Visualizing Production of Beta Interferon by Astrocytes and Microglia in Brain of La Crosse Virus-Infected Mice

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Beta interferon (IFN-β) is a major component of innate immunity in mammals, but information on the in vivo source of this cytokine after pathogen infection is still scarce. To identify the cell types responsible for IFN-β production during viral encephalitis, we used reporter mice that express firefly luciferase under the control of the IFN-β promoter and stained organ sections with luciferase-specific antibodies. Numerous luciferase-positive cells were detected in regions of La Crosse virus (LACV)-infected mouse brains that contained many infected cells. Double-staining experiments with cell-type-specific markers revealed that similar numbers of astrocytes and microglia of infected brains were luciferase positive, whereas virus-infected neurons rarely contained detectable levels of luciferase. Interestingly, if a mutant LACV unable of synthesizing the IFN-antagonistic factor NSs was used for challenge, the vast majority of the IFN-β-producing cells in infected brains were astrocytes rather than microglia. Similar conclusions were reached in a second series of experiments in which conditional reporter mice expressing the luciferase reporter gene solely in defined cell types were infected with wild-type or mutant LACV. Collectively, our data suggest that glial cells rather than infected neurons represent the major source of IFN-β in LACV-infected mouse brains. They further indicate that IFN-β synthesis in astrocytes and microglia is differentially affected by the viral IFN antagonist, presumably due to differences in LACV susceptibility of these two cell types.

Viruses can trigger pattern recognition receptors of infected hosts which initiate signaling cascades that culminate in transcriptional activation of type I and type III interferon (IFN) genes. Type I and type III IFNs are cytokines that use distinct receptor complexes for signaling and which, thereby, induce an antiviral state in uninfected cells. The family of type I IFNs includes more than 10 different IFN-α subtypes, IFN-β, and minor subtypes such as IFN-ω or IFN-δ, whereas type III IFNs include IFN-λ1, -λ2, and -λ3 (8, 19). These various IFN genes are typically co-induced in response to virus infection although the kinetics and the degree of activation of the different IFN genes differ considerably depending on the producer cell type and nature of challenge virus (6, 15). In the mouse, IFN-β is the first type I IFN subtype being expressed after viral infection and, together with IFN-α4, is considered to prime cells for the production of other type I IFN family members (2).

Pattern recognition receptors which can recognize RNA viruses include cytoplasmic retinoic acid-induced gene (RIG)-like helicases and membrane-anchored Toll-like receptors (TLR). Cell culture studies indicate that most if not all nucleated mammalian cells can synthesize IFN in response to signals from RIG-like helicases when cells are infected with replication-competent viruses (21). Further, certain immune cells such as macrophages and dendritic cells readily synthesize IFN when receiving signals from TLRs which recognize engulfed virus-derived nucleic acids (14). The situation after infection of an intact organism is much more complex. For viruses that cause viremia, plasmacytoid dendritic cells (pDC), which are mainly present in blood and spleen of mammals, are responsible for most of the circulating IFN (1, 7, 24). During influenza virus infection of the lung, pDC seem to play far less important roles (12). Similarly, classical immune cells including pDC are not present in healthy brains (10, 13), suggesting that other cell types are mainly responsible for IFN synthesis in this organ. However, previous attempts to unambiguously identify these alternative IFN-producing cells did not yield a clear picture. Experiments were either performed with isolated brain cells or were focused on certain cell types without addressing the question of the contribution of such cells to the overall IFN response in the central nervous system (10, 22, 23, 26). One difficulty with these experimental approaches was that IFNs are quickly secreted and are not accumulating to high intracellular levels in producer cells, thus complicating their detection in tissue slices by IFN-specific antibodies.

