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2003 Pioneer in NeuroVirology Review

Future trends in neurovirology: Neuronal survival during virus infection and analysis of virus-specific T cells in central nervous system tissues

Michael BA Oldstone

Division of Virology, Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California, USA

The recent ability to quantitate and localize the interaction of antigen-specific T cells in the central nervous system during infectious and autoimmune diseases will greatly enhance our understanding of the molecular and biologic basis of virus-induced diseases of the nervous and other systems. *Journal of NeuroVirology* (2004) **10,** 207–215.

Keywords: MMHC expression; neurons; TAP expression; TCR: T cell receptor; trafficking of antigen-specific T cells in the CNS1; virus

Setting the stage

Viral diseases of the central nervous system (CNS) have been known since antiquity. Over 2300 years before the first viruses were isolated, Homer mentioned the Greek word for rabies, "lyssa," in *The Illiad* (reviewed in Kaplan and Meslin, 1996; Waterson and Wilkinson, 1978). About 400 years later, Aristotle presented a clear picture of rabies in animals, although he failed to realize that humans could be similarly infected. But not until the 16th century did Fracastoro (1546) provide a clear description of rabies infection of man. Even before those early published depictions of viral disease, the first suspected case of poliomyelitis came from an Egyptian stele dating from the 18th dynasty of 1580 to 1350 BC (reviewed in Koprowski, 1996; Oldstone, 1998). Ancient hieroglyphic records describing the pharaoh Ramses with a limb withered during childhood suggest that poliomyelitis virus infection caused the deformity, although trauma, birth defect, or vascular insufficiency remain as possibilities. Isolation of the rabies and poliomyelitis agents and identification of both as a virus would await the early 20th century. The understanding that disease occurred in organs or tissues such as the brain rather than disturbing the balance among the four humors—fluids of the body: blood, phlegm, and yellow and black bile—derived from anatomic observations of only 233 years ago. So the science of pathology and modern medicine began, as exemplified by the work of Giovanni Battista Morgagni in 1761. His publication, "On the sites and causes of diseases as disclosed by anatomic dissection," compared alterations in the disease-ridden tissues of patients to the state of healthy, normal tissues.

Neurovirology: virus-neuron interactions and major histocompatibility complex (MHC) expression in the CNS

Neurotropic viruses can enter the CNS by two main routes, as outlined in Table 1. Most viruses gain access to the CNS by a hematogenous, or blood-borne route, which requires them to cross from a blood vessel into the brain parenchyma via the blood-brain barrier. These viruses can travel in plasma, bound to cells (i.e., red blood cells) or be contained inside cells (i.e., lymphocytes and/or macrophages). Other viruses, such as rabies, travel in a retrograde fashion along axon processes, transmitted trans-synaptically into the CNS.

Neurons are essential to the host's survival and are irreplaceable once destroyed, the exception being olfactory neurons. Therefore, neurotropic viral infections that directly cause neuronal death, or induce an aggressive antineuronal or antiviral immune response should permanently deplete the body's store of vital cells. Likely through selection and because of their essential role in host survival, CNS

Address correspondence to Michael B. A. Oldstone, MD, Division of Virology, Department of Neuropharmacology, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037, USA. E-mail: mbaobo@scripps.edu

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Table 1 Examples of the route taken by neurotropic viruses to access the central nervous system

Hematogenous	Neural pathway
Herpes simplex	Herpes simplex
Cytomegalovirus	Varicella zoster
Epstein-Barr virus	Rabies
Poliovirus	Poliovirus
Measles	Reovirus
Coxsackieviruses	
Human immunodeficiency virus (HIV)	
Mumps	
Echoviruses	
Lymphocytic choriomeningitis	
Arboviruses	

neurons rarely express class I or II MHC molecules (Horwitz *et al*, 1996, 1997; Joly *et al*, 1991; Keane *et al*, 1992; Mucke and Oldstone, 1992). Some exceptions are a limited number of selected neurons that express MHC molecules at minimal levels (Redwine *et al*, 2001), MHC molecules expressed during neuron development and believed to play a role in synaptic plasticity (Corriveau *et al*, 1998; Huh *et al*, 2000), MHC expression in mature neurons that have lost electrical activity (i.e., dying or dead) (Hausmann *et al*, 2001; Neumann *et al*, 1995), and those identified in graft-versus-host studies (Hickey and Kimura, 1987).

