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Innate Immune Response Induced by Theiler's Murine Encephalomyelitis Virus Infection

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Abstract

Although the causative agents of human multiple sclerosis (MS) are not known, it is suspected that a viral infection may be associated with the initiation of the disease. Several viral disease models in mice have been studied to understand the pathogenesis of demeylination. In particular, Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) has been extensively studied as a relevant model. Various cytokines and chemokines are produced upon viral infection by different cell types, including antigen-presenting cells (APCs) such as macrophages; dendritic cells (DCs); and glial cells, such as astrocytes, microglia, and oligodendrocytes. The upregulation of the corresponding molecules are also found in MS and are likely to play an important role in the protection and/or pathogenesis of chronic inflammatory demyelinating disease. In this review, the type of cells and molecules, gene-activation mechanisms as well as their potential roles in protection and pathogenesis will be discussed.

Key Words

Demyelination
Theiler's virus
Cytokines
Chemokines
Signal transduction

Introduction

Multiple sclerosis (MS) is a chronic inflammatory immune-mediated neurological disease leading to demyelination of the white matter of the brain and spinal cord *(1,2)*. It is suspected that the initial tissue damage caused by infectious agents yields autoimmunity to myelin components *(3,4)*. Several virus-induced models have been used to study the underlying mechanisms of this disease *(5,6)*. In particular, the Theiler's murine encephalomyelitis virus (TMEV)-induced demyelination system has been extensively studied as a relevant model *(7)*. After susceptible mice are intracerebrally infected with TMEV, they develop a chronic immune-mediated demyelinating disease similar to human

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MS involving strong autoimmunity to myelin antigens *(8)*. Viral persistence is closely associated with the progression of demyelinating disease *(9–11)*. In addition, the various proinflammatory chemokines and cytokines found in the central nervous system (CNS) of infected mice are similar to those found in human MS. More recently, it has been shown that various chemokines and cytokines are directly activated by TMEV infection in many different cells types including glial cells and antigen-presenting cells (APCs) *(12–14)*. These findings suggest that these proinflammatory molecules affect the initiation and establishment of inflammatory responses. In this article, we will review current and ongoing studies in order to understand the role and mechanisms of activation of these cellular genes in the protection and pathogenesis of demyelination.

TMEV-induced demyelinating disease (TMEV-IDD) has been recognized as an immune-mediated disease based on several experimental approaches. The treatment of host with either anti-thymocyte, anti-Ia (MHC class II), or anti-CD4 antibodies delays the onset of disease *(15–17)*, suggesting the involvement of $CD4^+$ T cells. T helper type 1 (Th1) cells preferentially producing interferon-γ (IFN-γ) are found within demyelinating lesions of the CNS and appear to be involved in the pathogenesis of demyelination *(18–21)*. Furthermore, the genetic association between susceptibility to TMEV-IDD and the major histocompatibility complex (MHC) *(22,23)*, as well as the T-cell receptor (TCR) β-chain *(24,25)*, further supports the involvement of such immune responses in pathogenesis. These associations have also been identified in human MS *(26)*. Attempts to correlate antibody response to viral antigens and pathogenesis have been made *(27)*. However, there is no clear critical role of the antibody response in developing demyelinating disease. A strong preventive role is established if TMEV-specific antibodies are present prior to viral infection, but these antibodies provide weak protection after viral persistence is established. The role of $CD8^+$ T cells is not yet clear. It is generally believed that virus-specific $CD8^+$ T cells are important for viral clearance and consequent resistance to TMEV-induced demyelinating disease *(7)*. Owing to the inflammatory nature of immune responses in the CNS following TMEV infection, the majority of the previous studies have been focused on the cytokines associated with Th1 and/or Th2 responses.

