Journal of NeuroVirology, 10: 372–382, 2004

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280490521122

Taylor & Francis
Taylor & Francis Group

Up-regulation of Fas ligand (FasL) in the central nervous system: A mechanism of immune evasion by rabies virus

Leïla Baloul, Serge Camelo, and Monique Lafon

Unité de Neuroimmunologie Virale, Institut Pasteur, Paris, France

Following its injection into the hindlimbs of mice, CVS, a highly pathogenic strain of rabies virus, invades the spinal cord and brain resulting in the death of the animal. In contrast, central nervous system (CNS) invasion by PV, a strain of attenuated pathogenicity, is restricted to the spinal cord and mice infected with this virus survive. Lymphocytes display transient migration into the infected CNS in fatal rabies and sustained migration in nonfatal rabies. The transient migration of T cells in fatal rabies is associated with an increase in T-cell apoptosis. We found that the early production of Fas ligand (FasL) mRNAs was up-regulated only in fatal rabies. FasL is produced by several neuronal cells and mainly in infected neurons. In mice lacking FasL (gld), infection with the neuroinvasive rabies virus strain was less severe, and the number of CD3 T cells undergoing apoptosis was smaller than that in normal mice. These data provide strong evidence that fatal rabies virus infection involves the early triggering of FasL production leading to the destruction of migratory T cells by the Fas/FasL apoptosis pathway. This mechanism could be in part responsible for the fact that T cells cannot control neuroinvasive rabies infection. Thus, rabies virus seems to use an immunosubversive strategy that takes advantage of the immune privilege status of the CNS. *Journal of NeuroVirology* (2004) **10,** 372–382.

Keywords: apoptosis; CNS; FasL; immune evasion; rabies virus

Introduction

Several viruses, including herpes simplex virus type 1 (HSV-1), lymphocytic choriomeningitis virus (LCMV), human cytomegalovirus (HCMV), and mouse mammary tumor virus (MMTV), use immunosubversion to facilitate host invasion (Acha-Orbea and MacDonald, 1993; Alcami and Koszinowski, 2000; Lucas *et al*, 2001; Wall *et al*, 1998). MMTV has been shown to subvert the local immune response (Held *et al*, 1993). This virus manipulates the intestinal mucosal immune system to favor its replication. However, little is known about the nature of the immunosubversion strategies of viruses that infect sites of immune privilege, such as the central nervous system (CNS).

The rules governing immune function are subject to regional tissue specialisation. As well as mucosal tissues that develop a specific immune system, the "eyes/brain and nerves" are regarded as having a different regional immune system (Miller, 1999). The immune status of the CNS is conditional. Inflammation is suppressed in the healthy brain. However, after selective injury or infection of the CNS parenchyma, proinflammatory cytokines and chemokines attract lymphocytes, activated as well as nonactivated, from the periphery and these cells are able to cross the blood-brain barrier (BBB) (Brabb *et al*, 2000; Hickey *et al*, 1991). The action of these cells is limited by several factors, including the destruction of migrating lymphocytes by apoptosis mediated by Fas and

Address correspondence to Monique Lafon, Unité de Neuroimmunologie Virale, Institut Pasteur, 25, rue du Dr Roux, 75724 Paris Cedex 15, France. E-mail: mlafon@pasteur.fr

The present address of Serge Camelo is School of Anatomy and Human Biology, The University of Western Australia, Perth, Australia.

This work was supported by institutional grants from the Institut Pasteur. L.B. is supported by an ARC fellowship. The authors are grateful to Danielle Voegle for the gift of FITC-conjugated anti-CD3 mAb.

Received 23 March 2004; accepted 25 June 2004.

its ligand, FasL, and tolerance induction in the CNS. Constitutive expression of FasL by eye and testis is thought to be a key component of the maintenance of immune privilege of these tissues (Green and Ferguson, 2001; Griffith *et al*, 1995; O'Connell *et al*, 2001). Fas is a molecule of the tumor necrosis factor-alpha (TNF-α) family. Its interaction with FasL drives death signaling in cells bearing FasL (Suda and Nagata, 1994). Fas is expressed on the surface of lymphocytes. Cells resident in the CNS cells, including neurons, produce FasL (Bechmann *et al*, 1999; Flugel *et al*, 2000; Bonetti and Raine, 1997; Dowling *et al*, 1996; D'Souza *et al*, 1996; Kohji and Matsumoto, 2000; French and Tschopp, 1996; Choi *et al*, 1999). FasL expression by neurons is involved in protection against cytotoxic T cells (Medana *et al*, 2001). Thus, lymphocytes entering the CNS could be triggered to death by Fas/FasL apoptosis pathway. Strong evidence for the potential role of FasL as an inducer of T-cell apoptosis has been provided after injury of nerves (Flugel *et al*, 2000) and in experimental autoimmune encephalitis (EAE) (Sabelko-Downes *et al*, 1999). However, contribution of FasL in the migratory T-cell apoptosis has not yet been demonstrated to be the case during CNS viral infection.

Rabies virus (RABV) is an enveloped RNA virus of the rhabdoviridae group that uses the neuronal network to spread within the host. Several strains with different levels of pathogenicity have been selected in the laboratory. Both pathogenic virus strain and those with attenuated pathogenicity are neurotropic and both replicate equally well in neurons. However, pathogenic virus strains cause fatal acute myeloencephalitis, with infection of both spinal cords and brains, whereas abortive infection results in a nonfatal myelitis involving infection of the spinal cord only once the virus is inoculated by a peripheral route of inoculation. We have shown that lymphocytes do not play any role in control of the fatal rabies because the severity of rabies is similar in nude and immunocompetent mice (Camelo *et al*, 2001b). In contrast, lymphocytes play an essential role in the protection against abortive rabies because nude mice injected with attenuated virus strain develop a fatal encephalitic form of rabies (Galelli *et al*, 2000). In this study, we investigated why T cells cannot control acute rabies infection by analyzing (1) the kinetics of T-cell migration into the CNS of mice infected with CVS and PV; (2) the kinetics of virus and FasL mRNA production by real-time polymerase chain reaction (PCR) in spinal cord extracts from mice infected with PV or CVS; (3) the production of FasL by infected neurons; (4) the apoptosis of CD3 T cells in mice deficient in functional FasL (gld); and (5) the severity of acute rabies in FasL-deficient mice.

We show *in vivo* that a pathogenic virus that successfully invades the nervous system triggers the upregulation of FasL, the absence of which decreases virus pathogenicity. This suggests that pathogenic strains of virus highly adapted to the CNS could have selected immune deviation as a strategy to favor their invasion of the CNS.

Results

Characterisation of CNS invasion and disease caused by a highly pathogenic RABV strain, CVS, and a less pathogenic strain, PV, in BALB/c mice

The development of the clinical signs induced in BALB/c mice by infection with CVS and PV viruses was analysed as a function of CNS invasion. After injection of 10^7 p.f.u of the CVS or PV strain of RABV in both hind legs of the mice, mean clinical score (aggravation of the disease; Figure 1A), mortality (Figure 1B), and levels of rabies-specific genomic RNA in the spinal cord (Figure 1C) and the brain (Figure 1D) were monitored until day 10 or 13. In CVS infection, the first signs of hind limb paralysis appeared as early as day 5 p.i. (Figure 1A). The development of paralysis occurred simultaneously and progressed with the invasion of the spinal cord (Figure 1C). RABV-specific genomic RNA levels peaked at days 5 to 7, decreased thereafter, and were no longer detected after day 9. In PV-infected mice, the kinetics of hindlimb weakness and paralysis development were similar to those in CVS-infected mice, but sequence of events began 1 day later. Nevertheless at day 7 post infection, the spinal cord was comparably infected by either PV or CVS virus (not shown).