MHC class I molecules must be present on virally infected target cells for their recognition and destruction by cytotoxic T lymphocytes (CTLs). In the absence of MHC antigen presentation, CTLs fail to see virally infected neurons despite the presence of viral proteins on their surfaces. Interestingly, even when interferon (IFN)-γ, a strong up-regulator of MHC molecules is overexpressed in the CNS in experiments that use a transgenic technique (Horwitz *et al*, 1999, 1997), neither MHC class I nor MHC class II protein molecules can be induced on neurons. Moreover, these MHC protein molecules, although not constitutively expressed on either astrocytes or oligodendrocytes, can be markedly induced by IFN-γ only on oligodendrocytes but not astrocytes. Thus, in murine models only vital oligodendrocytes, but not neurons nor astrocytes, would likely have the ability to act as targets for antigen-specific T cells. MHC molecules are constitutively expressed on microglia and blood vessels and up-regulated by IFN. In addition to failing to express MHC proteins on their surfaces, neurons lack other molecules necessary for antigen processing, as shown *in vitro*. When a virus infects a cell, the virus uncoats and synthesizes viral proteins, which are either incorporated into progeny virus or degraded into small peptides of 9 to 11 amino acids, mediated by the proteasome complex. These peptides are then shuttled into the endoplasmic reticulum by TAP1 and TAP2 proteins, where they encounter the host cell–encoded class I MHC: the heavy chain and the light chain (β2-microglobulin). The complex consisting of the class I heavy chain, the β2-microglobulin, and the viral peptide is transported to the cell surface, where the receptor of a specific CTL recognizes the complex, leading to the infected cell's death (reviewed in Whitton and Oldstone, 2001).

Study of OBL-21 neuronal cells *in vitro* indicates that at least two defects have the power to make antigen presentation of viral peptides to the CTLs unlikely (Joly *et al*, 1991; Joly and Oldstone, 1992). These neuronal cells fail to transcribe the mRNA encoding the class I MHC heavy chain and fail to transcribe the mRNA encoding the TAP1 and TAP2 molecules (Figure 1). The consequence is that these cultured neurons are unable to present viral peptide in the context of class I MHC and, therefore, do not serve as targets for CTL-mediated lysis (Joly *et al*, 1991; Joly and Oldstone, 1992). However, when OBL-21 cells are treated with IFN-γ, a molecule that up-regulates endogenous MHC, or are transfected with a retroviral vector encoding a heterologous MHC molecule (D^b), surface expression of the class I MHC molecule occurs so that CTL-mediated lysis becomes possible. Thus, reconstitution of class I MHC *in vitro* restores lysability of neurons by CTLs. However, *in vivo* treatment with excessive amounts of IFN-γ–induced focally in the CNS fails to induce MHC molecules (Horwitz *et al*, 1999, 1997). Further, unlike the situation *in vitro*, complementation of MHC class I molecules into and on neurons by means of transgenic techniques fails to make such virally infected neurons expressing MHC class I molecules susceptible to CTL-mediated lysis *in vivo* (Rall *et al*, 1995).

We generated transgenic mice expressing a class I MHC molecule driven by a neuronal-specific enolase promoter (Rall *et al*, 1995). Our initial thought was that mouse neurons now expressing a functional class I MHC molecule should serve as targets for virus-specific CTL-mediated lysis. Further, such a model would allow one to address questions of sensitized CTLs' movement across the blood-brain barrier and the consequences to a host of expressing MHC molecules in neurons with and without viral infection and in the presence of an antiviral CTL response. The MHC class I D^b mRNA was detected in brains of transgenic mice but not in their other tissues such as thymus, spleen, pancreas, lung, liver, or testes, or in the peripheral nervous system (sciatic and optic nerves). Protein synthesis of the transgene was identified in primary hippocampal neurons with a monoclonal antibody that recognized the correct folding of D^b-containing D^b-restricted peptides. When cultured, isolated neurons were placed onto cover slips precoated either with an antibody directed against the transgene (D^b) or against a heterologous class I MHC gene (L^d), only transgenic neurons in contact with the anti-D^b antibody adhered and aborized (Rall *et al*, 1995). Nontransgenic neurons did not stick to either the anti-D^b– or the anti-L^d–coated slips, and transgenic neurons did not