La Crosse virus (LACV) is a mosquito-borne pathogen that infects up to 300,000 people in the United States and can cause encephalitis in children and young adults (3). LACV belongs to the genus Orthobunyavirus, family Bunyaviridae. These viruses have a trisegmented single-stranded RNA genome of negative polarity and replicate in the cytoplasm of infected cells. The smallest genome segment of LACV codes for the viral nucleoprotein and a nonstructural protein, termed NSs, which efficiently inhibits the IFN system of infected mammalian hosts. NSs of LACV induces degradation of cellular RNA polymerase II, which, in turn, results in reduced transcription of many cellular genes, including the genes for type I and type III IFNs (27). A mutant of LACV lacking a functional NSs gene (LACV-ΔNSs) was generated (5). As expected if NSs served as an IFN antagonist, LACV-ΔNSs induced significantly more IFN in cultured cells and brains of infected mice than wild-type LACV (LACV-wt) (4, 18). Further, the LACV-ΔNSs mutant was less virulent in mice than wild-type LACV al-

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though both viruses caused encephalitis and death after 5 to 10 days if administered intraperitoneally into juvenile mice (4). Using conventional in situ hybridization and immunostaining techniques, we previously identified cells with macrophage and ependymal markers as major sources of IFN-α and -β in the brain of mice with acute LACV encephalitis, and we observed that neurons represent a minor but substantial source of IFN during viral encephalitis (10).

Transgenic mice in which reporter genes are inserted into the coding regions of the IFN-α or -β genes are promising new tools for studying virus-induced expression of IFN genes in vivo. A reporter mouse which expresses green-fluorescent protein (GFP) in place of IFN-αβ was successfully used to demonstrate that alveolar macrophages contribute to IFN synthesis in virus-infected lungs (17). Cells from a reporter mouse with a modified IFN-β locus encoding GFP were used to demonstrate the stochastic nature of type I IFN gene expression (28). We recently employed another reporter mouse in which the IFN-β coding region was replaced by luciferase to visualize IFN synthesis in virus-infected animals by in vivo imaging (18). We now used the same luciferase reporter mouse to analyze the contribution of various brain cell types to IFN-β synthesis in mice with LACV encephalitis. We visualized IFN-β-producing cells by staining brain sections with antibodies that simultaneously recognize luciferase and marker proteins of neurons, astrocytes, and microglia, respectively. We further took advantage of the possibility that theloxP-flanked luciferase gene in this reporter mouse can be rearranged in defined cell types by crossing these animals with mice that express Cre recombinase in a cell-type-specific manner. Using these two approaches, we identified astrocytes and microglia as the main IFN-β producers in LACV-infected brains. We further observed that the LACV-encoded IFN-α/β antagonistic factor NSs strongly impairs IFN production by astrocytes but not microglia.

MATERIALS AND METHODS

Mice. Mice were bred in the animal facility of the Department of Virology at the University of Freiburg. All mice used in this study were on the C57BL/6 background or backcrossed onto C57BL/6 for at least five generations. IFN-βloxP/loxP-luc and conditional reporter IFN-βloxP/FRT/loxP-luc mice have been described previously (18, 25). IFN-βloxP/FRT/loxP-luc mice were crossed to LysM-Cre (25), Thy1-Cre (11) (stock number 006143; Jackson Laboratory), and Synapsin1-Cre mice (29) to generate mice that express the reporter gene in microglia and macrophages, astrocytes and neurons, and neurons, respectively.

Viruses and infection of mice. Wild-type LACV and mutant LACV-ΔNSs that cannot express the IFN-α/β antagonistic factor NSs were previously described (5). Virus stocks were generated in Vero cells. Juvenile mice (12 to 15 days old) were infected intraperitoneally with 10^4 PFU of wild-type or mutant LACV diluted in 100 μL of phosphate-buffered saline (PBS). Animals were checked for neurological symptoms at 8-h intervals and sacrificed when the first signs of ataxia were noted. Brains were collected without fixation for virus titration and measurement of luciferase activity or were perfusion fixed for immunohistochemical analyses as described below.

Virus titrations. Plaque assays were performed in Vero cells using six-well plates. Serial dilutions of lysates were applied for 1 h. Supernatants were then removed and replaced by a 1:1 mixture of 3% Avicel cellulose (FMC BioPolymer) and double-concentrated Dulbecco’s modified Eagle’s medium (DMEM; Gibco). Vero cells were incubated for 72 h at 37°C in 5% CO₂ before supernatants were removed. Cells were fixed with 4% paraformaldehyde, and plaques were visualized by staining with 0.5% crystal violet.

