**Journal of NeuroVirology,** 10: 171–180, 2004

ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280490444119

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# Murine gammaherpesvirus-68 elicits robust levels of interleukin-12 p40, but not interleukin-12 p70 production, by murine microglia and astrocytes

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**Murine gammaherpesvirus-68 (γHV-68) is a tractable model to investigate the pathophysiology of human gammaherpesvirus infections, including Epstein-Barr virus (EBV). Herpesvirus infections are thought to play a role in the pathology of damaging, inflammatory diseases states of the central nervous system (CNS), such as multiple sclerosis. The ability of the host to mount a strong cell-mediated immune response is critical in determining the outcome of viral infections. Interleukin (IL)-12 is an important inflammatory cytokine that plays a pivotal role in the development of protective cell-mediated immune responses to viral infections. Given recent reports of associations between gammaherpesvirus infections and inflammatory disorders of the CNS, the authors investigated the ability of γHV-68 to induce the production of bioactive IL-12 in resident CNS cell types. In the present study, the authors demonstrate that γHV-68 infection is a potent stimulus for IL-12p40 production by murine microglia and astrocytes. However, despite the elevated expression of mRNA encoding IL-12p40 subunit, concomitant with robust secretion of IL-12p40 protein, γHV-68 failed to elicit the production of the bioactive IL-12p70 heterodimer. This failure did not result from an absence of T lymphocyte–derived signals or interactions between CNS cell types as determined by coculture studies. Taken together, these data suggest that the resident CNS cell types, astrocytes and microglia, are not significant sources of proinflammatory IL-12p70 in response to gammaherpesvirus infection. Indeed, the production of IL-12p40 may point to an anti-inflammatory role for these cells during herpesvirus infections of the CNS.** *Journal of NeuroVirology* (2004) **10,** 171–180.

**Keywords:** brain; glial cells; human herpesvirus 4; inflammation

## Introduction

Viral infection of the central nervous system (CNS) results in damaging inflammation and has commonly been associated with severe demyelinating disease states (Sweet *et al*, 2002; Matthews *et al*, 2002). Specifically, herpesvirus infections of the CNS have been implicated in the pathology of a variety of devastating, inflammatory diseases of the CNS, including multiple sclerosis (MS) (Ascherio *et al*, 2001; Ascherio and Munch, 2000). Recently, murine gammaherpesvirus-68 (γHV-68) has provided a tractable model to investigate the pathophysiology of human infection with two herpesviruses, Epstein-Barr virus (EBV) and Kaposi's sarcoma–associated herpesvirus/human herpesvirus-8 (KSHV/HHV-8) (Doherty *et al*, 1997; Simas and Efstathiou, 1998; Speck and Virgin, 1999; Virgin and Speck, 1999). Genetic analysis has determined that γHV-68 is a type 2 gammaherpesvirus that induces a nonfatal, mononucleosis-like disease in immune-competent rodents (Sunil-Chandra *et al*, 1992; Doherty *et al*, 1997). Importantly, γHV-68 infection closely reproduces many of the features of γ-herpesvirus infections in humans, including latency establishment

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This work is supported by grant NS40307 to K.B. from the National Institutes of Health and by a grant to I.M. from the Foundation for the Carolinas.

Received 17 September 2003; revised 21 November 2003; accepted 26 January 2004.

and maintenance, lytic replication, and viral reactivation, thereby mirroring the pathophysiology of human infection with viruses such as EBV.

Interleukin (IL)-12 has been shown to be a critical factor in viral immunity, activating both innate and specific immune responses (for review, see Gately *et al*, 1998). IL-12 has been shown to activate natural killer cells and promote proliferation and differentiation of Th1 CD4+ cells and stimulate them to produce interferon (IFN)-γ (for review, see Gately *et al*, 1998). Importantly, viral infection of the CNS has been associated with enhanced expression of IL-12 in nervous tissue (Keogh *et al*, 2002; Olson *et al*, 2001; Vollstedt *et al*, 2001). Although IL-12 may be required for protective cell-mediated responses against viral infections, it may also contribute to CNS demyelination via activation of CD4+ T cells. For example, inhibition of IL-12 production, IL-12 signaling, and Th1 differentiation has been shown to result in decreased severity of CNS inflammation and demyelination during an animal model of MS (Natarajan and Bright, 2002). As such, although IL-12 is critical for controlling viral replication in the host CNS, it may also contribute to the damaging inflammation associated with viral infections.

Several studies have demonstrated the ability of herpesviruses, including EBV, to infect resident cells of the CNS (Menet *et al*, 1999; Cheeran *et al*, 2001; Lecointe *et al*, 1999). In keeping with its similarities to EBV, γHV-68 has been shown to replicate and persist within the mouse CNS (Terry *et al*, 2000). Recently, a study conducted in our laboratory demonstrated the ability of γHV-68 to readily infect murine microglia and astrocytes (Taylor *et al*, 2003). Interestingly, infected microglia and astrocytes respond to γHV-68 infection by the production of the key inflammatory cytokines, IL-6 and tumor necrosis factor (TNF)-α (Taylor *et al*, 2003). Although it has been reported that microglia and astrocytes produce IL-12 (Constantinescu *et al*, 1996; Aloisi *et al*, 1997), the ability of resident CNS cells to produce bioactive IL-12p70 upon γHV-68 infection has not been investigated.

In the present study, we demonstrate the inability of γHV-68 to induce the production of the inflammatory cytokine, IL-12p70, by murine microglia and astrocytes. We show that infection of these cell types with γHV-68 elicits marked up-regulation in the levels of mRNA encoding the p40 subunit of IL-12. In contrast, γHV-68 infection did not elicit the induction of mRNA encoding the p35 subunit, or the p40-related cytokine, IL-23, in either microglia or astrocytes. In agreement with these findings, γHV-68 infection elicits marked IL-12p40 secretion by both cell types, yet fails to induce bioactive IL-12p70 secretion. Further, microglia cocultured in the presence of CD4+ T cells, splenocytes, or astrocytes do not produce IL-12p70. Taken together, these studies indicate that microglia and astrocytes are not sources of inflammatory IL-12p70 during γHV-68 infection *in vitro,* suggesting that infiltrating leukocytes are a more likely source of this important Th1-type cytokine during gammaherpesvirus infections of the CNS. Indeed, given the previously documented ability of IL-12p40 homodimers to act in an anti-inflammatory manner (Ling *et al*, 1995; Gillessen *et al*, 1995; Heinzel *et al*, 1997), the present demonstration of γHV-68–infected glial cells secreting significant quantities of IL-12p40 may indicate an immunosuppressive role for these cell types during viral CNS infections.

