivac™). In developing countries, where HRIG may not be available, purified equine RIG (ERIG) may be used. If neither HRIG nor purified ERIG is available, unpurified antirabies equine serum may be used, although this is associated with higher rates of adverse reactions (194).

Several human studies have assessed the effectiveness of rabies PEP. One such study consisted of 32 adults and 10 children that received HRIG (Hyperrab®) and 5 IM doses of HDCV following exposure to confirmed or suspected rabid animals. All individuals remained rabies-free during a 5-year observation (20). A second study investigated the clinical outcomes of 90 individuals with bites from confirmed rabid animals, wild carnivores, and bats. Following treatment with HRIG and 5 IM doses of HDCV, all patients remained rabies-free after 10-18 months of follow-up (15). Finally, a third study found no human rabies cases among 45 individuals receiving HRIG and 6 IM doses of PCECV after contact with confirmed rabid animals (40).

If a person is bitten by a dog or cat displaying signs of rabies, PEP should begin immediately. If the animal appears healthy, they should be kept under observation for 10 days and persons should not begin prophylaxis until the animals develops clinical signs of rabies. If an individual is bitten or scratched by a skunk, raccoon, fox, or bat, they should be regarded as rabid and PEP should commence immediately, unless lab tests confirm the animal is negative for rabies (62). Petting or handling animals, contact with blood, urine, or feces, and contact of saliva with intact skin do not constitute exposures

and PEP should not be administered.

Milwaukee Protocol

In 2003, a group of researchers and physicians proposed a new therapeutic approach for the treatment of rabies. Specifically, a combination therapy of rabies vaccine, HRIG, ribavirin, IFN-α, and ketamine was proposed. Ribavirin, a nucleoside analogue of guanosine, is a broad-spectrum antiviral commonly used in the treatment of chronic hepatitis C virus (HCV) and respiratory syncytial virus (RSV). Several mechanisms of action have been suggested including viral polymerase inhibition and RNA mutagenesis (344). Although *in vitro* activity of ribavirin against RABV has been demonstrated, *in vivo* efficacy was not achieved in a mouse model of RABV infection (58-60). Furthermore, several experimental mouse models of viral encephalitis have demonstrated that ribavirin does not readily cross the blood brain barrier (BBB) (189; 205; 325). In 1989, intrathecal and intravenous ribavirin proved to be ineffective in the treatment of a patient with rabies encephalitis (367). Interferons (IFNs) are a family of cytokines that play an important role in antiviral immunity. IFN-α, a type I IFN, is induced by viral infection and interferes with viral replication. In addition to inducing an antiviral state in cells, IFN-α also exhibits immunoregulatory effects, including induction of natural killer (NK) cell cytotoxicity as well as promotion of protective adaptive immune responses to viruses (44). While the post-exposure treatment of RABV-infected cynomolgus monkeys with IFN-α was shown to be protective, intrathecal and intravenous IFN-α therapy failed to cure three patients with rabies encephalitis (367; 370). The use of ketamine, a noncompetitive antagonist of the N-methyl-D-aspartate (NMDA) receptor and a dissociative anesthetic, was also suggested as a potential therapy for human rabies.

In addition to *in vitro* inhibition of viral replication, administration of ketamine to RABV-infected rats led to reduced infection in several regions of the brain (237; 238).

In 2004, a 15-year-old girl from Wisconsin developed clinical rabies one month after being bitten on her left index finger by a bat. Her wound was washed with peroxide but no medical attention was sought and thus rabies PEP was not administered (380). Initial symptoms included fatigue, tingling in her left hand, nausea, vomiting, double vision, and ataxia. Four days after symptom onset, the girl developed blurred vision, slurred speech, tremors of the left arm, and an abnormal gait. On day 1 of hospitalization, she began salivating and experienced uncoordinated swallowing. Serum, cerebrospinal fluid (CSF), skin from the nape of the neck, and saliva samples were collected for the diagnostic testing of rabies. While virus could not be isolated from skin or saliva samples, anti-RABV antibody was detected in CSF and serum (380). Coma was induced with ketamine and midazolam. In addition to treatment with ribavirin, the girl also received amantadine, an NMDA receptor antagonist that has shown *in vitro* activity against RABV (288; 338). On day 8, a lumbar puncture revealed increased levels of anti-RABV antibody in serum and CSF. Upon motor examination, the girl exhibited complete flaccidity. Starting on day 12, she began to regain muscle movement and became more alert and responsive. The patient was considered clear of transmissible rabies on day 31 and was subsequently placed in rehabilitation until day 76, at which point she was discharged to her home. The patient ultimately survived, but with neurological impairment such as involuntary muscle movement, abnormal gait, and fine motor difficulties. This therapeutic strategy was ultimately named the Milwaukee Protocol (MP). Several clinical reports hypothesized that death from rabies is due to neuronal

dysfunction rather than neuronal death (39; 138; 157; 179; 305) and in cases of survival, the individuals' immune response cleared the virus (39; 112; 239; 305). Therefore, the main rationale for using the MP is to protect the brain from injury while the individual mounts an immune response to clear the virus. However, it is unclear why the young girl survived as there is no clear scientific rationale supporting therapeutic coma in the treatment of rabies of other CNS infections (395). Furthermore, it is not known whether ribavirin, ketamine, and amantadine exhibited any antiviral activity in this particular case. Subsequent use of ketamine in the treatment of RABV-infected mice has cast further doubt on its therapeutic value (374). Since the first use of the MP in 2004, there have been at least 28 failed reports using this approach (21; 182).

ADAPTIVE IMMUNITY

Infection with lyssaviruses, particularly RABV, typically fail to provoke a robust immunological response, despite the period between virus exposure and disease onset. However, the adaptive immune response has been shown to be efficacious in preventing rabies in the context of vaccination. Indeed, it is well known that high titer neutralizing antibody responses induced by vaccination have been shown to successfully prevent rabies in humans and animals (190).

When a viral infection persists for at least 5-7 days, the adaptive immune response offers a highly specialized defense against the virus. During a viral infection, antigen presenting cells (APCs) collect viral antigen at the site of infection and migrate to the most accessible secondary lymphoid tissue. For example, during infections of the skin and connective tissue, viral antigen passively drains through the lymphatic system to local lymph nodes (LNs) in concert with active transport of antigen by dendritic cells

(DCs) (4). Professional APCs present in LNs include dendritic cells, macrophages, and B cells. DCs are particularly important for the initiation of T cells against viral infections. DCs acquire viral antigens by taking up viral particles from extracellular fluid or other cells that have been infected or by becoming infected themselves. Prior to an infection, T cells recirculate between blood, LNs, and the lymphatic system. Following an infection, T cells collect in LNs that drain the infected tissue and encounter DCs.

Activation of T cells

In order for a naïve T cell to become activated, a major histocompatibility complex (MHC) molecules must be loaded with antigen-derived peptide on the APC and the T-cell receptor must be able to recognize the complex of a specific MHC allele and a specific peptide (6). While CD4 T cells bind to MHC II molecules, CD8 T cells bind to MHC I. In addition, binding of the co-stimulatory molecule B7 located on the APC to CD28 on the T cell is also required. A small number of T cells that are specific for the viral antigen will become activated upon engagement of their T-cell receptor. Subsequent proliferation and differentiation eventually leads to a large number of antigen-specific effector T cells. Activation of naïve T cells induces the synthesis of the cytokine IL-2 as well as the IL-2 receptor. Binding of IL-2 to its receptor induces the T cell to progress through cell division, allowing for production of large numbers of antigen-specific effector cells. Following activation, CD4 T cells differentiate into several helper subsets including T_H1 cells, T_H2 cells, T_H17 cells, T follicular helper cells (Tfh), and regulatory T cells (Treg) (8). Of these, T_H1 cells are the drivers of cellular immunity and most relevant for an effective antiviral response (197). Differentiation to T_H1 cells is favored when IL-12 and IFN-γ are the predominate cytokines produced during the innate immune response

whereas IL-4 favors differentiation to T_H2 cells (4). While most adaptive immune responses involve a mixture of T helper types, the response can become biased towards a single type of response. A T_H1 response, or cell-mediated immunity, involves macrophage activation, inflammation, B-cell activation, and production of opsonizing antibodies.

Naïve CD8 T cells can be activated by a virus-infected dendritic cell and much like CD4 T cells, are stimulated to produce IL-2 and its receptor, inducing proliferation and differentiation of CD8 T cells. In general, the effective activation of CD8 T cells requires help from CD4 T cells, consisting of synthesis of IL-2 and/or stimulation of increased B7 expression on the APC. Activation of naïve T cells and their subsequent differentiation into effector T cells takes approximately 4-5 days. Changes in the expression of cell-surface adhesion molecules enables them to leave secondary lymphoid tissues and travel to sites of infection (6).

Activation of B cells

Within the B cell area of the LN (primary follicle), B cells may become activated by their B cell receptor (BCR) recognizing antigen presented by follicular dendritic cells (FDCs). Next, activated B cells internalize and process complexes of BCR with antigen and present antigen on MHC II molecules. The activated B cells then move to the edge of the follicle to interact with and receive help from Tfh cells. Receptors on Tfh cells sample antigen presented by MHC II molecules on the activated B cells. Upon recognition of specific antigen, B and Tfh cells form a pair and move into the medullary cords, where they begin to divide and form the primary focus of clonal expansion. (5; 8). Tfh cells secrete IL-5 and IL-6, inducing a proportion of activated B cells to become

IgM-secreting plasma cells. Other pairs return to the primary follicle where they continue to proliferate and form the second focus for B cell expansion. A germinal center is formed by expanding B cells, which is where B cells undergo somatic hypermutation. Through this process, mutations in the variable regions of the immunoglobulin gives rise to immunoglobulins with differing affinities for antigen. B cells also undergo isotype switching. While the first immunoglobulins made by B cells are of IgM and IgD classes, B cells can switch heavy chain isotype to IgG, IgA, or IgE. Cytokines secreted by Tfh cells determine the isotype to which a switch is made (5).

Functions of effector T cells

Effector CD8 T cells are particularly important in the killing of virus-infected cells thereby preventing the spread of infection to healthy cells. A cytotoxic CD8 T cell (CTL) selectively targets infected cells and programs them to die via apoptosis. At the site of infection, CTLs recognize antigen:MHC I complexes on infected cells. The CTL then secretes lytic granules containing cytotoxins such as perforin, granulysin, and granzymes. Perforin and granulysin form pores in the target cell membrane allowing granzymes to enter and cleave cell proteins that leads to the activation of nucleases and the initiation of apoptosis (6). CTLs can also induce apoptosis by expressing Fas ligand which binds to Fas molecules on the surface of the target cell which subsequently leads to apoptosis of the infected cell. In addition to cytotoxicity, CD8 T cells secrete IFN-γ which inhibits the replication of viruses within infected cells and increases the processing and presentation of viral antigens (6).

Effector T_H cells are defined by the cytokines they produce, distinct homing properties, and their effector functions. A major function of T_H1 cells is to enhance the

function of macrophages and microglia through IFN-γ and CD40 ligand, thereby activating macrophages to destroy intracellular pathogens such as viruses (24; 211; 229). For example, T_H1 cells cause phagosomes containing microorganisms to more efficiently fuse with lysosomes for subsequent degradation of the microorganisms. T_H1 cells can also cause macrophages to increase the synthesis of oxygen radicals, nitric oxide, and proteases which kill engulfed pathogens. TNF-α and TNF-β secreted by T_H1 cells can activate the vascular endothelium to induce macrophage adhesion and exit from blood vessels at the site of infection. Macrophage activation by T_H1 cells helps to amplify the immune response by increasing the expression of MHC II and B7 on macrophages, thereby increasing antigen presentation to naïve T cells. Additionally, T_H1 can kill chronically infected macrophages using Fas ligand or TNF-β (6). T_H1 cells secrete IFN-γ which induce several IFN-inducible antiviral mechanisms. For instance, antiviral proteins Mx and PKR inhibit transcription of viral RNA and protein synthesis for a variety of neurotropic viruses, respectively (82). In addition, T_H1 cells help activate cytotoxic T cells by activating APCs to express more costimulatory proteins. T_H1 cells also enhance killing of virus-infected cells by cytotoxic T cells by secreting IFN-γ, which enhances the processing and presentation of viral antigen by target cells to cytotoxic T cells (12). While CD8 T cells and T_H1 cells leave the lymphoid tissue and travel in lymph and blood to infected tissues, Tfh cells remain in the lymphoid tissue. Here, they activate pathogen-specific B cells and drive somatic hypermutation and isotype switching of immunoglobulin (see *Activation of B cells*).

Functions of effector B cells

IgG produced by pathogen-specific B cells is actively transported across the

endothelium to extracellular spaces in tissues. On the apical side of the endothelial cell, IgG is ingested by fluid-phase pinocytosis. Within the endocytic vesicle, IgG binds to the neonatal Fc receptor (FcRn) via the fragment crystallizable (Fc) domain of IgG. This receptor transports IgG to the basolateral surface of the cell where it is subsequently released into the extracellular space. One of the first steps in the infection of a host cell by a virus is attachment to the host cell. Neutralizing antibodies produced by plasma cells coat the virus and can inhibit the initial binding of the virus to the host cell or its subsequent entry (5). Antibodies bound to virus via their antigen-binding (Fab) fragment can bind to Fcγ receptors (FcγRs) on macrophages and neutrophils via their Fc domains. The opsonized virus is then endocytosed and becomes enclosed in a phagosome. Subsequent fusion of the phagosome with lysosomes results in degradation of the virus. Natural killer (NK) cells, effector lymphocytes that circulate in the blood, also express an Fc receptor that recognize antibody bound to antigens on target cells. Cross-linking of Fc receptors signals the NK cell to kill the target cell via antibody-dependent cell-mediated cytotoxicity (ADCC). Antibody bound to viruses can also activate the complement system, covering the pathogen with complement proteins and facilitating the uptake and degradation of the virus by phagocytes (187).

Immunity within the CNS

Neurotropic viruses use various routes to infect the CNS. For instance, West Nile virus (WNV) and tick-borne encephalitis virus infect the CNS vascular endothelium while human immunodeficiency virus (HIV) hijacks monocytes during homeostatic immune surveillance (13; 250; 356). In addition, RABV, vesicular stomatitis virus (VSV), poliovirus, and herpesviruses infect nerves in the periphery and gain entry into

the CNS via retrograde axonal transport (209). Following viral infection of the CNS, viral antigens are produced and antigen-specific lymphocytes are recruited to the CNS to clear the infection. Depending on the route of infection, lymphocytes are primed in LNs draining a peripheral site of inoculation or within draining LNs of the CNS, the latter occurring when infection of the CNS occurs without sufficient priming in the periphery. Within approximately 1-3 days following infection of the CNS, virally infected and/or activated astrocytes, microglia, and endothelial cells secrete chemokines to attract myeloid cells to the CNS. For example, neutrophils expressing CXCR2 are attracted to the chemokine CXCL1 while monocytes are attracted to the chemokine CCL5 and its receptor CCR5. As the infection progresses, activated CD4 and CD8 T cells bearing the receptor CXCR3 and/or CCR5 enter the CNS via chemokines CXCL10 and CCL5 (172). Antiviral function of T cells in the CNS depends on MHC presentation of viral antigen by infected cells. Neurons, astrocytes, and microglial cells in the parenchyma have only been shown to express antigen to T cells in the context of MHC I. However, perivascular macrophages and microglia within the perivascular space and microglia within meningeal sites can express MHC II (75; 167; 209). Therefore, it is thought that CD8 CTLs function as the main antiviral immune cells within the CNS while CD4 T cells function in a helper context. CD8 T cells have been shown to control viral replication by perforin-dependent cytolysis and Fas-mediated apoptosis of infected glial cells and neurons, respectively (235; 254). Non-cytolytic mechanisms of viral clearance from neurons include IFN-γ and/or virus-specific antibodies (130; 148; 279; 280). Galea et al. demonstrated that the presence of plasma cells within the CNS parenchyma is not required for antibodies to reach virus-infected cells. Rather, antibodies produced by plasma cells in the CSF can

diffuse into the parenchyma (130). Given that cells of the CNS do not self-renew, non-cytolytic mechanisms are particularly important in reducing damage to the infected CNS (235).

Adaptive immune responses to lyssavirus infection

Once lyssaviruses enter the CNS, they are capable of evading the host immune response. However, immune subversion appears to be strain-dependent (106; 137; 223; 377). For example, pathogenic strains replicate and lower rates thereby preserving neurons that the virus uses to ascend to the brain. Furthermore, lower expression of glycoprotein, the major antigen responsible for inducing protective immunity, aids in evasion of the host immune response (261). While pathogenic strains evade, it has been suggested that attenuated strains of RABV induce the host immune response (304; 365). Indeed, recent studies in mice have demonstrated that infection with attenuated RABV induces increased permeability of the BBB and subsequent clearance of CNS-resident RABV. While CD8 T cells were not found to be essential in these mice, CD4 T cells and virus neutralizing antibodies (VNAs) produced by infiltrating B cells were found to be essential (118; 303; 304; 363). In addition, attenuated RABV expresses greater amounts of viral glycoprotein and demonstrates higher rates of replication in mice (118; 233; 363). Greater levels of VNAs were also evident in mice infected with attenuated RABV (363), which are known to be essential for survival (168; 183; 244). Regarding human rabies cases, many patients do not develop VNAs prior to death which is thought to be due to the majority of cases (>90%) being associated with the highly pathogenic SHBRV (161; 309).

Antiviral monoclonal antibodies

In the treatment of viral infections, it is important to distinguish between active and passive immunity. Active immunity is the host-dependent production of antibodies to a virus following exposure to the virus, whether it be via infection with the virus itself or vaccination with a killed or attenuated form of the virus. Conversely, passive immunity is provided when an individual receives exogenously produced antibodies to a virus. While vaccines require time for the individual to mount an immune response, passive immunization can provide immediate protection (51). The earliest application of antibodies to treat viral infections consisted of the use of serum from individuals who had recovered from the same viral infection. The serum from these individuals contained polyclonal antibodies which recognize multiple epitopes on a virion. In 1975, development of the hybridoma enabled isolation from monoclonal antibodies from immunized mice (206). Monoclonal antibodies (mAbs) are identical immunoglobulins generated from a single B cell clone that recognize a single epitope, allowing for specific targeting of an antigen of interest. In addition, mAbs have a higher degree of consistency among manufactured lots as well as a reduced risk of serum sickness and anaphylaxis often seen with polyclonal serum from animals (361).

Monoclonal antibodies are classically known for blunting viral infections by neutralizing viruses via binding of their Fab fragments to virus surface antigens required for host cell entry. However, the Fc domain also plays an important role in the antiviral activity of mAbs. Following binding of mAbs to virions, complement can bind to the Fc domain of mAbs, facilitating lysis of free virions. Furthermore, opsonized virions can bind to FcγRs and complement receptors via the Fc domain of mAbs, leading to

phagocytosis by innate immune cells such as macrophages. FcγRs are expressed on several immune cells such as NK cells, B cells, DCs, and macrophages (54). Monoclonal antibodies can also bind to infected cells expressing viral antigens on their surface facilitating elimination of infected cells by ADCC, antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) (226).

Several methods have been developed to generate therapeutic human monoclonal antibodies. In one method, known as antibody phage display, immunoglobulin heavy and light chain genes from B cells of naïve, immunized, or infected individuals are cloned as Fab or single chain variable fragments (scFv) and displayed on phage. A viral antigen is then used to pan the library for virus specific antibodies and the Fab or scFv are subsequently cloned into a vector encoding a complete human IgG (257; 347). This method has been used to isolate neutralizing antibodies against influenza virus, ABLV, Nipah virus (NiV), and Hendra virus (HeV) (257; 347; 398). Another method includes isolating memory B cells from infected or immunized individuals, and either directly cloning antibody-encoding genes or culturing memory B cells then screening supernatant against viral antigen. The former method has been used to isolate neutralizing monoclonal antibodies against Zika virus (ZIKV), RSV, HIV, and human papilloma virus (HPV) (136; 314; 315; 364) while the latter led to the discovery of broad neutralizing mAbs against HIV (362). Given that antigen-specific plasma cells account for 40-90% of IgG-secreting cells in peripheral blood seven days-post boost immunization, methods for antibody gene cloning from plasma B cells have been developed (38). For example, mAbs have been cloned from plasma cells from human donors immunized or infected with dengue virus (DENV) and H1N1 influenza virus (100; 385). Additionally, a large

panel of mAbs were cloned from plasma cells from rhesus macaques immunized with an experimental DENV vaccine (256).

There are several mAbs in clinical trials for the prevention and treatment of viral infections (312). Synagis (palivizumab) is a humanized IgG1 mAb that has been approved as a prophylactic therapy in infants at high risk for RSV (48; 191). Similarly, researchers hope to advance a ZIKV mAb candidate to clinical trials for the prophylactic treatment of pregnant women in ZIKV endemic areas. The mAb targets the viral envelope protein and was shown to be protective to mother and fetus prior to ZIKV challenge in mice (313). During the 2014-2016 Ebola outbreak in West Africa, ZMapp, a triple mAb cocktail of mouse-human chimeric antibodies demonstrated efficacy in nonhuman primates. This therapy was also approved for compassionate use in the outbreak and is currently in Phase 1 trials (152; 292). VRC01 is a broadly neutralizing mAb that targets the CD4-binding site of the HIV glycoprotein, gp120. This mAb was isolated from memory B cells of HIV-1-infected individuals and is currently in multiple Phase 2 clinical trials (386). Finally, MHAA4549A is a human IgG1 mAb that targets a highly conserved epitope on the stalk of influenza A hemagglutinins (HA) (Group 1 and 2) thereby blocking HA-mediated endosomal membrane fusion and subsequent release of ribonucleoprotein. MHAA4549A was cloned from a single human plasmablast from an influenza-vaccinated individual and is currently in Phase 2 clinical trials (154).

Lyssavirus monoclonal antibodies

Monoclonal antibodies represent a highly specific alternative to HRIG, which consists of polyspecific antibody purified from volunteers hyperimmunized with rabies antigen. Despite established PEP regimens, less than 1% of all PEP administered in

developing countries consists of HRIG, due to high cost and limited availability (2) . HRIG also has many undesirable characteristics, including inherent lot-to-lot variability of constituent antibodies, the majority of which are not specific for rabies antigen. Furthermore, existing data strongly suggest that HRIG likely has no therapeutic value once virus enters the nervous system (50). Several studies have assessed rabies virus-neutralizing mAbs as an alternative to HRIG in the post-exposure treatment of rabies. For instance, a human IgG2 mAb (CR57) was shown to prevent lethal RABV infection in mice and hamsters when treated 24h prior- and 24h post-infection, respectively (104). In a separate study, Syrian hamsters were infected with a lethal dose of RABV and treated with rabies vaccine plus human anti-RABV mAb (CR57, CR4098, or CR4144) 24h post-infection. Each of the mAbs tested were found to protect hamsters equally as well as HRIG (26).

Cocktails of mAbs are often desired if the target epitope of a mAb is not conserved among all strains of a virus. This is especially true of RABV, given the variation in strains circulating in various animal reservoirs. When designing a cocktail of mAbs, several criteria should be met. For instance, mAbs should: (1) target a wide range of viral variants, (2) target distinct, non-overlapping epitopes, and (3) escape mutants of one mAb should be neutralized by the other mAb (51). In one study, a cocktail of five mouse mAbs (509-6 (IgG2a), 1112-1 (IgG1), 523-11 (IgG2a), 802-1 (IgG2b), 502-2 (IgG2a)) targeting RABV G and N were tested in an experimental mouse model of RABV infection. The mAb cocktail demonstrated protection of CVS-24-infected mice when administered 24h pre-, 2h pre-, and 2h post-virus inoculation. In addition, the mAb cocktail protected Syrian hamsters from death when administered 2, 3, and 36h post-

infection with a street RABV isolate (317). In a separate study, a combination of anti-rabies G human mAbs SOJA (IgG1), SOJB (IgG3), and SO57 (IgG1) prevented lethal RABV infection in mice and hamsters when administered 1 or 4h post-infection, respectively (289). In 2008, clinical data from two Phase 1 studies assessing the safety, tolerability, and neutralizing activity of a mAb cocktail in humans was reported. The cocktail consisted of two anti-rabies G human IgG1 mAbs, CR57 and CR4098 (26; 144; 247). In the first study, subjects receiving 20 IU/kg of the mAb cocktail on day 0 had detectable levels of serum RABV neutralizing antibody (RVNA) by day 3 with maximal titers at day 7, and was consistent with serum antibody concentrations in individuals receiving 20 IU/kg of HRIG (227). While none of the study participants experienced fever, redness, or swelling, a proportion experienced pain at the injection site. In the second study, the mAb cocktail was administered in conjunction with PCEC rabies vaccine. Consistent with current PEP regimens (HDCV + HRIG), 67% and 100% of subjects seroconverted on day 7 and 14, respectively with RVNA levels at or above 0.5 IU/ml, the level considered acceptable by WHO (7; 27; 227). A similar study assessed the efficacy of an anti-rabies G human IgG1 mAb (SII RMab) that was shown to neutralize a geographically diverse range of rabies isolates as well as in vivo protection in hamster studies (140; 327). In a Phase 2/3 study, a PEP regimen of rabies vaccine and SII RMab was found to be a safe and potent alternative to PEP with HRIG (141).

