

Neurons produce type I interferon during viral encephalitis

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Type I interferons, also referred to as IFN- α/β , form the first line of defense against viral infections. Major IFN- α/β producers in the periphery are the plasmacytoid dendritic cells (pDCs). Constitutive expression of the IFN regulatory factor (IRF)-7 enables pDCs to rapidly synthesize large amounts of IFN- α/β after viral infection. In the central nervous system (CNS), pDCs are considered to be absent from the parenchyma, and little is known about the cells producing IFN- α/β . The study presented here aimed to identify the cells producing IFN- α/β in the CNS *in vivo* after infection by neurotropic viruses such as Theiler's virus and La Crosse virus. No cells with high constitutive expression of IRF-7 were detected in the CNS of uninfected mice, suggesting the absence of cells equivalent to pDCs. Upon viral infection, IFN- β and some subtypes of IFN- α , but not IFN- ϵ or IFN- κ , were transcriptionally up-regulated. IFN- α/β was predominantly produced by scattered parenchymal cells and much less by cells of inflammatory foci. Interestingly, in addition to some macrophages and ependymal cells, neurons turned out to be important producers of both IFN- α and IFN- β . However, only 3% of the infected neurons produced IFN- α/β , suggesting that some restriction to IFN- α/β production existed in these cells. All CNS cell types analyzed, including neurons, were able to respond to type I IFN by producing Mx or IRF-7. Our data show that, *in vivo*, neurons take an active part to the antiviral defense by being both IFN- α/β producers and responders.

central nervous system | viruses | innate immune response | cytokines | interferon regulatory factor 7

Type I interferons, further considered as “interferons” (IFNs), were discovered for their ability to protect cells against viral infection. These inducible cytokines mediate their activity by binding to the common type I IFN receptor. Signaling from this receptor activates a multitude of IFN-stimulated genes with antipathogenic, antiproliferative, and immunomodulatory activities. The type I IFN family is multigenic. Human and mouse genomes carry one IFN- β , one IFN- κ , one IFN- ϵ/τ , and multiple IFN- α , IFN- ω (human), and *limitin* (mouse) genes.

Two members of the IFN regulatory factor (IRF) family, IRF-3 and IRF-7, were shown to be the crucial players in transcriptional induction of IFN genes (1). IRF-3, which is expressed constitutively, is activated by virus-induced kinases (2, 3) and participates to the transcriptional induction of IFN- β and IFN- $\alpha 4$ genes. The immediate-early IFNs produced by these genes signal through the IFN receptor in an autocrine and/or paracrine fashion and up-regulate the transcription of many IFN-stimulated genes, notably of IRF-7. Upon viral infection of cells primed by IFN, IRF-7 and IRF-3, which are activated by the same kinases (2, 3), cooperate to induce the transcription of the other IFN- α genes (namely late IFNs) (4).

In vitro, virtually any nucleated cell type can synthesize both IFN- α and IFN- β . *In vivo*, however, for both humans (5, 6) and mice (7–9), the major IFN-producing cells were identified as being the plasmacytoid dendritic cells (pDCs). Large amounts of

IFN are produced by human and mouse pDCs in response to a wide range of viruses, parasites, and bacteria.

Many previous investigations focused on IFN-producing DCs in the periphery. In the central nervous system (CNS), however, DCs are reportedly limited to perivascular cells of peripheral origin (10), and pDCs were reported to be absent from the brain (11). Few data are available on cells responsible for IFN production in the CNS. *In vitro* experiments in primary cell cultures agree that astrocytes and microglia can produce type I IFNs but are conflicted regarding possible IFN production by neurons (12, 13). A recent study showed that postmitotic neurons differentiated *in vitro* from the human NT2-N cell line were able to produce IFN- β in response to rabies virus infection (14). *In vivo*, very few studies tried to identify the IFN-producing cells in the CNS (15–18), and no general conclusion was reached.

In this work, we used two neurotropic viruses, Theiler's virus, a murine picornavirus, and La Crosse virus, a bunyavirus, to investigate type I IFN production and response in the CNS *in vivo*. Theiler's virus (or Theiler's murine encephalomyelitis virus, TMEV) strains are divided into two subgroups according to the disease they produce. The neurovirulent strain (GDVII) causes an acute lethal encephalomyelitis, whereas the persistent strain (DA) causes a mild transient encephalitis that resolves and is followed by viral persistence in the spinal cord white matter (19). La Crosse virus (LACV) massively infects neurons and causes fulminant encephalitis. A mutant of LACV lacking a functional *NSs* gene (LACVdelNSs) was used in this study (20). Because the *NSs* gene product is an IFN antagonist (21), LACVdelNSs induces high amounts of IFN in infected cells. We used *in situ* hybridization (ISH) and double immunostaining to identify IFN-producing cells in the CNS. Our data show that IFN is largely produced by infected resident cells of the CNS. Interestingly, neurons accounted for a substantial proportion of IFN-producing cells. Neurons also responded to IFN by expressing Mx and IRF-7.