## Results

### *Expression of mRNA encoding IL-12p35, IL-12p40, and IL-23p19 in γHV-68–infected microglia and astrocytes*

To begin to determine whether γHV-68 can elicit IL-12 production by murine microglia and astrocytes, we investigated the effect of γHV-68 infection on levels of mRNA encoding the p40 and p35 subunits of IL-12. Murine microglia or astrocytes were cultured in the absence or presence of γHV-68 for a 1-h adsorption period. At the indicated times post infection, RNA was isolated, and semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) was performed for the presence of IL-12p40 and IL-12p35 mRNA expression. As shown in Figures 1 and 2, there was little or no constitutive expression of mRNA encoding IL-12p40 in resting microglia and astrocytes, respectively. Interestingly, γHV-68 stimulation induced robust expression of mRNA encoding IL-12p40 as rapidly as 2 h post infection (Figures 1 and 2). In contrast, γHV-68 failed to elicit induction of IL-12p35 mRNA in either microglia or astrocytes (Figures 1 and 2). In addition, we have investigated the effect of γHV-68 infection on the expression of mRNA encoding the p19 subunit of IL-23. IL-23 is a newly discovered p40-related heterodimeric

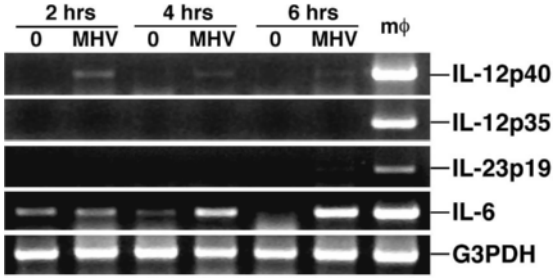


**Figure 1** Elevated expression of mRNA encoding IL-12p40, but not IL-12p35 or IL-23p19, in γHV-68–infected primary murine microglia. Microglia ($5 \times 10^5$) were untreated or infected with γHV-68 (5 PFU/cell) (MHV) for 1 h and RNA was isolated at the indicated times post infection for semiquantitative RT-PCR for the presence of mRNA encoding IL-12p40, IL-12p35, IL-6, or the housekeeping gene, G3PDH. As a positive control for the presence of IL-12p40, IL-12p35, and IL-23, mRNA was isolated from a similar number of murine peritoneal macrophages (mϕ) stimulated with 100 ng/ml LPS and 100 pg/ml IFN-γ. These studies were performed three times with similar results.

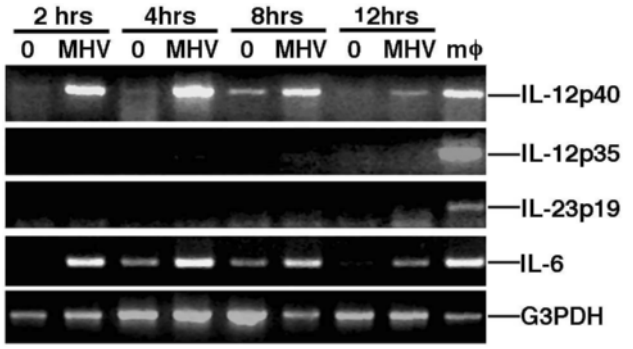


**Figure 2** Elevated expression of mRNA encoding IL-12p40, but not IL-12p35 or IL-23p19, in γHV-68–infected primary murine astrocytes. Astrocytes ($2 \times 10^6$) were untreated or infected with γHV-68 (5 PFU/cell) (MHV) for 1 h and RNA was isolated at the indicated times post infection for semiquantitative RT-PCR for the presence of mRNA encoding IL-12p40, IL-12p35, IL-6, or the housekeeping gene, G3PDH. As a positive control for the presence of IL-12p40, IL-12p35 and IL-23, mRNA was isolated from a similar number of murine peritoneal macrophages (mϕ) stimulated with 100 ng/ml LPS and 100 pg/ml IFN-γ. These studies were performed three times with similar results.

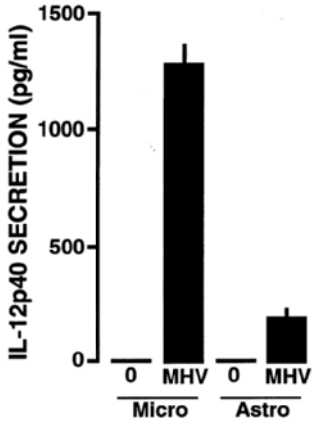


**Figure 3** Secretion of IL-12p40 by γHV-68–infected microglia and astrocytes. Cultured microglia ($5 \times 10^5$) and astrocytes ($2 \times 10^6$) were untreated or infected with γHV-68 (5 PFU/cell) (MHV) for 1 h. Culture supernatants were taken at 12 h post infection and a specific capture ELISA was performed to quantify IL-12p40 secretion. Results are presented as the mean of three separate experiments ± SEM.

cytokine composed of the IL-12p40 subunit and a novel p19 subunit (Oppmann *et al*, 2000). Because γHV-68 induced IL-12p40 mRNA expression by microglia and astrocytes (Figures 1 and 2), we investigated the ability of γHV-68 to induce expression of mRNA encoding the p19 subunit, and hence, IL-23. As shown in Figures 1 and 2, there was no constitutive expression of mRNA encoding IL-23 in murine microglia and astrocytes. Importantly, γHV-68 stimulation did not induce expression of IL-23 mRNA in murine microglia or astrocytes (Figures 1 and 2).

To confirm the ability of γHV-68 to induce proinflammatory cytokine production in microglia and astrocytes, PCR was performed to detect the presence of mRNA encoding IL-6. As shown in Figures 1 and 2, γHV-68 infection markedly induced expression of IL-6 mRNA, as previously described by our laboratory (Taylor *et al*, 2003). For comparison purposes and as a positive control for the presence of IL-12p40, IL-12p35, and IL-23, mRNA was harvested from a similar number of murine peritoneal macrophages (mϕ) isolated and stimulated with lipopolysacchariole (LPS) and IFN-γ as described previously (Marriott and Bost, 1998), and subjected to RT-PCR for each subunit (Figures 1 and 2). To ensure that differences in mRNA levels could not be attributed to differences in input RNA, or efficiencies of reverse transcription, RT-PCR amplification of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH), was performed for each sample (Figures 1 and 2). Taken together, these results demonstrate that although γHV-68 infection induces murine microglia and astrocytes to express mRNA encoding the p40 subunit of IL-12, the p35 subunit and the related heterodimeric cytokine IL-23 are not expressed by either of these cell types.