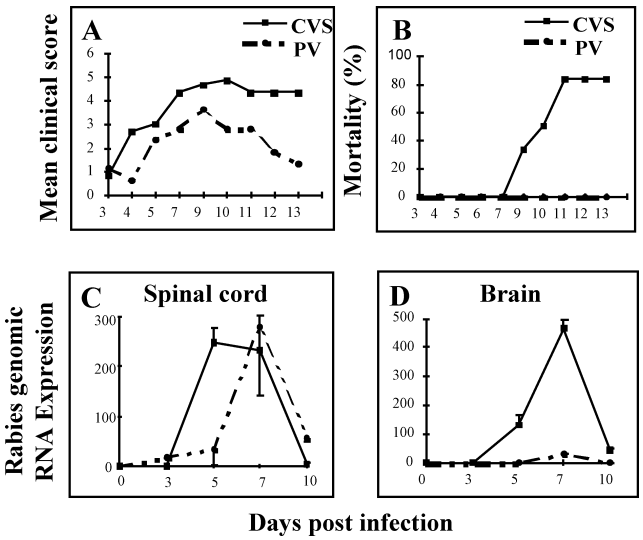

Figure 1 Characterisation of CVS and PV infection in BALB/c mice. After i.m. injection of 10^7 p.f.u of RABV strain CVS or PV into both hind-legs, (**A**) morbidity (n = 6) and (**B**) mortality (n = 6) were recorded daily. Invasion of BALB/c spinal cord (**C**) and brain (**D**) by CVS (*solid line*) and PV (*dotted line*) was measured by real-time PCR. Relative RABV nucleoprotein genomic RNA expression, expressed as $2^{-\Delta\Delta Ct}$ (see Materials and Methods) was evaluated at days 0, 3, 5, 7, and 10 post infection in spinal cord and brain of either CVS or PV infected mice compared to noninfected mice. Each point represents the arithmetic mean of results from three to four mice. Error bars indicate the SD.

By day 9, considerable aggravation of clinical signs was observed in CVS-infected mice, with the onset of hunchback and prostration, followed by generalized paralysis and death by day 11. In contrast, the clinical signs improved or remained at a constant level in PV-infected mice, which suffered irreversible paralysis but survived. In PV infection, brain invasion was limited to few Purkinje cells in the cerebellum (Galelli *et al*, 2000), whereas at the same time infection by CVS was widespread in the cerebellum and the brain of CVS-infected mice was entirely infected (not shown). The fact that PV infection produces only limited infection in the brain whereas CVS successfully invades the entire CNS suggests that these two viruses differ in their intrinsic capacity to invade the brain or to be controlled by the immune response.

Kinetics of cell infiltration and apoptosis in the CNS of CVS-infected mice

A previous study in our laboratory showed that in PV-infected mice, $CD4^+$ and $CD8^+$ T cells migrated into the CNS, with $CD8^+$ T cells outnumbering $CD4^+$ T cells from day 10 onwards (Galelli *et al*, 2000). In this study, the kinetics of CNS infiltration in PV- and CVS-infected mice was analyzed by measuring the number of mononuclear cells collected on a percoll gradient (PV, Figure 2A, and CVS, Figure 2B). In the CNS (brain and spinal cord) of both PV- and CVS-infected mice, the number of mononuclear cells had begun to increase by day 3 post infection (p.i.) This indicates that an early infiltration of mononuclear cells occurs in CVS as well as in PV infection, and thus the lack of immune control of CVS did not result from a lack of migratory T cells. However, in the CVS-infected CNS, the number of infiltrating mononuclear suddenly decreased after day 6, resulting in much lower numbers of these cells by day 9 p.i. (Figure 2B). In contrast, in PV infection, the number of infiltrating T cells increased steadily after day 6 (Figure 2A). These data indicate that after 6 days of infection, $CD3^+$T cells infiltrating the CNS have very different fates in CVS- and PV-infected mice.

To test whether the decrease in the number of infiltrating mononuclear cells in CVS infection could

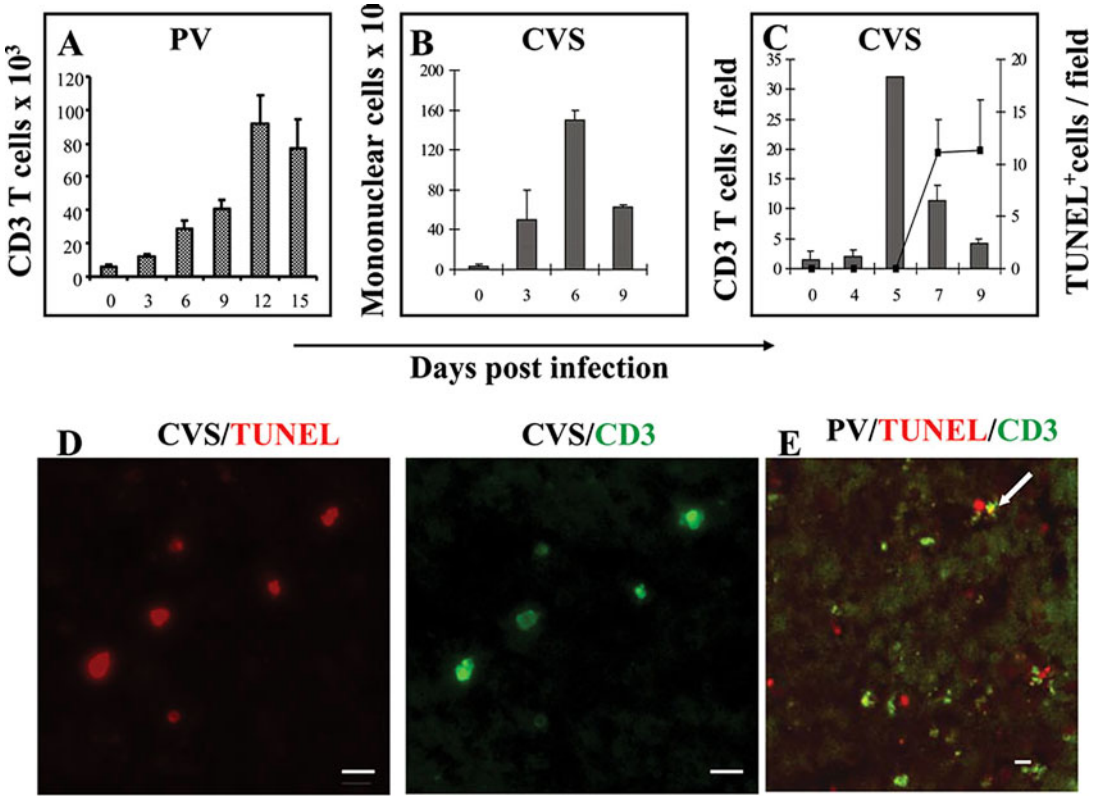

Figure 2 Transient infiltration of mononuclear cells into the CNS of CVS-infected mice. Kinetics of infiltrating $CD3^+$ T cells and mononuclear cells extracted from the CNS of PV (**A**) and CVS (**B**)-infected mice and purified on Percoll gradient. Each point represents the arithmetic mean of data from two to four experiments performed on pooled infiltrating cells isolated from the CNS (brain and spinal cord) from three mice per group. (**C**) Kinetics of $CD3^+$ T cell infiltration (*black histograms, left Y axis*) and apoptotic $TUNEL^+$cells (*line, right Y axis*) in spinal cords sections of CVS-infected mice were analyzed by immunohistochemistry in at least 20 different blinded fields. Reduction of the number of infiltrating $CD3^+$ T cells at days 7 and 9 in CVS-infected mice is concomitant of an increase of the number of cells encountering apoptosis. (**D**) $CD3^+$ T cells encounter apoptosis in the CVS-infected spinal cord as shown by immunohistochemistry. $CD3^+$ T cells (*green*) are $TUNEL^+$ (*red*). This picture is representative of several observations. Photographed with a 40× objective. Bar represents 10 μm. (**E**) Immunohistochemical analysis of spinal cord sections of day 7-PV-infected mice show that with the exception of one cell (yellow), the $TUNEL^+$ cells are not $CD3^+$ T cells (*green*). Bar represent, 50 μm.