Ex vivo luciferase measurement. Brains were homogenized in 800 μL of PBS using FastPrep-24 equipment and lysing matrix A (MP Biomedicals). Samples (200 μL) were treated with 50 μL of 5 × Cell Culture Lysis Buffer (Promega), and luciferase activity was measured in a Sirius Tube Luminometer (Berthold Technologies) using a single luciferase assay system (Promega) according to the manufacturer’s protocol.

Immunohistochemistry. Animals were sacrificed with a mixture of ketamine (3.7%), xylazine (0.2%), and acepromazine (0.02%) and transcardially perfused with 0.9% NaCl, followed by 4% buffered paraformaldehyde in PBS. Brains were postfixed in the same solution for 6 more hours. Fixed brains were cut horizontally into 50-μm-thick sections on a Leica vibratome. Free-floating tissue sections were blocked and permeabilized in PBS containing 5% normal donkey serum and 0.1% Triton X-100 for 30 min. Sections were then incubated with rabbit anti-luciferase antibody (70C-CR2020RAP; Fitzgerald), mouse anti-NeuN (MAB377; Millipore), rat anti-F4/80 (MCA497R; AbD Serotec), mouse anti-glial fibrillary acidic protein ([GFAP] G3893; Sigma-Aldrich), or mouse anti-LACV-G2 (18752; QED Bioscience) in PBS containing 3% normal donkey serum at 4°C overnight. For detection of luciferase, signal amplification with a TSA (tyramide signal amplification) Fluorescein System (PerkinElmer) was performed according to the manufacturer’s instructions using a biotin-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch). For cellular markers, appropriate DyLight488-, DyLight549-, Cy3-, or Cy2-conjugated secondary antibody (Jackson ImmunoResearch) was used. Slides were mounted in 4’,6-diamidino-2-phenylindole (DAPI)-containing IS Mounting Medium (Dianova). Digital images were taken with an ApoTome fluorescence microscope (Zeiss) using AxioVision software.

RESULTS

Identification of IFN-β-producing cells in LACV-infected brains. To visualize IFN-β-producing cells in the brain of LACV-infected heterozygous reporter mice, we stained tissue slices from animals exhibiting clinically apparent encephalitis with luciferase-specific antibodies. Because luciferase expression levels were expected to be low and because paraffin or cryo-embedding might affect epitope recognition, we decided to work exclusively with paraformaldehyde-fixed, free-floating slices prepared by vibratome sectioning. We observed that luciferase signals were very faint if standard histological staining techniques were applied. Consequently, signal amplification was routinely employed for better visualization of IFN-β-producing cells.

Luciferase-positive cells were typically observed in distinct clusters which were present in all parts of virus-infected brains. Double-staining experiments revealed that the luciferase-positive cell clusters exclusively mapped to brain regions in which virus-infected cells were highly abundant (Fig. 1). As expected from the fact that LACV encodes the IFN-α/β antagonist factor NSs, the number of luciferase-positive cells was at least 10-fold higher in brains of mice infected with the LACV-ΔNSs mutant (Fig. 1A to D) than in brains infected with LACV-wt (Fig. 1E to H). Detailed inspection revealed that although mostly found in close proximity to virus-infected cells, luciferase-positive cells were usually not positive for viral antigen (Fig. 1B, D, F, and H). It was shown previously that LACV predominantly infects neurons (16). Shape and distribution of luciferase-positive cells indicated that they might be mostly astrocytes. This was particularly obvious in the cerebellum, where luciferase-positive Bergmann glia cells were typically observed in immediate vicinity of virus-infected Purkinje cell somata (Fig. 1C and D).

