T Cell-, Interleukin-12-, and Gamma Interferon-Driven Viral Clearance in Measles Virus-Infected Brain Tissue[▽]

Samantha R. Stubblefield Park,^{1*} Mi Widness,^{1†} Alan D. Levine,² and Catherine E. Patterson^{1‡}

Departments of Molecular Biology and Microbiology¹ and Medicine, Pathology, and Pharmacology and Case Comprehensive Cancer Center,² 10900 Euclid Avenue, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-4952

Received 16 July 2010/Accepted 18 January 2011

Genetic studies with immunocompetent mice show the importance of both T cells and gamma interferon (IFN- γ) for survival of a measles virus (MV) challenge; however, the direct role of T cells and IFN- γ within the MV-infected brain has not been addressed. Organotypic brain explants represent a successful *ex vivo* system to define central nervous system (CNS)-specific mechanisms of leukocyte migration, activation, and MV clearance. Within the heterogeneous, brain-derived, primed leukocyte population which reduced MV RNA levels in brain explants by 60%, CD3 T cells are the active antiviral cells, as purified CD3-positive cells are highly antiviral and CD3-negative leukocytes are unable to reduce the viral load. Neutralization of CCL5 and CXCL10 decreases leukocyte migration to areas of infection by 70%. However, despite chemokines directing the migration of T cells to infected neurons, chemokine neutralization revealed that migration is not required for viral clearance, suggesting a cytokine-mediated antiviral mechanism. In accordance with our hypothesis, the ability of leukocytes to clear the virus is abrogated when explants are treated with anti-IFN- γ neutralizing antibodies. IFN- γ applied to infected slices in the absence of primed leukocytes reduces the viral load by more than 80%; therefore, in brain tissue, IFN- γ is both necessary and sufficient to clear MV. Secretion of IFN- γ is stimulated by interleukin-12 (IL-12) in the brain, as neutralization of IL-12 results in loss of antiviral activity and stimulation of leukocytes with IL-12/IL-18 enhances their immune effector function of viral clearance. MV-primed leukocytes can reduce both West Nile and mouse hepatitis viral RNAs, indicating that cytokine-mediated viral clearance occurs in an antigen-independent manner. The IFN- γ signal is transduced within the brain explant by the Jak/STAT signaling pathway, as inhibition of Jak kinases results in a loss of antiviral activity driven by either brain-derived leukocytes or recombinant IFN- γ . These results reveal that primed T cells directly act to clear MV infection of the brain by using a noncytolytic IL-12- and IFN- γ -dependent mechanism in the CNS and that this mechanism relies upon Jak/STAT signaling.

A wide variety of RNA and DNA viruses, including measles virus (MV), West Nile virus (WNV), human immunodeficiency virus, human cytomegalovirus, herpes simplex virus type 1 (HSV-1) and HSV-2, rabies virus, poliovirus, and lymphocytic choriomeningitis virus, cross the blood-brain barrier, infect the central nervous system (CNS), and cause encephalitis in mammals (3, 15, 26, 29, 34, 35, 75, 82, 85). A suitable combination of host inflammatory factors and the blood-borne viral load enables most viruses to enter the CNS. However, in the vast majority of cases, neuronal infection does not lead to overt CNS disease (82). Viral encephalitis detection rates, even when symptoms are severe, are very low (46), due in part to the poor sensitivity of the tools used to detect infection (21). However, with the increase in the number of immunocompromised individuals, whether through increasing populations of AIDS

patients or pharmacologically compromised tissue transplant recipients, there has been a concomitant increase in viral encephalitis (11, 77). Some viral infections, rather than being cleared by the host's immune system, result instead in high morbidity or mortality, often accompanied by severe inflammation (18, 68, 81). The balance between whether the adaptive immune response to a specific viral infection is protective or harmful is delicate, as many of the mechanisms that mediate inflammation in the CNS in both settings are similar (6, 12, 31, 65).

MV infection manifests itself primarily as a childhood illness with a characteristic macropapular rash. Measles is associated with high morbidity and mortality in developing countries, mostly due to a transient immunosuppression that leaves infected individuals highly susceptible to secondary infections (61). In approximately 0.1% of the cases, MV also causes CNS complications; one of these, subacute sclerosing panencephalitis (SSPE), is a progressive fatal disease. MV-associated encephalitis is one of many viral brain infections that cause high morbidity and mortality (26, 34, 85). The delayed pathogenesis of SSPE, including the route of viral entry into the CNS, is poorly understood (49). Although it is widely accepted that MV infection in the brain leads to complications, this is not necessarily the case, as MV mRNA is detected in the brains of 20% of individuals with no CNS pathology (33, 34). It is currently hypothesized that SSPE is a result of mutated, aberrant

* Corresponding author. Mailing address: Department of Molecular Biology and Microbiology, Case Western Reserve University School of Medicine W205, 10900 Euclid Avenue, Cleveland, OH 44106. Phone: (216) 368-0386. Fax: (216) 368-3055. E-mail: samantha.stubblefield@case.edu.

† Present address: Department of Neurosciences, 9500 Euclid Avenue, Cleveland Clinic Lerner Research Institute, Cleveland, OH 44195.

‡ Present address: Department of Molecular Medicine, College of Medicine, University of South Florida, Tampa, FL.

[▽] Published ahead of print on 26 January 2011.

viruses and the deleterious immune responses to MV infection. Thus, understanding the cellular and molecular events of a beneficial immune response could allow the development of acute viral encephalitis management and provide clues to the treatment of chronic inflammatory diseases of the brain.

Viral infection of genetically engineered mice is a powerful model to identify the immune cell types and effector functions that support encephalitis or successfully resolve infection without pathogenesis (5, 12, 14, 41, 42). Transgenic mice have been engineered for MV susceptibility through the CNS neuron-specific expression of CD46 (NSE-CD46), the human receptor for the Edmonston B strain of MV (62). Despite MV replication in neurons of the brain and a robust immune infiltrate into the parenchyma of the neuron-rich regions of the brain, these mice remain healthy, do not exhibit any significant signs of neurological illness, and completely clear the virus within 15 days. In an effort to determine the mechanism of viral clearance, NSE-CD46 mice were further engineered to be deficient in various immune cells or effector molecules and challenged with MV (59). Although *in vivo* experiments revealed the requirement of T cells and gamma interferon (IFN- γ) for animal survival (59), they did not provide direct evidence for the function of T cells or IFN- γ . Experiments of this kind also do not address more subtle questions, such as the specific location of critical immune interactions. For instance, where and how T cells and IFN- γ function to control virus and whether IFN- γ is directly antiviral are important questions that were left unanswered. An *ex vivo* model of CNS infection allows for a more detailed study of cellular interactions and mechanism of action than that accomplished *in vivo*.

Neuron-rich long-term organotypic brain explants provide a system to examine immune cell function in CNS tissue *ex vivo* while maintaining the complex interactions among resident cells, virus, and infiltrating leukocytes (24). Organotypic virus-infected mouse brain explants, when cocultured with purified antiviral leukocytes, enable us to visualize the infiltrating leukocytes and modulate their responses. This includes the characterization of virus-primed antiviral immune cell migration to sites of infection, as well as definition of the contributions from infiltrating immune cells distinct from the intrinsic response of resident brain cells. This study used organotypic brain explants to demonstrate that in the brain parenchyma, both T cells and IFN- γ are necessary and sufficient to reduce the viral load. While brain-derived CD4 and CD8 T cells respond to chemokine signals, migration in proximity to the virus-infected neuron is not required for viral clearance. We show, instead, that freshly isolated leukocytes from MV-infected brains clear the virus from infected brain explants in an interleukin-12 (IL-12)- and Janus tyrosine kinase (Jak)-dependent manner. Furthermore, the cytokine-mediated antiviral activity of infiltrating leukocytes is antigen independent, as MV-primed brain-derived leukocytes effectively control both mouse hepatitis virus (MHV) and WNV.

MATERIALS AND METHODS

Ethics statement. Inbred C57BL/6 (*H-2^b*) and homozygous NSE-CD46 transgenic mice (line 18; *H-2^b*) were obtained from Glenn Rall (Fox Chase Cancer Center, Philadelphia, PA) and maintained in the closed animal colony of the Case Western Reserve University School of Medicine in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals

of the National Institutes of Health. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University (permit A3145-01). Breeding pairs were continuously monitored to minimize excess neonate numbers.

Virus and infections. MV strain Edmonston was purchased from the American Type Cell Collection (ATCC, Manassas, VA). Green fluorescent protein (GFP)-expressing MV (MVeGFP) was a gift from M. Billeter via R. Cattaneo (University of Zurich, Zurich, Switzerland) (23). WNV (NY99) was a gift from M. Cho (Iowa State University, Ames). The concentrated encephalitis strain of MHV (MHV-A59) used was a gift from C. Bergmann (Cleveland Clinic Foundation, Cleveland, OH). MV and WNV were passaged and titers were determined on Vero cells (purchased from ATCC) maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum and 2 mM L-glutamine (Invitrogen, Carlsbad, CA). MV and WNV stocks were concentrated 100-fold by high-speed centrifugation (17,500 rpm, 1 h, SW28 rotor, Sorvall RC-5 Superspeed centrifuge) and resuspended in phosphate-buffered saline (PBS). All cells were maintained at 37°C in a humidified incubator with 5% CO₂ and routinely tested for mycoplasma contamination (MD Biosciences, St. Paul, MN). Mice were infected with 2,000 PFU of MV. The inoculum was diluted in PBS and delivered intracerebrally to isoflurane-anesthetized mice along the midline in a volume of 20 μ l by using a sterile 27-gauge needle (Becton Dickinson, Franklin Lakes, NJ).

