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Tumor necrosis factor alpha and macrophages in the brain of herpes simplex virus type 1–infected BALB/c mice

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After uniocular anterior chamber (AC) inoculation of herpes simplex virus type 1 (HSV-1), virus and TNF alpha (TNF-α) are detected in the suprachiasmatic nuclei (SCN). The goal of this study was to investigate the role of TNF-α and macrophages in the brain of HSV-1–infected BALB/c mice. Mice were treated with thalidomide for TNF-α inhibition or injected with clodronate liposomes to deplete macrophages, and the AC of one eye (ipsilateral) was injected with HSV-1 (KOS). The location of HSV-1, macrophages, and TNF-α was determined by fluorescence immunohistochemistry and the titer of virus was determined by plaque assay. Inhibition of TNF-α was determined by reverse transcriptase–polymerase chain reaction (RT-PCR) and depletion of macrophages was assessed by flow cytometry. In thalidomide-treated mice, TNF-α RNA levels were reduced in the SCN. Both SCN were infected by day 5 post inoculation (p.i.) and the titer of virus in the SCN contralateral to the side of injection was increased. The number of splenic macrophages was significantly reduced in clodronate-treated mice compared with controls. In macrophage-depleted mice, both SCN were infected at day 6 p.i. and the titer of virus in the SCN of these mice was increased at days 6 and 7 p.i. compared with controls. The titer of virus in the contralateral (uninoculated) eye of macrophage-depleted mice was increased at day 7 p.i. Fewer F4/80$^+$ cells were observed in the SCN of macrophage-depleted mice. The results of these studies suggest that TNF-α plays a role in limiting virus replication in the SCN of euthymic BALB/c mice and that one source of TNF-α is macrophages. *Journal of NeuroVirology* (2006) **12,** 443–455.

Keywords: cytokine; eye; herpesvirus; immunomodulator; suprachiasmatic nucleus

Introduction

Acute retinal necrosis syndrome (ARN) is a potentially blinding disease that is characterized by necrotizing retinitis, optic neuropathy, retinal arteritis, vitritis, and retinal vasculitis (Lewis *et al*, 1989; Duker *et al*, 1990; Culbertson and Atherton, 1993). ARN can be unilateral, involving one eye, or bilateral, involving both eyes (Duker *et al*, 1990; Culbertson and Atherton, 1993). ARN occurs primarily in immunocompetent and otherwise healthy individuals of all ages (Lewis *et al*, 1989; Duker *et al*, 1990; Culbertson *et al*, 1991). Several members of the human herpesvirus family, including herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) as well as varicella-zoster virus (VZV), have been implicated as causes of ARN (Culbertson *et al*, 1991). Neurotropic human herpesviruses persist in human hosts by establishing latent infection in sensory neurons, and most cases of ARN probably result from virus reactivation (Culbertson and Atherton, 1993).

In a mouse model of ARN, after inoculation of HSV-1 (KOS) into the anterior chamber (AC) of one eye, virus enters the optic nerve and retina of the contralateral (uninoculated) eye by spreading from the

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suprachiasmatic nucleus (SCN) ipsilateral to the side of injection (Vann and Atherton, 1991; Matsubara and Atherton, 1997). Although virus infects the SCN opposite the side of injection (contralateral), it does not spread from there to the optic nerve and retina of the injected eye in immunocompetent mice. In contrast, in T cell–depleted BALB/c mice, HSV-1 (KOS) also spreads from the contralateral SCN to the optic nerve and retina of the inoculated eye, resulting in bilateral retinitis in BALB/c mice following uniocular AC inoculation (Azumi and Atherton, 1994; Matsubara and Atherton, 1997). Further studies suggested that T cell–dependent protection takes place in or near the SCN in the hypothalamus of euthymic BALB/c mice (Matsubara and Atherton, 1997).

Many kinds of cells are capable of producing tumor necrosis factor alpha (TNF-α), such as T cells, macrophages, astrocytes, and glial cells (Kodukula *et al*, 1999; Lokensgard *et al*, 2001; Andersson *et al*, 2005). *In vivo* neutralization of TNF-α results in increased virus replication in the trigeminal ganglion of BALB/c mice (Kodukula *et al*, 1999). Other studies have shown that TNF-α directly decreases viral gene expression *in vivo* during HSV-1 infection (Shimeld *et al*, 1995). TNF-α has multiple functions in the immune response, such as containment of local infections, synergy with antiviral cytokines such as the interferons, and participation in both neuroprotective and neurodestructive processes in chronic and acute neurodegenerative disorders (Chen *et al*, 1993; Fontaine *et al*, 2002; Minagawa *et al*, 2004). Thalidomide ($\alpha - N$-phthalimidoglutarimide) is a psychoactive drug that readily crosses the blood-brain barrier (Moreira *et al*, 1993; Lokensgard *et al*, 2000). Thalidomide has been used in the treatment of many disorders such as erythema nodosum leprosum and Crohn's disease (Sampaio *et al*, 1993; Ginsburg *et al*, 2001). Thalidomide has been shown to effectively inhibit TNF-α production by enhancing the degradation of TNF-α mRNA and selective inhibition of TNF-α produced by stimulated human monocytes (Sampaio *et al*, 1991; Moreira *et al*, 1993).

Macrophages play a central role in antigen presentation. In addition to producing TNF-α, they produce a variety of other proinflammatory chemokines and cytokines such as interferon (IFN)α/β (Rossol-Voth *et al*, 1991; Cheng *et al*, 2000). Macrophages also play a role in extrinsic resistance to HSV, which is defined as the ability of these cells to influence extracellular virus through suppression of virus growth and selective lysing of HSV-infected cells (Baskin *et al*, 1997; Scieux, 1997; Benencia and Courreges, 1999). For example, peritoneal macrophages infected with HSV-2 impair viral production when cocultured with HSV-1–or HSV-2–infected Vero cells (Benencia and Courreges, 1999). Prior to T cell arrival, macrophages are one of the primary defenses against infectious agents and foreign substances (Shimeld *et al*, 1995; Kodukula *et al*, 1999). Macrophages are active participants in host resistance to HSV-1 infection and have the potential to produce chemokines, which play a major role in the infiltration of inflammatory cells (Johnson, 1964; Zisman *et al*, 1970; Widmer *et al*, 1993). In an HSV-1 chorioretinitis model, it has been shown that peripheral blood macrophages as well as those in the spleen participate in the immune mechanisms that lead to retinal destruction (Berra *et al*, 1994). Liposome-encapsulated dichloromethylene-biphosphate liposomes (Cl_2MBP or clodronate) have been shown to effectively deplete macrophages *in vivo* (Van Rooijen and Sanders, 1994). Injection of liposomes containing Cl_2MBP eliminates macrophages without affecting other immunocompetent cells (Van Rooijen, 1989). In an HSV-1–induced stromal keratitis (HSK) model, macrophage depletion using clodronate liposomes decreased the incidence and severity of stromal disease (Bauer *et al*, 2001).

The purpose of these studies was to test the hypothesis that TNF-α and macrophages play a role in the pathogenesis of virus infection of the brain following uniocular AC inoculation of HSV-1.

