

Tumor Necrosis Factor- α and Interleukin-1 β Play a Critical Role in the Resistance against Lethal Herpes Simplex Virus Encephalitis

Yan Sergerie,¹ Serge Rivest,² and Guy Boivin¹

¹Research Center in Infectious Diseases and ²Molecular Endocrinology, Centre Hospitalier Universitaire de Québec-Centre Hospitalier de l'Université Laval and Laval University, Quebec City, Quebec, Canada

Background. The innate immune response after herpes simplex type 1 (HSV-1) encephalitis could be protective or, paradoxically, implicated in neuronal damage. We investigated the role of the innate immune response in such infection using a C57BL/6 mouse knockout (KO) model for tumor necrosis factor (TNF)- α and/or interleukin (IL)-1 β .

Methods. Encephalitis was induced by intranasal infection with a clinical strain of HSV-1 in 1-month-old KO or wild-type (WT) mice. Mice were monitored for survival, brain viral load was quantified by real-time polymerase chain reaction, and the inflammatory response was assessed by in situ hybridization in groups of mice killed on days 3–7.

Results. WT mice had a significantly higher mean life expectancy ($P = .0001$, log-rank test) than other groups. IL-1 β and TNF- α KO mice had a similar mean life expectancy, and encephalitis was lethal to all TNF- α /IL-1 β -deficient mice. Brain viral loads were lower in WT than in KO mice that had disseminated viral replication in the pons and medulla. Moreover, TNF- α and IL-1 β KO mice failed to initiate an adequate immune response, as shown by the virtual absence of expression of proinflammatory molecules in the brain.

Conclusion. These data clearly demonstrate the importance of TNF- α and IL-1 β in protection against HSV-1 encephalitis in this mouse model.

Herpes simplex virus type 1 (HSV-1) is the most frequent cause of sporadic and potentially fatal viral encephalitis in Western countries [1, 2]. Although acyclovir blocks viral replication and significantly reduces the mortality induced by HSV-1 encephalitis, many treated patients have severe neurological sequelae. The mechanisms responsible for the neuronal damage and mortality attributable to HSV-1 encephalitis appear to involve both virus- and immune-related processes.

Microglial cells are the first line of defense against viral infection within the central nervous system (CNS)

and play a crucial role in the innate neuroimmune response [3, 4]. During a nonproductive infection with HSV-1, human microglial cells depict robust in vitro expression of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , RANTES, and C-X-C motif ligand 10 (CXCL10) chemokine [5]. We previously reported a profound inflammatory response in cerebral tissue of mice infected with a thymidine kinase (TK)-competent virus involving both TNF- α and macrophage chemoattractant protein (MCP)-1 in numerous regions of the pons and medulla [6]. However, whether such immune innate/inflammatory responses are protective for the host or, paradoxically, contribute to neuronal damage has not been elucidated.

TNF- α is a multifunctional cytokine critical for microglia activation and immune response to many bacterial and viral pathogens. Moreover, this proinflammatory cytokine regulates peripheral lymphoid tissue organogenesis and differentiation of NK and lymphoid cells [7, 8]. TNF- α pretreatment has been reported to confer significant protection from lethal intraperitoneal HSV-1 challenge of C57BL/6 mice by a mechanism

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Reprints or correspondence: Dr. Guy Boivin, Research Center in Infectious Diseases, CHUQ-CHUL, 2705 Laurier Blvd. (RC-709), Quebec City, QC, Canada, G1V 4G2 (guy.boivin@crchul.ulaval.ca).

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independent of interferon (IFN) production or NK cell activation [9].

Although TNF- α represents a very well characterized innate immune molecule during HSV infection, the direct or indirect role of IL-1 β against this pathogen remains controversial. Treatment with IL-1 α has been reported to protect against HSV-1 infection, resulting in a significantly increased survival rate in 7-day-old C57BL/6 mice [10]. IL-1 β demonstrated therapeutic activity against the Sendai virus (a paramyxovirus) when administered on the same day as intranasal infection [11]. By contrast, transgenic mice overexpressing IL-1 receptor antagonist protein are resistant to herpetic stromal keratitis [12]. Thus, the exact role of this cytokine during experimental HSV encephalitis needs to be clarified.