Innate Immunity and TMEV-IDD

NK/NKT Cells

In general, viral infection and subsequent pathogenesis can be greatly affected by different innate immune responses. These include natural killer (NK) and natural killer T (NKT) cell responses, as well as various cytokines and chemokines directly induced upon viral infection. Because TMEV infection also induces various innate immune responses including a wide-range of chemokines and cytokines *(28,29)*, these chemokines/cytokines and NK/NKT cell responses are likely to affect each other, leading to the induction of virus-specific adaptive immune responses. The balance of various cytokines produced by glial cells, APCs, NK cells, as well as $CD4^+$ and $CD8^+$ T cells, determines the outcome of either protection or pathogenesis. Not many studies on NK/NKT cells associated with TMEV-IDD have been reported. One early study *(30)* attempted to remove the NK cell population with anti-NK1.1 or anti-asialo-GM1 antibodies. This study found that the lack of NK population in resistant C57BL/10 mice renders susceptibility to acute encephalitis. In our preliminary studies, NK-deficient mice (Ly49A Tg C57BL/6 mice obtained from Dr. W.

Yokoyama) with resistant background genes are also susceptible (3/6 mice) to the early gray matter disease, suggesting the importance of NK response in viral clearance, hence protection from disease.

Interferon (IFN)-α/β

Administration of IFN-α/β is a widely used and effective treatment for human MS *(31,32).* However, its mechanism of action is poorly understood. It is known that IFN-α/β (type I) plays an important role in the reduction of blood–brain barrier (BBB) permeability as well as induction of the NK response *(33,34).* Type I IFN is induced via Toll-like receptor (TLR)-mediated activation leading to NF-κB activation upon microbial/viral infections, which results in further induction of chemokine/cytokine expression *(35).* Infection of IFN-α/β receptor-deficient mice (IFN-α/βR knockout [KO]) with TMEV results in rapid gray matter disease and subsequent death *(36).* Furthermore, type I IFN can induce Th2 cytokines such as interleukin (IL)-10 and IL-4 and downregulate Th1-associated genes such as IL-12 receptor β2 *(37,38).* It is also interesting to note that the induction of IFN-α/β gene in macrophages or astrocytes after TMEV-infection is significantly decreased or delayed compared to other proinflammatory cytokines *(28,39).* Thus, this suggests that type I IFN plays a significant role in the protection against TMEV-IDD. Type I IFN is also a strong anti-viral agent that directly inhibits viral replication. This delayed IFN-α/β induction may result in viral persistence, leading to subsequent early establishment of inflammatory responses and eventual Th1-mediated demyelinating disease in susceptible SJL mice.

Other Cytokines

Many investigations have examined the production of various cytokines in the CNS throughout the course of TMEV-IDD. Both Th1- and Th2-associated cytokines are detected during early infection. These include IFN-γ, IL-1, IL-6, IL-12, tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) *(40–43).* In addition, previous studies indicate that administration of anti-TNFα or anti-IL-12 antibody to susceptible mice significantly inhibits both disease progression and severity, strongly supporting the importance of these cytokines in the pathogenesis of disease *(44,45).* In MS, Th1-type cytokines are associated with relapses, whereas Th2-type cytokines are associated with remission *(46–48).* However, the pathogenesis of TMEV-IDD is significantly enhanced in mice pretreated with anti-IFN-γ antibodies or in IFN-γ receptor KO mice *(36,49),* whereas administration of IFN-γ also exacerbates the disease *(49).* These results suggest that this cytokine can be either protective or pathogenic, depending on the time present with respect to viral infection. Therefore, these studies imply that the balance between Th1 and Th2 responses may be important in the development of pathogenesis or protection.

Chemokines

The production of chemokines has also been detected in the CNS of mice following a variety of viral infections, including TMEV *(50–55).* Chemokines have multiple biological functions in inflammatory responses, such as chemo-attraction of a variety of cell types, activation of certain cell populations, as well as angiogenesis and BBB dysfunction *(56,57).* Our results concur with these findings. The brains (initial site of infection), but not the spinal cords, from susceptible SJL/J mice infected with TMEV show prominent expression of RANTES and IP-10 as early as 1 and 3 d