Results

No Detection by ISH of Cells with Constitutive High Expression of IRF-7 in the CNS. In human and murine pDCs, IRF-7 is constitutively expressed to ensure a strong and rapid IFN production after detection of virus infection (22, 23). The CNS is reportedly devoid of pDCs (11). Therefore, we asked whether a specific cell type of the CNS constitutively produces IRF-7 and may functionally replace pDCs in this organ.

Conflict of interest statement: No conflicts declared.

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Abbreviations: IRF, IFN regulatory factor; ISH, *in situ* hybridization; LACV, La Crosse virus; LACVdelNSs, LACV lacking the *NSs* gene; pDC, plasmacytoid dendritic cells; TMEV, Theiler's virus; DA, persistent strain of TMEV; GDVII, neurovirulent strain of TMEV.

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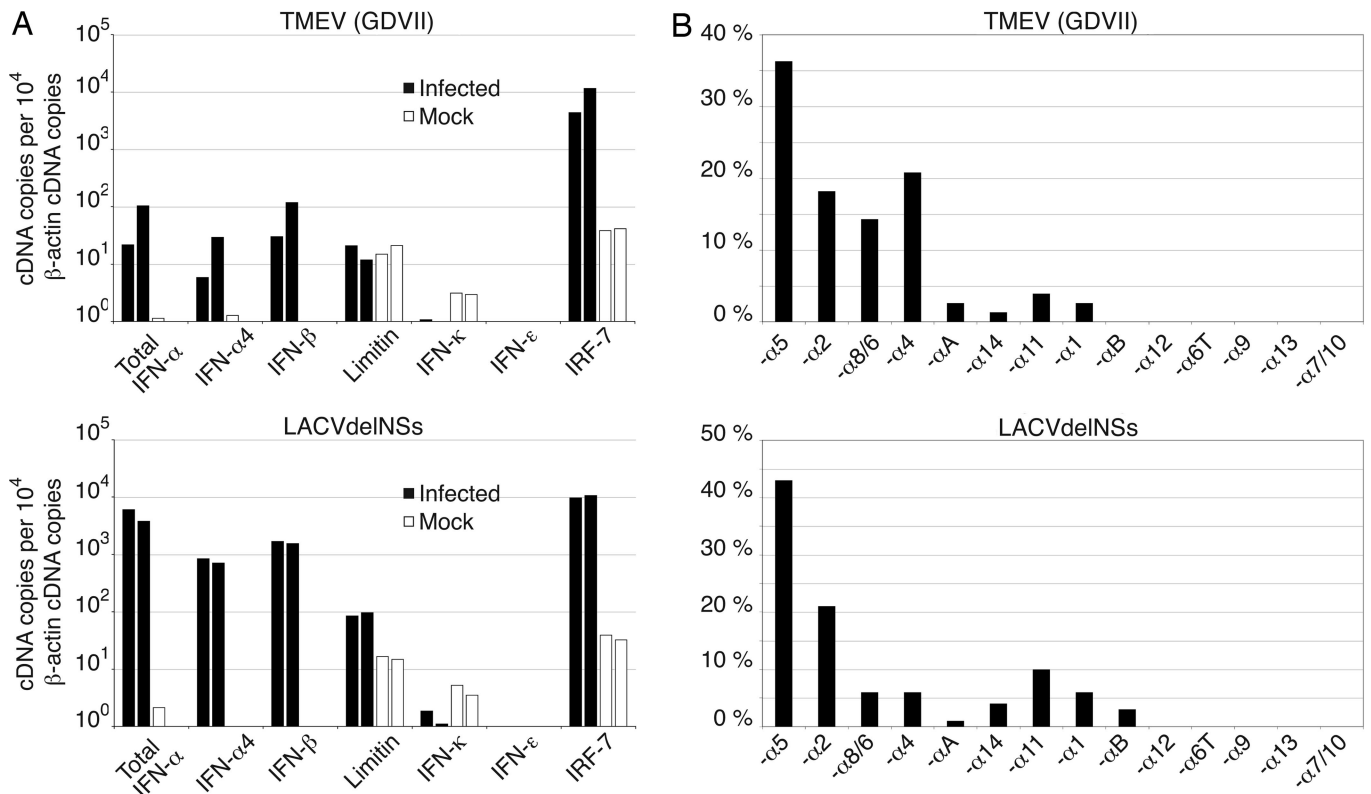