In the present article, we demonstrate the critical role of the innate immune response during HSV-1 encephalitis in mice that are knocked out (KO) for the gene encoding TNF- α and/or IL-1 β . Although C57BL/6 wild-type (WT) mice were almost completely resistant to HSV-1 intranasal infection, the majority of cytokine-deficient mice died of the encephalitis because of uncontrolled viral replication in the CNS.

MATERIALS AND METHODS

IL-1 β KO mice (C57BL/6 background) were provided by Dr. H. Zheng (Merck Research Laboratories, Rahway, NJ) and generated as described elsewhere [13, 14]. TNF- α KO and WT mice were obtained commercially (Jackson Laboratory) and generated through breeding procedures. TNF- α and IL-1 β double-KO mice were generated, genotyped, and maintained as described elsewhere [15]. BALB/c mice were obtained commercially (Charles River Canada). All mice were infected at the age of 4 weeks. Animal experimentation guidelines in use at the Center Hospitalier de l'Université Laval were followed.

Mice were infected intranasally as described elsewhere [6] using 3×10^6 and 1×10^3 pfu of HSV-1 clinical strain H25 for C57BL/6 and BALB/c mice, respectively. In the first experiment (survival curve), 4 groups (WT, TNF- α KO, IL-1 β KO, and double KO) of 9–20 mice were infected, monitored for 10 days or until the appearance of HSV-1 encephalitis-related symptoms, and perfused intracardially before brain removal [6]. The primary end point for this experiment consisted of the apparition of all symptoms typical of encephalitis, namely, ruffled fur, weight loss, ocular swelling, and shaking movements.

In the second experiment (viral load quantification), 4 groups of 8–16 mice were infected as described above and killed by saline perfusion 3–4 days after infection. All mice were perfused intracardially before brain homogenization. Viral DNA extraction and quantification by real-time polymerase chain reaction (PCR) were done as described elsewhere [16]. Briefly, 200 μ L of brain homogenate was processed for DNA extraction using the Qiagen DNA Blood Mini Kit (Qiagen). DNA was

eluted in 50 μ L of water. A real-time PCR assay for detection and quantification of a fragment (477 nt) of the DNA polymerase gene of HSV-1 was performed on the LightCycler platform (Roche Molecular Systems). PCR amplification was done using 5 μ L of extracted materials, 0.5 μ mol/L of each primer, 0.4 μ mol/L of the fluorescein-labeled probe, 0.8 μ mol/L of the LCRed 640 probe, 5% dimethyl sulfoxide, 2 mmol/L MgCl₂, and 2 μ L of the LightCycler Fast Start DNA master hybridization probes (Roche Molecular Systems) in a final volume of 20 μ L. Each PCR batch included a set of 8 external HSV-1 controls (10-fold dilutions of a plasmid containing the target DNA polymerase gene) for construction of a standard curve and a negative PCR control (water).

Detection and localization of MCP-1, IL-12, IFN- β , IFN- γ , Toll-like receptor (TLR) 2, and viral TK transcripts were performed on brain slices using specific cRNA probes for these genes as described elsewhere [6].

Immunocytochemistry was combined with in situ hybridization [6] to analyze TNF- α and IL-1 β production by microglial cells and infiltrating monocytes/macrophages in the brain of HSV-1-infected mice. The presence of the proinflammatory cytokines TNF- α and IL-1 β was indicated by the agglomeration of silver grains within cell ramifications (microglia or monocyte/macrophage) labeled with *iba-1* (brown coloration).

Life expectancies between groups of mice were estimated by the Kaplan-Meier method and were compared using a log-rank test. Quantitative real-time PCR data were analyzed using Student's *t* test for independent samples. All statistical analyses were performed using SPSS software (version 11.0; SPSS).

RESULTS

Strong expression of TNF- α and IL-1 β during HSV-1 encephalitis in BALB/c mice. In this model of HSV-1 encephalitis, BALB/c mice exhibited clinical signs of sickness (weight loss, ruffled fur, ocular swelling, and shaking movements) 5–8 days after infection. Figure 1 depicts representative examples of IL-1 β and TNF- α gene expression. As reported elsewhere [6, 17], the expression levels of those cytokines were particularly intense in the caudal brain. TNF- α transcripts were localized within microglia and infiltrating monocytes/macrophages, as shown in the bottom left panels. By contrast, very few IL-1 β -expressing cells were colocalized with *iba1*-immunoreactive cells, except for few infiltrating macrophages closely associated with blood vessels (figure 1, *bottom right*). These data suggest that microglia/macrophages are the main type of cells that produce TNF- α but that this is not the case for the cytokine IL-1 β . Nonetheless, there was a strong innate immune response, involving IL-1 β and TNF- α , associated with the onset of clinical symptoms in this murine model of HSV-1 encephalitis.

