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Expression of Interferon Gamma by a Recombinant Rabies Virus Strongly Attenuates the Pathogenicity of the Virus via Induction of Type I Interferon.

Darryll A Barkhouse
Thomas Jefferson University

Samantha A Garcia
Thomas Jefferson University

Emily K Bongiorno
Thomas Jefferson University

Aurore Lebrun
Thomas Jefferson University

Milosz Faber
Thomas Jefferson University

See next page for additional authors

Authors

Darryll A Barkhouse; Samantha A Garcia; Emily K Bongiorno; Aurore Lebrun; Milosz Faber; and D Craig Hooper, PhD

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Running Title: Attenuation of RABV via co-expression of IFNγ

Darryll A. Barkhouse[1,2], Samantha A. Garcia[1,2], Emily K. Bongiorno[1,2], Aurore Lebrun[1,2], Milosz Faber[2] and D. Craig Hooper[1,2,3 #]

Department of Cancer Biology[1], Center for Neurovirology[2], Department of Microbiology and Immunology[3], and Department of Neurological Surgery[4], Thomas Jefferson University, Philadelphia, PA, USA

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[#]Corresponding author. Mailing address:

Thomas Jefferson University, 1020 Locust Street, Jefferson Alumni Hall, Room 454, Philadelphia, PA 19107

Phone: 215.503.1559

Email: douglas.hooper@jefferson.edu

ABSTRACT

Previous animal model experiments have shown a correlation between interferon gamma (IFNγ) expression and both survival from infection with attenuated rabies virus and reduction of neurological sequelae. Therefore, we hypothesized that rapid production of murine IFNγ by the rabies virus itself would induce a more robust antiviral response than would occur naturally in mice. To test this hypothesis, we used reverse engineering to clone the mouse IFNγ gene into a pathogenic rabies virus backbone, SPBN, to produce the recombinant rabies virus designated SPBNγ. Morbidity and mortality were monitored in mice infected intranasally with SPBNγ or SPBN(-) control virus to determine the degree of attenuation caused by the expression of IFNγ. Incorporation of IFNγ into the rabies genome highly attenuated the virus. SPBNγ has an LD_{50} more than 100 fold greater than SPBN(-). *In vitro* and *in vivo* mouse experiments show that SPBNγ infection enhances the production of type I interferons. Furthermore, knockout mice lacking the ability to signal through the type I interferon receptor ($IFNAR^{-/-}$) cannot control the SPBNγ infection and rapidly die. These data

suggest that IFNγ production has antiviral effects in rabies, largely due to the induction of type I interferons.

IMPORTANCE

Survival from rabies is dependent upon the early control of virus replication and spread. Once the virus reaches the CNS this becomes highly problematic. Studies of CNS immunity to RABV have shown that control of replication begins at the onset of T cell entry and IFNγ production in the CNS prior to the appearance of virus-neutralizing antibodies. Moreover antibody deficient mice are able to control but not clear attenuated RABV from the CNS. We find here that IFNγ triggers the early production of type I interferons with the expected antiviral effects. We also show that engineering a lethal rabies virus to express IFNγ directly to the infected tissue reduces rabies virus replication and spread limiting its pathogenicity in normal and immunocompromised mice. Therefore, vector delivery of IFNγ to the brain may have the potential to treat individuals who would otherwise succumb to infection with rabies virus.

INTRODUCTION

Rabies virus (RABV) is the type species of the *lyssavirus* genus in the rhabdoviridae family. Its small, negative-stranded RNA genome encodes only five true genes (1, 2). Although relatively simple, this zoonotic virus has a devastating impact worldwide. The majority of human rabies deaths occur in

children in the developing world, and it is estimated that at least 55 000 humans die of rabies each year in Africa and Asia alone (3).

Although RABV infection historically has been viewed as a death sentence once the virus reaches the brain, there is a small but growing number of humans who survived rabies even though the virus entered the brain (4, 5). Due to such cases and to research using animal models of RABV infection (6–8), many believe that the immune system may be capable of clearing RABV from the brain without causing irreparable immunopathology. It is clear, however, that therapeutic intervention will be necessary in the vast majority of cases. Some theoretical or experimental rabies treatments involve slowing virus replication and/or spread by: induction of hypothermia (9); the highly controversial use of therapeutic coma (5); enhancing immune cell entry into the CNS (10); superinfection with an attenuated RABV (11, 12); and systemic or intrathecal administration of anti-viral drugs and interferons (IFN) (13). For decades while many researchers have been studying the effects of type I IFN (predominantly IFNα and β) during RABV infection our work has focused primarily on the role(s) of IFNγ during RABV clearance from the CNS.