Fig. 1. Type I IFNs and IRF-7 expression in brains of mice infected with TMEV(GDVII) or LACVdelNSs. Total RNA was extracted from brains of uninfected animals (FVB mice), mice infected for 5 days with TMEV(GDVII) (FVB mice), or mice infected for 7 days with LACVdelNSs (B6.A2G-Mx1 mice). (A) Quantitative RT-PCR was performed to quantify total IFN-α, IFN-α4, IFN-β, limitin, IFN-κ, IFN-ε, and IRF-7 transcripts. The results are expressed as cDNA copy numbers per 10⁴ copies of β-actin cDNA. (B) Expression profile of IFN-α subtypes. IFN-α was amplified by RT-PCR, by using a mix of primers amplifying all IFN-α subtypes. PCR products from two independent experiments were subcloned and sequenced to identify the IFN-α subtypes expressed. For TMEV(GDVII) and LACVdelNSs, 77 and 100 individual clones, respectively, were analyzed.

In agreement with the recent data of Ousman *et al.* (24), real-time RT-PCR showed low-level expression of IRF-7 in uninfected mice (4×10^{-3} cDNA copies per copy of β-actin cDNA) (Fig. 1). Examination of >60 sagittal or coronal sections from uninfected mice by ISH failed to show a specific cell population with detectable IRF-7 expression (Fig. 2). Thus, the low expression detected by RT-PCR likely results from a low basal expression by many cells and not by the existence of a cell population with high constitutive IRF-7 expression.

In contrast, IRF-7 expression was strongly up-regulated (100- to 200-fold) in mice infected with strain GDVII of TMEV or with LACVdelNSs. ISH showed a clear up-regulation of IRF-7 expression in virtually all cells including neurons (Fig. 2). IRF-7 expression was, however, slightly more prominent in some inflammatory cells (Fig. 2).

Expression Pattern of Type I IFN Subtypes in the CNS. We identified the type I IFN subtypes that are transcribed in the CNS in response to infection with either the GDVII strain of TMEV or LACVdelNSs. Real-time RT-PCR analysis showed that transcription of both *IFN-α* and *IFN-β* genes was induced after infection with TMEV(GDVII) and even more after infection with LACVdelNSs (Fig. 1A). In contrast, levels of IFN-ε and IFN-κ mRNA were unaffected by viral infection. *Limitin* gene expression was not affected by TMEV infection but up-regulated some 5-fold in response to LACVdelNSs infection.

Because the mouse genome potentially encodes 14 slightly different IFN-α subtypes, we used an RT-PCR cloning-sequencing strategy to analyze whether all IFN-α subtypes were equally induced. Interestingly, only some *IFN-α* genes were

strongly up-regulated after TMEV(GDVII) and LACVdelNSs infection (Fig. 1B). In TMEV(GDVII)-infected brains, IFN-α5, IFN-α2, IFN-α8/6, and IFN-α4 transcripts represented >80% of all IFN-α transcripts. In LACVdelNSs-infected brains, transcripts from *IFN-α2* and *IFN-α5* genes were most prominent (Fig. 1B). IFN-α4, IFN-α2, and IFN-α5 were also reported to be the most abundantly transcribed IFN-α subtypes in L929 cells treated with polyinosinic-polycytidylic acid (poly IC) (25), suggesting little tissue-specificity for the IFN-α subtype expression.

Spatial Correlation Between IFN-α- and IFN-β-Producing Cells and Viral Infection. To localize the IFN-producing cells of the brain, we analyzed *IFN-α* and *IFN-β* gene expression by ISH. IFN detection was performed on sections from mice infected with TMEV(GDVII), TMEV(DA), or with LACVdelNSs. In uninfected mice, neither IFN-α nor IFN-β transcripts were detected. However, upon TMEV or LACVdelNSs infection, both IFN-α and IFN-β transcripts were readily detected (Fig. 3A). Hybridization of adjacent sections from TMEV(GDVII)-infected brains with probes for TMEV, IFN-α, and IFN-β revealed a spatial correlation between viral RNA, IFN-α, and IFN-β expression (Fig. 3B).

Infection of mice with the DA strain of TMEV induces an intense inflammatory response in the brain. Nevertheless, the analysis of such brains revealed that only a few cells present in the inflammatory foci produced IFN-α or IFN-β. For all viruses tested, the majority of IFN-positive cells were scattered in the parenchyma and likely corresponded to resident cells of the CNS (Fig. 3C).