Isolation and labeling of leukocytes from the brain. Eight- to 10-week-old mice inoculated intracerebrally with 2,000 PFU of MV were saline perfused and sacrificed at 7 dpi, their brains were removed, and lymphocytes were isolated for subsequent use. To obtain lymphocytes from brain tissue, brains were diced and washed with Hanks balanced salt solution (HBSS)–1% D-glucose (HBSS; Invitrogen; D-glucose, Acros, Geel, Belgium). Debris was allowed to settle, and supernatant was transferred to a fresh 50-ml conical tube. This process was repeated to allow maximum dissociation between cells and brain tissue. Cells were harvested by centrifugation and resuspended in 5 ml 70% Percoll (in PBS; Amersham Pharmacia Biotech, Piscataway, NJ) with 5 ml of 30% Percoll overlaid. Tubes were centrifuged at 4°C at 1,300 $\times$ g for 30 min. Each sample was aspirated down to the layer of cells at the interface, which was harvested, transferred to a new tube, and brought to 12 ml with DMEM. These cells were lysed of red blood cells, washed by centrifugation, and used for immunofluorescent labeling with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR). Cells isolated from individual brains were examined for their likelihood of recovery, determined by the lack of debris, cell morphology, trypan blue viability, and the final cell number. Final counts per brain ranged from 250,000 to 1,000,000 immune cells comprising T cells, dendritic cells, macrophages, NK cells, some NK T cells, microglia cells, and astrocytes. The best preparations were applied directly to the MVeGFP-infected brain slices at 2,000 immune cells per slice for imaging and 40,000 cells per slice for viral clearance studies.

Hippocampus isolation. Healthy 6- to 10-day-old mice were used for primary organotypic brain cultures. Pups were decapitated, and the skin removed from each head by cutting from the base of the skull up to the nose. Without cutting into the brain, the tip of the scissors was gently slipped under the flap of the skull, which was snipped up toward the nose, and the skull was removed with forceps. The exposed brain was scooped out of the cavity into a petri dish containing filter paper and HBSS-glucose.

The hippocampal region was isolated using a spatula under a dissecting microscope. Dissection and slicing were similar to previous descriptions (24, 72). Positioning the rostral end of the brain with the dorsal surface facing up, several progressive cuts were made approximately 3 mm thick and 45° from the coronal plane of the brain toward the boundaries of each of the lateral ventricles to expose the hippocampus. Nonspecific tissue and the adjacent cortex were removed to further hippocampal separation. The isolated hippocampus, approximately 1 by 2 by 1 mm in size, was then sliced as described below.

Slicing, plating, and culturing of brain tissue. Two sheets of filter paper were placed on the cutting pad of a McIlwain Tissue Chopper (The Mickel Laboratory Engineering Co. Ltd., Guildford, Surrey, United Kingdom), followed by a 5- by 5-cm piece of ethanol-cleaned ACLAR film (Ted Pella, Inc., Redding, CA). A new razor blade was used for each cut (American Safety Razor Company, Verona, VA). Samples with a thickness of 400 μ m were generated while handling the tissue as little as possible. Subsequent to cutting, the slices were submerged in an HBSS-glucose solution in a clean petri dish. To aid the separation of slices, a wide-bore plastic disposable transfer pipette was used to plunge the slices up and down.

Membrane inserts (Minicell-CM; Millipore, Billerica, MA) were placed in each well of a six-well plate with 600 μ l of brain tissue medium underneath the membrane. Brain tissue medium was defined as 50% 1 $\times$ stock minimum essen-

tial medium (Invitrogen), 25% stock 1× HBSS (Invitrogen), 24% horse serum (Invitrogen), and 1% D(+)-glucose (50% stock; Amcos). Employing a transfer pipette, the individual brain slices were dropped onto the membrane using as little HBSS-glucose solution as possible. The slices were then incubated at 37°C in 5% CO₂. Slices in which tissue died in the center because the slices were too thick or wide or touched other slices, which was discernible by opaqueness in phase microscopy, were discarded. Slices were examined daily to monitor their health, as indicated by flattening of the edges. Every 3 days, the old brain tissue medium was removed and replaced with new medium.

Virus infection of brain slices. To introduce a virus infection into the brain explants, ~5 µl (fewer than 10⁴ PFU) of purified MV or MVEGFP was placed on the center of each brain slice using a pipette tip. WNV (10³ PFU) and MHV-A59 (10² PFU) were applied similarly to brain explants. The effects of infection were studied by direct live imaging, harvesting or fixation of tissue, and immunofluorescence imaging with a TE200 inverted microscope (Nikon, Tokyo, Japan). Low-resolution MVEGFP images were collected with a Spot RT digital camera and image acquisition software (Diagnostic Instruments, Inc., Sterling Heights, MI). Brain explants were infected for 3 days before treatment or coculturing.

Imaging of fixed immune cells within MV-infected brain slices. Slices with immune cells added for overnight incubation were fixed in 1% formaldehyde solution and subjected to a series of freezings and thawings in 30% sucrose-PBS. Tissue was processed on the membranes and mounted tissue side down. Virus-infected neurons were detected with an anti-MV matrix (M) protein antibody (Millipore/Chemicon, Temecula, CA) and an Alexa Fluor 488-conjugated secondary (Invitrogen). CD4 and CD8 T cells were probed with rat anti-CD4 and anti-CD8 antibodies, respectively (BD Biosciences Pharmingen, San Diego, CA). Both T cell antibodies were detected with a goat anti-rat Alexa Fluor 594-conjugated secondary antibody (Invitrogen). 4',6-Diamidino-2-phenylindole (DAPI) staining was used to visualize nuclei. Dried slides were imaged on an Olympus IX71 epifluorescence microscope fitted with an automated stage (DeltaVision system), and images were captured in z series on a CoolSNAP HQ digital camera. Out-of-focus light was digitally removed with Softworks deconvolution software (Applied Precision, Inc.).

Migration of immune cells and neutralization of chemokines. Viral infections of adult mice for antiviral leukocyte preparations were coordinated to coincide with the virus infection of the brain slice *ex vivo*. Antiviral leukocytes at 7 dpi were isolated from the adult mice and added to MV-infected organotypic brain slices at 3 dpi. To inhibit the chemokines CCL5 and CXCL10, previously identified as targets for T cell infiltration (57), infected slices were treated with neutralizing anti-CCL5 and anti-CXCL10 antibodies (0.05 mg MAB466 and 5 mg MAB478, in accordance with the manufacturer's neutralization assay instructions; R&D Systems, Minneapolis, MN) or the matched isotype control (MAB006; R&D Systems) in PBS. Antibodies were preincubated for 20 min in a humidified 37°C incubator with 5% CO₂. CFSE-labeled antiviral brain leukocytes were then added to the antibody-treated slices. Slices were viewed to collect fluorescent images over time. The migrating leukocytes were counted from images taken after overnight (16-h) incubation. The same 250- by 325-µm oval was superimposed on a random selection of eight GFP-positive neurons from each antibody treatment. All potential CFSE-labeled cells were counted after image enhancement in Adobe Photoshop CS2 by sharpening the image once and adjusting the brightness to -2 and the contrast to +69.

Calculation of distances and migration velocities. Formaldehyde-fixed slices incubated with brain-derived leukocytes for the previous 16 h were immunostained for either CD4 or CD8 T cell antigen and viral M protein. Six different infected neurons from more than three different slices were chosen at random from each condition, and fluorescence images were collected on a DeltaVision deconvolution microscope. DeltaVision Softwork software (Applied Precision, Issaquah, WA) was used to measure the distances between the T cells and infected neurons. For measurement of distances from the cell soma, T cells were measured from the leading edge to the middle of the neuronal soma. For measurement of distances from infected neurons, the distance between the leading edge of responding T cells and the closest MV-infected neuronal projection was measured. To determine migration velocities, a series of time-lapse images of leukocytes migrating within antichemokine antibody and isotype antibody-treated slices were taken. Images in sequence were used to measure the distance traveled over the time elapsed, and migration velocities were calculated from those values. Migration depth was calculated in a similar manner; however, only the distance traveled from 0 to 2 h postaddition in the z plane was calculated, ignoring the lateral (x- and y-plane) movements used for migration velocities. All distances and velocities were analyzed for the mean and standard deviation of the mean.

RNA harvesting, cDNA generation and RT-PCR. Brain slice tissue was harvested from six-well plates by cutting out individual slices on the membrane and submerging them, without the membrane, in 500 µl Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was extracted in accordance with the manufacturer's instructions with the addition of glycogen during RNA precipitation. One-tenth of each brain slice RNA preparation was used for reverse transcription (RT)-PCR, and primers were designed to specifically amplify MV N gene (3'-ATGG CCACACTTTTAAAGGAGCTTAG-5', 5'-CGGTCCAGAAGTCTGGATCGA G-3') and control hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene (3'-CCCAGCGTCGTGATTAGCGATG-5', 5'-GTGATGGCCTCCCCT CTCCTTCATGAC-3') transcripts or WNV NS3 gene (3'-TGAGATGGCTGA AGCACTGA-5', 5'-ACCCTGTGAGGAGACATCA-3') or MHV-A59 N gene (3'-ACCAGGTGACGGAATTGGGGCT-5', 5'-GGCAACCATGTGGTCAAT AACTGC-3') transcripts. Standards of viral gene fragments were diluted over 8 logs for the calculation of viral copy numbers. These standard curves were used to select the optimal fluorescence for the extrapolation of mean copy numbers. Amplification cycles were as follows: denaturing at 94°C for 45 s, annealing at 55°C for 30 s, and extension at 72°C on a thermocycler (MyIQ iCycler; Bio-Rad, Hercules, CA). Each cycle included quantitation by SYBR green labeling in accordance with the manufacturer's instructions in the iScript One-Step RT-PCR kit (Bio-Rad). At the end of 40 cycles, further extension was done for 5 min at 72°C and an analysis of the DNA melting curve was included. The resultant PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide; amplified fragments were visualized by UV illumination to confirm that the real-time data matched the sample band intensity. Real-time RT-PCR of the samples was used to determine the viral RNA load (N gene fragment amplification). Samples were normalized using HPRT transcript levels to control for variations in brain explant size. Within each experiment, the control group value was expressed as 100% virus replication, and each experimental group was compared to the control to determine the level of inhibition by the treatment.

