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*J Immunol* 2009; 182:2297-2304; doi: 10.4049/jimmunol.0800596
http://www.jimmunol.org/content/182/4/2297
Local Type I IFN Receptor Signaling Protects against Virus Spread within the Central Nervous System

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Several neurotropic viruses such as vesicular stomatitis virus (VSV), because this virus has been shown to infect neuroepithelial cells (3–5). VSV is a negative-strand RNA virus that is a member of the family rhabdoviridae. It is highly cytopathic (6). Upon intranasal (i.n.) instillation, within 12–24 h VSV is transported via the olfactory nerves to the olfactory bulb. Interestingly and unlike other neurotropic viruses, VSV enters the CNS via the olfactory bulb and does not seem to use the route via the trigeminal system (7). Activation of astrocytes and astrocytosis are observed in the olfactory bulb as early as 1 day postinfection (p.i.) reaching a peak at ~ day 8. On the contrary, significant microgliosis is usually not observed before day 3 p.i. (8). Viral proteins are found in deeper layers of the olfactory bulb and the ventricular system by day 4 (9). Having reached the olfactory bulb, VSV spreads transsynaptically using both anterograde and retrograde transport to other regions of the brain (7) and most likely via cerebrospinal fluid (10). Virus-associated pathology can be recognized in the spinal cord and is associated with hind limb paralysis (9, 11).

Compared with i.n. infection, upon i.p. or i.v. infection the susceptibility for lethal disease is decreased by 3–4 orders of magnitudes. The higher sensitivity upon i.n. infection is most likely due to the fact that upon i.n. inoculation VSV can readily infect olfactory receptor neurons (7, 9). Because the cell body of olfactory receptor neurons is located in the nasal mucosa, whereas their axons project into the olfactory bulb, virus can easily enter the CNS (12). In contrast to i.n.- or i.v.-infected mice, intracerebrally inoculated mice rapidly develop fatal disease (13), probably because once virus entered the brain it cannot easily brought under control. The successful clearance of viral infection from the CNS requires the elimination of virus from its intracellular location without damaging nonrenewable neurons. The activation of the innate immune system inhibits viral replication but does not always completely eliminate viral components (14). Nevertheless, the mechanisms of virus clearance within the CNS and host recovery are still poorly understood.

Among cytokines that are induced upon VSV infection, IFN plays a critical role for virus control. IFN constitutes a family of cytokines consisting of several IFN-α subtypes, single IFN-β, -ε, -κ, and -α (15, 16). IFN exhibits pleiotropic effects. In many viral infections, increased IFN-α and -β levels can be detected in the

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1 Abbreviations used in this paper: VSV, vesicular stomatitis virus; IFNAR, type I IFN receptor; p.i., postinfection; i.n., intranasal; WT, wild type; eGFP, enhanced GFP; BBB, blood-brain barrier; RIG-I, retinoic acid-inducible gene I.

Received for publication March 3, 2008. Accepted for publication December 18, 2008.

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1 This study was supported by the German Research Council (Grants SFB432, B15, and ME 1648/2-1), the Volkswagen Foundation, and the Vereinigung von Freunden und Förderern der Johann Wolfgang Goethe-Universität Frankfurt am Main.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0800596
serum. Despite the fact that most cell types are able to express IFN upon in vitro infection, plasmacytoid dendritic cells are important IFN producers upon VSV infection in vivo (17, 18). IFN binds to a common receptor, the type I IFN receptor (IFNAR) that is ubiquitously expressed. Genetically modified mice lacking the IFNAR1 subunit (IFNAR1−/−) are devoid of a functional IFN system and show an enhanced susceptibility for lethal disease upon infection with VSV and a number of other viruses (19). Whereas lethally infected wild-type (WT) mice show productive virus infection exclusively in the CNS, in IFNAR−/− mice the virus is universally spread (20). Thus, IFNAR expression does seem to play a role in determining viral tropism (21).

