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STAT1-Independent Control of a Neurotropic Measles Virus Challenge in Primary Neurons and Infected Mice

Lauren A. O’Donnell,* Stephen Conway,* R. Wesley Rose,† Emmanuelle Nicolas,* Michael Slifker,* Siddharth Balachandran,* and Glenn F. Rall*

Neurons are chiefly nonrenewable; thus, cytolytic immune strategies to clear or control neurotropic viral infections could have lasting neurologic consequences. IFN-γ is a potent antiviral cytokine that is critical for noncytolytic clearance of multiple neurotropic viral infections, including measles virus (MV); however, the downstream pathways through which IFN-γ functions in neurons have not been defined. Unlike most cell types studied to date in which IFN-γ affects gene expression via rapid and robust activation of STAT1, basal STAT1 levels in primary hippocampal neurons are constitutively low, resulting in attenuated STAT1 activation and consequently slower kinetics of IFN-γ–driven STAT1-dependent gene expression. Given this altered expression and activation of STAT1 in neurons, we sought to determine whether STAT1 was required for IFN-γ–mediated protection from infection in neurons. To do so, we evaluated the consequences of MV challenge of STAT1-deficient mice and primary hippocampal neurons explanted from these mice. Surprisingly, the absence of STAT1 did not restrict the ability of IFN-γ to control viral infection either in vivo or ex vivo. Moreover, the canonical IFN-γ–triggered STAT1 gene expression profile was not induced in STAT1-deficient neurons, suggesting that IFN-γ regulates neuronal STAT1-independent pathways to control viral replication. The Journal of Immunology, 2012, 188: 1915–1923.

Central nervous system invasion is a rare, but typically severe, consequence of several human viral infections that include most herpesviruses, poliovirus, measles virus, flaviviruses (e.g., West Nile virus), and HIV (1). When CNS infections do occur, complications typically result in lasting neurologic damage and often in death, especially in the young, elderly, and immunocompromised (2). The severity of neurotropic virus pathogenicity may be due to the delicate balance that must be achieved by the antiviral immune response: effective immunity must control the infection while minimizing CTL-mediated lysis of this chiefly nonrenewable cell population. Whereas multiple animal models have demonstrated that cytokines, including IFN-γ, can limit viral spread in the brain via noncytopathic mechanisms (3–6), the downstream signaling pathways that result in clearance remain largely undefined. This becomes clinically significant in those instances in which viral clearance fails, as in many cases of pediatric encephalitis including measles and rubella, and neurotropic infections of the elderly, including West Nile virus and St. Louis encephalitis virus (1, 7–10).

IFN-γ is the only type II member of the IFN family, which also consists of type I IFNs (IFN-αβ) and type III IFNs. Unlike type I IFNs, which are expressed by most cells soon postinfection, IFN-γ is chiefly produced by activated immune cells such as NK cells and T cells. IFN-γ initiates a cellular response by binding to the IFN-γ receptor complex (consisting of a heterotetramer of IFN-γR1 and R2 subunits), which triggers activation of receptor-associated JAK-1/2 and subsequent tyrosine phosphorylation of the cytoplasmic tail of the IFN-γR1 subunits. STAT1 is recruited to the phosphorylated R1 subunit, where it is phosphorylated, homodimerizes, and translocates to the nucleus. The phosphorylated STAT1 homodimer binds to γ-activated sequence elements within IFN-γ–responsive genes to initiate transcription. Over 250 genes are induced in this manner to inhibit viral spread (11). Whereas STAT1 is central to a classical IFN-γ response, a substantial number of studies has also demonstrated the presence of IFN-γ–dependent, STAT1-independent pathways (12–20).

IFN-γ is required for viral clearance of many neurotropic viral and bacterial infections (4, 6, 21–26). Of note, however, distinct immune strategies may be employed to resolve CNS infections, depending on the cell type that is infected. For example, mouse hepatitis virus can infect CNS resident astrocytes, microglia, and oligodendrocytes (27); whereas perforin is sufficient to mediate viral clearance from astrocytes and microglia, IFN-γ is sufficient for mouse hepatitis virus control in oligodendrocytes (28, 29). In addition, certain subsets of neural cells, such as neural precursors in the retina, preferentially use STAT3 instead of STAT1 in response to IFN-γ (30). Numerous studies have also shown that both STAT1–dependent and -independent pathways are likely to be important for IFN-γ-mediated viral clearance in other regions of the body (13, 18, 19, 31, 32). For example, STAT1 plays a biphasic role in control of systemic dengue infection in mice: STAT1–dependent pathways are required for early viral control, but STAT1–independent pathways are required for later control and eventual viral clearance (13,
Thus, although IFN-γ has been conclusively linked with noncytolytic clearance of multiple neurotropic infections, the role of STAT1, particularly in neurons, remains less well defined.