AUSTRALIAN BAT LYSSAVIRUS

Isolation and Characterization

In 1996, a retrospective study investigating the host reservoir of HeV (267), inadvertently led to the discovery of ABLV. Antibody to HeV has been detected in black

flying fox fruit bats (*Pteropus alecto*), and this species was therefore thought to be a reservoir of HeV (394). However, brain samples from two juvenile black flying foxes collected near Ballina, in northern New South Wales, Australia, tested negative for HeV (128). The first sample, collected in 1996, was from a female flying fox under a tree, unable to fly. Following euthanasia, fresh blood, lung, kidney, and spleen were collected. Histological analysis of formalin-fixed, paraffin-embedded brain tissue revealed severe nonsuppurative encephalitis. The second sample was an archived paraffin-embedded tissue sample collected from a hyperaggressive female flying fox in 1995. Eosinophilic, cytoplasmic inclusion bodies consistent with Negri bodies, were found in several areas of the brain. Through indirect immunoperoxidase testing using an anti-rabies mAb, evidence of rabies was detected in the hippocampus, medulla oblongata, and the trigeminal nerve nuclei in the 1996 bat and in virtually all areas of the brain in the 1995 bat. Furthermore, cytoplasmic inclusion bodies were found in the hippocampus of the 1996 bat via electron microscopy, and staining with an anti-rabies mAb and gold-labeled rabbit anti-mouse serum revealed these bodies contained viral nucleocapsids.

Neutralizing antibodies to RABV (CVS-11) could not be detected in blood from the 1996 bat. Furthermore, lung, kidney, and spleen homogenates from the 1996 bat were added to mouse neuroblastoma cells, but virus could not be isolated after several cell passages. Therefore, tissue homogenates were individually inoculated intracerebrally into weanling mice or pooled and inoculated into day-old suckling mice. The virus (Ballina isolate) was isolated from one mouse inoculated with kidney homogenate, which developed hind-limb paralysis 16 days post-infection while all other mice remained healthy. Brain smears from the affected mouse tested positive for a lyssavirus using a

fluorescein-labeled mAb and histological examination of brain tissue showed nonsuppurative encephalitis. Additionally, brain material from this mouse was repassaged intracerebrally into 3-week-old mice, which developed neurological signs by day 11 post-infection. Electron microscopy of brain homogenate from these mice showed bullet-shaped rhabdoviruses. While the virus was shown to be neutralized by antisera to RABV (Evelyn-Rokitnicki-Abelseth strain), mAb binding patterns tested against a panel of lyssavirus nucleoprotein mAbs revealed that the isolate was serologically distinct from known lyssaviruses.

Polymerase chain reaction (PCR) was performed to amplify the nucleoprotein from brain, lung, kidney, and spleen from the 1996 bat as well as paraffin-embedded formalin-fixed brain tissue from the 1995 and 1996 bats. Positive PCR products were obtained from tissue culture virus and paraffin-embedded formalin-fixed brain tissues and sequence analysis revealed these products were identical. Sequence comparisons of known lyssavirus nucleoproteins and the novel lyssavirus (provisionally termed pteropid lyssavirus (PLV)) nucleoprotein revealed PLV was most closely related to EBLV1 and RABV, with 93% and 92% amino acid sequence homology, respectively (128). Percent relatedness of the virus compared to RABV (Pasteur vaccine strain) was 92, 75, 87, and 75% for N, P, M, and G proteins, respectively. Additionally, phylogenetic analyses of N sequences confirmed that PLV is a member of the *Lyssavirus* genus and represents a new 7^{th} genotype (145).

Several months after its isolation from the black flying fox fruit bat, PLV was isolated from a yellow-bellied sheathtail (YBST) (*Saccolaimus flaviventris*), a species of insectivorous microbat, and was subsequently renamed Australian bat lyssavirus (170).

Sequence comparison of the *Saccolaimus* isolate to the *Pteropus* isolate at both the nucleotide and amino acid sequence level revealed the most variation between L, P, and G while M and N were most conserved (146). Sequence comparisons of N also showed the *Saccolaimus* isolate to be of the same genotype (genotype 7) as the *Pteropus* isolate. Based on phylogenetic analysis of N nucleotide sequences, *Saccolaimus* and *Pteropus* isolates separated into distinct clades, representing two variants of ABLV, ABLVs and ABLVp. Furthermore, mAb binding profiles differed between these variants, providing a useful tool for distinguishing between ABLVs and ABLVp (146).

ABLV tropism

While RABV has both bat and terrestrial reservoirs, bats are the only known reservoir of ABLV. Mainland Australia is home to four species of flying fox fruit bats (Order *Chiroptera*, Suborder *Megachiroptera*, Genus *Pteropus*): the spectacled flying fox (*Pteropus conspicillatus*), the little red flying fox (*Pteropus scapulatus*), the grey-headed flying fox (*Pteropus poliocephalus*), and the black flying fox (*Pteropus alecto*). ABLV has been isolated from all four of these species (249). While the grey-headed flying fox is restricted to Australia, the black flying fox and spectacled flying fox can be found in Indonesia and the black flying fox, spectacled flying fox, and little red flying fox can be found in Papa New Guinea (371). Although ABLV has not been detected in bat samples from either of these countries, ABLV neutralizing antibodies have been detected in several bat species in the Philippines, suggesting the presence of ABLV in Asia (22). Australia is also home to six families containing 65 species of insectivorous bats (Order *Chiroptera*, Suborder *Microchiroptera*, Genus *Saccolaimus*). To date, ABLV has been isolated from one species, the YBST bat. In addition to mainland Australia, the YBST bat

is also native to Papa New Guinea (371). Serological evidence of ABLV infection has been reported in five of the six families of *Microchiroptera* in Australia and thus all Australian bat species are thought to be potential reservoirs for ABLV (121).

While the prevalence of ABLV in healthy bats is thought to be <1%, the prevalence is significantly higher in orphaned, injured or sick bats (34; 121). For example, approximately 60% of little red flying foxes displaying neurological impairment tested positive for ABLV antigen (34). Furthermore, seroprevalence of lyssavirus antibody in sick or injured flying foxes and YBST bats can reach 20% and 62.5%, respectively (121; 371). The high seroprevalence in YBST bats suggests this bat plays a crucial role in ABLV ecology (121).

ABLV host cell entry

Recent examination of the entry pathway utilized by ABLV found that ABLV G-mediated entry uses a clathrin-dependent pathway and that similar to RABV, ABLV entry requires actin for a productive infection (372). Furthermore, Rab5 was shown to be essential for productive ABLV infection, suggesting ABLV G fuses with early endosomes facilitating release of viral genome into the cytoplasm. While the receptor for ABLV remains to be determined, proposed receptors for RABV do not permit entry of ABLV into resistant cell lines. Therefore, it is likely that ABLV utilizes alternative receptor(s) from that of RABV (373).

ABLV infection in humans and horses

Humans

Transmission of ABLV occurs by the bite or scratch from infected bats (Figure 4). There have been three confirmed cases of ABLV infection in humans, all of which

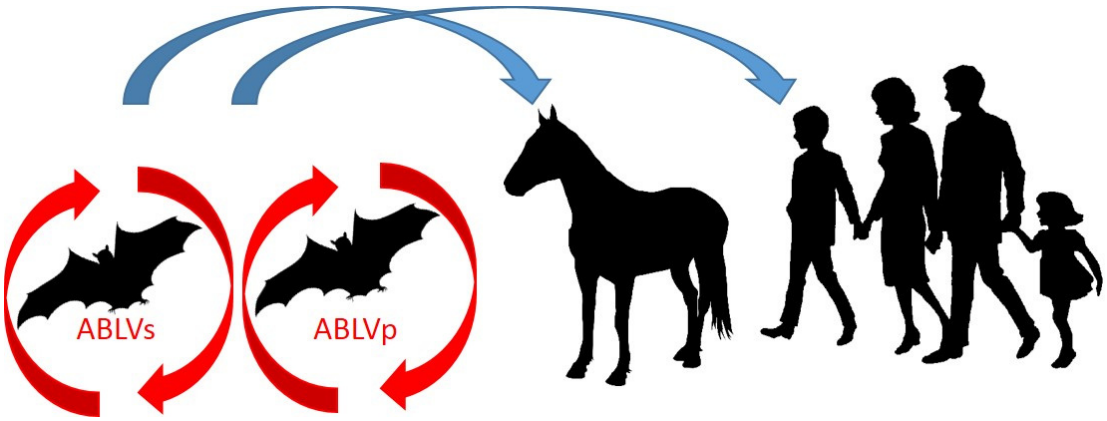


Figure 4. ABLV transmission. Two genetically distinct variants of ABLV are currently known. The *Saccolaimus* variant (ABLVs) circulates among insectivorous bats while the *Pteropus* variant (ABLVp) circulates among flying fox fruit bats. Both ABLVs and ABLVp have been implicated in human cases of ABLV infection. Documented cases of ABLV infection in horses were due to ABLVs.

occurred in Queensland, Australia. The first case occurred in 1996, when a 39-year-old female developed pain and numbness in her left arm. Approximately 2-4 weeks prior, the woman had been caring for several fruit bats and sustained several scratches to her left arm although there was no record of a bat bite (14). Prior to caring for the fruit bats, she had been caring for cockatoos, dogs, cats, marsupials, and an insectivorous bat. Within 3 days, she was admitted to the hospital with fever, headaches, dizziness, and vomiting. Although a lumbar puncture revealed an elevated white blood cell count, organisms could not be detected via microscopy and no growth of organisms was observed in culture. Despite treatment with broad-spectrum antibiotics, the woman developed double vision and difficulty swallowing. Over the next 10 days of her illness, she developed progressive weakness in all limbs and decreased consciousness. Neuroimaging revealed no abnormalities and lumbar punctures continued to test negative. By day 11, she unresponsive and ventilator dependent. An electroencephalogram revealed diffuse encephalitis and her serum tested positive for antibodies to a lyssavirus. Serology for viruses common to Australia were all negative. A positive PCR product was obtained from cerebrospinal fluid (CSF) using primers specific ABLV nucleoprotein. Despite administration of rabies immunoglobulin, she continued to deteriorate and died approximately 20 days following symptom onset. It was later determined that the woman was bitten by a yellow-bellied sheathtail bat approximately 4.5 weeks prior to symptom onset (155). PCR and sequence analysis performed on CSF, brain tissue, and virus isolated from brain tissue all suggested that the infection originated from an insectivorous bat and mAb binding profiles of the isolated virus confirmed infection with the *Saccolaimus* variant (146).

The second case occurred in 1998 when a 37-year-old woman presented to the hospital with a five day history of anorexia, fever, vomiting, left shoulder pain, sore throat, difficulty swallowing, and tingling in her left hand (155). She was drooling saliva and examination of her throat induced spasmodic attempts to swallow. Within 12 hours, the woman began to experience agitation, increased difficulty swallowing and speaking, and her muscle spasms became more violent. She became paralyzed and was placed on a ventilator. On day 2 of hospitalization, marked fluctuations in blood pressure and body temperature became evident and attempts to cease ventilation were unsuccessful. After the staff learned of a history of a bat bite, PCR was performed on CSF and saliva and a positive ABLV product was detected in the woman's saliva. She ultimately died 19 days after symptom onset. This case had a particularly long incubation period of 27 months. In August of 1996, while attending a barbeque, the woman attempted to remove a flying fox that had landed on the back of a young child and was bitten at the base of her left little finger. As expected based on the patient's exposure to a flying fox, the woman was infected with the flying-fox or *Pteropus* variant (155). Two days after being bitten, she visited her general practitioner and was given antibiotics and tetanus toxoid. After 6 months, the woman returned to her doctor inquiring about a blood test for the "bat virus" and was advised to receive PEP, but she declined.

The third reported case of ABLV infection in humans occurred in 2013 (127). An 8-year-old boy presented to the hospital with a two day history of fever, anorexia, and worsening abdominal pain. Approximately 8 weeks prior, the boy had been scratched by a bat on his left forearm. Soon after admittance, he began behaving abnormally and experienced several aggressive outbursts. In addition to muscle spasms of the lower

limbs, abdomen, and face, the boy developed hypertension and an elevated heart rate. While he did not develop hydrophobia, excessive drooling and difficulty swallowing were apparent. As a result, tracheal intubation was performed to control throat spasms and maintain an open airway. Encephalopathy, a disease affecting brain function, was suggested following an abnormal electroencephalography but the causative agent remained unknown. Microscopy and biochemistry of CSF was normal and PCR assays of blood and CSF were negative for lyssavirus. In addition, serum lyssavirus antibodies were not detected via enzyme-linked immunosorbent assay (ELISA). The boy was subsequently treated with vancomycin, acyclovir, and tetanus immunoglobulin, although he was fully immunized against tetanus. After bacterial and fungal infections were ruled out, the child received methylprednisolone and pooled tetanus immunoglobulin. Encephalopathy ensued as well as fluctuating body temperature and an irregular heartbeat. On day 12 of hospitalization, lyssavirus testing was repeated and reverse transcription PCR (RT-PCR) and a specific *Pteropid* ABLV real-time TaqMan assay were both positive from saliva and CSF samples. Sequence analysis revealed the boy was infected with the flying-fox or *Pteropus* variant, as it closely matched a Pteropid-ABLV sequence from 1999 as well as a 1998 human case (127). Subsequently, supportive care measures from the Milwaukee protocol such as fluid and electrolyte control were administered. The boy was sedated with ketamine and benzodiazapenes and treated with the antiviral amantadine. In addition to progressive neurological deterioration, reflexes became absent and pupils became fixed and dilated. The boy was extubated on day 28 of hospitalization and died shortly after.

Treatment

Although ABLV infection in humans is rare, all three confirmed cases described here have proved fatal. Clinical presentation in all three cases were identical to rabies, specifically with the encephalitic or "furious" form. There are no established treatments for ABLV and current PEP consist of rabies vaccination and HRIG. Consistent with RABV infection, PEP was not effective against ABLV infection following symptom onset (14; 182; 379). Furthermore, the Milwaukee protocol was unsuccessful in the treatment of rabies in the third case of ABLV infection (127). Failure of current rabies PEP to effectively treat post-symptomatic ABLV infection in humans underscores the need for improved therapeutics capable of treating CNS-resident lyssavirus infections.

Horses

In May 2013, ABLV infection was reported in a species other than bats and humans. Two confirmed cases of ABLV infection occurred in horses in southeast Queensland and both cases signify the risk of ABLV spillover to domestic animals, animal handlers, and veterinarians in Australia (19). In the first case, an 18-month-old female began interacting less with paddock mates and developed subtle behavioral changes and signs of hind limb ataxia. The horse shared a paddock with two other horses of the same breed and age. Ataxia worsened and she developed increased sensitivity to stimuli and mild fever. Hematology did not reveal any abnormalities and the horse was administered electrolytes, vitamins, and anti-inflammatory drugs. She was isolated in a stable and the two paddock mates were also isolated. At 24h post-symptom onset, the horse became lethargic and severely ataxic and protrusions of nictitating membranes (third eyelid) were evident. Nasal, rectal, and oral swabs, as well as blood samples were

sent to Biosecurity Queensland for HeV exclusion testing. She continued to receive fluids and anti-inflammatory drugs, as well as penicillin and tetanus antitoxin. Within hours, the horse was recumbent and made several attempts to stand. Oral mucoid discharge was present, potentially due to increased salivation and difficulty swallowing. At 54h post-symptom onset, she remained recumbent and demonstrated uncontrollable movement of all four limbs, head and neck, and rapid eye movement. The horse was subsequently euthanized. Following exclusion of HeV, nasal, rectal, and oral swabs were tested for ABLV by real-time RT-PCR. Only the oral swab tested positive for the *Saccolaimus* variant of ABLV.

The morning after horse 1 became sick, one of her paddock mates that had been isolated, also developed subtle hind limb ataxia (19). The 18-month-old male had no prior history of abnormal behavior. Over the next 48h, the horse showed no further clinical abnormalities. However, at 72h, he began to demonstrate mild hind limb ataxia similar to horse 1. With 12h of clinical onset, the horse was severely lethargy and developed head pressing, photophobia, and severe ataxia. Upon examination, dilated pupils, fever, and increased heart rate were evident. Similar to horse 1, he was administered vitamins, fluids, and anti-inflammatory drugs. At 36h post-symptom onset, the horse became recumbent and was unable to stand. Samples were sent to Biosecurity Queensland for HeV exclusion. Within hours, he demonstrated worsening head pressing and tachycardia, as well as difficulty swallowing and overextension of all four limbs. The horse developed clinical signs similar to horse 1, consisting of lateral recumbency, head nodding, involuntary limb and eye movements, and increased oral discharge. He was euthanized 54h post-clinical onset. Histopathology of brain and spinal cord revealed

diffuse, nonsuppurative encephalitis. Furthermore, Negri bodies were prominent in Purkinje neurons of the cerebellum (19). Brain tissue was negative for HeV and equine herpesvirus-1 and -4 (EHV-1, EHV-4) via real-time PCR. However, fluorescent antibody testing of brain tissue smears demonstrated the presence of lyssavirus antigen (19). Furthermore, ABLV RNA was detected in brain tissue and from an oral swab via RT-PCR. Sequence analysis revealed a 97.7% identity to the *Saccolaimus* variant (322).

Regarding potential routes of exposure, insectivorous Gould's wattled bats (*Chalinolobus gouldii*) were found roosting in a feed shed on the same property as the horse stables. Furthermore, serological evidence of ABLV exposure has been reported in this genus (121). In the horses' paddock, there were trees suitable for housing microbats (19). A team of bat epidemiologists made sound recordings for 72h, which identified 10 species of microbat, but not the YBST bat. Ultimately, the vector responsible for transmission and the timing of when the infection occurred could not be identified. However, the close timing of disease onset between the two horses suggests similar incubation periods and that a single bat may have infected both horses. Finally, clinical disease in these two horses was consistent with that of equine RABV outside of Australia (147; 277; 375).

SPECIFIC AIMS

Currently, recommended PEP for a suspected exposure to rabies or a rabies-like lyssavirus consists of wound cleansing, rabies virus-vaccination, and administration of HRIG (50; 246). Lyssaviruses are considered invariably fatal following CNS involvement, and PEP is thought to be effective only if undertaken within the first day following an exposure. Notably, several cases of failed PEP have been reported despite

prompt care (164; 321; 348; 378). The most publicized treatment for symptomatic rabies, the "Milwaukee protocol", consists of therapeutic coma induction and nonspecific antiviral administration. However, this regimen is now generally disfavored as a treatment for rabies, due to a lack of efficacy and absence of clear scientific rationale underpinning its use as a lyssavirus therapy (21; 180-182). Thus, there is no effective treatment for symptomatic lyssavirus infections, and even immediate treatment does not always prevent fatal disease.

Monoclonal antibodies represent a highly specific alternative to HRIG, which consists of polyspecific antibody purified from volunteers hyperimmunized with rabies antigen. Despite established PEP regimens, less than 1% of all PEP administered in developing countries consists of HRIG, due to high cost and limited availability (1). Furthermore, this therapeutic product has many undesirable characteristics, including inherent lot-to-lot variability of constituent antibodies, the majority of which are not specific for rabies antigen. Existing data strongly suggest that HRIG likely has no therapeutic value once virus enters the nervous system (50). A small number of studies have assessed rabies virus-neutralizing mAbs in the post-exposure treatment of rabies. Although these studies suggested that neutralizing mAbs may be of therapeutic value, administration of mAbs was performed within hours of RABV infection (289; 317). Therefore, the efficacy of lyssavirus-neutralizing mAbs following CNS involvement remains to be determined. **We hypothesized that mAbs could be used as a therapeutic to treat CNS-resident lyssavirus infection.**

Aim 1: Generate a luciferase-tagged ABLV reporter virus to develop a preclinical model of ABLV infection in mice.

Subaim 1A. Optimize the lethal dose of ABLV-luc in mice. B6(cg)-*Tyr*$^{c\text{-}2J}$/J mice will be infected with a recombinant ABLV that expresses firefly luciferase (ABLV-luc) at various doses and bioluminescence imaging will be performed to determine the dose that results in productive CNS infection and uniform lethality.

Subaim 1B. Compare the virulence ABLV-luc to that wild-type ABLV. B6(cg)-*Tyr*$^{c\text{-}2J}$/J mice will be infected with ABLV-luc or wild-type ABLV (WT ABLV) and disease score, body weight, and survival will be assessed to confirm ABLV-luc is not attenuated in virulence.

Aim 2: Assess the efficacy of mAb therapy against CNS-resident ABLV-luc infection in mice. B6(cg)-*Tyr*$^{c\text{-}2J}$/J mice will be infected with ABLV-luc and treated with a human IgG1 mAb, F11, at 3, 5 and 7 days post-infection. Mice will be imaged to longitudinally trace viral infection in response to treatment and measure viral burden by quantifying luminescence. Additionally, disease score, body weight, and survival will be assessed and compared to infected, untreated mice.

CHAPTER 2: Materials and Methods

STUDY DESIGN

For mouse studies, the objectives were to assess the therapeutic efficacy of a human IgG1 mAb (F11) against established lyssavirus infection in mice. Given that F11 mAb-treated mice cleared ABLV-luc from the brain beyond day 30pi (>4 human mAb half-lives later), we then explored the hypothesis that F11 mAb facilitates an adaptive immune response that ultimately mediates viral clearance. Sample sizes for experimental groups and their respective controls are indicated in the figure legends. For all mouse infection studies, relative viral load was measured by bioluminescence imaging and presence of virus was confirmed by immunofluorescence microscopy of brain tissue, as further detailed in the Materials and Methods. Wherever possible, B6(cg)-*Tyr*$^{c\text{-}2J}$/J mice were used for bioluminescence imaging, as melanin in the hair, skin, and eyes has been shown to attenuate luminescent signal (94). However, we found that shaving C57BL/6J mice immediately prior to imaging yielded comparable luminescent signal to that of B6(cg)-*Tyr*$^{c\text{-}2J}$/J mice. For F11 mAb treatment studies, mice were randomized into treated and control groups. Viral load, body weight, cumulative disease scores, and survival of F11 mAb-treated mice were compared to untreated controls. Immune cell infiltration of brain tissue was assessed by immunofluorescence microscopy. For CD8 T cell depletion studies, mice were randomized into treated or control groups. Antibody blockade of CD8 T cells was compared to isotype control antibody-treated mice and CD8 T cell depletion was assessed by flow cytometry. For all mouse infection studies, endpoint criteria consisted of ≥ 20% weight loss. Isotype control antibody-treated C57BL/6J mice in Figures 18-20 were repeated as the control group for the experiment in Figures 22-24.

MICE

All animal experiments were performed under ABSL-2 conditions in accordance with and approval of Uniformed Services University Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee guidelines. Seven to nine week-old C57BL/6J, B6(cg)-*Tyr*$^{c\text{-}2J}$/J, B6.129S2-*Ighm*^{tm1Cgn}/J (muMt^{-}), NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG), NOD.CB17-*Prkdc*^{scid}/J (NOD scid), B6.129S2-H2$^{d/Ab1\text{-}Ea}$/J (MHCII^{-}) mice were obtained from Jackson Laboratories. muMt^{-} mice have a targeted mutation in the membrane exon of the gene encoding the mu chain constant region (*Ighm*). By lacking this chain, B cell development is arrested, resulting in a complete lack of B cells (201). MHCII^{-} mice have a targeted deletion in the entire class II region (*H2*) and therefore lack all four of the classical murine MHC II genes. As a result, these mice have a nearly complete absence of CD4 T cells (245). NOD scid mice have a spontaneous point mutation in the *Prkdc* gene, which encodes the catalytic subunit of a protein kinase involved in resolving DNA double stranded breaks during V(D)J recombination. Without V(D)J recombination of the T cell receptor in T cells and the Ig gene in B cells, maturation of these cell types does not occur and therefore T and B cells are absent in these mice (324). NSG mice also have the spontaneous point mutation in the *Prkdc* gene, making them devoid of T and B cells. In addition, these mice have a targeted mutation of the *Il2rg* gene, which encodes the gamma chain of the interleukin 2 receptor. As a result, signaling pathways for multiple cytokines, including IL-15, are blocked. Without IL-15 signaling, functional NK cells are absent in these mice.

RESCUE AND PRODUCTION OF RECOMBINANT ABLV EXPRESSING FIREFLY LUCIFERASE

To generate a luciferase-expressing ABLV construct (ABLV-luc), the codon-

optimized luc2 variant of firefly luciferase was PCR-cloned from pGL4.10 (Promega), in a manner that replaced the C-terminal degradation sequence with a stop codon. This fragment was then cloned into the recombinant ABLV cDNA plasmid via standard cloning techniques and confirmed by DNA sequencing. HEK293T cells (ATCC® CRL-3216) were transfected with 4µg of DNA and 10µl Lipo2000® (Thermo Fisher Scientific) diluted in OptiMEM® media (Thermo Fisher Scientific) per manufacturer instructions. The 4µg of DNA was comprised of plasmids expressing full-length ABLV-luciferase cDNA (2µg), ABLV N (1µg), ABLV P (0.5µg), and ABLV L (0.5µg). To detect ABLV-luc virions, HEK293T cells used in the virus rescue were stained with mouse anti-rabies G (clone 1C5, Novus Biologicals) and Alexa Fluor 647 goat anti-mouse IgG2a (Thermo Fisher Scientific) and analyzed via confocal microscopy.

