Article
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# Role of IL-4 in an experimental model of encephalitis induced by intracranial inoculation of herpes simplex virus-1 (HSV-1)

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## ABSTRACT

Herpes simplex virus-1 (HSV-1) is a pathogen that may cause severe encephalitis in humans. In this study, we aimed to investigate the role of interleukin-4 (IL-4) in a model of HSV-1 brain infection. IL-4 knockout (IL-4$^{-/-}$) and wild type (WT) C57BL/6 mice were inoculated with $10^4$ plaque-forming units of HSV-1 by the intracranial route. Histopathologic analysis revealed a distinct profile of infiltrating cells at 3 days post-infection (dpi). Infected WT mice presented mononuclear inflammatory cells while IL-4$^{-/-}$ mice developed meningoencephalitis with predominance of neutrophils. IL-4$^{-/-}$ mice had diminished leukocyte adhesion at 3 dpi when compared to infected WT animals in intravital microscopy study. Conversely no differences were found in cerebral levels of CXCL1, CXCL9, CCL3, CCL5 and TNF-α between WT and IL-4$^{-/-}$ infected mice. IL-4 may play a role in the recruitment of cells into central nervous system in this acute model of severe encephalitis caused by HSV-1.

**Key words:** herpes simplex virus type 1, IL-4, neuroinflammation.

**Papel da IL-4 em modelo experimental de encefalite induzida pela inoculação intracraniana do herpes simplex vírus-1 (HSV-1)**

## RESUMO

O vírus herpes simplex-1 (HSV-1) é um patógeno que pode causar encefalite grave em humanos. Neste estudo, buscamos investigar o papel da interleucina-4 (IL-4) no modelo de infecção intracerebral por HSV-1. Camundongos C57BL/6 selvagens (WT) e deficientes no gene IL-4 (IL-4$^{-/-}$) foram inoculados com $10^4$ unidades formadoras de placas de HSV-1 por via intracraniana. A análise histopatológica revelou um padrão distinto de infiltrado leucocitário. Camundongos WT infectados apresentaram infiltrado de células mononucleares, enquanto camundongos IL-4$^{-/-}$ desenvolveram meningoencefalite com predomínio de neutrófilos 3 dias pós-infecção (dpi). Animais IL-4$^{-/-}$ tiveram menor adesão de leucócitos 3 dpi quando comparados aos animais WT infectados à microscopia intravital. Em contrapartida, não foram encontradas diferenças nos níveis cerebrais de CXCL1, CXCL9, CCL3, CCL5 e TNF-α entre camundongos WT e IL-4$^{-/-}$ infectados. Esses resultados sugerem que IL-4 pode desempenhar um papel no recrutamento de células no sistema nervoso central neste modelo agudo de encefalite grave causada pelo HSV-1.

**Palavras-chave:** vírus herpes simplex tipo 1, IL-4, neuroinflamação.

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Interleukin-4 (IL-4) is a pleiotropic cytokine synthesized primarily by $CD4^+$ T lymphocytes in response to their activation[1,2]. Studies have reported that IL-4 may have either detrimental or protective effects during viral infection[3-6]. For instance, the expression of IL-4 by mousepox virus, due to the insertion of a copy of mouse IL-4 cDNA in viral genome, turns this virus lethal to mice that are usually resistant to the infection[3]. Similarly, the expression of IL-4 by myxoma virus enhances virulence and overcomes genetic resistance of rabbits to viral infection[4]. IL-4 knockout ($IL\text{-}4^{-/-}$) mice challenged with herpes simplex virus type 1 (HSV-1) by ocular route had reduced virus load in their eyes when compared with wild type (WT) mice[5]. Conversely one study demonstrated that a recombinant HSV-1 expressing IL-4 had a great decrease in its pathogenic potential[6].

HSV-1 is a neurotropic virus known to cause infection in the central nervous system (CNS). Herpes simplex encephalitis is a common sporadic viral disease of the brain[7,8]. Although antiviral treatment has greatly reduced mortality due to herpetic encephalitis, the majority of survivors presents residual neuropsychological deficits and/or neuropsychiatric symptoms[9]. Our group has developed an experimental model of severe HSV-1 encephalitis[10-12]. We observed an increase in the levels of rolling and adhered leukocytes in meningeal vessels of infected mice in parallel with the increase of the expression of several cytokines in the CNS[12]. Nonetheless, the role of IL-4 on the early inflammatory response to HSV-1 brain infection has not been investigated yet.