The observation that 2–3 days after i.n. high-dose infection of WT mice, VSV can be detected in brains of virtually all infected animals, whereas only 50% of the animals eventually died, indicated that virus infection was controlled within the CNS (9). Although administration of high-dose IFN after i.n. VSV infection improved the overall survival rate (22), an enhanced sensitivity to lethal disease observed in T cell-deficient and anti-CD4- or anti-CD8-treated mice was interpreted as evidence that T cells play a critical role to limit viral replication and are important for survival, whereas NK cells and IFN do not provide protection sufficient to enable recovery (3). In the same line, a more recent study showed that upon i.n. VSV infection IFN-α/β mRNA was not induced in the CNS and thus did not seem to play a critical role for protection (23).

To readdress the question of whether IFN is a critical factor for the control of virus infection within the CNS, we used a new approach and generated mice with a CNS-specific IFNAR deletion. Here we show that despite normal innate and adaptive immune responses in the periphery, such mice succumbed to i.n. VSV infection with 10^3 PFU. Furthermore, only in the olfactory bulb, the site of VSV entry, disease-associated IFNAR signaling was observed as indicated by increased STAT1 phosphorylation. Collectively, our data point toward the olfactory bulb as a region of the CNS in which IFNAR triggering is critically required to limit spatial spread of virus infection.

Materials and Methods
Mice and viruses
NesCre+/− IFNARfloxflox mice were obtained by intercrossing conditional IFNAR mice (IFNARfloxflox; Ref. 24) and transgenic mice that express Cre specifically in neuroectodermal cells of the CNS (NesCre+/−; Ref. 25). Both mouse strains were 10-fold backcrossed to the C57BL/6 background before intercrossing. IFNAR-deficient mice (IFNAR−/−; Ref. 20) were 20-fold backcrossed to the C57BL/6 background (26). Mice were kept under specific pathogen-free conditions in the central mouse facility of the Paul-Ehrlich-Institut. Unmutated C57BL/6 mice, also referred to as WT, were purchased at Harlan-Winkelmann or bred at Paul-Ehrlich-Institut. Mice were screened for tissue-specific deletion of exon 10 by PCR using the primers ΔExon10 forward (5′-GGTTAAGCTTGGGCTATGCA-3′) and ΔExon10 reverse (5′-GGTTAAGCTTGGGCTATGCA-3′). This method was sensitive enough to detect 1 deleted allele among 100 nondeleted ones. Animal experiments were conducted under specific pathogen-free conditions in compliance with German federal and state legislation on animal experiments. For experiments, 8- to 16-wk-old mice were used. VSV-Indiana (Mudd-Summers isolate), originally obtained from Dr. D. Kolakofsky (University of Geneva, Geneva, Switzerland), and VSV-enhanced GFP (eGFP; Ref. 27) were grown on BHK-21 cells. Tissues were harvested from cerebellum, olfactory bulb, and tibers were determined by plaque formation on Vero cells.

Intranasal VSV infection and perfusion
For i.n. infection, mice were anesthetized with Ketamin/Rompun and a total of 10 μl containing 10^6 PFU of VSV in PBS were pipetted into both nostrils. Mice were perfused with ~5 ml of 0.9% NaCl and for subsequent immunohistochemical staining with additional 5 ml of 4% formaldehyde-supplemented saline.

VSV plaque assay
Tissues were homogenized in 1 ml of medium. Serial 10-fold dilutions of homogenates were transferred onto Vero cell monolayers in six-well plates and incubated for 1 h at 37°C. Monolayers were overlaid with 2 ml of MEM containing 1% methylcellulose and incubated for 24 h at 37°C. Then the overlay was removed, and the monolayer was fixed and stained using 0.5% crystal violet dissolved in 5% formaldehyde, 50% ethanol, and 4.25% NaCl.

VSV neutralization assay
VSV neutralization assay was performed as described previously (28).