ABLV-luc amplification and purification

ABLV-luc rescue cells were co-cultured with mouse neuroblastoma (N2a) cells (ATCC® CCL-131) for 11 days at 37ºC, 5% CO_2. Viral supernatant was collected and added to HEK293T cells grown to 80% confluency in 175cm^2 cell culture flasks. After 14 days of incubation at 37ºC, 5% CO_2, supernatant was collected and layered on a 20% sucrose gradient and spun at 27,000 rpm on a Beckman Ultracentrifuge using a SW28 rotor (Beckman Coulter). Ten-fold serial dilutions of purified ABLV-luc were then added to N2a cells and incubated for 72h at 37 ºC, 5% CO_2. Cells were stained overnight with FITC anti-rabies G (FDI Fujirebio Diagnostics) at 37 ºC, 5% CO_2. To determine the viral titer, fluorescent foci were counted with a 20× objective on a Zeiss Observer.A1 microscope. The titer was calculated as infectious units (IU)/mL of ABLV-luc.

Luciferase assays

Following virus rescue, expression of virus-encoded luciferase was confirmed via a SteadyGlo® luciferase assay (Promega) per manufacturer's instructions. Briefly, 48h post-transfection, supernatant was removed and transfected HEK293T cells were treated with 1X Glo Lysis Buffer. Lysates were aliquoted into triplicate wells of a 96 well-plate and treated with SteadyGlo® Luciferase Assay Substrate. Following a 5 min incubation at room temperature, luminescence was measured using a GloMax® Multi-Detection System plate reader (Promega). Relative luminescence intensity was determined by dividing each reading by the reading from a well containing lysis buffer and luciferase assay substrate only. HEK293T cells transfected with a plasmid constitutively expressing a codon-optimized luc2 variant of firefly luciferase served as a positive control.

FOOTPAD DOSE RESPONSE CHALLENGE

B6(cg)-*Tyr*$^{c\text{-}2J}$/J mice were inoculated in the right rear footpad with $2x10^3$, $2x10^4$, or $2x10^5$ infectious units (IU) of ABLV-luc in a volume of 20 µl Dulbecco's Modified Eagle's Medium (DMEM). Uninfected control mice received 20 µl DMEM only. All mice were monitored twice daily and scored for disease progression with an internal scoring protocol approved by the USU IACUC. Cumulative disease scores were calculated as 0 = healthy, 1 = lethargy, 2 = weight loss, 3 = tremors, 4 = uncoordinated movement, and 5 = hind-limb paralysis. To longitudinally trace the progression of the viral infection, *in vivo* imaging was performed (see *In vivo* bioluminescence imaging). Endpoint criteria for euthanasia consisted of a 20% loss in body weight.

ABLV-LUC VS. WILD-TYPE ABLV CHALLENGE

B6(cg)-*Tyr*$^{c\text{-}2J}$/J mice were inoculated in the right rear footpad with $2x10^4$ IU of

ABLV-luc or wild-type ABLV in a volume of 20 µl Dulbecco's Modified Eagle's Medium (DMEM). Uninfected control mice received 20 µl DMEM only. All mice were monitored twice daily and scored for disease progression with an internal scoring protocol approved by the USU IACUC. Cumulative disease scores were calculated as 0 = healthy, 1 = lethargy, 2 = weight loss, 3 = tremors, 4 = uncoordinated movement, and 5 = hind-limb paralysis. To longitudinally trace the progression of the viral infection, *in vivo* imaging was performed (see *In vivo* bioluminescence imaging). Endpoint criteria for euthanasia consisted of a 20% loss in body weight.

ISOLATION AND CHARACTERIZATION OF HUMAN MAB F11

Human mAb F11 was isolated and characterized using methods previously described (398). Briefly, a recombinant vesicular stomatitis virus (rVSV) encoding the ABLVs G gene from an isolate of ABLV derived from a yellow-bellied sheathtail microbat (373) was used to screen a naïve human Fab phage display library for ABLVs G-specific Fabs. ELISAs were performed to identify clones of Fabs with a high binding affinity for ABLVs-G. The variable regions (VH and VL) of positive clones were sequenced and used to express and purify Fabs, which were then cloned into a human IgG1 expression vector.

IN VITRO NEUTRALIZATION ASSAYS

ABLV-luc or rabies challenge-virus standard (CVS-11) (ATCC VR-959) was added to ten-fold serial dilutions of mAb F11 at a multiplicity of infection (MOI) of 0.1. Following a 1.5h incubation at 37 ºC, 5% CO_2, the virus-antibody mixture was added to N2a cells (triplicate wells) and incubated for an additional 48h at 37 ºC, 5% CO_2. Cells were then stained with mouse anti-rabies G (clone 1C5, Novus Biologicals) and Alexa

Fluor 647 goat anti-mouse IgG2a (Thermo Fisher Scientific) and analyzed via confocal microscopy. Neutralization titer is expressed as the first dilution at which no anti-rabies G fluorescence is observed in any of the triplicate wells.

F11 MAB TREATMENT STUDY

Mice were inoculated in the right rear footpad with $2x10^5$ IU of ABLV-luc or CVS-11 in a volume of 20 µl DMEM. Uninfected control mice received 20 µl DMEM only. Mice were treated with a single dose of F11 mAb on day 3, 5, or 7 post-infection. F11 mAb was administered intraperitoneally at a dose of 10 mg/kg. CVS-11-infected mice were treated with F11 mAb on days 5 and 7 post-infection. NSG, NOD scid, $MHCII^-$, $muMt^-$, and CD8-depleted C57BL/6J mice were treated with F11 mAb on day 5 post-infection.

All mice were monitored twice daily and scored for disease progression with an internal scoring protocol approved by the USU IACUC. Cumulative disease scores were calculated as 0 = healthy, 1 = lethargy, 2 = weight loss, 3 = tremors, 4 = uncoordinated movement, and 5 = hind-limb paralysis. To longitudinally trace the progression of the viral infection, *in vivo* imaging was performed (see *In vivo* bioluminescence imaging). Endpoint criteria for euthanasia consisted of a 20% loss in body weight.

IN VIVO CD8 T CELL DEPLETION

To optimize the dosing regimen for CD8 T cell depletion, C57BL/6J mice received 300 µg of anti-CD8α mAb (rat IgG2b, clone 2.43; BioXCell) or isotype control mAb via the intraperitoneal route on days -3, -1, 6, 13, and 20. Spleens were harvested on days 5, 10, 17, and 25 and single-cell suspensions were stained with APC-H7 rat anti-mouse CD8α (clone 53-6.7, BD Biosciences), PerCP-Cy5.5 rat anti-mouse CD45 (clone

30-F11, BD Biosciences), and PE rat anti-mouse CD4 (clone RM 4-5, BD Biosciences). To confirm $CD8^+$ T cell depletion following ABLV-luc infection, spleens were harvested from an isotype control mouse and a CD8 T cell-depleted mouse on day 5 post-infection. Single-cell suspensions were stained with Alexa Fluor 647 rat anti-mouse CD8α (clone 53-6.7, purified from hybridoma supernatant and labeled in-house), PerCP-Cy5.5 rat anti-mouse CD45 (clone 30-F11, BD Biosciences), and PE rat anti-mouse CD4 (clone RM 4-5, BD Biosciences). Flow cytometry was performed with a BD LSRII flow-cytometer and data were analyzed with FlowJo Software (FlowJo, LLC).

IN VIVO BIOLUMINESCENCE IMAGING

Following virus infection, *in vivo* bioluminescence imaging was performed. Imaging time points are indicated in respective figures. Ten minutes prior to imaging, mice were administered D-Luciferin, Potassium Salt (GoldBio) via the intraperitoneal route. Stock solutions of D-luciferin were prepared in DPBS at 15 mg/ml and each mouse received 150 mg Luciferin/kg body weight. During each imaging session, mice were anesthetized with isoflurane and imaged in groups of three for 1 minute using the In-Vivo Xtreme II system (Bruker). Contrast levels of x-ray images were adjusted using MI software (Bruker). Scaling of the luminescence overlays was standardized for all groups of mice and mean luminescence intensity (MLI) values were calculated using MI software (Bruker). Images were exported to Adobe Photoshop for cropping.

IMMUNOHISTOCHEMISTRY

Brains from infected mice were harvested and fixed with 4% paraformaldehyde for 48 h then soaked in 18% sucrose overnight at 4°C. Tissues were embedded in OCT compound (Fisher Healthcare), flash frozen on dry ice, and 16-µm sections were cut

using a Leica CM1850 UC Cryostat (Leica Biosystems). Sections were stained with Alexa Fluor 555 mouse anti-neuronal nuclei (NeuN) (clone A60, Millipore Sigma), mouse anti-rabies G (clone 1C5, Novus Biologicals), and Alexa Fluor 647 goat anti-mouse IgG2a (Thermo Fisher Scientific) to detect ABLV-luc and CVS-11. To detect the presence of T and B lymphocytes within the CNS, sections were stained with DAPI (Thermo Fisher Scientific) or Alexa Fluor 555 mouse anti-neuronal nuclei (NeuN) (clone A60, Millipore Sigma), Alexa Fluor 647 rat anti-mouse CD8α (clone 53-6.7, purified from hybridoma supernatant and labeled in-house), Oregon Green rat anti-mouse B220 (clone RA3-6B2, purified from hybridoma supernatant and labeled in-house), and Alexa Fluor 647 rat anti-mouse CD4 (clone RM 4-5, BD Biosciences). Images were obtained on a Zeiss Axio Scan.Z1 slide scanner and Zen software. Contrast levels were adjusted in Zen and exported to Adobe Photoshop for cropping.

CONFOCAL MICROSCOPY

Confocal images were obtained with a 40× 1.4NA oil objective, a Zeiss 710 confocal microscope, and Zen software. Contrast levels were adjusted in Zen and images were exported to Adobe Photoshop for cropping.

STATISTICAL ANALYSIS

Graphical data presented throughout are expressed as means ± SEMs. For Figure 5, p values for the difference in mean between control and treated cells were calculated by one-way ANOVA with Dunnett's multiple comparison post-test. For Figure 17, p values for the difference in mean between isotype and CD8 T cell-depleted mice were calculated by two-way ANOVA with Bonferroni's multiple comparison post-test. For Figure 20, p values for the difference in mean between isotype and CD8 T cell-depleted

mice over measured over time were calculated by two-way repeated measures ANOVA with Sidak's multiple comparison post-test. Kaplan-Meier survival curves presented throughout were calculated using Graphpad Prism 7.00 software, and differences in survival were assessed using the Log-rank Mantel Cox test. All statistical graphs were generated using Graphpad Prism 7.00.

Chapter 3: Results

ESTABLISHMENT OF A LONGITUDINAL PRE-CLINICAL MODEL OF LETHAL LYSSAVIRUS INFECTION

Current knowledge of lyssavirus pathogenesis is based primarily on histological analysis of timed infections in animal models. While such data are extensive, they are also limited by the inability to anatomically trace infections in individual animals over the course of infection (207; 278; 334). Reporter viruses represent a powerful approach to trace viral infections *in vitro* and *in vivo*. For example, investigators have utilized fluorescence-based reporter RABVs to assess cell entry and replication *in vitro* (122; 202) and to trace neuronal circuits *in vivo* (274). Additionally, several studies have described luciferase-based reporter systems for the *in vivo* study of neurotropic viral infections (90; 234; 241; 242; 281). Additionally, *in vivo* imaging of animals infected with luciferase reporter viruses can be used to assess the effectiveness of therapeutics, by measuring changes in luciferase signal and spread of virus (241; 242; 281). To better understand the pathogenesis and efficacy of therapies *in vivo*, we established a longitudinal model of fatal lyssavirus infection by generating an ABLV reporter virus expressing firefly luciferase (henceforth termed ABLV-luc) (Figure 5A). Following successful insertion of the luciferase gene into the ABLV genome, ABLV-luc was rescued using a reverse genetics approach. An *in vitro* assay to detect virally encoded luciferase showed significantly higher levels of luminescence compared to a no DNA control (Figure 5B). To confirm production of infectious virions, packaging cells were stained with antibodies directed against the viral glycoprotein, and confocal microscopy analysis revealed virions in the cytoplasm (Figure 5C).

For initial *in vivo* studies, B6(cg)-*Tyr*$^{c\text{-}2J}$/J mice were inoculated via footpad

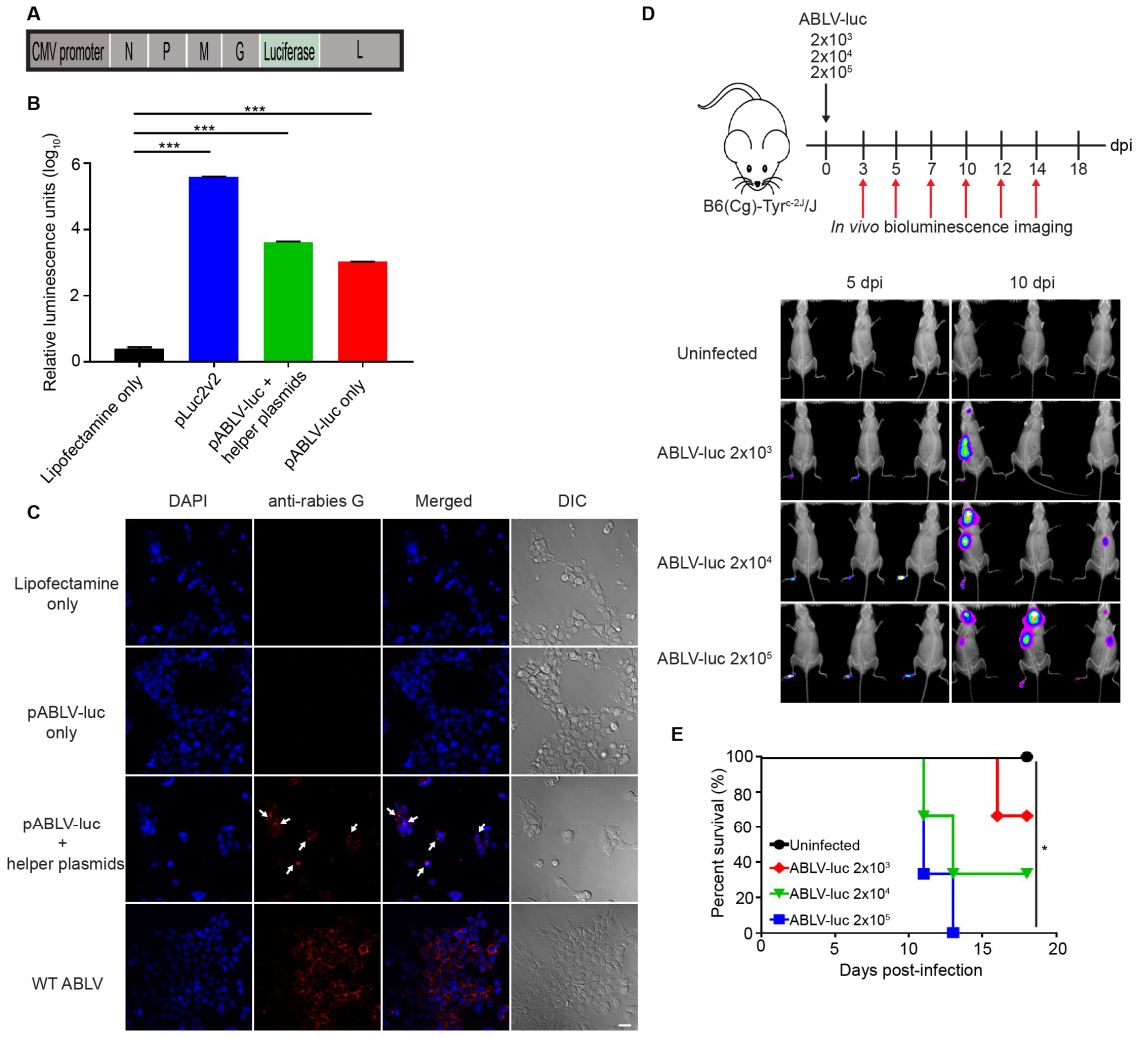


Figure 5. ABLV-luc infects the CNS of B6(cg)-*Tyr*$^{c\text{-}2J}$/J mice, resulting in clinical disease and mortality. (A) Schematic of recombinant ABLV genome encoding firefly luciferase between the G and L genes. (B) HEK293T cells containing rescued virus were lysed, incubated with luciferin and quantified for luminescence. Values are mean luminescence generated from triplicate samples (***p=0.0001, One-way ANOVA of log-transformed data, Dunnett's Multiple Comparisons Test, error bars, SEM). (C) HEK293T cells containing rescued virus were stained with anti-rabies G to detect virions. Bar, 20µm. (D) Mice were infected with 2×10^3, 2×10^4, or 2×10^5 IU of ABLV-luc on day 0 (n=3 mice/group) and bioluminescence imaging was used to detect virus. See also Figure 6. (E) Kaplan-Meier survival plot of mice infected with increasing doses of ABLV-luc (*p=0.0224, Log-rank Mantel Cox test).

injection with 2×10^3, 2×10^4, or 2×10^5 infectious units (IU) of ABLV-luc to determine a uniformly lethal dose (Figure 5D). Via bioluminescence imaging, we detected virus in the footpad of all infected mice, regardless of dose, by day 5 post-infection. By day 10 post-infection, virus was present in the CNS of all mice infected with 2×10^5 IU (Figure 5D) compared to mice infected with 2×10^3 or 2×10^4 IU, in which only a proportion developed CNS infection (Figure 6A-C). All mice infected with 2×10^5 IU lost up to 20% of their body weight whereas only a proportion of mice infected with 2×10^3 and 2×10^4 IU exhibited weight loss (Figure 6D). Ultimately, a dose of 2×10^5 IU resulted in 100% lethality by day 13 post infection compared to 67% and 33% survival in mice infected with 2×10^3 and 2×10^4 IU, respectively (Figure 5E). To determine a more precise location of ABLV-luc infection, mice were imaged in 45º increments at day 10 post-infection. Using this approach, ABLV-luc could be detected within the upper lumbar region of the spinal cord as well as the hindbrain (Figure 7).

We next compared the pathogenicity of ABLV-luc to that of wild-type (WT) ABLV. B6(cg)-*Tyr*$^{c\text{-}2J}$/J mice were infected with the sub-maximal lethality dose 2×10^4 IU of ABLV-luc or WT ABLV and monitored for signs of disease. Weight loss and disease scores were comparable between mice infected with ABLV-luc or WT ABLV (Figure 8A, B). Importantly, while there was a significant difference in survival between uninfected mice and mice infected with WT ABLV, we observed no significant difference in survival between mice infected with ABLV-luc or WT ABLV (Figure 8C) and therefore concluded that our luciferase-tagged ABLV-luc is not attenuated for virulence *in vivo*.

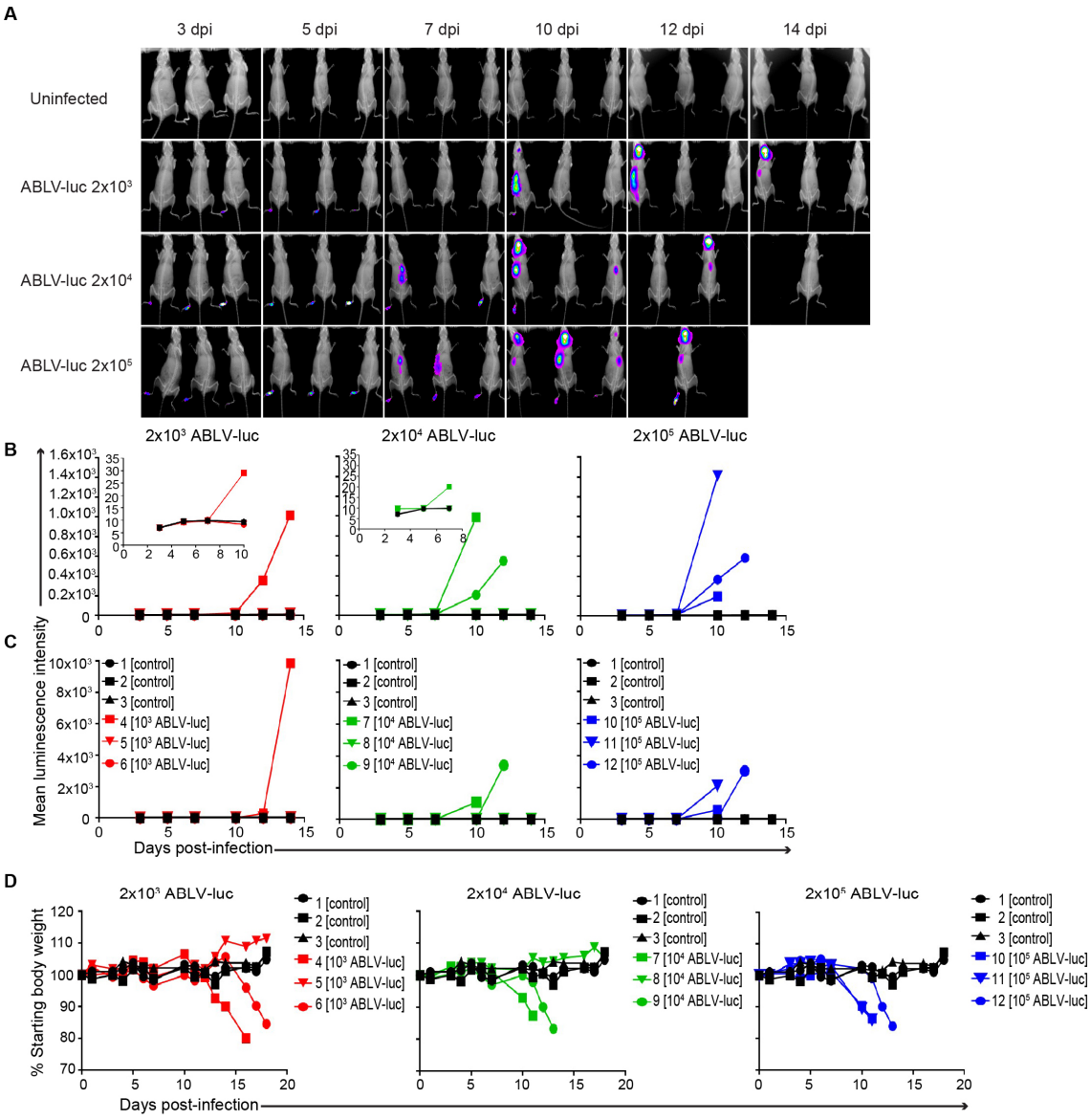


Figure 6. ABLV-luc dose response in B6(cg)-*Tyr*$^{c\text{-}2J}$/J mice. (A) Bioluminescence imaging of mice infected with 2×10^3, 2×10^4, or 2×10^5 IU of ABLV-luc. Viral burden was quantified as mean luminescence intensity in the spines (B) and brains (C) of infected mice. (D) Percent starting body weight as an indicator of disease.

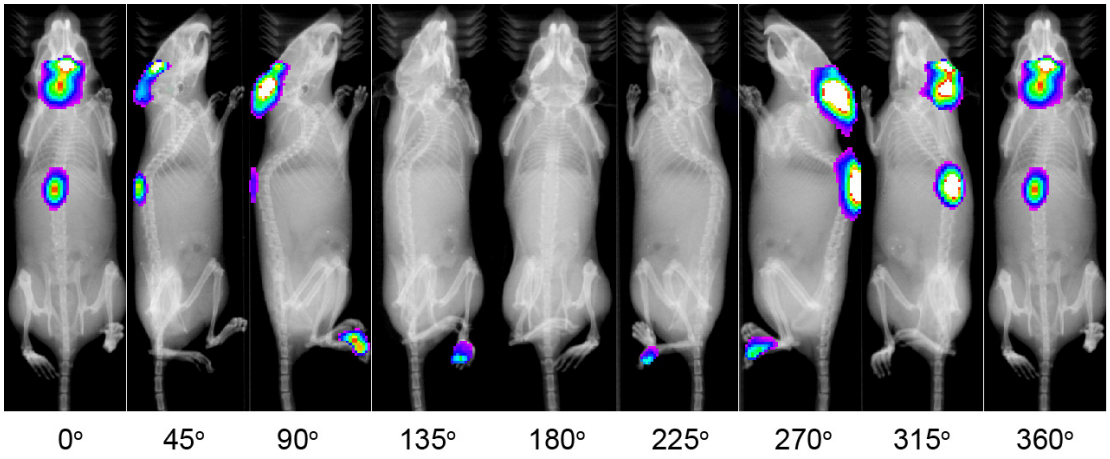


Figure 7. Rotational imaging of ABLV-luc infected mice. Bioluminescence imaging of ABLV-luc infection in a mouse infected with 2×10^5 IU ABLV-luc at 10 days post-infection. Images were taken in 45^0 increments to more precisely localize the anatomical origin of the luminescence signals.