result from T-cell apoptosis, the number of terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL)-positive and $CD3^+$ cells were counted in spinal cord sections of CVS-infected mice. The decrease in the number of infiltrating $CD3^+$ T cells on days 6 to 7 coincided with a sharp increase in the number of TUNEL-positive cells (Figure 2C). Immunohistochemical analysis of CVS-infected spinal cords at day 7 p.i. revealed that infiltrating $CD3^+$ T cells (green) stained positive in TUNEL detection (red) (Figure 2D). This indicates that the loss of $CD3^+$ T cells infiltrating the CNS could be due to apoptosis in the CVS-infected spinal cord. In contrast, as shown in Figure 2E, in PV-infected mice, very few $CD3^+$ T cells (green) were costained by TUNEL technique (yellow), indicating that $CD3^+$ T cells (green) rarely encounter apoptosis in PV infection. Additional analysis indicate that TUNEL-positive cells in PV-infected spinal cords sections correspond to neurons (data not shown). The number of $CD3^+$ T cells and TUNEL-positive cells were numbered in 20 fields of spinal cords sections of CVS- and PV-infected mice. In PV-infected spinal cords sections, only 4.8% $\pm$ 0.7% of $CD3^+$cells encounters apoptosis, whereas, 40% $\pm$ 0.4% of $CD3^+$ T cells were TUNEL positive in CVS-infected spinal cord sections. This difference was statistically significant.

Altogether, these results indicate that infiltrating $CD3^+$ T cells encounter apoptosis in CVS-infected spinal cords. This process may account for the decline of $CD3^+$ T cells in the CNS of CVS-infected mice 7 days post inoculation.

Production of FasL mRNA in CVS- and PV-infected mouse CNS

In order to test whether migratory T-cell apoptosis could use the Fas/FasL pathway, the expression of FasL mRNA was compared in spinal cord extracts fom CVS-infected and PV-infected mice. As shown in the left panel of Figure 3A, the CVS infection induced an early production of FasL mRNA (day 5 p.i.) in the spinal cord, whereas PV infection did not. In the CVS-infected spinal cord, FasL and virus mRNA reached a maximum on day 5 p.i., indicating that the up-regulation of FasL is linked to spinal cord invasion. In PV-infected spinal cord, the FasL mRNA was detected only late in infection (day 10 p.i.), at a time at which viral-specific genomic RNA were no longer detected in PV-infected spinal cord extracts. FasL was also detected in PV-infected brains until day 10 p.i. (data not shown) despite the very low level of infection with this virus observed in the brain. Thus, in contrast to CVS infection, the FasL production was not correlated with PV infection and late FasL production observed in PV infection may be linked to a factor other than the infection itself. The kinetics of FasL production and regulation therefore differed considerably between the two types of infections: (i) In CVS infection, FasL mRNA levels peak early and were correlated with infection of the spinal cord,

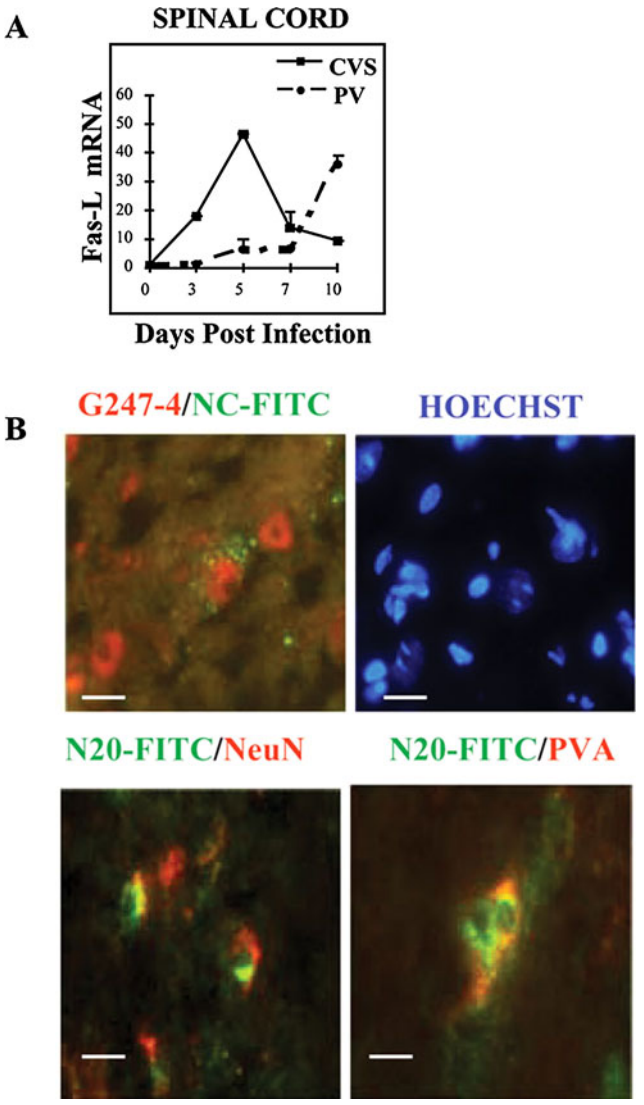

Figure 3 FasL is expressed by neurons in the CNS of CVS-infected mice. (**A**) Kinetics of FasL in the CNS of RABV-infected mice. Relative FasL mRNAs expression was evaluated at days 0, 3, 5, 7, and 10 post infection in spinal cord of either CVS (*solid line*) or PV- (*dotted line*)-infected mice. Each point represents the arithmetic mean of results from three to four mice. Error bars indicate the s.d. (**B**) Expression of FasL by infected neurons was determined by immunohistochemistry. RABV-infected cells (NC positive, *green*) express FasL (mAb G247-4, red) (*upper left panel*). Hoechst stains nuclei in blue (*upper right panel*). Neurons (Ab NeuN, *red*) express FasL (Ab N20, *green*) (*bottom left panel*). RABV-infected cells (PVA-3–positive cells, *red*) express FasL (N20, *green*) (*bottom right panel*). Bar represents 10 μm.

whereas (ii) in PV infection, FasL was produced late in the infection and was not directly associated with the infection.

Expression of FasL by infected neurons in CVS-infected CNS

To test the nature of the cells that produce FasL, spinal cord sections from CVS-infected mice were immunostained with a combination of rabbit antibody

(Ab) N20 or mouse monoclonal antibody (mAb) G247-4 directed against FasL, anti-NeuN (neuron marker), and anti-RABV protein (PVA-3 or NC-FITC) (Figure 3B). The detection of FasL cells (green for N20 and red for G247-4) double-stained with anti-RABV protein Ab (red for PVA-3 and green for NC-FITC) or with Neu-N (red) indicated that, in the course of CVS infection of the spinal cord, neurons and infected cells expressed FasL protein in the cytoplasm. As RABV infects only neurons, we can conclude that infected neurons produce FasL. However, some noninfected cells could also produce FasL (left upper panel in Figure 3B). The observation that both infected and uninfected neurons produce FasL rules out the possibility that the kinetics of FasL mRNA production in CVS-infected CNS reflects T-cell migration alone.