Cytokine and anticytokine antibody treatments. Brain slices were treated with cytokines (1 ng/ml IL-12 [BD Biosciences Pharmingen] and 100 ng/ml IL-18 [R&D Systems] or 100 U/ml IFN-γ [eBioscience]) 30 min before leukocyte coculture and allowed to incubate for the entire 48 h before samples were harvested for RNA isolation. IFN-γ was blocked using 2.5 µg of a neutralizing antibody (eBioscience). The same concentration of a matched IgG1 antibody (BD Biosciences Pharmingen) was used as a control. The shared p40 subunit was blocked using 6.2 µg of a neutralizing antibody in accordance with the manufacturer's instructions (eBioscience) and compared to equal concentrations of a matched IgG2a control antibody (eBioscience). The p19 subunit of IL-23 was blocked using 12.5 µg antibody in accordance with the manufacturer's instructions (eBioscience) and compared to the matched IgG1 control antibody (BD Biosciences Pharmingen).

Jak Inhibitor 1. Jak Inhibitor I (Calbiochem, La Jolla, CA) was dissolved in dimethyl sulfoxide to produce a 1 mM stock and then diluted in brain explant medium to produce working solutions of 0.1 µM, 1.0 µM, and 10 µM. Medium containing inhibitor was added to brain explants at 16 h prior to leukocyte addition.

Flow cytometry and cell sorting. Splenocytes and brain-derived leukocytes were isolated as described above. Cell surface markers were stained with antibodies against CD3, CD11b, CD8, F4/80 (BD Biosciences Pharmingen), CD4, NK1 (eBioscience), and GFAP (Millipore/Chemicon) for 30 min at room temperature and then analyzed using an LSRII (BD Immunocytometry Systems, San Jose, CA). Gates were drawn to exclude dead cells and contaminating red blood cells, and percentages of gated cells were recorded (see Table 1). For CD3⁺ T cell isolation, freshly harvested brain-derived leukocytes were incubated for 30 min at room temperature with a fluorescein isothiocyanate (FITC)-conjugated anti-CD3 antibody (BD Biosciences Pharmingen) and analyzed via FACSaria (BD Immunocytometry Systems). The lymphocytes within the total population were gated and then sorted into two populations by FITC positivity (relative to an isotype control) or FITC negativity.

Statistics. Normalized viral loads from viral inhibition experiments were calculated as the mean and the standard error of the mean (SEM). The percentage of the viral load [i.e., (normalized viral load/mean viral load of MV group) × 100] from each experiment (tissues from the same mouse) was also reported as the mean ± SEM. The difference in the normalized viral load and percent inhibition between two treatments was examined by a mixed-model approach (22, 83). Measurements using the tissues from the same mouse were assumed to be correlated, and an unstructured covariance structure was used for inference. The mixed-model approach takes the variation within the same mouse (tissues from the same mouse) and variation across the treatments into account. All tests were two sided, and *P* values of less than 0.05 were considered significant.

FIG. 1. Migration of antiviral immune cells in virus-infected hippocampal brain explants. (A) Hippocampal slices were infected with MVeGFP, and live fluorescent images were recorded at 2, 5, and 12 dpi (top). Corresponding phase-contrast images are shown below. (B) MV-infected brain slices were fixed at 3 dpi and immunostained for MV M protein (green) and nuclei (DAPI, blue). High-resolution (magnification, $\times 1,000$) deconvolution microscopy of a z stack volume projection reveals infected neurons connected by neurites in the brain explant. (C) CFSE-labeled, brain-derived leukocytes (2×10^3) were imaged live over time within infected (black line) or uninfected (red line) brain slices, and measurements in the z plane were quantified for 2 h immediately after coculture. The average migration depth of >10 cells from seven tissue blocks $\pm$ the standard deviation is shown.

RESULTS

Brain explants support MV infection. To validate organotypic brain explants as a model system of CNS infection by MV and the subsequent leukocyte response, we evaluated the migration and effector function of MV-primed brain-derived leukocytes in an MV-infected brain slice culture. Brain explants from the hippocampuses of neonatal NSE-CD46/BL6 transgenic mice were infected with 2×10^3 PFU of GFPeMV, a GFP-expressing MV (23), applied to the top of the slices. Live images of the GFPeMV-infected brain slices show that infection spread to neurons over the course of days and was sustained for at least 12 days. GFP-positive infection foci were observed throughout each hippocampal brain slice. Initial infections appeared as single fluorescent neurons at 1 and 2 days postinfection (dpi) (Fig. 1A). After 5 dpi, virus spread was visualized as an increase in the number of infected neuronal cells, with two to four GFP-positive clusters of cells, containing two or three cells per cluster, per field (Fig. 1A). GFP expres-

sion depended upon the viral infection level; however, the protein itself was free to diffuse into the nucleus, as shown in Fig. 1A, as it is not linked to a virus polypeptide. Late in infection, viral spread from neuron to neuron was obvious, as all infected cells were connected through neurites to nearby infected cells (Fig. 1A, 12 dpi, and B). At high resolution, the connections between infected neurons (green) were even more apparent within the three-dimensional structure of the brain explant (Fig. 1B). When wild-type MV was used, visualization was accomplished by the use of an anti-MV M protein antibody and an FITC-conjugated secondary antibody (Fig. 1B), which restricted the fluorescence signal to the cytosol. Viral spread between neurons was not attributed to cell-free virus because the applied virus is washed off at 1 h postinfection and MV does not spread via budding between neurons (37). Viral infection was not observed in nontransgenic wild-type brain explants or nonneuronal cell types (data not shown).

Brain-derived leukocytes migrate through MV-infected brain explants. The migration of exogenous leukocytes through virus-infected brain explants was investigated with primed antiviral leukocytes isolated from MV-infected transgenic mouse brains. These leukocytes are a heterogeneous population composed of $22\% \pm 7\%$ CD4 T cells, $17\% \pm 3.5\%$ CD8 T cells, $\sim 15\%$ F4/80-positive cells (macrophages and microglia cells), and $<10\%$ NK cells (Table 1). Monocytes, neutrophils, dendritic cells, and astrocytes make up the remainder of the brain-derived cell population isolated (Table 1). Uninfected and mock-infected mouse brains have no adaptive immune cells and thus were unavailable for use as a control. Two thousand leukocytes labeled with the vital dye CFSE were added to each infected brain slice. Explants were MV infected for 3 days before leukocyte addition to mimic the kinetics of leukocyte infiltration of adult brains after a viral challenge *in vivo* (59). Using high-resolution microscopy, live-cell migration was followed for 2 h after leukocyte addition and quantification of the average depth of cells within brain tissue explants was done for six cells from three separate brain slices (Fig. 1C). By 2 h, leukocytes migrated an average of $80 \mu\text{m}$ into the brain slices, as measured from the top of the infected explants (Fig. 1C, black line). Leukocytes from infected adult brains continued to migrate through slices for at least 2 days (data not shown). Brain-derived leukocytes did not migrate deeply into unin-

TABLE 1. Flow cytometric analysis of the cellular composition of a brain-derived leukocyte population

Cell marker(s)	Avg % of total brain leukocyte population $\pm$ SD ^a	Cell type(s)
CD3	30.30 $\pm$ 3.15	T cells
CD4	22.24 $\pm$ 7.32	CD4 T cells
CD8	16.99 $\pm$ 3.70	CD8 T cells
NK	9.01 $\pm$ 2.45	NK cells
F4/80	15.44 $\pm$ 4.19	Microglia cells, macrophages
GFAP	6.05 $\pm$ 4.62	Astrocytes
CD11b	36.53 $\pm$ 3.48	Monocytes, macrophages, granulocytes
CD4 + F4/80	<1	CD4 ⁺ macrophages

^a Percentages were calculated by determining the number of marker-positive cells and dividing by the total number of cells. Positivity was determined by comparison to an isotype control antibody.

FIG. 2. $CD3^+$ cells within brain-derived leukocytes are required for noncytolytic clearance of MV from brain tissue. (A) Leukocytes (4×10^4) isolated from the spleens of naïve or infected mice or the brains of infected mice were cocultured with MV-infected brain slices (3 dpi, infected with 10^4 PFU). Total RNA was collected 48 h later and assayed for viral transcript level by real-time RT-PCR. MV Nucleoprotein gene transcripts were normalized to HPRT in order to control for variations in explant size. Samples not treated with leukocytes were set to 100% (black bars) and used as a comparison for leukocyte-treated samples (white bars). All bars represent the mean, and error bars indicate the standard deviation (*, $P \leq 0.015$ compared to the untreated control; $n = 5$). (B) $CD3^+$ cells within the brain-isolated leukocyte population were sorted by fluorescence-activated cell sorting. Postsorting, the purity of the $CD3^+$ and $CD3^-$ populations was determined by flow cytometry. $CD3^+$, $CD3^-$, or a 70:30 ratio of $CD3^+$ and $CD3^-$ brain-derived leukocytes (10^4) were added to brain slices at 3 dpi (white bars). Viral transcript levels were quantified and analyzed as described for panel A. Non-leukocyte-treated slices (black bar) were set to 100%. All bars represent the mean, and error bars indicate the standard deviation. (C) Brain explants cocultured with brain-derived leukocytes as described were fixed and costained for MV M and the T cell receptor. DNA cleavage products were end labeled with terminal

deoxynucleotidyltransferase and DIG-labeled nucleotides. DIG-labeled DNA termini were visualized using an FITC-conjugated anti-DIG antibody. As a positive control, the number of apoptotic cells in brain explants 24 h after a 10-min UV light exposure is shown. No significant difference was observed between samples with or without virus or leukocytes. The percentage of apoptotic cells was calculated by counting the apoptotic cells and dividing by the total number of cell nuclei per field of view. Ten fields of view per experimental condition were analyzed. All bars represent the mean, and error bars indicate the standard deviation ($n = 3$).

infected tissue (Fig. 1C, red line), nor did they survive for more than 10 to 16 h within uninfected explants. We hypothesize that the lack of chemokines and cytokines associated with productive infection results in the death of the activated immune cells. Leukocytes from an uninfected brain could not be used as a control, as there are essentially no leukocytes resident in an uninfected brain.