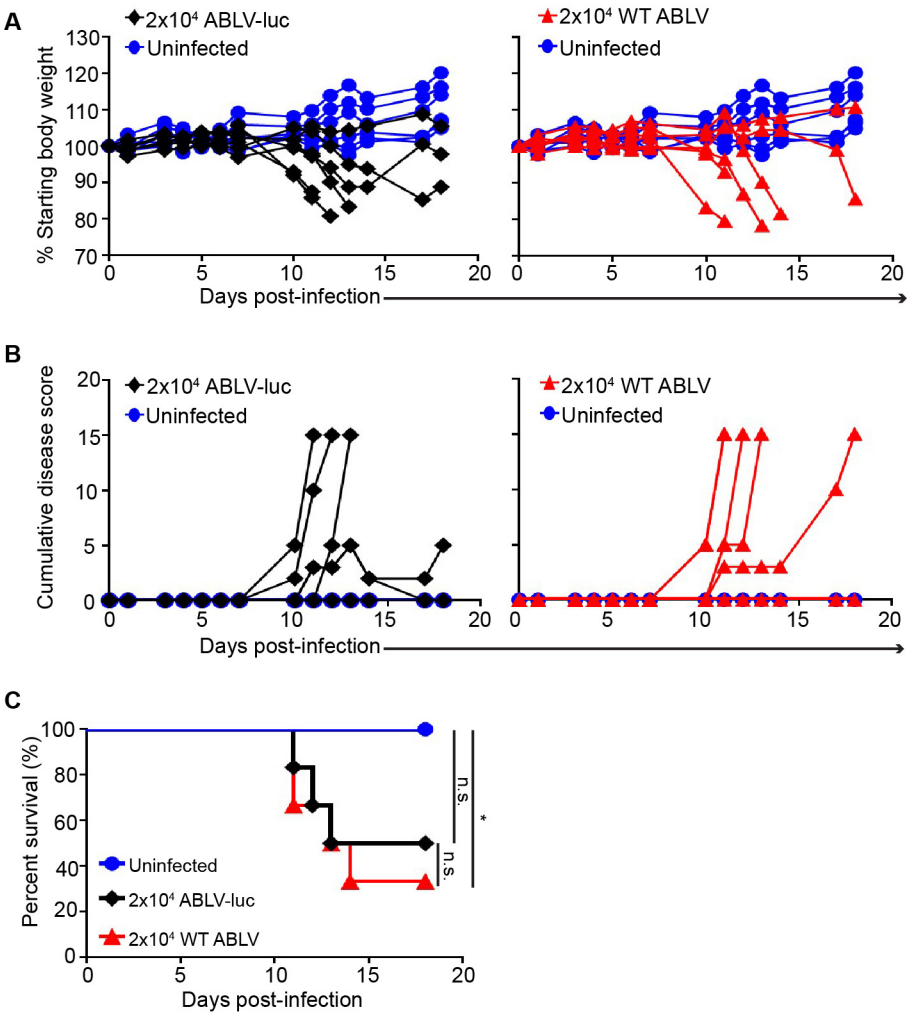


Figure 8. Comparison of virulence between ABLV-luc and WT ABLV. Percent starting body weight (A), cumulative disease score (B), and Kaplan-Meier survival plot (C) of B6(cg)-*Tyr*$^{c\text{-}2J}$/J mice infected with ABLV-luc or WT ABLV (n.s., not significant, *p=0.0179, Log-rank Mantel Cox test).

CLEARANCE OF ABLV-LUC BY HUMAN MAB F11, POST-VIRUS REPLICATION IN THE CNS

F11 is a recombinant Human IgG1 mAb that targets the ABLV glycoprotein G (see *Materials and Methods*). As shown in Figure 9A, F11 mAb demonstrates potent *in vitro* neutralization of ABLV-luc with a neutralization titer of 0.267 μg/ml. To evaluate the *in vivo* efficacy of mAb therapy on established lyssavirus infection, mice were infected with a lethal dose (2×10^5 IU) of ABLV-luc and treated with a single injection of F11 mAb (10 mg/kg body weight) on day 3, 5, or 7 post-infection (Figure 10A). In addition to bioluminescence imaging, the presence of virus within the CNS was confirmed via immunofluorescence microscopy (Figure 9B). Treatment initially appeared to clear virus from day 3-treated mice prior to CNS infection. However, in these mice, virus appeared in the brain with delayed kinetics (day 14) and was ultimately lethal in the majority of animals by day 26 post-infection (Figure 10B, 11).

While unable to prevent virus from infecting the CNS, treatment with F11 mAb on day 5 or 7 completely cleared virus from the spines and brains by 38 dpi (Figure 10B, 11). Furthermore, while virus was cleared from the footpad of all mice treated on days 5 and 7 and in the majority of mice treated on day 3, viral clearance in the foot was not observed in untreated mice (Figure 10B). Similar to untreated animals, mice treated on days 5 and 7 began exhibiting signs of clinical disease on day 8 post-infection (Figure 10C, D). However, weight loss and clinical disease resolved in all of the mice treated on day 5 and in the majority of mice treated on day 7, by days 25 and 18, respectively. Hind limb paralysis did remain in a proportion of day 7-treated mice despite viral clearance (Figure 10C, D). By contrast, with the exception of one mouse that began exhibiting signs of illness on day 8, clinical disease did not become apparent in day 3-treated mice

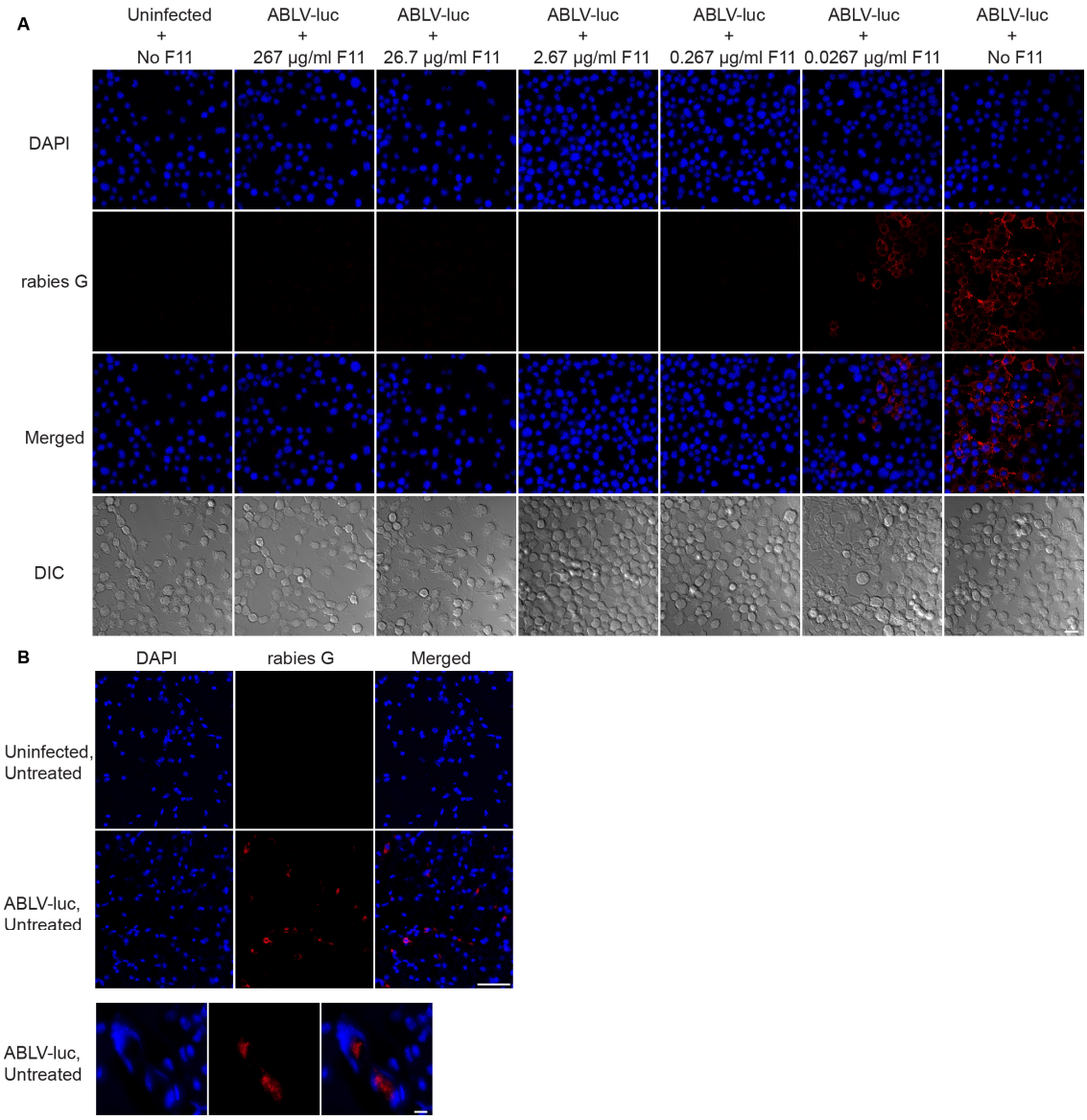
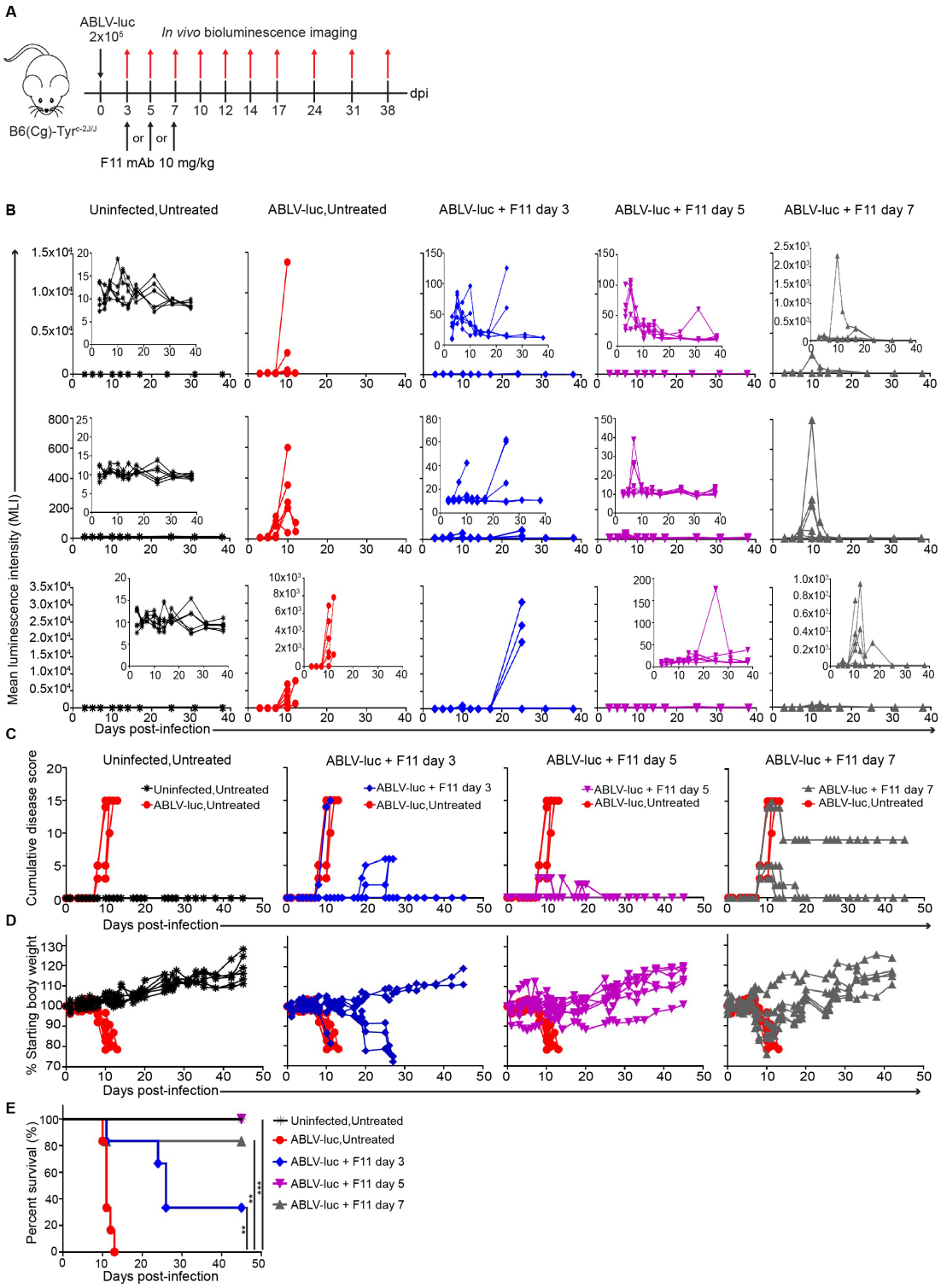


Figure 9. *In vitro* neutralization assay and confirmation of ABLV-luc in brain. (A) Ten-fold serial dilutions of F11 mAb were incubated with 8×10^4 IU ABLV-luc, added to mouse N2a cells, and cells were stained with anti-rabies G to detect virions. Bar, 20 µm. (B) Coronal sections of brain stem from ABLV-luc-infected B6(cg)-*Tyr*$^{c\text{-}2J}$/J mice were stained to confirm the presence of virus on day 11 post-infection. Bar, top set 50 µm, bottom set 10 µm.

Figure 10. Single dose F11 mAb therapy clears ABLV-luc from CNS and promotes survival. (A) B6(cg)-*Tyr*$^{c\text{-}2J}$/J mice were infected with 2×10^5 IU of ABLV-luc on day 0 and F11 mAb (10 mg/kg) was administered on day 3, 5, or 7 (n= 6 mice/group). (B) Viral burden was quantified as mean luminescence intensity within the footpads (top row), spines (middle row), and brains (bottom row) of mice. See also Figure 11. (C) Cumulative disease scores were determined by clinical presentation following ABLV-luc challenge. (D) Percent starting body weight as an indicator of disease (E) Kaplan-Meier survival plot (**p=0.0044, ***p=0.0005, Log-rank Mantel Cox test).


A
ABLV-luc
2x10^5
In vivo bioluminescence imaging
dpi
0 3 5 7 10 12 14 17 24 31 38
B6(Cg)-Tyr^c-2J/J
or or
F11 mAb 10 mg/kg
B
Uninfected,Untreated
ABLV-luc,Untreated
ABLV-luc + F11 day 3
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ABLV-luc + F11 day 7
Mean luminescence intensity (MLI)
Days post-infection
C
Cumulative disease score
Uninfected,Untreated
ABLV-luc,Untreated
ABLV-luc + F11 day 3
ABLV-luc + F11 day 5
ABLV-luc + F11 day 7
Days post-infection
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% Starting body weight
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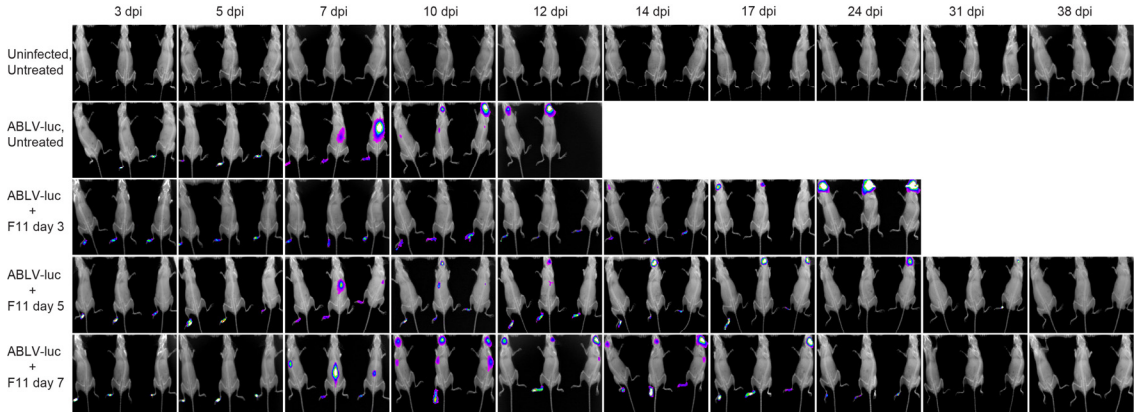


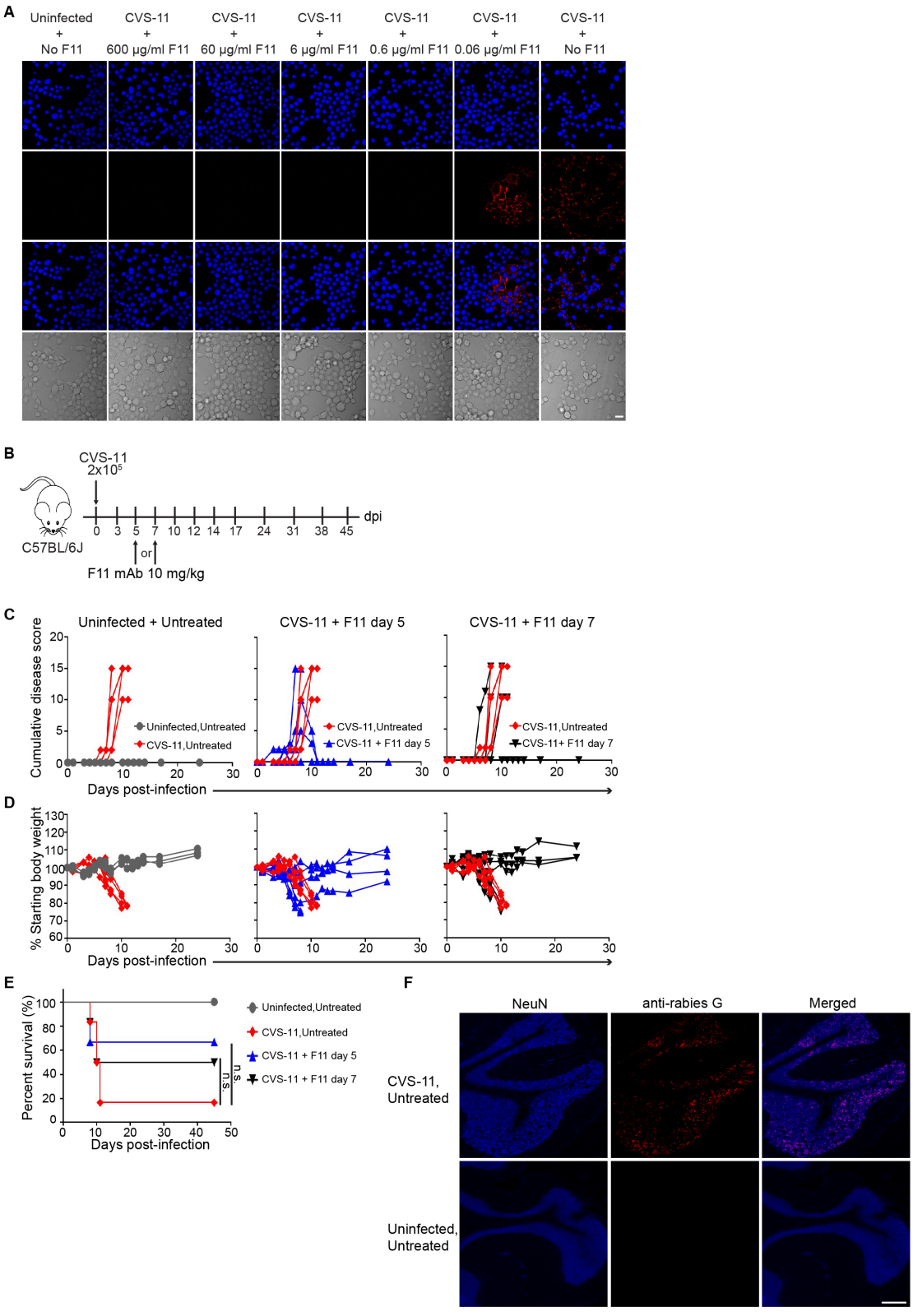
Figure 11. F11 mAb therapy clears ABLV-luc infection. Bioluminescence imaging of B6(cg)-*Tyr*$^{c\text{-}2J}$/J mice infected with 2×10^5 IU of ABLV-luc and treated with F11 mAb on day 3, 5, or 7 post-infection.

until day 19 post-infection, including rapid weight loss in the days prior to death (Figure 10C, D). Finally, survival analysis showed that treatment of mice with F11 mAb on days 5 or 7 resulted in 100% and 83% survival, respectively, whereas only 33% of mice treated on day 3 survived (Figure 10E). These data demonstrate that F11 mAb therapy is highly effective for treatment of ABLV infections, even when delivered post-virus replication in the CNS. Moreover, within the groups of mice treated on days 5 and 7, most F11 mAb-treated animals that experience severe virus-induced symptoms ultimately recover and survive.

F11 MAB EFFECTIVELY TREATS ESTABLISHED RABV INFECTION

To assess whether F11 mAb could be used to treat other related viruses within the *Lyssavirus* genus, mice were infected with 2×10^5 IU of the mouse-adapted lethal RABV, challenge virus standard (CVS)-11. As shown in Figure 12A, like ABLV, CVS-11 is also potently neutralized by F11 mAb *in vitro*, exhibiting a neutralization titer of 0.6 μg/ml. Infected mice were treated with a single dose of F11 mAb on day 5 or 7 post-infection and monitored for clinical signs of disease (Figure 12B). Clinical disease and weight loss resolved in the majority of mice treated on day 5 and in half of the mice treated on day 7 (Figure 12C, D). Survival analysis showed that F11 mAb-treatment of CVS-11-infected mice resulted in 67% and 50% survival when treated on day 5 or day 7, respectively (Figure 12E). Importantly, the highly pathogenic CVS-11 strain induced clear symptoms as early as day 3 post-infection, and three out of six mice with symptoms on or before day 5 exhibited symptom reversal and survival, post-F11 mAb treatment. The presence of CVS-11 within the brains of infected, untreated animals was confirmed via immunofluorescence microscopy (Figure 12F). These data suggest that F11 mAb may be

Figure 12. F11 mAb is effective against rabies virus. (A) Ten-fold serial dilutions of F11 mAb were incubated with 1×10^5 IU CVS-11, added to mouse N2a cells, and cells were stained with anti-rabies G to detect virions. Bar, 20 μm. (B) Mice were infected with 2×10^5 IU of CVS-11 on day 0 and F11 mAb (10 mg/kg) was administered on day 5 or 7 (n= 6 mice/group, n=3 Uninfected, Untreated mice). (C-E) Cumulative disease scores, percent starting body weight, and Kaplan-Meier survival plot of mice following CVS-11 challenge and F11 mAb treatment (n.s., not significant, Log-rank Mantel Cox test). (F) Coronal sections of cerebellum from CVS-11-infected mice were stained to confirm the presence of virus on day 8 post-infection. Bar, 200 μm.


A
Uninfected + No F11
CVS-11 + 600 µg/ml F11
CVS-11 + 60 µg/ml F11
CVS-11 + 6 µg/ml F11
CVS-11 + 0.6 µg/ml F11
CVS-11 + 0.06 µg/ml F11
CVS-11 + No F11
B
CVS-11 2x10^5
C57BL/6J
0 3 5 7 10 12 14 17 24 31 38 45
dpi
or
F11 mAb 10 mg/kg
C
Uninfected + Untreated
CVS-11 + F11 day 5
CVS-11 + F11 day 7
Cumulative disease score
Uninfected,Untreated
CVS-11,Untreated
CVS-11 + F11 day 5
CVS-11+ F11 day 7
Days post-infection
D
% Starting body weight
Days post-infection
E
Percent survival (%)
Uninfected,Untreated
CVS-11,Untreated
CVS-11 + F11 day 5
CVS-11 + F11 day 7
n.s.
Days post-infection
F
NeuN
anti-rabies G
Merged
CVS-11, Untreated
Uninfected, Untreated

effective for the treatment of a broad range of rabies-like lyssaviruses, and that F11 mAb therapy can be effective even after clear symptom onset.