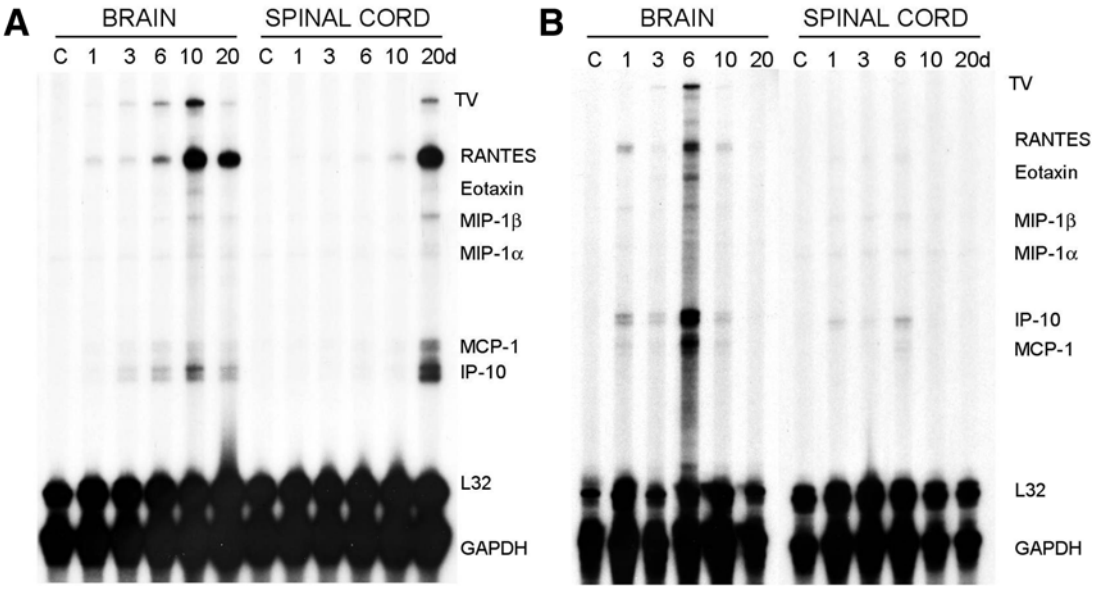

Fig. 1. Chemokine and viral gene expression in pooled brains and spinal cords of susceptible SJL/J **(A)** and resistant C57BL/6 **(B)** mice (four to eight mice per group) analyzed by RNase protection assay (RPA) at different time points post-TMEV-infection.

after viral infection when analyzed by RPA (Fig. 1A). At the peak of viral RNA levels (10 d postinfection), a corresponding increase of RANTES and IP-10 transcripts was observed in the brain. As the viral message was reduced (20 d postinfection), chemokine expression was only slightly reduced, which may reflect continuous chemokine gene activation by cytokines produced by infiltrating T-cell populations. In the spinal cord, however, the level of viral and RANTES messages was barely detectable at 6 d post viral infection, followed by a rapid increase in viral, RANTES, and IP-10 messages at 20 d postinfection. These data strongly suggest that early upregulation of chemokines in the brain and spinal cord is subsequent to TMEV-infection and coincides with the level of viral replication. Such a correlation between viral persistence and chemokine production has also recently been suggested by others in TMEV-infected SJL mice *(58)*. We have also determined the levels of viral and chemokine messages in the brain and spinal cord of the prototypically resistant C57BL/6 strain, during TMEV infection (Fig. 1B). RANTES and IP-10 (and to some level, eotaxin, MIP-1β, and MCP-1) genes are similarly induced at the early stage of viral infection (up to 6 d). However, overall levels of viral as well as chemokine messages were markedly reduced later in the brain of resistant C57BL/6 mice, in sharp contrast to SJL/J mice. Interestingly, no significant expression of chemokine or viral messages was detectable in the spinal cord except for IP-10 at 6 d postinfection. This lack of significant upregulation correlates well with the low inflammatory response in C57BL/6

mice. Therefore, the lack of sustained chemokine induction in the CNS of resistant C57BL/6 mice may reflect poor viral persistence, critically important for initiating and promoting inflammatory responses, including cytokine/chemokine expression leading to demyelination. Recently, it was shown that transgenic mice expressing MCP-1 (CCL2) in the CNS show increased severity and accelerated onset of demyelinating disease *(59).* Together, these studies strongly suggest that chemokines are likely to play an important role in the initiation of TMEV-IDD.