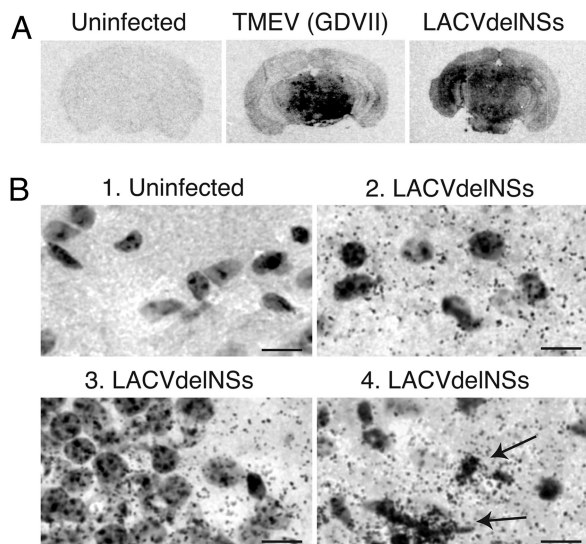


Fig. 2. IRF-7 mRNA detection in infected and uninfected mouse brains. ISH with an IRF-7-specific probe was performed on mouse brain sections from uninfected and TMEV(GDVII)-infected FVB mice or from LACVdelNSs-infected B6.A2G-Mx1 mice. (A) Macroscopic analysis showing absence of IRF-7-expressing areas in the CNS of uninfected mice and strong up-regulation of IRF-7 transcription in infected mice. (B) Microscopic analysis: IRF-7 mRNA was not detected in uninfected mice (1, thalamic area). IRF-7 was clearly detectable in most cells of LACVdelNSs-infected areas: thalamus (2) and hippocampal neurons (3). IRF-7 up-regulation was slightly more prominent in some inflammatory cells (arrows) (4). (Scale bar: 10 μ m.)

Neurons, Macrophages, and Epithelial Cells Can Produce IFN- α . To further identify the IFN-producing cells in the CNS, we performed double immunohistofluorescence by using a polyclonal antibody directed against IFN- α together with various antibodies for markers of specific cell types. First, we tested the sensitivity of the anti-IFN- α antibody by performing immunolabeling on COS-7 cells transfected with plasmids expressing the 14 different murine IFN- α genes. Using this assay, we found that the anti-IFN- α antibody readily recognized all of the 14 murine IFN- α

subtypes (data not shown). This recognition was specific to IFN- α members because no signal was observed for IFN- β - or limitin-producing cells or cells transfected with the empty vector. Second, we tested the specificity of the antibody by blocking experiments. The IFN- α labeling completely disappeared when the antibody was preincubated with supernatants of COS-7 cells expressing IFN- α , but not with supernatants containing identical amounts of IFN- β , or with supernatant from empty vector-transfected cells (data not shown). Thus, the anti-IFN- α antibody used for the further experiments seemed to specifically recognize IFN- α .

In infected, as well as in uninfected, mouse brains, a strong IFN- α signal was detected by immunohistofluorescence, but not by ISH, on endothelial cells both in the choroid plexus and the parenchyma (data not shown), presumably because of cross-reactivity of the IFN- α antibody to an unknown antigen of endothelial cells.

Upon infection with TMEV (DA or GDVII strain), IFN- α staining appeared in infected areas. Combined analysis of IFN- α and TMEV capsid protein VP1 showed that many of the IFN- α producers were virus-infected cells (Fig. 4 A and B).

At the time point used (5 days postinfection), both the DA and the GDVII strains of TMEV are known to infect predominantly neurons (19). For these viruses, double immunostaining performed with anti-NeuN (a neuron-specific marker), and anti-IFN- α antibodies suggested that neurons were important IFN-producing cells (Fig. 4 C and D). Besides neurons, macrophages (CD11b/Mac-1, MOMA-2, or F4/80 positive cells) (Fig. 4E) were found to produce IFN- α after TMEV infection. Epithelial cells bordering the ventricles were also identified morphologically by immunohistofluorescence, as well as by ISH, as producers of IFN (Fig. 4F). Production of IFN- α by neurons, macrophages, and ependymal cells was also observed in BALB.A2G-Mx1 and SJL mice (data not shown), suggesting that the nature of the IFN-producing cells did not depend on the genetic background of the mice.