### *γHV-68 elicits IL-12p40 secretion by cultured murine microglia and astrocytes*

To address whether the elevations in mRNA encoding IL-12p40 in murine microglia and astrocytes exposed to γHV-68 translate into secretion of IL-12p40, a specific capture enzyme-linked immunosorbent assay (ELISA) was performed to detect the presence of this molecule. Culture supernatants of untreated microglia or astrocytes or cells exposed to γHV-68 (5 plaque-forming units [PFU]) were taken 12 h after infection and assayed for IL-12p40. As shown in Figure 3, γHV-68 elicited the production of significant levels of IL-12p40 by murine astrocytes and was a potent stimulus for IL-12p40 secretion by microglia.

### *T lymphocytes cocultured with γHV-68–infected murine microglia do not induce IL-12p70 secretion as measured indirectly by IFN-γ production*

Our inability to detect the presence of mRNA encoding the p35 subunit of IL-12 in microglia suggests that γHV-68 does not elicit the production of bioactive IL-12p70 heterodimers by these cells. In order to assay for the presence of IL-12p70 in γHV-68–stimulated microglia supernatants, we utilized a bioassay to indirectly detect the presence of IL-12 in cell culture supernatants by measuring IFN-γ production by splenic T cells. Isolated murine microglia ($5 \times 10^5$) were stimulated with γHV-68 (5 PFU) for a 1-h adsorption period. Twelve hours later, γHV-68 stimulated microglia were cocultured with T lymphocytes ($1 \times 10^6$) isolated from the spleens of uninfected mice and 12 or 24 h later, culture supernatants were harvested and assayed for the presence of IFN-γ by specific capture ELISA. As shown in Figure 4, T lymphocytes cocultured with γHV-68–stimulated murine microglia failed to elicit detectable levels of IFN-γ production at 12 and 24 h, respectively. To confirm the ability of

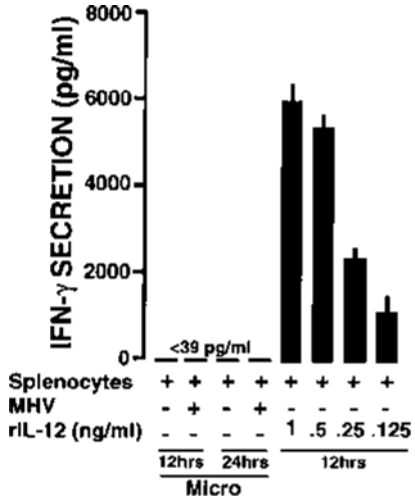


**Figure 4** T lymphocytes cocultured with γHV-68–infected microglia do not induce IL-12p70 secretion as determined by bioassay. Isolated murine microglia ($5 \times 10^5$) were stimulated with γHV-68 (5 PFU) (MHV) for a 1 h adsorption period (+). γHV-68 stimulated microglia were then cocultured with $1 \times 10^6$ T lymphocytes (+) isolated from the spleens of uninfected mice. 12 and 24 hrs later, culture supernatants were harvested and assayed for the presence of IFN-γ by a specific capture ELISA. In addition rIL-12 (1 ng/ml, 0.5 ng/ml, 0.25 ng/ml, 0.125 ng/ml) was added to T lymphocyte cultures alone to assess the ability of splenic T cells to respond to IL-12. After 12 h, culture supernatants were harvested and assayed for the presence of IFN-γ. Where indicated, levels were below the detectable range of the assay. Results are presented as the mean of 3 separate experiments ± SEM.

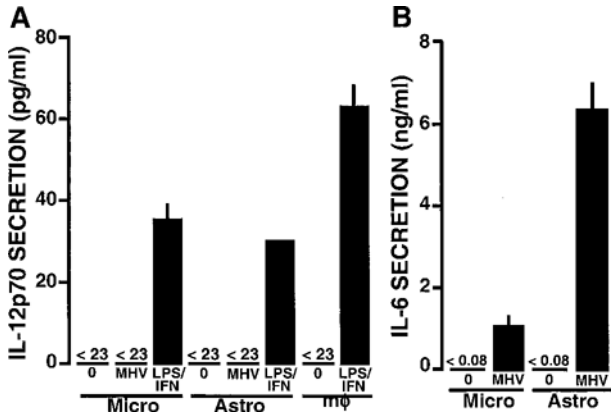


**Figure 5** Absence of IL-12p70 secretion by γHV-68–infected microglia and astrocytes as determined by specific capture ELISA. Cultured microglia ($5 \times 10^5$) and astrocytes ($2 \times 10^6$) were untreated or infected with γHV-68 (5 PFU/cell) (MHV) for 1 h. Panel A: Culture supernatants were taken at 12 h post infection and a specific capture ELISA was performed to quantify IL-12p70 secretion. As positive controls for the ability of these cells to produce L-12p70, supernatants were isolated from microglia and astrocytes maximally stimulated with 10 ng/ml LPS and 100 ng/ml IFN-γ. In an additional positive control, murine peritoneal macrophages (mφ) were stimulated with 100 ng/ml LPS and 100 pg/ml IFN-γ and culture supernatants were assayed for the presence of IL-12p70. Panel B: To ensure that cells were responsive to γHV-68–infection, a specific capture ELISA was performed on the same samples for the presence of IL-6. All results are presented as the mean of three separate experiments ± SEM.

splenic T cells to respond to IL-12, recombinant IL-12 (rIL-12) was added to T-cell cultures to elicit IFN-γ production in a dose-dependent manner (Figure 4).

### *γHV-68–infected murine microglia or astrocytes do not secrete IL-12p70 as measured by specific capture ELISA*

In order to confirm the inability of glial cells to produce the bioactive IL-12p70 heterodimer in response to γHV-68 infection, a specific capture ELISA for IL-12p70 was employed. In agreement with the results from the bioassay (Figure 4), γHV-68 failed to elicit detectable levels of IL-12p70 by murine microglia after 12 h (Figure 5A) or 24 and 48 h (data not shown). Furthermore, similar experiments with astrocytes failed to elicit detectable IL-12p70 (Figure 5A). To demonstrate that murine microglia and astrocytes can produce IL-12p70 following appropriate stimulation, secretion of IL-12p70 by LPS + IFN-γ–stimulated cells was assayed and is shown in Figure 5A. For comparison purposes and to demonstrate the ability of the capture ELISA to detect IL-12p70, secretion of IL-12p70 by resting (0) and LPS + IFN-γ–stimulated murine peritoneal macrophages was assayed and is shown in Figure 5A (mφ). In addition, to confirm that the cells used in this assay were responsive to γHV-68 stimulation, we quantified the amount of IL-6 present in the same culture supernatants (Figure 5B). Taken together, although isolated cultures of microglia and astrocytes respond to γHV-68 stimulation by producing robust levels of IL-6 and IL-12p40, these cells are not a source of bioactive IL-12p70.