Cellular Source of Cytokines and Chemokines

Glial Cells

Previous studies have indicated that the main reservoir of viral replication is microglia/macrophages in the CNS *(60).* Our initial studies indicate that the majority (>50%) of microglias in the CNS of TMEV-infected SJL/J mice produce TNF-α, compared to a minor population (<5%) of CNS-infiltrating macrophages, suggesting that microglia rather than infiltrating macrophages/monocytes are a major contributor of pro-inflammatory chemokines and cytokines (Mohindru and Kim, unpublished data). Infection of other glial cells (e.g., astrocytes, oligodendrocytes) within the CNS is also crucial for viral persistence *(61).* However, isolation and maintenance of these glial cells from infected adult mice are rather difficult. As an alternate source of glial cells, astrocytes, oligodendrocytes, and microglia derived from neonatal brains have been utilized to investigate the effects of viral infection on the production of chemokines and cytokines. Owing to the abundance and ease of isolation, primary astrocyte cultures have been most frequently used. These studies indicate that various cytokines such as IL-12, IL-6, TNF-α, IL-1, and IFN-β are directly induced following TMEV infection in primary astrocytes *(28).* Similarly, TNF-α, IL-6, IL-18, type I IFNs, and IL-12 genes are activated in microglia cultures upon TMEV infection *(13).* It is interesting to note that the levels of key proinflammatory cytokines (e.g., IL-12 and IL-1) are much reduced following infection with a low-pathogenic variant virus *(14),* strongly suggesting the important pathogenic role of these initial proinflammatory cytokines directly induced after viral infection in developing demyelinating disease.

In addition to cytokines, various chemokines are also produced in these glial cells following TMEV infection. Previously, we have demonstrated that several chemokine genes are activated upon TMEV infection in various glial cells *(12).* The scope of chemokine genes that are activated at 6–8 h post-TMEV-infection has been determined using a mini-array system (SuperArray, Bethesda, MD) *(29).* The results clearly indicate that only select chemokine genes (9 out of >30 tested) are significantly (>fivefold) activated in astrocyte cultures. These include MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), MCP-3 (CCL7), MCP-5 (CCL12), GRO-1 (CXCL1), MIP-2 (CXCL2), and IP-10 (CXCL10). It is interesting to note that the level of GRO-1 (KC) chemokine protein secreted by astrocytes after TMEV infection is >20-fold higher than MCP-1 or MIP-1α. However, the significance of this difference in the pathogenesis of the initial inflammatory response and establishment of chronic demyelinating disease is not yet clear. It has been reported that GRO-1 is a potent chemoattractant of neurophils and an angiogenic factor. Nevertheless, overlapping chemokines *(29)* and cytokines (unpublished results) are

also induced in astrocyte cultures after treatment with proinflammatory cytokines. Thus, chemokines and cytokines produced in a delayed fashion (after 24 h viral infection) may include those indirectly stimulated by the initial proinflammatory cytokines produced in culture.

Antigen-Presenting Cells

The initial commitment of Th1/Th2 differentiation is most likely affected by professional APCs in the periphery. Thus, the possibility that TMEV infection may also directly induce proinflammatory cytokines in professional APCs has been explored *(14).* Infection of isolated DCs and macrophages with pathogenic TMEV results in the preferential upregulation of Th1-promoting IL-12 production over Th2-promoting IL-10, whereas nonpathogenic variant virus preferentially activates IL-10 over IL-12. In addition to these cytokines, additional proinflammatory cytokines (e.g., TNF-α, IL-6, IL-1) as well as chemokines (RANTES, MCP-1, MIP-2, MCP-1, IP-10) are also induced in APCs upon TMEV infection (unpublished observation). These results suggest the importance of cytokines directly induced in these cells by TMEV infection in subsequent inflammatory immune responses.