In the present study we aimed to investigate the possible involvement of IL-4 in the inflammatory response to HSV-1, assessing the recruitment of leukocytes by intravital microscopy, the chemokine and cytokine profile and the histopathological changes in $IL\text{-}4^{-/-}$ and WT mice infected with an intracerebral inoculum of HSV-1.

## METHOD

### Mouse strains

Male C57BL/6 mice and $IL\text{-}4^{-/-}$ mice on a C57BL/6 background, aged 6-9 weeks, were obtained from Animal Care Facilities of the Institute of Biological Sciences (ICB), Federal University of Minas Gerais (UFMG). All experiments were approved by the Animal Ethics Committee of UFMG.

### Virus

HSV-1 strain EK[13] was allowed to multiply in Vero cells and was maintained with minimal essential medium (GIBCO, Grand Island, NY) containing 5% fetal bovine serum (FBS) (GIBCO) and 25 μg/μL of ciprofloxacin (Cellofarm, Carapina, ES, Brazil) at 37°C in 5% $CO_2$. Virus was purified in sucrose gradient and the titers determined in Vero cells as previously described[14,15]. The virus titers obtained were $1.1\times10^8$ plaque-forming cells (PFU)/mL for HSV-1.

### Vero cells

Vero cells were maintained in minimal essential medium (GIBCO) supplemented with 5% heat-inactived FBS and antibiotics in 5% $CO_2$ at 37°C. These cells were used for virus multiplication.

### Infection with HSV-1

Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (150 mg/kg) and xylazine (10 mg/kg). A $10^4$ plaque-forming units (PFU) inoculum of HSV-1 resuspended in 10 μL of phosphate-buffered saline (PBS) was injected intracranially in the right side of sagittal suture at the level of the eye[10-12]. Control mice received PBS.

### Intravital microscopy

At 1 and 3 days post-infection (dpi) intravital microscopy of the mouse brain microvasculature was performed[12,16,17]. Briefly, WT injected with PBS (control), infected WT and infected $IL\text{-}4^{-/-}$ mice (n=4 for each group) were anesthetized by intraperitoneal injection and the tail vein was cannulated for administration of fluorescent dyes. A craniotomy was performed using a high-speed drill and the dura mater was removed to expose the underlying pial vasculature. Throughout the experiment, the mouse was maintained at 37°C with a heating pad and the exposed brain was continuously superfused with artificial cerebrospinal fluid buffer.

Leukocytes were fluorescently labeled by intravenous administration of Rhodamine 6G-Sigma (0.5 mg/kg body weight) and were observed using a microscope (Olympus B201, 10× objective lens, corresponding to 100 μm of area) outfitted with a fluorescent light source (epi-illumination at 510-560nm, using a 590 nm emission filter). The number of rolling and adherent leukocytes was determined offline during video playback analyses. Leukocytes were considered adherent to the venular endothelium if they remained stationary for a minimum of 30s. Rolling leukocytes were defined as white cells moving at a velocity lower than that of erythrocytes within a given vessel.

### Enzyme-linked immunosorbent assay (ELISA) for cytokines in the CNS

Brains from control and infected WT and $IL\text{-}4^{-/-}$ mice (n=4 in each group) were collected after intravital microscopy at 3 dpi and divided in two pieces by the interhemispheric fissure. Thereafter, the left fragment of the brain (opposite to the inoculation site) was homogenized in extraction solution containing aprotinin. Brain

homogenate was centrifuged at 3000g for 10 min at 4ºC, and the supernatants were collected and stored at –20ºC. The concentration of chemokines CXCL1, CXCL9, CCL3 and CCL5 and cytokine TNF-α was determined using ELISA. The supernatants of brain tissue were assayed in an ELISA setup using commercially available antibodies, according to the procedures provided by the manufacturer (R&D Systems, Minneapolis, MN).

### Histopathology

For histological analysis, the right fragment of the brain was preserved in 10% buffered formalin (infected WT n=5, infected IL-4$^{-/-}$ n=4). Sections 5 µm thick were cut and mounted for routine haematoxylin and eosin staining. These sections were examined at the optical level in the Olympus microscopy. Digital images were acquired for documentation.

### Statistical analysis

Data are shown as mean±standard error. A one-way ANOVA with Tukey's correction was used for multiple comparisons. Statistical significance was set at $p<0.05$.

## RESULTS

### HSV-1 infection induced milder meningitis in IL-4$^{-/-}$ mice

WT animals infected with $10^4$ PFU showed progressive inflammatory infiltrate in the meninges composed of neutrophils, lymphocytes and macrophages (Fig 1A). At day 3, the inflammation was more widespread and composed mainly of mononuclear cells. Inflammatory infiltrates were observed at the meninges (Fig 1B) and around some small cerebral blood vessels. Focal cerebral degenerative changes were also visualized adjacent to these inflamed areas.