Neurons Can Produce IFN- α and IFN- β . Neurons are reportedly immune-privileged cells and were not expected to produce IFN. Thus, to further confirm the production of IFN by neurons, we performed ISH to detect either IFN- α or IFN- β , combined with

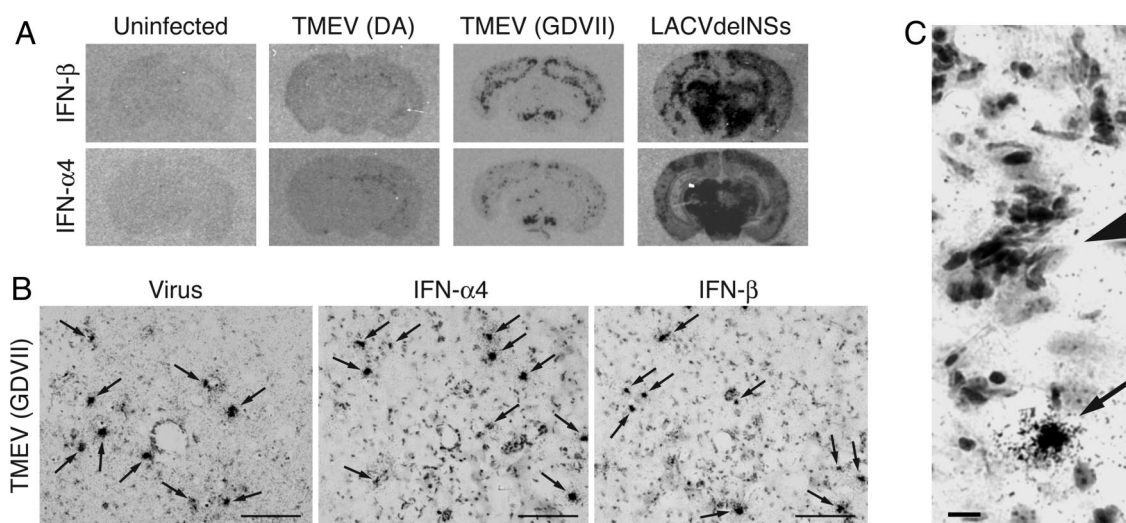


Fig. 3. Localization of IFN- α / β -expressing cells in the brain of mice infected by neurotropic viruses. ISH with IFN- α 4 or IFN- β probes was performed on brain sections from uninfected, TMEV(DA)-infected, or TMEV(GDVII)-infected FVB mice. B6.A2G-Mx1 mice were infected with LACVdelNSs. (A) Macroscopic analysis. (B) Microscopic analysis of TMEV(GDVII)-infected mice showing, on adjacent sections, colocalization of areas with viral RNA, IFN- α , and IFN- β transcripts. (Scale bar: 100 μ m.) (C) ISH with a probe for IFN- β and toluidine blue coloration, in an inflamed brain area. (Scale bar: 10 μ m.) The cell positive for IFN- β (arrow) is out of the inflammatory focus (arrowhead).