$CD3^+$ T-cell infiltration and apoptosis in the CNS of CVS-infected B6 and gld mice

We observed that migratory $CD3^+$ T cells underwent apoptosis and that FasL protein production was up-regulated during CVS RABV infection. We then investigated whether $CD3^+$ T cells died by apoptosis by determining whether $CD3^+$ apoptosis occurred in the CNS of mice lacking a functional FasL. Groups of gld mice and of normal B6 mice were infected with CVS. The infiltrating $CD3^+$ T cells that underwent apoptosis (TUNEL) were compared in brain sections of CVS-infected B6 and gld mice on day 5 p.i. (Figure 4). Despite similar levels of brain infection (data not shown), we found that there were three times as many $CD3^+$/TUNEL double-positive cells in B6 mice infected with CVS than in infected gld mice (7.28% ± 1.14% versus 22.50% ± 1.8%). The lower levels of T-cell death in mice lacking functional FasL strongly supports the notion that most of the T cells that undergo apoptosis in the CVS-infected CNS follows the Fas/FasL apoptotic pathway.

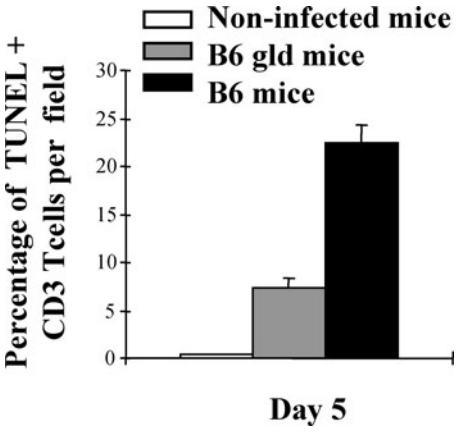

Figure 4 Migrating $CD3^+$ T cells survive in Fasl deficient (gld) mice infected by CVS. Percentage of TUNEL-positive $CD3^+$ T cells was determined by immunohistochemistry in brain sections in noninfected (*white bar*) and in CVS-infected FasL deficient gld (*grey bar*) or B6 mice (*black bar*) at day 5 post infection. Analysis was performed in at least 20 different blinded fields. Results are expressed as means ± SD % of $TUNEL^+$ $CD3^+$ T cells per field.

Characterization of CVS rabies disease in B6 and gld mice

The early expression of FasL, which is up-regulated by CVS infection, is required for the death of migratory T cells. We therefore investigated whether the early up-regulation of FasL was involved in CVS pathogenesis. The level of infection and the production of TNF-α, monocyte chemoattractant protein (MCP)-1, interleukin (IL)-6, and IL-1β mRNAs in the spinal cord were identical in the two types of mice on day 5 (Figure 5A). This indicates that the absence of functional FasL does not interfere with the capacity of CVS to invade the spinal cord or to develop a local inflammatory response. Similarly, FasL mRNA levels were up-regulated in the spinal cord of both CVS-infected B6 and gld mice (Figure 5A). The detection of FasL mRNA in gld mice is not surprising because the gld mice express as much FasL mRNAs as the wild-type mice but the FasL they produce is not functional (Takahashi *et al*, 1994).

The involvement of FasL in CVS pathogenesis was assayed by comparing the morbidity (Figure 5B) and mortality (Figure 5C) of CVS infection in B6 and gld mice from day 3 until day 14. The first signs of hind limb weakness appeared as early as day 5 p.i. and paralysis of one hind leg appeared as early as day 6 p.i. in B6 mice. In contrast in CVS-infected gld mice, there was a 2-day delay in the development of clinical signs. Moreover, the clinical symptoms that developed were less severe in infected gld mice than in their normal counterparts, as mean clinical scores on day 9 were 2.8 for gld mice and 4.5 for B6 (Figure 5B). On day 14 p.i., all the infected B6 mice were dead, whereas only 60% of the infected gld mice had died (Figure 5C). It is noteworthy that CVS-infected gld mice that survived present only transient minor clinical signs of rabies. These data indicate that CVS infection is less severe in FasL-deficient mice than in normal mice and that the deletion of FasL favors the survival of mice infected with CVS. These data strongly support the hypothesis that FasL up-regulation is a key factor in rabies pathogenesis.

Discussion

The aim of this study was to identify the factors that render T cells nonprotective in fatal rabies infection. We found that infection with a highly pathogenic RABV results in the early increase in FasL mRNA levels in spinal cord extracts and the transient migration of T cells into the CNS. We observed that fewer $CD3^+$ T cells undergo apoptosis in CVS-infected gld mice, which display lower rates of mortality and delayed signs, than do normal mice. Altogether, these data suggest that the up-regulation of FasL production could be one component of the immunosubversive strategy of the RABV to reduce the protective immune response.

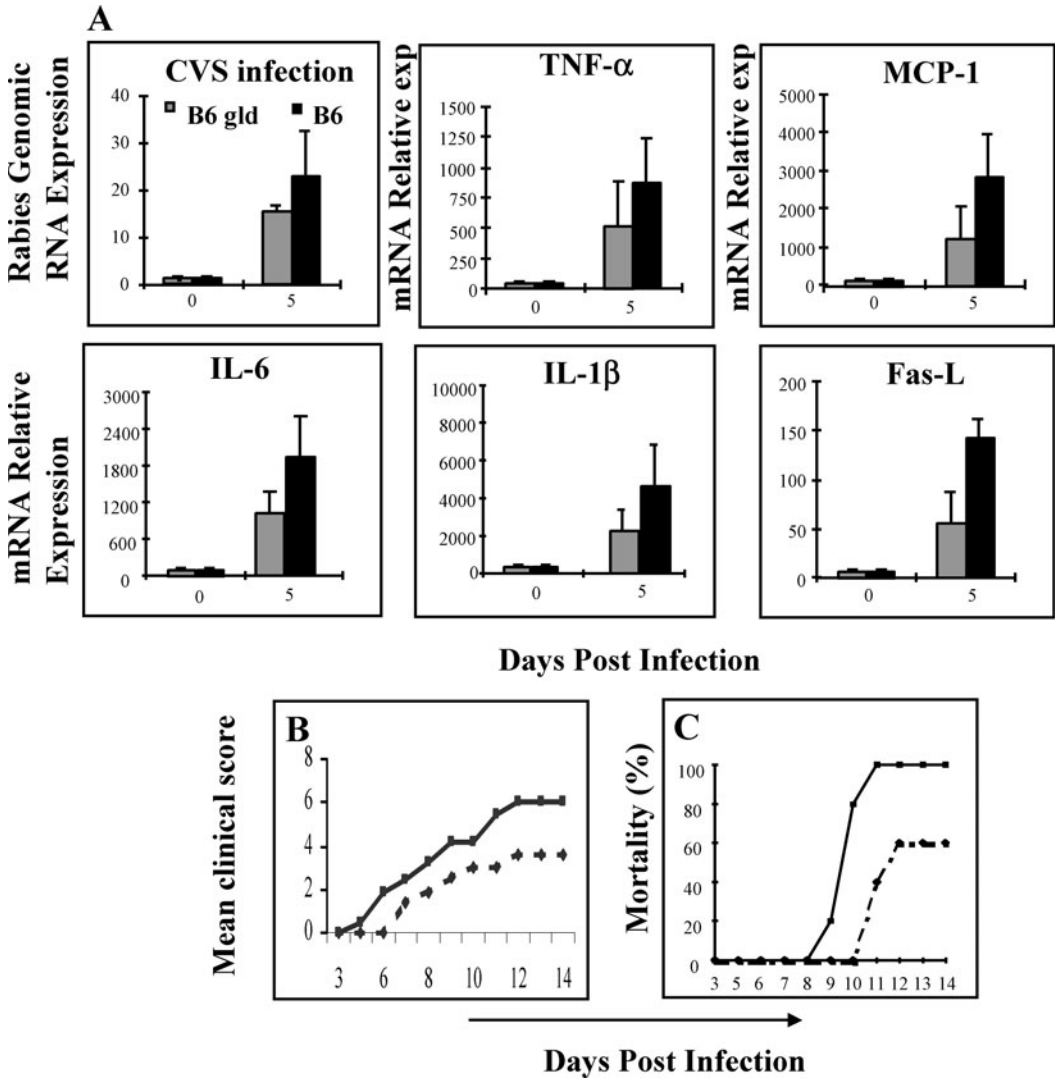

Figure 5 Comparison of CVS virus infection in C57Bl/6 (B6) and FasL deficient (gld) mice. After injection with 10^7 p.f.u of RABV strain CVS by i.m. injection into both hindlegs, (**A**) detection of virus-specific genomic RNA, mRNAs of TNF-α, IL-6, MCP-1, FasL, and IL-1β by real-time PCR in the spinal cord of infected B6 (*black bar*) and gld mice (*grey bar*) at days 0 (noninfected) and 5 post infection. Each bar represents the arithmetic means ± SD of results from 2 mice. (**B**) Clinical score and (**C**) mortality were recorded daily for 14 days. Experiments were performed twice with n = 5 and n = 8 mice in each group. Dotted lines represent morbidity or mortality of C57BL/6. Dashed lines represent data for B6 mice.