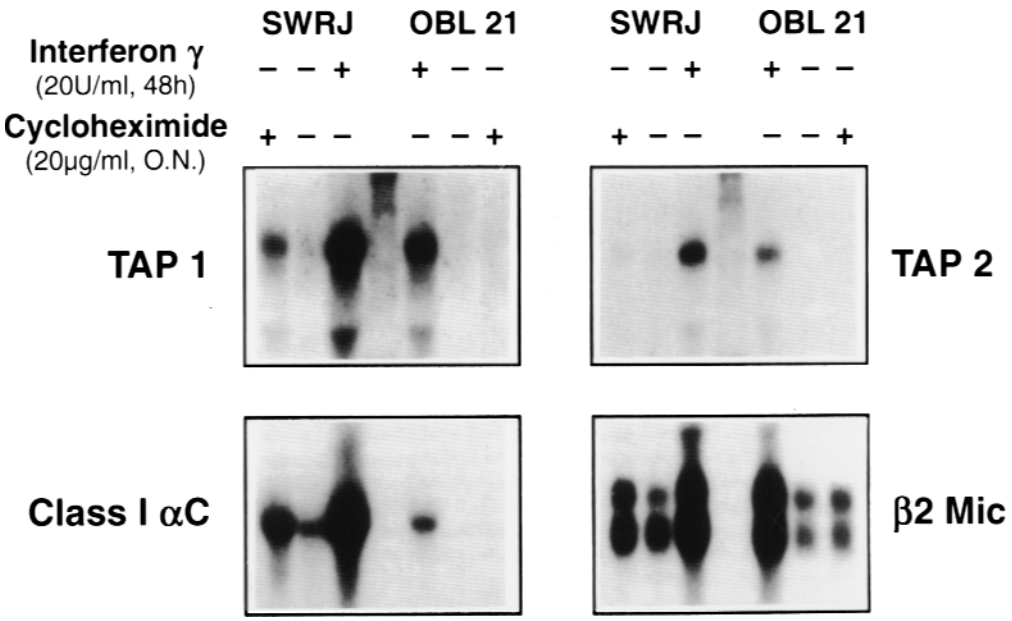

Figure 1 OBL-21 neuronal-like cell line fails to express TAP 1 or 2, or class I MHC heavy chain. This figure shows Northern blot analysis of the levels of mRNA for TAP1, TAP2, and MHC heavy or light (β2-mic) chains. Both TAP1, TAP2, and MHC heavy chain mRNA are induced *in vitro* by IFN-γ in OBL-21 neuronal cells and SWR/J mouse fibroblasts. However, in the absence of IFN-γ, there is no transcription of TAP1, TAP2, or MHC class I heavy chain. See Joly *et al*, 1991; Joly and Oldstone, 1992, for details.

stick to the heterologous (anti-L^d) antibody. When D^b-restricted CD8 CTLs harvested 7 to 8 days after primary infection with lymphocytic choriomeningitis virus (LCMV) or anti-LCMV D^b-restricted CD8 T-cell clones were added to uninfected D^b-expressing neurons, lysis did not occur. In contrast, when these effector anti-LCMV CD8 CTLs were added *in vitro* to D^b-expressing neurons infected by LCMV, virus-specific MHC lysis followed. Hence, results from these experiments performed *in vitro* mirrored those from cultured OBL-21 neuronal cells. Virus-specific lysis of primary neurons by CTLs occurred *in vitro* only when those neurons expressed the MHC restriction class I molecule and were infected by virus.

We then turned our attention to whether CTL-mediated lysis would occur *in vivo* in neurons also infected with LCMV and expressing MHC D^b molecules. Interestingly and importantly, no such lysis by CTLs of virus-infected neurons expressing MHC molecules was detectable *in vivo* (Rall *et al*, 1995). Thus, *in vivo*, neurons have evolved unique strategies to evade immune recognition, thereby allowing viruses to infect and persist, yet resist attack by antiviral CTLs. The molecular basis of how this occurs is not yet clear. Perhaps immunosuppressive molecules are present in the CNS that accomplish this function. One such candidate might be transforming growth factor (TGF)-β. TGF-β is found in heightened concentrations in the brain compared to other tissues outside the CNS (Cook *et al*, 1998). Recent studies from Flavell's laboratory (Gorelik and Flavell, 2001) indicated that TGF-β expression on/by tumors prevents their lysis of anti-tumor CTLs. Once the TGF-β environment is removed, the same CTLs effectively lyse the tumor. However, our preliminary studies with TGF-β knockout mice has not implicated TGF-β in the failure of antiviral CTLs to lyse virus-infected neurons (Tishon and Oldstone, 2003).

The wave of the future: trafficking of antigen-specific T cells into the CNS and study of molecules at the interface of virus-infected CNS cells and antigen-specific T cells *in vivo*

Early informative studies by Hickey (Hickey *et al*, 1991) and Wekerle (Wekerle *et al*, 1986) clearly document the ability of activated CTLs to enter and to percolate through the CNS. These findings, coupled with observations of focal up-regulation of immune regulatory chemokines and cytokines suggest that the immune response could play a role in either the resolution of viral infections of the CNS or in the precipitation of CNS disease. The limitations of these studies were the inability to study and quantitate antigen-specific T cells instead of total T cells (antigen-specific and nonspecific bystander) (Allan *et al*, 1987) and the insufficient resolution of CNS tissue to determine what occurs at the immunologic synapse where the antigen-specific T cells interact with the virus-infected CNS cell. The interaction in need of resolution is shown in Figure 2, where an inflammatory response of lymphoid cells in the CNS following virus infection (upper panels) is currently resolved into CD8 or CD4 T cells in the infiltrate (lower panels) by use of specific monoclonal antibodies and immunohistochemical assay. Yet, as shown in