Majority of IFN-β-producing cells in LACV-infected brains are astrocytes and microglia. To assess the extent to which the various cell types in LACV-infected brains might contribute to
IFN-β synthesis, we determined which fractions of luciferase-positive cells could unambiguously be classified as astrocytes, neurons, or microglia by double staining with antibodies that recognize cell-type-specific markers. This analysis was performed with both wild-type LACV and the NSs-deficient mutant virus to address the question of whether the IFN-antagonistic factor NSs might act predominantly in certain cell types. In agreement with previous results (4), we found that luciferase levels in brains of IFN-β-producing cells are found near LACV-infected cells in various brain regions. Brain sections from cortex (A, B, E, and F) and cerebellum (C, D, G, and H) of diseased IFN-β−/− mice infected with LACV-ΔNSs or LACV-wt were simultaneously stained for luciferase, viral antigen (LACV-G2), and cell nuclei (DAPI). Note that although in close proximity to infected cells, luciferase-positive cells did not usually stain for viral antigen. Scale bar, 50 μm.
mice infected with the ΔNs virus were about 6-fold enhanced although the mutant virus replicated substantially less well than wild-type virus, with about 30-fold reduced peak brain titers (Fig. 2). A large number of luciferase-positive cells in brains of mice infected with wild-type or NSs-deficient LACV expressed GFAP, indicating that they represent astrocytes (Fig. 3A and B). Another fraction of luciferase-positive cells expressed the macrophage/microglia marker protein F4/80 (Fig. 3C and D). A very small proportion of luciferase-expressing cells expressed the neuron marker NeuN (Fig. 3E). Interestingly, luciferase-positive neurons were detected at low frequency in brains of mice infected with LACV-ΔNs (Fig. 3E) but not LACV-wt (Fig. 3F).

To make this analysis more quantitative, we compiled the results from a detailed inspection of tissue slices from three or more severely diseased animals per virus strain. At least 300 luciferase-positive cells were evaluated individually for each double-staining experiment listed in Table 1. In brains of mice infected with the LACV-ΔNs mutant, the vast majority (89%) of luciferase-positive cells expressed GFAP, suggesting that they represent astrocytes. The F4/80 marker, which is present on microglia and infiltrating macrophages, was expressed by approximately 5% of the luciferase-positive cells in brains of mice infected with LACV-ΔNs. The number of luciferase-positive cells expressing NeuN was approximately 1%, suggesting that only very few neurons can synthesize large amounts of IFN-β. A strikingly different picture emerged when the staining data from wild-type LACV-infected mice were compiled. In this case, the frequency of luciferase-positive astrocytes was only 35%, whereas the frequency of luciferase-positive microglia/macrophages was 62% (Table 1). No luciferase-positive cells expressing NeuN were detected in brains which were infected with wild-type LACV.

LACV replicates predominantly in neurons. As the majority of IFN-producing cells were seemingly uninfected astrocytes and microglia, we carefully analyzed if LACV can establish productive infections in these cell types. To do this, we subjected brains infected with LACV-wt or LACV-ΔNs to simultaneous staining for viral antigen and specific markers for neurons, astrocytes, or microglia. In line with previous data (16), we found that LACV-wt and LACV-ΔNs replicated almost exclusively in neurons (Fig. 4A and B). We could not detect any virus-positive microglia (data not shown), and less than 1% of the virus-positive cells in the infected brains were astrocytes (Fig. 4C and D). Thus, astrocytes are sus-

<table>
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<tr>
<th>Virus</th>
<th>Astrocytes</th>
<th>Microglia/macrophages</th>
<th>Neurons</th>
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<tbody>
<tr>
<td>LACV-ΔNs</td>
<td>89 (536/603)</td>
<td>5 (29/561)</td>
<td>1 (4/424)</td>
</tr>
<tr>
<td>LACV-wt</td>
<td>35 (134/379)</td>
<td>62 (189/306)</td>
<td>0 (0/337)</td>
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aData in parentheses represent the number of cells positive for the corresponding marker/number of luciferase-positive cells analyzed.

Astrocytes are defined here as GFAP-positive cells.

The microglia/macrophage population is defined here as F4/80-positive cells.

Neurons are defined here as NeuN-positive cells.

![FIG 2](image2.png) LACV-ΔNs activates the IFN-β reporter gene more efficiently than LACV-wt although it replicates less well in the brain. Brains of infected IFN-β/ΔH-100 mice showing signs of neurological symptoms were collected and homogenized. Viral titers (A) and luciferase activity (B) in brain homogenates were determined. ***, P < 0.0005.

![FIG 3](image3.png) Astrocytes, microglia, and neurons of LACV-infected mouse brains express IFN-β reporter gene. Results of double staining of brain slices for luciferase (luc) and the astrocyte marker GFAP, microglia/macrophage marker F4/80, and neuron marker NeuN are shown. Luciferase-positive neurons were detectable only in LACV-ΔNs-infected (E) but not in LACV-infected brains (F). Cells were counterstained with DAPI. Single-channel and merged pictures of the same frames are shown. Scale bar, 10 μm.
ceptible to LACV although infection of such cells might mostly be nonproductive.