TNF- α and IL-1 β needed for protection of C57BL/6 mice against HSV-1 encephalitis. To evaluate the role of TNF- α

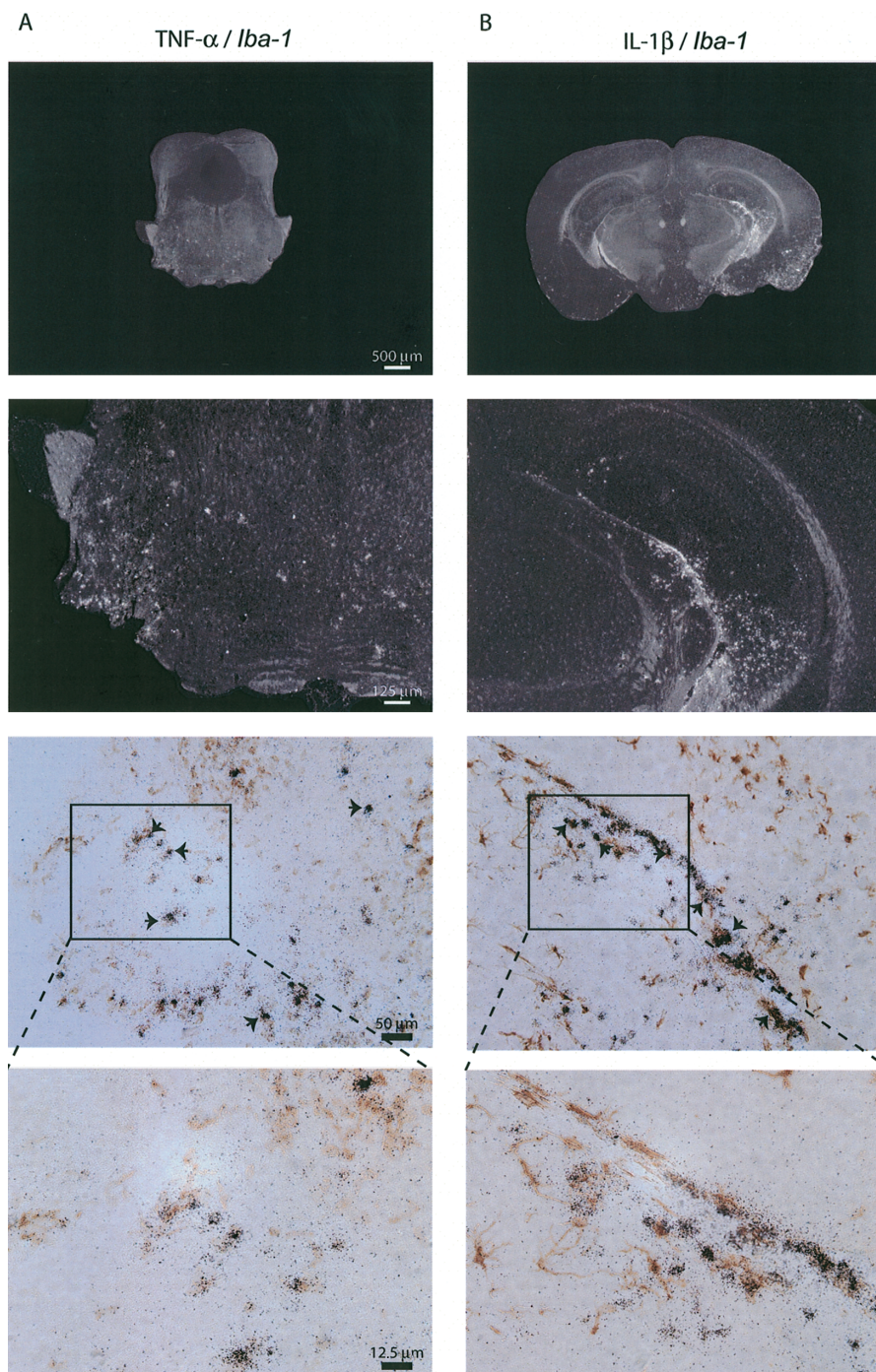


Figure 1. Expression of tumor necrosis factor (TNF)- α (A) and interleukin (IL)-1 β (B) in the brains of BALB/c mice developing encephalitis in response to intranasal herpes simplex virus type 1 (HSV-1) inoculation. The gene encoding TNF- α is essentially expressed in microglia and/or infiltrating macrophages. Parenchymal microglial cells and infiltrating monocytes/macrophages were labeled by an immunoperoxidase technique using an antiserum directed against ionized calcium-binding adapter molecule 1 (*iba-1*) in brain sections of mice killed 5–8 days after intranasal infection with 1×10^3 pfu of HSV-1 in 20 μ L of MEM. IL-1 β and TNF- α mRNAs were thereafter hybridized on the same sections by means of a radioactive in situ hybridization technique (silver grains). Note that most cells with a positive hybridization signal for TNF- α are specifically detected in microglia, whereas very few parenchymal *iba-1* cells expressed IL-1 β mRNA.

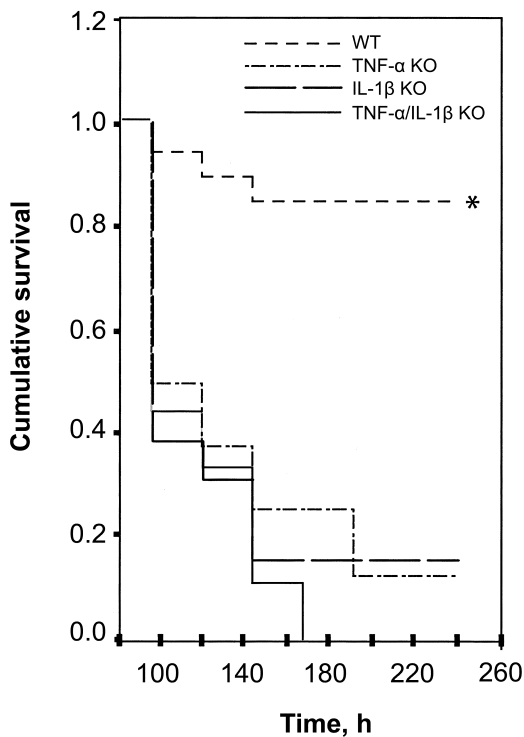


Figure 2. Survival curve of herpes simplex virus type 1 (HSV-1)-infected C57BL/6 wild-type (WT) and tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and TNF- α /IL-1 β knockout (KO) mice. The different groups of mice were inoculated with HSV-1 (3×10^6) and monitored for 10 days. Mice were carefully examined twice a day, and obvious signs of sickness (>20% weight loss, isolation with no social interaction, and limited movement) were