We have developed a mouse model of neuron-restricted measles virus (MV) infection, in which CNS neurons express the MV receptor (CD46) under the control of the neuron-specific enolase (NSE) promoter (NSE-CD46+ mice) (33). Using this model, we previously showed that adult NSE-CD46+ mice clear MV infection from CNS neurons without neurologic damage or neuronal loss in an IFN-γ-dependent and T cell-dependent manner (3, 34). IFN-γ can act directly upon neurons to induce an antiviral state, as demonstrated by the direct antiviral ability of IFN-γ to limit MV replication in purified CD46+ neurons cultured ex vivo (3). Surprisingly, however, the neuronal signaling response to IFN-γ treatment was distinct from that observed in control mouse embryonic fibroblasts (MEFs) (35). Specifically, neurons responded to IFN-γ with delayed and attenuated STAT1 expression and activation kinetics in comparison with control fibroblasts. This was reflected in the delayed and reduced expression of classical IFN-γ-dependent genes. These previous studies suggest that IFN-γ activates a critical antiviral program in neurons, but that STAT1 may play a subordinate role in this response.

In this report, we examined the requirement for STAT1 in the IFN-γ-mediated control of MV infection and spread using both an ex vivo model (infected primary hippocampal neurons), as well as infection of NSE-CD46 mice on a STAT1-deficient background. We observed that STAT1 was dispensable for the resolution of a MV CNS infection and that the canonical gene profile induced by IFN-γ was not activated in neurons. These data indicate that IFN-γ-triggered STAT1-independent pathways are most likely operative in CNS neurons.

Materials and Methods

Cells, viruses, mice, and infections

Primary hippocampal neurons were prepared from embryonic (E15–16) mice, as previously described (35–38). Neurons were plated on 15-mm glass coverslips or in 12-well plates coated with poly-l-lysine (Sigma-Aldrich) at a density of 2.5 × 105 cells/well, unless otherwise noted. Neuronal cultures were routinely >95% MAP2 positive. Neurons were plated and incubated at 37°C in a humidified incubator with 5% CO2 for 5 d prior to IFN-γ treatment or infection to allow for full differentiation.

Primary astrogyltes were prepared from E16 cortices, as described previously (35, 39), and were routinely >95% glial fibrillary acidic protein (GFAP) positive. After 7 d in culture, monolayers were trypsinized, re-plated in poly-l-lysine-coated 6-well plates (2 × 105 cells/well), and incubated for 48 h before treatment.

MV-Edmonston (vacine strain) was purchased from American Type Culture Collection and passed and tittered in Vero cells. Passages 2 or 3 of the MV stock were used for intracerebral (IC) injections and in vitro infection assays.

Inbred C57BL/6 (H-2b) and homozygous NSE-CD46+ transgenic mice (line 18, H-2b) (33) were maintained in the closed breeding colony of the Fox Chase Cancer Center. All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee. Homozygous NSE-CD46+ and haplotype-matched homozygous immune-knockout (KO) mice were intercrossed for three or more generations to obtain NSE-CD46+ mice on the desired KO background. STAT1-KO mice (40) were obtained from the Jackson Laboratory. Genotypes of all mice used in these experiments were confirmed by either PCR analysis of tail biopsy DNA, or by flow cytometry of PBLS immunostained with fluorochrome-conjugated Abs to mouse B and T cell markers.

Anafane-anesthetized mice were infected with MV via IC inoculation (2 × 107 PFU in a volume of 20 μl, delivered along the midline using a 27-g needle). Mice were monitored daily postinfection for signs of illness, including weight loss, ruffled fur, ataxia, and seizures. Moribund mice were euthanized in accordance with Institutional Animal Care and Use Committee guidelines.

Primary neuron infections and IFN-γ treatments

Five days postplating, primary hippocampal neurons from NSE-CD46+ mice and NSE-CD46+/STAT1-KO mice were infected with MV (multiplicity of infection [MOI] 1.0) for 1 h. Thereafter, the inoculum was removed, the neurons were washed twice in Dulbecco’s PBS (DPBS), and the cells were placed in conditioned Neurobasal media.

For cells treated with IFN-γ, murine IFN-γ (BD Transduction Laboratories) was diluted in B27-free Neurobasal media, added to the cultures (100 U/ml final concentration), and incubated for the indicated times prior to collection.