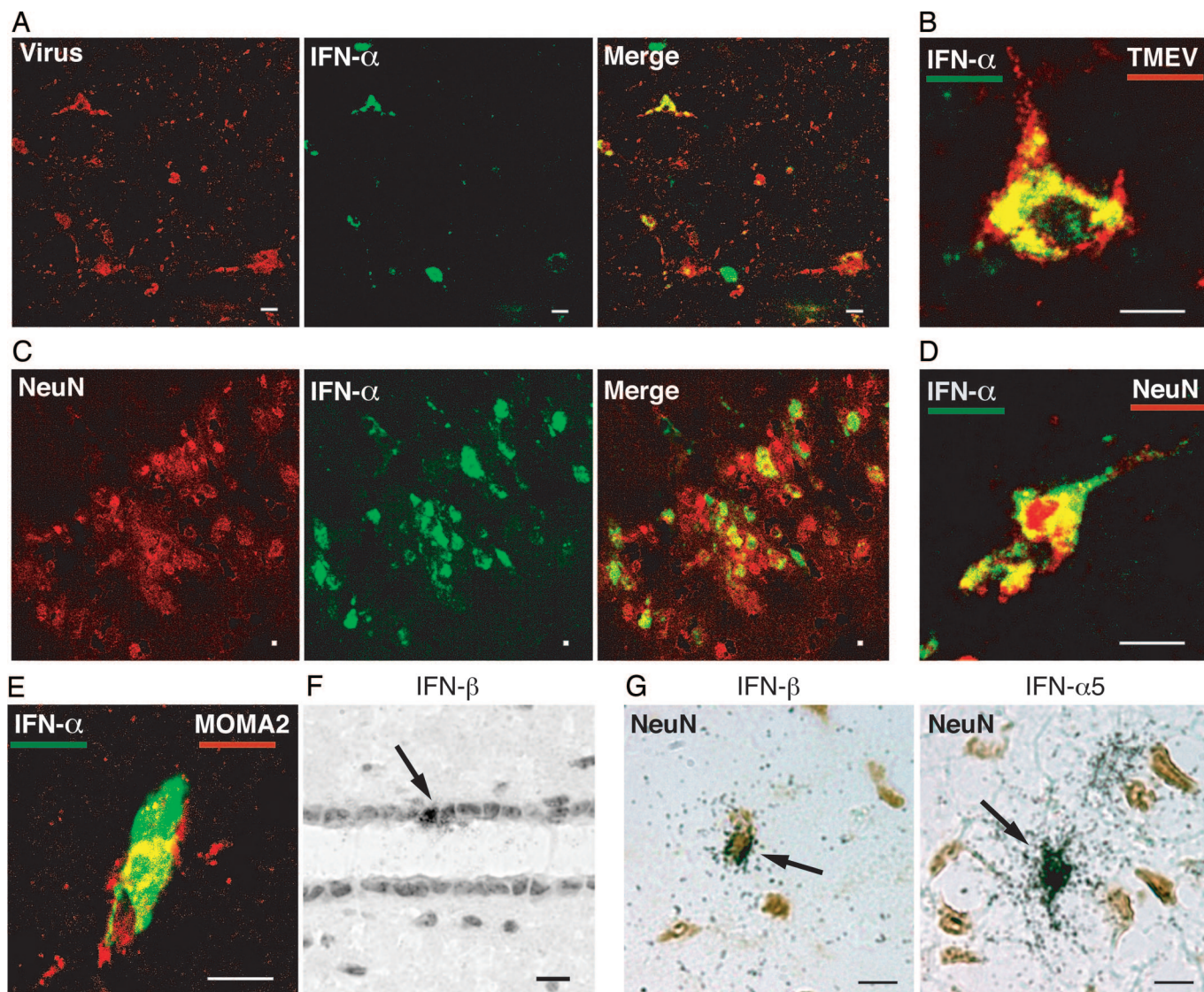


Fig. 4. Identification of IFN- α -producing cells in brain sections of infected mice. (A–E) Confocal microscopy. (Scale bar: 10 μ m.) (A, B, D, and E) TMEV(GDVII)-infected FVB mice. (A) Colocalization of viral TMEV antigen staining and IFN- α staining. (B) Higher magnification of double TMEV (red)/IFN- α (green) staining. (C) TMEV(GDVII)-infected SJL mice. Colocalization of a neuronal marker (NeuN) and IFN- α staining in the region of the hippocampus. (D) High magnification of double NeuN (red)/IFN- α (green) staining. (E) Macrophages, detected by a MOMA2 staining (red), can also produce IFN- α (green). (F) ISH with IFN- β probes were performed on brain sections from LACVdelNSs-infected mice. Epithelial cell positive for IFN- β (arrow). (G) Immunohistochemistry of neurons (NeuN, in brown) in combination with ISH with IFN- α 5 or IFN- β probes. Brain sections are from B6.A2G-Mx1 mice infected with LACVdelNSs.

immunohistochemistry to detect either neurons or LACVdelNSs-infected cells (which are nearly 100% neurons). In the case of both TMEV(GDVII) and LACVdelNSs infections, neurons turned out to be producers of IFN- α as well as IFN- β (Fig. 4G).

LACVdelNSs infection was prominent in many areas of the brain (and particularly extensive in the brainstem, thalamus, and hypothalamus), allowing some countings of double positive cells (Tables 1–3, which are published as supporting information on the PNAS web site). IFN- α and IFN- β positive cells were scattered in all infected areas. Among the cells that were positive by ISH for IFN- α or for IFN- β , 16% were NeuN-positive cells, 27–34% were NeuN-negative, and 50–57% could not conclusively be identified because of technical limitations. Very similar proportions of IFN-positive cells were positive for LACV (22–23%) or for NeuN (16%), suggesting that the neurons producing IFN were infected. Interestingly, only a small fraction (2.5–3.2%) of the infected cells (i.e., the neurons) were IFN-positive by ISH. This proportion appeared to be even lower for infected neurons

of the hippocampus where very few IFN-positive neurons could be detected.

Taken together, this analysis of IFN synthesis in the brain (i) confirms the production of both IFN- α and IFN- β by neurons, (ii) shows that IFN can be produced by infected neurons as well as by noninfected (or cells with undetectable infection) nonneuronal cells, and (iii) demonstrates that only a small part of the infected cells produced significant levels of IFN.