Results

HSV-1 and TNF-α in the SCN

To determine whether TNF-α is produced in the SCN, HSV-1 (KOS) was injected into the AC of one eye of euthymic BALB/c mice. In agreement with previously published results, the ipsilateral (on the side of injection) SCN was HSV-1 positive at day 5 post inoculation (p.i.) (Figure 1A) and both SCN were virus positive by day 7 p.i. (Figure 1B) (Vann and Atherton, 1991; Matsubara and Atherton, 1997). At day 5 p.i., a small amount of TNF-α was detected in the ipsilateral SCN (Figure 1C) whereas at day 7 p.i., the amount of TNF-α had increased and TNF-α wasdetected in both SCN (Figure 1D).

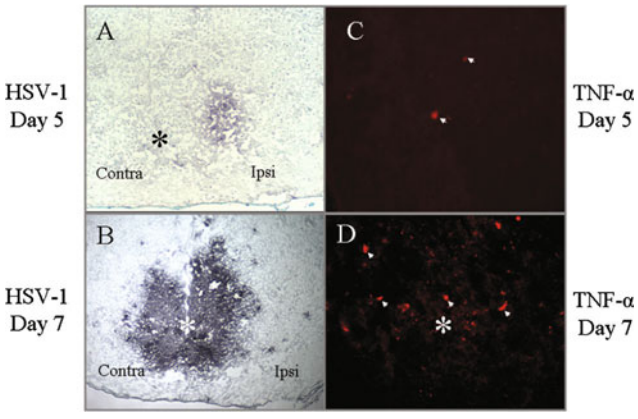

Figure 1 Photomicrograph of HSV-1 and TNF-α in the SCN of BALB/c mice infected with HSV-1 (KOS). At day 5 p.i. the ipsilateral (side of virus injection) SCN was HSV-1 positive (**A**). At day 7 p.i. both SCN were HSV-1 positive (**B**). TNF-α was detected in the ipsilateral SCN at day 5 p.i. (**C**) and in both SCN at day 7 p.i. (**D**). *Arrows* = TNF-α; * = midline of the brain.

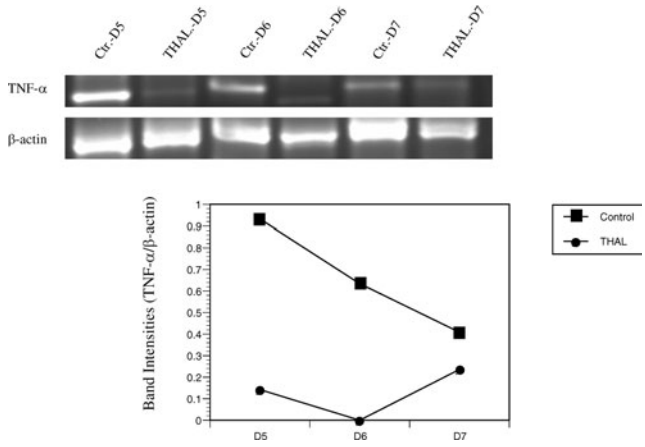

Figure 2 TNF-α depletion in the SCN of thalidomide treated (THAL) mice at day 5, 6, and 7 p.i. All mice were injected with HSV-1 via the AC route on day 0 and RNA from the SCN of THAL-treated mice and control (Ctr.) mice was amplified for TNF-α or for β-actin.

Down-regulation of TNF-α mRNA after thalidomide treatment

Thalidomide selectively inhibits TNF-α production by enhancing degradation of TNF-α mRNA (Moreira, 1993). To determine whether thalidomide depleted TNF-α mRNA, experimental mice were injected intraperitoneally with thalidomide whereas control mice received an equivalent amount of vehicle alone dimethyl sulfoxide [DMSO] and phosphate-buffered saline [PBS]). Reverse transcriptase–polymerase chain reaction (RT-PCR) was used to determine the effect of thalidomide treatment on the level of TNF-α mRNA in the SCN of HSV-1–infected BALB/c mice. As shown in Figure 2, TNF-α mRNA levels in the SCN were decreased in thalidomide-treated mice compared with control mice on days 5, 6, and 7 p.i. in HSV-1–infected BALB/c mice.

Effect of thalidomide treatment on virus spread

To determine if thalidomide treatment and concomitant reduction of TNF-α affected virus spread in the SCN, the brains of experimental and control mice were removed, frozen, sectioned, and the area of the SCN was stained for HSV-1. Viral antigen was detected in the ipsilateral SCN in both thalidomide-treated mice and the control group on day 5 p.i. (Figure 3A, B). However, virus was also detected in the contralateral SCN at day 5 p.i. in the thalidomide-treated group (Figure 3B). On days 6 and day 7 p.i., virus was detected in both the ipsilateral and contralateral SCN in both groups (data not shown).

To determine whether detection of viral antigen at day 5 p.i. in the contralateral SCN of thalidomide-treated mice correlated with an increase in virus, the titer of virus in the ipsilateral and contralateral SCN of mice treated with thalidomide was compared to mice treated with DMSO and PBS. The titer of virus in the ipsilateral SCN of mice treated with thalidomide

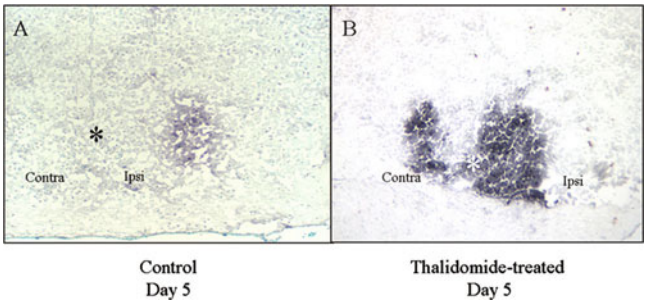

Figure 3 Photomicrograph of HSV-1 infection of the SCN of mice infected with HSV-1 (KOS). At day 5 p.i., only the ipsilateral SCN was infected in HSV-1 (KOS)–infected control mice treated with DMSO and PBS (**A**), whereas both the ipsilateral and contralateral SCN were infected in THAL treated mice (**B**). *= midline of the brain; ipsi = ipsilateral to side of injection; contra = contralateral to side of injection.

was not significantlydifferent from the mice treated with DMSO and PBS at all time points (Figure 4A). In agreement with the viral antigen results, the titer of virus in the contralateral SCN of thalidomide-treated mice was significantly higher than the titer of virus in the contralateral SCN of mice treated with DMSO and PBS on day 5 p.i. (Figure 4B). On days 6 and 7