The CNS is regarded as an immunologically privileged site in which immune responses are restricted (Griffith *et al*, 1995). However, in pathological situations or in cases of viral infection of the CNS, the immune privilege is weakened. In particular, infections lead to the production of cytokines and inflammatory molecules such as IL-1α-β, IL-6, IL-10, TNF-α, interferons (IFNs), and nitric oxide (NO) (Benveniste, 1997). Pathogenic strains of RABV do not escape this reaction because IL-1β, IL-6, and NO can be detected in spinal cords and brains of CVS-infected mice (Baloul and Lafon, 2003; Camelo *et al*, 2000; Marquette *et al*, 1996; Akaike *et al*, 1995). The triggering of TNF-α mRNA production by CVS infection reported here is consistent with the previous detection of this cytokine by enzyme-linked immunosorbent assay (ELISA) and immunochemistry in the spinal cords and brains of CVS-infected mice (Camelo *et al*, 2000; Marquette *et al*, 1996). TNF-α is known to attract inflammatory cells (Seabrook and Hay, 2001), as illustrated by the lower level of influx of inflammatory cells into the CNS of CVS-infected mice lacking the TNF-α receptor (p55TNFR−/−) than into the CNS of their normal counterparts (B6 mice) (Camelo *et al*, 2001b). In addition, mRNA of MCP-1 chemokine were detected in CVS-infected spinal cord extracts as early as 2 days after virus inoculation (Figure 4 and data not shown). Thus, the neuroinvasiveness of CVS does not result from a lack of early CNS inflammation. Following the injection of CVS into the footpad, the number of popliteal lymph node cells and cytokine-producing cells, particularly of those that produced IFN-γ, increased (data not shown). In addition, increase in spleen cell number during infection

and proliferation of splenocytes following addition of ConA were similar in the first 7 days of the CVS and PV infection (Camelo *et al*, 2001b). Thus the inefficiency of T cells to protect against the CVS-infection is unlikely the result of a lack of immune activation in the periphery. Moreover, the triggering of an immune activation in the periphery during the CVS infection rules out the possibility that CNS of CVS-infected mice was infiltrated by non activated T cells as observed in EAE (Brabb *et al*, 2000).

The divergence between the two types of virus infection happens later on day 7, when a sharp decline in the number of migratory T cells occurs in the CVS-infected CNS, following the peak in FasL mRNA levels on day 5. The destruction of recruited inflammatory cells by apoptosis has been observed in the CNS of mice infected with Sindbis virus (Havert *et al*, 2000) or with Theiler virus (Schlitt *et al*, 2003; Oleszak *et al*, 2003), or in retinas infected with HCMV (Raftery *et al*, 2001) and with RABV (Camelo *et al*, 2001a). The observation in this study that fewer infiltrating $CD3^+$ T cells undergo apoptosis in gld than in B6 mice provides the first evidence that the apoptosis of infiltrating CD3 T cells during viral infection of the CNS occurs by the Fas/FasL pathway.

Lower virulence of CVS infection in mice expressing a nonfunctional FasL, and the higher survival of migratory T cells in these mice, provide evidence that migratory T cells have a protective function. Further evidence that migratory T cells play a protective role is provided by the observation that nude mice do not resist PV infection and develop acute rabies, similar to that induced by the neuroinvasive CVS strain. Cytotoxic IFN-γ $CD8^+$ T cells could be derived from the CNS of mice infected with neurotropic viruses such as the neurotropic strain of mouse hepatitis virus (Marten *et al*, 2003). In rabies, T cells could also be directly involved in cytolysis because they have been found to produce mRNAs encoding IFN-γ, macrophage inflammatory protein (MIP)-1α, IP10, and RANTES (Galelli *et al*, 2000), mediators which have been shown to be directly involved in cytolysis (Biddison *et al*, 1997; Price *et al*, 1999). In addition, T cells may also be protective effectors by secreting IFN-γ, possibly through the antiviral activity of this cytokine (Camelo *et al*, 2000; Hooper *et al*, 1998). Based on our results, we propose a model to account for the success of CVS infection. Following the injection of CVS into the hind limbs, progressive infection of the spinal cord and the brain is accompanied by the production of the inflammatory cytokines and chemokines that attract lymphocytes, resulting in their migration across the BBB. However, CVS up-regulates FasL. The protective T cells specifically triggered in the periphery are inefficient at controlling acute RABV encephalitis because they are destroyed by apoptosis shortly after their entry into the CNS. This facilitates the propagation of the neuroinvasive virus (CVS) through the CNS to the brain, resulting in the death of the mice. Similar mechanism could be evoked to explain why mice lacking functional FasL showed delayed and reduced mortality after infection with the Murray Valley encephalitic flavivirus (Licon Luna *et al*, 2002).

Residual apoptosis of CD3 T cells was observed in gld mice. The percentage of CD3 cells undergoing apoptosis was low, but this may indicate the existence of a Fas-dependent but FasL-independant pathway of apoptosis. Mechanisms of Fas apoptosis that do not require FasL have been described in ultraviolet (UV)-treated keratinocytes and in cancer cells treated with cytotoxic drugs (Aragane *et al*, 1998; Micheau *et al*, 1999). Fas-dependent, FasL-independent apoptosis has also been reported in FasL mutant mice in which TNF-α could replace FasL (Price *et al*, 1999; Suzuki *et al*, 1999). This may account for the death of some of the CVS-infected gld mice, despite the much higher level of protection against rabies infection. In addition, the residual apoptosis of CD3 T cells observed in RABV-infected gld mice could also occur as a result of the partial functional impairment of FasL in gld mice (Karray *et al*, 2004).

Besides the triggering of T cell death by the Fas/FasL pathway, other factors can participate to render T cells nonprotective in fatal rabies. The existence of such factors is strongly supported by the residual death of CVS-infected gld mice. Endogenous immunosuppressive factors are secreted by glial cells, such as transforming growth factor beta (TGF-β), vasointestinal peptide (VIP), alpha-melanocyte-stimulating hormone (α-MSH), or calcitonin gene–related peptide (CGRP) (Irani *et al*, 1997; Stanisz, 1994). Role of TGF-β is unlikely because levels of mRNA of TGF-β in spinal cord extracts of CVS and PV infected mice are very low (Baloul and Lafon, 2003). The implication of such immunosuppressive factors requires further investigations.

We found that together with the up-regulation of FasL mRNA in the CNS, neurons produced FasL protein during the course of CNS infection. These results are consistent with other studies indicating that neurons produce FasL protein (Bechmann *et al*, 1999; Bonetti *et al*, 1997; Dowling *et al*, 1996; Flugel *et al*, 2000; French and Tschopp, 1996). FasL was also produced in non-neuronal cells (NeuN-negative). We cannot exclude the possibility that some of these cells are migratory T cells, which were found to produce FasL as well as Fas, at least in PV infection (data not shown). However, most of the non-neuron cells of the RABV-infected nervous system that produce FasL do not have lymphocyte morphology. They should be glial cells, known to express FasL (Bechmann *et al*, 1999; Bonetti *et al*, 1997; Choi *et al*, 1999; Dockrell *et al*, 1998; Kohji and Matsumoto, 2000). The production of FasL by uninfected cells may be a normal process in the context of immune privilege (Bechmann *et al*, 2000).