Immunofluorescence assay

At the indicated time points post–IFN-γ addition, cells on coverslips were fixed in a 1:1 solution of methanol:acetone for 5 min at room temperature (RT). Cells were washed twice in DPBS. To prevent nonspecific Ab binding, cells were incubated in a 10% goat serum/20% FBS solution in DPBS for 1 h at room temperature (RT). A primary Ab was then added in blocking solution for 1 h at RT. Abs, sources, and concentrations used included the following: monoclonal anti–phospho-STAT1 (pY701), BD Pharmingen (1:100); rabbit polyclonal anti-MAP2, Chemicon (1:250); rabbit polyclonal anti-GFAP, Novus Biologicals (1:250); and Keller anti-MV human serum, a gift of M. Oldstone (The Scripps Research Institute) (1:1000). After three washes in DPBS, the cells were incubated in the appropriate secondary Ab in blocking solution for 1 h at RT; anti-mouse AlexaFluor-555 (1:10,000) or anti-rabbit AlexaFluor-488 (1:10,000) with Hoechst 33,342 (10 μM) nuclear stain, all from Molecular Probes. After three washes in DPBS, coverslips were mounted onto glass slides using Citi-Fluor AF1 (Electron Microscopy Sciences). Images were captured using an Invitrode TE2000 (Nikon C1 confocal scanhead (original magnification ×40 objective) in the Fox Chase Cancer Center Confocal Facility.

Immunoblots

Cells were treated with IFN-γ and collected at the indicated times, and immunoblots were performed, as previously described (35). Abs used included the following: anti-STAT1 (1:1000); phospho-specific STAT1 (pY701; 1:1000); Keller anti-MV (1:1000); and anti–GAPDH (1:10,000; Chemicon). All were diluted in PBS-Tween 20 (PBS-T) containing 5% milk. After three washes in PBS-T (10 min each), the blots were incubated in secondary Ab solution for 1 h at RT. Abs included the following: goat anti-rabbit HRP (1:1000; Vector Laboratories) for anti-STAT1; goat anti-mouse HRP (1:2000; Santa Cruz) for anti-pSTAT1; and anti-human-HRP for Keller anti-MV (1:10,000), all diluted in PBS-T with 5% milk. For quantitative analysis of immunoblots, densitometric analysis was performed using National Institutes of Health Image software (v. 1.63). When reprobing was necessary, blots were stripped in an acidic glycine/SDS solution for 2 h at RT.

Immunohistochemical analysis of mouse tissues

Brains from four to five mice/time point were removed, immersed in tissue-embedding compound (Fisher), snap frozen in a dry ice-isopentane bath, and stored at −70°C. Horizontal cryosections (10 μm) were air dried and stored at −70°C. Standard immunohistochemistry using diaminobenzidine precipitation (3, 41) was performed. To detect MV-infected cells, Keller anti-MV serum was used at a dilution of 1:2000, followed by a biotinylated anti-human secondary Ab (1:300; Vector). Rat anti-mouse CD4 (clone RM4-5; 1:200; BD Pharmingen) or rat anti-mouse CD8α/CD8β Abs (clones 53-6.7 and 53-5.8, respectively; 1:100 each; BD Pharmingen) were used to identify T lymphocyte subsets. Sections were then sequentially incubated for 1 h at RT with a biotinylated anti-rat IgG secondary Ab (1:200; Vector Laboratories), 30 min with a streptavidin-biotin-peroxidase conjugate (ABC Elite; Vector Laboratories), and, finally, for 5–10 min with diaminobenzidine (0.7 mg/ml in 60 mM Tris) and urea-H2O2 (0.2 mg/ml), purchased as preweighed tablets (Sigma-Aldrich). All were counterstained with hematoxylin and mounted with aqueous mounting medium. Uninfected tissues or omission of the primary Ab served as negative controls. For all histological analyses, at least three sections per brain were examined from three different horizontal levels, and at least four mice per experimental group were assessed.

Quantitative RT-PCR

Mouse brains were snap frozen in liquid nitrogen and stored at −80°C. RNA was isolated by TRizol, according to the manufacturer’s instructions (Sigma-Aldrich). Contaminating DNA was removed from RNA preparations using DNase I treatment (Inviogen). Purified RNA was quantified using a Nanodrop instrument. RNA was reverse transcribed using Mlooney murine leukemia virus reverse transcriptase (Ambion) and a mixture of anchored oligo-RT and random decamers. For each sample, two reverse transcriptase reactions were performed with inputs of 100 and 20 ng. An aliquot of the cDNA was used for 5′-nuclease assays using TaqMan chemistry. For IFN-γ expression, Assay-on-Demand Mm00801778_m1

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was used. A TaqMan set specific for the N gene of MV (GenBank sequence AB046218) was used for detecting viral RNA. Sequences were as follows: forward, 5′-CCGACGATGCTGGAAAGTCA-3′; reverse, 5′-TTCCGAGATTCCCTGGCAATG-3′; probe, 5′-6-Fam-TGACGCCCTGTTAAGCTGCAA-BHQ1-3′. Assays were used in combination with Universal Master mix and run on a 7900 HT sequence detection system (Applied Bio-systems). Cycling conditions were 95°C, 15 min, followed by 40 (two-step) cycles (95°C, 15 s; 60°C, 60 s). The assay for MV-N was validated with a 4-fold five-points dilution curve of cDNA. The slope was −3.54, corresponding to a PCR efficiency of 95%. For each sample, the values are averaged and SD of data are derived from two independent PCRs. Relative quantification to the control was done using the comparative cycle threshold method.