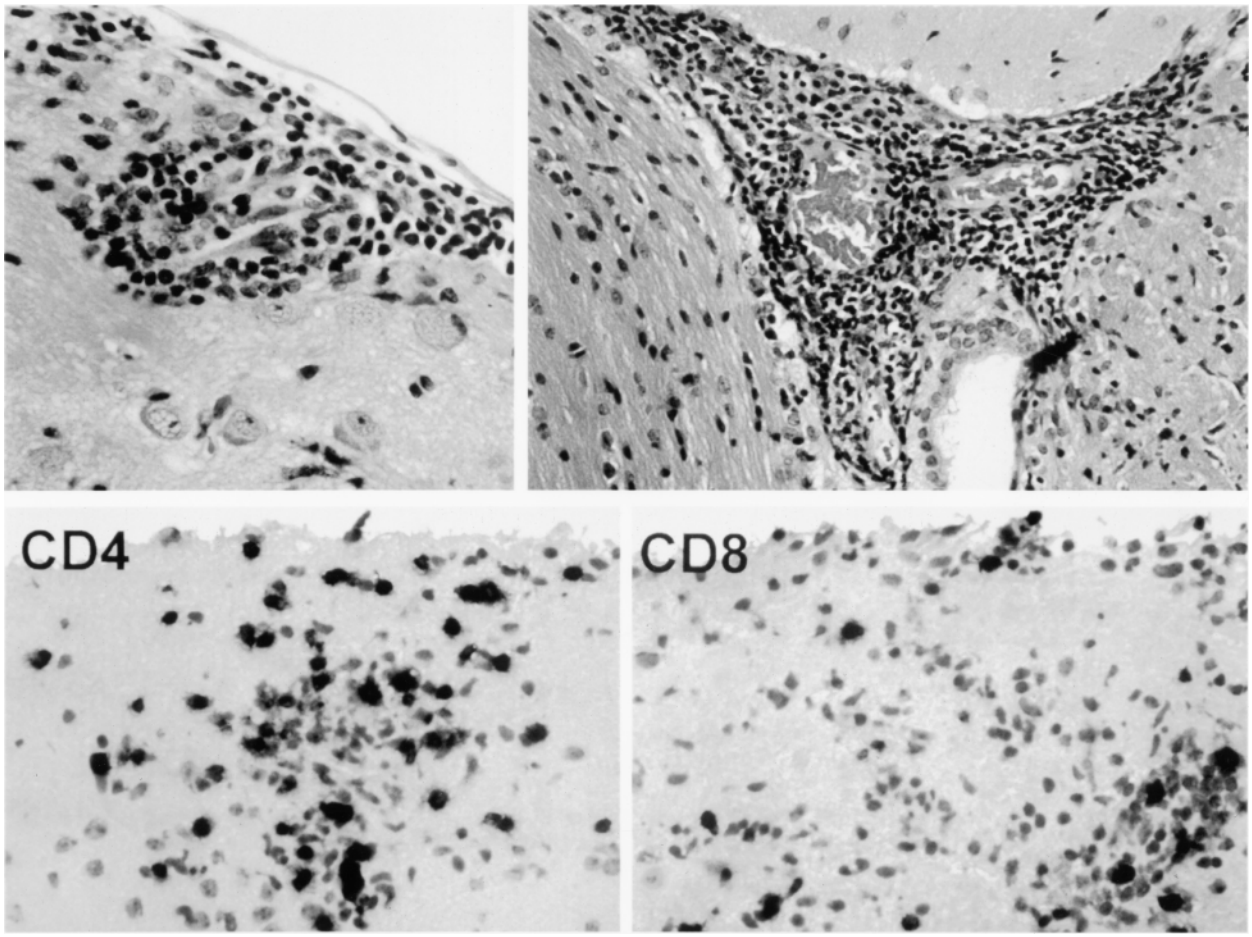

Figure 2 Histologic picture of cells infiltrating the CNS as judged by H and E stain (upper panels) and immunohistochemical analysis of total CD4 or CD8 T cells (lower panels). Missing is knowledge of which of the cells is antigen-specific to the disease process under study as compared to nonspecific T cells or macrophages, NK cells, etc., that are called into the inflammatory nidus.

the lower panel of Figure 3, when the number of antigen (virus)-specific T cells is defined, they make up only a minority, between 2% to 4%, of the total T-cell response to a single immunodominant T-cell epitope (Figure 3) or up to 15% to 20% for all the dominant and subdominant T-cell epitopes restricted by the MHC allele. The importance of these antigen-specific T cells is that, when they are blocked in the appropriate model, immunopathologic disease does not occur. Hence, only these few cells are the focus of our attention, not the vast majority of nonactive, nonparticipating bystander T cells (McGavern and Truong, 2004). It is these antigen (virus)-specific T cells that one must follow in their generation, expansion, and trafficking to the CNS and as they interact with virus-infected cells in the CNS. Identifying the molecules required to turn on or turn off effector function is the challenge of the future. The novel technology developed and refined just over a year ago in 2002 solely by Dorian McGavern in my laboratory (McGavern *et al*, 2002a; McGavern and Truong, 2004; McGavern *et al*, 2002b) (Figure 4) allows the visualization and identification at high resolution of interactions between either antigen (virus)-specific CD8 or CD4 T cells and virus-infected cells *in vivo* (Figures 3, 4).