$CD3^+$ T cells clear the virus from organotypic brain explants without cell loss. In order to determine if exogenous leukocytes could clear MV from brain explants, 40,000 Percoll-isolated leukocytes from the spleens or brains of infected adult mice were cocultured with infected explants at 3 dpi. The total RNA was harvested from samples after 48 h. Real-time RT-PCR of the samples was used to determine viral RNA loads (N gene fragment amplification), and the results were normalized against HPRT transcript levels to control for variations in brain explant size. Within each experiment, the control group was expressed as 100% virus replication and each experimental group was compared to the control to determine the level of inhibition. Leukocytes isolated from the spleen of an uninfected mouse had a modest effect on the viral loads within brain explants ($P = 0.01$). Splenocytes from an infected mouse had a greater effect, reducing virus RNA levels to ~70% of those of untreated explants ($P = 0.0002$). Brain-derived leukocytes from an infected mouse were most efficient at clearing MV from brain explants, decreasing the viral load to 45% of that of the untreated control ($P < 0.0001$) (Fig. 2A). Again, leukocytes from an uninfected brain were not available for use as a control.

In vivo genetic experiments highlighted the importance of T cells for the survival of an animal after a viral challenge (59) but were unable to show where and how T cells contributed to viral clearance and ultimately animal survival. To determine if viral clearance by leukocytes in brain tissue was a T cell-dependent event, brain-derived, Percoll-isolated mononuclear cells were stained with an FITC-conjugated anti- $CD3$ antibody and sorted into two populations, a $CD3^+$ population and a $CD3^-$ population (Fig. 2B). A total of 10,000 $CD3^+$ or $CD3^-$ cells or a reconstituted mixture of $CD3^+$ and $CD3^-$ cells was applied directly to infected brain explants and cocultured for 48 h. Quantitative RT-PCR was performed to determine the amount of MV RNA remaining after treatment (Fig. 2B). The $CD3^+$ population of brain leukocytes reduced the viral RNA load to 11% of the untreated-control levels ($P < 0.0001$), while the $CD3^-$ population was not able to decrease viral RNA levels. The reconstituted population, which was 70% $CD3^-$ and 30% $CD3^+$ cells, reflecting the actual distribution of T cells within the brain-derived leukocytes, behaved similarly to

FIG. 3. T cells migrate toward infected neurons and can make contact. The mononuclear fraction of leukocytes from MV-infected mouse brains was added to an MV-infected brain slice. At 24 h later, the slices were fixed and stained for MV M (green) and CD4 or CD8 (red) antigens. (A) A volume projection (magnification, $\times 600$) demonstrates that CD4 T cells are found on the neurites of infected neurons. (B and C) Immunofluorescence reveals that CD4 T cells interact closely with infected neurites (magnification, $\times 1,000$). (D) Immunofluorescence of a CD8 T cell near an infected neuronal soma (left side magnification, $\times 1,000$; right side, inset). (E) Infected brain slices and brain-derived leukocytes ($n = 2,000$) were cocultured for 48 h, and the distance between an infected neuron and all CD4 or CD8 T cells within 30 μm (>10 images per experiment; >5 cells per image) was recorded using DeltaVision software. Bars represent the mean, and error bars indicate the standard deviation (*, $P = 0.002$; $n = 3$).

freshly isolated brain-derived leukocytes, reducing the viral load to 55% of the untreated-control level ($P = 0.001$; Fig. 2B).

Viral clearance occurs without concomitant cell lysis. Non-cytolytic clearance, immune system removal of virus without killing of infected cells, is a hallmark of MV infection and resolution in the brains of NSE-CD46 mice (59). While brain-derived leukocytes are able to clear the virus from brain slice cultures, it was important to determine the fate of infected neurons in the presence of antiviral T cells by assaying for cell death. MV-infected or uninfected organotypic explants were cocultured with freshly isolated brain-derived leukocytes from infected transgenic mice. After 48 h, the tissue was fixed and costained for neuron markers and cell death-associated DNA cleavage products. DNA cleavage products were end labeled by deoxynucleotidyltransferase with digoxigenin (DIG)-labeled nucleotides. DNA termini were visualized using an FITC-conjugated anti-DIG antibody. No significant increase in apoptotic cells was observed in either MV-infected slices or slices treated with brain-derived leukocytes (Fig. 2C). Quantification of microscopic imaging was performed on >10 fields of view from more than five explants.

T cells migrate toward MV-infected neurons. The observation that $\text{CD}3^+$ T cells were the dominant antiviral cell population within brain-derived leukocyte populations led to the use of immunofluorescence to colocalize T cells with MV-infected neurons within brain explants. MV-infected brain explants cultured with brain-derived leukocytes for 24 h were fixed and immunostained to determine the distribution of T cells and virus-infected neurons. CD4 T cells selectively decorate the infected neuronal processes and do not appear to be attracted to the cell body itself ($n = 27$, Fig. 3A to C). A z stack image within an infected brain slice treated with leukocytes captured at a magnification of $\times 400$ highlights the accumulation of individual CD4 T cells (CD4 membrane clusters of a single T cell in red) at infected neuronal processes (MV antigen in green) (Fig. 3A). High-resolution images of CD4 T cells (red CD4 clusters) interacting closely with infected neurites (green MV antigen) are shown as a volume projection in Fig. 3B and as an individual z plane image in Fig. 3C, where the cell body is not in the field of view. In contrast to CD4 T cell localization, CD8 T cells (CD8 clusters in red) migrate closer to infected neuronal cell bodies (MV-infected neurons in

green) (Fig. 3D). Quantification of distances between T cells and neurons for 22 fields of view from eight immunostained brain slices demonstrates that CD4 T cells migrate much closer to infected neurons than CD8 T cells do (Fig. 3E).

Accumulation of leukocytes at sites of infection within brain explants is chemokine dependent. It was previously reported (36, 57) that MV-infected primary neurons express increased levels of chemokine RNAs. Therefore, we investigated the role of chemokines in lymphocyte migration within MV-infected brain slices. As T cells contribute to MV immune protection in brain explants, we focused our study on the T cell-tropic chemokines CCL5 and CXCL10 (16, 25, 57) as possible stimuli for migration within brain tissue. Neutralizing antibodies to each chemokine alone or in combination were added to infected brain slices before coculturing with antiviral leukocytes. Explants were imaged 16 h later, and immune cell infiltration was quantified by demarcating individual neurons with a fixed-area oval, giving a 150- μ m by 100- μ m range around each neuron cell body in which the neuron occupied approximately one-third of the image. The number of infiltrating CFSE-labeled immune cells for at least 10 representative neurons was recorded. Infiltration of leukocytes was dramatically reduced by the addition of antichemokine antibodies. Isotype-treated controls exhibited the highest number of brain-derived leukocytes infiltrating toward MVEGFP-infected neurons, with an average of 42 cells per field surrounding the neuron (Fig. 4A). The average number of leukocytes in each field decreased to 26 with the addition of the CCL5 neutralizing antibody, a decline of 38% ($P = 0.0012$). The CXCL10-specific antibody reduced the number to 14 cells per neuron, a reduction of 66% compared to the isotype treatment ($P < 0.0001$). A mixture of antibodies to neutralize both CCL5 and CXCL10 reduced the average number of cells in each neuron field to 11, a decline of 72% in comparison to the isotype control ($P < 0.0001$). In addition to preventing accumulation, neutralization of CXCL10 and CCL5 in brain slices reduced the velocity of migrating leukocytes by more than 30-fold compared to that in isotype control-treated infected brain explants. Velocity was determined by live, time-lapse microscopy of MV-infected explants cocultured with leukocytes (Fig. 4B; $P = 0.005$). When leukocytes were added to uninfected slices, they did not penetrate the tissue, in part because the uninfected slices did not synthesize the tested chemokines (Fig. 1C). These results indicate that locally secreted immune chemokines attract adaptive immune cells to virus-infected target cells within CNS tissue.