### *γHV-68–infected murine microglia fail to produce IL-12p70 in the presence of activated CD4+ T-lymphocyte signals*

Previous studies have suggested that microglia may be induced to express IL-12p70 by microbial components only when signals derived from activated T lymphocytes are present (Aloisi *et al*, 1997; Stalder *et al*, 1997). To determine whether such signals might induce IL-12p70 production by γHV-68–infected microglia, we have utilized transgenic mice bearing a T-cell receptor having a single specificity for ovalbumin (OVA 323–339). Uninfected microglia, or cells infected with γHV-68 (5 PFU) were untreated or preincubated with 10 μg/ml ovalbumin peptide (323–339) for 2 h. Microglia were then cocultured in the presence of DO11.10-derived $CD4^+$ T cells at a final ratio of 50:1 $CD4^+$ T cells to microglia. After 24 and 48 h, cell supernatants were harvested and analyzed for the presence IL-12p70 and IFN-γ protein by specific capture ELISAs. As shown in Figure 6, γHV-68–stimulated microglia cocultured with $CD4^+$ T cells did not produce detectable levels of IL-12p70 after 24 or 48 h. Importantly, γHV-68–infected microglia successfully presented this antigen to T cells, resulting in T-cell activation as assessed by IFN-γ production

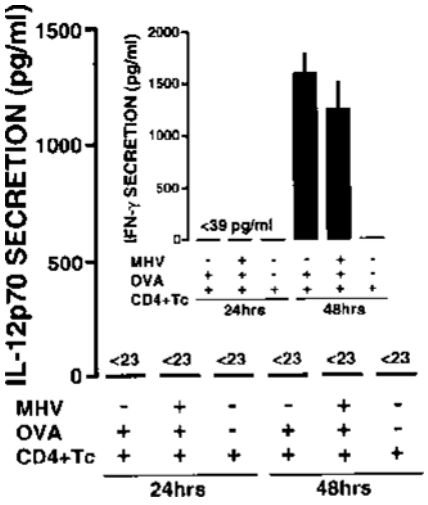


**Figure 6** Absence of IL-12p70 secretion by γHV-68–infected microglia in the presence of signals from T-lymphocytes activated in an antigen specific manner. Uninfected microglia, or cells infected with γHV-68 (5 PFU) were untreated or pre-incubated with 10 μg/ml ovalbumin peptide (323–339) for 2 h. Microglia were then cocultured in the presence of DO11.10-derived $CD4^+$ T cells (50:1 $CD4^+$ T cells to microglia). After 24 and 48 h, cell supernatants were harvested and analyzed for the presence of IL-12p70 by specific capture ELISA. To ensure that T cells were responsive to antigen stimulation, a specific capture ELISA was performed on the same samples for the presence of IFN-γ (inset). Results are presented as the mean of three separate experiments ± SEM.

(Figure 6, *inset*). As such, these data suggest that γHV-68–infected microglia fail to secrete IL-12p70 even in the presence of T cell–derived signals, such as IFN-γ.

### *γHV-68–infected murine microglia cocultured with astrocytes do not produce IL-12p70*

*In vivo*, it is probable that astrocyte and microglia functions are interrelated during inflammatory responses within the CNS. To address whether astrocytes could contribute to microglia-derived IL-12p70 production in response to γHV-68 stimulation, we employed mixed glial cultures obtained from 2-day-old neonates. Mixed glial cells were untreated or exposed to γHV-68 (5 PFU) and cell culture supernatants were harvested 12 and 24 h after exposure and assayed for the presence of IL-12p70. As shown in Figure 7, coculture fails to result in the production of IL-12p70 by either cell type at any of the time points indicated. However, the cells were able to respond to γHV-68 stimulation with a marked increase in IL-6 production after 12 h (Figure 7, *inset*) or 24 h (data not shown). Again, for comparison purposes, and to demonstrate the ability of the capture ELISA to detect IL-12p70, secretion of IL-12p70 by resting (0) and LPS + IFN–stimulated murine peritoneal macrophages was assayed at 12 and 24 h and is shown in Figure 7 (mϕ).

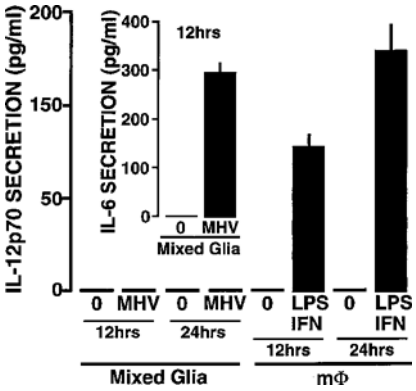


**Figure 7** γHV-68–infected microglia cocultured with astrocytes do not produce IL-12p70. Mixed glial cultures were obtained from 2 day old Balb/c neonates. Mixed glial cultures were untreated (0) or exposed to γHV-68 (MHV) (5 PFU). 12 and 24 h later, culture supernatants were harvested and assayed for the presence of IL-12p70 by a specific capture ELISA. To demonstrate that the mixed glial cells were responsive to γHV-68, culture supernatants harvested at 12 h were also analyzed for IL-6 secretion by ELISA (inset). For comparison purposes, and to demonstrate the ability of the capture ELISA to detect IL-12p70, culture supernatants were harvested from resting (0) and LPS + IFN-γ treated peritoneal macrophages (mϕ) after 12 and 24 h. Results are presented as the mean of three separate experiments ± SEM.

## Discussion

The involvement of viral infections in inflammatory diseases of the CNS has recently been the subject of much interest following reports of associations between EBV infection and the risk of MS (Villoslada *et al*, 2003; Levin *et al*, 2003; Ascherio *et al*, 2001). Such a hypothesis is attractive given that latent infections caused by herpesviruses, such as EBV, periodically reactivate in a manner that resembles the relapsing-remitting nature of MS (Sunil-Chandra *et al*, 1992). The possible link between herpesvirus infection of the CNS and the development of inflammatory disease states has necessitated an animal model of herpesvirus infection to study the CNS host response. The murine γHV-68 has provided such a model, whereby the pathogenesis of human herpesvirus infections can be reliable reproduced (Doherty *et al*, 1997; Simas and Efstathiou, 1998; Speck and Virgin, 1999; Virgin and Speck, 1999). To date, the identity of the specific CNS cell populations that are targets for herpesvirus infection, and the nature of the cellular responses evoked by such a challenge within the CNS, have been poorly categorized. Recent work from our laboratory has indicated that the resident glial cell types, astrocytes and microglia, can be infected by, and harbor, the murine herpesvirus, γHV-68 (Taylor *et al*, 2003).