IFNγ is a pleiotropic cytokine and is the only known form of type II interferon. Although originally discovered by its ability to “interfere” with virus infection (14), its immunomodulatory functions were quickly recognized, and it is now widely accepted that IFNγ has many important functions in both innate and adaptive

immunity. Some of these include: upregulation of adhesion molecules; activation of macrophages and NK cells; T cell activation and differentiation; upregulation of MHC molecules; antibody isotype switching; as well as induction of reactive oxygen species and reactive nitrogen intermediates (15). These are merely a sample of what IFNγ induces as it is known to affect the expression of hundreds of genes (16). Additionally, cross-talk between type I and type II IFN has been shown (17), suggesting IFNγ can amplify its anti-viral effects via the induction of type I IFN. Furthermore, it is known that IFNγ can potentiate the action of type I IFN (18) and act synergistically with them (19). Since its discovery, researchers and clinicians have been trying to harness IFNγ for its potential therapeutic effects. It has been tested experimentally as an adjuvant in vaccines (20, 21) and as treatment in animal models of disease such as tuberculosis (22), each with varying degrees of success. Currently the only FDA-approved uses for IFNγ in humans are treatments for chronic granulomatous disease (23) and osteopetrosis (24), but IFNγ is also prescribed by veterinarians to treat canine atopic dermatitis (25). These clinical uses of IFNγ highlight its pleiotropic nature as the mechanisms of action in these diseases are through enhanced innate immunity, activation of osteoclasts and suppression of the adaptive immune response, respectively.

Our previous work has shown that IFNγ mRNA expression in the brains of mice infected with attenuated RABV strongly correlates with blood-brain barrier (BBB) permeability changes and clearance of RABV from the CNS (6, 26). Recent

experiments confirm the importance of IFNγ during RABV clearance from the CNS, supporting the concept that IFNγ is at the center of the signaling pathway that alters tight junction protein expression and leads to increased BBB permeability and survival from RABV infection (27). These rabies-specific data, as well as the more commonly known roles of IFNγ in the innate and adaptive arms of the immune response, suggest that therapeutically enhancing IFNγ expression in the CNS during a rabies infection may prevent virus spread and aid the clearance of RABV from CNS tissues through a number of mechanisms. Therefore, to more precisely determine the effects of IFNγ expression in the CNS on pathogenic RABV infection, we engineered a pathogenic RABV to express the murine IFNγ gene. We report that RABV-expressed IFNγ highly attenuates a pathogenic RABV in mice, and that this attenuation is due, at least in part, to upregulation of type I IFN.

MATERIALS AND METHODS:

Mice. Female Swiss Webster mice were purchased from Taconic Farms (Germantown, NY). Female C57Bl/6J mice and IFNγ$^{-/-}$ mice on a C57Bl/6 background were purchased from Jackson Laboratories (Bar Harbor, ME). WT129 and IFNAR$^{-/-}$ mice were from investigator breeding colonies maintained at Thomas Jefferson University and were originally provided by Michel Aguet (28). All mouse experiments were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee.

Cell lines. BSR cells (a BHK-21 clone) (29) grown in DMEM (Mediatech, Manassas, VA) supplemented with 10% FBS (Atlanta Biologicals, Flowery Ranch, GA) were used to grow virus stocks. Mouse neuroblastoma cells (NA) were grown in RPMI medium 1640 (Mediatech, Manassas, VA) and used for growth curves, virus titers and immunofluorescence. Mouse astrocytoma (AS) cells and monocyte lineage (MC) cells were grown in DMEM supplemented with 10% FBS and were used for growth curves.

Viruses. SPBN, the prototype recombinant RABV was derived from the SADB19 strain (30, 31). Although SPBN is often referred to as a vaccine vector, when administered to mice intranasally (i.n.), it is highly pathogenic. The wild type RABV strain DRV4 was isolated from a human rabies victim and propagated in NA cells as previously described (32).

Construction of recombinant viruses. The recombinant RABVs, SPBN(-) and SPBNγ, were engineered as described previously (33). For SPBNγ, murine IFNγ DNA was PCR-amplified from mRNA extracted from RABV-infected mouse brain tissue using the custom primers (IDT, Coralville, IA) below and DeepVent polymerase (NEB, Ipswich, MA). The forward primer, 5'ATAGAATTC**CGTACG**AAGATG*AACGCTACACACTGCATCTTGGCT*3', contains a **BsiWI** restriction site, start codon, and the *gene specific sequence*. The reverse primer, 5'ATTCTCTAGATA**GCTAGC**TCA*GCAGCGACTCCTTTTCCGCTTCCT*3',

contains a **NheI** restriction site, stop codon, and the *gene specific sequence*. The resultant IFNγ DNA was cloned into the pSPBN plasmid, creating the pSPBNγ plasmid. Standard transformation and transfection methods were used to complete the virus rescue of SPBNγ as outlined above.

Growth Curves. NA, AS or MC cells were infected at a multiplicity of infection (MOI) of 0.01 for multi-step and 1 for single-step growth curves. 100 ul supernatant aliquots were taken at 0, 24, 48 and 72 hours post-infection. Virus titers from the supernatants were calculated as described below.