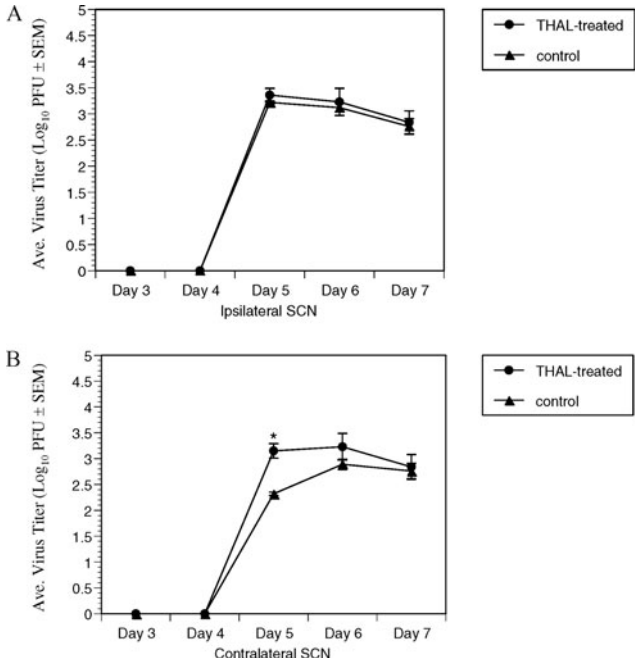

Figure 4 Titer of virus (average PFU ± SEM) in the ipsilateral SCN (**A**) and contralateral SCN (**B**) of mice infected with HSV-1 (KOS). Mice were inoculated in the AC of the right eye with 2×10^4 PFU of HSV-1 (KOS) and treated with thalidomide (THAL) or DMSO and PBS daily. Mice were sacrificed on days indicated. Ipsilateral and contralateral SCN were separated from the brains, the SCN were pooled, homogenized, and the titer of virus determined by plaque assay. The titers represent the average ± SEM of five animals in each group. The minimum level of detection was 0.7 Log_{10} PFU/tissue. *Significantly different from control group ($P < .05$).

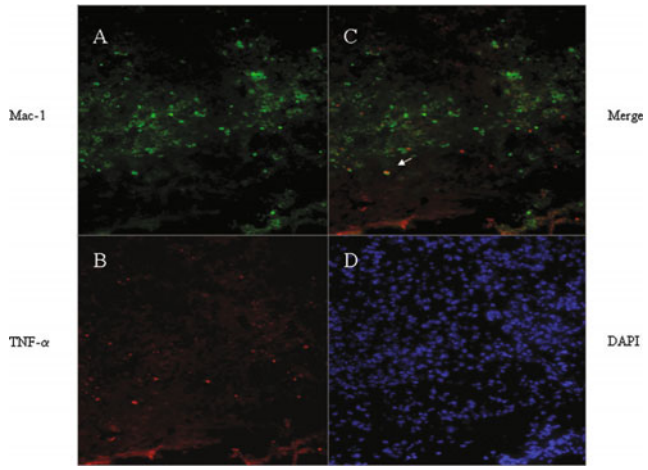

Figure 5 Photomicrograph of Mac-1$^+$ cells and TNF-α in the SCN after AC inoculation of HSV-1. Mac-1$^+$ cells (**A**) and TNF-α (**B**) were observed in the SCN of HSV-1 infected mice and as shown in **C** (merge), Mac-1$^+$ cells and TNF-α colocalized in the SCN of HSV-1–infected mice (**C**). DAPI (**D**). Colocalization of Mac-1+ cells and TNF-α indicated by arrows.

p.i., therewas no significant difference in either the ipsilateral or contralateral SCN of experimental and control mice (Figure 4A, B). Taken together, the viral antigen detection studies and the virus recovery studies suggest that TNF-α in the SCN plays a role in limiting virus spread and/or viral replication during early infection of the SCN (day 5 p.i.).

Macrophages and virus spread

Although the results of the thalidomide treatment studies suggested that TNF-α was involved in limitation of virus spread in the SCN after AC inoculation of HSV-1, the source of the TNF-α remained to be determined. Because macrophages produce TNF-α and have been implicated in early control of virus infection, experiments were performed to determine whether macrophages were the source of TNF-α detected in the SCN. As shown in Figure 5, macrophages and TNF-α colocalized in the SCN of HSV-1–infected mice, suggesting that TNF-α in the SCN was produced by macrophages.

To investigate the role macrophages play in HSV-1 infection of the SCN, depletion experiments were performed by intravenous injection of clodronate containing liposomes. The extent of macrophage depletion in splenocytes of HSV-1–infected BALB/c mice, PBS liposome–treated HSV-1–infected mice, clodronate liposome–treated HSV-1–infected mice and uninfected mice was assessed on days 2, 4, and 6 p.i. by flow cytometry (Figure 6). After treatment with clodronate liposomes, >99% of F4/80$^+$ cells in the spleen were depleted on day 2 p.i.; after continued treatment with clodronate liposomes, >99% of F4/80$^+$ cells were depleted on day 4 p.i. and >98% of F4/80$^+$ cells were depleted on day 6 p.i. (Table 1, Figure 6A). After treatment with clodronate liposomes, 90.4%, 76.2%, and 85.8% of Mac-1$^+$ cells in the spleen were depleted on day 2, 4, and 6 respectively (Table 1, Figure 6B).

To determine the effect of macrophage depletion on virus spread, clodronate liposome–treated and control HSV-1–infected mice were sacrificed and the brain and contralateral eye were removed and prepared for immunohistochemistry or virus titration. At day 5 p.i., the ipsilateral SCN of clodronate liposome–treated mice and PBS-treated control mice was viral antigen positive (data not shown). However by day 6 p.i., more viral antigen was detected in the ipsilateral SCN as well as in the contralateral SCN of macrophage depleted mice (compare Figure 7A with Figure 7B). On day 7 p.i., both the ipsilateral and contralateral SCN of macrophage-depleted mice and control mice were HSV-1 antigen positive (data not shown). At day 7 p.i. (the time when virus is first observed in the retina of the uninoculated eye; Vann

Table 1 Macrophage depletion at day 2, 4, and 6 p.i. in the spleen of HSV-1–infected BALB/c mice

Day	*Group*	*Spleen cells (n)*	*F4/80$^+$(n)*	*% F4/80$^+$(% depletion)**	*Mac1$^+$ (CD11b) (n)*	*% Macl$^+$(% depletion)**
2	1	6.0×10^7	2.1×10^6	3.56/—	3.2×10^6	5.34/—
	2	5.9×10^7	2.3×10^6	3.91/—	3.1×10^6	5.25/—
	3	4.8×10^7	2.6×10^6	5.39/—	2.3×10^6	4.89/—
	4	7.0×10^6	1.8×10^4	0.26/99.4%	2.2×10^5	3.26/90.4%
4	1	7.3×10^7	1.5×10^6	2.09/—	2.1×10^6	2.85/—
	2	6.8×10^7	3.1×10^6	4.59/—	3.8×10^6	5.60/—
	3	5.4×10^7	2.7×10^6	4.97/—	3.2×10^6	5.93/—
	4	2.4×10^7	4.8×10^3	0.02/99.8%	7.6×10^5	3.15/76.2%
6	1	3.7×10^7	1.5×10^6	4.30/—	1.7×10^6	4.58/—
	2	9.5×10^7	5.9×10^6	6.30/—	8.3×10^6	8.68/—
	3	4.6×10^7	2.4×10^6	5.30/—	3.6×10^6	7.78/—
	4	9.5×10^6	4.7×10^4	0.50/98.1%	5.1×10^5	5.32/85.8%

Group 1: noninfected BALB/c mice.
Group 2: HSV-1–infected BALB/c mice without treatment.
Group 3: PBS liposome–treated HSV-1–infected BALB/c mice.
Group 4: Clodronate liposome–treated HSV-1–infected BALB/c mice.
*% depletion compared with HSV-1–infected, PBS liposome–treated control mice (group 3).