Earlier strategies to mark antigen-specific lymphocytes as they moved to the CNS or other tissues (Flugel *et al*, 2001; Flugel and Bradl, 2001; Reinhardt *et al*, 2001; Skinner *et al*, 2000) primarily used one of two techniques. The first was *in vitro* manipulation to label T cells of interest, i.e., CD4 T cells that cause allergic encephalitis were transduced with a retroviral vector engineered to express green fluorescent protein (GFP) (Flugel *et al*, 2001). The second implemented a model in which ovalbumin (OVA)-specific CD4 Th1.1 T cells were transferred into Th1.2 congenic mice then treated with a monoclonal antibody to detect the Th1.1 marker (Reinhardt *et al*, 2001). Other studies used tetramers to visualize OVA-labeled T cells (Skinner *et al*, 2000). These studies provided the foundation for the innovative method later developed by McGavern (McGavern *et al*, 2002a) (Figure 5). The strength of McGavern's studies is twofold. First, he has extended the earlier tetramer strategy (Skinner *et al*, 2000) by developing techniques to use six micron sections

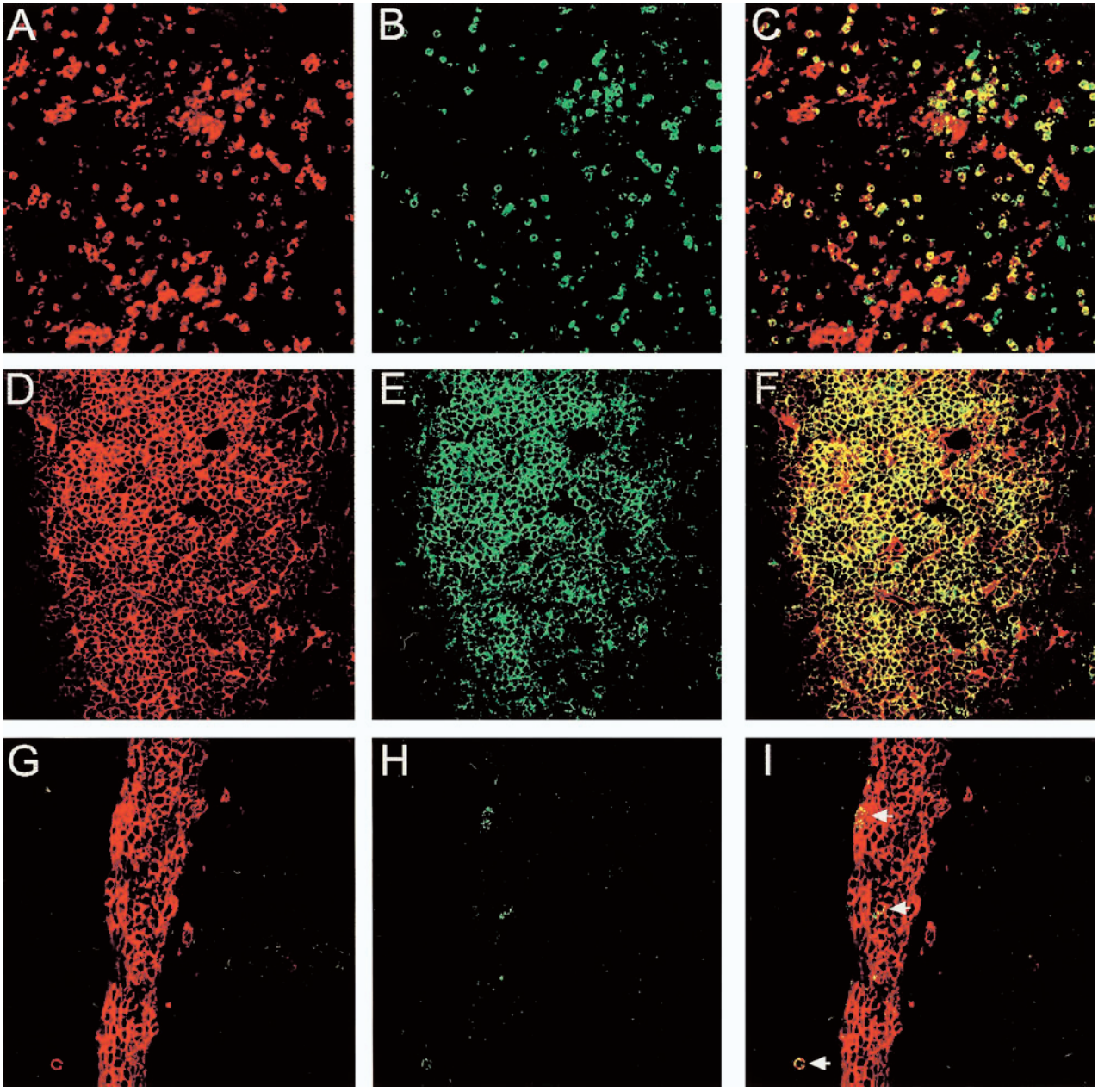

Figure 3 Use of tetramer technique to study antigen-specific T cells in CNS tissues. Visualization of H-2D^b-restricted GP_{33-41}-specific CD8 T cells by use of *in situ* MHC class I tetramer staining. MHC class I tetramers containing the GP_{33-41} peptide of LCMV conjugated to the fluorophore allophycocyanin were used to visualize antigen-specific