Virus Titers. To determine virus titers, NA cells were seeded into 96 well plates, grown for two days and then infected with virus in serial ten-fold dilutions. Two days post-infection (d.p.i.), the cells were fixed with 80% acetone and stained with FITC-conjugated anti-RABV RNP antibody (Fujirebio Diagnostics, Malvern, PA). Virus titers of triplicate samples were determined using a fluorescence microscope.

Immunofluorescent staining. NA cells were grown in culture well slides for 24 hours and then infected with RABV at an MOI=0.1. 24 hours later, cells were stained for the presence of RABV and mouse IFNγ using FITC-conjugated anti-RABV RNP antibody and PE-conjugated anti-mouse IFNγ antibody (Sigma, St. Louis, MO), respectively. Slides were mounted using ProLong Gold with DAPI

(Life Technologies, Eugene, OR). Photos were taken using a DM6000B fluorescent microscope (Leica, Buffalo Grove, IL).

Infection of mice. Groups of mice were infected under isoflurane (Vedco, St. Joseph, MO) anesthesia: intracranially (i.c.) with 10^3 focus-forming units (ffu) in 10 ul PBS or intranasally (i.n.) with 10^3-10^6 ffu in 20 ul PBS. Mice were observed for at least 30 days for appearance of clinical signs of rabies such as limb paralysis, tremors and weight loss. Mice were humanely euthanized when moribund or after losing greater than 30% of starting body weight.

IFNγTreatment. Cells were treated with 10, 100 or 1000U of recombinant mouse IFNγ (BD Biosciences, San Jose, California) at 24 and 48 hours post-infection by addition to the culture medium.

Enzyme-Linked Immunosorbent Assay (ELISA). 50 ul aliquots of virus-infected supernatants were assayed for IFNγ using an OptEIA Mouse IFNγ Kit II (BD Biosciences, San Jose, CA) or for IFNα using a Mouse IFN-alpha Platinum ELISA kit (eBioscience, Vienna, Austria) per manufacturers' instructions. The plates were then scanned in a Synergy H1 plate reader (BioTek, Winooski, VT) and absorbance at 450 nm was recorded.

Quantitative PCR. RNA_{tot} was extracted from brain samples using a Qiagen RNeasy kit (Qiagen, Valencia, CA) and modified Qiagen protocol previously

described (6). Briefly, tissues were homogenized by aspirating through a sterile, 20 gauge needle approximately 15 times in TriReagent (MRC, Cincinnati, OH). Homogenized samples were spun in a Heraeus Biofuge pico benchtop centrifuge (Thermo Scientific, Langenselbold, Germany) at 12 000 rpm for 15 min at RT. The aqueous layer was removed and mixed with an equal portion of 70% ethanol and then added to the Qiagen spin column. All subsequent steps are outlined in the Qiagen manual, including the optional on-column DNAse I digestion which was used. After RNA_{tot} extraction, cDNA was prepared using oligo dT_{15} primers, dNTP and Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI) as described previously (6). Finally, each sample was analyzed for the presence of specific mRNAs by qPCR using a Biorad iCycler, iQ Supermix or iQ Sybr (BioRad, Hercules, CA), and primer/probe sets (IDT, Coralville, IA) designed as follows: CD4 F AGGTCTCGCTTCAGTTTGCT, R AGCCACTTTCATCACCACCA, Pr TGGCAACCTGACTCTGACTCTGGACA; CD8 F CATCCTGCTTCTGCTGGCATT, R TGGGCGCTGATCATTTGTGAAA, Pr TGTGTGCGGAGGAGAGCCCGAATTCA; CD19 F GAGCTCAGAGCCATGAAACA, R CAAGGTTGGAGTCGTTCTCA, Pr CCAGACAGCGAGGAGGGCTCTGAAT; IFNγ F AGCAACAACATAAGCGTCATT, R CCTCAAAACTTGGCAATACTCA, Pr ACCTTCTTCAGCAACAGCAAGGGC; SPBN-N F AGAAGGGAATTGGGCTCTG, R TGTTTTGCCCGGATATTTTG, Pr CGTCCTTAGTCGGTCTTCTCT; L13 F CTACAGTGAGATACCACACCAAG, R TGGACTTGTTTCGCCTCCTG, Pr ATCCACAAGAAAGTGGCTCGCACCAT;

IFNα F ATTTTGGATTCCCCTTGGAG, R TGATGGAGGTCATTGCAGAA; IFNβ F CACAGCCCTCTCCATCAACT, R GCAACCACCACTCATTCTGA.

Statistical Analysis. All calculations were performed using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA) with the exception of the Habel Test for Potency (34).

RESULTS

Recombinant rabies virus expresses murine IFNγ. Recombinant RABVs were constructed using the pathogenic SPBN backbone vector. Fig. 1A shows a wild-type RABV genome while 1C depicts the IFNγ-expressing SPBN, termed SPBNγ. To control for any changes in replication efficiency that might occur due to increased size of the RABV genome, we used the previously constructed SPBN(-) (Fig. 1B). SPBN(-) has an inactivated tumor necrosis factor α (TNFα) gene in lieu of murine IFNγ.