considered to be end points for the mean life expectancy after HSV encephalitis. The survival curves were estimated using the Kaplan-Meier method and were compared using a log-rank test. Statistical analyses were performed using SPSS software (version 11.0; SPSS). As expected, C57BL/6 mice were resistant to HSV-1 infection, whereas the C57BL/6 TNF- α and/or IL-1 β KO mice developed lethal HSV-1 encephalitis. *Significantly different ($P < .0001$) from all other groups of infected mice.

and IL-1 β in HSV-1 encephalitis, 4 groups of C57BL/6 mice, including those with WT, TNF- α KO, and/or IL-1 β KO genotypes, were infected intranasally with HSV-1 and monitored for 10 days. TNF- α KO, IL-1 β KO, and double-KO groups exhibited signs of sickness after 2–3 days and had mean \pm SD life expectancies of 135.00 ± 19.21 , 127.38 ± 14.15 , and 117.33 ± 9.33 h, respectively. By contrast, the WT group exhibited no clinical symptoms except for a slight weight loss, and its mean life expectancy was significantly higher (222.00 ± 9.73 h; $P = .0001$), compared with the other groups (censored at 240 h). Most WT mice recuperated well after developing mild clinical signs, whereas cytokine KO mice had to be euthanized because of severe sickness. The survival curves showed a higher percentage of mice surviving HSV-1 infection in the WT group (85%) than in the TNF- α KO (12%), IL-1 β KO (15%), and double-KO (0%) groups ($P = .0001$, for WT

vs. other groups) (figure 2 and table 1). These compelling data highlight the neuroprotective effect of TNF- α and IL-1 β in HSV-1-induced encephalitis.