IFN-α/β determination by ELISA
For determination of IFN levels in brain homogenates, brains of perfused mice were homogenized in 20 mM Tris-HCl (pH 7.3), 140 mM NaCl, 0.5% Triton X-100, complete protease inhibitors (Roche), and 2 mM sodium orthovanadate. Upon centrifugation, clear supernatants of brain homogenates and serum samples prepared from blood were analyzed for IFN-α or IFN-β by ELISA following the manufacturer’s instructions (PBL Biomedical Laboratories).

Immunohistochemistry and FACS analysis
Serial sections of 10 μm were cut from paraffin-embedded tissue blocks. For labeling of phospho-STAT1, a polyclonal rabbit anti-mouse Ab was used that reacts specifically with phosphotyrosine 701 (Cell Signaling). For pan-STAT1 staining, the rabbit anti-mouse Ab C-24 (Santa Cruz Biotechnology) was used. Staining of CD3+ T cells was performed as previously described (29). For immunofluorescence, brains of VSV-eGFP-infected mice were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with Alexa Fluor-488-conjugated secondary Abs (Dako) and ECL (Amersham).

Western blotting
Extracts from the olfactory bulb or the cerebrum of VSV-inoculated mice were prepared by incubating tissue samples for 30 min in 50 μl of ice-cold lysis buffer (10 mM KCl, 20 mM HEPES (pH 7.4), 0.2% Nonidet P-40, 0.1 mM EDTA, 10% glycerol, 0.1 mM sodium orthovanadate, 0.1 mM PMSF, 2 mM DTT, and complete protease inhibitors from Roche). After centrifugation, supernatants were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membrane and probed with the phosphotyrosine-specific STAT1 Ab. After stripping, the blot was reprobed with the anti-CD19, anti-CD3 (BD Biosciences), and anti-IFNAR1 monoclonal Ab (kindly provided by Dr. R. Schreiber) (30).

Electrophoretic mobility shift assay
Tissue extracts were prepared as described above. Four microliters of each extract were incubated with 1 ng of 32P-labeled M67 probe (31) for 15 min at room temperature. The assay was conducted on 4% 29:1 acrylamide-bisacrylamide gels at 4°C, as described previously (32). Binding activity was visualized with a phoshimaging system (Storm 820; Molecular Dynamics).

Results
Brain-specific IFNAR signaling controls VSV infection
Conditional NesCre+/− IFNARfloxflox mice with an IFNAR deletion specifically in neuroectodermal cells of the CNS, including neurons, oligodendrocytes, and astrocytes but not microglia, showed >90% deletion efficiency in the brain, whereas other tissues such as spleen did not show deletion (33). In NesCre+/− IFNAR−/− mice, residual deletion in peripheral cells was below the detection limit of <0.01% of the analytical PCR used (Fig. 1, A and B, and data not shown). Accordingly, FACS analysis revealed normal IFNAR expression on B and T cells of NesCre+/− IFNARfloxflox mice, whereas IFNAR−/− mice did not show detectable IFNAR expression (Fig. 1C).

Upon i.n. infection with 10^5 PFU of VSV, NesCre+/− IFNARfloxflox mice did not show signs of disease until day 5 or 6. Then they became hemiplegic and died within hours. In contrast, WT controls tolerated the infection well, whereas conventional
IFNAR−/− mice developed significant disease symptoms 2–3 days after infection and died (Fig. 1D). The increased sensitivity of NesCre−/−/IFNARlox/lox mice was not due to impaired Ab responses as indicated by high levels of VSV-neutralizing serum Abs found 4 days after i.n. infection with 10^3 PFU of VSV of WT and NesCre−/−/IFNARlox/lox mice (Fig. 1E).