All CNS Cell Types Can Respond to IFNs. We further analyzed the cell types in the CNS that are able to respond to type I IFNs. To this aim, we used expression of Mx1 as a marker for the IFN response. *Mx1* is an antiviral IFN-stimulated gene specifically induced by type I IFNs. It is not expressed under physiological conditions (26). BALB.A2G-Mx1 mice were infected with the GDVII strain of TMEV. Double immunohistofluorescence was performed on brain sections with antibodies directed against Mx (27), IFN- α , viral antigen, or specific cell type markers. In

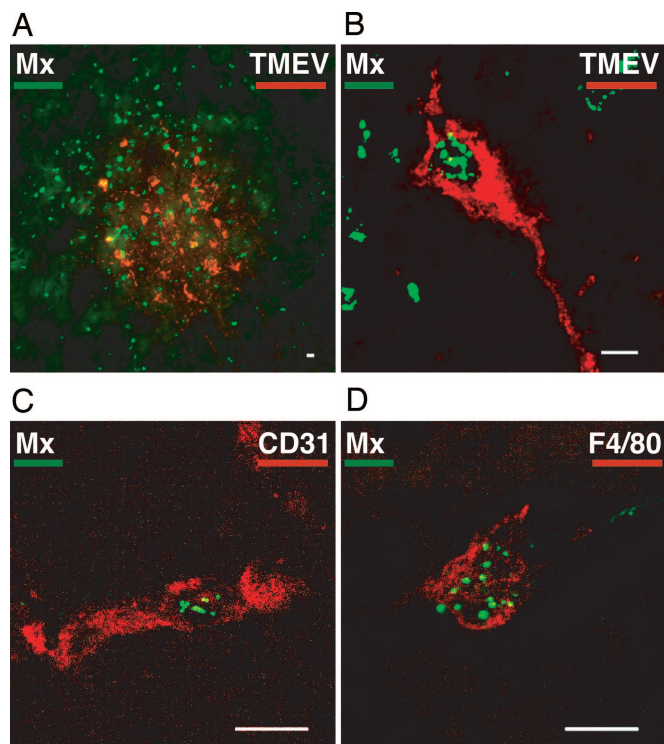


Fig. 5. Identification of IFN- α -responding cells in brain sections of infected mice. (A–D) Expression of Mx1 in TMEV(GDVII)-infected BALB. A2G-Mx1 mice (confocal microscopy). Mx1 staining appears in green as a nuclear dotted pattern. (Scale bars: 10 μ m.) (A and B) Viral TMEV antigen (red). (C) Endothelial cell detected by a CD31 staining (red). (D) Macrophage detected by a F4/80 staining (red).

uninfected mice, very few Mx-positive cells were detected. After infection with TMEV, areas of Mx-positive cells were spatially associated with virus-infected cell foci (Fig. 5 *A* and *B*) and with areas of IFN production (data not shown). However, as is the case for IRF-7, Mx-positive cells were much more abundant than both virus-infected cells and IFN-producing cells. All cell types analyzed (neurons, astrocytes, oligodendrocytes, macrophages, ventricular epithelial cells, vascular endothelial cells, cuboidal epithelial, and endothelial cells of the choroid plexus) responded to the presence of IFN by producing Mx (Fig. 5 *C* and *D* and data not shown).

Discussion

In peripheral organs, pDCs have been shown to be the major type I IFN producers. The rapid response of these cells was ascribed to their capacity to express IRF-7 in a constitutive fashion, in contrast to other cells that only produce IRF-7 when they are primed with type I IFNs. In agreement with the recent report of Ousman *et al.* (24), we detected a weak constitutive IRF-7 mRNA level in the CNS of uninfected mice. However, no single cell type constitutively expressed IRF-7 mRNA in sufficient amounts to be detected by ISH, suggesting the absence of professional IFN-producing cells.

After viral infection, virtually all cells present in infected areas, including neurons, expressed detectable levels of IRF-7 mRNA. Accordingly, neurons appeared to be important IFN-producing cells *in vivo*, after infection with two neurotropic viruses belonging to different viral families (bunyaviruses are enveloped, negative-stranded RNA viruses, and picornaviruses are nonenveloped, positive-stranded RNA viruses). Neurons are of strategic importance for the organism. Owing to their postmitotic nature and to their limited regenerative capacity, neu-

rons were found to be somehow protected from immunity. For instance, MHC class I expression on neurons was shown to be restricted to cells that lost electrical activity (28). A similar restriction might account for the fact that only 2.5–3% of the neurons infected by LACVdeINs produced IFN.

Our data show that, *in vivo*, neurons can produce both IFN- α and IFN- β . It is not known, however, whether infected neurons alone can initiate the production of IFN- β and of IFN- α or whether they require priming by IFN to express sensors and signal transduction molecules that enable them to produce IFN. Prehaud *et al.* (14) showed that neurons derived *in vitro* from NT2-N cells produced IFN- β mRNA as soon as 1 h after infection, suggesting that neurons might be equipped to initiate IFN production. Further work is required to test whether this ability to initiate IFN production applies to the *in vivo* situation. In our analysis (5–7 days after infection), IRF-7 transcription was clearly up-regulated in neurons (Fig. 2), in agreement with the fact that they produced both IFN- α and IFN- β .