Increased HSV-1 replication in the brain in the absence of TNF- α and IL-1 β . Figure 3 depicts an increased HSV-1 DNA load in the brains of single- and double-KO mice, compared with that in WT mice, at 3–4 days after infection (figure 3A). However, these data did not reach statistical significance because of a high degree of variability among viral loads in the brains of WT mice. Notably, the viral load was very similar between the single IL-1 β and TNF- α KO groups of mice (figure 3A). Although viral DNA was detected by real-time PCR in the brains of infected WT mice, in situ hybridization with the HSV TK cRNA probe failed to detect any signal in the CNS of these mice. By contrast, high levels of TK mRNA were found in numerous regions of cytokine-deficient mice (figure 3B). These results suggest that viral replication is enhanced in the brains of IL-1 β and/or TNF- α KO mice.

Suppressed innate immune response in the CNS of mice lacking TNF- α and IL-1 β . The robust neurovirulence may depend on an altered expression of key proinflammatory molecules by microglia or infiltrating macrophages. Such an innate immune response is determinant to restrict viral replication and for transfer to an adaptive immune response. Of interest is the expression of TLR2, which is a reliable marker of microglia/macrophage activation [18–21]. A very intense hybridization signal was detected in the CNS of WT mice, whereas this transcript was barely detectable in the brains of infected TNF- α and/or IL-1 β KO mice (figure 4A). It is interesting to note that, despite having a strong microglial reaction to HSV-1 inoculation, WT mice had mild clinical symptoms and recuperated rapidly from the infection. This indicates that C57BL/6 mice are quite resistant to HSV-1-induced encephalitis, because they can mount a proper innate immune reaction and clear the virus from the CNS. Mice lacking IL-1 β and/or TNF- α failed to mount an inflammatory response to HSV-1, which had lethal consequences for the infected host.

The pattern of MCP-1 mRNA was also similar to that of TLR2 in the CNS of WT mice inoculated with the virus (figure

Table 1. Mean life expectancy of herpes simplex virus type 1 (HSV-1)-infected C57BL/6 wild-type (WT) and tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and TNF- α /IL-1 β knockout (KO) mice.

Group	Life expectancy, mean \pm SD, h	Died/total in group, no.	Survival, %
WT	222.00 ± 9.73^a	3/20	85
TNF- α KO	135.00 ± 19.21	7/8	12
IL-1 β KO	123.38 ± 14.15	11/13	15
TNF- α /IL-1 β KO	117.33 ± 9.33	9/9	0

^a Significantly different ($P < .0001$) from all other groups of infected mice.