To determine the viral load in various different tissues, mice were i.n. infected with 10^3 PFU of VSV, and 3 or 6 days later tissues were analyzed for the presence of virus in a plaque assay. In WT mice, only in the brain minimal amounts of VSV were detected by day 3 that were further decreased by day 6 (Fig. 1F). In contrast, 3 days after VSV infection of IFNAR−/− mice, virus was detected in all tissues analyzed, reaching amounts of up to 10^6 PFU/g of tissue. Three days after infection of NesCre−/−/IFNARlox/lox mice, minimal amounts of VSV were detected in the brain and lung. By day 6, virus infection was completely cleared from peripheral tissues, whereas in the brain up to 10^6 PFU/g were detected (Fig. 1F). Taken together, these data point toward a pivotal role of brain-specific IFNAR signaling for limiting virus spread within the CNS.

Peripheral type I IFN is retained by the blood-brain barrier (BBB)

To determine peripheral IFN responses in VSV-infected mice, serum samples were analyzed by ELISA. WT and NesCre−/−/IFNARlox/lox mice displayed significant IFN-α responses that reached maximal levels of ~100–750 pg/ml by day 2. Then, serum IFN-α declined and returned to background levels within the following days (Fig. 2A). Similar data were obtained for the analysis of IFN-β responses (data not shown). To study IFN responses in the CNS, whole brains from perfused and virtually blood-free animals were extracted, and the IFN content of brain homogenates was determined. Irrespective of whether brain homogenates of VSV-infected WT, IFNAR−/− mice showed significant IFN-α responses that were detected in all tissues analyzed, reaching amounts of up to 10^6 PFU/g of tissue. Three days after infection of NesCre−/−/IFNARlox/lox mice, minimal amounts of VSV were detected in the brain and lung. By day 6, virus infection was completely cleared from peripheral tissues, whereas in the brain up to 10^6 PFU/g were detected (Fig. 1F). Taken together, these data point toward a pivotal role of brain-specific IFNAR signaling for limiting virus spread within the CNS.

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Because even minimal amounts of IFN-α/β added to brain homogenate were recovered (Fig. 2C), the above results suggested that in brains of VSV-infected mice IFN was absent or below the detection limit and that peripheral IFN did not easily cross the BBB.

To address whether the death of VSV-infected NesCre^{+/-} IFNAR^{−/-} mice was exclusively mediated by virus infection or whether immune pathology also played a role, mice were depleted of CD8^{+} T cells before inoculation with VSV. Depletion of CD8^{+} T cells did not affect the course of disease, neither in WT nor in NesCre^{+/-} IFNAR^{−/-} mice (Fig. 2D), indicating that CD8^{+} T cells played no relevant disease-modulating role.

In VSV-infected WT mice, viral infection, T cell infiltration, and IFNAR signaling are restricted to the olfactory bulb

Immunohistological analysis of brain sections of WT mice 6 days after i.n. infection with VSV showed that only in the olfactory bulb, but not in other brain regions tested, massive lymphocyte infiltration of 406 ± 184 CD3^{+} cells/mm² was present. Surprisingly, VSV-infected NesCre^{+/-} IFNAR^{−/-} mice showed reduced lymphocyte infiltration in the olfactory bulb with 63 ± 37 CD3^{+} cells/mm² (Fig. 3A and B). Furthermore, and unlike WT mice, lymphocyte infiltration was also detected in the white and gray matter of the cerebellum and submeningeal as well as in parenchymal areas of the brain stem (Fig. 4A). To analyze virus infection of brain subregions, mice were infected i.n. with 10^5 PFU of VSV and brains of perfused animals were isolated on days 2, 4, and 6 and dissected into the olfactory bulb, cerebrum, cerebellum, brain stem, and spinal cord. Two days after VSV infection, WT mice showed virus only in the olfactory bulb, whereas in other brain regions no virus was detected (Fig. 3C). Virus infection of the olfactory bulb was reduced by day 4 and was completely resolved by day 6 (Fig. 3C). Minimal amounts of virus detected in the serum of one of three WT mice tested on day 2 p.i. were resolved by day 4. In contrast, 2 days after i.n. infection, IFNAR^{−/-} mice showed high VSV titers of up to 10^8 PFU/g in all brain regions. These mice showed also viremia (Fig. 3C). Two days after VSV infection of NesCre^{+/-} IFNAR^{−/-} mice, virus was found in the olfactory bulb as observed in WT mice. Furthermore, two of three mice showed minimal amounts of virus in cerebrum, and one of three mice showed this in the brain stem, whereas no virus was found in the cerebellum or spinal cord (Fig. 3C). On days 4 and 6, the picture changed, and high virus quantities of up to 10^9 PFU/g were detected in all brain regions analyzed, whereas no virus was detected in the serum.