Microglia are resident immune cells of the CNS and like other professional antigen-presenting cells, such as macrophages and dendritic cells, are of myeloid lineage. As such, these cells are likely to play important roles in either the development of protective

immune responses to invading pathogens or aiding the development of damaging inflammation during CNS disease states (Stoll and Jander, 1999). Microglia are known to be facultative phagocytes expressing major histocompatibility complex (MHC) class II molecules (Hickey and Kimura, 1988). Studies have reported that microglia are capable of producing IL-12 subunits in response to viral infection of the CNS (Olson *et al*, 2001). However, IL-12p70 production by resident immune cells, such as astrocytes and microglia, may occur in a T cell/IFN-γ–dependent manner. For example, Aloisi and coworkers (1997) demonstrated that LPS alone is not an efficient stimulus to elicit IL-12p70 production by murine microglia; however, LPS stimulation in combination with IFN-γ led to marked increases in IL-12p70 production by these cells (Aloisi *et al*, 1997). As such, although this cell type may be capable of making IL-12p70, the production of this cytokine appears to be highly regulated, requiring T cell–dependent signals.

Previous studies from our laboratory have demonstrated the ability of microglia and astrocytes to respond to γHV-68, a gammaherpesvirus that closely mimics the pathology of EBV infections in humans, by the production of the key inflammatory cytokines, TNF-α, and IL-6 (Taylor *et al*, 2003). In the present study, we demonstrate the failure of γHV-68 infection to induce the production of IL-12p70 by murine microglia and astrocytes. Importantly, we demonstrate that this failure occurs even in the presence of signals derived from T lymphocytes activated in an antigen-dependent manner. Interestingly, we show that γHV-68 infection of microglia and astrocytes does elicit enhanced expression of mRNA encoding the p40 subunit of IL-12 (Figures 1 and 2) and IL-12p40 secretion (Figure 3), although it must be noted that the robust expression of mRNA encoding IL-12p40 in astrocytes results in relatively modest secretion of the protein product (Figure 3). Such a finding is surprising given the important role for IL-12p70 in the generation of optimal cell-mediated immune responses against viral pathogens (for review, see Gately *et al*, 1998).

Recently, a novel p40-related cytokine, IL-23, has been described (Oppmann *et al*, 2000). IL-23 is a heterodimeric cytokine composed of the IL-12p40 subunit and a novel p19 subunit and is produced by myeloid cell types, including macrophages and dendritic cells (Belladonna *et al*, 2002; Oppmann *et al*, 2000). Importantly, this cytokine has been shown to perform many of the same functions attributed to IL-12 (Belladonna *et al*, 2002) and its expression has been detected within the CNS (Broberg *et al*, 2002; Cua *et al*, 2003). Indeed, one recent report has suggested that it is IL-23, and not IL-12p70, that plays a crucial role in the development of CNS inflammation (Cua *et al*, 2003). As such, we have investigated the expression of IL-23 (p19 subunit) mRNA in both astrocytes and microglia. In contrast to earlier studies, we failed to detect expression of IL-23p19 mRNA in either cell type, whether constitutively or following γHV-68 infection. However, the present results do not preclude the possibility that other resident CNS cell types, such as perivascular macrophages, may be a source of this cytokine.

The present finding that γHV-68 induces significant IL-12p40 secretion by both glial cell types raises intriguing possibilities. IL-12p40 homodimers have previously been shown to antagonize IL-12p70 bioactivity by competing for receptor occupancy (Ling *et al*, 1995; Gillessen *et al*, 1995; Heinzel *et al*, 1997). As such, the possibility exists that glial cells themselves can limit the progression of potentially devastating inflammation within the confines of the CNS by producing anti-inflammatory IL-12p40 homodimers. Alternatively, these findings may indicate a role for the virus in subverting antiviral cellular responses. Intriguingly, EBV infection has been shown to induce the production of EBI3, an EBV-induced cytokine homologous to the IL-12p40 subunit that can heterodimerize with IL-12p35, thereby antagonizing bioactive IL-12p70 production (Niedobitek *et al*, 2002). Although such a product has not been identified in γHV-68, the possibility that the virus induces a mediator analogous to EBI3 cannot be ruled out.

Taken in concert, the present study demonstrates that microglia and astrocytes do not express IL-12p70 or IL-23 *in vitro* upon γHV-68 infection. In contrast, virally infected microglia and astrocytes are a significant source of IL-12p40, a potentially immunosuppressive molecule that might antagonize the inflammatory effects of IL-12p70. Although the reasons for the apparent disparity between the present results and the findings of others (Olson *et al*, 2001) in virally challenged glial cells remain unclear, these differences may arise from the inference of IL-12p70 production from detection of the p40 subunit in some earlier work, use of cell lines rather than primary glial cell cultures, or from the nature of the virus itself. Furthermore, we cannot preclude the possibility that other resident cell types, such as perivascular macrophages or infiltrating leukocytes, may be sources of inflammatory IL-12p70 and/or IL-23 production during CNS disease progression.

## Materials and methods

*Virus isolation*

γHV-68 was provided by Tony Nash (University of Edinburgh, UK) and Peter Doherty (St. Jude's Hospital, Memphis, TN, USA). Virus stock was prepared by infecting BHK-21 cells (ATCC CCL 10) with γHV-68 at a low multiplicity of infection (m.o.i) (0.1 PFU per cell). Twenty-four h later, the cells were trypsinized and centrifuged. The resulting pellet was pulse sonicated (Vibra Cell) at an output of 5 W to ensure release of intact virions. The sonicated material was centrifuged to remove cellular debris and the supernatant containing virions was aliquoted and stored at −80°C. Virus was quantified by making threefold

serial dilutions on NIH-3T3 cell (ATCC CRL 1658) monolayers and the number of PFU was determined was quantified as described previously (Taylor *et al*, 2003; Stevenson *et al*, 1999; Stewart *et al*, 1998) using a plaque-forming assay. Briefly, cells were pulse sonicated to release intracellular virus. After sonication, lysates were centrifuged at 2500 r.p.m. to remove cellular debris. Limiting dilutions of the lysates were placed on NIH-3T3 monolayers for 1 h followed by washing and overlaying with 0.15% agar (Difco) in RPMI 1640 medium supplemented with 30% fetal calf serum (FCS). After 5 days, overlays were removed and cell monolayers were stained with crystal violet. The number of PFU were quantified in duplicate at several dilutions. NIH-3T3 cells were maintained in RPMI (Gibco-BRL, Gaithersburg, MD) supplemented with 10% FCS at 37°C and 5% $CO_2$.