T cells (green) in tissue sections from GP_{33-41} T-cell receptor transgenic mice (TCR-tg) (**A–F**) or B6 mice infected intracerebrally with LCMV Armstrong (**G–I**). Sections were colabeled with an anti-CD8 antibody (red) and analyzed by confocal microscopy. Tetramer-positive cells were easily visualized in 200-μm unfixed vibratome sections from the spleen of a naïve TCR-tg mouse (**A–C**). However, when compared with 6-μm frozen sections (**D–F**), significant distortions in the anatomy were observed. **A** to **F** were captured in splenic white pulp. Each ring of staining represents a single cell; overlapping fluorescence between tetramer and CD8 cells appears in yellow. Separation between individual cells was observed with vibratome sections. At day 6 after an intracerebral infection with LCMV, GP_{33-41}-specific CD8 T cells were visualized and quantified in CNS meninges, choroid plexus, and ependyma. **G** to **I**, Virus-specific cells (arrows) in dense meningeal infiltrate. Quantitative analyses revealed that during the terminal phase of disease, 4% to 5% of CNS CD8 T cells were GP_{33-41}-specific. Comparable results were obtained by flow cytometry. This percentage reflects only one of seven possible virus-specific CD8 T-cell populations that expand after LCMV infection of C57BL/6 mice. Thus, the total percentage of virus-specific cells in the CNS is higher (15% to 20% total). See McGavern *et al*, 2002a, 2002b, for details.