These experiments indicated that WT mice were able to constrain VSV replication to the olfactory bulb and to resolve the infection. Similarly, in NesCre^{+/-} IFNAR^{−/-} mice, VSV was found primarily in the olfactory bulb, whereas at later time points the infection spread to the entire brain. Thus, despite the observation that local IFN responses were not detectable by an ELISA method in brain homogenates of VSV-infected mice, IFNAR expression in the brain played a critical role to limit VSV infection to the olfactory bulb and to eventually clear the infection.

To further address whether IFNAR signaling takes place locally in the primary infected olfactory bulb, STAT1 induction and phosphorylation were analyzed. Upon IFNAR stimulation, STAT1 is phosphorylated and subsequently translocated to the nucleus to modulate target gene expression. In VSV-infected WT mice, tyrosine phosphorylation of STAT1 was resolved by day 4 (Fig. 3C). Minimal amounts of virus detected in the serum of one of three WT mice tested on day 2 p.i. were resolved by day 4. In contrast, 2 days after i.n. infection, IFNAR^{−/-} mice showed high VSV titers of up to 10^8 PFU/g in all brain regions. These mice showed also viremia (Fig. 3C). Two days after VSV infection of NesCre^{+/-} IFNAR^{−/-} mice, virus was found in the olfactory bulb as observed in WT mice. Furthermore, two of three mice showed minimal amounts of virus in cerebrum, and one of three mice showed this in the brain stem, whereas no virus was found in the cerebellum or spinal cord (Fig. 3C). On days 4 and 6, the picture changed, and high virus quantities of up to 10^9 PFU/g were detected in all brain regions analyzed, whereas no virus was detected in the serum.

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the olfactory bulb of WT mice. As expected, in VSV-infected IFNAR<sup>−/−</sup> and NesCre<sup>+/−</sup> IFNAR<sup>flox/flox</sup> mice, no STAT1 up-regulation or tyrosine phosphorylation was found (Fig. 4A).

DNA binding activity of STAT1, as measured by gel shift assays, increased in the olfactory bulb of infected WT mice with similar kinetics as already observed for STAT1 phosphorylation (Fig. 4B). In the cerebrum, no binding of STAT1 to the high-affinity M67 binding site was detected. In contrast, in IFNAR<sup>−/−</sup> mice, no DNA-binding activity of STAT1 was found in any neuronal tissue.

These observations were confirmed by immunohistochemical analyses. Only in VSV-infected WT mice, but not in uninfected

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**FIGURE 3.** Aberrant T cell distribution in the brain and disturbed virus clearance in the absence of IFNAR in the CNS. WT controls and NesCre<sup>+/−</sup> IFNAR<sup>flox/flox</sup> mice were infected i.n. with 10<sup>3</sup> PFU of VSV. A, On day 6 after infection, brains were analyzed immunohistologically for CD3<sup>+</sup> cells. Arrowheads, Single infiltrating T cells. B, For quantification of histological analyses, infiltrating cells in the olfactory bulb were counted and related to overall tissue area. C, Blood samples were taken at the indicated days postinfection. Upon perfusion, brains were dissected into olfactory bulb, cerebrum, cerebellum, brain stem, and spinal cord. For determination of viral load, homogenates and serum samples were plaqued on Vero cells. Three animals per group were analyzed. One of two similar experiments is shown. <, Not detected; --, not determined.