To ensure that SPBNγ can produce murine IFNγ, we infected NA cells grown on culture well slides with SPBNγ or SPBN(-) at an MOI =0.1. 24 hours p.i. the cells were fixed, permeabilized and stained for evidence of RABV infection and murine IFNγ production. Fig. 2 shows the results of immunostaining for RABV and IFNγ of uninfected (A), SPBN(-) (B) and SPBNγ (C) infected NA cells. While cells infected with SPBN(-) are positive only for RABV, the SPBNγ-infected cells stained positive for both RABV and IFNγ, demonstrating that our recombinant

SPBNγ produces IFNγ. We next used ELISA to analyze the supernatants of NA, AS and MC cell lines infected with SPBNγ or SPBN(-) for the presence of IFNγ. Fig. 2D shows that by 12 hours p.i. significant amounts of IFNγ are produced and secreted into the supernatants of all cell types infected with SPBNγ but not SPBN(-).

Virus-encoded IFNγ has differential effects on RABV replication *in vitro*. To determine the effects of IFNγ expression on RABV replication in relevant cell types, we infected NA, AS and MC cell lines with SPBNγ or SPBN(-) (Fig. 3) As shown in Fig. 3A and 3D, there is less than one $\log_{10}$ difference in virus replication and spread between SPBNγ and SPBN(-) in NA cells by 48 hours p.i. Both viruses grow to relatively high titers (>10^7 ffu/ml). In AS cells there is a similar result with less than one $\log_{10}$ difference in growth by 48 hours p.i. in the single-step growth curve (Fig. 3B). IFNγ production may slow the spread of SPBNγ in AS cells, however, as there is greater than one $\log_{10}$ difference between the virus titers at 72 hours p.i. (Fig. 3E). In contrast to the minor differences seen in NA and AS cell lines, the replication rate and spread of SPBNγ in MC cells is several $\log s_{10}$ lower as compared to SPBN(-) (Fig. 3C, 3F). The antiviral mechanisms in the MC cells appear to be very rapidly activated, as this multi-log difference in replication is evident as early as 24 hours p.i. This significant decrease in SPBNγ replication as compared to SPBN(-) indicates that the IFNγ produced by SPBNγ and measured by ELISA (Fig. 2D) is indeed functional. Although SPBNγ replication is hampered in MC cells, substantial

numbers of progeny virions (~10^3 ffu/ml) were still produced, indicating that even in an immune cell line highly responsive to IFNγ, SPBNγ is able replicate and spread.

IFNγ attenuates pathogenic RABV. After demonstrating the ability of SPBNγ to replicate well in permissive cells and produce IFNγ *in vitro*, we examined the pathogenicity of SPBN(-) and SPBNγ *in vivo*. Groups of ten Swiss-Webster mice were infected i.n. with 10^3 – 10^6 ffu of SPBNγ or SPBN(-). Mice were monitored for mortality and morbidity for 30 days. The resultant survivorships (Fig. 4A and 4C) clearly demonstrate the attenuation of the pathogenic SPBN vector by the addition of IFNγ to its genome. The most striking difference in pathogenicity occurs at 10^5 ffu where 90% of SPBNγ mice survive infection while 100% of mice infected with SPBN(-) succumb within 15 days, but SPBNγ is significantly attenuated at all doses compared to SPBN(-). To quantify the attenuation of pathogenicity due to IFNγ, we calculated the LD_{50} of SPBNγ and SPBN(-) and found greater than a two $\log_{10}$ difference between the viruses (Fig. 4B). It should be noted that the exact difference could not be calculated because infection with all doses of SPBN(-) killed more than 50% of the mice. Thus, the expression of IFNγ from the RABV genome decreased the pathogenicity of SPBN by more than 100 fold when administered i.n.

SPBNγ replication is diminished while IFNγ expression is elevated in the CNS of infected mice. Having demonstrated that IFNγ expression by RABV has

differential effects on virus replication in various cell types *in vitro*, we next infected groups of C57BL/6 mice with SPBNγ or control SPBN(-) to quantify virus replication and IFNγ gene expression in the CNS. At 10 d.p.i. viral nucleoprotein (N) RNA expression in both the cerebellum and cortex is approximately ten fold lower in mice infected with SPBNγ than SPBN(-) (Fig. 5A). Despite the reduced replication, the cerebellum and cortex of the SPBNγ-infected mice showed approximately ten fold greater IFNγ mRNA levels than in SPBN(-)-infected mice (Fig. 5B). Taken together, these data show that at 10 d.p.i. SPBNγ induces greater than 100 fold more IFNγ expression per viral N message in the CNS of mice than SPBN(-) (Fig. 5C).