SEVERELY IMMUNOCOMPROMISED MICE ARE UNABLE TO CLEAR ABLV FROM THE CNS FOLLOWING F11 MAB TREATMENT

As shown above, F11 mAb treatment administered on day 7 post-infection successfully clears virus from the CNS, even though ABLV is already robustly replicating within the spine by this time point. However, our data strongly suggest that the mode of clearance is more complicated than simple virus neutralization. More specifically, given that the half-life of human mAbs in mice is 6-8 days (357), the amount of F11 mAb is reduced to less than $1/16^{th}$ of the initial dose when viral clearance within the brain is observed beyond 30 dpi (Figure 10, 11). Therefore, we hypothesized that F11 mAb facilitates cellular or humoral immune responses that ultimately mediate viral clearance within the brain. A prediction of this model is that F11 mAb would not be effective at clearing infections in immunocompromised mice. We first tested NSG mice, which are deficient in T cells, B cells, and functional natural killer (NK) cells. We infected NSG mice with 2×10^5 IU of ABLV-luc and treated animals with F11 mAb on day 5 (Figure 13A). Using bioluminescent imaging, we found that F11 mAb-treated NSG mice were unable to clear ABLV-luc from the CNS (Figure 13B). Furthermore, F11 mAb treatment did not reduce viral burden in the brains or spines of NSG mice, although amplification and anatomical progression of the virus was delayed compared to untreated mice (Figure 14A, B). In addition, virus was not cleared from the footpads of untreated mice and remained at low levels in F11-treated NSG (Figure 14C).Weight loss and mortality were also delayed, relative to untreated animals. Ultimately, all F11 mAb-treated NSG mice succumbed to infection by day 26 (Figure 14D, E). Similar results

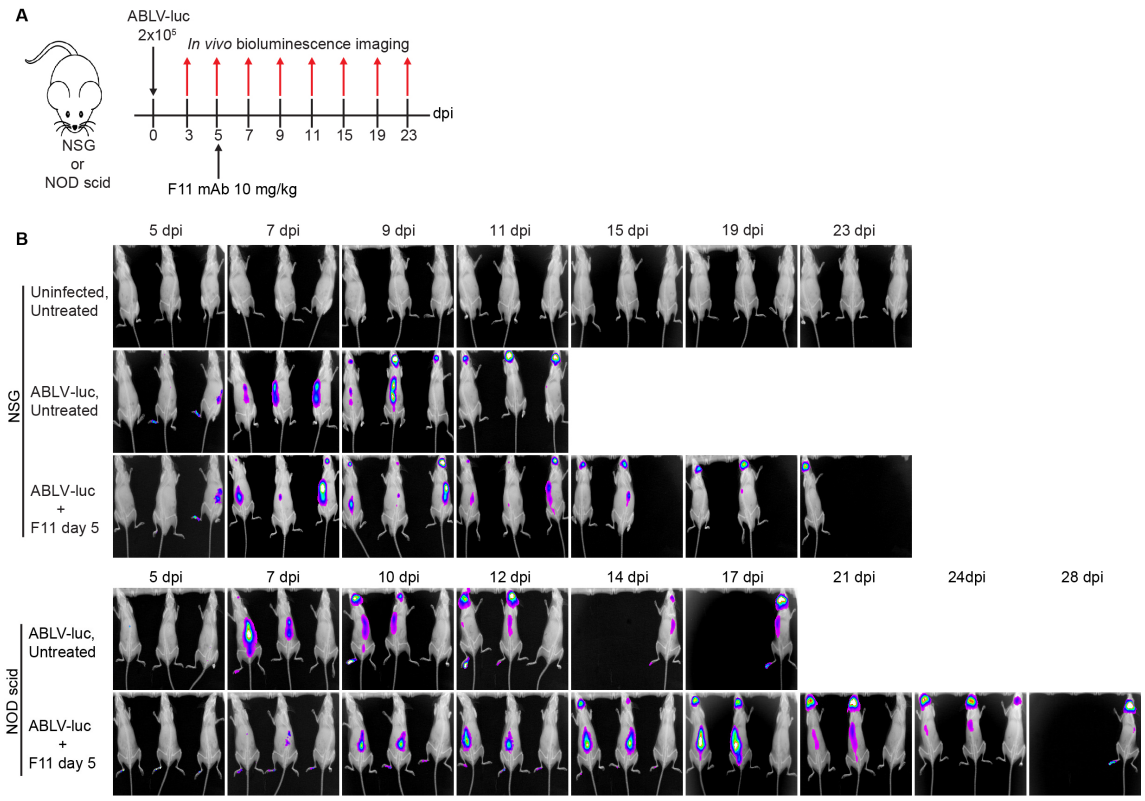
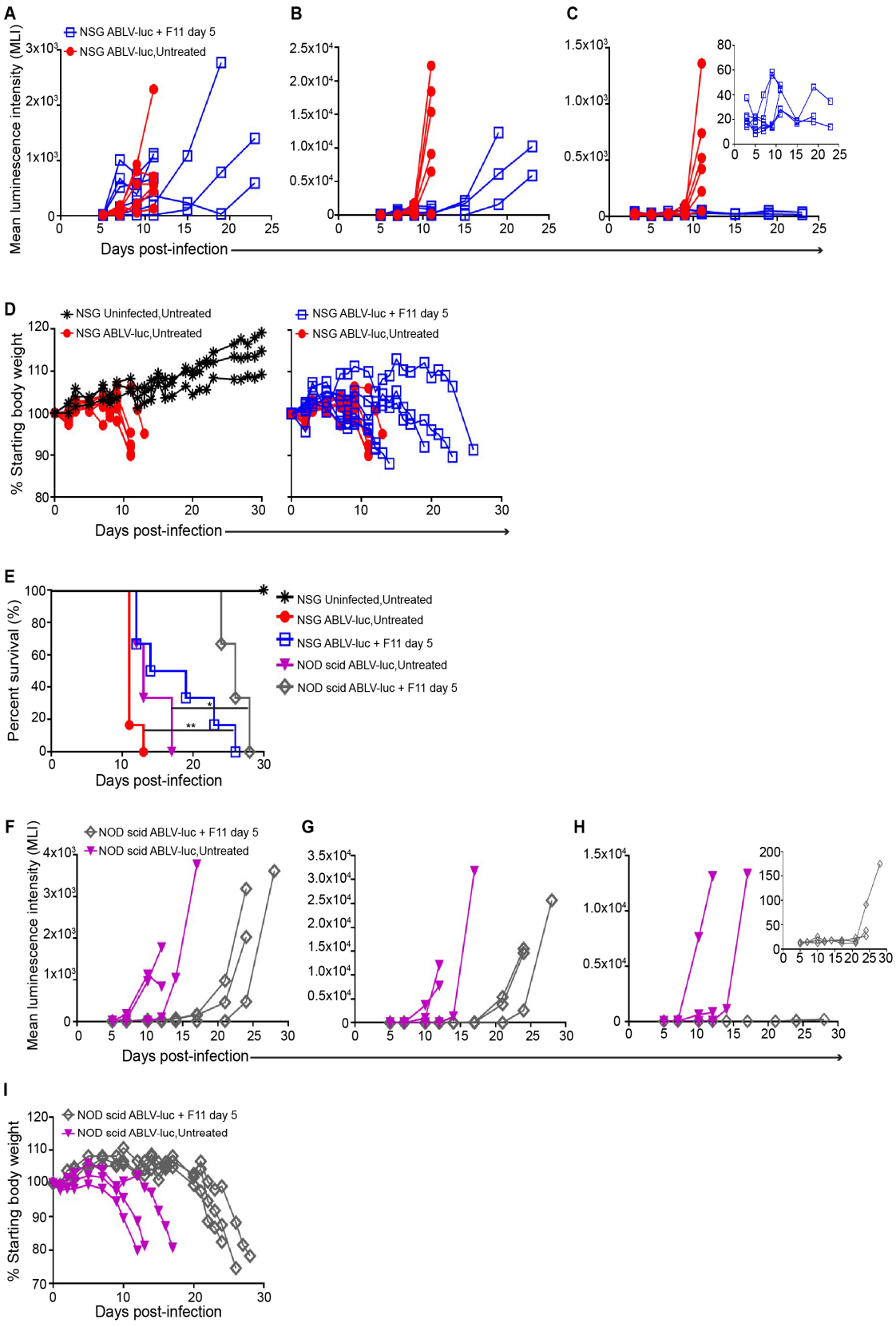


Figure 13. Adaptive immunity is essential for viral clearance from the CNS. (A) Severely immunocompromised mice (n=3 Uninfected, Untreated NSG mice, n=6 NSG mice/group, n=3 NOD scid mice/group) were infected with 2×10^5 IU of ABLV-luc on day 0 and F11 mAb (10 mg/kg) was administered on day 5. (B) Bioluminescence imaging of mice following infection.

Figure 14. F11 mAb does not ameliorate viral burden or clinical disease in severely immunocompromised mice. Viral burden was quantified as mean luminescence intensity in the spines (A), brains (B), and footpads (C) of NSG mice. (D) Percent starting body weight in NSG mice. (E) Kaplan-Meier survival plot of all mice following ABLV-luc challenge and F11 mAb treatment (**p=0.0051, *p=0.0246, Log-rank Mantel Cox test). Viral burden was quantified as mean luminescence intensity in the spines (F), brains (G), and footpads (H) of NOD scid mice. (I) Percent starting body weight of NOD scid mice.


A
NSG ABLV-luc + F11 day 5
NSG ABLV-luc,Untreated
Mean luminescence intensity (MLI)
Days post-infection
B
C
D
NSG Uninfected,Untreated
NSG ABLV-luc,Untreated
NSG ABLV-luc + F11 day 5
% Starting body weight
Days post-infection
E
Percent survival (%)
NSG Uninfected,Untreated
NSG ABLV-luc,Untreated
NSG ABLV-luc + F11 day 5
NOD scid ABLV-luc,Untreated
NOD scid ABLV-luc + F11 day 5
Days post-infection
F
NOD scid ABLV-luc + F11 day 5
NOD scid ABLV-luc,Untreated
Mean luminescence intensity (MLI)
Days post-infection
G
H
I
NOD scid ABLV-luc + F11 day 5
NOD scid ABLV-luc,Untreated
% Starting body weight
Days post-infection

were obtained with NOD-scid mice, which lack T and B cells but have functional NK cells (Figure 13A, B, 14E-I). Taken together, the data in Figures 13 and 14 show that an adaptive immune response is essential for viral clearance, and that the presence of NK cells is not sufficient to clear ABLV-luc from the brain. Furthermore, although F11 mAb appears to potently neutralize lyssavirus, delaying the kinetics of high CNS replication and disease manifestations, enough virus remains to ultimately cause lethality in the absence of intact adaptive immunity. Thus, these data strongly suggest that the key therapeutic activity of F11 mAb is facilitation of an adaptive immune response that clears virus post-CNS replication.

B CELLS ARE DISPENSABLE FOR ABLV CLEARANCE

One possible explanation for the efficacy of F11 mAb is simply delaying virus replication for long enough that the host humoral (B cell) immune response is able to produce high amounts of neutralizing antibody, ultimately clearing the infection. We thus assessed the requirement for host B cells in virus clearance from the CNS. To accomplish this, B cell-deficient mice (muMt$^-$) were infected with 2×10^5 IU of ABLV-luc and treated with F11 mAb on day 5. Clearance of ABLV from the CNS and resolution of clinical disease was observed in muMt$^-$ mice (Figure 15, 16). Similar to WT mice, all F11 mAb-treated muMt$^-$ mice maintained their body weight and survived infection, with no significant difference in survival observed between F11 mAb-treated WT and muMt$^-$ mice (Figure 16A, C). Thus, these data demonstrate that host B cells do not measurably contribute to the therapeutic efficacy of F11 mAb.

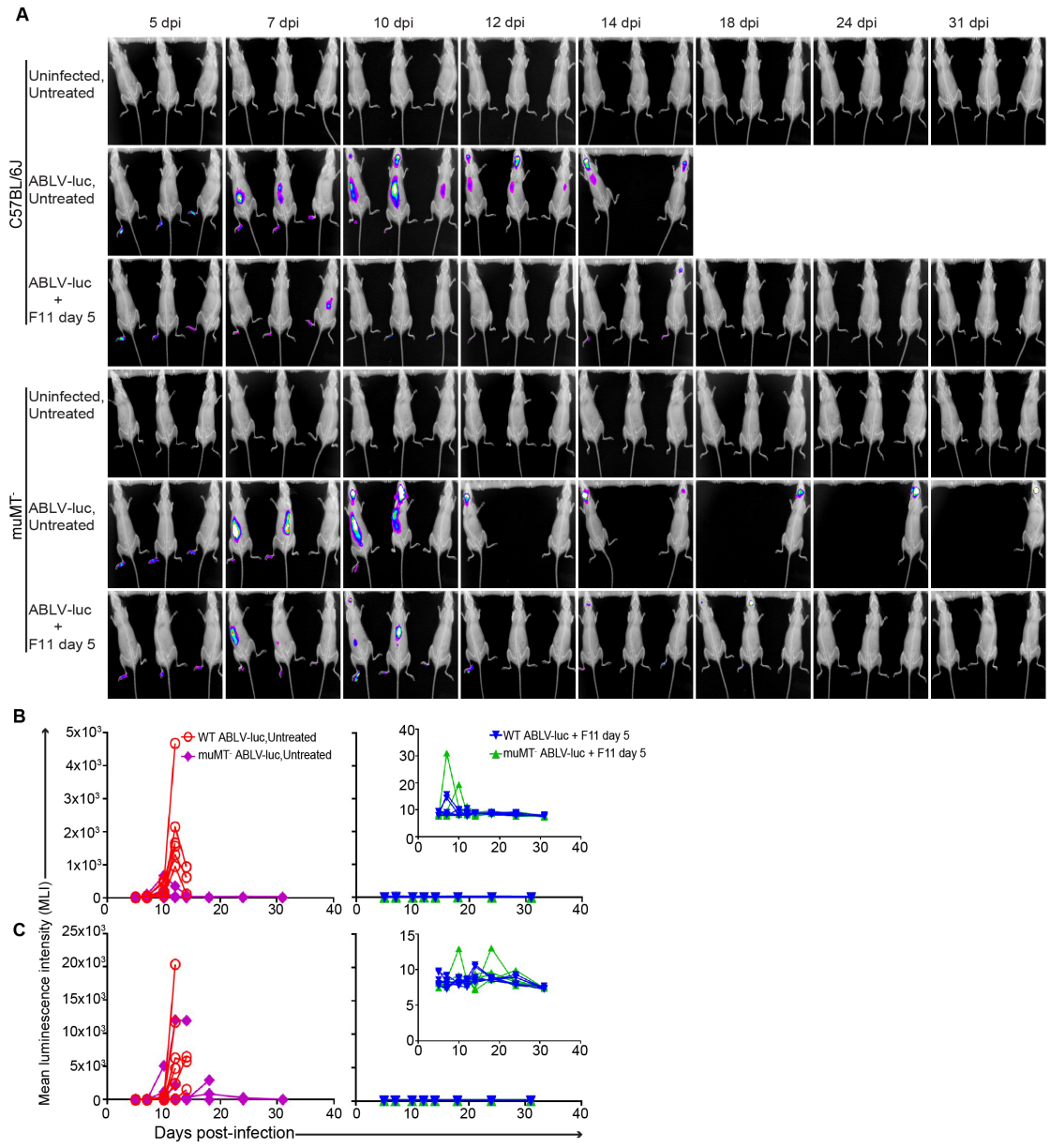


Figure 15. B cells do not play an essential role in viral clearance. (A) Bioluminescence imaging of C57BL/6J and muMt$^-$ mice infected with 2×10^5 IU ABLV-luc on day 0 and treated with F11 mAb on day 5 (n=6 mice/group, n=3 Uninfected, Untreated mice/group). Viral burden was quantified as mean luminescence intensity (MLI) in the spines (B) and brains (C) of infected mice.

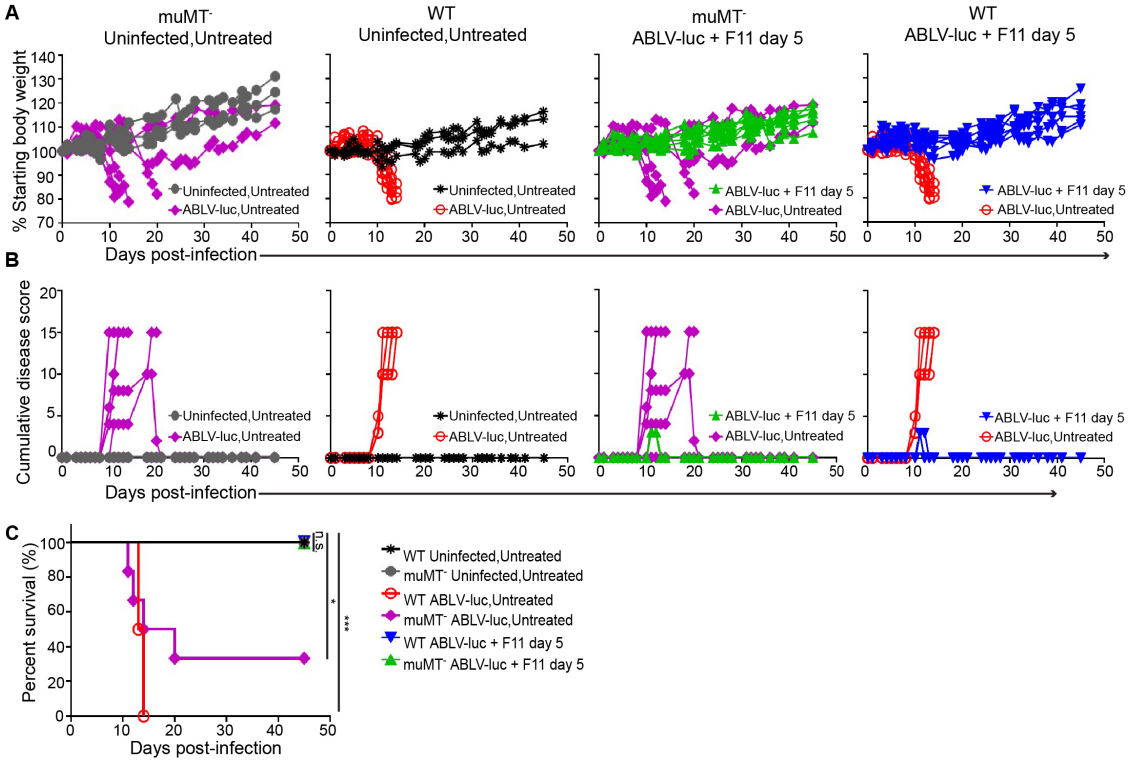


Figure 16. F11 mAb ameliorates clinical disease in B cell-deficient mice. Percent starting body weight (A) and cumulative disease scores (B) following ABLV-luc challenge and F11 mAb treatment of C57BL/6J and muMt$^-$ mice. (C) Kaplan-Meier survival plot (n.s., not significant, *p=0.0183, ***p=0.0009, Log-rank Mantel Cox test).

CD4 T CELLS BUT NOT CD8 T CELLS ARE REQUIRED FOR ABLV CLEARANCE FROM THE CNS

Because B cell-deficient mice showed no reduction in F11 mAb efficacy in comparison to WT mice, we concluded that the key effector population must be a T cell subset. We first addressed the role of CD8 T cells, as the primary function of this T cell subset is the killing of cells infected with intracellular pathogens, such as viruses. Although we initially intended to use a genetic model in which mice fail to differentiate the CD8 T cell lineage, the class I-deficient (β2-microglobulin -/-) model could not be used, because this model also lacks functional FcRn, which is required for IgG transport to extracellular spaces and which greatly extends the *in vivo* half-life of IgG (10; 133; 192; 300). The CD8-/- model is also not appropriate for these studies, as it has been shown that these mice still produce functional cytotoxic cells in quantities sufficient to clear certain viral infections (18; 95).

Having ruled out a genetic approach, we instead used *in vivo* mAb-depletion of CD8 T cells to determine whether CD8 T cells were required for viral clearance. Preliminary studies confirmed an appropriate dose and timeline of anti-CD8α mAb administration for ≥ 95% CD8 T cell depletion in mice through at least day 20 (Figure 17). Mice were infected with 2×10^5 IU of ABLV-luc and treated with F11 mAb on day 5 (Figure 18A). While complete viral clearance was delayed in CD8 T cell-depleted mice relative to mice given isotype control mAb (Figure 19, 20A, B), all mice survived and no significant difference in survival was observed between F11-treated isotype control and CD8 T cell-depleted mice (Figure 18B). Interestingly, low levels of viral signal could still be detected in the spines and brains of CD8 T cell-depleted mice until days 18 and 36 post-infection, respectively (Figure 20A-D), suggesting that CD8 T cells contribute to

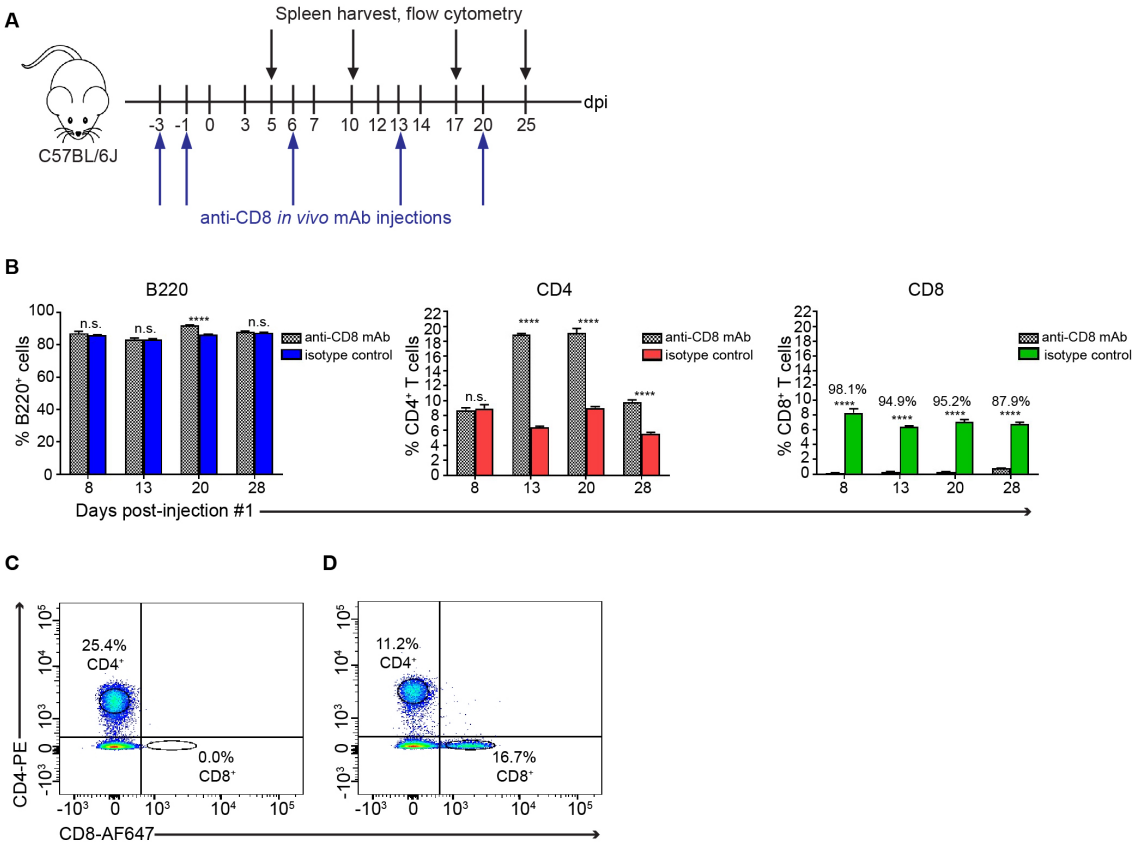


Figure 17. CD8 T cell depletion in C57BL/6J mice. (A) Anti-CD8α mAb (300 µg) was administered to mice on days -3, -1, 6, 13, and 20. Spleens were harvested on days 5, 10, 17, and 25 to assess CD8 T cell depletion. (B) Values are mean percentage of B220+ cells, CD4+ T cells, and CD8+ T cells on days 8, 13, 20, and 28 following the first dose of anti-CD8α mAb (B220: ****p<0.0001, CD4:****p<0.0001, CD8:****p<0.0001, n.s., not significant, Two-way ANOVA, Bonferroni's multiple comparisons test, error bars, SEM). Percent CD8 T cell depletion shown above each set of bars in CD8 panel. Representative flow plots from mice that received anti-CD8α mAb (C) or isotype control (D) on day 5.

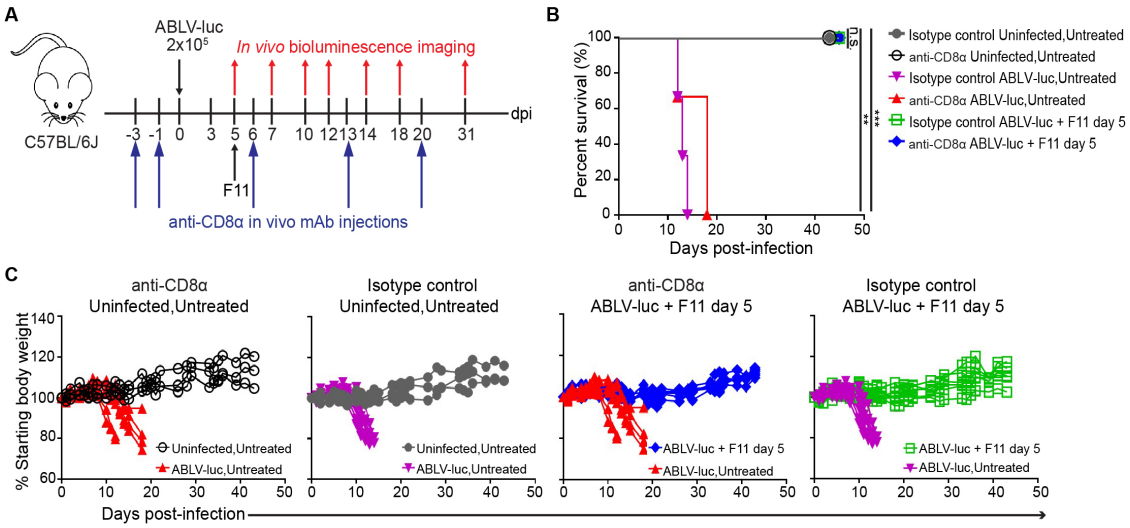


Figure 18. CD8 T cells are dispensable for viral clearance. (A) Isotype control and CD8 T cell-depleted C57BL/6J mice were infected with 2×10^5 IU of ABLV-luc on day 0 and F11 mAb (10 mg/kg) was administered on day 5 (n=6 mice/group, n=3 Uninfected, Untreated mice/group). Kaplan-Meier survival plot (n.s., not significant, **p=0.0012, ***p=0.0007, Log-rank Mantel Cox test) (B) and percent starting body weight (C) of CD8 T cell-depleted mice.