Molecular Mechanisms of Chemokine/Cytokine Gene Activation

NF-κB Requirement

To identify the mechanism(s) involved in TMEV-induced cytokine expression in glial cells, the activation of NF-κB has been investigated in astrocytes by immunohistochemistry and electrophoretic mobility shift assay (EMSA) *(28).* Rapid NF-κB nuclear translocation is observed within 5–15 min after TMEV infection. The activation of NF-κB is also apparent based on EMSA experiments with nuclear extracts from TMEV-infected astrocytes. The molecular weights of NF-κB subunits involved in the binding to NF- κB-specific oligonucleotides suggest that p65/p50 and p50/p50 complexes are involved in the activation. These results conclusively demonstrate the activation of NF-κB in astrocytes after TMEV infection. To correlate NF-κB activation and cytokine gene expression induced by TMEV, chemical inhibitors for the NF-κB pathway (caffeic ester phenyl ester and MG132), as well as recombinant-adenovirus expressing a dominant-negative form of I-κB, were used. Pretreatment of astrocytes with these inhibitors suppress most, if not all, of the cytokines and chemokines *(28,29).* These results indicate that TMEV-induced NF-κB activation is required for cytokine and chemokine gene expression in primary astrocyte cultures.

MAPK, PKR, and IFN-α/β Dependence

Many pathways, including the double-stranded RNA-dependent protein kinase (PKR), can lead to NF-κB activation, resulting in the production of proinflammatory cytokines *(62–68).* Pretreatment of astrocytes with 2-aminopurine (AP), a serine threonine protein kinase inhibitor of PKR *(69),* was able to only partially reduce the level of some (i.e., IL-1, TNF-α, and IL-6) of the TMEV-induced cytokines *(28,29).* In addition, TMEV-induced cytokine expression was not significantly compromised in a PKR-deficient fibroblast cell line. These results strongly suggest that NF-κB activation induced by TMEV can be independent of the PKR pathway. The possibility that MAPK is necessary for the activation of chemokine/cytokine genes after TMEV infection was also examined ([*29*] and unpublished results) using specific inhibitors for MEK and p38 (U0126 and SB2024190, respectively). These treatments partially inhibited both chemokine and cyto-

kine gene expression, suggesting that activation of MAPK may also contribute to the activation of chemokine gene expression at some level.

The type I interferons (IFN-α/β) induced following various infections, including viruses, further induce numerous other cytokines (TNF-α, IL-12, IL-1, and IL-6) and chemokines *(63–65,70)*. To determine the potential involvement of IFN-α/β in the induction of cytokines and chemokines by TMEV infection, the levels of cytokine and chemokine gene expression in astrocytes from control and IFN-α/β receptor-deficient (IFN-α/βR-KO) mice were examined *(28, 29)*. The overall kinetics of the initial gene expression was similar to astrocytes with intact IFN-α/β receptor. However, the levels of some cytokines critically important for inflammatory responses (IL-12p40 and TNF-α) are significantly lower in the IFN-α/βR KO astrocytes during the late time periods (12–24 h). The lack of additional stimulation secondarily induced by Type I IFNs may result in a reduction in cytokine gene expression at late time points, because IFN-α/β treatment induces significant levels of cytokines and chemokines in astrocyte cultures *(28)*. These results indicate that the induction of cytokines/chemokines by TMEV does not require the IFN-α/β pathway, which is important for amplifying and sustaining the subsequent protective immune response *(29)*.

Differential Gene Activation in Different Species

Infection with different picornaviruses such as Coxsackievirus can cause meningitis/encephalitis in humans and experimental animals. Potential chemokine gene activation in human astrocytes by TMEV has been investigated along with the human picornaviruses, Coxsackievirus B3, or Coxsackievirus B4 *(71)*. Interestingly, all of these viruses induce the expression of IL-8 and MCP-1 genes in primary human astrocytes as well as in an established astrocyte cell line. The pattern of activated chemokine genes in human astrocytes is quite restricted compared to that in mouse astrocytes infected with the same viruses, suggesting distinct mechanisms of gene activation in cells from different species (Fig. 2). Further studies indicate that both AP-1 and NF-κB transcription factors are required for the activation of chemokine genes in human astrocytes, whereas only NF-κB activation is sufficient for mouse astrocytes *(71)*. Such a difference in the activation pathway and pattern of chemokine/ cytokine production may result in potential differences in the pathogenic outcome in different species following infection with the same virus.