Luciferase production in virus-infected conditional reporter mice. To confirm the IFN-β expression patterns observed by immunohistochemistry, we created reporter mice in which luciferase is expressed exclusively in predetermined cell types. Such animals may be generated by breeding reporter mice which contain strategically positioned loxP sites with mice that selectively express Cre recombinase in defined cell types (25). For the current study we used Synapsin1-Cre mice to generate animals in which expression of luciferase is restricted to neurons (29). Further, we used Thy1-Cre mice to produce reporter mice which express luciferase in both neurons and astrocytes (11). Finally, we employed LysM-Cre mice to generate reporter mice in which luciferase expression is restricted to microglia and macrophages (9, 20).

To verify the predicted luciferase gene expression patterns in our conditional reporter mice, we performed double-staining experiments of brain sections from LACV-ΔNSs-infected LysM-Cre, Thy1-Cre, or Synapsin1-Cre reporter mice with antibodies recognizing luciferase and the corresponding markers F4/80, GFAP, and NeuN, respectively. As expected, if the reporter mice expressed the luciferase gene with desired cell type specificity, we found that luciferase-positive cells in brains of infected LysM-Cre mice expressed F4/80, an antigen of microglia and macrophages (Fig. 5A), but not the astrocyte marker GFAP (data not shown). Similarly, the luciferase-positive cells observed in brains of Thy1-Cre mice expressed GFAP (Fig. 5B) but not F4/80 (data not shown). Further, the few luciferase-positive cells that we detected in Synapsin1-Cre reporter mice expressed the neuron marker NeuN (Fig. 5C). Thus, the conditional reporter mice used here represent suitable tools for the assessment of the relative contributions of the various cell types to overall IFN synthesis in LACV-infected brains.

Since luciferase expression is driven by the virus-inducible IFN-β promoter in our mice, a direct comparison of reporter gene activity in individual animals will yield meaningful quantitative data only if virus replication rates in brains are similar. Although the different conditional reporter mice showed only slight differences in onset of symptoms or course of disease (Fig. 6), we tried to minimize errors resulting from such variation by restricting this comparison to animals having matching viral brain titers (Fig. 7A). Luciferase activity in individual animals will yield meaningful quantitative data only if virus replication rates in brains are similar. Although the different conditional reporter mice showed only slight differences in onset of symptoms or course of disease (Fig. 6), we tried to minimize errors resulting from such variation by restricting this comparison to animals having matching viral brain titers (Fig. 7A). Luciferase activity in brain extracts from Thy1-Cre mice infected with LACV-ΔNSs was only slightly reduced, suggesting that at least 71.4% of the luciferase signal originates from virus-mediated stimulation of astrocytes and neurons (Fig. 7B). Luciferase activity in extracts from brains of Synapsin1-Cre mice was 9.4% of the level in global mice, whereas luciferase activity in extracts of brains from LysM-Cre mice infected with LACV-ΔNSs was comparatively low. Compared to the level in global mice, the signal in LysM-Cre mice was only about 1.7%, on average (Fig. 7B).

Thus, astrocytes, neurons, and microglia together accounted for ~75% of luciferase activity of global reporter mice. The cellular origin of the missing ~25% of activity (Fig. 7B, white sector) remains unclear. It most likely indicates incomplete Cre-mediated recombination of the loxP-tagged target gene in our mice.

FIG 4 LACV replicates predominantly in neurons. Brains of mice infected with LACV-ΔNSs or LACV-wt were stained simultaneously for viral antigen (LACV-G2) and either the neuron marker NeuN or the astrocyte marker GFAP. Scale bar, 20 μm.