Flow cytometric analysis of brain infiltrates
On the indicated day postinfection (dpi), mice were deeply anesthetized with 400 μl 3.8% chloral hydrate in PBS, delivered i.p. Once animals were confirmed to be nonresponsive, the mice were perfused with 30 ml PBS. Following perfusion, each brain and spleen were removed and pressed through a nylon mesh cell strainer in PBS. Dissociated tissue was run over 0.84% ammonium chloride to remove contaminating RBCs, and washed again. Mononuclear cells were recovered from the interface, washed with PBS, treated with 0.84% ammonium chloride to remove contaminating RBCs, and washed again. Collected mononuclear cells were counted using a standard hemocytometer and plated into a V-bottom 96-well plate for subsequent Ab staining for multicolor flow cytometry. The following Abs (eBioscience) were used: PE-Cy5-CD8α, allophycocyanin-Ax750-CD4, PE-CD3e, FITC-CD11c, allophycocyanin-CD161c (NK1.1), PB-CD19, PE-Cy5.5 Gr-1 (Ly-6G), PE-Cy7-CD49b (DX5), and Ax700-CD11b. Cells were allowed to incubate with Ab for 1 h at 4°C and then washed following the incubation period. Pelleted, stained cells were resuspended and read in a BD LSR II system. Percentages obtained from flow cytometry were combined to calculate total cell numbers.

Microarray
Primary hippocampal neurons were plated in poly-L-lysine–coated 6-well plates at a density of 1 × 10⁶ cells/well. RNA was purified from whole-cell lysates using the RNaseasy mini kit (Qiagen), and contaminating DNA was removed using a RNase-free DNase set. A quantity amounting to 500 ng total RNA was amplified and labeled using the low RNA input linear amplification kit (Agilent). Labeled cRNA targets were hybridized onto Agilent 4×44k mouse whole genome arrays. Microarray images were processed using Agilent Feature Extraction software (version 9.5). Data were background corrected using the normexp method (PMID: 17720982) implemented in the Bioconductor package limma (42, 43), and quantile normalized. Identification of differentially expressed genes was performed with empirical Bayes moderated t tests using limma. Biological pathways and networks were examined with Ingenuity Pathway Analysis software (www.ingenuity.com). The dataset is at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE33057.

Results
IFN-γ-mediated STAT1 signaling is attenuated in CNS neurons
We previously showed that IFN-γ is required for control of MV in a transgenic mouse model (NSE-CD46⁺), in which infection is neuron restricted (3). To define the mechanism by which IFN-γ functions, we determined the efficacy of IFN-γ-mediated control of MV replication in primary neurons. Explanted CD46⁺ hippocampal neurons were treated with murine rIFN-γ (100 U/ml) or an equivalent amount of heat-inactivated IFN-γ; 18 h thereafter, the neurons were infected with MV-Ed (MOI = 1), and relative infection levels were determined by quantifying viral protein load. Note that standard plaque assays cannot be used to measure viral levels because no infectious virus is released from infected neurons (44). Neuronal lysates were harvested 48 hours postinfection (hpi) for Western blot analysis. As shown in Fig. 1A and 1B, viral protein levels were >75% reduced in the presence of IFN-γ, whereas heat-inactivated IFN-γ had no effect. These results confirm that IFN-γ limits MV replication in primary neurons.