Association With Viral Replication

Several lines of experimental data suggest that there is an association between viral replication and chemokine/cytokine gene activation. First, UV-inactivated virus fails to activate these genes in both mouse and human astrocytes *(12,71)*. Second, the number of astrocytes showing viral message is similar to that displaying NF-κB nuclear translocation *(28)*. Third, NF-κB inhibitors that inhibit chemokine/cytokine gene activation following TMEV-infection completely suppress viral replication in both mouse and human astrocytes (unpublished results). These results strongly suggest that cellular gene activation is required for TMEV infection and replication. Supporting this notion, viral replication is also significantly enhanced in cell lines and primary astrocytes that are preactivated with lipopolysaccharide (LPS), which is known to be a potent activator for many different cell types, including APCs (unpublished data). In addition, we have also observed that

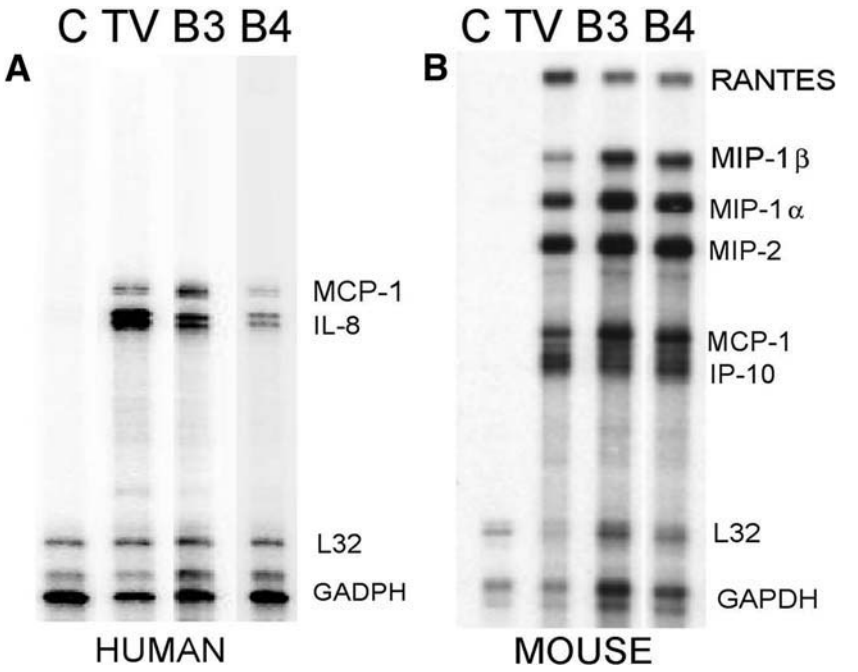

Fig. 2. Activation of chemokine genes in human **(A)** and mouse **(B)** astrocytes following infection with TMEV (TV), CVB3 (B3), and CVB4 (B4). U373 human astroglioma cells and mouse (SJL/J) primary astrocytes were used. The chemokine specific mRNA levels at 8 h after viral infection were assessed by RPA using chemokine mutiprobe sets (mCK-5 and hCK-5, respectively; Pharmingen). Adapted with permission from ref. *71*.

administration of LPS or IL-1β peptide permits viral persistence in resistant mice and renders them susceptible to disease *(11)*.

Role of the Initial Innate Immunity in Pathogenesis

As discussed earlier, a variety of chemokines and cytokines appear to play critical roles in early cellular infiltration, viral persistence, development of adaptive immunity, and consequent pathogenesis of viral demyelinating disease. It appears that viral replication is required for the initial activation of chemokine and cytokine genes and these are dependent on NF-κB activation. It is not yet clear which pathway triggers NF-κB activation. Because dsRNA generated during TMEV replication may activate NF-κB via PKR and TLR-3, it is conceivable that these receptors are involved in delivering initial signals for NF-κB activation, leading to the production of chemokines and cytokines. The initial proinflammatory chemokines and cytokines produced in various virus-infected glial cells and APCs may further activate adjacent cell populations that are not infected by virus (Fig. 3). The specific inhibition or delay in IFN-α/β gene activation in virus-infected cells may allow continuous viral replication initially. The newly released virus may have easy access to adjacent cells that are pre-activated by proinflammatory cytokines from virus-infected cells. In particular, TNF-α and IL-1, which are potent NF-κB activators,