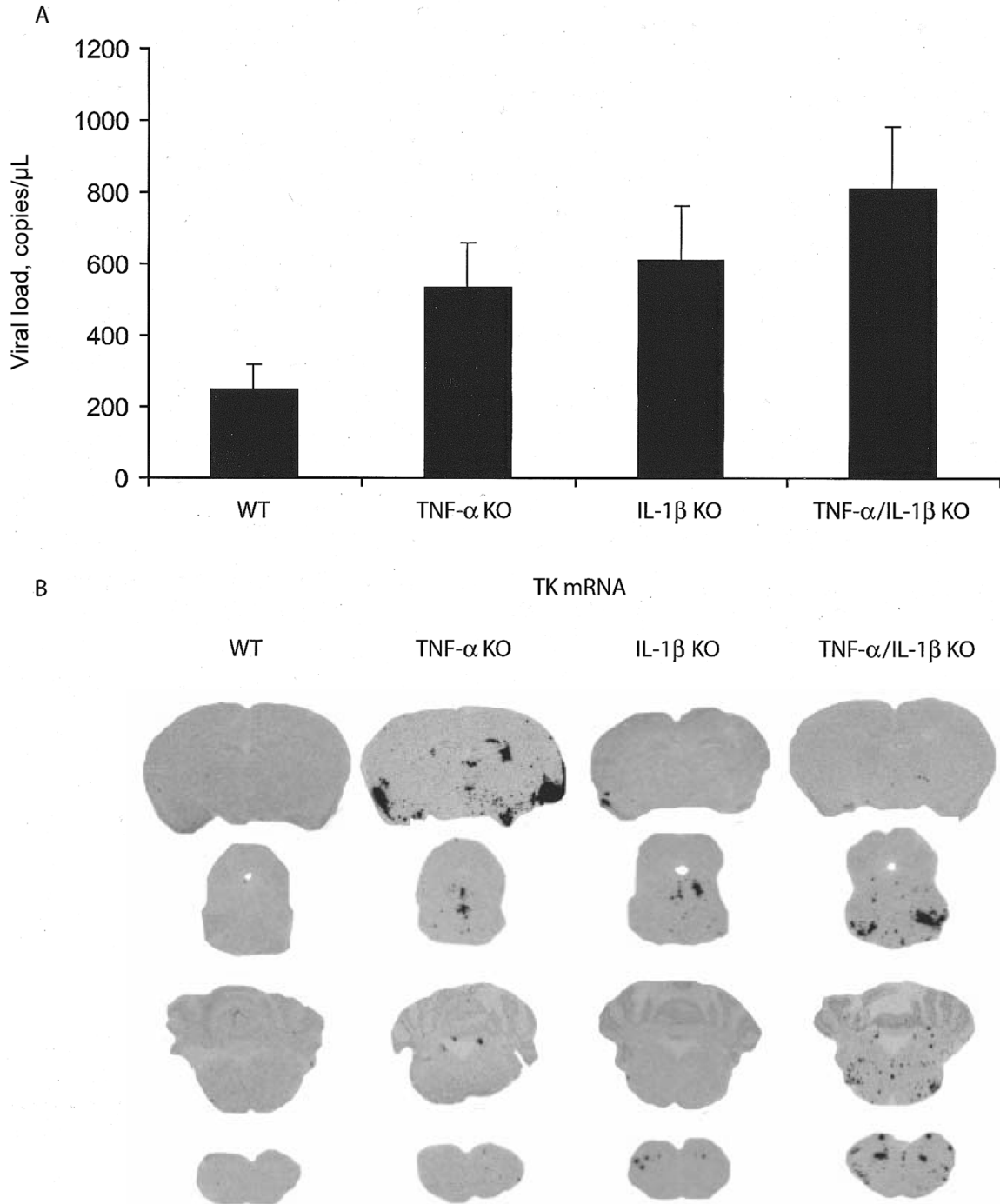


Figure 3. Herpes simplex virus type 1 (HSV-1) replication in the brains of wild-type (WT) littermates and tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and TNF- α /IL-1 β knockout (KO) C57BL/6 mice. *A*, Mice infected with HSV-1 and perfused transcardially with cold saline solution (0.9%) before brain removal and homogenization. Total DNA was extracted and quantified with a real-time polymerase chain reaction assay targeting HSV-1 DNA polymerase. *B*, Rostrocaudal coronal sections from HSV-1-infected mice on day 3 (KO mice) or 10 (WT mice) after infection. Please note the positive signal on x-ray film (Biomax, exposed at 4°C for 2 days) for thymidine kinase (TK) mRNA in various regions throughout the brain, especially in the midbrain, pons, and medulla of TNF- α , IL-1 β , and TNF- α /IL-1 β KO mice that developed lethal HSV-1 encephalitis.