The up-regulation of FasL by a virus has been observed in human immunodeficiency virus/simian

immunodeficiency virus (HIV/SIV)-infected macrophages and lymphocytes (Xu *et al*, 1997; Badley *et al*, 1996; Dockrell *et al*, 1998), in feline immunodeficiency virus (FIV)-infected lymphocytes (Mizuno *et al*, 2003), in Epstein-Barr virus (EBV)-infected neutrophils (Tanner and Alfieri, 1999), as well as in HCMV human retinal pigment epithelial cells or dendritic cells (Chiou *et al*, 2001; Raftery *et al*, 2001). Some evidence has been provided that Tat and Nef of HIV-1 directly up-regulates the expression of FasL (Westendorp *et al*, 1995; Xu *et al*, 1997; Ghorpade *et al*, 2003; Li-Weber *et al*, 2000). In the case of RABV infection, similar direct regulation can take place. However, cytokines including IL-6 whose expression is up-regulated in the CNS during RABV infection (Baloul and Lafon, 2003; Camelo *et al*, 2000) can also play a role in this process (Choi *et al*, 2002; Ghorpade *et al*, 2003). Thus it cannot be ruled out that CVS induces FasL production by an indirect mechanism with the virus triggering in cells other than those that it infects. This should be consistent with the observation that noninfected cells express FasL. Whether CVS infection up-regulates FasL production in resident cells of the CNS by a direct or an indirect mechanism deserves further attention.

The destruction of migratory T cells via the Fas-FasL pathway has been demonstrated in previous studies of EAE (Bonetti and Raine, 1997; Dowling *et al*, 1996; D'Souza *et al*, 1996). This study provides the first *in vivo* demonstration that a neurotropic virus modulates FasL production in CNS and that successful neurotropic virus infection may involve a strategy of immunosubversion, taking advantage of the immune privilege status of the CNS and the Fas-FasL apoptosis of migratory T cells in particular.

Materials and methods

Mice, infection, and assessment of clinical signs
Experiments were performed with 6-week-old female mice. C57BL/6 (B6) and C57BL/6-gld (gld) mice, presenting a generalized lymphoproliferative disease due to a point mutation in *FasL*, were from CDTA CNRS (Orléans, France) and BALB/c mice were from Janvier (St. Berthevin, France). Mice were inoculated intramuscularly in both hind legs, with 1×10^7 infectious RABV particles of Challenge Virus Standard, CVS-11 (American Type Cell Collection, Rockville, MD [Vr959], or the Pasteur Virus strain, PV [Lafon *et al*, 1988]). Disease progression was evaluated by scoring mobility and mortality as follows: 0 = normal mice; 1 = ruffled fur; 2 = loss of agility; 3 = one paralyzed hind leg; 4 = two paralyzed hind legs; 5 = total loss of mobility; and 6 = death. At various times after infection, groups of two or three mice were perfused by intracardiac injection of 50 ml phosphate-buffered saline (PBS). Spinal cords and brains were removed separately and stored at −80°C before to be processed for RNA extraction or immunohistochemistry.

RNA extraction and reverse transcription
Total RNA was isolated from the spinal cord and the brain, and reverse transcription was performed as previously described (Galelli *et al*, 2000). RABV genomic RNA was detected by reverse transcription with 1 μM RABV N protein–specific sense primer (Shankar *et al*, 1991).

Real-time PCR
Real-time PCR was carried out with an ABI prism 7700 Sequence Detection System (TaqMan; Perkin Elmer/Applied Biosystems), using the DNA binding dye SYBR Green I. The PCR product was detected directly by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded (ds) DNA. PCR was set up using 12.5 μl of SYBR Green PCR Master Mix (PE Biosystems, Foster City CA, USA), an amount of cDNA equivalent to 100 ng total RNA and 0.5 μM of each primer (Table 1). Gene expression was measured by quantification of the cDNA with respect to the cDNA from uninfected mice as a calibrator. All quantifications

Table 1 Primer sequences for mouse cytokine gene and for the GAPDH and rabies N protein genes

	Name	*Sequence (5′ → 3′)*	*Accession number*
TNF-α	TNF-α+	GTA-TGA-GAT-AGC-AAA-TCG-GC	M13049
	TNF-α−	CTG-AAC-TTC-GGG-GTG-ATC-GG	
IL-6	IL-6+	CCG-GAG-AGG-AGA-CTT-CAC-AG	M20572
	IL-6−	CAG-AAT-TGC-CAT-TGC-ACA-AC	
IL1-β	IL1-β+	GCA-ACT-GTT-CCT-GAA-CTC-A	X04964
	IL1-β−	CTC-GGA-GCC-TGT-AGT-GCA-G	
MCP-1	MCP-1+	AGGTCCCTGTCATGCTTCTG	J04467
	MCP-1−	GTGCTGAAGACCTTAGGGCA	
Fas-L	Fas-L+	CAC-AAA-TCT-GTG-GCT-ACC-G	U06948
	Fas-L−	GCC-CAT-ATC-TGT-CCA-GTA-G	
GAPDH	GAPDH+	CTC-AGT-GTA-GCC-CAG-GAT-GC	M32599
	GAPDH−	ACC-ACC-ATG-GAG-AAG-GCT-GG	
Rabies N protein	Sense	GGA-ATT-CTC-CGG-AAG-ACT-GGA-CCA-GCT-ATG-G	AF033905
	Antisense	AGA-ATT-CCC-ACT-CAA-GCC-TAG-TGA-ACG-G	

Note. Forward (+) and reverse primers (−) were selected from different exons.

were also normalized with respect to an endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative value obtained for quantification is expressed as $2^{-\Delta\Delta Ct}$, where ΔCt represents the difference between the Ct value of a sample and that of GAPDH in the same sample and $\Delta\Delta Ct$ is the difference between the ΔCt value of a sample and that of the calibrator. Because SYBR Green is nonspecific and thus any double-stranded DNA is detected, the formation of specific product was checked by electrophoresis.

Purification of mononuclear cells infiltrating the CNS

CNS mononuclear cells were collected from homogenates of brains and spinal cords samples onto percoll gradients as described (Galelli *et al*, 2000).

Immunohistochemistry and detection of TUNEL-positive cells

Dissected brains and spinal cords were embedded in tissue-Tek OCT compound (Miles, Elkhart, IN, USA). Cryostat sections (8 μm) were fixed in 4% paraformaldehyde (PFA) for 10 min, washed, permeabilized in PBS–0.5% Triton X-100 for 15 min, incubated for 30 min with blocking buffer (5% goat serum and 2% bovine serum albumin (BSA) in PBS) and treated with anti-FasL mouse mAb G247-4 1/50 (Pharmingen Becton-Dickinson Biosciences, Le Pont de Claix, France) or polyclonal rabbit Ab N20 1/200 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse mAb anti-neuronal nuclei, NeuN 1/200 (Chemicon, Temecula, CA, USA) to visualize neurons, mouse PVA-3 mAb 1/100 directed against rabies N protein (Libeau *et al*, 1984), or rabbit Ab conjugated to fluorescein isothiocyanate (FTIC) directed against RABV NC for RABV antigen detection, or anti-CD3-FITC 1/350 for migratory T-lymphocyte staining (gift from D Voegle). Secondary antibody was biotinylated goat anti-rabbit Ab 1/200 (Vector, Biosys, Compiègne, France) for N20, FITC or Cy3-conjugated anti-mouse IgG (Jackson Immuno-Research Laboratories) 1/250 for G247-4, NeuN, or PVA-3 detection. The slides were incubated for 30 min at room temperature with streptavidin-DTAF (1/30) (Immunotech, Marseille, France) for FasL visualization with N20. The Ab N20 has been suspected to give false-positive reactivity on Jurkat T-cell lines (Strater *et al*, 2001), whereas G247-4 specifically recognized FasL and is suitable for immunohistochemistry (Herr *et al*, 2000; Strater *et al*, 2001). In our hands, the reactivity of the two Abs gave similar staining. For the detection of infiltrating apoptotic $CD3^+$ T cells, brain sections were first labelled by the TUNEL technique, as previously described (Gavrieli *et al*, 1992), and then stained with FITC-conjugated anti-CD3 mAb and with Hoechst (catalogue number 33258, 10 μg/ml) (Sigma). The slides were then examined by fluorescence UV microscopy.