FIGURE 1. IFN-γ treatment limits MV neuronal infection, despite low basal levels of STAT1 and delayed STAT1 phosphorylation. A. Hippocampal neurons explanted from NSE-CD46⁺ embryos were treated with IFN-γ (100 U/ml, 18 h) or heat-inactivated (ΔH) IFN-γ (100 U/ml, 18 h) and infected with MV-Ed (MOI = 1). At 48 hpi, neurons were lysed in protein solubilization buffer and subjected to Western blot for MV Ag and GAPDH as a loading control. B. Western blots from A were quantified by densitometry using ImageJ software. MV signal was normalized to GAPDH as loading control. Statistical analysis was applied using a paired t test (n = 4, *p < 0.05 versus MV infected). C and D. Hippocampal neurons and cortical astrocytes from NSE-CD46⁺ embryonic mice were treated with IFN-γ (100 U/ml) for the indicated times and collected for Western blot analysis (C) or fixed for immunofluorescence (D). For Western blots (C), whole-cell lysates were subjected to Western blot for phosphorylated STAT1 (STAT1-P; tyrosine 701), total STAT1, and GAPDH. D. Coverslips were stained with a mAb against STAT1-P (red), and a polyclonal Ab against GFAP or MAP2 as astrocyte or neuronal markers, respectively (green). All experiments were conducted in triplicate. Original magnification ×400.
Our previous work (35) suggested that the downstream signaling response of primary neurons following IFN-γ engagement was distinct from classical IFN-γ-activated STAT1-dependent signaling (11). To determine whether this altered signaling profile was true for other brain parenchymal cells, primary hippocampal neurons were compared with primary astrocytes, which are derived from the same pool of neural precursor cells (45). Both primary cultures were consistently >95% pure, as defined by MAP2 and GFAP staining, respectively (Fig. 1D). Cultures were treated with IFN-γ (100 U/ml) for the indicated times, and samples were then analyzed for both total and phosphorylated STAT1 by Western blot (Fig. 1C) and by immunofluorescence (Fig. 1D). Like MEFs (35), astrocytes expressed abundant endogenous levels of STAT1, which was rapidly phosphorylated after IFN-γ treatment (Fig. 1C, 1D, top row). In contrast, primary hippocampal neurons expressed low basal levels of STAT1, and phosphorylated STAT1 was detectable in neurons only after extended IFN-γ stimulation (24 h), coincident with an increase in available STAT1 protein (Fig. 1C). These data demonstrate that delayed STAT1 phosphorylation is not a general characteristic of resident brain parenchymal cells, but rather is unique to neurons.

**STAT1 is not required for MV control in primary neurons**

Despite reduced expression and activation of neuronal STAT1, it was possible that these low levels were still sufficient to initiate an effective antiviral signal. Thus, to determine what role STAT1 played in IFN-γ-mediated MV clearance, CD46⁺ and CD46⁻/STAT1-KO neurons were treated with IFN-γ and infected with MV, as described above; neurons were then harvested for Western blot analysis for MV Ag or fixed for immunofluorescence. Quantified results are shown in Fig. 2A, and representative images are shown in Fig. 2B. Without IFN-γ, increasing levels of MV Ag were detectable in both CD46⁺ and CD46⁻/STAT1-KO neurons from 24 to 72 hpi, evidenced by the increasing size of viral Ag-positive foci (Fig. 2B). In contrast, infected CD46⁺ and CD46⁻/STAT1-KO neurons treated with IFN-γ showed equivalently less MV Ag at each time point; there were no statistical differences between STAT1-expressing and KO neurons. These data suggest that STAT1 is not required for IFN-γ-mediated control of MV in neurons.

IFN-γ could abate MV growth either by directly eliminating MV from infected neurons, by limiting trans-synaptic spread (44), or by some combination of the two. To assess this, receptor-expressing CD46⁺ neurons were infected with MV, and treated with IFN-γ either 24 h before, 24 h after, or coincident with MV infection. IFN-γ-mediated control was most effective when neurons were pretreated with IFN-γ (Fig. 2C, compare lanes 2 and 1); the antiviral effect was negligible when IFN-γ was added post-infection (compare lanes 4 and 1). Moreover, in the presence of IFN-γ, MV Ag-positive neurons were typically found as single immunopositive cells, in contrast to abundant multineuronal networks in the absence of IFN-γ (Fig. 2B, quantified in Fig. 2D), suggesting that IFN-γ may play a role in blocking viral spread rather than directly resolving the infection.

**FIGURE 2.** Primary neurons use STAT1-independent pathways during IFN-γ-mediated viral control. A, NSE-CD46⁺ and NSE-CD46⁻/STAT1-KO hippocampal neurons were treated with IFN-γ (100 U/ml) and infected with MV (MOI = 1) for 24 or 48 h. Whole-cell lysates were harvested, subjected to Western blot analysis, and quantified on ImageJ software, with MV signal normalized to GAPDH. The average MV signal is plotted with MV infection at 24 h set to 100%, and error bars represent SEM (n = 3). B, Immunofluorescence analysis of MV Ag in CD46⁺ and CD46⁻/STAT1-KO neurons. Neurons were treated as in A and stained for MV Ag (red), MAP2 (green), and Hoescht (blue). Original magnification ×400. C, Primary CD46⁺ neurons were treated with IFN-γ either 24 h before infection (lane 2), coincident with infection (lane 3), or 24 h postinfection (lane 4). Lysates were collected from infected (and corresponding uninfected) neurons, and blots probed for MV Ag and GAPDH, as described. D, CD46⁺ neurons were either treated with IFN-γ (100 U/ml) or left untreated and, 24 h later, infected with MV. At 48 hpi, coverslips were collected and immunostained for MV Ags, and nuclei within immunopositive clusters were counted. Clusters were “binned” into groups of 1–3, 4–6, 7–10, or >10 (n = 3). SDs are shown from three such experiments.
STAT1-deficient mice survive viral infection in CNS neurons