Leukocyte proximity to neuronal targets is not a requirement for clearance. Once it was determined that the migration of leukocytes within infected brain tissue was chemokine dependent, we investigated whether the location of the leukocytes was an important factor in the ability of immune cells to clear the virus. Infected brain slices were preincubated with a neutralizing antibody against CXCL10, the most potent chemoattractant assayed, or an isotype control antibody before antiviral leukocytes were added. Quantitative RT-PCR for MV N was normalized using the gene for HPRT, a housekeeping gene, and compared to the untreated controls. As shown earlier, brain-derived leukocytes clear the virus from brain slices efficiently ($P = 0.01$) (Fig. 4C). Treatment with either anti-CXCL10 antibody or an isotype control antibody does not

FIG. 4. Chemokines promote leukocyte migration within infected brain slices but are not required for leukocyte-mediated viral clearance. (A and B) CFSE-labeled mononuclear leukocytes (2×10^3) from infected brains were added to MVEGFP-infected brain slices. Slices were pretreated for 30 min with neutralizing anti-CCL5 or anti-CXCL10 antibodies alone or in combination or with the matching isotype control antibody. (A) Accumulation of migrating cells in the absence or presence of the neutralizing antibodies was determined by counting the leukocytes that fell within an ~ 150 - μ m-diameter circle around 10 individual neurons for each treatment $\pm$ the standard deviation ($n = 3$). (B) Live cocultures were imaged every 10 min for 8 h. Migration velocities were determined using DeltaVision software by tracking individual cells in three dimensions over time ($n = 3$). (C) Brain-derived leukocytes (4×10^4) were cocultured with MV-infected brain slices alone or in the presence of an anti-CXCL10 neutralizing antibody or the matched isotype control antibody (white bars). Viral RNA was quantified and analyzed as described in the legend to Fig. 2, with untreated control samples normalized to 100% (black bars). All bars represent the mean, and error bars indicate the standard deviation. (*, $P \leq 0.01$ compared to the untreated control; $n = 3$).

abrogate the clearance capacity of the leukocytes (Fig. 4C), indicating that the mechanism of viral clearance does not require the responding leukocytes to be in direct contact with or proximal to the infected neuronal targets. While surprising, this finding is consistent with a lack of neuronal cell death

FIG. 5. IFN- γ is necessary and sufficient for MV clearance from brain explants. Splenocytes or brain-derived leukocytes (4×10^4) isolated from infected adult mice were cocultured for 48 h with MV-infected brain slices alone or with cytokine or anticytokine antibody treatment (white bars). Viral transcripts were quantified and analyzed as described in the legend to Fig. 2, with untreated samples normalized to 100% (black bar). (A) Infected explants were treated with splenocytes, with brain-derived leukocytes (Leuk) alone, with leukocytes pretreated with IL-12 (1 ng/ml) and IL-18 (100 ng/ml), or with recombinant IFN- γ (100U/ml) (*, $P \leq 0.0002$ compared to the untreated control; $n = 5$). (B) MV-infected brain explants were treated with brain-derived leukocytes alone or with leukocytes with an anti-IFN- γ neutralizing antibody (2.5 μ g) or an isotype control antibody (white bars) (*, $P = 0.005$ compared to the untreated control; $n = 3$). (C) MV-infected brain slices were treated with brain-derived leukocytes alone or with leukocytes with neutralizing antibodies against p40 (6.2 μ g) or p19 (12.5 μ g) or the matched isotype control antibodies (white bars) (*, $P \leq 0.001$ compared to the untreated control; $n = 3$). NS, no statistically significant difference.

during viral clearance and suggests a role for cytokine-mediated mechanisms (2, 28, 56).

IFN- γ is necessary and sufficient for viral clearance. Due to the importance of IFN- γ in animal health during MV challenge (59) and our observation that T cells mediate viral clearance without obvious direct cell contact, the role of IFN- γ in brain tissue was determined. MV-infected organotypic brain explants were cultured with brain-derived leukocytes to determine the baseline level of viral inhibition. Brain-derived leukocytes were also cultured with infected explants in the presence of the IFN- γ stimulating cytokines IL-12 and IL-18. With IL-12 and IL-18 stimulation, the leukocytes cleared the virus more efficiently than leukocytes without stimulation ($P = 0.0002$), reducing viral RNA levels to 10% of the untreated-control level (Fig. 5A). Recombinant IFN- γ served as a positive control and, by itself, resulted in a 97% decrease in the viral load (Fig. 5A), indicating that IFN- γ alone is all that is required to clear the virus from MV-infected CNS tissue. It was also established that IFN- γ is the mediator of brain-derived leukocyte viral clearance. Brain-derived leukocytes reduced viral loads by 60% when cocultured with MV-infected explants alone ($P = 0.005$) or with an isotype control antibody ($P = 0.005$). When cocultures were treated with an anti-IFN- γ neutralizing antibody, viral RNA levels did not decrease by more than 20%, emphasizing the necessity of IFN- γ in the leukocyte-mediated antiviral response (Fig. 5B).

IFN- γ -mediated viral clearance from the brain slice is dependent upon IL-12 function. In accordance with cytokine release independent of target cell contact, IFN- γ production and secretion were hypothesized to be stimulated by another effector molecule. The observation that a cocktail of IL-12 and

IL-18 increased the ability of brain-derived leukocytes to clear the virus within explant cocultures (Fig. 5A) implicated one or both cytokines as potential candidates that would stimulate IFN- γ production in the absence of sustained T cell receptor ligation. To test this hypothesis, neutralizing antibodies were employed to block the function of secreted IL-12, a heterodimer of a p35 and p40 chain. Due to a lack of specific neutralizing antibody to p35, an antibody against the p40 subunit was used. Neutralizing the p40 subunit fully abrogated the ability of brain-derived leukocytes to clear the virus from infected explants, while a matched isotype control antibody did not affect clearance (Fig. 5C). To rule out possible cross-neutralization of IL-23, which also uses the p40 subunit of IL-12, an IL-23-specific neutralizing antibody was also tested. Neutralizing p19 (IL-23-specific subunit) did not affect the ability of brain-derived leukocytes to clear the virus from infected explants. These results demonstrate that the anti-p40 effects are fully attributable to IL-12 (Fig. 5C).

IFN- γ -mediated viral clearance is dependent on the Jak/STAT pathway. To identify the pathway involved in propagating the IFN- γ antiviral signal in MV-infected brain explants, recombinant IFN- γ or brain-derived antiviral leukocytes were administered to MV-infected brain explants in the presence of an inhibitor of Jak kinases, Jak Inhibitor I, which, while initially thought to target Jak I, inhibits all Janus kinases (76). The highest dose (10 μ M) of Jak Inhibitor I did not affect explant viability (data not shown) or viral replication within brain explants ($P = 0.64$) (Fig. 6A). The potent antiviral effect of IFN- γ is completely disrupted by the inhibitor at 10 μ M (Fig. 6A), demonstrating the critical role of the Jak/STAT pathway in IFN- γ signal transduction and resulting antiviral

FIG. 6. Janus kinase signaling is required for IFN- γ -mediated viral clearance. (A) MV-infected brain explants were left untreated or treated with 10 μ M Jak Inhibitor I (Jak Inhib), IFN- γ , or both IFN- γ and 10 μ M Jak Inhibitor I. Total RNA was harvested from the explant cultures 48 h later and assayed for viral transcript levels by real-time RT-PCR. (*, $P \leq 0.001$ compared to the untreated control). (B) MV-infected brain explants were cocultured with 4×10^4 brain-derived leukocytes (leuk) isolated from MV-infected adults alone or leukocytes in the presence of various concentrations of Jak Inhibitor I. Total RNA was harvested from the explant cultures 48 h later and assayed for viral transcript levels by real-time RT-PCR (*, $P \leq 0.04$ compared to untreated infected slices; #, $P < 0.05$ compared to leukocyte-treated infected explants; NS, no statistically significant difference).

mechanisms. Furthermore, the reduction of viral RNA observed when infected explants are treated with MV-primed, brain-derived leukocytes, which we show is IFN- γ dependent, can be reversed in a dose-dependent manner by treatment with 1 μ M or 10 μ M Jak Inhibitor I ($P < 0.05$ and $P < 0.005$, respectively; Fig. 6B). These results demonstrate the contribution of the Jak/STAT transduction pathway to the antiviral effects of either recombinant or leukocyte-derived IFN- γ .

MV-primed brain leukocytes exert antigen-independent antiviral activity. The ability of leukocyte-derived and exogenous IFN- γ to clear MV in brain explants suggests that this immune protection is antigen independent. To determine if activated MV-primed leukocytes are able to clear an unrelated virus from brain tissue, MV-primed, brain-derived leukocytes and primed and unprimed splenocytes, as a control, were cocultured with brain explants infected with MHV-A59 or WNV. Total RNA was collected after 48 h of coculturing. Quantitative RT-PCR determined the level of viral RNA within each sample. Brain-derived leukocytes harvested from an MV-infected brain, though naïve to MHV-A59 and WNV, effectively cleared MV, MHV-A59, and WNV from brain tissue ($P < 0.01$ and $P \leq 0.02$; Fig. 7A and B). While less effective than brain-

FIG. 7. Viral clearance mediated by MV-primed, brain-derived leukocytes is antigen nonspecific. (A) Brain-derived leukocytes (Leuko), splenocytes (Spl) isolated from MV-infected adults, naïve splenocytes (4×10^4), and IFN- γ were added to brain explants infected 3 days previously with either MV or MHV. Total RNA was harvested from the coculture 48 h later and assayed for viral transcript levels by real-time RT-PCR (*, $P \leq 0.01$; ##, $P < 0.01$, leukocyte-mediated clearance of MV or MHV [compared to untreated brain slices]). (B) Brain-derived leukocytes (4×10^4) isolated from MV-infected adults or IFN- γ were added to brain explants infected 3 days previously with either MV or WNV. Total RNA was harvested from the coculture 48 h later and assayed for viral transcript levels by real-time RT-PCR (*, $P < 0.001$; #, $P < 0.005$ [leukocyte-mediated clearance of MV or WNV, respectively, compared to that from untreated brain slices]; $n = 3$). ND, levels not detected.

derived leukocytes, and not statistically significantly different from the control, splenocytes from MV-infected mice also reduced MV, as expected, and MHV-A59 RNA in explants ($P < 0.01$ and $P < 0.09$, respectively, Fig. 7A). Naïve splenocytes did not reduce MV or MHV-A59 RNA in explants, indicating a requirement for initial activation of cells for specific and non-specific viral clearance (Fig. 7A). Furthermore, consistent with the critical role of IFN- γ in the clearance of MV, direct administration of recombinant IFN- γ dramatically reduced MV, MHV, and WNV RNAs ($P \leq 0.005$; Fig. 7A and B).