Statistical analysis

Data are analyzed with the regular standard deviation and the estimated variance method.

References

Acha-Orbea H, MacDonald HR (1993). Subversion of host immune responses by viral superantigens. *Trends Microbiol* **1:** 32–34.

Akaike T, Weihe E, Schaefer M, Fu ZF, Zheng YM, Vogel W, Schmidt H, Koprowski H, Dietzschold B (1995). Effect of neurotropic virus infection on neuronal and inducible nitric oxide synthase activity in rat brain. *J NeuroVirol* **1:** 118–125.

Alcami A, Koszinowski UH (2000). Viral mechanisms of immune evasion. *Trends Microbiol* **8:** 410–418.

Aragane Y, Kulms D, Metze D, Wilkes G, Poppelmann B, Luger TA, Schwarz T (1998). Ultraviolet light induces apoptosis via direct activation of CD95 (Fas/APO-1) independently of its ligand CD95L. *J Cell Biol* **140:** 171–182.

Badley AD, McElhinny JA, Leibson PJ, Lynch DH, Alderson MR, Paya CV (1996). Upregulation of Fas ligand expression by human immunodeficiency virus in human macrophages mediates apoptosis of uninfected T lymphocytes. *J Virol* **70:** 199–206.

Baloul L, Lafon M (2003). Apoptosis and rabies virus neuroinvasion. *Biochimie* **85:** 777–788.

Bechmann I, Lossau S, Steiner B, Mor G, Gimsa U, Nitsch R (2000). Reactive astrocytes upregulate Fas (CD95) and Fas ligand (CD95L) expression but do not undergo programmed cell death during the course of anterograde degeneration. *Glia* **32:** 25–41.

Bechmann I, Mor G, Nilsen J, Eliza M, Nitsch R, Naftolin F (1999). FasL (CD95L, Apo1L) is expressed in the normal rat and human brain: evidence for the existence of an immunological brain barrier. *Glia* **27:** 62–74.

Benveniste EN (1997). Cytokines: influence on glial cell gene expression and function. *Chem Immunol* **69:** 31–75.

Biddison WE, Kubota R, Kawanishi T, Taub DD, Cruikshank WW, Center DM, Connor EW, Utz U, Jacobson S (1997). Human T cell leukemia virus type I (HTLV-I)-specific CD8+ CTL clones from patients with HTLV-I-associated neurologic disease secrete proinflammatory cytokines, chemokines, and matrix metalloproteinase. *J Immunol* **159:** 2018–2025.

Bonetti B, Pohl J, Gao YL, Raine CS (1997). Cell death during autoimmune demyelination: effector but not target cells are eliminated by apoptosis. *J Immunol* **159:** 5733–5741.

Bonetti B, Raine CS (1997). Multiple sclerosis: oligodendrocytes display cell death-related molecules in situ but do not undergo apoptosis. *Ann Neurol* **42:** 74–84.

Brabb T, von Dassow P, Ordonez N, Schnabel B, Duke B, Goverman J (2000). In situ tolerance within the central nervous system as a mechanism for preventing autoimmunity. *J Exp Med* **192:** 871–880.

Camelo S, Castellanos J, Lafage M, Lafon M (2001a). Rabies virus ocular disease: T-cell-dependent protection is

under the control of signalling by the p55 tumor necrosis factor alpha receptor, p55TNFR. *J Virol* **75:** 3427–3434.

Camelo S, Lafage M, Galelli A, Lafon M (2001b). Selective role for the p55 Kd TNF-alpha receptor in immune unresponsiveness induced by an acute viral encephalitis. *J Neuroimmunol* **113:** 95–108.

Camelo S, Lafage M, Lafon M (2000). Absence of the p55 Kd TNF-alpha receptor promotes survival in rabies virus acute encephalitis. *J NeuroVirol* **6:** 507–518.

Chiou SH, Liu JH, Hsu WM, Chen SS, Chang SY, Juan LJ, Lin JC, Yang YT, Wong WW, Liu CY, Lin YS, Liu WT, Wu CW (2001). Up-regulation of Fas ligand expression by human cytomegalovirus immediate-early gene product 2: a novel mechanism in cytomegalovirus-induced apoptosis in human retina. *J Immunol* **167:** 4098–4103.

Choi C, Gillespie GY, Van Wagoner NJ, Benveniste EN (2002). Fas engagement increases expression of interleukin-6 in human glioma cells. *J Neurooncol* **56:** 13–19.

Choi C, Park JY, Lee J, Lim JH, Shin EC, Ahn YS, Kim CH, Kim SJ, Kim JD, Choi IS, Choi IH (1999). Fas ligand and Fas are expressed constitutively in human astrocytes and the expression increases with IL-1, IL-6, TNF-alpha, or IFN-gamma. *J Immunol* **162:** 1889–1895.

Dockrell DH, Badley AD, Villacian JS, Heppelmann CJ, Algeciras A, Ziesmer S, Yagita H, Lynch DH, Roche PC, Leibson PJ, Paya CV (1998). The expression of Fas Ligand by macrophages and its upregulation by human immunodeficiency virus infection. *J Clin Invest* **101:** 2394–2405.

Dowling P, Shang G, Raval S, Menonna J, Cook S, Husar W (1996). Involvement of the CD95 (APO-1/Fas) receptor/ligand system in multiple sclerosis brain. *J Exp Med* **184:** 1513–1518.

D'Souza SD, Bonetti B, Balasingam V, Cashman NR, Barker PA, Troutt AB, Raine CS, Antel JP (1996). Multiple sclerosis: Fas signaling in oligodendrocyte cell death. *J Exp Med* **184:** 2361–2370.

Flugel A, Schwaiger FW, Neumann H, Medana I, Willem M, Wekerle H, Kreutzberg GW, Graeber MB (2000). Neuronal FasL induces cell death of encephalitogenic T lymphocytes. *Brain Pathol* **10:** 353–364.

French LE, Tschopp J (1996). Constitutive Fas ligand expression in several non-lymphoid mouse tissues: implications for immune-protection and cell turnover. *Behring Inst Mitt***:** 156–160.

Galelli A, Baloul L, Lafon M (2000). Abortive rabies virus central nervous infection is controlled by T lymphocyte local recruitment and induction of apoptosis. *J NeuroVirol* **6:** 359–372.

Gavrieli Y, Sherman Y, Ben-Sasson SA (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* **119:** 493–501.

Ghorpade A, Holter S, Borgmann K, Persidsky R, Wu L (2003). HIV-1 and IL-1 beta regulate Fas ligand expression in human astrocytes through the NF-kappa B pathway. *J Neuroimmunol* **141:** 141–149.

Green DR, Ferguson TA (2001). The role of Fas ligand in immune privilege. *Nat Rev Mol Cell Biol* **2:** 917–924.

Griffith TS, Brunner T, Fletcher SM, Green DR, Ferguson TA (1995). Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* **270:** 1189–1192.