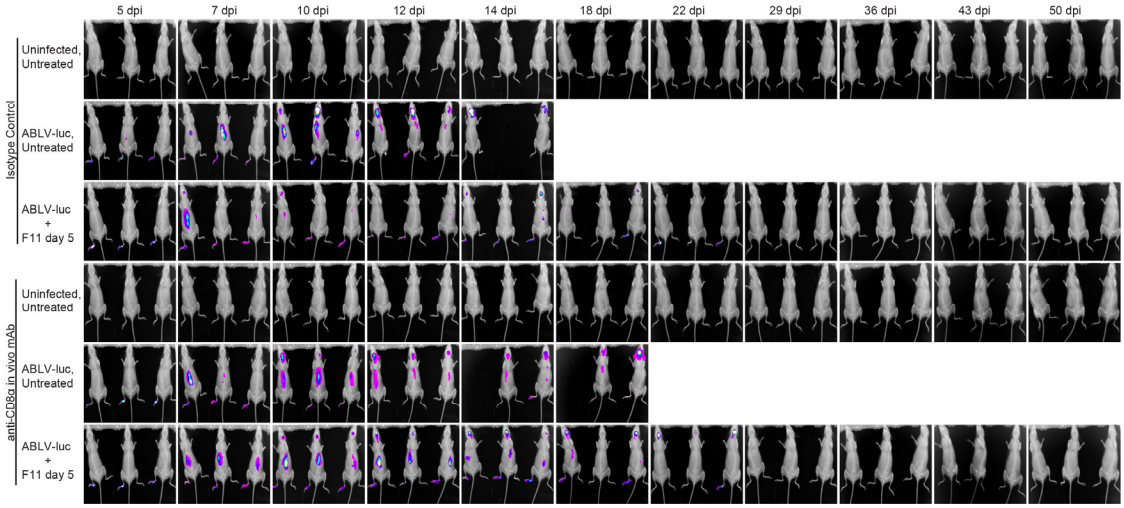
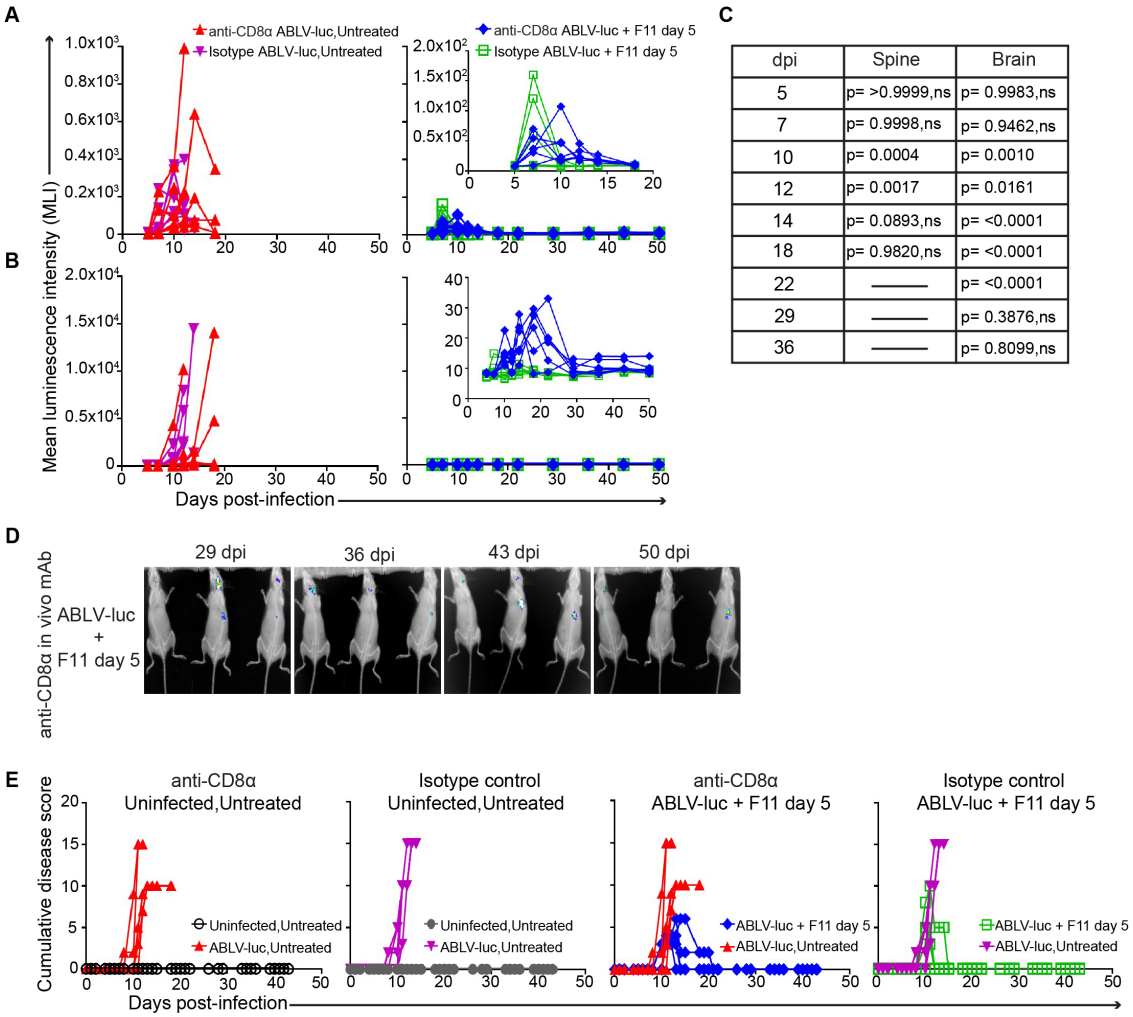


Figure 19. CD8 T cells do not play an essential role in viral clearance.
Bioluminescence imaging of isotype control and CD8 T cell-depleted mice infected with 2×10^5 IU ABLV-luc and treated with F11 mAb on day 5.



dpi	Spine	Brain
5	p= >0.9999,ns	p= 0.9983,ns
7	p= 0.9998,ns	p= 0.9462,ns
10	p= 0.0004	p= 0.0010
12	p= 0.0017	p= 0.0161
14	p= 0.0893,ns	p= <0.0001
18	p= 0.9820,ns	p= <0.0001
22	———	p= <0.0001
29	———	p= 0.3876,ns
36	———	p= 0.8099,ns

Figure 20. F11 mAb ameliorates viral burden and clinical disease in CD8 T cell-depleted mice. Viral burden was quantified as mean luminescence intensity (MLI) in the spines (A) and brains (B) of infected mice. (C) Two-way repeated measures ANOVA on log-transformed MLI values. (D) Bioluminescence imaging of CD8 T cell-depleted mice maintaining low levels of virus within brain. (E) Cumulative disease scores were determined by clinical presentation following ABLV-luc challenge.

elimination of virus-infected cells. No differences in body weight were observed between CD8 T cell-depleted mice and control mice (Figure 18C) and resolution of clinical disease was observed in both depleted and control animals (Figure 20E). To determine if mice that recovered from ABLV-luc infection developed protective immunity, we rechallenged day 5-treated mice on day 82 post-infection with 2×10^5 IU of ABLV-luc. Upon rechallenge, virus could not be detected in any tissue in isotype control or CD8 T cell-depleted mice, and clinical disease did not develop (Figure 21A-E). Importantly, 100% of isotype control and CD8 T cell-depleted mice survived (Figure 21F). Together, these data suggest that F11 mAb-treated mice develop a protective immune response capable of preventing infection upon re-challenge with a lethal dose of ABLV-luc. Although CD8 T cells contribute to virus clearance, our data suggest this population is not essential for elimination of lyssavirus infection or for development of protective memory.

To investigate the contribution of CD4 T cells to viral clearance, we infected CD4 T cell-deficient mice ($MHCII^-$) with 2×10^5 IU of ABLV-luc. Mice were treated with F11 mAb on day 5 (Figure 22A). Through bioluminescence imaging, we found that $MHCII^-$ mice are unable to clear ABLV from the CNS (Figure 23, 24A, B) and do not survive infection (Figure 22B). Although the kinetics of virus amplification in the CNS were delayed in F11 mAb-treated $MHCII^-$ mice (Figure 24A, B), 100% of these animals exhibited weight loss and failed to resolve clinical disease (Figure 22C, 24C). Together, the data in Figures 13, 14, and 22-24 demonstrate that F11 mAb-mediated clearance of lyssavirus infection from the CNS requires an adaptive immune response, with CD4 T cells being required for this activity.

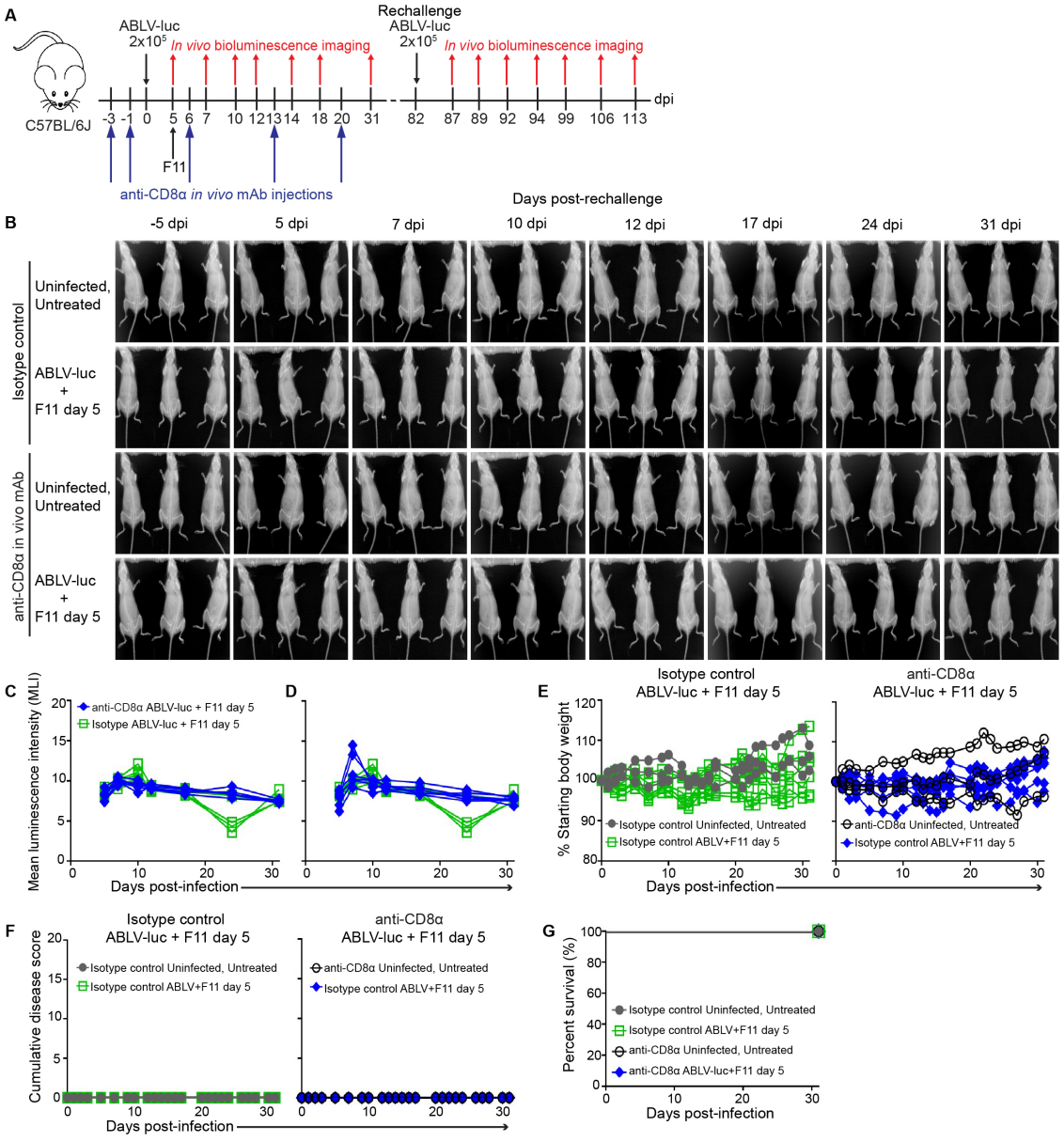


Figure 21. F11 mAb protects mice against rechallenge. (A) Day 5-treated mice were rechallenged with 2x10^5 IU of ABLV-luc on day 82. (B) Bioluminescence imaging of mice on days -5, 5, 7, 10, 12, 17, 24, and 31 post-rechallenge. Viral burden was quantified as mean luminescence intensity (MLI) in the spines (C) and brains (D) of infected mice. Percent starting body weight (E), cumulative disease scores (F), and Kaplan-Meier survival plot (G) of rechallenged mice.

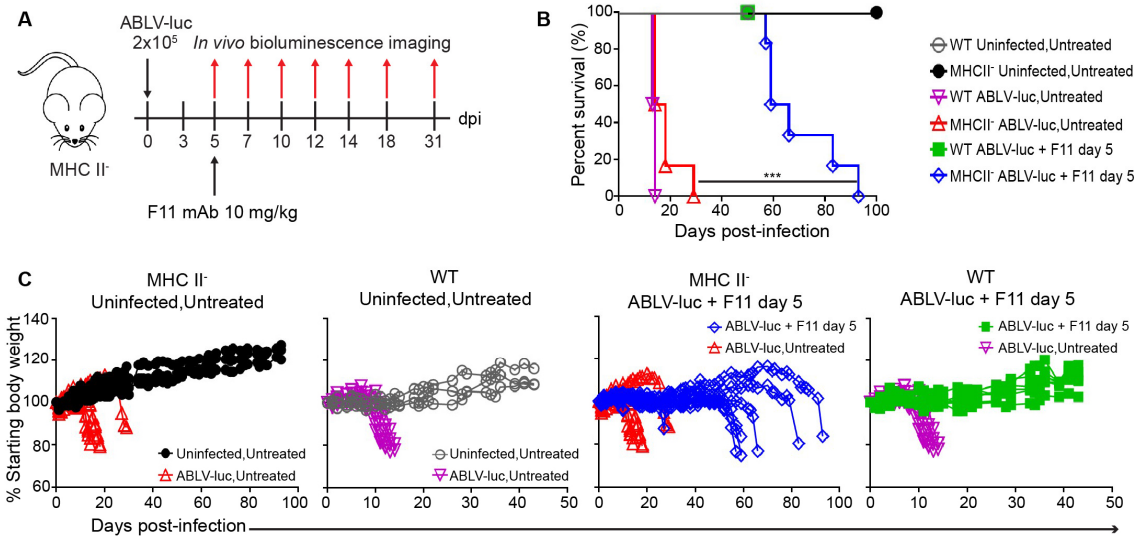


Figure 22. CD4 T cells are essential for viral clearance. (A) C57BL/6J and MHCII$^-$ mice were infected with 2×10^5 IU of ABLV-luc on day 0 and F11 mAb (10 mg/kg) was administered on day 5 (n= 6 mice/group, n=3 Uninfected, Untreated mice/group). Kaplan-Meier survival plot (B) and percent starting body weight (***p= 0.0005, Log-rank Mantel Cox test) (C) of mice following ABLV-luc challenge.

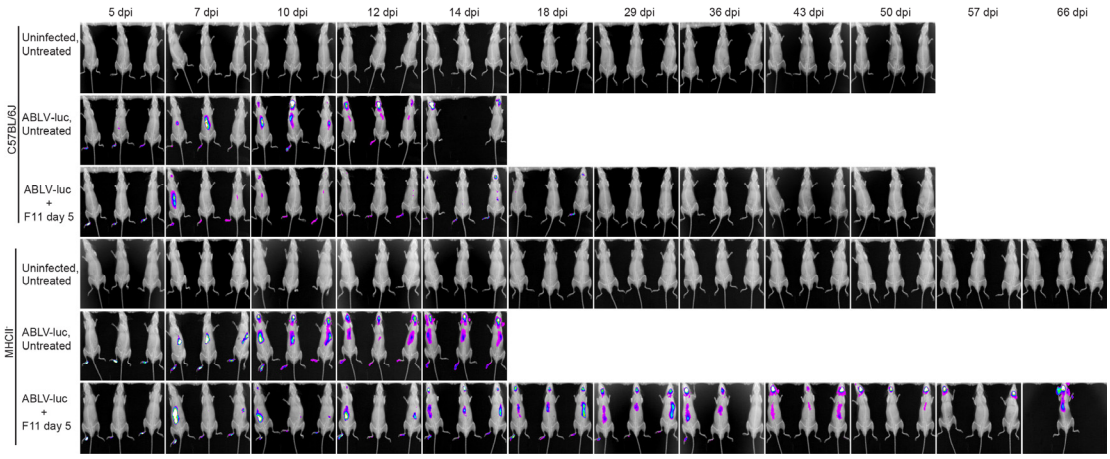


Figure 23. CD4 T cells are essential for viral clearance within the CNS. Bioluminescence imaging of C57BL/6J and MHCII^{-} mice infected with 2×10^{5} IU ABLV-luc and treated with F11 mAb on day 5.

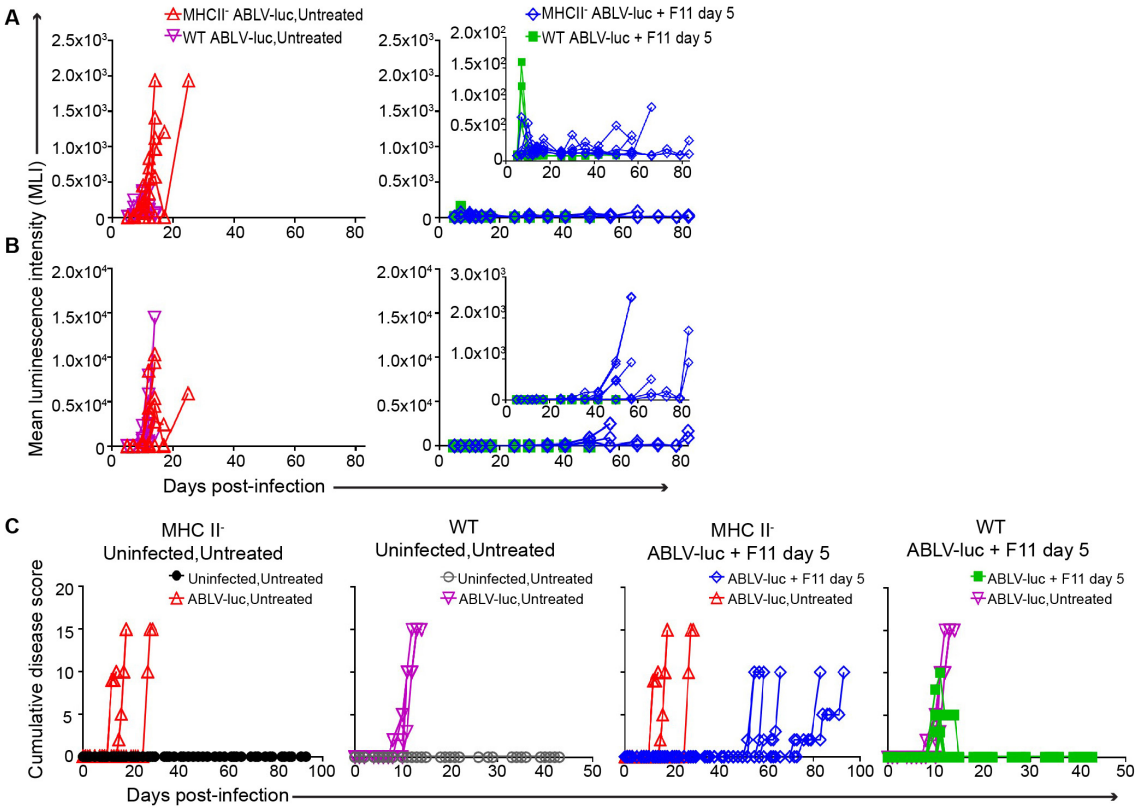
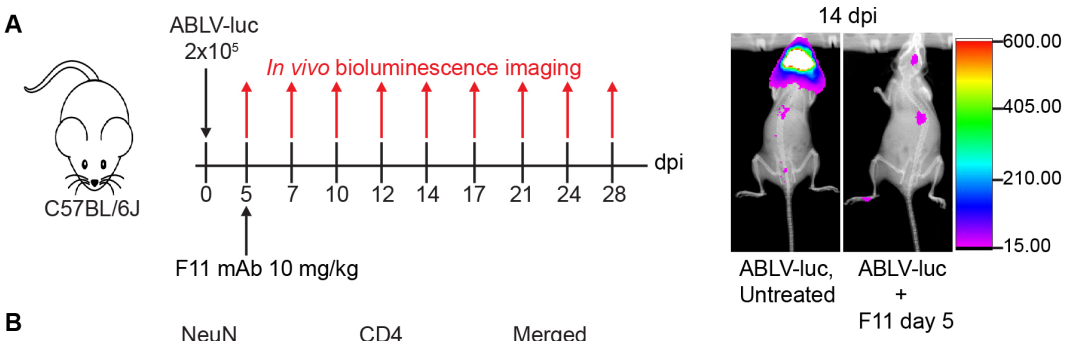


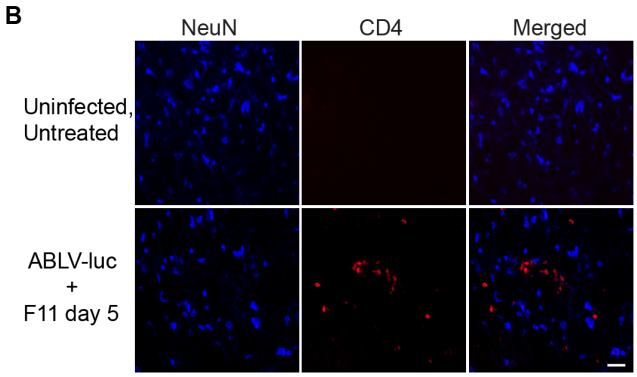
Figure 24. Viral burden and clinical disease are not resolved in F11mAb-treated MHCII$^-$ mice. Viral burden was quantified as mean luminescence intensity (MLI) in the spines (A) and brains (B) of infected mice. (C) Cumulative disease scores were determined by clinical presentation following ABLV-luc challenge.

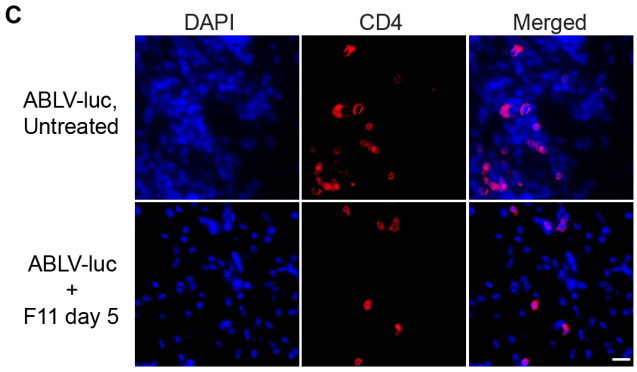
CD4 T CELLS INFILTRATE BRAINS OF F11 MAB-TREATED MICE

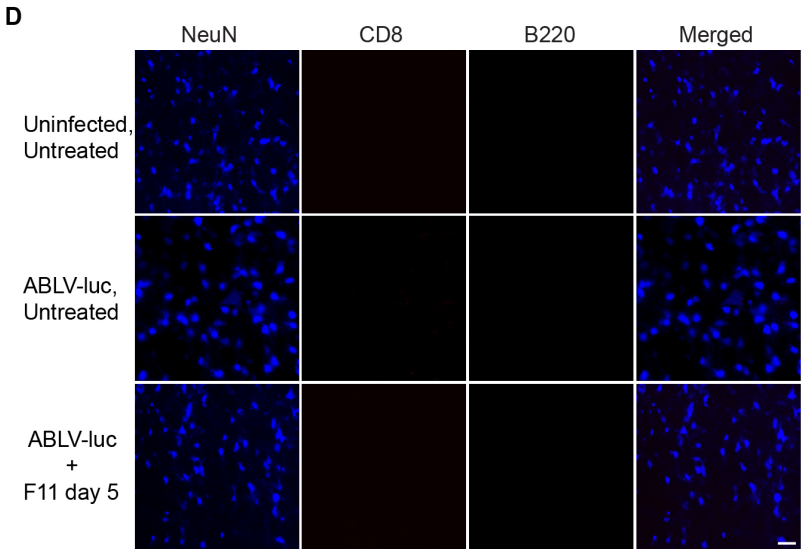
The simplest explanation for the above genetic data is that CD4 T cells infiltrate the brain of F11 mAb-treated, lyssavirus infected mice to facilitate virus clearance. Although normally excluded from the brain by the BBB, cells of the adaptive immune system can be efficiently recruited to the brain in specific circumstances, including viral infection (23; 113). To determine whether one or more adaptive immune cell types infiltrate the brain following lyssavirus infection, C57BL/6J mice were infected with 2×10^5 IU of ABLV-luc and treated with F11 mAb on day 5 (Figure 25A). Immunofluorescence staining of CD4 T cells, CD8 T cells, and B cells on day 14 revealed the presence of CD4 T cells within the brains of both infected, F11 mAb-treated mice and infected, untreated mice (Figure 25B, C). Importantly, viral loads in the brain were reduced at this time point relative to untreated mice, as evidenced via *in vivo* imaging data (Figure 25A). Consistent with our data regarding the lack of an essential role for B cells and CD8 T cells in ABLV clearance, these populations were not detected within the brains of infected mice at this time point (Figure 25D), although staining of spleen sections with the same antibodies yielded robust staining of these populations (Figure 26). Thus, these histological data support the conclusions of Figures 22-24 that CD4 T cells are the key adaptive immune population mediating F11 mAb-dependent lyssavirus clearance.