*Isolation of primary murine microglia and astrocytes and* in vitro *stimulation*

Murine neonatal brain microglia were isolated and cultured as described previously by our laboratory (Rasley *et al*, 2002a, 2002b; Taylor *et al*, 2003) and maintained in RPMI 1640 medium supplemented with 10% FCS and 20% conditioned medium from LADMAC cells as a source of colony stimulating factor (CSF-1) (O'Keefe *et al*, 2001; Rasley *et al*, 2002a, 2002b; Taylor *et al*, 2003). Astrocytes were isolated and cultured as described previously by our laboratory (Taylor *et al*, 2003; Bowman *et al*, 2003) and maintained in RPMI 1640 medium supplemented with 10% FCS. Cells isolated in this fashion were previously shown to be greater than 95% authentic microglia or astrocytes due to their characteristic morphology and the presence of the microglia cell surface markers CD11b and F4/80 or the presence of the astrocyte marker glial fibrillary acidic protein (GFAP) as determined by fluorescence-activated cell sorting (FACS) analysis and confocal microscopy, respectively (Rasley *et al*, 2002b; Taylor *et al*, 2003; Bowman *et al*, 2003).

Microglia and astrocytes were infected with γHV-68 at m.o.i of 5 PFU and the virus was allowed to adsorb for 1 h. A dose of γHV-68 at 5 PFU per cell was previously determined by our laboratory to be optimal for expression of viral DNA and the presence of lytic viral particles in exposed microglia and astrocytes (Taylor *et al*, 2003). After 60 min, nonadherent viral particles were removed by washing, followed by the addition of RPMI 1640 medium supplemented with 10% FCS and 20% LADMAC (microglia) or 10% FCS (astrocytes). Cultures were maintained for 4, 8, 12, 24, or 48 h for isolation of poly(A)$^+$ RNA and culture supernatants as indicated.

*Isolation of poly(A)$^+$ RNA and semiquantitative RT-PCR*

Total RNA was isolated from microglia and astrocytes using TRIzol reagent (GIBCO-BRL) as previously described (Rasley *et al*, 2002a, 2002b; Taylor *et al*, 2003; Bowman *et al*, 2003). Poly(A)$^+$ RNA was then isolated from total RNA using polystyrene latex–oligo dT beads (Oligotex-dT) (Qiagen, Chatsworth, CA) as described previously (Rasley *et al*, 2002a, 2002b; Taylor *et al*, 2003; Bowman *et al*, 2003). Poly(A)$^+$ RNA was reverse transcribed in the presence of random hexamers using 200 U RNase H−, Moloney leukemia virus reverse transcriptase (RT) (Promega, Madison, WI) in the buffer supplied by the manufacturer, as described previously by our laboratory (Rasley *et al*, 2002a, 2002b; Taylor *et al*, 2003; Bowman *et al*, 2003).

PCR was performed on the reverse-transcribed cDNA product to determine the expression of mRNA encoding IL-12 and IL-23, essentially as described previously (Rasley *et al*, 2002a, 2002b; Taylor *et al*, 2003; Bowman *et al*, 2003). Positive- and negative-strand primers used, respectively, were GCACCAA ATTACTCCGGACGGTTC and GCAAGTTCTTGGGC GGGTCTG to amplify mRNA encoding the murine p40 subunit of IL-12; AAGACATCACACGGGACC AAACCA and CGCAGAGTCTCGCCATTATGATTC to amplify mRNA encoding the murine p35 subunit of IL-12; CTGCTTGCAAAGGATCCGCCAAGG and CTCAGTCAGAGTTGCTGCTCCGTG to amplify mRNA encoding the murine p19 subunit of IL-23; and CCATCACCATCTTCCAGGAGCAGCGAG and CACAGTCTTCTGGGTGGCAGTGAT to amplify mRNA encoding murine G3PDH. PCR primers were derived from the published sequences of IL-12 (Schoenhaut *et al*, 1992) and IL-23 (Oppmann *et al*, 2000). These primers were designed using oligo 4.0 primer analysis software (National Biosciences, Plymouth, MA) based on their location in different exons of the genomic sequences for IL-12 and IL-23 in addition to their lack of significant homology to sequences present in GenBank (MacVector Sequence analysis software) (IBI, New Haven, CT). All amplifications were performed in the linear range for that reaction that was determined as previously described by our laboratory (Bost and Clements, 1995).

Following PCR, 15% of each amplified sample was electrophoresed on ethidium bromide–stained agarose gels and visualized under ultraviolet (UV) illumination. PCR amplification of the housekeeping gene, G3PDH, was performed on cDNA from each sample to insure equal input of RNA and similar efficiencies of reverse transcription. The identities of the PCR-amplified fragments were verified by size comparison with DNA standards.

*Bioassay for IL-12p70 production*

The bioassay is based on the ability of IL-12 to induce the secretion of IFN-γ by mononuclear splenocytes in a quantitative manner (Bost and Clements, 1997). Adherent microglia were infected with γHV-68 at an m.o.i of 5 PFU and the virus was allowed to adsorb for 1 h. After 60 min, nonadherent viral particles were removed by washing, followed by the addition of RPMI 1640 medium supplemented with

10% FCS and 20% LADMAC and cultured for 12 or 24 h. Total T lymphocytes were enriched from splenic leukocytes by passage through a 30 gauge wire-mesh screen as previously described by our laboratory (Elhofy *et al*, 2000). Cells isolated in this manner were previously determined to be >80% T lymphocytes using flow cytometry (Elhofy *et al*, 2000) to detect the presence of CD3 (clone 145-2C11; BD PharMingen, San Diego, CA). Total T lymphocytes, at $1 \times 10^6$, isolated in this manner were added to $5 \times 10^5$ virally infected microglia. At 12 h after coculture, cell supernatants were harvested and analyzed for the presence of IFN-$\gamma$ protein by specific capture ELISA. To assess the ability of splenic T cells to respond to IL-12p70 by IFN-$\gamma$ production, known concentrations of recombinant IL-12p70 (BD PharMingen) were added to equal numbers of splenic T cells.