**FIGURE 4.** In VSV-infected mice STAT1 is tyrosine phosphorylated exclusively in the olfactory (Olf) bulb but not in other brain regions. WT, IFNAR<sup>−/−</sup> and NesCre<sup>+/−</sup> IFNAR<sup>flox/flox</sup> mice (three mice per group) were infected i.n. with 10<sup>3</sup> PFU of VSV. Brains were dissected and lysed at the indicated time points following VSV inoculation. A, Time course of STAT1 tyrosine phosphorylation, and protein expression was measured by Western blotting. Lysates from olfactory bulb and cerebrum were examined for expression of phospho-STAT1 using an Ab that specifically reacts with STAT1 phosphorylated at tyrosine residue 701. The membranes were then re-exposed to a pan-STAT1 Ab. One representative experiment of three is shown. dpi, Dots per inch. B, Induction of STAT1 DNA-binding activity during the course of experimental VSV infection. Tissue extracts were incubated with a radioactively labeled M67 probe that contains a single high-affinity STAT1 binding site. As a positive control, IFN-γ-treated HeLa cells were used in lane 1. Supershifts with a pan-STAT1 Ab (S) added to the reaction mix revealed the identity of the band containing phosphorylated STAT1 homodimers (arrow). Values are representative of two independent experiments.
controls (Fig. 5, A and E), tyrosine-phosphorylated STAT1 was detected in the olfactory bulb. At day 4 after VSV inoculation, focal areas of phospho-STAT1 expressing cells became visible in which positive immunoreactivity was restricted to nuclei of periglomerular cells (Fig. 5B). Only a minor population of cells showed nuclear accumulation of phosphorylated STAT1, whereas the majority of cells, in particular those in the mitral cell layer and granule cell layer, remained unstained. In IFNAR−/− and NesCre+/−IFNARlox/lox mice, no phospho-STAT1 expressing cells were detected (Fig. 5F and data not shown). Using a pan-STAT1-specific Ab, decoration of STAT1 was found in WT as well as IFNAR−/− and NesCre+/−IFNARlox/lox mice where it was preferentially localized in the cytoplasm (Fig. 5, C, D, G, and H, and data not shown). However, only in WT mice was STAT1 up-regulated during the course of infection, where it was localized predominantly in the cytoplasm of periglomerular cells (Fig. 5, C and D). Collectively, these results showed that upon VSV infection signal transmission via the IFNAR took place selectively in the olfactory bulb and that local IFNAR signaling visualized by STAT1 phosphorylation was sufficient to constrain virus infection to the olfactory bulb.

Four days after intranasal instillation of WT mice with 10^3 PFU of VSV-eGFP, GFP expression was found exclusively in the axonal layer and in glomerular structures (G; see Fig. 6) of the glomerular layer, whereas in the periglomerular cells and in deeper layers of the olfactory bulb, such as the external plexiform layer, the mitral cell layer or the granule cell layer, no virus was detected. This indicated that upon intranasal VSV challenge, virus infected only olfactory nerves that projected into glomerular structures. Nevertheless, within the glomerular layer, virus was arrested at the interface between glomerular structures and periglomerular cells.

**Discussion**

Here we show that upon i.n. VSV infection, IFNAR signaling in the CNS is critically required to limit viral spread within the brain and to clear the infection. VSV-infected mice with a cell type-specific IFNAR deletion in neurons of the CNS show massive virus replication in the brain, whereas in peripheral tissues no virus was found. In contrast, in VSV-infected WT mice virus was found only in the olfactory bulb where it was cleared within 1–2 wk after infection. Interestingly, in infected WT mice IFNAR signaling was only found in brain areas from which virus could be reisolated, i.e., the olfactory bulb, and not in other brain regions. Obviously, the olfactory bulb, the part of the CNS that is most prominently exposed to environmental factors, is specialized to control viral spread and to prevent virus infection. This mechanism is in accordance with the regeneration capacity of olfactory neurons that are originated in the olfactory epithelium and project into the olfactory bulb (35, 36).