DISCUSSION

The classical depiction of an antiviral adaptive immune response includes the activation of helper CD4 T cells to mobilize the immune system and the subsequent entry of cytolytic CD8 T cells into the affected tissue to eliminate virus-infected cells (51). However, viral clearance from the CNS, which is composed of nonrenewable resident cells, is likely to be quite distinct (44, 64). In this report, we demonstrate that primed T cells are able to clear MV from infected neurons of the CNS. Brain-derived CD4 T cells make intimate contact with MV-infected neurons in CNS tissue, while CD8 T cells remain in abeyance, positioned greater than 10 μ m from the infected cell. Interestingly, while T cells are attracted to infected neurons by the local synthesis of chemokines and migrate as ex-

pected, chemokines are not required for leukocyte-mediated viral clearance. Thus, the juxtaposition of leukocytes in proximity to infected neurons is not critical for their antiviral function. This clearance is, instead, IFN- γ - and IL-12 dependent in that recombinant and leukocyte-derived IFN- γ exhibits a potent, antigen-independent antiviral activity in CNS tissue. IFN- γ -facilitated viral clearance is mediated via activation of the Janus family of tyrosine kinases. We therefore propose that the T cell plays a direct role in noncytolytic antiviral immunity in the CNS.

While *in vivo* studies are elegant, they are confounded by the diverse and varied populations of responding cells and inflammatory mediators that make up an immune response and thus such studies cannot distinguish between direct and indirect mechanisms of viral clearance (17, 27, 31, 32, 47). In addition, *in vivo* studies do not allow a facile study of the migration of specific immune effector cells through an organ because of an inability to access the tissue experimentally in real time. The shortcomings of an *in vivo* study can be overcome by working in three-dimensional tissue explants populated with resident cell types in their native microenvironment. Studies of host-pathogen interactions, cellular interactions within complex organs, cell migration, and regeneration have all benefited by advancing from a two-dimensional cell monolayer to three-dimensional tissue systems (1, 48, 52, 66, 86). Virus infection of cultured murine brain explants provides a means to investigate the migration of immune cells to infected neurons in an intact-tissue model. Brain slices are moderately easy to culture, survive *ex vivo* for over a month, and are permissive to infection by viruses of many unrelated families.

The results reported here confirm previously published work done *in vivo* (59) and extend it by demonstrating that both T cells and IFN- γ directly contribute to viral clearance from the brain in an IL-12- and Jak-dependent manner. Experiments with immune knockout mice had shown that T cells and IFN- γ are important for animal survival of infection; however, it was impossible to conclude whether IFN- γ has an immunologically supportive role or an active antiviral role. Similarly, it remained unclear where the cytokine was acting. Neutralization studies with infected brain explants demonstrate that leukocyte-mediated viral clearance is directly dependent upon IFN- γ within the CNS.

This is not the only example of noncytolytic viral clearance from the CNS. In many neurotropic viral infections, activated T cells and IFN- γ play pivotal roles in viral clearance (8, 67). Specifically, in another murine model of encephalitis, clearance of Sindbis virus from neurons occurs in an IFN- γ -dependent manner (9, 13). Lymphocytic choriomeningitis virus (LCMV) is effectively cleared from CNS neurons by T cells secreting IFN- γ (78, 79). MHV has also been shown to be susceptible to IFN- γ antiviral function and, further, is dependent upon T cell function in the brain (5). In fact, the virulence of MHV strains can be correlated with their ability to resist IFN- γ (70). MHV infects nonneuronal cells of the CNS as well, and it was found that IFN- γ was able to effectively clear the virus from oligodendrocytes (38), indicating that IFN- γ plays a pivotal role in viral clearance when the preservation of cells or tissue is advantageous for the survival of the animal, such as with neurons or in the delicate structure of the brain.

IFN- γ binds to the IFN- γ receptor (IFN- γ R), which consists

of two subunits and is expressed on many cell types, including neurons (73). Both subunits are required for signal transduction and associate with Jak1 and Jak2 (73). Upon IFN- γ engagement and Jak-mediated phosphorylation of the IFN- γ R, STAT1 (signal transducers and activators of transcription 1) transcription factors, in turn, are phosphorylated by Jak (73), causing STAT1 to dissociate from the receptor and form homodimers, which translocate to the nucleus, bind specific elements in gene promoters called gamma-activated sequences (GAS) (30), and initiate gene expression. GAS regulatory elements have been identified in more than 200 genes (73). Signals from the IFN- γ R can, less commonly, be propagated by heterotrimers of STAT1, STAT2, and IFN response factor 9 (IRF-9) (43). This STAT1/2/IRF-9 trimer is a transcription factor called ISGF-3 (IFN-stimulated gene factor 3) which, upon nuclear translocation, binds IFN response element sequences in targeted promoters (10, 43). The alternative signaling by IFNs also requires initial Jak function. Our findings that a universal Jak inhibitor reverses the ability of either recombinant or brain-derived leukocyte IFN- γ to clear MV confirms the requirement of IFN- γ R signaling and defines the mechanism by which IFN- γ signaling functions during CNS viral clearance.

IFN- γ affects immune responses by activating proteins regulating antigen processing and presentation (69). IFN- γ stimulates major histocompatibility complex (MHC) class I expression and function by increasing proteosomal degradation and peptide loading (69) and MHC class II chain expression and presentation capabilities in professional and nonprofessional antigen-presenting cells (7, 40, 69). IFN- γ also induces the expression of many genes considered to be antiviral. For example, IFN- γ induces protein kinase R, a double-stranded RNA (dsRNA)-activated kinase that phosphorylates eIF-2 (eukaryotic translation initiation factor 2), thereby rendering eIF-2 nonfunctional and blocking all protein synthesis in the cell (45, 74). IFN- γ also induces the expression of the antiviral dsRNA-specific adenosine deaminase (ADAR) (60, 69). ADAR catalyzes the deamination of adenosine in dsRNA to form inosine, which is read by the ribosome as a guanine, thereby facilitating nucleotide changes in viral RNA. This aberrant editing of viral RNA results in decreased viral protein synthesis and the production of noninfectious viral particles (60). The functions of many other IFN- γ -inducible genes remain to be elucidated.

Similar questions concerning the direct and sole contribution of T cells also surface from the *in vivo* results. By sorting leukocytes into CD3⁺ T cells and CD3⁻ non-T cells and testing the antiviral capacity of these two populations, we determined that 10,000 T cells alone are able to clear the virus better than 40,000 cells of a mixed population of leukocytes (of which ~10,000 are T cells [Table 1]). Interestingly, a mixed population of 7,000 CD3⁻ non-T cells and 3,000 CD3⁺ T lymphocytes was able to clear the virus at the same efficiency as 40,000 unsorted leukocytes, suggesting that the number of cells used in our studies is far higher than what is required to achieve significant viral clearance. It was somewhat surprising that the CD3-negative population was unable to clear the virus, given the relatively high number of NK cells (Table 1), which are also normally able to produce IFN- γ . Perhaps the mechanism of leukocyte priming in the MV-infected brain initiates

IFN- γ secretion specific to the T cell. One mechanism for inducing IFN- γ synthesis from T cells is exposure to the cytokine IL-12, which is known to be secreted by both astrocytes and microglia cells (19).

This study also extends the current field of knowledge by demonstrating that leukocyte-mediated MV clearance is dependent upon IL-12, suggesting that noncytolytic viral clearance is brain-derived cytokine induced, as well as cytokine mediated. The requirement for IL-12 induction of IFN- γ may explain our observation that leukocyte proximity to infected neurons is not necessary for viral clearance. IL-12 was inhibited by an anti-p40 antibody. However, the p40 subunit is shared between IL-12 and IL-23; thus, we included an experimental condition where IL-23-specific subunit p19 was also neutralized. IL-23 neutralization had no effect upon leukocyte-mediated viral clearance, despite reported evidence that IL-23 modulates T cell function in the brain (4, 39). Therefore, the effect of p40 neutralization can be directly attributed to IL-12.

We demonstrate a critical role for IL-12 in cytokine-mediated MV clearance from neurons. We hypothesize that IL-12 is responsible for IFN- γ secretion from responding primed T cells, regardless of the antigen involved or the status of viral antigen presentation. IL-12 is involved in MV clearance from brain tissue. It would be logical to assume that IFN- γ -mediated clearance of both WNV and MHV is also dependent upon IL-12. However, the role of IL-12 in neuronal infection is unclear. IL-12 is induced by many viral infections of the CNS (20, 50, 55) and is a proinflammatory cytokine that plays a role in the development and maturation of a robust Th1, antiviral, adaptive immune response. However, while IL-12, a known inducer of IFN- γ (80), is required for resolution of murine cytomegalovirus infection (53), IL-12 is not critical during LCMV infections, even though IFN- γ function is necessary (54). To determine the exact role of IL-12 in IFN- γ -mediated antiviral function in MV, it will be important to determine the cellular source of IL-12 and what stimuli promote its production. Our experiments investigating the role of IL-12 were not appropriate for the identification of the source of IL-12, as IL-12 could have originated within cells within the leukocyte population or a resident brain cell.

In accordance with an immune mechanism that involves cytokine-mediated viral clearance and cytokine induction of IFN- γ secretion, MV clearance from mouse brain tissue occurs in an antigen-independent manner. Recombinant IFN- γ alone, or MV-primed leukocytes with IFN- γ -secreting potential, was able to clear unrelated RNA viruses (MHV and WNV) from brain tissue *ex vivo*. Thus, activated T cells, capable of secreting IFN- γ , are fully capable of responding to and controlling infections with viruses never before encountered, providing, of course, that these viruses are susceptible to IFN- γ .