*ELISA for quantification of IL-12p40, IL-12p70, and IFN-$\gamma$ secretion*

Capture ELISAs were performed to quantify IL-12p40, IL-12p70, and IFN-$\gamma$ production, essentially as described previously (Rasley *et al*, 2002a, 2002b; Taylor *et al*, 2003; Bowman *et al*, 2003). Pairs of capture and detection antibodies were used to quantify IL-12p40 (clones MP5-20F3 and MP5-32C11; BD Pharmingen). IL-12p70 was quantified using a Quantikine assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. IFN-$\gamma$ was quantified using an OptEIA assay kit (BD Pharmingen) according to the manufacturer's instructions.

*Isolation of splenic CD4$^+$ lymphocytes from transgenic mice expressing an ovalbumin-specific T-cell receptor*

DO11.10 mice on a BALB/c background expressing a single T-lymphocyte receptor specific for a peptide derived from ovalbumin (OVA peptide 323–339), and restricted to expression by class II MHC for ovalbumin, were used in this study. Spleens were removed and single-cell suspensions were prepared. CD4$^+$ T lymphocytes were then magnetically separated using a ferritin-conjugated anti-CD4 antibody according to instructions supplied by the manufacturer (Miltenyi Biotech, Auburn, CA) and using the VARIO MACS magnetic separator (Miltenyi Biotech). Cells not expressing CD4 were washed through with excess buffer, and cells retained within the column were eluted with buffer after removal of the column from the magnetic field. Cells isolated in this manner were >98% CD4$^+$ as determined by FACS analysis.

*Cocultures of γHV-68–infected microglia with DO11.10-derived CD4$^+$ T cells*

Uninfected microglia, or cells infected with γHV-68 (5 PFU) were untreated or preincubated with 10 $\mu$g/ml ovalbumin peptide (323–339) for 2 h. Microglia were then cocultured in the presence of DO11.10-derived CD4$^+$ T cells at a final ratio of 50:1 CD4$^+$ T cells to microglia. At 24 and 48 h, cell supernatants were harvested and analyzed for the presence IL-12p70 protein by specific capture ELISA.

*Statistical analysis*

Results of the present studies were tested statistically using Student's paired *t* test or one-way analysis of variance (ANOVA) using commercially available software (GraphPad Prism) (GraphPad Software, San Diego, CA). Results were determined to be statistically significant when a probability of $<.05$ was obtained.

## References

Aloisi F, Penna G, Cerase J, Menendez Iglesias B, Adorini L (1997). IL-12 production by central nervous system microglia is inhibited by astrocytes. *J Immunol* **159:** 1604–1612.

Ascherio A, Munch M (2000). Epstein-Barr virus and multiple sclerosis. *Epidemiology* **11:** 220–224.

Ascherio A, Munger KL, Lennette ET, Spiegelman D, Hernan MA, Olek MJ, Hankinson SE, Hunter DJ (2001). Epstein-Barr virus antibodies and risk of multiple sclerosis: a prospective study. *JAMA* **286:** 3083–3088.

Belladonna ML, Renauld JC, Bianchi R, Vacca C, Fallarino F, Orabona C, Fioretti MC, Grohmann U, Puccetti P (2002). IL-23 and IL-12 have overlapping, but distinct, effects on murine dendritic cells. *J Immunol* **168:** 5448–5454.

Bost KL, Clements, JD (1995). In vivo induction of interleukin-12 mRNA expression after oral immunization with *Salmonella dublin* or the B subunit of *Escherichia coli* heat-labile enterotoxin. *Infect Immun* **63:** 1076–1083.

Bost KL, Clements JD (1997). Intracellular *Salmonella dublin* induces substantial secretion of the 40-kilodalton subunit of interleukin-12 (IL-12) but minimal secretion of IL-12 as a 70-kilodalton protein in murine macrophages. *Infect Immun* **65:** 3186–3192.

Bowman CC, Rasley A, Tranguch SL, Marriott I (2003). Cultured astrocytes express toll-like receptors for bacterial products. *Glia* **43:** 281–291.

Broberg EK, Setala N, Eralinna JP, Salmi AA, Roytta M, Hukkanen V (2002). Herpes simplex virus type 1 infection induces upregulation of interleukin-23 (p19) mRNA expression in trigeminal ganglia of BALB/c mice. *J Interferon Cytokine Res* **22:** 641–651.

Cheeran MC, Hu S, Yager SL, Gekker G, Peterson PK, Lokensgard JR (2001). Cytomegalovirus induces cytokine and chemokine production differentially in microglia and astrocytes: antiviral implications. *J NeuroVirol* **7:** 135–147.

Constantinescu CS, Frei K, Wysocka M, Trinchieri G, Malipiero U, Rostami A, Fontana A (1996). Astrocytes

and microglia produce interleukin-12 p40. *Ann NY Acad Sci* **795:** 328–333.

Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, Lucian L, To W, Kwan S, Churakova T, Zurawski S, Wiekowski M, Lira SA, Gorman D, Kastelein RA, Sedgwick JD (2003). Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* **421:** 744–748.

Doherty PC, Tripp RA, Hamilton-Easton AM, Cardin RD, Woodland DL, Blackman MA (1997). Tuning into immunological dissonance: an experimental model for infectious mononucleosis. *Curr Opin Immunol* **9:** 477–483.

Elhofy A, Marriott I, Bost KL (2000). Salmonella infection does not increase expression and activity of the high affinity IL-12 receptor. *J Immunol* **165:** 3324–3332.

Gately MK, Renzetti LM, Magram J, Stern AS, Adorini L, Gubler U, Presky DH (1998). The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Annu Rev Immunol* **16:** 495–521.

Gillessen S, Carvajal D, Ling P, Podlaski FJ, Stremlo DL, Familletti PC, Gubler U, Presky DH, Stern AS, Gately MK (1995). Mouse Interleukin-12 (IL-12) p40 homodimer: a potent IL-12 anatagonist. *Eur J Immunol* **25:** 200–206.

Heinzel FP, Hujer AM, Ahmed FN, Rerko RM (1997). In vivo production and function of IL-12 p40 homodimers. *J Immunol* **158:** 4381–4388.

Hickey WF, Kimura H (1998). Perivascular microglial cells of the CNS are bone-marrow-derived and present antigen in vivo. *Science* **239:** 290–292.