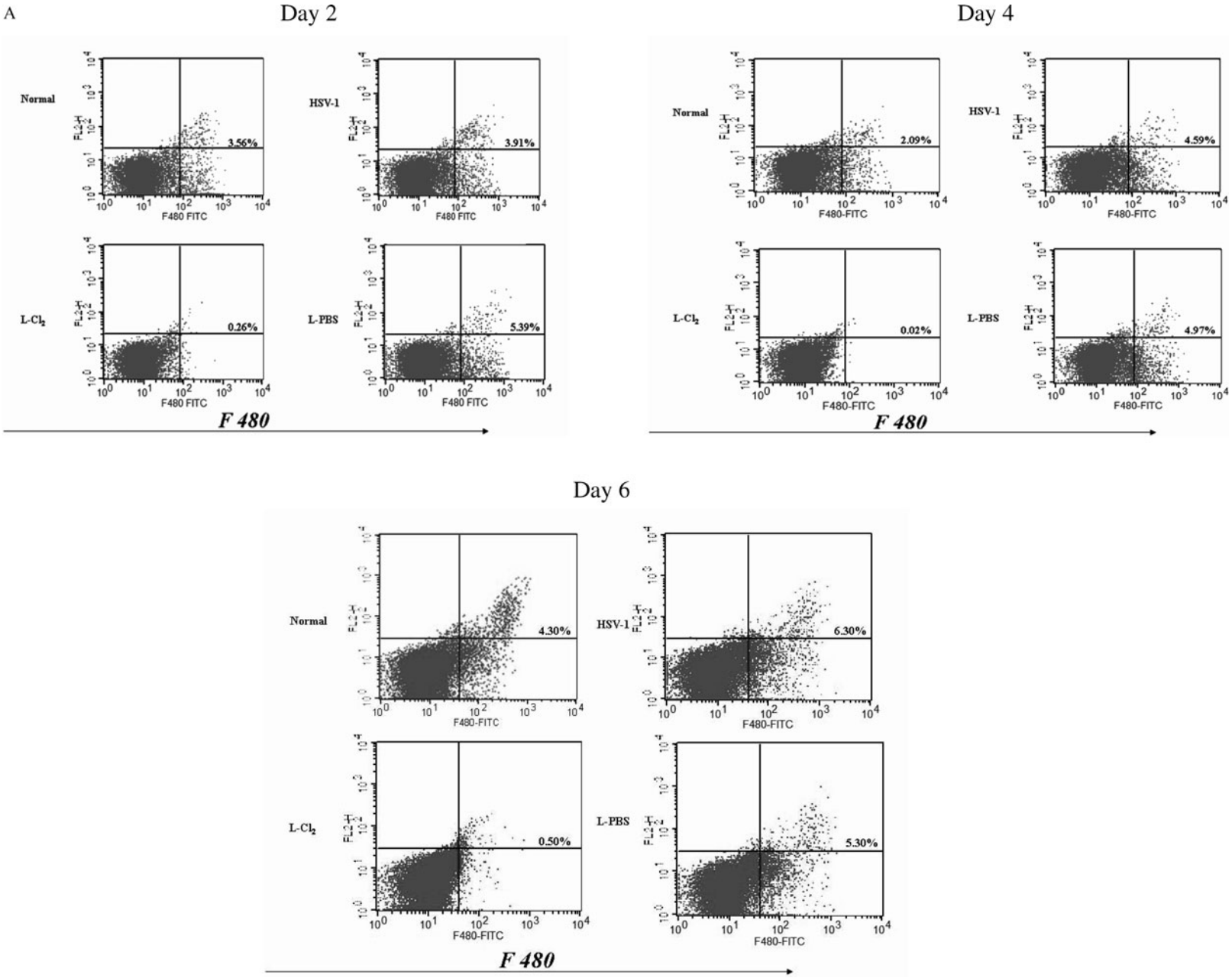

Figure 6 Flow cytometric profiles of splenocytes isolated from HSV-1 (KOS)–infected mice (HSV-1), clodronate liposome–treated HSV-1 (KOS)–infected mice (L-Cl_2), PBS liposome–treated HSV-1 (KOS)–infected mice (L-PBS), and uninfected mice (normal). On days 2, 4, and 6 p.i., splenocytes were stained with FITC-conjugated anti-mouse F4/80 (**A**) and FITC-conjugated anti-mouse Mac-1 (**B**). Results were analyzed by flow cytometry. *(Continued)*