FIG 5 Conditional reporter mice express the luciferase reporter gene in the predicted cell types. (A) LysM-Cre+/− IFN-β−/loxP−loxP-lucmice were infected with LACV-ΔNSs, and brains of diseased mice were simultaneously stained for luciferase (green) and the microglia/macrophage marker F4/80 (red). (B) Thy1-Cre+/− IFN-β−/loxP−loxP-lucmice were infected with LACV-ΔNSs, and brains of diseased mice were simultaneously stained for luciferase (green) and the astrocyte marker GFAP (red). (C) Synapsin1-Cre+/− IFN-β−/loxP−loxP-lucmice were infected with LACV-ΔNSs, and brain sections of diseased mice were simultaneously stained for luciferase (green) and the neuron marker NeuN (red). Scale bar, 50 μm.
Diseased animals were killed, and brain titers were determined. No significant differences were observed between the different mouse strains. p.i., postinfection.

A different picture emerged when LACV-wt was used for the infection study. First, as discussed above, luciferase activity in brain extracts of global mice infected with LACV-wt was about 6-fold lower than that in global mice infected with LACV-ΔNSs and, on average, reached values of only $9 \times 10^4$ RLU per µl of brain extract (Fig. 7C). Second, compared to infection with LACV-ΔNSs, luciferase activity in brains of wild-type LACV-infected LysM-Cre mice was significantly increased. On average, it accounted for 41.4% of the signal observed in global mice infected with LACV-wt (B) were monitored for neurological symptoms. Diseased animals

**DISCUSSION**

Employing transgenic mice that express a luciferase reporter gene under the control of the IFN-β promoter, we established a staining protocol that can identify single IFN-β-producing cells in virus-infected brain tissue. To quantify the relative contribution of specific cell types to overall IFN-β synthesis in the brain, we took advantage of the Cre-lox system and generated reporter mice that express the luciferase transgene either in astrocytes and neurons, neurons only, or microglia and macrophages. When using La Crosse virus as a model for viral encephalitis, we found that astrocytes and microglia were the main producers of IFN-β in the infected brain, whereas the contribution of infected neurons was relatively small. Interestingly, when a mutant virus that cannot synthesize the IFN-antagonistic factor NSs was used for challenge, the balance was shifted, and astrocytes became the dominant IFN-β producers. Our work demonstrates that, in addition to infected cells, seemingly uninfected cells also contribute massively to IFN synthesis in the central nervous system. Our work further demonstrates that virus-encoded antagonistic factors can affect IFN production by acting selectively on distinct cell types.

Previous attempts to characterize the production of IFN during viral encephalitis by in situ hybridization technology had already indicated that various cell types, including neurons, contain detectable levels of type I IFN (10). The authors had used specific antisera to visualize IFN-α- and IFN-β-producing cells in virus-infected brains. However, signals were weak, and quantitative analyses were dependent on visual interpretation of histological data. This difficulty presumably originates from the fact that type I IFN is quickly secreted from producer cells and fails to accumulate to high intracellular levels, thus complicating detection by immunostaining. The reporter mice that we used in this study overcome this problem as the luciferase molecule lacks export signals and thus accumulates in the cytoplasm of the producer cells. Nevertheless, staining of tissue for luciferase in virus-infected reporter mice was challenging as standard histological protocols failed to produce detectable signals. We could eventually overcome these problems by using mild conditions for sectioning and staining and by including a signal amplification step.

Our immunostaining approach is well supported by results from experiments with conditional reporter mice that express the

![FIG 6](http://jvi.asm.org)  
**FIG 6** Conditional reporter mice show no significant differences in susceptibility to LACV. Global reporter mice ($\Delta\beta$-luc) and conditional Thy1-Cre $^{+/\text{luc}}$ (thy), LysM-Cre $^{+/\text{luc}}$ (lys), and Synapsin1-Cre $^{+/\text{luc}}$ (syn) reporter mice infected with LACV-ΔNSs (A) or LACV-wt (B) were monitored for neurological symptoms. Diseased animals