As early as 1998, it was recognized that 2–3 days after i.n. VSV infection, a dose that causes lethal disease in ~50% of the
animals, virus was found in the brains of all mice. Because virus could not be reisolated from surviving mice, these results proved clearance of virus in the brains of the surviving mice (9). Thus far, it is unclear how exactly virus clearance is accomplished within the brain. It is assumed that clearance of RNA viruses from neurons is initiated by the expression of IFN-β and that it is primarily mediated by neutralizing Abs that effectively inhibit the production of new virus but do not necessarily eliminate virus RNA from cells. This concept is in line with the observation that CD4-depleted WT mice, which mount only short-lived neutralizing Ab responses, show an increased susceptibility to lethal VSV infection (14, 37).

Here we studied sublethal VSV infections using an i.n. dose of 10^6 PFU that was usually well tolerated by WT animals. Similar to studies using up to 3 orders of magnitude higher i.n. infection doses (3, 8), also under these conditions virus was detected in the brains of all infected WT animals, i.e., in the olfactory bulb, but not in other regions of the brain, where the virus was cleared within 1–2 wk. To investigate the role of IFN in the CNS, mice were generated that had an IFNAR deletion only in the CNS but not in other tissues (NesCre+/− IFNAR^flox/flox, Ref. 33). Such mice showed an efficient IFNAR deletion in the CNS, whereas lymphocytes showed normal IFNAR expression (see Ref. 33 and Fig. 1).

Infection experiments, NesCre+/− IFNAR^flox/flox mice succumbed to infection within 1 wk, whereas WT control mice usually survived. Both VSV-infected WT and NesCre+/− IFNAR^flox/flox mice mounted normal IFN and VSV-neutralizing Ab responses in the serum. Thus, local IFNAR signaling within the brain was critically required to restrict virus replication to the olfactory bulb and to clear infection. Furthermore, upon depletion of CD8^+ T cells, WT mice survived VSV infection as well as undepleted controls, and NesCre+/− IFNAR^flox/flox mice died with similar kinetics as undepleted NesCre+/− IFNAR^flox/flox mice. Thus, CD8^+ T cells did not critically contribute to limit viral replication and did not cause immune pathology. These observations are in line with a previous study also showing that MHC class I-deficient mice showed a similar disease course upon i.n. VSV infection compared with WT mice (37). However, another study showed that in the absence of CD8^+ T cells, a reduced survival of VSV infection was observed (3). This discrepancy is probably due to differences in the experimental setup, i.e., an infection dose of 2 × 10^6 PFU vs 10^3 PFU and BALB/c mice vs C57BL/6 mice analyzed in the above cited study and this study, respectively.

Our observation that despite the high IFN serum levels 2 days after VSV infection IFN was not detected in brain homogenates of infected mice indicated that serum IFN cannot easily cross the BBB to enter the CNS. This notion is in line with an earlier study infected mice indicated that serum IFN was not detected in brain homogenates of BALB/c mice vs C57BL/6 mice analyzed in the above cited study, respectively.

Both VSV-infected WT and NesCre+/− IFNAR^flox/flox mice succumbed to infection within 1 wk, whereas WT control mice usually survived. Both VSV-infected WT and NesCre+/− IFNAR^flox/flox mice mounted normal IFN and VSV-neutralizing Ab responses in the serum. Thus, local IFNAR signaling within the brain was critically required to restrict virus replication to the olfactory bulb and to clear infection. Furthermore, upon depletion of CD8^+ T cells, WT mice survived VSV infection as well as undepleted controls, and NesCre+/− IFNAR^flox/flox mice died with similar kinetics as undepleted NesCre+/− IFNAR^flox/flox mice. Thus, CD8^+ T cells did not critically contribute to limit viral replication and did not cause immune pathology. These observations are in line with a previous study also showing that MHC class I-deficient mice showed a similar disease course upon i.n. VSV infection compared with WT mice (37). However, another study showed that in the absence of CD8^+ T cells, a reduced survival of VSV infection was observed (3). This discrepancy is probably due to differences in the experimental setup, i.e., an infection dose of 2 × 10^6 PFU vs 10^3 PFU and BALB/c mice vs C57BL/6 mice analyzed in the above cited study and this study, respectively.