SPBNγ infection does not increase immune cell infiltration into the CNS. In other animal models, immune cell infiltration into the CNS has been shown to be essential for RABV clearance (eg. 8, 35). To determine whether enhanced immune cell infiltration could play a role in the attenuation of SPBNγ, we measured the expression of mRNA specific for CD4+, CD8+ and CD19+ cells as an estimation of T and B cell influx into the CNS of mice infected with SPBNγ or SPBN(-) (Fig.6). At 10 d.p.i. we found no significant increase in immune cell markers in the brains of SPBNγ-infected as compared to SPBN(-)-infected mice. In fact, the SPBN(-)-infected mice showed increased expression of T cell marker CD8 in both the cerebellum (CB) and cortex (CX).

SPBNγ attenuation occurs in the absence of endogenous IFNγ or antibodies. Previously it has been suggested that IFNγ-producing T cells as well as virus-neutralizing antibodies (VNA) are important for the clearance of RABV from the brain (36, 37). To determine if endogenously produced IFNγ or VNA are essential for SPBNγ attenuation, we infected groups of IFNγ$^{-/-}$ mice, having no endogenous IFNγ, and groups of $J_HD^{-/-}$ mice, lacking functional B cells and antibodies, with SPBNγ or SPBN(-). At 16 d.p.i. 100% of SPBNγ-infected IFNγ$^{-/-}$ mice were alive compared to only 11% of those infected with SPBN(-) (Fig. 7A). We continued to monitor the mice until 35 d.p.i., at which time 75% of IFNγ$^{-/-}$ mice infected with SPBNγ remained alive while only 11% of SPBN(-)-infected mice survived. As seen in Fig. 7B, SPBN(-)-infected IFNγ$^{-/-}$ mice also begin to lose weight earlier than their SPBNγ-infected counterparts.

The importance of antibody to RABV clearance from the brain is well established. Therefore, we infected $J_HD^{-/-}$ mice to determine if IFNγ overexpression could prevent or delay death in the absence of antibody. Infection of $J_HD^{-/-}$ mice yields a similar outcome to that seen with IFNγ$^{-/-}$ mice. By 17 d.p.i. all SPBN(-)-infected $J_HD^{-/-}$ mice succumbed to rabies infection while 100% of those infected with SPBNγ remained alive (Fig. 7C). By the end of the experiment, at 32 d.p.i., 92% of $J_HD^{-/-}$ mice infected with SPBNγ survived, despite having no functional, circulating antibodies. As can be expected, the mice infected with SPBN(-) lost weight faster than those infected with SPBNγ (Fig. 7D). Although most of the

mice were sacrificed at day 32, we continued to monitor five SPBNγ-infected JHD$^{-/-}$ mice. Surprisingly, 5 of 5 of these mice survived for 75 days, 4 of 5 survived 87 days, and 3 of 5 remained alive for 88 days, at which time this experiment was concluded (data not shown). Thus even this severe immune deficit does not prevent long-term control of SPBNγ infection by the host.

IFNAR$^{-/-}$ mice do not survive SPBNγ infection. IFNAR$^{-/-}$ mice are unresponsive to type I IFN because the IFNAR1 chain of the heterodimeric receptor is knocked out (28). Based on knowledge that IFNγ can potentiate type I IFN effects as well as act synergistically with type I IFN, we infected groups of IFNAR$^{-/-}$ mice, as well as their background control WT129 mice, with SPBNγ or SPBN(-) to determine the role of type I IFN in attenuation of SPBNγ. 100% of IFNAR$^{-/-}$ die from infection with SPBNγ by 14 d.p.i., and this is not significantly different from the control SPBN(-)-infected IFNAR$^{-/-}$ mice which all die by 13 d.p.i.. The WT129 background does not significantly alter the strong attenuation of SPBNγ seen in Swiss-Webster mice as 86% of SPBNγ-infected WT129 mice survive for 31 days while 100% of these animals infected with SPBN(-) die.

SPBNγ induces early Type I IFN expression *in vitro* and *in vivo*. There is substantial overlap in the regulation of type I and type II IFN (38). The early or increased expression of IFNγ by SPBNγ may increase the expression of type I IFN, thereby enhancing the innate immune response to RABV and protecting mice from death during SPBNγ infection. To test this theory, we infected flasks of

MC cells with SPBNγ or SPBN(-) and measured virus- and IFN-specific mRNA levels in these cells at various timepoints post-infection. Infection of MC cells with SPBNγ leads to an early and sustained overexpression of IFNγ (Fig. 9A) and induces a significant increase in IFNα expression as compared to SPBN(-) infection from 6 to 48 hours p.i. (Fig. 9B). This effect is also seen when measuring IFNβ induction (Fig. 9C), however, IFNβ expression continues to rise in the SPBN(-)-infected MC cells, becoming significantly higher in these cultures by 24 hours p.i. The increase in type I IFN expression in SPBN(-)-infected MC cells during the later timepoints is most likely the result of more rapid virus replication, as evidenced by the viral N message levels (Fig. 9A-C). SPBNγ replication remains very low throughout the experiment but induces relatively high type I and type II IFN expression. The addition of IFNγ to cultures of SPBN(-)-infected MC cells reduces viral replication (Fig. 9D) in concert with increasing IFNα levels (Fig. 9E) at early timepoints. Elevated IFNα levels, which are only seen in the presence of both IFNγ and virus infection, are particularly evident at 24 hrs of culture (Fig. 9F).