Our results also show that all cell types of the CNS can respond to IFN by expressing the Mx protein. This finding is in agreement with previous studies showing up-regulation of *MHC class I* genes or of the *Mx1* gene in many cell types in response to IFN (29, 30).

In summary, our data show that, *in vivo*, neurons take an active part to the antiviral defense by being both IFN producers and responders.

Materials and Methods

Mice. Three- to four-week-old female SJL/J and FVB/N mice were purchased from Charles River Laboratories or from the animal facility of the University of Louvain (Brussels). BALB.A2G-Mx1 and B6.A2G-Mx1 mice carrying functional *Mx1* alleles (31) were used for the TMEV and LACV experiments, respectively. These animals were from the breeding colony maintained in Freiburg, Germany. Handling of mice and experimental procedures were conducted in accordance with national and institutional guidelines for animal care and use.

Viruses and Infections. TMEV's DA (DA1 molecular clone), GDVII strains, and LACVdelINs were produced as described (20, 32).

TMEV infections were done by intracranial injection of 40 μ l of serum-free medium containing 10³ pfu of TMEV(GDVII) or 10⁶ pfu of TMEV(DA). Control mice were injected with 40 μ l of serum-free culture medium. B6.A2G-Mx1 mice were infected by i.p. injection of 10⁴ pfu of LACVdelNsS. Mice were anesthetized before being euthanized for organ harvest at day 5 after TMEV infection and at day 5–7 after LACV infection.

RNA Extraction and Real-Time Quantitative RT-PCR for Type I IFN mRNA. For real-time RT-PCR detection of cytokine mRNA and analysis of IFN- α subtypes expression, RNA was isolated from mouse brain tissue as described (32). Quantitative analysis of PCR amplification was assessed by incorporation of SYBR green (Molecular Probes) into dsDNA (core kit, Eurogentec, Belgium), and performed with the Icyler or the MyIQ apparatus (Bio-Rad). Standards consisted of 10-fold dilutions of known concentrations of murine genomic DNA or of plasmid pTM796. The latter plasmid is a pCR4-Topo (Invitrogen) derivative carrying the 351-nt IRF-7 PCR fragment, amplified with the IRF-7 primers. Primer sequences for IRF-7, IFN- β , and IFN- κ were as described (25, 33, 34). Primers sequences and PCR conditions used are presented in Table 4, which is published as supporting information on the PNAS web site.

IFN- α Subtypes Expression Analysis. Total IFN- α transcripts of mouse brains were amplified by RT-PCR by using a mixture of primers for *IFN- α* sequences (primers IFN- α total). To deter-

mine the relative proportions of the different IFN- α subtypes expressed, the amplified PCR fragments were cloned, and a series of individual clones were sequenced.

In Situ Hybridization Studies. For the detection of IFN- α mRNA, we used probes for IFN- α 4 and IFN- α 5, which are two of the most abundantly produced IFN- α subtypes. Plasmids encoding IFN- α 4, IFN- α 5, and IFN- β were described (25, 35). Plasmid pTM469, used to synthesize the TMEV probe, contained a 2-kb fragment of the viral genome (nucleotides 1,733–3,883 of the DA1 sequence). The IRF-7 probe was synthesized from plasmid pTM796. Mice were perfused with 4% paraformaldehyde in PBS. Brain samples preparation and ISH were performed as described by Tissir *et al.* (36). Sections were cut at 8 or 12 μ m. Control hybridizations performed with positive-sense probes instead of antisense probes failed to yield any signal.

For ISH in combination with immunohistochemistry of neurons, sections were treated for the immunohistochemistry immediately after the last washes of the hybridization. Sections were incubated with the primary antibody directed against neuron-specific nuclear protein (NeuN, MAB377, Chemicon) or the nucleoprotein (N) of LACV, for 2–12 h at room temperature. The DAKO CSA system (K1500) or Envision kit

(K4006/K4010) were used for detection and diaminobenzidine staining.

Immunohistofluorescence Analysis. Mice were perfused with PBS and dissected. Freshly collected brains were immersed in Tissue-Tek optimal cutting temperature (OCT) compound (Sakura) and frozen at -80°C . Tissue sections of 7 μ m in thickness were cut in a cryostat, placed on SuperFrost Plus slides, and dried at 37°C overnight. Sections were fixed with ice-cold acetone for 10 min and washed before processing for immunohistofluorescence. Data on the antibodies used are supplied in Table 5, which is published as supporting information on the PNAS web site.

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