Keogh B, Atkins GJ, Mills KH, Sheahan BJ (2002). Avirulent Semliki Forest virus replication and pathology in the central nervous system is enhanced in IL-12-defective and reduced in IL-4-defective mice: a role for Th1 cells in the protective immunity. *J Neuroimmunol* **125:** 15–22.

Lecointe D, Hery C, Janabi N, Dussaix E, Tardieu M (1999). Differences in kinetics of human cytomegalovirus cell-free viral release after in vitro infection of human microglial cells, astrocytes and monocyte-derived macrophages. *J NeuroVirol* **5:** 308–313.

Levin LI, Munger KL, Rubertone MV, Peck CA, Lennette ET, Spiegelman D, Ascherio A (2003). Multiple sclerosis and Epstein-Barr virus. *JAMA* **289:** 1533–1536.

Ling P, Gately MK, Gubler U, Stern AS, Lin P, Hollfelder K, Su C, Pan YC, Hakimi J (1995). Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. *J Immunol* **154:** 116–127.

Marriott I, Bost KL (1998). Substance P diminishes lipopolysaccharide and interferon-gamma-induced TGF-beta 1 production by cultured murine macrophages. *Cell Immunol* **183:** 113–120.

Matthews AE, Lavi E, Weiss SR, Paterson Y (2002). Neither B cells nor T cells are required for CNS demyelination in mice persistently infected with MHV-A59. *J NeuroVirol* **8:** 257–264.

Menet A, Speth C, Larcher C, Prodinger WM, Schwendinger MG, Chan P, Jager M, Schwarzmann F, Recheis H, Fontaine M, Dierich MP (1999). Epstein-Barr virus infection of human astrocyte cell lines. *J Virol* **73:** 7722–7733.

Natarajan C, Bright JJ (2002). Peroxisome proliferator-activated receptor-gamma agonists inhibit experimental allergic encephalomyelitis by blocking IL-12 production, IL-12 signaling and Th1 differentiation. *Genes Immun* **3:** 59–70.

Niedobitek G, Pazolt D, Teichmann M, Devergne O (2002). Frequent expression of the Epstein-Barr virus (EBV)-induced gene, EBI3, an IL-12 p40-related cytokine, in Hodgkin and Reed-Sternberg cells. *J Pathol* **198:** 310–316.

O'Keefe GM, Nguyen VT, Tang LP, Benveniste EN (2001). IFN-$\gamma$ regulation of class II transactivator promoter IV in macrophages and microglia: involvement of the suppression of cytokine signaling-1 protein. *J Immunol* **166:** 2260–2269.

Olson JK, Girvin AM, Miller SD (2001). Direct activation of innate and antigen-presenting functions of microglia following infection with Theiler's virus. *J Virol* **75:** 9780–9789.

Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, Vega F, Yu N, Wang J, Singh K, Zonin F, Vaisberg E, Churakova T, Liu M, Gorman D, Wagner J, Zurawski S, Liu Y, Abrams JS, Moore KW, Rennick D, de Waal-Malefyt R, Hannum C, Bazan JF, Kastelein RA (2000). Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* **13:** 715–725.

Rasley A, Anguita J, Marriott I (2002a). *Borrelia burgdorferi* induces inflammatory mediator production by murine microglia. *J Neuroimmunol* **130:** 22–31.

Rasley A, Bost KL, Olson JK, Miller SD, Marriott I (2002b). Expression of functional NK-1 receptors in murine microglia. *Glia* **37:** 258–267.

Schoenhaut DS, Chua AO, Wolitzky AG, Quinn PM, Dwyer CM, McComas W, Familletti PC, Gately MK, Gubler U (1992). Cloning and expression of murine IL-12. *J Immunol* **148:** 3433–3440.

Simas JP, Efstathiou S (1998). Murine gammaherpesvirus 68: a model for the study of gammaherpesvirus pathogenesis. *Trends Microbiol* **6:** 276–282.

Speck SH, Virgin HW (1999). Host and viral genetics of chronic infection: a mouse model of gammaherpesvirus pathogenesis. *Curr Opin Microbiol* **2:** 403–409.

Stalder AK, Pagenstecher A, Yu NC, Kincaid C, Chiang CS, Hobbs MV, Bloom FE, Campbell IL (1997). Lipopolysaccharide-induced IL-12 expression in the central nervous system and cultured astrocytes and microglia. *J Immunol* **159:** 1344–1351.

Stevenson PG, Belz GT, Castrucci MR, Altman JD, Doherty PC (1999). A gamma-herpesvirus sneaks through a CD8(+) T cell response primed to a lytic-phase epitope. *Proc Natl Acad Sci U S A* **96:** 9281–9386.

Stewart JP, Janjua NJ, Pepper SD, Bennion G, Mackett M, Allen T, Nash AA, Arrand JR (1998). Identification and characterization of murine gammaherpesvirus 68 gp150: a virion membrane glycoprotein. *J Virol* **70:** 3528–3535.

Stoll G, Jander S (1999). The role of microglia and macrophages in the pathophysiology of the CNS. *Prog Neurobiol* **58:** 233–247.

Sunil-Chandra NP, Efstathiou S, Arno J, Nash AA (1992). Virological and pathological features of mice infected with murine gamma-herpesvirus 68. *J Gen Virol* **73:** 2347–2356.

Sweet TM, Valle LD, Khalili K (2002). Molecular biology and immunoregulation of human neurotropic JC virus in CNS. *J Cell Physiol* **191:** 249–256.

Taylor WR, Rasley A, Bost KL, Marriott I (2003). Murine gammaherpesvirus-68 infects microglia and induces

high levels of pro-inflammatory cytokine production. *J Neuroimmunol* **136:** 75–83.

Terry LA, Stewart JP, Nash AA, Fazakerley JK (2000). Murine gammaherpesvirus-68 infection of and persistence in the central nervous system. *J Gen Virol* **81:** 2635–2643.

Villoslada P, Juste C, Tintore M, Llorenc V, Codina G, Pozo-Rosich P, Montalban X (2003). The immune response against herpesvirus is more prominent in the early stages of MS. *Neurology* **60:** 1944–1948.

Virgin HW, Speck SH (1999). Unraveling immunity to gamma-herpesviruses: a new model for understanding the role of immunity in chronic virus infection. *Curr Opin Immunol* **11:** 371–379.

Vollstedt S, Franchini M, Alber G, Ackermann M, Suter M (2001). Interleukin-12- and gamma interferon-dependent innate immunity are essential and sufficient for long-term survival of passively immunized mice infected with herpes simplex virus type 1. *J Virol* **75:** 9596–9600.