To determine if SPBNγ also induces higher levels of type I IFN in the brain than SPBN(-), we infected groups of C57BL/6 mice with either virus. We sacrificed the mice at early timepoints during infection and analyzed the samples by qPCR for the presence of viral RNA as well as type I and type II IFN mRNA. It is widely held that IFN expression levels increase as virus replication increases (39). In fact, mice dying from RABV and other related viruses, often have highest levels

of circulating IFN just before death (40). Therefore, we compared IFN expression levels to the amount of virus replication in the olfactory bulb (OB) (Fig. 10A,D,G), cerebellum (CB) (Fig. 10B,E,H) and cerebral cortex (CX) (Fig. 10C,F,I) of infected mice. After normalizing the IFN message levels to RABV N mRNA, it is clear that SPBNγ induces significantly higher levels of IFNγ (Fig. 10A-C) and type I IFN (Fig. 10D-I) per viral message than does SPBN(-). Relative levels of IFNγ, IFNα and IFNβ expression were significantly increased in SPBNγ-infected OB at 4, 6 and 8 d.p.i. as compared to SPBN(-)-infected OB (Fig. 10A,D,G). In the CB and CX, expression of type I IFN was significantly increased at 6 and 8 d.p.i. (Fig. 10E,F,H,I). No SPBNγ virus was detected in these samples at day 4 p.i. likely due to the time required for the spread of SPBNγ to those brain regions from the OB. The relative level of IFNγ was significantly increased in SPBNγ-infected CB at 8 d.p.i. (Fig. 10B) and both 6 and 8 d.p.i. in the CX (Fig. 10C), as compared to SPBN(-)-infected tissues.

DISCUSSION.

IFNγ is a pleiotropic cytokine capable of modulating both the innate and adaptive immune responses and, as hypothesized, the addition of murine IFNγ to the genome of SPBN highly attenuated the pathogenicity of this virus in mice. To determine which IFNγ-inducible antiviral mechanism(s) led to the strong attenuation of the RABV, we infected cell lines relevant to RABV infection as well as wild-type mice and knock-out mice with specific immune deficits. Together the data support the concept that IFNγ production by SPBNγ attenuates the virus

by enhancing the production of type I IFNs early in the infection. Thus IFNγ, an important product of adaptive immunity, inhibits RABV replication by activating well-established innate antiviral mechanisms.

We previously showed a significant correlation exists among IFNγ expression, BBB permeability and RABV clearance from the CNS (6, 26). The changes in BBB permeability are necessary to allow immune effectors to enter the CNS and eliminate the RABV infection. Various studies suggest that the mechanism involves IFNγ-mediated induction of radicals (26, 41), which culminates in the alteration of tight-junction proteins (27). In the present study, however, we did not observe an increase in BBB permeability due to IFNγ overexpression. While pathogenic when administered intranasally, the control SPBN(-) virus, unlike wild-type RABV (42), induces BBB permeability and allows immune cells to infiltrate the brain. In fact, we showed that SPBN(-) infection resulted in a significant increase in immune cell markers in some brain regions as compared to SPBNγ infection, despite having much lower levels of IFNγ expression. This seemingly paradoxical result is most likely the result of decreased SPBNγ replication necessitating the influx of fewer immune cells to clear the infection, as compared to the SPBN(-) infection. Since SPBN(-) is pathogenic, despite triggering functional changes in the BBB and immune cell invasion into the infected CNS, we can conclude that viral IFNγ expression has additional therapeutic effects on RABV infection.

Since it is known that production of VNA is important for protection against RABV infection, and IFNγ is capable of affecting the humoral adaptive response against viruses, we studied SPBNγ infection in $J_HD^{-/-}$ mice which have no functional B cells and produce no antibodies. Interestingly, the substantial attenuation of SPBNγ is not the result of earlier or greater production of antibodies, as evidenced by the survival of 92% of SPBNγ-infected B cell deficient mice 32 d.p.i. In actuality, addition of IFNγ to a vaccine RABV strain could even suppress VNA induction due to lower viral replication. Our data support the concept that circulating antibody may not be the best indicator of vaccine efficacy as IFNγ could be affecting T cell memory or memory in other cell types that are not evident when judging vaccine efficacy only by the induction of serum VNA (43). It should be noted that antibody is not required for long-term survival from RABV in the context of IFNγ overexpression in our experiments. Nevertheless, these data do not refute the numerous reports that VNA are essential for the clearance of RABV in the absence of such therapeutic intervention.