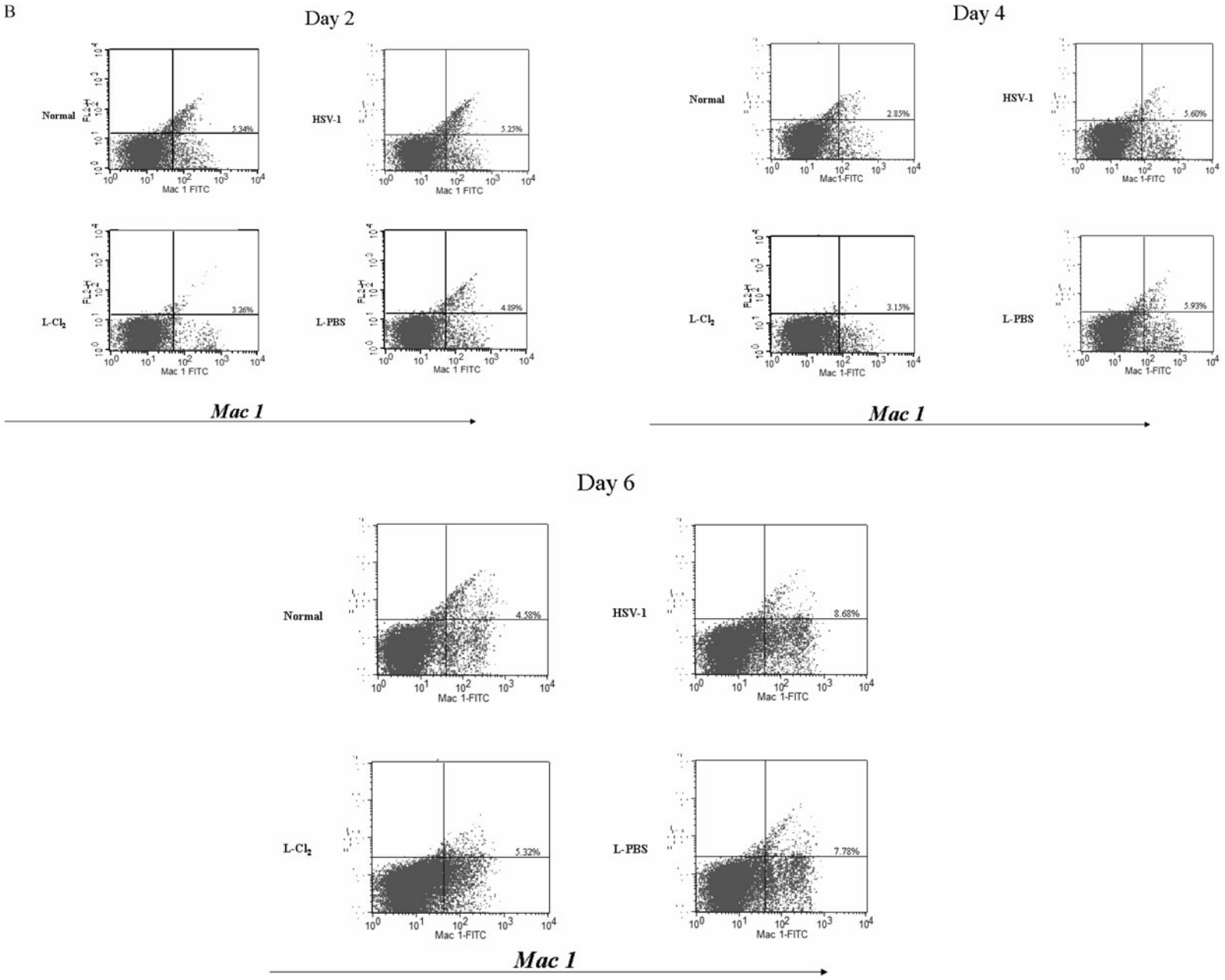

Figure 6 (Continued).

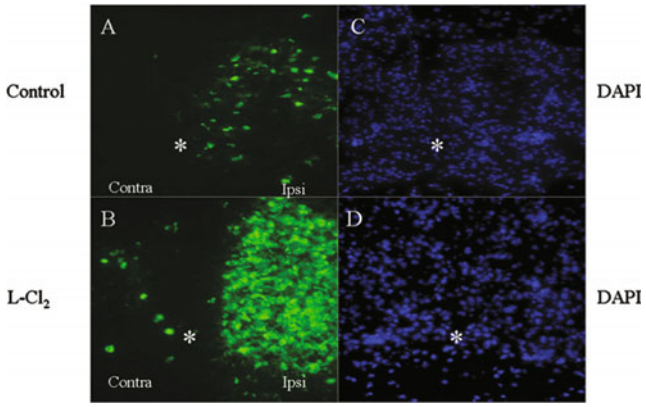

Figure 7 Photomicrograph of HSV-1 antigen–positive cells in the SCN of mice infected with HSV-1 (KOS). At day 6 p.i., only the ipsilateral (side of virus injection) SCN was virus positive in mice treated with PBS liposomes (L-PBS) (**A**), whereas, both SCN were virus positive in mice treated with clodronate liposomes (L-Cl_2) (**B**). DAPI (**C**, **D**). *= midline of the brain.

and Atherton [1991]), morevirus infected cells were observed in the retina of the uninoculated contralateral eye of clodronate-treated mice than in the retina of the uninoculated eye of control mice (compare Figure 8A with Figure 8B).

To determine the effect of macrophage depletion on the amount of virus in the brain and uninoculated contralateral eye, the titer of virus in the brain of mice treated with clodronate liposomes was compared to that of mice treated with PBS liposomes. No significant difference in virus titer between macrophage-depleted and control mice was observed in the ipsilateral SCN (Figure 9A). In contrast, the titer of virus in the contralateral SCN of clodronate liposome–treated mice was significantly higher than that in the contralateral SCN of PBS liposome–treated mice on days6 and 7 p.i. (Figure 9B). The titer of virus in the

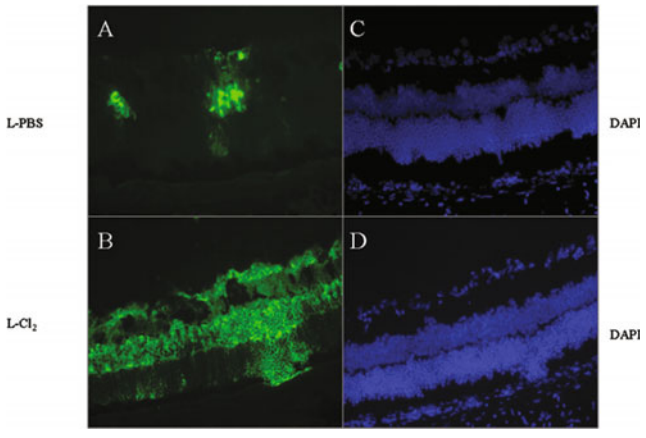

Figure 8 HSV-1 infection of the retina of the uninoculated contralateral eye of mice infected with HSV-1 (KOS) at day 7 p.i. More viral antigen was observed in the retina of the contralateral eye of macrophage depleted mice (**B**) than in the retina of nondepleted mice (**A**). DAPI (**C**, **D**).

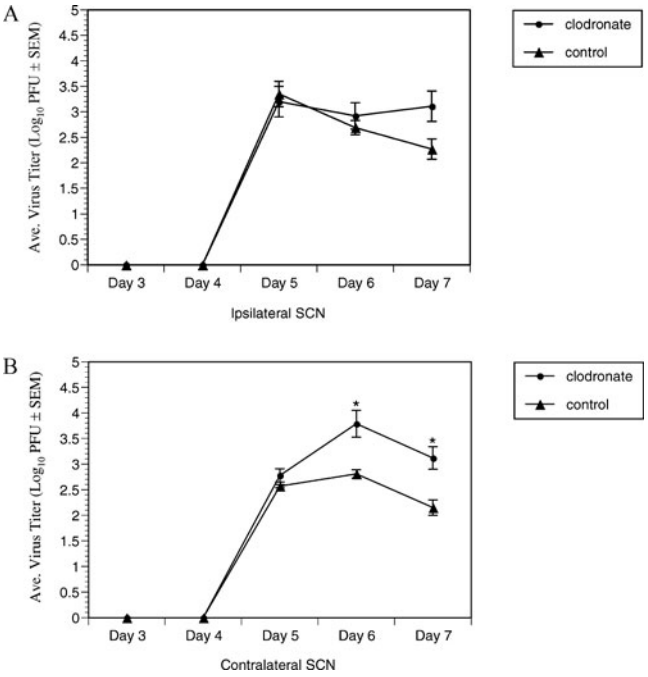

Figure 9 Titer (average PFU ± SEM) of virus recovered from the ipsilateral SCN (**A**) and contralateral SCN (**B**) of mice infected with HSV-1 (KOS). Mice were inoculated in the AC of the right eye with 2×10^4 PFU of HSV-1 (KOS) and treated with clodronate liposomes or PBS liposomes on days –1, 2, and 5. Mice were sacrificed on days indicated. The ipsilateral and contralateral SCN were separated from the brains of HSV-1–infected mice and the titer of virus was determined by plaque assay of tissue homogenates. Plaques were stained with crystal violet, counted, and differences between groups were analyzed for statistical significance. The minimum level of detection was 0.7 Log_{10} PFU/tissue. *Significantly different from nondepleted control group ($P < .05$). Ipsilateral = side of injection; contralateral = uninjected side.

uninoculated contralateral eye of mice treated with clodronate liposomes was also compared to that of mice treated with PBS liposomes. At day 7 p.i., the titer of virus in the contralateral eye was significantly increased in clodronate-treated mice (Figure 10).