Brains of IL-4$^{-/-}$ infected animals presented mild to moderate meningitis. At day 1, lymphocytes, macrophages and rare neutrophils were detected in the meninges (Fig 1C). At day 3, only leptomeninges were focally infiltrated by neutrophils and occasional mononuclear cells (Fig 1D). The inflammation was restricted to the meninges and no degenerative changes were detected in the brain from IL-4$^{-/-}$ mice. Histological sections obtained from brains of control animals showed no alteration.

### IL-4$^{-/-}$ infected mice had a diminished adherence of leukocytes to meningeal vessels at day 3 p.i.

Both infected groups (WT and IL-4$^{-/-}$) presented increase of leukocyte rolling and adhesion in meningeal vessels when compared with control group. In comparison with infected WT mice, lower leukocyte adhesion was observed in IL4$^{-/-}$ infected mice at 3 dpi (Fig 2).

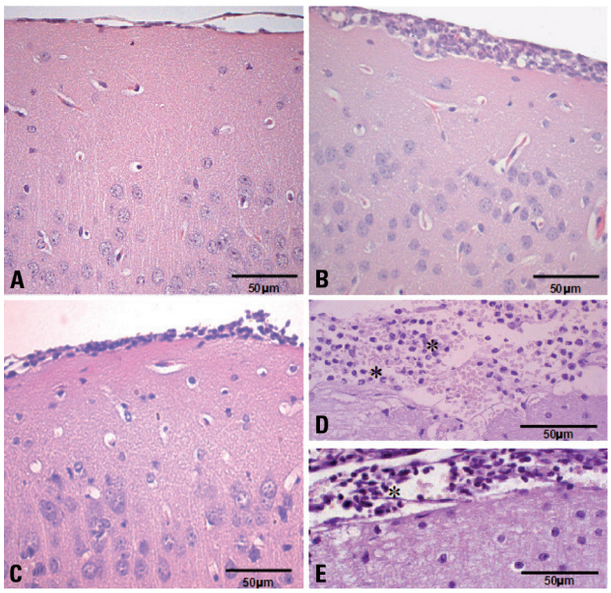


**Fig 1.** Histological changes in the brain after intracerebral inoculation with $10^4$ PFU of HSV-1 in C57BL/6 and IL-4$^{-/-}$ mice. H&E-stained sections of the brain. [A] Control mice with normal histological tissue. [B] Intense meningitis of WT infected mice, 1 dpi. [C] Mild meningitis of IL-4$^{-/-}$ mice, 1 dpi. [D] Infiltration of mononuclear cells at the meninges of C57BL/6, 3 dpi. [E] Meningitis with predominance of neutrophils of IL-4$^{-/-}$ mice, 3 dpi.

### Absence of IL-4 did not alter chemokines and cytokines levels in the brain tissue of HSV-1 infected mice

Brain tissue extracts were obtained from control animals, infected WT and IL-4$^{-/-}$ mice to evaluate the expression of chemokines (CXCL1, CXCL9, CCL3 and CCL5) and cytokine (TNF-α) by ELISA. WT and IL-4$^{-/-}$ infected mice presented significant increase in these molecules levels relative to controls at 3 dpi. However, there were no significant differences in cytokines or chemokines levels in brain tissue of infected IL-4$^{-/-}$ mice when compared to the infected WT group (Fig 3).

## DISCUSSION

The present study investigated the role of the cytokine IL-4 in the inflammatory response to HSV-1 cerebral infection. We found that the absence of IL-4 interferes in the response to HSV-1 infection in mice. Histopathological analysis revealed a delayed cellular infiltration and predominance of distinct leukocyte types at 3 dpi. With the progression of the disease, IL-4$^{-/-}$ mice showed diminished leukocyte adhesion, one relevant step for cellular recruitment into inflamed tissues. By contrast, there was no alteration in the levels of some chemokines and the cytokine TNF-α in brain tissue from IL4$^{-/-}$ when compared to controls.