Havert MB, Schofield B, Griffin DE, Irani DN (2000). Activation of divergent neuronal cell death pathways in different target cell populations during neuroadapted sindbis virus infection of mice. *J Virol* **74:** 5352–5356.

Held W, Waanders GA, Shakhov AN, Scarpellino L, Acha-Orbea H, MacDonald HR (1993). Superantigen-induced immune stimulation amplifies mouse mammary tumor virus infection and allows virus transmission. *Cell* **74:** 529–540.

Herr I, Posovsky C, Bohler T, Debatin KM (2000). mAb33 from transduction laboratories specifically binds human CD95-L. *Cell Death Differ* **7:** 129–130.

Hickey WF, Hsu BL, Kimura H (1991). T-lymphocyte entry into the central nervous system. *J Neurosci Res* **28:** 254–260.

Hooper DC, Morimoto K, Bette M, Weihe E, Koprowski H, Dietzschold B (1998). Collaboration of antibody and inflammation in clearance of rabies virus from the central nervous system. *J Virol* **72:** 3711–3719.

Irani DN, Lin KI, Griffin DE (1997). Regulation of brain-derived T cells during acute central nervous system inflammation. *J Immunol* **158:** 2318–2326.

Karray S, Kress C, Cuvellier S, Hue-Beauvais C, Damotte D, Babinet C, Levi-Strauss M (2004). Complete loss of Fas ligand gene causes massive lymphoproliferation and early death, indicating a residual activity of gld allele. *J Immunol* **172:** 2118–2125.

Kohji T, Matsumoto Y (2000). Coexpression of Fas/FasL and Bax on brain and infiltrating T cells in the central nervous system is closely associated with apoptotic cell death during autoimmune encephalomyelitis. *J Neuroimmunol* **106:** 165–171.

Lafon M, Bourhy H, Sureau P (1988). Immunity against the European bat rabies (Duvenhage) virus induced by rabies vaccines: an experimental study in mice. *Vaccine* **6:** 362–368.

Libeau G, Lafon M, Rollin PE (1984). [Specificity of monoclonal antibodies obtained with the Pasteur PV rabies virus strain]. *Rev Elev Med Vet Pays Trop* **37:** 383–394.

Licon Luna RM, Lee E, Mullbacher A, Blanden RV, Langman R, Lobigs M (2002). Lack of both Fas ligand and perforin protects from flavivirus-mediated encephalitis in mice. *J Virol* **76:** 3202–3211.

Li-Weber M, Laur O, Dern K, Krammer PH (2000). T cell activation-induced and HIV tat-enhanced CD95(APO-1/Fas) ligand transcription involves NF-kappaB. *Eur J Immunol* **30:** 661–670.

Lucas M, Karrer U, Lucas A, Klenerman P (2001). Viral escape mechanisms–escapology taught by viruses. *Int J Exp Pathol* **82:** 269–286.

Marquette C, Van Dam AM, Ceccaldi PE, Weber P, Haour F, Tsiang H (1996). Induction of immunoreactive interleukin-1 beta and tumor necrosis factor-alpha in the brains of rabies virus infected rats. *J Neuroimmunol* **68:** 45–51.

Marten NW, Stohlman SA, Zhou J, Bergmann CC (2003). Kinetics of virus-specific CD8+—T-cell expansion and trafficking following central nervous system infection. *J Virol* **77:** 2775–2778.

Medana I, Li Z, Flugel A, Tschopp J, Wekerle H, Neumann H (2001). Fas ligand (CD95L) protects neurons against perforin-mediated T lymphocyte cytotoxicity. *J Immunol* **167:** 674–681.

Micheau O, Solary E, Hammann A, Dimanche-Boitrel MT (1999). Fas ligand-independent, FADD-mediated activation of the Fas death pathway by anticancer drugs. *J Biol Chem* **274:** 7987–7992.

Miller DW (1999). Immunobiology of the blood-brain barrier. *J NeuroVirol* **5:** 570–578.

Mizuno T, Goto Y, Baba K, Momoi Y, Endo Y, Nishimura Y, Masuda K, Ohno K, Tsujimoto H (2003). Quantitative analysis of Fas and Fas ligand mRNAs in a feline T-lymphoid cell line after infection with feline immunodeficiency virus and primary peripheral blood mononuclear cells obtained from cats infected with the virus. *Vet Immunol Immunopathol* **93:** 117–123.

O'Connell J, Houston A, Bennett MW, O'Sullivan GC, Shanahan F (2001). Immune privilege or inflammation? Insights into the Fas ligand enigma. *Nat Med* **7:** 271–274.

Oleszak EL, Hoffman BE, Chang JR, Zaczynska E, Gaughan J, Katsetos CD, Platsoucas CD, Harvey N (2003). Apoptosis of infiltrating T cells in the central nervous system of mice infected with Theiler's murine encephalomyelitis virus. *Virology* **315:** 110–123.

Price DA, Klenerman P, Booth BL, Phillips RE, Sewell AK (1999). Cytotoxic T lymphocytes, chemokines and antiviral immunity. *Immunol Today* **20:** 212–216.

Raftery MJ, Schwab M, Eibert SM, Samstag Y, Walczak H, Schonrich G (2001). Targeting the function of mature dendritic cells by human cytomegalovirus: a multilayered viral defense strategy. *Immunity* **15:** 997–1009.

Sabelko-Downes KA, Cross AH, Russell JH (1999). Dual role for Fas ligand in the initiation of and recovery from experimental allergic encephalomyelitis. *J Exp Med* **189:** 1195–1205.

Schlitt BP, Felrice M, Jelachich ML, Lipton HL (2003). Apoptotic cells, including macrophages, are prominent in Theiler's virus-induced inflammatory, demyelinating lesions. *J Virol* **77:** 4383–4388.

Seabrook TJ, Hay JB (2001). Intracerebro ventricular infurions of TNF-α preferentially recruit blood lymphocytes and induce a perivascular leukocyte infiltrate. *J Neuro Immunol* **113:** 81–88.

Shankar V, Dietzschold B, Koprowski H (1991). Direct entry of rabies virus into the central nervous system without prior local replication. *J Virol* **65:** 2736–2738.

Stanisz AM (1994). Neuronal factors modulating immunity. *Neuroimmunomodulation* **1:** 217–230.

Strater J, Walczak H, Hasel C, Melzner I, Leithauser F, Moller P (2001). CD95 ligand (CD95L) immunohistochemistry: a critical study on 12 antibodies. *Cell Death Differ* **8:** 273–278.

Suda T, Nagata S (1994). Purification and characterization of the Fas-ligand that induces apoptosis. *J Exp Med* **179:** 873–879.

Suzuki A, Tsutomi Y, Shimizu M, Matsuzawa A (1999). Another cell death induction system: TNF-alpha acts as a ligand for Fas in vaginal cells. *Cell Death Differ* **6:** 638–643.

Takahashi T, Tanaka M, Brannan CI, Jenkins NA, Copeland NG, Suda T, Nagata S (1994). Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* **76:** 969–976.

Tanner JE, Alfieri C (1999). Epstein-Barr virus induces Fas (CD95) in T cells and Fas ligand in B cells leading to T-cell apoptosis. *Blood* **94:** 3439–3447.

Wall EM, Cao JX, Upton C (1998). Subversion of cytokine networks by viruses. *Int Rev Immunol* **17:** 121–155.

Westendorp MO, Frank R, Ochsenbauer C, Stricker K, Dhein J, Walczak H, Debatin KM, Krammer PH (1995). Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. *Nature* **375:** 497–500.

Xu XN, Screaton GR, Gotch FM, Dong T, Tan R, Almond N, Walker B, Stebbings R, Kent K, Nagata S, Stott JE, McMichael AJ (1997). Evasion of cytotoxic T lymphocyte (CTL) responses by nef-dependent induction of Fas ligand (CD95L) expression on simian immunodeficiency virus-infected cells. *J Exp Med* **186:** 7–16.