Figure 25. CD4 T cells are present within the CNS of F11 mAb-treated mice. (A) C57BL/6J mice were infected with 2×10^5 IU of ABLV-luc on day 0 (n= 4 mice/group) and F11 mAb (10 mg/kg) was administered on day 5. Brains were harvested as reduction of viral load was observed relative to untreated controls via *in vivo* imaging (day 14 post-infection). The heat-map scale represents luminescence intensity. Coronal sections of brain stem were stained to detect CD4 T cells (B, C) or CD8 T cells and B cells (D). Scale bars, 50μm.


A
ABLV-luc
2x10⁵
In vivo bioluminescence imaging
C57BL/6J
0 5 7 10 12 14 17 21 24 28
dpi
F11 mAb 10 mg/kg
14 dpi
600.00
405.00
210.00
15.00
ABLV-luc, Untreated
ABLV-luc + F11 day 5


B
NeuN
CD4
Merged
Uninfected, Untreated
ABLV-luc + F11 day 5


C
DAPI
CD4
Merged
ABLV-luc, Untreated
ABLV-luc + F11 day 5


D
NeuN
CD8
B220
Merged
Uninfected, Untreated
ABLV-luc, Untreated
ABLV-luc + F11 day 5

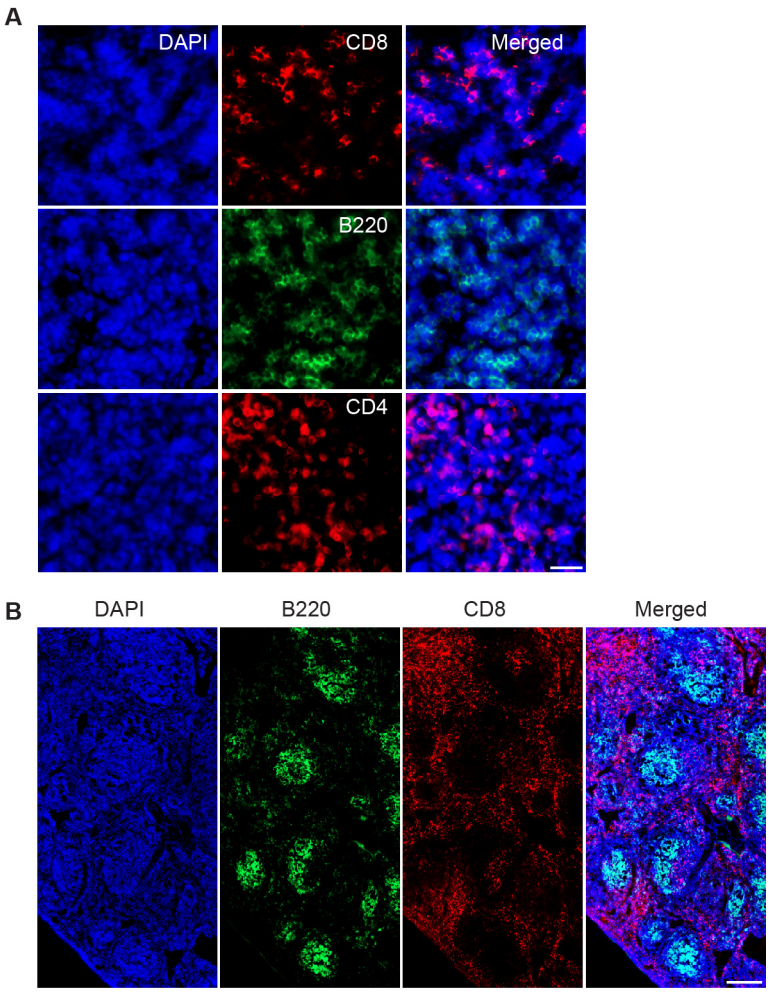


Figure 26. Validation of CD4, CD8, and B220 antibodies using staining of spleen sections. (A) Sagittal sections of spleens from C57BL/6J mice were used to optimize staining of B cells, CD8 T cells, and CD4 T cells. Bar, 20 µm. (B) Distribution of CD8 T cells and B cells within the spleen. Bar, 200 µm.

Chapter 4: Discussion

Rabies and rabies-like lyssaviruses are fatal neurotropic viruses that continue to pose a threat to global health, particularly in developing countries. While established PEP regimens of rabies vaccination and HRIG successfully prevent rabies, there are no effective therapeutics capable of treating established rabies and rabies-like lyssavirus infections of the CNS. While anti-rabies mAbs have proven successful in the prevention of rabies in animal models, the efficacy of mAbs in the treatment of CNS-resident lyssavirus infection remains to be investigated. To assess the efficacy of mAb therapy on established lyssavirus infection in mice, we developed a preclinical mouse model of ABLV infection. This was accomplished by generating and rescuing a firefly luciferase-expressing ABLV reporter virus (ABLV-luc). Using this reporter virus, we demonstrate that ABLV-luc infection can be traced from the site of inoculation into the CNS in real-time via longitudinal *in vivo* bioluminescence imaging. Furthermore, we show distinct areas of viral localization in the footpad, the lumbar and thoracic regions of the spine, and the hindbrain. This is consistent with experimental infections of mice, skunks, and hamsters with RABV as well as documented cases of rabies in wildlife and domestic animals (70; 207; 240; 264; 278; 334). There is one report of a luciferase-expressing RABV reporter virus (234). However, this group only compared *in vitro* and *in vivo* virulence between their reporter virus and WT RABV and did not perform any *in vivo* bioluminescence imaging to trace viral infection. Therefore, this work represents the novel use of a luciferase reporter virus to assess lyssavirus infection *in vivo* through longitudinal bioluminescence imaging. In addition to providing an anatomical location of the virus, bioluminescence imaging allowed us to measure viral burden within the

footpad and CNS through quantification of the luminescent signal. Therefore, we utilized our model to assess the *in vivo* efficacy of a lyssavirus neutralizing human mAb, F11, by measuring changes in viral load in response to F11 mAb treatment. Here, we demonstrate that treatment of mice with a single dose of F11 mAb on days 5 or 7 post-infection effectively clears ABLV-luc from the CNS and resolves the majority of clinical symptoms, following a lethal challenge. F11 mAb was also effective in treating mice infected with the lethal mouse-adapted RABV strain, CVS-11, underscoring the applicability of F11 mAb for the treatment of genetically diverse lyssavirus infections.

The use of anti-rabies mAbs has been suggested as a therapeutic alternative to HRIG, which is designed to neutralize virus in the periphery before CNS involvement, a point at which the virus can more easily evade the host immune response (308). Indeed, previous studies have demonstrated the effective use of human mAbs in the prevention of rabies in mice when administered before or within 24h of virus inoculation (104; 105; 289; 317). For instance, a human IgG2 mAb (mAb 57) targeting the viral glycoprotein was found to protect mice from a lethal challenge of CVS-24 when administered 24h prior to viral challenge. Presumably, most if not all of the virus was neutralized before entering peripheral nerves, as mAb 57 was administered at the same site (left gastrocnemius muscle) of virus inoculation. In the same study, Syrian hamsters were infected with a pathogenic strain of RABV (street isolate from a rabid fox) and treated with mAb 57 24h or 72h post-infection. As a result, 5/5 and 3/5 hamsters survived when treated 24h and 72h post-infection, respectively (104). In a separate study, the efficacy of a cocktail of mouse anti-rabies mAbs in the prevention of rabies was investigated. The mAb cocktail consisted of five mouse anti-rabies G antibodies with demonstrated

neutralization of fixed and street RABV strains *in vitro*. When mice were treated 24h and 2h prior to infection with a lethal dose of an attenuated strain of RABV (CVS-24), a lethal RABV infection was successfully prevented. However, delaying treatment until 2h post-infection resulted in decreased survivorship (317). Conversely, treatment of Syrian hamsters with the mAb cocktail prevented rabies when administered 2, 3, and 36h post-infection, following challenge with a pathogenic strain of RABV (NYC strain). The post-exposure efficacy observed in the hamster model may be attributed to a longer incubation period. Indeed, time to symptom onset in hamsters is 10-14 days compared to mice, which have an incubation period of approximately 5-6 days. This longer incubation period observed in hamsters presumably provides a greater therapeutic window for mAb-mediated neutralization prior to virus entering the peripheral nerves of the gastrocnemius muscle (208). Similarly, a cocktail of three human anti-rabies mAbs prevented lethal RABV infection in mice and hamsters when administered 1h and 4h post-infection, respectively (289). While these studies provide evidence for mAbs as an efficacious alternative to HRIG in the prevention of rabies, they fail to address a current and important problem of PEP failure post-CNS involvement. Specifically, these groups did not investigate whether mAb therapy can effectively clear an established lyssavirus infection in mice, particularly after the virus has entered the CNS.

CLEARANCE OF CNS-RESIDENT ABLV INFECTION BY AN ADAPTIVE IMMUNE RESPONSE

We found that mice treated with F11 mAb on days 5 or 7 were able to completely clear ABLV-luc from the CNS. In particular, clearance was observed in day 7-treated mice even though virus was present in the spine at the time of treatment. Despite the fact that the typical half-life of human mAbs in mice is approximately 6-8 days, we observed

clearance of ABLV-luc from the CNS of mice beyond 30 days post-infection. Therefore, we hypothesized that an adaptive immune response was ultimately clearing ABLV-luc from the CNS. To test this, we utilized NSG mice, which are deficient in T, B, and functional NK cells (see *Materials and Methods*). We found that while F11 mAb treatment delayed disease progression and mortality, these mice were unable to clear ABLV from the CNS and did not survive infection. While these results demonstrated that an adaptive immune response was essential for viral clearance, they did not tell us which specific immune subset(s) was responsible. Therefore, subsequent experiments were performed to determine whether T cells, B cells, or NK cells were mediating ABLV-luc clearance.

Natural killer cells

While direct evidence linking NK cells to clearance of RABV infection is lacking, antiviral functions of NK cells have been well described. In addition to cytolytic killing of virus-infected cells via perforin and granzymes, NK cells secrete IFN-γ, which is capable of limiting viral replication. NK cells are also potent regulators of antiviral T and B cell responses (360). For instance, NK-cell depletion in mice rendered them more susceptible to murine cytomegalovirus (MCMV), as evidenced by significantly higher viral titers and more extensive hepatitis (56; 343). Additionally, NK cells have been shown to limit replication of the myocarditic variant of coxsackievirus B3 ($CVB3_m$) within mouse heart tissue thereby limiting the number of myocarditic lesions found in these mice. Furthermore, depletion of NK cells in these mice resulted in increased viral replication and increased disease severity (139).

To determine whether NK cells were sufficient to mediate ABLV-luc clearance

from the CNS, we utilized NOD scid mice, which lack T and B cells but have NK cells (see *Materials and Methods*). We hypothesized that if NK cells were essential for viral clearance, NOD scid mice would successfully clear virus from the CNS and survive infection, in contrast to NSG mice which lack NK cells. We found that while the presence of NK cells extended the survival of NOD scid mice, they were unable to clear virus from the CNS and ultimately succumbed to infection. Therefore, NK cells are not sufficient to mediate ABLV-luc clearance and survival. Based on these results, we performed subsequent experiments using B cell, CD8 T cell, and CD4 T cell-deficient mice to determine which of these immune cell subsets was essential for viral clearance from the CNS.

B cells

To assess the role of B cells in F11 mAb-mediated lyssavirus clearance, we utilized muMt$^-$ mice (see *Materials and Methods*). Some studies have suggested that a RABV-specific antibody response is essential for clearance of pathogenic and attenuated RABV strains from the CNS (168; 169; 285; 302). For instance, Hooper et al. found that mice lacking B cells (J_HD and RAG2 knockout mice) were unable to clear an established RABV infection of the CNS and ultimately succumbed to infection following intranasal inoculation of an attenuated strain of RABV (CVS-F3) (168). J_HD mice cannot assemble Ig heavy chain genes due to a targeted deletion of JH gene segments. As a result, these mice lack surface Ig-positive (sIg+) B cells and B cell differentiation is blocked (80). Furthermore, C57BL/6 mice infected with CVS-F3 were protected against a lethal challenge with a pathogenic strain of RABV (CVS-N2c) with viral clearance from the brain dependent on RABV-specific antibody-secreting B cells infiltrating the CNS (169).

However, in our studies of F11 mAb therapy, we found that muMt$^-$ mice, which completely lack B cells, were able to effectively clear ABLV-luc from the brain. Furthermore, we did not detect B cells within the brains of F11 mAb-treated mice by histological methods. Our findings may reflect the fact that the administration of F11 mAb circumvented the need for VNAs produced by host B cells. In considering our data together with these previous studies, antibody-mediated reduction in virus titers in the periphery may be an essential component of successful immune-clearance of lyssavirus infection. However, host antibody apparently plays no role in the context of the therapeutic clearance of lyssavirus by F11 mAb.

CD8 T cells

We found CD8 T cells to be dispensable for viral clearance, consistent with previous reports of mice infected with attenuated strains of RABV. For example, beta-2 microglobulin (β2m) knockout mice that do not express MHC I and therefore do not select for effector CD8 T cells, survive infection with CVS-F3. However, complete clearance of virus from the CNS was delayed in these mice compared to C57BL/6 control mice. Virus was cleared from the brains of WT and β2m mice on days 13 and 21 post-infection, respectively (168). In a separate study, clearance of CVS-F3 was associated with enhanced BBB permeability and invasion of CD4 T cells and B cells but not CD8 T cells (285). Similarly, we observed that clearance of ABLV-luc from the CNS was delayed in our CD8-deleted mice compared to C57BL/6J mice (50 days vs. 18 days, respectively) with significantly higher levels of virus remaining in the spine and brain of CD8-depleted mice until days 12 and 22, respectively. However, while our data suggest that CD8 T cells contribute to control of infection within the brain, several observations

argue that this population is not an absolute requirement for viral clearance. First, although CD8-depleted mice were slow to resolve ABLV-luc infection in the CNS, the fact that virus is eventually cleared in these mice suggests that CD8 T cells are not essential. Additionally, we were unable to detect CD8 T cells in the brains of F11 mAb-treated mice that were actively clearing ABLV-luc infection. Also, MHCII$^-$ mice, which have an intact CD8 T cell compartment, all succumb to ABLV infection, despite F11 mAb treatment. Lastly, RABV infection of the CNS has been shown to upregulate expression of immunoinhibitory molecules such as B7 homolog 1 (B7-H1) and Fas ligand (FasL) on neurons, resulting in apoptosis of migratory CD8 T cells (29; 224; 225). Thus, several lines of evidence suggest that lyssaviruses have evolved to effectively circumvent the CD8 T cell response.

CD4 T cells

In contrast to results with B cell- and CD8 T cell-deficient animals, we observed uniform lethality following ABLV infection of mice lacking CD4 T cells (MHCII$^-$ mice, see *Materials and Methods*), due to failure to clear ABLV-luc from the brain. Complementing our MHCII$^-$ study, we found that viral clearance in F11 mAb-treated mice was accompanied by the presence of CD4 T cells within the CNS. Thus, our data strongly suggest that a CD4-dependent adaptive immune response is ultimately responsible for F11 mAb-mediated lyssavirus clearance from the CNS. In this regard, future studies should include immunofluorescence staining of brain tissue sections from muMt$^-$ and CD8 T cell-depleted mice for CD4 T cells to bolster our findings that CD4 T cells are indeed the immune population responsible for clearance. Notably, data from other groups support a key role for CD4 T cells in resolution of lyssavirus infection,

including observations of infiltrating CD4 T cells during clearance of a pathogenic RABV variant (SHBRV-17) from mice (304), and conversion of the RABV-resistant phenotype of mouse strains SJL/J and BALB/cByJ to 100% lethality following CD4 T cell depletion (283). A separate study investigated the antiviral role of T-bet, a transcription factor required for commitment of CD4 T cells to the Th1 lineage, in response to an attenuated RABV infection (230). The authors found that T-bet $^{-/-}$ mice experienced less efficient clearance of virus from the CNS due to delayed, low-level antibody production and prolonged viral replication, which they attributed to a lack of Th1 help to B cells in CNS tissues.

Blood-brain barrier permeability

While BBB permeability in response to ABLV-luc infection was not addressed in this work, several groups have reported RABV-induced permeability of the BBB. Indeed, several groups have reported enhanced BBB permeabilization following RABV infection (168; 169; 284; 285; 302; 304), with clearance from the CNS attributed to infiltration of CD4 T cells and B cells (169; 285; 302; 304). In fact, several studies have suggested that RABV clearance from the CNS is due to infiltrating B cells producing VNAs, rather than circulating antibodies crossing the BBB (169; 285; 302). Indeed, several groups have demonstrated that passive transfer of RABV-neutralizing antibodies via intraperitoneal or intravenous injection could not clear RABV from the CNS of immunocompetent and immunocompromised mice without enhanced BBB permeability (169; 176). In the absence of BBB permeabilization, clearance of virus from the brain was dependent on Ab production within the CNS by infiltrating B cells (169).

Interestingly, a study using 129/SvEv mice, an inbred strain of mice, found that

these mice were unable to survive infection with SHBRV despite developing a normal virus-specific immune response in peripheral lymphoid organs. This is attributed to the fact that increased permeability of the BBB does not occur nor does the invasion of immune effector cells into the CNS. Therefore, viral clearance from the brain does not occur and the mice do not survive infection. Conversely, Roy and Hooper demonstrated that PLSJL mice are less susceptible to SHBRV infection due to enhanced BBB permeability. PLSJL mice have an SJL (Swiss Jim Lambert) genetic background and are known to have reduced hypothalamus-pituitary-adrenal (HPA) axis activity and thus have an increased ability to facilitate inflammatory responses within the CNS (16). Indeed, increased BBB permeability and infiltration of immune effectors resulted in greater viral clearance and survival (302). Interestingly, administration of the steroid hormone dehydroepiandrosterone (DHEA) to PLSJL mice resulted in reduced BBB permeability, assessed by Na-fluorescein (Na-F) levels in the brain (284), and decreased survival. Another group found that 129/SvEv mice infected with SHBRV or CVS-F3 have different disease outcomes. For instance, mice infected with SHBRV do not survive infection compared to CVS-F3-infected mice despite equivalent virus-specific antibody and cell-mediated responses. However, enhanced BBB permeability and infiltration of T cells, B cells, and macrophages into the brain was markedly reduced in SHBRV-infected mice. Therefore, future studies should address potential permeabilization of the BBB in response to ABLV-luc infection (see *Future Studies*).

MONOCLONAL ANTIBODIES ENHANCE THE ANTIVIRAL IMMUNE RESPONSE

The current consensus opinion is that lyssavirus infection is not treatable once the virus has infected the CNS and symptoms have begun (50). For example, in 2014, a 6-

year-old boy from Tunisia was bitten by a stray dog on the forehead. After 14 days, the boy presented to the hospital with fever and vomiting. Three days later, the boy was transferred to a nearby children's hospital with conjunctivitis, febrile seizures, ataxia, and agitation. He was promptly administered ERIG and four doses of PCEC on days 0, 3, 7, and 14. Within two weeks of initiating PEP, the boy developed seizures and cardiac arrest and died on the same day. Biopsies of the cerebral cortex, cerebellum, hippocampus, and medulla were positive for rabies antigen. Several days before the boy was administered to the hospital, the stray dog died and brain biopsies were positive for rabies antigen. Failure of PEP was most likely due to delayed treatment, especially given the short incubation period due to close proximity of the bite site to the brain (348).

Despite the poor outcome of current rabies PEP post-CNS involvement, we have demonstrated that mice treated with F11 mAb on days 5 and 7 post-infection, when virus has infected the CNS, were able to completely clear ABLV-luc from the CNS by days 33 and 17 post-treatment, respectively. We also observed that mice treated with F11 mAb on days 5 and 7 post-infection, shortly before mice begin to experience tremors and weight loss, exhibit resolution of disease manifestations within a week of treatment. While the simplest mechanism to explain ABLV-luc clearance is that this mAb simply neutralizes lyssavirus infection within the CNS, several lines of evidence argue strongly against this possibility. First, given that the half-life of human IgG1 in adult mice is approximately 6-8 days (357), there would presumably be insufficient amounts of antibody remaining to effectively clear virus within the brain by the time that virus is ultimately eliminated in day 5- and day 7-treated mice (more than four half-lives later, in some animals). Second, such a mechanism is entirely inconsistent with the observations that mice treated on days

5 and 7 successfully eliminate infection as long as 4 weeks after antibody administration, whereas mice treated at day 3 exhibit a dramatic viral expansion followed by mortality within 3 weeks of mAb treatment. Third, the inability of F11 mAb to clear virus infections from NSG and NOD scid mice demonstrates that the host adaptive immune response plays an essential role in viral clearance. Thus, our data strongly support the conclusion that F11 mAb-mediated neutralization, by itself, is insufficient to eliminate lyssavirus infection. However, experiments will need to be performed to confirm that F11 mAb is not getting into the brain (see *Future Studies*). Rather, we hypothesize that in addition to neutralizing virus in the periphery, F11 mAb may promote an adaptive immune response that ultimately clears virus from the CNS.

Immune complexes modulate the host antiviral response

Binding of mAbs to virions or infected cells expressing viral antigens on their surface can lead to the formation of immune complexes that are recognized by FcγRs on APCs. Recently, several studies have investigated the induction of antiviral immune responses by immune complexes, shedding further light on the clinical potential of antiviral mAbs. For instance, hepatitis B virus (HBV) mAbs complexed with hepatitis B surface antigen (HBsAg) is a more potent stimulator of HBV-specific T cell proliferation than HBsAg alone due to an increased uptake of the HBsAg-mAb complex by monocytes for subsequent processing and presentation of antigen. Interestingly, DCs incubated with these immune complexes showed increased expression of MHC II as well as increased production of IL-12. When immune complex-treated DCs were co-cultured with T cells, increased production of IL-2 and IFN-γ by T cells was observed (63; 397). In a separate study, immune complexes comprised of simian immunodeficiency virus SIV Gag p55

and anti-p55 IgG enhanced MHC I cross presentation of Gag to CD8 T cells *in vitro*, resulting in higher frequencies of Gag-specific CTLs (358). Furthermore, stimulation of DCs with immune complexes of whole SIV virions and neutralizing antibodies triggered enhanced virus-specific CD4 T cell responses in an Fc-dependent manner. Indeed, subsequent immunization of rhesus macaques with neutralizing antibodies 7 days post-SIV challenge was associated with a rapid induction of Gag-specific CD4 T cells and sustained virus control even after neutralizing antibodies could no longer be detected (389; 390). Finally, Kruijsen et al. showed that mice primed with palivizumab-RSV complexes develop substantial virus specific-CD4 and CD8 T cell responses compared to mice primed with UV-inactivated RSV (213).

As previously mentioned, we detected CD4 T cells within the brains of ABLV-luc-infected, F11 mAb-treated mice. However, we also observed CD4 T cells within the CNS of ABLV-luc-infected, untreated mice, yet these mice were unable to clear ABLV-luc from the CNS. Therefore, potential immunomodulatory properties of F11 mAb may explain why untreated mice do not clear ABLV-luc from the CNS despite CD4 infiltration into the brain. During the initial phase of lyssavirus infection, antigen presenting cells (APCs) such as dendritic cells (DCs), collect viral antigen and migrate to draining LNs (396). For instance, RABV can be detected in the gastrocnemius muscle and draining LNs for up to 4 days following intramuscular injection of mice with a live-attenuated RABV variant (TriGAS) (318). In LNs, DCs expressing viral antigen on their MHC class II molecules prime naïve CD4 T cells, leading to their proliferation and differentiation into effector T cells, with peak priming occurring 5-7 days post-infection (84; 85; 255; 260; 301). Perhaps the administration of F11 mAb on day 5 or day 7 results

in delivery of a large number of viral antigen-loaded APCs to differentiating CD4 T cells during a time period critical to completing their differentiation into the mature subtype required for elimination of infection. Therefore, fluorescence immunostaining of hind brain tissue sections should be performed to determine whether F11 mAb-treated mice have greater numbers of CD4 T cells present in the CNS compared to untreated mice. An alternative hypothesis is that the slowed kinetics of viral expansion in the CNS following F11 mAb treatment may enable the anti-lyssavirus CD4 T cell response to control and ultimately neutralize the infection.