To confirm that systemic macrophage depletion correlated with depletion of macrophages in the brain, the brains of clodronate liposome–treated mice and control HSV-1–infected mice were removed, sectioned, and stained for F4/80^+ cells. At day 6 p.i., fewer F4/80^+ cells were observed in the SCN of clodronate liposome–treated mice than in the SCN of control mice (compare Figure 11A with Figure 11B).

Discussion

The interaction between immune effector cells and virus in patients with ARN is a complex process that has yet to be fully understood. The murine model of ARN has provided insights into the interaction between the immune cells and the pathogenesis of human disease. Previous studies of the BALB/c model of ARN have shown that after inoculation of HSV-1

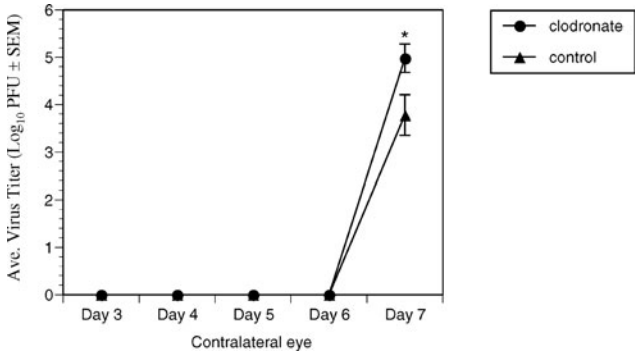

Figure 10 Titers (average PFU ± SEM) of virus recovered from the uninoculated contralateral eye of mice infected with HSV-1 (KOS). Mice were inoculated in the AC of one eye with 2×10^4 PFU of HSV-1 (KOS) and treated with clodronate liposomes or PBS liposomes on days −1, 2, and 5. Mice were sacrificed on days indicated. The uninoculated eye was removed and the titer of virus was determined by plaque assay of tissue homogenates. Plaques were stained with crystal violet, counted, and results were analyzed for statistical significance. Minimum level of detection was 0.7 Log_{10} PFU/tissue. *Significantly different from non-depleted control group ($P < .05$).

(KOS) into the anterior chamber (AC) of one eye, virus enters the optic nerve and retina of the contralateral eye by spreading from the ipsilateral suprachiasmatic nucleus (Vann and Atherton, 1991; Matsubara and Atherton, 1997). Results from previous studies using a mouse model of ARN have suggested that both $CD4^+$ and $CD8^+$ T cells are important in preventing viral spread from the brain to the optic nerve and retina of the injected eye of HSV-1–infected mice (Azumi and Atherton, 1994; Matsubara and Atherton, 1997). However, the role of other immunomodulatory cells and molecules remains to be determined. In this study we investigated the effect of depletion of TNF-α and macrophages on viral spread in the brain and contralateraleye of HSV-1–infected BALB/c mice.

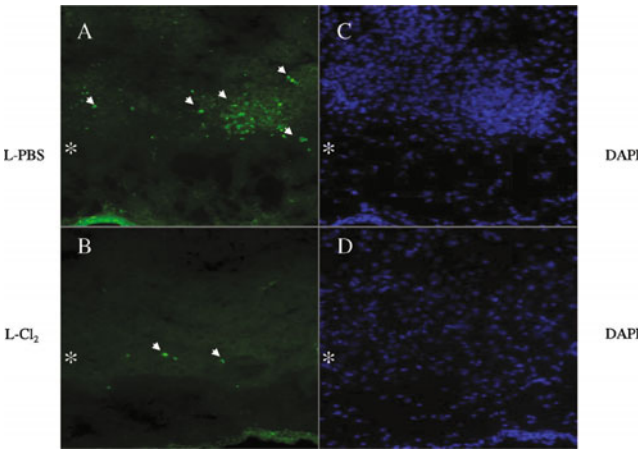

Figure 11 Photomicrograph of $F4/80^+$ cells in the SCN 6 days after AC inoculation of HSV-1. At this time, fewer $F4/80^+$ cells were observed in the SCN of clodronate liposome-treated mice (L-Cl_2) (**B**) than in the SCN of control mice (L-PBS) (**A**). DAPI (**C**, **D**). * = midline of the brain; arrows = F4/80+ cells.

As previously reported, thalidomide exerts its inhibitory action on TNF-α by enhancing mRNA degradation (Moreira *et al*, 1993). TNF-α production has been shown to be decreased *in vivo* in male ICR mice treated with thalidomide (Lee *et al*, 2004). In the studies reported herein, treatment with thalidomide reduced TNF-α mRNA levels in the brain of HSV-1–infected BALB/c mice.

Clodronate liposomes (L-Cl_2 MBP) have been shown to selectively deplete macrophages in *in vivo* animal models (Van Rooijen and Sanders, 1994). In these studies, treatment with clodronate liposomes depleted splenic macrophages when injected intravenously into HSV-1–infected BALB/c mice. Treatment with clodronate liposome resulted in >98% depletion of $F4/80^+$ macrophages and of 90.4%, 76.2%, and 85.8% of Mac-1^+ cells on days 2, 4, and 6 p.i., respectively. The Mac-1 antigen is expressed on macrophages, microglia, dendritic cells, natural killer cells, and neutrophils after activation (Springer *et al*, 1979; Kishimoto *et al*, 1989; Vremec *et al*, 1992). Because the Mac-1 antigen is expressed not only by macrophages but by other cell types, depletion of Mac-1^+ cells appears to be lower than that of $F4/80^+$ cells in clodronate–liposome treated mice. Therefore, although our initial observations were of Mac-1–expressing cells in the spleen and brain, the depletion studies focused on F4/80–expressing cells because most $F4/80^+$ cells are systemic macrophages.

In this study, treatment with thalidomide resulted in increased viral spread in the SCN on day 5 p.i. in HSV-1–infected BALB/c mice. Treatment of mice with thalidomide also resulted in increased viral titers in the contralateral SCN on day 5 p.i. when compared to control mice. Although there was a slight increase in the titer of virus in the contralateral SCN on days 6 and 7 p.i., there was no significant difference between the thalidomide-treated mice and control mice. There was also no difference in viral titer in the ipsilateral SCN of mice in both groups. Collectively, these results suggest that TNF-α plays a role in limiting early viral spread/replication in the SCN of HSV-1–infected BALB/c mice. Although thalidomide has been used in previous studies to inhibit TNF-α production, other studies suggest that in addition to inhibition of TNF-α, thalidomide may have other effects (Keifer et al, 2001; Majumdar et al, 2002). Although we believe that the results of our study support a role for TNF-α in early limitation of virus spread in the hypothalamus, the effect of thalidomide on other immunomodulators, especially at later time points of infection, cannot be determined from these studies. Limitation might occur during spread of virus to the SCN from the Edinger-Westphal nucleus, but this cannot be determined from the experiments done during these studies.

TNF-α is a pleiotropic proinflammatory cytokine that has a broad range of biological activities such as containment of local infections (Chen and Goeddel, 2002). TNF-α is also required to initiate the immune

response to certain antigens (Trevejo *et al*, 2001; Kasahara *et al*, 2003). In this study, production of TNF-α seemed to be important in the containment of HSV-1 infection in the ipsilateral SCN, which delayed spread of virus into the contralateral SCN. This conclusion is supported by the result that in the PBS- and DMSO-treated mice, virus was observed in the ipsilateral SCN only on day 5 p.i., whereas in the thalidomide-treated mice, virus was observed in both SCN. Thus, production of TNF-α seems to play an important role in limiting virus spread and/or virus replication in the SCN of HSV-1–infected mice.