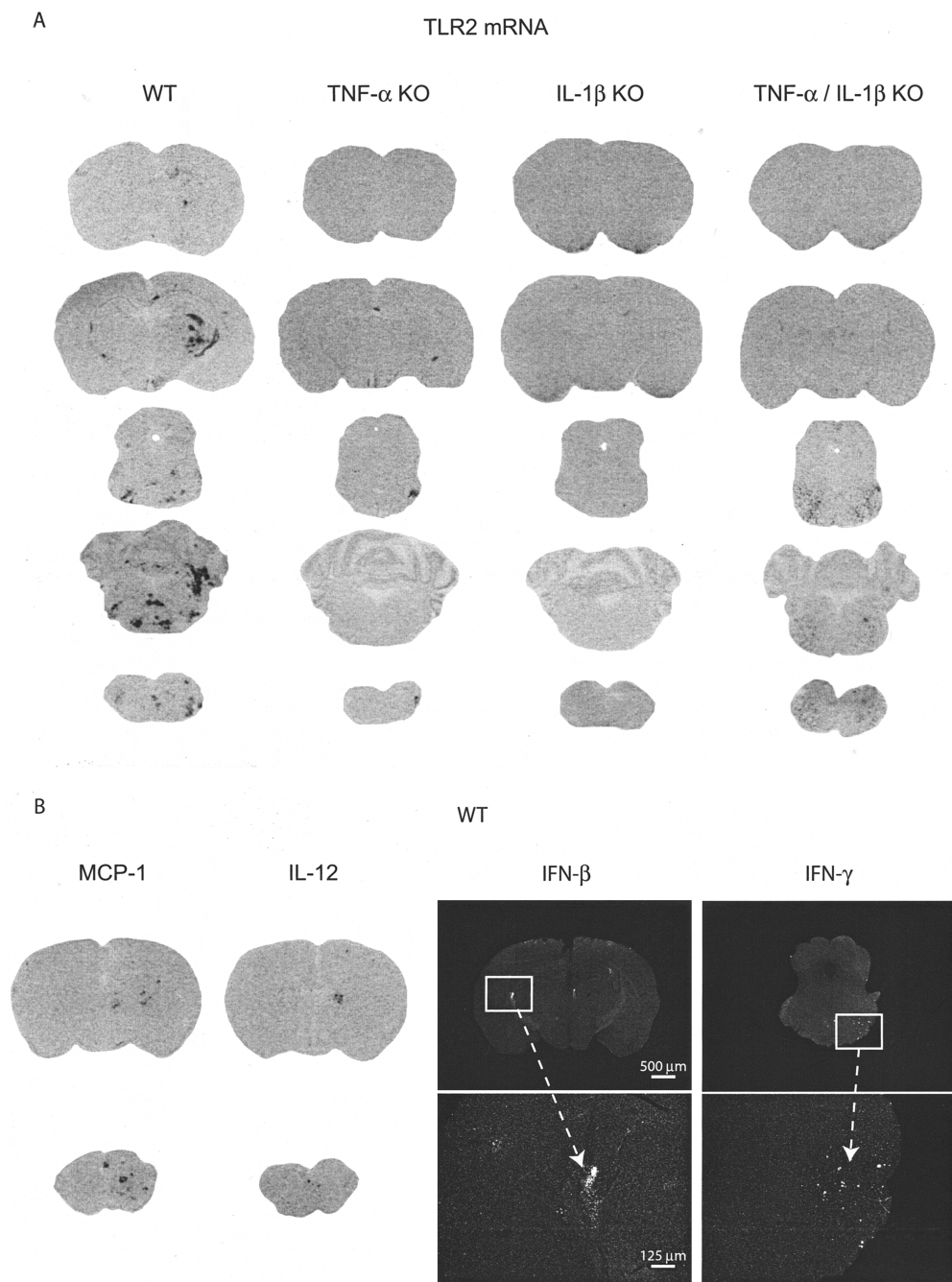


Figure 4. Representative distribution of proinflammatory genes in the brains of mice that received a single intranasal herpes simplex virus type 1 (HSV-1) inoculation. *A*, Rostrocaudal distribution of the mRNA expressing Toll-like receptor 2 (TLR2) on x-ray film (exposed at 4°C for 2–3 days) in wild-type (WT) and tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and TNF- α /IL-1 β knockout (KO) mice. This suggests a strong and widespread activation of microglia in the WT mice but not in cytokine-deficient mice. Cytokine-deficient mice exhibited severe clinical signs of encephalitis and had to be euthanized around day 3 after infection, whereas most WT mice were resistant to the infection, had no clinical signs up to day 10 after infection, and cleared the virus from the central nervous system (CNS). *B*, Localized expression of monocyte chemoattractant protein (MCP)-1 (CCL2), IL-12, interferon (IFN)- β , and IFN- γ in the brains of WT littermates in response to HSV inoculation. These mRNAs were not detectable in the CNS of infected TNF- α , IL-1 β , and TNF- α /IL-1 β KO mice (data not shown).

4B). However, very few clusters of IL-12 γ 1 $^{-/-}$, IFN- β $^{-/-}$, and IFN- γ -expressing cells were found in the brains of these mice, which suggests a more localized production of these cytokines. Such a restricted IFN response seems to be quite efficient in controlling HSV infection, because the majority of the WT mice were resistant to the infection and most cytokine KO mice developed lethal encephalitis. It is of interest to note that IFN- β , IFN- γ , and IL-12 genes were not expressed in the CNS of IL-1 β $^{-/-}$ and/or TNF- α $^{-/-}$ mice, which suggests that both innate and adaptive immune responses may be seriously compromised in these mice.