![FIG 7](http://jvi.asm.org)  
**FIG 7** IFN-β synthesis by astrocytes and neurons but not microglia is repressed by LACV-encoded IFN-antagonistic factor NSs. Reporter mice in which the luciferase gene is expressed exclusively in astrocytes and neurons (thy), neurons only (syn), or microglia/macrophages (lysM) were infected with LACV-ΔNSs (A and B) or LACV-wt (C and D). Global IFN-β $^{+/\text{luc}}$ reporter mice (Δβ-luc) served as the reference. Brains of diseased animals with very similar virus loads were selected for further analysis. Mean luciferase activities (with standard deviations) in brain samples from the various mouse strains infected with either LACV-ΔNSs or LACV-wt are shown. The average contributions of different cell types to luciferase activity in brains of mice infected with LACV-ΔNSs or LACV-wt are shown as pie charts; the activity of global IFN-β $^{+/\text{luc}}$ reporter mice was set to 100%.
IFN-β promoter-regulated luciferase gene exclusively in defined cell types. We stringently evaluated the specificity of Cre-mediated recombination in these mice and excluded the possibility that reporter gene expression by undesired cell types clouded the picture. As predicted, LysM promoter-driven expression of Cre recombinase seemed to rearrange the IFN-β locus exclusively in cells that were positive for the microglia/macrophage marker F4/80. Similarly, Thy1 promoter-driven expression of Cre recombinase seemed to activate the loxP-tagged reporter gene only in astrocytes and presumably neurons. Synapsin1-driven expression of Cre recombinase resulted in selective rearrangement of the loxP-tagged IFN-β locus in neurons.

The results of our immunostaining experiments and those of our Cre-LoxP approach showed a very good correlation in the case of astrocytes and microglia but no clear correlation in the case of neurons. This discrepancy can easily be explained by the high detection threshold of the immunostaining technique. Most likely, the IFN-β promoter-driven luciferase gene was activated to a low extent in LACV-infected neurons, but luciferase levels in individual cells remained too low for detection by antibody staining. Since a large percentage of neurons got productively infected with LACV in our mice, it is likely that the small contribution of individual neurons did add up considerably. These considerations may explain why experiments with our Synapsin1-Cre reporter mice indicated a more substantial contribution of neurons to luciferase activity in LACV-infected brains than the histological analysis.

A remarkable finding of our study was that astrocytes contribute substantially to IFN synthesis in the virus-infected brain. This result was not expected, as we (Fig. 4A and B) along with others (4, 10, 16) showed that productive replication of LACV is largely restricted to neurons. Since the contribution of astrocytes was much more pronounced if mutant LACV that cannot synthesize the IFN-antagonistic factor NSs was used for infection, we assume that a high number of brain astrocytes actually got infected by LACV although such infections usually remained nonproductive. In line with this hypothesis, our immunofluorescence analysis (Fig. 4C and D) demonstrated that LACV can, indeed, establish productive infection of astrocytes although this may occur only rarely.

We noted that the luciferase signal in microglia was not negatively regulated by NSs. In fact, IFN production by microglia was even higher if wild-type virus was administered instead of LACV-ΔNSs. Therefore, IFN production by microglia correlated directly with the virus load in the brain, irrespective of whether the challenge virus coded for NSs or not. Since the two viruses used here differ slightly with regard to the kinetics of disease induction, it remains possible that the kinetics of virus-induced cytokine gene activation in astrocytes and microglia also differed somewhat and thus contributed to the difference which we observed between LACV-wt and LACV-ΔNSs. To minimize variations of this sort, we restricted our analysis to animals that showed similar signs of neurological disease. Taking all these caveats into account, the most coherent explanation of the various observations reported here seems to be that, in contrast to astrocytes, IFN production by microglia is not triggered by intracellular virus sensors such as RIG-I but rather by alternative sensors such as TLR which can detect viral components in the extracellular space. Interestingly, recent LACV infection experiments with TLR7-deficient mice failed to reveal a prominent role of this virus sensor in our system (unpublished data), suggesting that several virus sensors might get activated simultaneously in the LACV-infected mouse brain.

Taking our results together, our study demonstrates that three cell types are responsible for the bulk of IFN synthesis during acute encephalitis after LACV infection, namely, productively infected neurons, abortively infected astrocytes, and uninfected microglia. Interestingly, if IFN-β is not inhibited by the viral IFN-antagonistic factor NSs, the amount of IFN-β expressed by neurons exceeds the quantity of IFN-β produced by microglia and macrophages. In line with these findings, IFN synthesis by astrocytes was much more pronounced if mutant LACV that cannot synthesize the IFN-antagonistic factor NSs, whereas microglia proved to be a more robust source of IFN synthesis.

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