There are many mechanisms by which thalidomide treatment of HSV-1–infected BALB/c mice may have resulted in increased virus spread and replication. For example, TNF-α is known to be involved in leukocyte migration into tissue (Wan *et al*, 2001). Because treatment with thalidomide resulted in a decrease in TNF-α production, a decrease in the amount of TNF-α might affect leukocyte migration into the brain, which, in turn, might reduce the immune response to infection. TNF-α is known to induce cell surface expression of intercellular cell adhesion molecule (ICAM)-1 (Sasaki *et al*, 2001). So by reducing the amount of TNF-α, thalidomide treatment could also have an affect on the expression of this cell surface receptor, which, in turn, would limit leukocyte migration into infected tissue. TNF-α also is known to cause apoptosis of virus-infected cells by binding to the TNF receptor, which is present on most cell types (Galvan and Roizman, 1998). The triggering of apoptosis is initiated when factors such as virus disrupt the orderly program of cellular events (Galvan and Roizman, 1998). Treatment with thalidomide could alter TNF-induced apoptosis in the SCN of HSV-1 (KOS)-infected BALB/c mice by allowing more cells to survive, which could result in increased virus spread.

Treatment of mice with clondronate liposomes resulted in increased viral spread at day 6 p.i. when compared to PBS liposome–treated control group. Treatment of mice with clondronate liposomes also resulted in increased viral titers on days 6 and 7 p.i. Viral spread seemed to increase in mice treated with clondronate liposomes when compared to PBS liposome–treated controls. Viral titers increased in the contralateral eye of mice treated with clondronate liposomes on day 7 p.i. Collectively these results suggest that infiltration of F4/80$^+$ macrophages into the SCN plays a role in limiting viral spread in the SCN which, in turn, might reduce the amount of virus that can spread to the optic nerve and retina of the uninoculated eye. Alternatively, F4/80$^+$ macrophages might play a role in limiting virus replication in the contralateral eye during the acute phase of retinal infection in HSV-1–infected BALB/c mice.

Macrophages have been shown to play a crucial role in host resistance to HSV-1 infection (Zisman *et al*, 1970; Cheng *et al*, 2000). The observation that virus spread and titer were altered on day 6 p.i. upon depletion of systemic macrophages supports the idea that F4/80$^+$ macrophages play a role in limiting virus spread in the SCN. Early-phase response to host infection depends on the innate arm of the immune response. There are a variety of mechanisms that contribute to this response and macrophages are among the most important immunomodulatory cell types in the early phase response. Macrophages serve as antigen-presenting cells and as effector cells in humoral and cell-mediated immunity. These cells are migratory and are able to travel to and then infiltrate infected host tissue. The results of the present study suggest that macrophages play a role by infiltration into the SCN upon virus infection because in macrophage-depleted mice, the amount of virus in the ipsilateral and contralateral SCN was increased on day 6 p.i. and spread within the SCN seemed to increase. Macrophages are likely recruited to the site of infection by cytokines such as TNF-α, produced early in infection, i.e., by day 5 p.i. Macrophages were observed in the SCN on day 6 p.i. One arm of protection against virus infection in the SCN is likely cell-mediated protection by macrophages upon entering the brain, which, in turn, leads to antigen presentation to other leukocytes, which initiates the adaptive immune response to virus.

In conclusion, these findings support the idea that TNF-α and systemic macrophages play role in limiting HSV-1 (KOS) infection in the SCN of BALB/c mice. Although the mechanism of protection in the SCN by TNF-α remains to be elucidated, the results presented here suggest that this cytokine plays a critical role in limiting viral spread in the brain of HSV-1–infected BALB/c mice. Macrophages seem to exert their effect through a systemic infiltration into the SCN upon which they contribute to the antiviral response against HSV-1 infected cells. Additional studies will be required to differentiate the exact mechanism of TNF-α protection/signaling and macrophage protection in the SCN and to determine the effects that thalidomide treatment has on other immunomodulators, especially in the hypothalamus.

Materials and methods

Animals

Adult female BALB/c mice, 6 to 8 weeks old (Taconic, Germantown, NY), were used in all experiments. The mice were housed in accordance with National Institutes of Health guidelines. All study procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the Medical College of Georgia. Mice were maintained on a 12-h light-dark cycle and were given unrestricted access to food and water. Mice were anesthetized with 0.5 to 0.7 ml/kg of a mixture of 42.9 mg/ml ketamine, 8.57 mg/ml xylazine, and 1.43 mg/ml acepromazine before all experimental

manipulations. A minimum of five animals per group were used in each experiment. Experiments were repeated at least once.

Anterior chamber (AC) inoculation
Mice were anesthetized and inoculated using the AC route. The right eye was proptosed, aqueous humor was removed by paracentesis, and 2 μl containing 2×10^4 plague-forming units (PFU) of HSV-1 (KOS) was injected into the anterior chamber with a 30-gauge needle attached to a 100-μl microsyringe (Hamilton, Reno, NV). The inoculum was prepared by diluting virus stock in Dulbecco's modified Eagle's medium (DMEM) with antibiotics. Control mice were inoculated in the AC with 2 μl of DMEM with antibiotics without virus.

Thalidomide treatment
For TNF-α depletion, euthymic BALB/c mice were injected intraperitoneally with 100 mg/kg^{-1} of thalidomide (THAL) (ICN Biomedicals, Aurora, OH) diluted in DMSO (Sigma-Aldrich) and PBS on days −1, 0, 1, 2, 3, 4, 5, 6, and 7. Control mice were injected intraperitoneally with DMSO (vehicle in which thalidomide is diluted) and PBS on days -1, 0, 1, 2, 3, 4, 5, 6, and 7. On day 0, mice in both groups were inoculated with 2×10^4 PFU HSV-1 (KOS) in the AC of one eye. Mice were sacrificed from the thalidomide-treated group and from the control group on days 3 to 7 p.i.

Macrophage depletion
For macrophage depletion, experimental mice were injected intravenously with 0.2 ml of dichloromethylene-biphosphate liposomes (Cl_2MBP, clodronate) on days –1, 2, and 5, a regimen that has been shown to deplete macrophages *in vivo* (Van Rooijen and Sanders, 1994). Control mice were injected intravenously with 0.2 ml of liposomes containing PBS on day –1, 2, and 5. On day 0, all mice were inoculated with 2×10^4 PFU HSV-1 (KOS) in the anterior chamber of one eye. Macrophage-depleted and control mice were sacrificed on days 2 to 7 p.i.

Perfusions
Mice were deeply anesthetized and perfused transcardially with PBS for approximately 3 min. After perfusion, the brain and both eyes were removed and immediately stored at −80°C.