To determine what role STAT1 plays in the resolution of a neuronal viral infection in vivo, we infected NSE-CD46+ transgenic mice that had been backcrossed to selective immune KO mice. NSE-CD46+ mice lacking T and B cells (NSE-CD46+/RAG2-KO), IFN-γ (NSE-CD46+/IFN-γ-KO), or STAT1 (NSE-CD46+/STAT1-KO) were infected IC with MV and monitored daily for signs of illness (Table I) and mortality (Fig. 3). Consistent with our published results (3), immunocompetent NSE-CD46+ mice remained disease free, and all survived, whereas >95% of NSE-CD46+/RAG2-KO mice and ~50% of NSE-CD46+/IFN-γ-KO mice died of unrestricted MV infection between 7 and 17 dpi, during the period of maximal T cell presence within the brain parenchyma (dotted horizontal line, Fig. 3) (34). Of note, surviving NSE-CD46+/IFN-γ–KO mice showed lasting signs of neurologic damage, including ataxia, piloerection, and hunched posture (Table I). Unexpectedly, NSE-CD46+/STAT1-KO mice segregated into two groups following MV infection. Consistently, a small proportion (~25%; 9 of 35 mice in the experiment shown) died between 4 and 6 dpi, earlier than any other KO mouse tested in this model system, and of three separate experiments were pooled (n = 10–35 mice/condition). Mice were monitored daily for signs of morbidity and were euthanized when signs of illness were apparent. The dotted horizontal line indicates the peak of T cell entry into the CNS parenchyma of NSE-CD46+ mice. Pairwise analysis, contingent on survival to 8 dpi, was performed between infected NSE-CD46+ mice and the other three genotypes. NSE-CD46+ versus NSE-CD46+/STAT1-KO: not significant, NSE-CD46+ versus NSE-CD46+/IFN-γ–KO: p < 0.05. NSE-CD46+ versus NSE-CD46+/RAG2-KO: p < 0.05.

FIGURE 3. STAT1 is not required for T cell-mediated resolution of a neuron-restricted MV infection. Kaplan–Meier survival plot of MV-challenged NSE-CD46+ mice on various KO backgrounds. NSE-CD46+, NSE-CD46+/STAT1-KO, NSE-CD46+/IFN-γ–KO, and NSE-CD46+/RAG2-KO mice were infected, as described in Materials and Methods. Results from three separate experiments were pooled (n = 10–35 mice/condition). Mice were monitored daily for signs of morbidity and were euthanized when signs of illness were apparent. The dotted horizontal line indicates the peak of T cell entry into the CNS parenchyma of NSE-CD46+ mice. Pairwise analysis, contingent on survival to 8 dpi, was performed between infected NSE-CD46+ mice and the other three genotypes. NSE-CD46+ versus NSE-CD46+/STAT1-KO: not significant, NSE-CD46+ versus NSE-CD46+/IFN-γ–KO: p < 0.05. NSE-CD46+ versus NSE-CD46+/RAG2-KO: p < 0.05.

T cell infiltration into the CNS and control and clearance of MV in neurons are STAT1 independent

Studies using STAT1 KO mice have implicated a role for STAT1 in both lymphocyte proliferation and migration (47–49). To address whether the absence of STAT1 affected the timing or magnitude of T cell infiltration into the infected brain parenchyma, immune cell profiles were determined in brain tissues of MV-infected NSE-CD46+ and NSE-CD46+/STAT1-KO mice by flow cytometry and immunohistochemistry. At 7 dpi, the profile of immune cells (including both innate and adaptive cell types) in the spleens of NSE-CD46+, NSE-CD46+/IFN-γ–KO, and NSE-CD46+/STAT1-KO mice was statistically similar (Fig. 4A, left panel). In brains, however, a lower percentage of CD4+ and CD8+ T cells was found in the CNS of IFN-γ–deficient mice despite overall numbers of infiltrating cells within the CNS, confirming IFN-γ as a chemoattractant (Fig. 4A, right panel). However, no differences in either profile (Fig. 4A, right panel) or localization (Fig. 4B) of intraparenchymal T cells were observed between MV-permissive wild-type and STAT1 KO mice.