Our histological analysis revealed differences between cellular infiltrates of WT and IL-4$^{-/-}$ mice. A model of

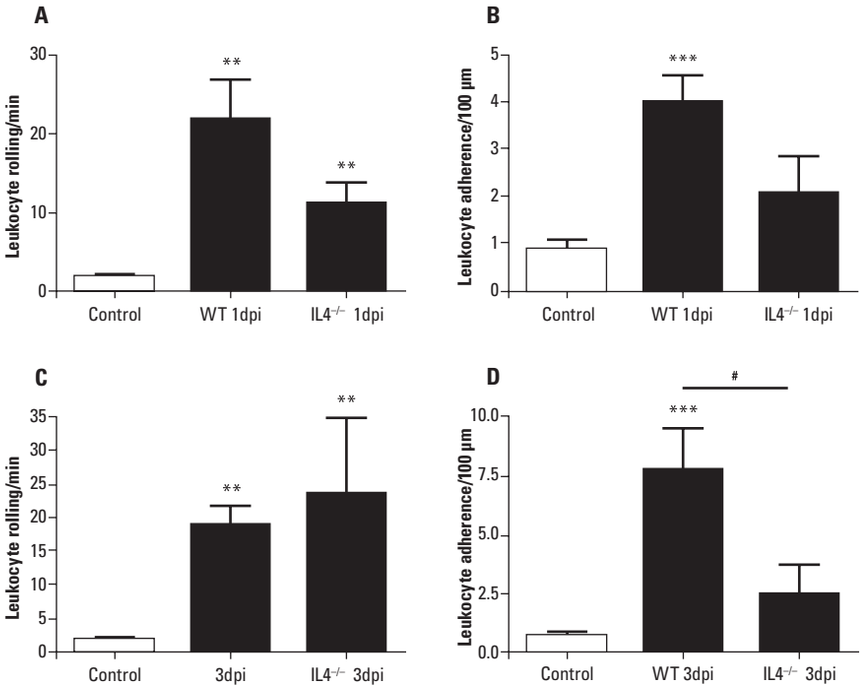


**Fig 2.** Visualization of leukocyte-endothelium interaction at 1 and 3 dpi with HSV-1. C57BL/6 and IL4$^{-/-}$ were intracranially inoculated with $10^4$ PFU of HSV-1. Intravital microscopy was used to assess rolling [A and C] and adhesion [B and D] of leukocytes in brain microvasculature, at 1 and 3 dpi. Data indicate mean±SEM of cells per minutes [A and C] and per 100 µm [B and D]. Intravital microscopy revealed a significant decrease of leukocyte adhesion in IL4$^{-/-}$ infected mice at 3 dpi (n=4; #p<0.01). No significant differences were found for leukocyte rolling in IL4$^{-/-}$ infected mice at 1 or 3 dpi (n=4; p>0.05). Statistical analysis used: one-way ANOVA with Tukey correction. (***p<0.001 and **p<0.01 relative to control).