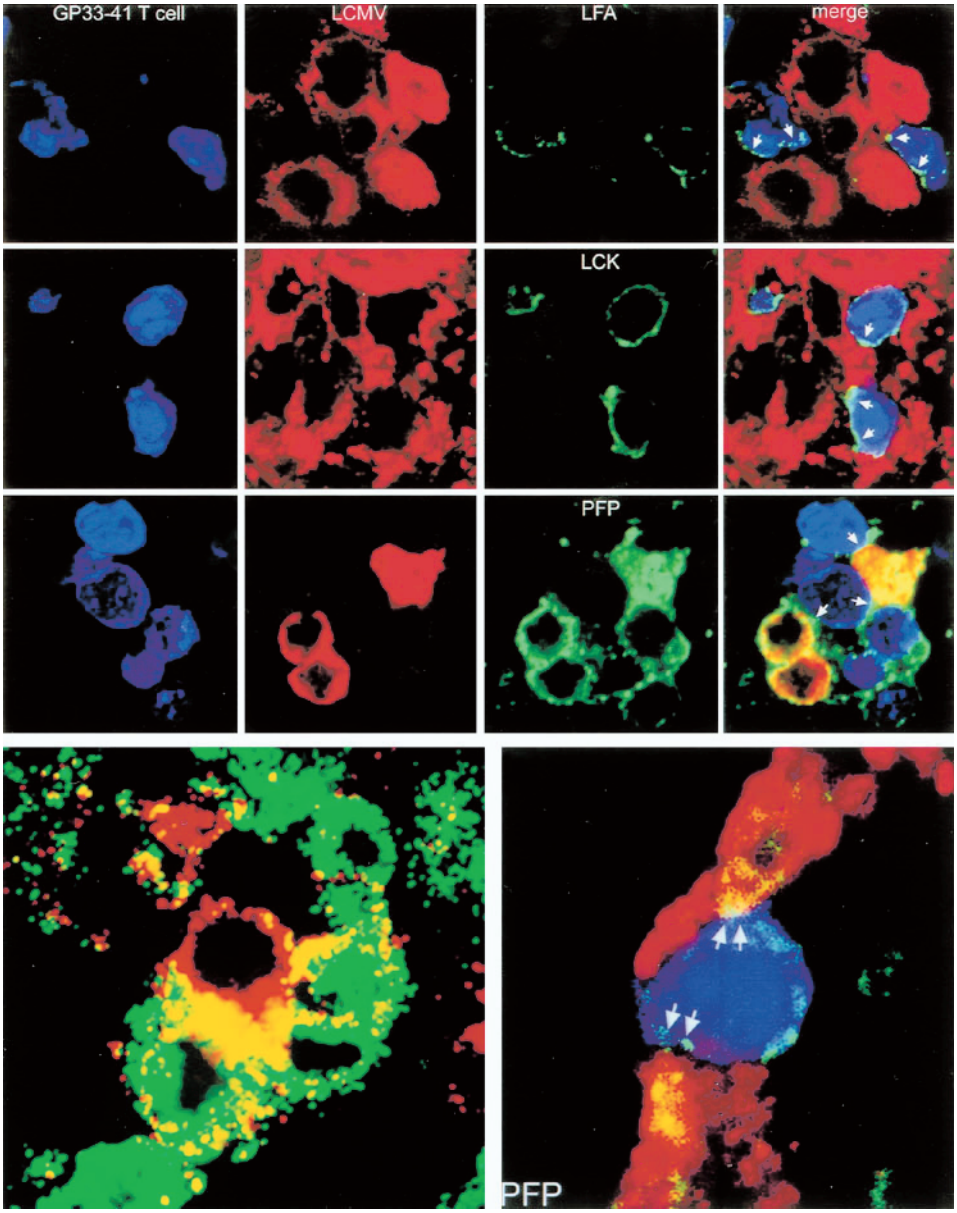

Figure 4 The use of GFP × TCR technique to study antigen-specific T cells in the CNS. The upper three panel rows show the antigen-specific CD8 T cells (first rows, in blue), the presence of viral antigen in CNS infected leptomeningeal cells (second row, in red), and staining for molecules of interest at the immunologic synapse in the CNS: adhesion molecule LFA-1, T-cell activation molecule LCK, and T-cell effector molecule perforin (PFP) (third row, in green) using monoclonal antibodies to these molecules labeled with a fluorochrome dye. The last row (right row) shows the merge. The two enlarged panels at the bottom provide the initial documentation that one antigen-specific CD8 T cells (lower left, in red) can engage multiple, in this case three, virus-infected target cells (lower right, in green). The immune synapse is the yellow color. The large panel on the lower right shows antigen-specific CD8 T cells (blue) that release perforin (PFP: yellow) onto two virus-infected target cells (red). Thus, antigen-specific T cells have a greater hit in this one snapshot in time than previously believed. See McGavern *et al*, 2002a, for details. Our and other *in vitro* kinetic studies have shown that one antigen-specific CD8 T cell can attack and lyse one target, then disengages to attack kinetically multiple targets, also emphasizing the effectiveness of a single antigen-specific T cell. See McGavern *et al*, 2002a, 2002b, for details.

(instead of 200-μ sections used by Skinner *et al*), selecting two dyes that enhance absorption and emission at the same or closely related wavelengths, thereby potentiating the signal and employing confocal microscopy, which dramatically improves the resolution of cells under investigation. The result is higher resolution microscopy than was previously possible, and this enabled fine dissection of the immune synapse *in vivo*. In addition, the invention of the GFP × TCR (T cell receptor) adoptive transfer procedure (Figures 4, 5), in conjunction with a peripheral virus infection (virus recognized by adoptively transferred TCR CD8 or CD4 T cell) allows enormous expansion of antigen (virus)-specific T cells (from <1% of the total T-cell repertoire to >75%). We now have the ability to dissect cellular trafficking and localize