To compare relative levels of IFN-γ, we quantified IFN-γ RNA in brain tissue at the peak of T cell infiltration (11 dpi) by quantitative RT-PCR (Fig. 5A) and compared MV RNA levels in the CNS at various dpi (Fig. 5B). As expected, NSE-CD46+/IFN-γ–KO mice did not produce IFN-γ (data not shown), and were unable to clear MV RNA to levels seen in NSE-CD46+ or NSE-CD46+/STAT1-KO mice (Fig. 5B). Brains of NSE-CD46+ and NSE-CD46+/STAT1-KO mice expressed significantly higher levels of IFN-γ RNA at 11 dpi than NSE-CD46+ mice, which correlated with the decrease in MV RNA seen in nonseizing NSE-CD46+/STAT1-KO brains between 4 and 11 dpi (Fig. 5B). Importantly, NSE-CD46+ mice had consistently low levels of MV RNA, indicating that STAT1 most likely plays a pivotal role in the type I IFN innate response at early times postinfection. Note also that, although ~20% of NSE-CD46+/STAT1-KO mice develop lethal seizures at 4 dpi, there was no statistical difference in viral load between seizing and healthy mice. Together, these data indicate that the adaptive immune response of NSE-CD46+/STAT1-KO mice is comparable to that made by wild-type mice, leading to viral resolution in the absence of the canonical STAT1-mediated pathway.

Canonical IFN-γ–dependent genes are not expressed in STAT1-KO neurons

Finally, to address the possibility that IFN-γ resulted in the expression of a classic profile of genes via an alternative (non-STAT1–mediated) pathway, we used a microarray strategy to identify the top 50 genes that were activated by IFN-γ in MEFs, and then determined whether these same genes were induced in IFN-γ–treated neurons, with and without STAT1. Microarrays were performed on both primary MEFs and primary NSE-CD46+ and NSE-CD46+/STAT1 KO neurons following IFN-γ exposure for 0, 3, 6, or 24 h. Three individual cultures were analyzed for each time point. Heat maps, reflective of expression levels, are shown in Fig. 6. Yellow represents the baseline for each gene in

Table I. Measles virus-induced CNS disease in mice of various immunodeficient backgrounds

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Average Illness Score (% Surviving)</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
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<td>0 (100)</td>
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<td>0 (100)</td>
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<tr>
<td>NSE-CD46+/RAG2-KO</td>
<td>0.6 (100)</td>
<td>2.8 (30)</td>
<td>2.3 (20)</td>
<td>2.5 (10)</td>
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<tr>
<td>NSE-CD46+/IFN-γ–KO</td>
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<td>1.9 (60)</td>
<td>1.8 (60)</td>
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</tr>
<tr>
<td>NSE-CD46+/STAT1-KO</td>
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<td>1.0 (70)</td>
<td>0.5 (70)</td>
<td>0 (70)</td>
<td></td>
</tr>
</tbody>
</table>

Mice were subjectively assessed for signs of morbidity every other day postinfection. Average scores (0–3) of surviving mice for a 4-wk observation interval are shown (n = 10 for each genotype), as follows: 0 = healthy; 1 = ruffled fur/piloerection, ataxic; 2 = ruffled fur/piloerection, ataxic, plus seizures; 3 = moribund.
each cell type; progression to red indicates induction of gene expression. The top 50 IFN-γ-activated genes in MEFs are shown in the first three columns; as expected, the majority of these genes achieved maximal levels by 3 h posttreatment. Primary STAT1+ neurons induced a similar profile of genes, although some genes were not induced at all, and most others showed delayed induction kinetics, consistent with the delayed activation of STAT1 in neurons. Importantly, despite the capacity to control MV as effectively as STAT1+ neurons, STAT1 KO neurons did not induce the majority of canonical IFN-γ-driven genes. Thus, it is likely that alternative genes are induced in STAT1 KO neurons that mediate viral control. A complete list of genes that were induced or suppressed in neurons following IFN-γ exposure, independent of STAT1 expression, is provided in Supplemental Figs. 1 and 2.

Discussion

In this manuscript, we make three key observations, as follows: 1) distinct from other parenchymal CNS cell types, the IFN-γ–triggered, STAT1-dependent signaling profile in neurons is delayed and restricted; 2) STAT1 is not required for IFN-γ–mediated control of MV either in primary neurons or in a permissive mouse model; and 3) alternative signaling pathways are most likely operative in STAT1-deficient neurons (and perhaps also in STAT1-positive neurons) to result in viral control.