DISCUSSION

The present study clearly demonstrates that TNF- α and IL-1 β play pivotal roles in conferring resistance to HSV-1 brain infection, at least in this model of encephalitis. C57BL/6 mice are quite resistant to HSV infection, but mice with the same genetic background that bear mutation in the IL-1 β and/or TNF- α gene are highly vulnerable to this infection. The encephalitis was lethal in most cytokine-deficient mice. Inhibition of the innate immune reaction may largely contribute to the strong viral replication and severity of the CNS infection.

HSV-1 is initially recognized by TLR2 and TLR3 [17], which ultimately leads to the release of a large number of innate/inflammatory molecules, including TNF- α and IL-1 β . Microglial cells have been identified as the major source of HSV-induced chemokines and cytokines such as TNF- α , IL-1 β , RANTES, and CXCL10 [5]. This robust proinflammatory cascade is responsible for the recruitment/infiltration of peripheral immune cells in the CNS. TNF- α and IL-1 β are essential to orchestrate such an immune response, which is obviously vital to control HSV-induced encephalitis, as shown by our results.

In the CNS, astrocytes in the dorsal root have been reported to produce TNF- α and IL-6 during clearance of HSV for up to 30 days after infection [22]. Moreover, TNF- α antiviral activity against HSV has been found by different groups [5, 23, 24]. As suggested by these reports and confirmed by our study, HSV-1-infected mice lacking TNF- α have an impaired immune response that is needed to ensure viral clearance. IL-1 β does not suppress viral replication in cultured human astrocytes [5]. Surprisingly, our results indicate that IL-1 β KO mice have a mean life expectancy similar to that of TNF- α KO mice (15% and 12%, respectively) and not significantly different from that of double-KO mice (0%). Overlapping effects between these 2 cytokines can be involved in the initiation of an innate immune response required for viral clearance. As recently reported, TNF- α is required for C57BL/6 mouse resistance against HSV-1 encephalitis, but TNF receptors 1 and 2 are dispensable [25]. How TNF- α precisely mediates this protection against HSV-1 remains unclear. As shown in our study, IL-1 β also confers HSV-1 resistance and should be considered as a compensatory

mediator to TNF- α , although the exact interaction between IL-1 β and TNF- α needs to be investigated. The data showing that both groups of IL-1 β $^{-/-}$ and TNF- α $^{-/-}$ mice were highly susceptible to HSV-1 are quite interesting, because no differences were found in the brains of both IL-1 β KO and WT groups in response to acute injury. Indeed, intracerebral infusion with the nitric oxide donor sodium nitroprusside caused neurodegeneration and demyelination in which the extent was markedly increased in the brains of TNF- α $^{-/-}$ and IL-1 β /TNF- α $^{-/-}$ mice, compared with that in WT and IL-1 β $^{-/-}$ mice [15]. Although TNF- α but not IL-1 β plays a major role in the microglial reactivity in this model of acute injury, both cytokines are involved in the control of the innate immune response to HSV-1 infection in the CNS.

In a recent study, microglial cells were reported to initiate vigorous yet nonprotective immune responses during HSV-1 infection in the brains of BALB/c mice [26]. The protective immune response seen in our study has been observed in mice generated in a C57BL/6 background, which are naturally resistant to HSV infection. Because virtually no inflammatory response was observed in mice with deficiency in TNF- α and IL-1 β , mortality among these mice may be related to increased viral replication, as demonstrated by *in situ* hybridization for the HSV TK transcript and real-time PCR analysis of viral DNA. These data highlight the temporal concept encountered during development of HSV encephalitis. In previous work, we emphasized the importance of controlling the innate immune response early after viral infection in a BALB/c mouse model of HSV-1-induced encephalitis. We showed that glucocorticoid treatment starting 3 days after intranasal infection significantly increased the survival rate of infected mice, whereas those treated with the same agent concomitant to viral infection had severe neuronal damage leading to increased mortality [17]. These data might be of clinical significance; although an early immune response mediated by TNF- α and IL-1 β is needed to block viral replication and transfer to acquired immunity, the use of anti-inflammatory drugs such as glucocorticoids could be needed to counteract an exaggerated immune response in severely affected individuals. Specific surrogate biomarkers indicating disease progression remain to be defined for optimal intervention therapies using antiviral agents and anti-inflammatory drugs.

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