Previous findings suggest that IFNγ-producing T cells play an important role in the clearance of RABV (6, 26, 36, 44), as well as other neurotropic viruses (45, 46), from the CNS of mice. Additionally, little is known about the role of NK-derived IFNγ during RABV infection. To determine if the cellular source of IFNγ is essential for a therapeutic effect, we infected $IFN\gamma^{-/-}$ mice with SPBN(-) or SPBNγ. We found that the majority of SPBNγ-infected mice survived while SPBN(-)-infected $IFN\gamma^{-/-}$ mice did not. Since we used i.n. infection, and RABVs

primarily infect neurons, it is not likely that infected T or NK cells were a major source of RABV-encoded IFNγ in the CNS. Therefore, during SPBNγ infection of mice, the production of IFNγ by classical immune cells does not appear to be essential for survival. Furthermore, the survival of SPBNγ-infected IFNγ$^{-/-}$ mice demonstrates that only virus-encoded IFNγ is necessary for protection and endogenous IFNγ production by NK or T cells is not required. Together, these IFNγ$^{-/-}$ and JHD$^{-/-}$ data show that attenuation of RABV by IFNγ is profound in adult mice and it is not dependent upon the early production of VNA or an immune source of IFNγ.

Analysis of our *in vitro* monocyte infection experiments leads us to consider that the stimulation of type I IFN expression by IFNγ contributes to survival from SPBNγ infection. Experiments performed over the last several decades have questioned the importance of type I IFN during RABV infection. Although some early experiments found type I IFN expression to be inconsequential to the fate of the RABV-infected animal (39) or equivocal (47), other reports suggest an important role for type I IFN (7, 48). Measuring IFN levels late in the course of infection as well as focusing on survival as an estimation of IFN efficacy may have contributed to the confusion as to the importance of type I IFN. One of the early experiments showed that there are two waves of type I IFN expression during wild-type RABV infection (40). Very early expression of type I IFN is important for survival, while the second wave of expression is not therapeutic and is likely the result of uncontrolled RABV replication (40). More recent work has

demonstrated that type I IFN can impede replication and spread of a highly neuroinvasive RABV both *in vitro* and *in vivo* and can delay/reduce mortality but cannot prevent death altogether (48). Furthermore, an engineered RABV-based HIV vaccine vector expressing IFNβ is attenuated by its IFNβ production (49). Still other experiments have shown that attenuated RABV induce high levels of type I IFN while wild-type RABV do not (7). Since type I IFN signaling is important for protection against infection, it is not surprising that the RABV virus itself dedicates substantial resources to blocking type I IFN signaling. Both the N and P genes of RABV express products that interfere with type I IFN signaling (50, 51) and mutating P can attenuate the virus (52). The blockade of type I IFN by RABV is a "leaky" process, however, and the host response is able to impede replication and spread to some degree despite this immunoevasion strategy (48).

The attenuation of SPBNγ is profound in a number of wild-type mouse strains as well as those with severe immune deficits. When we infected IFNAR$^{-/-}$ mice with SPBNγ, however, the overexpression of IFNγ could not compensate for this immune deficiency, and the mice quickly died. This result again underscores the importance of type I IFN and suggests the mechanism by which SPBNγ is attenuated. Together, our *in vitro* and *in vivo* data showing highly increased type I IFN expression from cell lines and brain tissue infected with SPBNγ over SPBN(-) as well as the lack of attenuation of SPBNγ in IFNAR$^{-/-}$ mice, suggest that expression of IFNγ induces the expression of type I IFN, and that enhancing type I IFN signaling plays a vital role in the attenuation of SPBNγ.

Although IFNγ induces hundreds of genes involved in other antiviral mechanisms, we have shown that a major mechanism of SPBNγ attenuation is through the induction of type I IFN. This rapid type I IFN expression only occurs when an IFNγ-responsive cell is exposed to the virus. This mechanism may circumvent the IFN evasion tactics of the RABV, leading to control of virus replication and spread and, ultimately, virus clearance.

Altogether, our results indicate that the expression of IFNγ by RABV renders it attenuated even in mice with severe immune deficits. The fact that the replication and spread of RABV in the CNS is controlled by immune mechanisms long before neutralizing antibody is produced has long been enigmatic in rabies and understanding the process may hold the key to therapy. Our work shows that IFNγ produced early in the immune response to RABV likely uses innate mechanisms to control the infection such that the later appearance of antibody can clear the infection.

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FIGURE LEGENDS

Figure 1. RABV constructs. A, The negative-stranded RNA genome of the wild-type (WT) parental vector, SAD B19, which was used as the backbone to construct two RABV viruses used for all subsequent experiments. The control virus, SPBN(-), which contains a cytokine gene (red crosshatch), inactivated by

the substitution of STOP codons for the initial seven START codons, is shown in B. C represents the interferon gamma-expressing RABV, SPBNγ. N, nucleoprotein; P, phosophoprotein; M, matrix protein; G, glycoprotein; L, large catalytic subunit of the viral polymerase; TGA, STOP codon; γ, murine IFNγ gene.