A number of points warrant further discussion. First, these data add to a growing literature that shows that the cellular response to extracellular cytokines is not monolithic (15, 30, 35, 50–52). Whereas signaling pathways and the genes they regulate are generally maintained across distinct cell populations, subtle differences in expression levels can exert major changes in the antiviral response. Specifically, the delayed and muted neuronal response to IFN-γ, in contrast to the rapid and robust signal seen in MEFs (35) and astrocytes (Fig. 1C), has a marked impact on the kinetics and magnitude of IFN-γ-responsive genes expressed in neurons. Relevant to published studies, these primary neurons are ∼95% pure cultures, and, in this report, we assessed pSTAT1 levels within 3–24 h post–IFN-γ treatment; whereas untreated neurons do have detectable levels of unphosphorylated STAT1, and eventually respond to IFN-γ [as shown in other studies (53–55)], the signature of the early neuronal response is appreciably different from that observed in control cells. Of note as well, whereas these data specifically compare primary hippocampal neurons with primary astrocytes, differences may also be present in neuronal subpopulations as well. The primary neurons used in these experiments are mainly derived from the embryonic hippocampus, although dissection of this substructure from day E15 to 16 embryos can be imprecise, and most likely includes some cortical tissue. Moreover, both glutamatergic and GABAAergic neurons are present in the hippocampus at this stage of development (36); thus, these cultures are most likely comprised of different neuronal subtypes. This may be relevant to the STAT1 activation profile seen in neurons since at 24 h posttreatment, about one-half of the neurons have a detectable pSTAT1 signal, whereas the remainder appears nonresponsive (Fig. 1D, lower right panel). Efforts are underway to determine whether there is a correlation between neuronal subtype in the CNS and IFN-γ responsiveness.

A second issue is the apparent dispensability of STAT1 in IFN-γ–mediated viral control. In both infected mice and infected primary neurons, IFN-γ plays a central role in protection; its absence from mice results in death of ∼50% of MV-challenged animals, and permanent neurologic impairment in the survivors. Similarly, addition of murine rIFN-γ to infected neurons restricts viral load, and may afford a survival advantage to these vulnerable cells. Given the crucial role of IFN-γ in survival from MV CNS disease, we were therefore surprised that, during the period of adaptive immune cell presence in the brain of infected mice (7–17 dpi), STAT1 played no role in mouse survival. This was in contrast to an extensive body of evidence that supports a central and pivotal
role for STAT1 in mouse survival following infection by many viruses. In these papers, STAT1 KO mice demonstrated greater viremia and mortality from both peripheral viral challenges (15, 55, 56) as well as from CNS infections (55), leading to the conclusion that STAT1 was a key feature of protection against multiple infections. Yet, evidence for STAT1-independent antiviral pathways also exists for other viral infections (15, 18, 19), suggesting that particular viruses or target tissues may not be as reliant on STAT1-dependent pathways for viral clearance. The difference between our results and those that underscore the importance of STAT1 in CNS infections may be because the only cell type infected in NSE-CD46+ mice is CNS neurons, which may allow neuronal STAT1-independent pathways to be effective against this limited viral tropism.

Whereas STAT1 is not required during the 7- to 17-dpi window when T cells are abundant in the CNS, there is a small, but reproducible effect of STAT1 deficiency at early stages postinfection (3–6 dpi), resulting in death of 20–30% of infected mice. We presume that this implies participation of STAT1 in the type I IFN response (mediated by IFNs α and β), in which, along with STAT2 and IFN regulatory factor-9, it is part of the IFN-stimulated gene factor 3 complex that binds to IFN-stimulated gene promoter elements. Whereas this hypothesis awaits confirmation, one issue that this model does not address is that neurons, in vivo, may encounter IFNs sequentially; that is, it is likely that cellular exposure to type I IFNs precedes IFN-γ exposure. As such, when a neuron encounters IFN-γ, it may already have made a transcriptional response to type I IFN. Thus, an ultimate appreciation of the role of IFNs and their subsequent signaling pathways in antiviral immunity will most likely require an integrated evaluation of the neuronal response to serial IFN encounters.
Finally, the uncoupling of STAT1 dependence from IFN-γ dependence makes it likely that a different constellation of genes is induced in neurons that influence viral control. As shown in Fig. 6, the standard IFN-γ profile is not induced in STAT1 KO neurons, and thus our current efforts are aimed at identifying what role IFN-γ-inducible, STAT1-independent genes may play in viral control.

From these data, we propose a signaling factor density model, as follows: in MEFs and astrocytes, the abundant cytokinome levels of STAT1 facilitate rapid and complete occupation of binding sites on the intracellular domain of the IFN-γ receptors, triggering a primarily STAT1-driven response. In contrast, neurons with lower constitutive expression of STAT1 may allow for other signal transduction factors to bind and eventually trigger transcription of a distinct subset of genes, creating a unique neuronal response profile. Indeed, the relevance of these alternative signaling pathways is supported by the impairment-free survival of STAT1 KO mice, implying that the canonical STAT1 signaling pathway plays a modest, perhaps negligible, role in viral control in neurons.

As the field of cell-specific cytokine responses progresses, one can envision that the use of cytokines as antivirals will need to be reassessed in the context of the disease or pathogen that is targeted and the cell populations affected.

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References