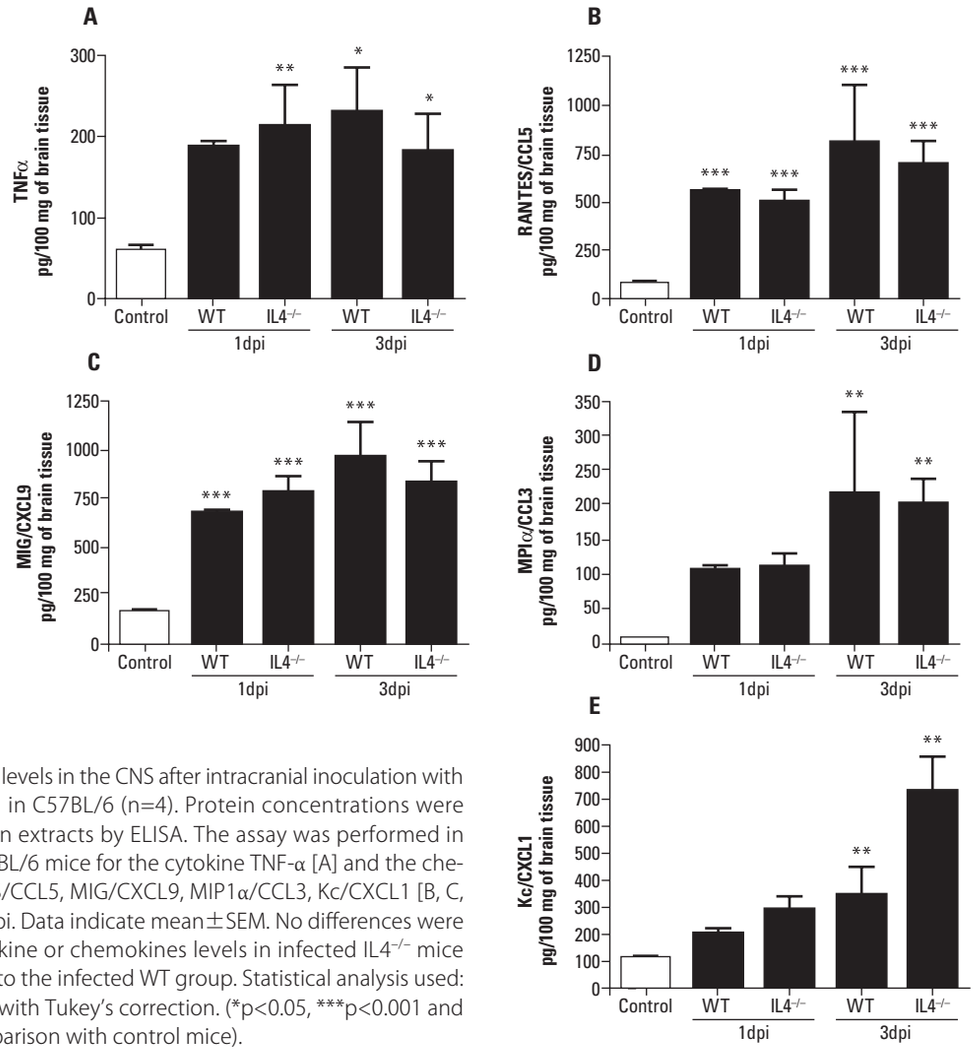


**Fig 3.** Chemokine levels in the CNS after intracranial inoculation with $10^4$ PFU of HSV-1 in C57BL/6 (n=4). Protein concentrations were measured in brain extracts by ELISA. The assay was performed in brain cells of C57BL/6 mice for the cytokine TNF-α [A] and the chemokines: RANTES/CCL5, MIG/CXCL9, MIP1α/CCL3, Kc/CXCL1 [B, C, D, E] at 1 and 3 dpi. Data indicate mean±SEM. No differences were observed in cytokine or chemokines levels in infected IL4$^{-/-}$ mice when compared to the infected WT group. Statistical analysis used: one-way ANOVA with Tukey's correction. (*p<0.05, ***p<0.001 and **p<0.01 in comparison with control mice).

HSV-1 corneal infection in mice revealed an elevated expression of IL-4 and IL-10 associated with massive $CD8^+$ T cell infiltration in trigeminal ganglion, but not in the cornea where inflammatory infiltrate was mainly composed of polymorphonuclear leukocytes. Therefore, these authors have suggested that IL-4 and IL-10 limit the infiltration of polymorphonuclear leukocytes and the destruction of neural tissues in this model[18]. In line with this, our results showed that when IL-4 is absent, the infiltration of neutrophils is delayed. Rare neutrophils were observed at 1 dpi in IL-$4^{-/-}$ mice brains. In contrast, they were the predominant cell type in WT infected mice at this same time point. At 3 dpi, neutrophils prevailed amongst cellular infiltrate in IL-$4^{-/-}$ mice whereas infected WT animals presented mainly mononuclear cells. Therefore the expression of IL-4 may prevent early infiltration of neutrophils in the brain after HSV-1 infection.

The migration of leukocytes from the vessels to brain parenchyma can be caused by the presence of a pathogen at this site and is composed of a series of events. Leukocytes must tether and roll along the venular wall before they can attach firmly and emigrate from the vasculature. These processes depend on multiple proteins interactions among leukocytes and vascular endothelial cells[19-21].

Our results revealed that infected IL-$4^{-/-}$ mice showed diminished leukocyte adhesion at 3 dpi when compared to infected WT mice. Previous studies have demonstrated that IL-4 regulates expression of adhesion molecules, with an upregulatory effect on vascular cell adhesion molecule-1 (VCAM-1)[22,23] and possibly on P-selectin[24,25] and a downregulatory effect on E-selectin[26,27]. It has also been reported that IL-4 can act synergistically with other cytokines, such as IL-1β and TNF-α, in order to promote lymphocyte binding to cultured microvascular endothelial cells[28,29]. It is possible that the absence of IL-4 modifies the profile of adhesion molecules expressed during infection causing the changes in leukocyte adhesion and cellular infiltrates observed in this study.

In conclusion, the lack of IL-4 gene in mice was associated with a delayed cellular infiltration and predominance of neutrophils. Overall leukocyte adhesion to brain microvasculature was diminished in the third day after infection. However, the lack of IL-4 did not abolish nor exacerbated the inflammatory response in mice brains. The results suggest that IL-4 plays a role in an acute model of severe encephalitis caused by HSV-1. Further studies are necessary to define the involvement of IL-4 in the outcome of the disease.

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