Figure 2. IFNγ Production. NA cells were infected at an MOI=0.1 and stained 24 hours later for the presence of RABV and IFNγ (A-C). NA, AS and MC cells were infected at an MOI=1 and the supernatants were analyzed by IFNγ-specific ELISA 12 hours p.i. A, uninfected control NA cells; B, SPBN(-)-infected cells; C, SPBNγ-infected cells. D, IFNγ concentration in supernatants. All photos are 280x magnification. Significance determined by student's t test. **, $p \leq 0.01$; ***, $p \leq 0.001$.

Figure 3. Virus Growth Curves. NA, AS and MC cell lines were infected with SPBN(-) or SPBNγ RABV at an MOI=1 for single step growth curves (A-C) and MOI=0.01 for multi-step growth curves (D-F). Supernatant virus titers were measured at the indicated times post-infection.

Figure 4. *In vivo* mouse infection. Groups of ten Swiss-Webster mice were infected i.n. with SPBNγ or SPBN(-) RABV and monitored for mortality. 4A shows absolute survival proportions for each dose of virus used to calculate the LD_{50} in 4B. 4C is the Kaplan Meier plot indicating both death and time to death.

LD_{50} was calculated by the Habel Test for Potency. Significant difference between survival curves was determined by the Mantel-Cox test. ***, $p \leq 0.001$. #, LD_{50} for SPBN(-) could not be accurately calculated because all SPBN(-) doses killed more than 50% of mice.

Figure 5. IFNγ expression and RABV replication in the brain. Groups of five C57BL/6 mice were infected i.n. with SPBNγ or SPBN(-) RABV. Ten d.p.i. brains were analyzed for the presence of specific mRNA. A shows copies of viral nucleoprotein mRNA, while B shows copies of IFNγ mRNA. C shows IFNγ/N expression illustrated as fold change in the brains of SPBNγ-infected as compared to control SPBN(-)-infected mice. All copy numbers have been normalized to the L13 housekeeping gene. Significance determined by the Mann-Whitney test. **, $p \leq 0.01$.

Figure 6. Adaptive immune cell markers in the brain. Groups of five C57BL/6 mice were infected i.n. with SPBNγ or SPBN(-) RABV. Ten dpi brains were analyzed for the presence of specific mRNA. Normalized mRNA copy numbers of CD4, CD8 and CD19 in the brains of RABV-infected mice are depicted in A, B and C, respectively. All copy numbers are normalized to the L13 housekeeping gene. Significance determined by the Mann-Whitney test. *, $p \leq 0.05$.

Figure 7. Infection of IFNγ$^{-/-}$ and JHD$^{-/-}$ mice. Groups of 8-9 IFNγ$^{-/-}$ mice (A and B) and groups of 13-14 JHD$^{-/-}$ mice (C and D) were infected i.n. with 10^5 ffu of SPBN(-) or SPBNγ and monitored for mortality and morbidity for 35 and 32 days, respectively. The survival curves are shown in A and C. Weight loss as an indicator of infection is shown in B and D. Significant difference between survival curves determined by the Mantel-Cox test. Significant differences in weight loss determined by the student's t test. **, $p \leq 0.01$; ***, $p \leq 0.001$.

Figure 8. Infection of IFNAR$^{-/-}$ mice. Groups of 6-7 WT129 (A) or IFNAR$^{-/-}$ (B) mice were infected i.n. with 10^5 ffu of SPBNγ or SPBN(-) and monitored for mortality and morbidity. Significance was determined by the Mantel-Cox test. ***, $p \leq 0.001$.

Figure 9. ***In vitro*** **induction of type I interferon**. MC cells were infected with SPBNγ or SPBN(-) at an MOI=10. In A, B, C cultures were analyzed at the indicated timepoints for levels of mRNA specific for RABV N versus IFNγ, IFNα and IFNβ. Bars represent normalized IFN mRNA levels plotted on the left Y axis and lines are normalized viral N mRNA expression plotted on the right Y axis. IFNγ was added to the cell cultures in D, E, F and virus titers (D) as well as IFNα levels (E, F) assessed. F shows IFNα levels in the cultures at 24 hrs p.i. Significant differences in cytokine levels between SPBN(-) and SPBNγ infected cultures (A-C) and control versus treated cultures (F) were determined by student's t test.

Figure 10. ***In vivo*** **induction of type I interferon**. Groups of C57BL/6 mice were infected i.n. with 10^5 ffu of SPBNγ or SPBN(-)and their brain sections were analyzed for the gene products denoted above. Expression levels are normalized to the L13 housekeeping gene. Significant differences were determined by the Mann-Whitney test. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.