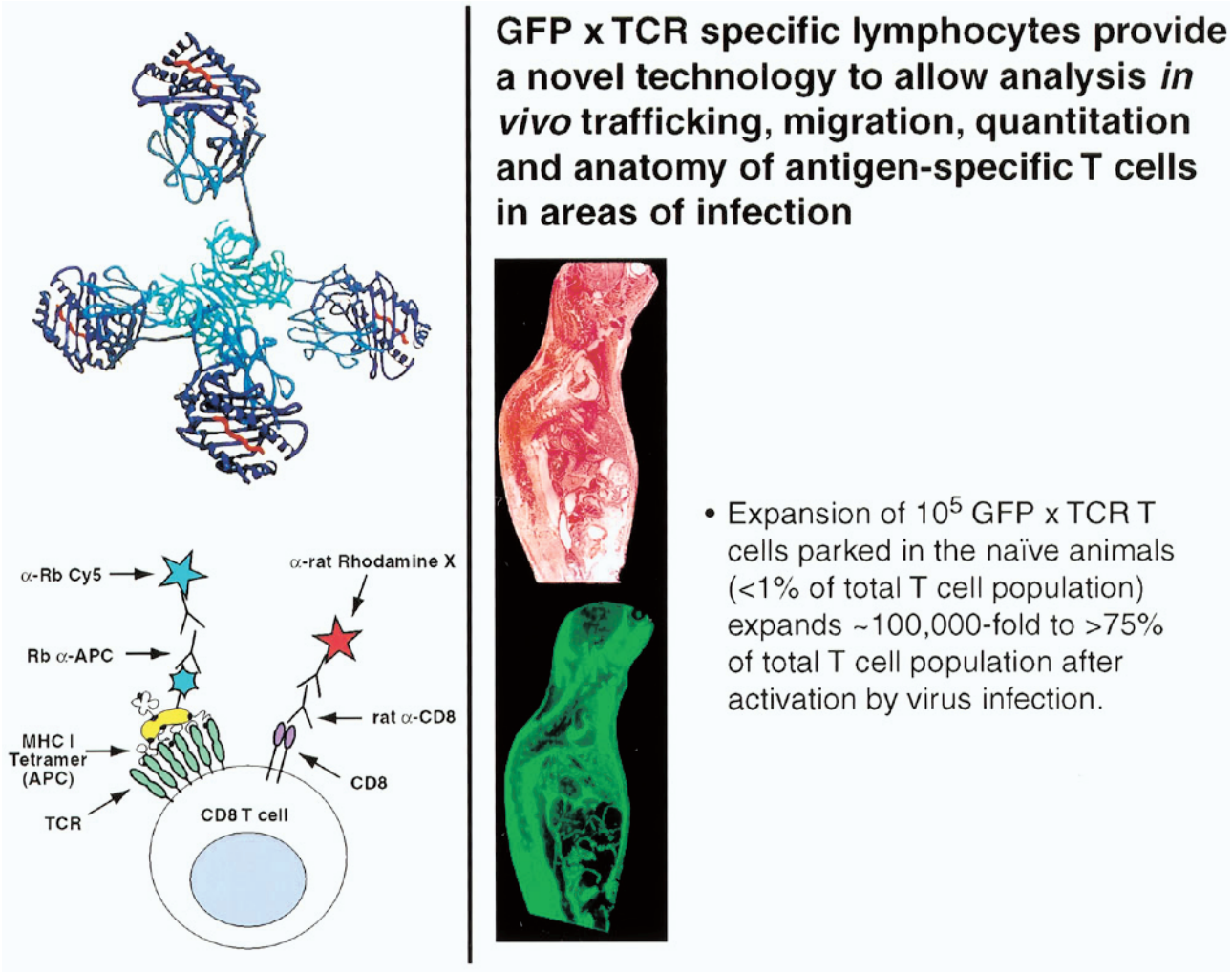

Figure 5 Display of the two techniques described in the legend to Figure 4 that allow detection of antigen-specific T cells *in vivo*. See McGavern *et al*, 2002a, for details.

antigen-specific T cells in any anatomic compartment of interest. Finally, the GFP × TCR assay (McGavern *et al*, 2002b) allows great flexibility for studying a wide variety of mouse models after cloning into the gene of interest the LCMV CD8 or CD4 T-cell epitope. That is, by inserting the LCMV T-cell epitopes into Theiler's virus to study virus-induced demyelination or inserting them into a plasmid causing a tumor for study of immune responses to expanding or contracting tumors. Further, the tetramer assay provides the opportunity to extend the identification of antigen-specific T cells from mouse models to human tissues. Towards the goal of analyzing human diseases, we (McGavern *et al*, 2004) have utilized these procedures to look at hepatitis B–specific CD8 T cells in liver biopsies from subhuman primates and are currently extending our observations to humans with virus infections, tumors, and autoimmune diseases. All that is needed for this epiphany is identification of the T-cell epitope (peptide) and the human leukocyte antigen (HLA) restriction in question.

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Biography

Michael BA Oldstone, MD, was presented the 2003 Pioneer in NeuroVirology Award at the 5th International

Symposium on NeuroVirology, which was held September 2–6, 2003 in Baltimore, Maryland, USA. The International Society for NeuroVirology presented this award of distinction to Dr. Oldstone for his leadership, outstanding scholarly accomplishments, and contributions to the field of neurovirology.

Dr. Oldstone received his medical degree from the University of Maryland School of Medicine, after which he completed his intern and residency training at University Hospital in Baltimore, Maryland. As a postdoctoral fellow, he trained under Dr. Frank Nixon at the Scripps Clinic and Research Foundation in La Jolla, California, currently known as The Scripps Research Institute. Dr. Oldstone is a Professor in the Departments of Neuropharmacology and Immunology at The Scripps Research Institute and the Head of the Division of Virology. The Pioneer in NeuroVirology Award represents an impressive list of honors bestowed upon Dr. Oldstone, among them, the J. Allyn Taylor International Prize in Medicine, Rouse-Whipple Award for Research Excellence, Abraham Flexner Award, and The Cotzias Award for Research Excellence in Nervous System Disease. Additionally, he was elected to the Institute of Medicine, the National Academy of Sciences, and the Association of American Physicians.

For more information and a list of past Pioneers, visit www.isnv.org.