In addition to a potential role in enhancing an antiviral CD4 T cell response, F11 mAb therapy may blunt priming of CD4 T cells when administered on day 3 post-infection. Specifically, the administration of F11 mAb on day 3 post-infection, when virus is primarily localized to the footpad, may act to limit exposure of viral antigen to APCs during later stages of CD4 T cell differentiation, thereby leading to ineffective or abortive priming (17; 110; 212; 282). Consequently, the small amount of virus that has already entered peripheral nerves on day 3 would proceed to the spine and ultimately to the brain, where it would then expand uncontrollably, due to failure to prime an effective CD4 T cell response.

The above studies demonstrate that mAb therapy can not only serve as a passive form of immunotherapy but also as a form of active immunization. Indeed, several studies have tested the use of immune complexes as vaccines to enhance protective immune responses. For instance, immunization of mice with HBV vaccine complexed with an anti-HBV mAb resulted in an enhanced humoral immune response compared to mice immunized with HBV vaccine alone (35). In a separate study, the administration of

mAb 667 to mouse pups infected with the FrCasE murine leukemia virus protects them from an otherwise lethal infection. The protective immune response was due primarily to enhanced humoral and cytotoxic CD8 T cell responses. Not only did all 667-treated mice survive infection, they also resisted viral challenge as late as 14 months following initial infection (149-151). Furthermore, treatment of African green monkeys with a human mAb (m102.4) resulted in enhanced humoral responses and recovery from both HeV and NiV (49; 132). Interestingly, we found that ABLV-luc infected mice were completely protected when challenged 82 days post-initial infection, such that virus could not be detected within the footpad 3 days post-virus inoculation. This suggests that F11 mAb treatment induces long-lasting protective antiviral immunity. Indeed, quantification of virus in the footpads of ABLV-luc-infected mice revealed persistent, low levels of virus that does not clear in the absence of F11 mAb treatment (Figure 10). Virus also remains at low levels in the footpads of ABLV-luc-infected NSG and NOD scid mice despite F11 mAb treatment, suggesting a role for adaptive immunity in lyssavirus clearance from the footpad (Figure 14). Furthermore, the rechallenge study was performed in CD8-depleted mice. Therefore, it is unclear whether memory CD8 T cells would have been successfully generated in these animals. Future studies will be required to determine whether such memory cells are generated and whether they are present at the time of rechallenge with ABLV-luc.

F11 MAB AS A THERAPEUTIC

Overall, our data demonstrate that treatment of mice with a single dose of the human mAb F11 effectively clears ABLV-luc from the CNS and resolves the majority of clinical symptoms, following a lethal challenge. F11 mAb was also effective in treating

mice infected with the lethal mouse-adapted RABV strain, CVS-11, underscoring the applicability of F11 mAb for the treatment of genetically diverse lyssavirus infections. Mechanistically, an adaptive immune response is essential for F11 mAb-dependent lyssavirus clearance from the brain, with a specific requirement for the CD4 T cell lineage. Most importantly, these pre-clinical data suggest that anti-lyssavirus mAb therapy may successfully treat human rabies cases that have progressed beyond the first day of exposure. There are two classes of patients who develop rabies: those with a known exposure who are aware that they may have been infected but do not receive PEP due to personal choice or circumstances (i.e. endemic areas with no available PEP), and those who are not aware they were exposed and do not seek treatment. These two classes of individuals would be excellent candidates for F11 mAb therapy that is effective after rabies has entered the CNS and symptoms have started to become apparent.

However, for clinical use, timing of this therapy would be a major concern. Specifically, although F11 mAb administration on days 5 and 7 effectively cleared ABLV from the CNS in our mouse model, therapy initiated on day 3 was ineffective. Thus, a rational approach to effective timing of F11 mAb administration in humans would need to be developed, as several factors can influence the time to CNS involvement and the time required for priming of the adaptive immune response. For example, the amount of virus in the inoculum, severity of the wound, and proximity of the wound to the CNS can all influence the time it takes for lyssaviruses to reach the brain (109). In addition, whereas a proportion of day 7 treated mice developed paralysis by day 10 post-infection, mice treated on day 5 did not. Consistent with previous reports of RABV pathogenesis in mice, we found that paralysis was confined to the virus-

inoculated limb (131; 336; 369). The aforementioned studies also implicated a pathogenic role for T cells in the development of paralysis, which is consistent with our observation that the majority of MHCII^{-}, NSG, NOD scid, and CD8 T cell-depleted mice do not develop paralysis. Interestingly, paralysis persisted in a proportion of day 7-treated WT mice, despite subsequent viral clearance. This observation suggests that while viral clearance from the CNS can be achieved with F11 mAb therapy even after robust replication of lyssavirus in the CNS, treatment that is delayed until this stage of infection may not reverse all symptoms, particularly paralysis. Some or all of these factors would likely also dictate the time required to produce enough lyssavirus antigen to prime CD4 T cells. Thus, the above uncertainties represent an important limitation when considering how to apply these findings to human cases of rabies.

In considering the possible use of F11 mAb as a therapeutic, it will be important to assess the efficacy of F11 mAb beyond day 7 in order to fully determine therapeutic efficacy. Given that many patients do not seek treatment until the appearance of symptoms, we need to demonstrate that F11 mAb can resolve disease after symptoms have become apparent to have clinical relevance. We found that virus could be cleared from the CNS when treated as late as day 7. However, clinical disease did not become apparent in these mice until day 8. Therefore, further studies should assess the efficacy of F11 mAb following symptom onset. Specifically, C57BL/6J mice will be infected with ABLV-luc and treated with F11 mAb when clinical disease first becomes apparent. In addition to monitoring viral clearance via *in vivo* imaging, body weight, disease scores, and survival will be assessed.

CD4 T cell-mediated clearance of ABLV-luc

To confirm that CD4 T cells are an essential mediator of viral clearance, CD4 T cells will be isolated from spleens and LNs of ABLV-infected mice and adoptively transferred to ABLV-luc-infected, F11-treated NOD scid mice via tail vein injection. Viral load, disease score, body weight, and survival will subsequently be assessed and compared to ABLV-luc-infected, F11-treated NOD scid mice receiving PBS only. Furthermore, additional studies will be required to determine the precise mechanism by which CD4 T cells participate in successful clearance of lyssavirus from the CNS. For instance, CD4 T cells have been shown to protect against viral pathogens through cytolytic activity facilitated by perforin and FAS (53; 177; 188). However, antiviral effects of cytotoxic CD4 T cells requires the direct recognition of virus-infected cells by the T cell. Given the absence of MHC II expression on neurons and the fact that RABV primarily infects neurons, cytolytic activity by CD4 T cells against ABLV-infected neurons is unlikely. Rather, we propose several alternative mechanisms of CD4 T cell-mediated clearance of ABLV from the CNS.

IFN-γ

IFN-γ production by CD4 T cells has been shown to limit replication of several viruses (330; 376). For instance, IFN-γ production by infiltrating CD4 T cells in the CNS can suppress cytomegalovirus (CMV) replication in astrocytes (76-78). Given that CD4 T cells were found to be an essential mediator of ABLV-luc clearance within the CNS, future studies should aim to identify whether CD4 T cell-mediated clearance of ABLV is dependent on IFN-γ. Since IFN-γ expression in the CNS can be attributed not only to

infiltrating CD4 T cells but also NK cells and CD8 T cells (82), C57BL/6J mice will be treated with mAbs to CD8α, NK cells, and IFN-γ and subsequently infected with ABLV-luc. CNS viral load, clinical disease, and survival of CD8/NK cell-depleted mice will be compared to CD8/NK/IFN-γ-depleted mice. Higher viral loads and mortality in mice depleted of CD8 T cells, NK cells, and IFN-γ vs. mice depleted of CD8 T cells and NK cells would suggest IFN-γ derived from CD4 T cells is required for ABLV-luc clearance from the CNS.

Additionally, IFN-γ is potent inducer of nitric oxide (NO) production by microglia. NO is a pro-inflammatory signaling molecule (273), formed by conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS). The NOS-2 (iNOS) isoform is expressed by microglia and astrocytes and is inducible by IL-12, IFN-γ, TNF-α, and lipopolysaccharide (LPS) while the NOS-1 isoform is constitutively expressed by neurons (82). Since IFN-γ is a potent inducer of NO, it is possible that IFN-γ secreted by CD4 T cells that have entered the CNS may be stimulating microglia to produce NO. To test this, CD4 T cells will be isolated from C57BL/6J mouse spleens and LNs using a Dynabeads™ Untouched™ mouse CD4 T cell isolation kit. CD4 T cells, recombinant IL-2, and soluble CD28 Ab will be added to 6-well plates coated with anti-CD3 Ab. To activate cells to a Th1 lineage, CD4 T cells will be activated in the presence of exogenous IL-12 and anti-IL-4 neutralizing Ab (9). Confirmation of IFN-γ production by activated CD4 T cells will be confirmed using an IFN-γ mouse ELISA kit. IFN-γ producing-CD4 T cells will be co-cultured with microglia isolated from C57BL/6J mice. Given that NO is a short-lived molecule, NO production by microglia will be assessed by measuring NOS-2 mRNA levels via RT-PCR. To confirm NO production, a Griess assay will be performed.

In this assay, the amount of nitrite (NO_2^-), the stable end product of NO, released from microglia will be measured by mixing equal parts cell supernatant with Griess reagent and measuring absorbance on a plate reader (55). NO production by microglia will be compared to microglia treated with GR-20, a mAb to the IFN-γ receptor, prior to co-culture with CD4 T cells (36).

Macrophages are not considered permissive to RABV infection, yet several *in vitro* studies show that some RABV strains are capable of infecting microglia, the resident macrophages of the brain (290; 295; 336). In addition, RABV virions and antigens have been detected in microglia within the CNS of mice (336). This suggests that microglia may phagocytose virions that are released from infected neurons and/or microglia can be productively infected by RABV. Furthermore, a recent *in vitro* study demonstrated that RABV virions stimulated macrophages to produce NO. Given that NO has been shown to limit virus replication both *in vitro* and *in vivo* (195; 298), future studies should assess whether ABLV-luc can induce NO production by microglia. Initial studies will determine whether microglia are susceptible to ABLV infection. To accomplish this, microglia isolated from C57BL/6J mice will be infected with ABLV-luc at an MOI of 1 (1 IU/cell) for 2 hours. Cells will be washed and incubated in culture media for an additional 24-48h. A luciferase assay will be performed to assess viral load and will be compared to uninfected microglia. In addition, cell supernatant will be collected and titrated on mouse N2a cells to quantify virus. To assess NO-production in response to ABLV-luc infection, microglia will be incubated with ABLV-luc for 2h, washed, and incubated for an additional 12-24h (251) and mRNA expression of NOS-2 will be measured by RT-PCR (166; 273). Additionally, NO production will be confirmed

with a Griess assay. To determine whether NO affects viral load, a luciferase assay will be performed to quantify virus. In addition, cell supernatant will be collected and titrated on mouse N2a cells to quantify virus. Results will be compared to microglia treated with the NOS inhibitor, L-NMA (359). *In vitro*, NO production by macrophages has been shown to inhibit Japanese encephalitis virus (JEV) replication not only within macrophages but in nearby neuronal cells (236). To test whether NO production by microglia inhibits ABLV replication in nearby neuronal cells, mouse N2a cells will be infected with ABLV-luc for 48h then co-cultured with primary microglia for 24h. Cell supernatant will be collected and titrated on mouse N2a cells to quantify virus. Results will be compared to microglia treated with L-NMA prior to co-culture with mouse N2a cells.

IFN-γ has also been shown to upregulate MHC II expression on microglial cells (319). To test the possibility that IFN-γ secreted by CD4 T cells that have entered the CNS may be enhancing MHC II expression on microglia, CD4 T cells will be isolated from C57BL/6J mouse spleens and LNs as described above. Confirmation of IFN-γ production by activated CD4 T cells will be confirmed using an IFN-γ mouse ELISA kit. IFN-γ producing-CD4 T cells will be co-cultured with microglia isolated from C57BL/6J mice for 12-24h and MHCII expression on microglia will be assessed via flow cytometry using anti-Iba1 (microglia marker) and anti-I-A/I-E antibodies (MHC II). MHCII expression on microglia will be compared to microglia treated with GR-20 prior to co-culture with CD4 T cells.

TNF-α

In addition to IFN-γ, CD4 T cells can protect against viral pathogens by secreting

the proinflammatory cytokine, TNF-α (116; 323; 393). Indeed, TNF-α has been shown to limit viral infections through direct antiviral effects, modulating lymphocyte infiltration into the CNS, and enhancing MHC I and II expression and antigen presentation by APCs (37). Indeed, Shrestha et al. found that disruption of TNF-α signaling reduced the accumulation of macrophages and CD8 T cells within the brains of mice infected with West Nile virus (WNV), suggesting that TNF-α dependent signals are required for infiltration of these cells into the brain and subsequent clearance of virus (323). In addition, *in vivo* administration of TNF-α to mice infected with encephalomyocarditis virus (EMC-M) resulted in decreased clinical paralysis and reduced viral burden in the brain (332). Finally, TNF-α expression in the brain was shown to be upregulated in mice infected with CVS-F3, and was associated with infiltration of monocytes and lymphocytes (285).

To determine if TNF-α is essential for ABLV-luc clearance from the CNS, experiments will be performed with TNF receptor-1 (TNF-R1)-deficient mice (323). Mice will be infected with ABLV-luc and treated with F11 on day 5 post-infection. Disease score, body weight, and survival will be assessed, as well as viral burden in the brain via *in vivo* imaging. Data obtained from these experiments will be compared to wild-type C57BL/6J mice.

Microglia

Microglia are capable of producing several cytokines in response to viral infection. Indeed, microglia are a major source of type I IFNs in response to HSV-1 infection with cytokine production dependent on the immune signaling protein STING. Notably, mice deficient in STING are susceptible to herpes simplex encephalitis (HSE)

which correlates with decreased IFN-α/β production (297). In a separate study, production of TNF-α by microglia inhibits CMV replication in astrocytes (79). Therefore, future studies should investigate antiviral cytokines that may be produced by microglia in response to ABLV infection. Specifically, microglia will be isolated from C57BL/6J mice and infected with ABLV-luc. Following a 48h incubation, cytokines produced by microglia will be assessed using a Bio-Plex Pro™ mouse cytokine multiplex assay. To determine whether cytokines produced by microglia affect viral load, a luciferase assay will be performed to quantify virus. In addition, cell supernatant will be collected and titrated on mouse N2a cells to quantify virus. Results from the cytokine and luciferase assays will be compared to uninfected (negative control) and phorbol 12-myristate 13-aceteate (PMA) + ionomycin-treated (positive control) microglia. To determine whether microglia confer an antiviral response *in vivo*, pharmacological depletion of microglia will be performed by administering liposome-encapsulated clodronate to mice via intracranial injection. Clodronate is a drug capable of inducing apoptosis and elimination of macrophages *in vivo* (119; 215). Mice will be infected with ABLV-luc and treated with F11 mAb on day 5 post-infection. Viral burden will be determined via *in vivo* bioluminescence imaging and body weight, clinical disease score, and survival will be compared to ABLV-luc-infected, F11 mAb-treated mice receiving PBS.

A recent study found that nonproductive infection of microglia by CMV induces production of the chemokine CXCL10 which recruits CD4 T cells to the site of infection. Interestingly, a recent study found that RABV infection induced expression of CXCL10 in neurons, astrocytes, and microglia within the mouse CNS and was associated with infiltration of inflammatory cells (68). Therefore, future studies should investigate

chemokine expression within the brains of ABLV-luc-infected mice and determine whether chemokine expression is associated with infiltration of CD4 T cells. To accomplish this, mice will be infected with ABLV-luc and treated with F11 mAb on day 5. Following confirmation of virus within the CNS, brains will be harvested and homogenized. Using the cell supernatant, a Bio-Plex Pro™ mouse chemokine multiplex assay will be performed to determine the profile of chemokines produced in response to ABLV infection. Given that CXCL10 has been shown to be predominant chemokine produced during RABV infection, fluorescent immunostaining of ABLV-luc-infected brains will be performed to assess CXCL10 expression in neurons, microglia, and astrocytes. Tissue sections from uninfected, untreated mice would serve as a negative control. Staining for CD4 T cells should also be performed to determine if CXCL10 expression is associated with infiltration of CD4 T cells. To confirm CXCL10 is an important mediator of lymphocyte infiltration, *in vivo* neutralization of CXCL10 using an anti-CXCL10 antibody will be performed. Brain tissue sections will be stained for CD4 T cells and compared to isotype control-treated mice.

Blood-brain barrier permeability changes in response to ABLV-luc infection

Future studies should assess whether ABLV-luc infection induces BBB permeability changes. Specifically, C57BL/6J mice will be infected with ABLV-luc and upon established viral infection of the CNS (7-10 days post-infection) will be administered Na-F via intraperitoneal injection. Mice will be anesthetized 10 minutes later and transcardially profused with PBS. Spinal cords and brains will be harvested and homogenized and clarified supernatant will be used to assess Na-F content using a plate reader. To determine whether increased BBB permeability in response to ABLV-luc

infection allows entry of F11 mAb into the CNS, brains of ABLV-luc-infected, F11 mAb-treated mice will be harvested following transcardial profusion with PBS. Frozen brain sections will be stained with an anti-human IgG1 antibody and compared to untreated mice (negative control) and F11 mAb-treated mice that received monocyte chemotactic protein-1 (MCP-1) (positive control) (176). Interestingly, Phares et al. showed a positive correlation between CD4 T cell infiltration, IFN-γ expression, and BBB permeability within the CNS of CVS-F3-infected mice (284; 285). Furthermore, Iijima and Iwasaki found that in response to HSV-2 infection, CD4 T cells migrate to the spinal cord and dorsal root ganglia. Once there, they secrete IFN-γ and mediate increased vascular permeability, allowing access of circulating antibodies to these neuronal tissues to clear virus. Given that CD4 T cells were detected in the CNS of ABLV-luc-infected mice, future studies should determine whether CD4 T cells are required to enhance BBB permeability. Specifically, NOD scid mice will be infected with ABLV-luc and Na-F uptake within the brain will be assessed as described above and compared to C57BL/6J mice. If BBB permeability is found to be markedly reduced, NOD scid mice will be reconstituted with CD4 T cells isolated from ABLV-luc-infected C57BL/6J donor mice to assess whether BBB permeability is restored. To determine whether IFNγ can directly induce BBB permeability, C57BL6/J mice will be infected with ABLV-luc and administered an anti-IFN-γ mAb on days 0, 2, and 4 post-infection to deplete IFN-γ. Na-F uptake will be compared to mice that received an isotype control mAb (67).

Delineating immunomodulatory properties of mAb F11

In depth analysis of CD4 T cell participation in F11 mAb-dependent lyssavirus clearance will be important in delineating the mechanism by which timing of mAb

administration affects therapeutic efficacy. To test whether CD4 T cell priming is being blocked when F11 mAb is administered on day 3, we will assess ovalbumin-specific T cell proliferation (83). To accomplish this, CD4 T cells will be isolated from spleens and LNs of OT-II TCR transgenic mice, which express TCR that pairs with CD4 co-receptor and is specific for chicken ovalbumin (33). CD4 T cells will be labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE). By covalent coupling to intracellular molecules, CFSE is often used to assess cell proliferation due to progressive dilution of the fluorescent dye following cell division (243). CFSE-labeled CD4 T cells ($5x10^6$ cells) will be adoptively transferred into C57BL/6J mice via tail vein injection on day -1 (83). Twenty-four hours later, mice will be infected with a recombinant OVA-tagged ABLV (ABLV-OVA) and treated with F11 mAb on days 3, 5, and 7. CD4 T cells will be isolated from spleens and LNs on day 10 and the extent of CD4 T cell proliferation will be assayed by flow cytometry. A lack of CD4 T cell expansion in day 3 treated mice would suggest that F11 mAb treatment on day 3 limits the amount of viral antigen present to prime a robust CD4 T cell response. Conversely, enhanced CD4 T cell proliferation following treatment on days 5 and 7 would suggest that F11 mAb enhances CD4 T cell priming. To test whether the absence of a CD4 T cell response in the day 3-treated mice is responsible for the observed lack of viral clearance and survival, an adoptive transfer experiment will be performed. Specifically, CD4 T cells will be isolated from spleens and LNs of ABLV-infected C57BL/6J mice and administered to day 3-treated mice via the intravenous route when virus becomes present in the CNS. The capacity of adoptively transferred CD4 T cells to clear virus from the brains of day 3 treated mice will be assessed via *in vivo* imaging, as well as clinical disease score, body

weight, and survival analysis.

As mentioned above, immune complexes of mAb and virions can stimulate robust proliferation of T cells. Therefore, future studies should investigate whether immune complexes of F11 mAb and ABLV enhance CD4 T cell proliferation. While the RABV glycoprotein is responsible for induction of a neutralizing antibody response, both RABV G and RNP have been shown to stimulate the proliferation of CD4 T cells (65). However, the ABLV antigens responsible for inducing T cell proliferation are not known. Therefore, we will investigate whether immune complexes of OVA-tagged ABLV + F11 mAb result in enhanced uptake and presentation to OVA-specific CD4 T cells by assessing the proliferative response of CD4 T cells. To accomplish this, CD4 T cells will be isolated from spleens and LNs of OT-II mice. CFSE-labeled CD4 T cells ($5x10^6$ cells) will be adoptively transferred into C57BL/6J mice via tail vein injection on day -1. Twenty-four hours later, mice will be infected with a recombinant OVA-tagged ABLV and treated with F11 mAb on day 5. CD4 T cells will be isolated from spleens and LNs on day 10 and CD4 T cell proliferation will be assessed via flow cytometry. Proliferation of CD4 T cells in mice receiving immune complexes of ABLV-OVA+F11 mAb will be compared to mice receiving ABLV-OVA only. To assess the effect of immune complexes on MHC II expression, DCs isolated from C57/BL/6J mouse spleens and LNs will be stimulated with ABLV+F11 mAb or ABLV for 12-24h and stained with an anti-MHC II (M5/114.15.2) antibody for flow cytometric analysis. Subsequent experiments should determine whether an enhanced CD4 T cell response is FcγR-dependent. To accomplish this, DCs isolated from the spleens and LNs of C57BL/6J mice will be stimulated with ABLV-OVA + F11 mAb in the presence or absence of anti-mouse CD64

(FcγRI), CD32 (FcγRII), and CD16 (FcγRIII). DCs will then be cultured with CD4 T cells isolated from OT-II mice and the number of OVA-specific CD4 T cells will be determined via flow cytometry. In addition, we are currently engineering F11 mAb with a mutated Fc domain. Specifically, a point mutation at a glycosylation site in the Fc domain prevents binding of F11 to Fc receptors (F11 N297G). C57BL/6J mice will be infected with ABLV-luc and administered F11 N297G on day 5 post-infection. Viral load, body weight, disease score, and survival will be compared to mice that receive WT F11 mAb.

Memory response following rechallenge

To determine whether B cells are important for protection against rechallenge, muMt$^-$ mice treated with F11 mAb will be rechallenged on day 82 and imaged to detect virus. Clearance and survival in these mice would suggest a humoral response is not required for rechallenge. To determine which cells are responsible for preventing viral infection upon rechallenge, ABLV-luc-infected, F11 mAb-treated mice will be infected with ABLV 82 days post-initial infection. Memory T and B cell subsets infiltrating the footpad will be assessed via homogenization of footpad tissue and flow cytometry.

CONCLUSIONS

Overall, this work suggests that it may be possible to successfully treat human lyssavirus infection, beyond the point of CNS involvement. Indeed, F11 mAb treatment of infections with the lethal RABV strain, CVS-11, clearly led to survival of a subset of animals that were already exhibiting disease symptoms. Thus, our data may suggest that post-symptomatic human infections (the most likely target patient population for this type of intervention) might be successfully treated by F11 mAb therapy.

Moreover, our findings may have broad implications for the treatment of other

neurotropic infections. Specifically, we have shown that mAb therapy can successfully eliminate a neurotropic viral infection, post-CNS involvement, via a mechanism that involves CD4 T cells. Although intravenous antibody therapy is generally not considered as a viable option for pathogens in the CNS parenchyma because of BBB exclusion, our data strongly suggest that antiviral mAbs should not be dismissed as a therapeutic option for the treatment of such infections. Thus, it would be prudent to investigate whether mAb therapy may be effective against other neurotropic pathogens, even beyond the point of CNS infection.

Mechanistically, we found that an adaptive immune response is essential for F11 mAb-dependent lyssavirus clearance from the brain, with a specific requirement for the CD4 T cell lineage. In this study, we have shown that a single dose of human mAb F11 can clear lyssavirus infection, even beyond the point of robust replication in the CNS. Importantly, F11 mAb treatment mediates resolution of clinical disease and long-term survival, with no return of viral infection (we have monitored F11 mAb-treated wild-type mice for as long as 140 days after virus became undetectable in the CNS, with no return of virus or disease symptoms). Most importantly, these pre-clinical data suggest that anti-lyssavirus mAb therapy may successfully treat human rabies cases that have progressed beyond the first day of exposure.

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