A pathogenic response provokes immune cells to infiltrate the infected tissue, although the processes involved vary among organs and individual cell types (63). Chemokines produced by infected neurons act as molecular homing beacons for immune cells to target viral clearance (17, 36, 57). Upon infection with MV, mouse brain neurons secrete T cell-tropic chemokines, as demonstrated *in vivo* and in cultured monolayers of primary neurons (16). Adding to the complexity of the immune response, the T cell-tropic chemokines CCL5 and CXCL10, which have been shown to play roles in the homing of T cells

to virus-infected cells, are also produced by T cells themselves (71). Blocking of these chemokines resulted in a significant decrease in leukocyte migration. However, the antiviral function of these leukocytes was not affected by antichemokine antibody treatment, indicating that leukocytes are able to clear the virus without direct target cell contact. This finding, in addition to the observation that CD4 T cells migrate close to neurites while CD8 T cells pause 10 μ m away from neurons, suggests that MHC class I is not playing an active role in the initiation of an immune response to virus-infected neurons. MHC class I presentation on CNS neurons has not been observed (data not shown), while MHC class II presentation has not been exhaustively studied.

These T cell localization studies suggest that a putative adhesion molecule mediating CD4⁺ T cell contact is expressed predominately on the dendrites of neurons or that the neuronal somas send inhibitory cell contact signals to CD4 T cells. It is also possible that small neurites, not visible by GFP excitation or viral immunostaining, may have halted the migration of CD4 T cells at a fixed distance from the very bright neuronal cell body. In contrast, CD8 T cells were never observed in such proximity or involved in intimate contact, as were CD4 T cells. This may explain the lack of cytolytic activity observed in the brain during MV infection, which contrasts with the visualized migration of CD8 T cells in many other viral infections of the CNS (5, 14, 58, 59, 84). Hence, a protective immune response to MV relies on the secretion of soluble mediators, thereby preserving the delicate microenvironment of brain tissue. Developing means by which we can bias antiviral immunity in the CNS toward a measles-like response may yield novel strategies that minimize the occurrence of virus-induced encephalitis.

ACKNOWLEDGMENTS

S.R.S.P. was supported by NIH grant T32-GM8056. A.D.L. was supported by NIH grants DK054213 and AI083609. This research was supported by the Cytometry and Light Microscopy Core Facility of the Comprehensive Cancer Center of Case Western Reserve University and University Hospitals of Cleveland (P30 CA43703).

We thank members of the S. Brady-Kalnay laboratory, J. Karn, A. Malouf, D. McDonald, P. Fu, D. Canaday, and C. Savarin for their support and time.

REFERENCES

- Abbott, A. 2003. Cell culture: biology's new dimension. *Nature* **424**:870–872.
- Abignani, S. 1997. Bystander activation by cytokines of intrahepatic T cells in chronic viral hepatitis. *Semin. Liver Dis.* **17**:319–322.
- Arpino, C., et al. 2002. Reactivation of human herpesvirus 6 infection in an immunocompetent host with chronic encephalopathy. *J. Infect. Dis.* **185**:1843–1844.
- Becher, B., B. G. Durell, and R. J. Noelle. 2003. IL-23 produced by CNS-resident cells controls T cell encephalitogenicity during the effector phase of experimental autoimmune encephalomyelitis. *J. Clin. Invest.* **112**:1186–1191.
- Bergmann, C. C., C. Ramakrishna, J. M. Gonzales, S. I. Tschen, and S. A. Stohlman. 2006. Coronavirus immunity: from T cells to B cells. *Adv. Exp. Med. Biol.* **581**:341–349.
- Berlin, A. A., C. M. Hogaboam, and N. W. Lukacs. 2006. Inhibition of SCF attenuates peribronchial remodeling in chronic cockroach allergen-induced asthma. *Lab Invest.* **86**:557–565.
- Billiau, A., et al. 1988. Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma. *J. Immunol.* **140**:1506–1510.
- Binder, G. K., and D. E. Griffin. 2003. Immune-mediated clearance of virus from the central nervous system. *Microbes Infect.* **5**:439–448.
- Binder, G. K., and D. E. Griffin. 2001. Interferon-gamma-mediated site-specific clearance of alphavirus from CNS neurons. *Science* **293**:303–306.
- Bluyssen, A. R., J. E. Durbin, and D. E. Levy. 1996. ISGF3 gamma p48, a specificity switch for interferon activated transcription factors. *Cytokine Growth Factor Rev.* **7**:11–17.
- Bossolasco, S., et al. 1999. Human herpesvirus 6 in cerebrospinal fluid of