Our observation that despite the high IFN serum levels 2 days after VSV infection IFN was not detected in brain homogenates of infected mice indicated that serum IFN cannot easily cross the BBB to enter the CNS. This notion is in line with an earlier study in which radioactively labeled IFN-β was used to study the ability of IFN to cross the BBB to enter the CNS. This notion is in line with the observation that CD4-depleted WT mice, which mount only short-lived neutralizing Ab responses, show an increased susceptibility to lethal VSV infection (14, 37).

Our study shows that STAT1 phosphorylation, which is a crucial and early event of the IFNAR-triggered JAK/STAT pathway (48, 49) and thus a good measure for biologically relevant IFNAR signaling, is observed as early as 2 days after VSV infection of WT mice. STAT1 phosphorylation was confined to the olfactory bulb and was not observed in other brain regions. This observation is in line with the above discussed finding that virus was found only in the olfactory bulb but not in other brain regions. Thus, there is a clear correlation of local IFN signaling and sites of inflammation. This finding suggests that indeed local virus infection triggered local induction of cytokines that in turn activated IFNAR. Thus, it is possible that IFN was undetectable in brain homogenates of VSV-infected mice by means of an ELISA approach because minute concentrations of locally produced IFN were below the detection threshold. Nevertheless, even if olfactory bulb homogenates of VSV-infected WT mice were tested, no IFN was detected (data not shown). Thus, at this moment it is unclear whether IFNAR triggering within the CNS was mediated by locally produced IFN or by peripherally induced IFN that crossed, although with low efficiency, the BBB.

By performing experiments with VSV-eGFP (27), we could clearly show that virus infection reached axons of olfactory neurons that project into glomerular structures of the outer layer of the olfactory bulb. However, virus was arrested within glomerular structures and was not found in periglomerular cells or other deeper layers of the olfactory bulb. Thus, periglomerular cells were IFN stimulated without being virus infected.

Our observation that 2 days after infection NesCre+/− IFNAR^flox/flox and WT mice showed virus primarily in the olfactory bulb but not in other brain regions suggests that the CNS is not easily accessible for virus infection. Furthermore, the data demonstrate that virus must follow similar infection routes, irrespective of whether cells in the CNS express IFNAR or fail to do so. Collectively, our results demonstrate the utmost importance of local IFNAR triggering within the glomerular layer of the olfactory bulb to limit viral spread and to support virus clearance. Although the pivotal role of IFN in virus clearance has been recognized earlier (19, 20, 50), here we show that local IFNAR triggering within the periglomerular cells of the olfactory bulb is critically required to protect against lethal disease. Our results point toward outer layers of the olfactory bulb as the part of the brain that is particularly exposed to the environment and that seems to have developed effective mechanisms to control transnasal virus entry. Because
VSV cannot easily enter the CNS by other routes than the olfactory nerves (10), local activation of the JAK-STAT1 pathway is an important antiviral defense strategy to restrict viral replication to the olfactory bulb, thus preventing uncontrolled propagation of the virus to adjacent regions of the brain. Furthermore, our data underscore that neuroectodermal cells of the CNS, including neurons, play a central role in modulating the IFNAR-mediated inflammatory reaction, thus shaping the innate immune response toward neurotropic viruses.

Acknowledgments
We thank Theresa Frenz, Paul-Ehrlich-Institut, for help with some experiments and Marlies Cronbach, University of Marburg, for expert experimental assistance.

Disclosures
The authors have no financial conflict of interest.

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