Flow cytometry
Single-cell suspensions were prepared from the spleens of KOS-infected mice, KOS-infected mice treated with clodronate liposomes, KOS-infected mice treated with PBS liposomes, and uninfected, untreated mice at different days post infection. Splenocytes were teased out with a 21-gauge bent needle in DMEM with antibiotics (1% Penicillin/Streptomycin solution) and filtered through a 70-μm nylon cell strainer (BD Falcon, Bedford, MA). Cells were suspended in Hank's balanced salt solution (Cellgro; Mediatech, Herndon, VA) and centrifuged at 400 $\times$ *g*, at 4°C for 5 min. The supernatant was removed and 2 to 3 ml of ACK lysing buffer (Cambrex Bio Science, Walkersville, MD) was added to lyse red blood cells. Cells were then centrifuged and resuspended in PBS containing 0.5% fetal bovine serum (FBS), 1 mM EDTA, and 25 mM HEPES (Cambrex Bio Science). The antibodies fluorescein isothiocyanate (FITC) anti-mouse F4/80 (Caltag Laboratories, Burlingame, CA), FITC mouse CD 11b (Integrin α_M chain, Mac-1 α chain; BD PharMingen) were used to determine the extent of macrophage depletion. Flow cytometry of stained cell samples was performed (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ) and the flow cytometry results were analyzed using Cellquest software (Becton Dickinson).

RNA isolation and RT-PCR
Brain tissue was homogenized in 1 ml of Trizol Reagent (Invitrogen, Carlsbad, CA). The sample was vortexed and 200 μl of chloroform added. The sample was incubated at room temperature for 10 min then centrifuged at 13,000 $\times$ *g*, for 15 min at 4°C. RNA contained in the aqueous phase was collected, precipitated with an equal volume of isopropanol, and washed twice with 70% ethanol. RNA pellets were dissolved in distilled water and quantified using a PerkinElmer MBA 2000 Spectrometer (PerkinElmer, Wellesley, MA). One microgram of total RNA was reverse transcribed and cDNA amplified using the SuperScript III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen). The specific primers for β-actin and for TNF-α were as follows:

β-Actin: (S) 5′-TCCTTCGTTGCCGGTCCACA-3′
(A) 5′-CGTCTCCGGAGTCCATCACA-3′
TNF-α: (S) 5′-TTCTGTCTACTGAACTTCGGGGTGAT CGGTCC-3′
(A) 5′-GTATGAGATAGCAAATCGGCTGACG GTGTGGG-3′

Using a Perkin Elmer Cetus DNA Thermal Cycler (PerkinElmer) DNA synthesis was done at 1 cycle at 50°C for 30 min. Denaturation was done for 1 cycle at 94°C for 2 min followed by 40 cycles of the following: 94°C for 15 s, 55°C for 30 s, and 68°C for 1 min. The products were separated by electrophoresis on a 1% agarose gel and visualized under ultraviolet (UV) light.

Plaque assay
The brain and eyes were removed from HSV-1–infected mice treated with thalidomide or clodronate, and from control mice. Frozen brain tissue was thawed slightly and placed in a rodent brain matrix (ASI Instruments, Warren, MI) that had been chilled on ice. When each brain was thawed, coronal slices containing the SCN (Sidman *et al*, 1971) were made using alcohol-sterilized disposable microtome blades (Accu-Edge blades; Sakura Finetek,

Torrance, CA). Each section was divided in the midline, and the right (ipsilateral) and left (contralateral) halves of each SCN-containing section were placed in separate sterile tubes and homogenized in 500 μl DMEM containing antibiotics. Each eye was homogenized in 500 μl DMEM containing antibiotics. For virus titration, each homogenate was serially diluted and plated on Vero cells (American Type Culture Collection [ATCC], Manassas, VA) that were 80% confluent. Adsorption of virus was carried out for 1 h at 37°C in a CO_2 incubator. Following adsorption, the cells were overlaid with a 1:1 solution of 2X DMEM containing 10% serum and antibiotics and 1% low-melt agarose (Life Technologies, Rockville, MD). After 5 days at 37°C, the cells were fixed with 10% buffered-formalin and stained with 0.13% crystal violet (Sigma). Plaques were counted, the titer of virus was calculated and analyzed for significant differences using Student's *t* test, and the results were plotted using Delta Graph (Delta Point, Monterey, CA).

Immunohistochemistry

The uninoculated (contralateral) eye and brain of each mouse were removed, snap-frozen, and embedded in Tissue-Tek O.C.T Compound (Electron Microscopy Sciences, Hatfield, PA). Eight-to 10-μm sections were prepared on positively charged slides (Fisher Scientific, Pittsburgh, PA) using a Microm HM505E Cryostat slicer (EquipNet, Canton, MA). The frozen sections of brains and uninoculated eyes were fixed with 4% paraformaldehyde, washed in PBS and blocked with normal goat serum (Vector Laboratories, Burlingame, CA.) diluted in PBS for 1 h. The sections were incubated with rabbit anti-HSV-1 polyclonal antibody (Accurate Chemicals, Westbury, NY). Sections were washed in PBS, incubated with a biotinylated anti-rabbit immunoglobulin (IgG) (Vector Laboratories), washed again and reacted with avidin-biotin solution (Vector Laboratories). Diaminobenzidine (DAB; Sigma, St. Louis, MO) was used as the chromogen to obtain a final color reaction. The color reaction was intensified by adding 0.4% nickel chloride to the DAB solution. Sections were then counterstained with methyl green (Sigma), dehydrated in a graded ethanol series, cleared with xylenes, coverslipped, and examined microscopically for blue-stained virus-positive cells.

For detection of HSV-1–and TNF-α–positive cells, frozen sections were fixed with 4% paraformaldehyde. The sections were washed in PBS and blocked with normal goat serum (Vector laboratories), bovine serum albumin (Fisher Scientific), and Triton X-100 (Sigma) for 30 min. Sections were incubated with FITC-conjugated HSV-1–specific polyclonal antibody (DAKO, Carpinteria, CA), washed with PBS, and mounted with VectorShield containing DAPI (Vector Laboratories). Sections were incubated with FITC-conjugated anti-mouse CD 11b (Integrin αM chain, Mac-1 α chain; BD PharMingen) to detect Mac-1 cells. To detect TNF-α, the hybridoma XT-311 producing anti-mouse TNF-α antibody, generously provided by Dr. Gary Klimpel (University of Texas Medical Branch, Galveston), was used. The antibody was purified from culture medium of the hybridoma using ammonium sulfate precipitation and then biotinylated with Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) according to the manufacturer's instructions. Sections were then incubated with the biotinylated anti-mouse TNF-α antibody, washed, and reacted with Fluorescein Avidin D (Vector Laboratories). Sections were washed with PBS and mounted with VectorShield containing DAPI (Vector Laboratories). To detect F4/80 cells, the hybridoma for F4/80 (anti-mouse macrophage) was obtained from ATCC. The antibody was purified from culture medium of the hybridoma using ammonium sulfate precipitation and then biotinylated with Sulfo-NHS-LC-Biotin (Pierce) according to the manufacturer's instructions. Sections were then incubated with the biotinylated anti-mouse F4/80 antibody, washed, and reacted with Fluorescein Avidin D (Vector Laboratories). Sections were washed with PBS and mounted with VectorShield containing DAPI (Vector Laboratories). Slides were examined using a fluorescence microscope connected to the computer program SPOT Advanced (Diagnostic Instruments, Sterling Heights, MI).

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