- patients infected with HIV: frequency and clinical significance. *J. Neurol. Neurosurg. Psychiatry* **67**:789–792.
12. Brooks, D. G., et al. 2006. Interleukin-10 determines viral clearance or persistence in vivo. *Nat. Med.* **12**:1301–1309.
 13. Burdeinick-Kerr, R., D. Govindarajan, and D. E. Griffin. 2009. Nontyrotolytic clearance of Sindbis virus infection from neurons by gamma interferon is dependent on Jak/STAT signaling. *J. Virol.* **83**:3429–3435.
 14. Burdeinick-Kerr, R., J. Wind, and D. E. Griffin. 2007. Synergistic roles of antibody and interferon in nontyrotolytic clearance of Sindbis virus from different regions of the central nervous system. *J. Virol.* **81**:5628–5636.
 15. Cannon, M. J. 2009. Congenital cytomegalovirus (CMV) epidemiology and awareness. *J. Clin. Virol.* **46**(Suppl. 4):S6–S10.
 16. Carr, D. J., J. Chodosh, J. Ash, and T. E. Lane. 2003. Effect of anti-CXCL10 monoclonal antibody on herpes simplex virus type 1 keratitis and retinal infection. *J. Virol.* **77**:10037–10046.
 17. Christen, U., D. B. McGavern, A. D. Luster, M. G. von Herrath, and M. B. Oldstone. 2003. Among CXCR3 chemokines, IFN-gamma-inducible protein of 10 kDa (CXCL10) but not monokine induced by IFN-gamma (CXCL9) imprints a pattern for the subsequent development of autoimmune disease. *J. Immunol.* **171**:6838–6845.
 18. Christensen, J. E., et al. 2009. Fulminant lymphocytic choriomeningitis virus-induced inflammation of the CNS involves a cytokine-chemokine-cytokine-chemokine cascade. *J. Immunol.* **182**:1079–1087.
 19. Constantinescu, C. S., et al. 2005. Astrocytes as antigen-presenting cells: expression of IL-12/IL-23. *J. Neurochem.* **95**:331–340.
 20. Coutelier, J. P., J. Van Broeck, and S. F. Wolf. 1995. Interleukin-12 gene expression after viral infection in the mouse. *J. Virol.* **69**:1955–1958.
 21. Debiase, R. L., and K. L. Tyler. 2004. Molecular methods for diagnosis of viral encephalitis. *Clin. Microbiol. Rev.* **17**:903–925.
 22. Diggle, P. J., K.-Y. Liang, and S. L. Zeger (ed.). 1994. Analysis of longitudinal data, vol. 13. Oxford University Press, New York, NY.
 23. Duprex, W. P., S. McQuaid, L. Hangartner, M. A. Billeter, and B. K. Rima. 1999. Observation of measles virus cell-to-cell spread in astrocytoma cells by using a green fluorescent protein-expressing recombinant virus. *J. Virol.* **73**:9568–9575.
 24. Ehrenguber, M. U., et al. 1999. Recombinant Semliki Forest virus and Sindbis virus efficiently infect neurons in hippocampal slice cultures. *Proc. Natl. Acad. Sci. U. S. A.* **96**:7041–7046.
 25. Glass, W. G., et al. 2004. Antibody targeting of the CC chemokine ligand 5 results in diminished leukocyte infiltration into the central nervous system and reduced neurologic disease in a viral model of multiple sclerosis. *J. Immunol.* **172**:4018–4025.
 26. Gray, F., et al. 1996. Neuropathology of early HIV-1 infection. *Brain Pathol.* **6**:1–15.
 27. Gremion, C., and A. Cerny. 2005. Hepatitis C virus and the immune system: a concise review. *Rev. Med. Virol.* **15**:235–268.
 28. Guidotti, L. G., et al. 1999. Nontyrotolytic clearance of lymphocytic choriomeningitis virus from the hepatocyte. *J. Exp. Med.* **189**:1555–1564.
 29. Gyorkey, F., J. L. Melnick, and P. Gyorkey. 1987. Human immunodeficiency virus in brain biopsies of patients with AIDS and progressive encephalopathy. *J. Infect. Dis.* **155**:870–876.
 30. Horvath, C. M. 2000. STAT proteins and transcriptional responses to extracellular signals. *Trends Biochem. Sci.* **25**:496–502.
 31. Howell, M. D., et al. 2006. Cytokine milieu of atopic dermatitis skin subverts the innate immune response to vaccinia virus. *Immunity* **24**:341–348.
 32. Kamal, S. M., and K. El Sayed Khalifa. 2006. Immune modulation by helminthic infections: worms and viral infections. *Parasite Immunol.* **28**:483–496.
 33. Katayama, Y., H. Hotta, A. Nishimura, Y. Tatsuno, and M. Homma. 1995. Detection of measles virus nucleoprotein mRNA in autopsied brain tissues. *J. Gen. Virol.* **76**(Pt. 12):3201–3204.
 34. Katayama, Y., et al. 1998. Detection of measles virus mRNA from autopsied human tissues. *J. Clin. Microbiol.* **36**:299–301.
 35. Kimberlin, D. W. 2007. Management of HSV encephalitis in adults and neonates: diagnosis, prognosis and treatment. *Herpes* **14**:11–16.
 36. Klein, R. S., et al. 2005. Neuronal CXCL10 directs CD8⁺ T-cell recruitment and control of West Nile virus encephalitis. *J. Virol.* **79**:11457–11466.
 37. Lawrence, D. M., M. M. Vaughn, A. R. Belman, J. S. Cole, and G. F. Rall. 1999. Immune response-mediated protection of adult but not neonatal mice from neuron-restricted measles virus infection and central nervous system disease. *J. Virol.* **73**:1795–1801.
 38. Li, J., Y. Liu, and X. Zhang. 2010. Murine coronavirus induces type I interferon in oligodendrocytes through recognition by RIG-I and MDA5. *J. Virol.* **84**:6472–6482.
 39. Li, Y., et al. 2008. Inducible IL-23p19 expression in human microglia via p38 MAPK and NF-kappaB signal pathways. *Exp. Mol. Pathol.* **84**:1–8.
 40. Mach, B., V. Steimle, E. Martinez-Soria, and W. Reith. 1996. Regulation of MHC class II genes: lessons from a disease. *Annu. Rev. Immunol.* **14**:301–331.
 41. Manchester, M., and G. F. Rall. 2001. Model systems: transgenic mouse models for measles pathogenesis. *Trends Microbiol.* **9**:19–23.
 42. Marques, C. P., S. Hu, W. Sheng, and J. R. Lokensgard. 2006. Microglial cells initiate vigorous yet non-protective immune responses during HSV-1 brain infection. *Virus Res.* **121**:1–10.
 43. Matsumoto, M., et al. 1999. Activation of the transcription factor ISGF3 by interferon-gamma. *Biol. Chem.* **380**:699–703.
 44. McCandless, E. E., B. Zhang, M. S. Diamond, and R. S. Klein. 2008. CXCR4 antagonism increases T cell trafficking in the central nervous system and improves survival from West Nile virus encephalitis. *Proc. Natl. Acad. Sci. U. S. A.* **105**:11270–11275.
 45. Meurs, E., et al. 1990. Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. *Cell* **62**:379–390.
 46. Michael, B. D., et al. 2010. Acute central nervous system infections in adults—a retrospective cohort study in the NHS North West region. *QJM* **103**:749–758.
 47. Munier, M. L., and A. D. Kelleher. 2007. Acutely dysregulated, chronically disabled by the enemy within: T-cell responses to HIV-1 infection. *Immunol. Cell Biol.* **85**:6–15.
 48. Nickerson, C. A., E. G. Richter, and C. M. Ott. 2007. Studying host-pathogen interactions in 3-D: organotypic models for infectious disease and drug development. *J. Neuroimmune Pharmacol.* **2**:26–31.
 49. Norrby, E., and K. Kristensson. 1997. Measles virus in the brain. *Brain Res. Bull.* **44**:213–220.
 50. Novelli, F., and J. L. Casanova. 2004. The role of IL-12, IL-23 and IFN-gamma in immunity to viruses. *Cytokine Growth Factor Rev.* **15**:367–377.
 51. Novy, P., M. Quigley, X. Huang, and Y. Yang. 2007. CD4 T cells are required for CD8 T cell survival during both primary and memory recall responses. *J. Immunol.* **179**:8243–8251.
 52. O'Brien, L. E., M. M. Zegers, and K. E. Mostov. 2002. Opinion: building epithelial architecture: insights from three-dimensional culture models. *Nat. Rev. Mol. Cell Biol.* **3**:531–537.
 53. Orange, J. S., and C. A. Biron. 1996. Characterization of early IL-12, IFN-alpha/beta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection. *J. Immunol.* **156**:4746–4756.
 54. Oxenius, A., U. Karrer, R. M. Zinkernagel, and H. Hangartner. 1999. IL-12 is not required for induction of type 1 cytokine responses in viral infections. *J. Immunol.* **162**:965–973.
 55. Parra, B., D. R. Hinton, M. T. Lin, D. J. Cua, and S. A. Stohlman. 1997. Kinetics of cytokine mRNA expression in the central nervous system following lethal and nonlethal coronavirus-induced acute encephalomyelitis. *Virology* **233**:260–270.
 56. Paschetto, V., L. G. Guidotti, K. Kakimi, M. Tsuji, and F. V. Chisari. 2000. Host-virus interactions during malaria infection in hepatitis B virus transgenic mice. *J. Exp. Med.* **192**:529–536.
 57. Patterson, C. E., J. K. Daley, L. A. Echols, T. E. Lane, and G. F. Rall. 2003. Measles virus infection induces chemokine synthesis by neurons. *J. Immunol.* **171**:3102–3109.
 58. Patterson, C. E., J. K. Daley, and G. F. Rall. 2002. Neuronal survival strategies in the face of RNA viral infection. *J. Infect. Dis.* **186**(Suppl. 2):S215–S219.
 59. Patterson, C. E., D. M. Lawrence, L. A. Echols, and G. F. Rall. 2002. Immune-mediated protection from measles virus-induced central nervous system disease is nontyrotolytic and gamma interferon dependent. *J. Virol.* **76**:4497–4506.
 60. Patterson, J. B., D. C. Thomas, S. L. Hans, and C. E. Samuel. 1995. Mechanism of interferon action: double-stranded RNA-specific adenosine deaminase from human cells is inducible by alpha and gamma interferons. *Virology* **210**:508–511.
 61. Rall, G. F. 2003. Measles virus 1998–2002: progress and controversy. *Annu. Rev. Microbiol.* **57**:343–367.
 62. Rall, G. F., et al. 1997. A transgenic mouse model for measles virus infection of the brain. *Proc. Natl. Acad. Sci. U. S. A.* **94**:4659–4663.
 63. Ransohoff, R. M., A. Glabinski, and M. Tani. 1996. Chemokines in immune-mediated inflammation of the central nervous system. *Cytokine Growth Factor Rev.* **7**:35–46.
 64. Richter, K., J. Hausmann, and P. Staeheli. 2009. Interferon-gamma prevents death of bystander neurons during CD8 T cell responses in the brain. *Am. J. Pathol.* **174**:1799–1807.
 65. Rodriguez-Galan, M. C., J. H. Bream, A. Farr, and H. A. Young. 2005. Synergistic effect of IL-2, IL-12, and IL-18 on thymocyte apoptosis and Th1/Th2 cytokine expression. *J. Immunol.* **174**:2796–2804.
 66. Rossi, M. I., et al. 2005. Multicellular spheroids of bone marrow stromal cells: a three-dimensional in vitro culture system for the study of hematopoietic cell migration. *Braz. J. Med. Biol. Res.* **38**:1455–1462.
 67. Rottenberg, M., and K. Kristensson. 2002. Effects of interferon-gamma on neuronal infections. *Viral Immunol.* **15**:247–260.
 68. Savarin, C., and C. C. Bergmann. 2008. Neuroimmunology of central nervous system viral infections: the cells, molecules and mechanisms involved. *Curr. Opin. Pharmacol.* **8**:472–479.
 69. Schroder, K., P. J. Hertzog, T. Ravasi, and D. A. Hume. 2004. Interferon-gamma: an overview of signals, mechanisms and functions. *J. Leukoc. Biol.* **75**:163–189.
 70. Smith, A. L., S. W. Barthold, M. S. de Souza, and K. Bottomly. 1991. The

- role of gamma interferon in infection of susceptible mice with murine coronavirus, MHV-JHM. *Arch. Virol.* **121**:89–100.
71. **Song, A., T. Nikolcheva, and A. M. Krensky.** 2000. Transcriptional regulation of RANTES expression in T lymphocytes. *Immunol. Rev.* **177**:236–245.
 72. **Stoppini, L., P. A. Buchs, and D. Muller.** 1991. A simple method for organotypic cultures of nervous tissue. *J. Neurosci. Methods* **37**:173–182.
 73. **Tau, G., and P. Rothman.** 1999. Biologic functions of the IFN-gamma receptors. *Allergy* **54**:1233–1251.
 74. **Taylor, S. S., N. M. Haste, and G. Ghosh.** 2005. PKR and eIF2alpha: integration of kinase dimerization, activation, and substrate docking. *Cell* **122**:823–825.
 75. **Tervo, T., et al.** 1986. Abnormal ocular motility as early sign of CNS involvement in HIV infection. *Lancet* **ii**:512.
 76. **Thompson, J. E., et al.** 2002. Photochemical preparation of a pyridone containing tetracycline: a Jak protein kinase inhibitor. *Bioorg. Med. Chem. Lett.* **12**:1219–1223.
 77. **Tiacci, E., et al.** 2000. Fatal herpesvirus-6 encephalitis in a recipient of a T-cell-depleted peripheral blood stem cell transplant from a 3-loci mismatched related donor. *Haematologica* **85**:94–97.
 78. **Tishon, A., M. Eddleston, J. C. de la Torre, and M. B. Oldstone.** 1993. Cytotoxic T lymphocytes cleanse viral gene products from individually infected neurons and lymphocytes in mice persistently infected with lymphocytic choriomeningitis virus. *Virology* **197**:463–467.
 79. **Tishon, A., H. Lewicki, G. Rall, M. Von Herrath, and M. B. Oldstone.** 1995. An essential role for type 1 interferon-gamma in terminating persistent viral infection. *Virology* **212**:244–250.
 80. **Trinchieri, G.** 1993. Interleukin-12 and its role in the generation of TH1 cells. *Immunol. Today* **14**:335–338.
 81. **Tsunoda, I., T. Tanaka, and R. S. Fujinami.** 2008. Regulatory role of CD1d in neurotropic virus infection. *J. Virol.* **82**:10279–10289.
 82. **van den Pol, A. N.** 2006. Viral infections in the developing and mature brain. *Trends Neurosci.* **29**:398–406.
 83. **Verbeke, G. (ed.).** 1997. Linear mixed models in practice: an SAS-oriented approach, vol. 126. Springer-Verlag New York, LLC, New York, NY.
 84. **Verjans, G. M., et al.** 2007. Selective retention of herpes simplex virus-specific T cells in latently infected human trigeminal ganglia. *Proc. Natl. Acad. Sci. U. S. A.* **104**:3496–3501.
 85. **Wang, T., et al.** 2004. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat. Med.* **10**:1366–1373.
 86. **Zhang, S.** 2004. Beyond the petri dish. *Nat. Biotechnol.* **22**:151–152.