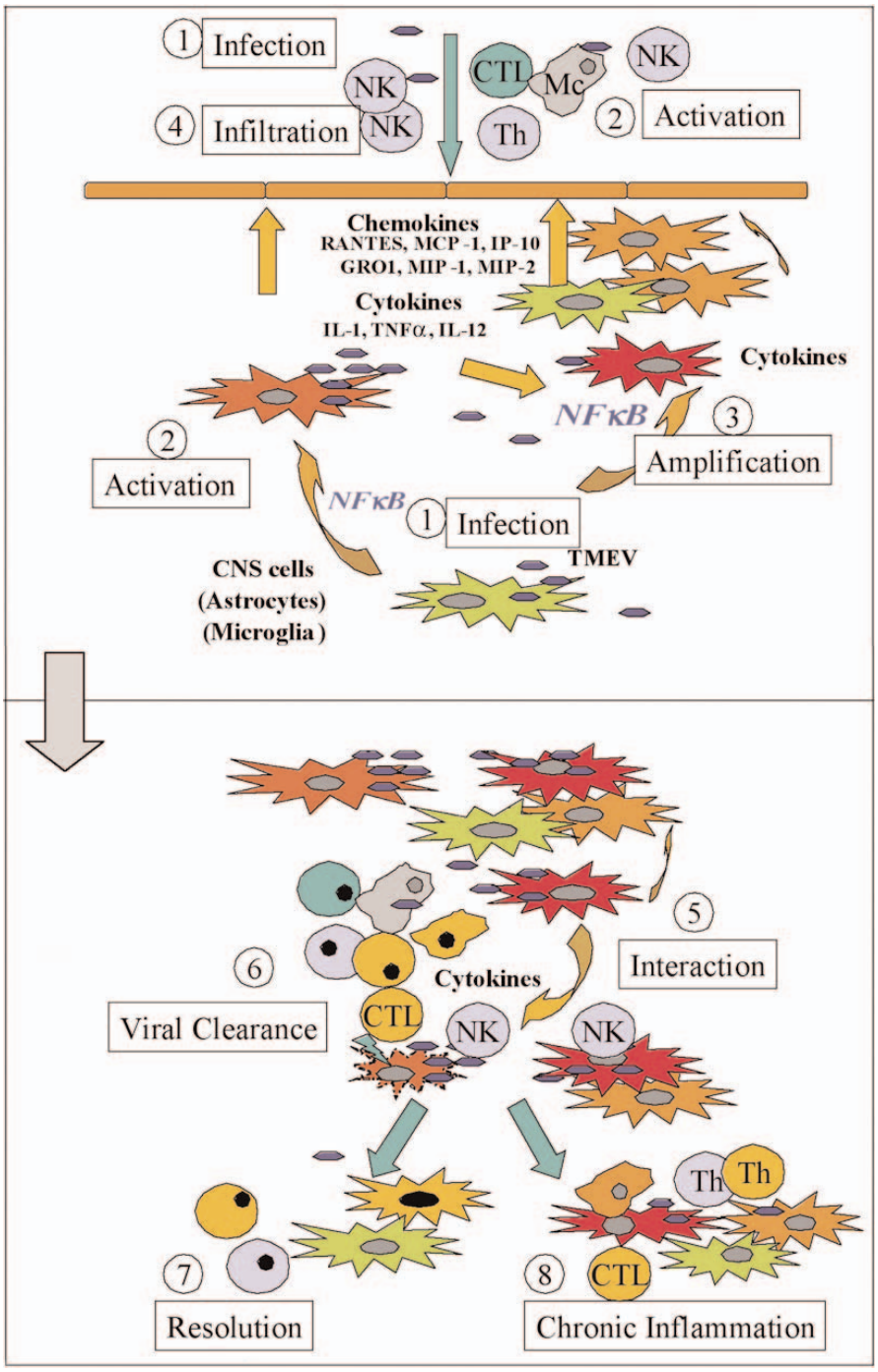

Fig. 3. Potential role of innate immune responses in immune-mediated demyelination induced following TMEV infection. Viral infection may activate various glial cells, including astrocytes, microglia via NF-κB to produce select chemokines, and proinflammatory cytokines. The secreted chemokines promote infiltration of various inflammatory cells to the CNS, including NK, Th, CTL, B, and monocytes/macrophages. The secreted cytokines activate adjacent glia cells and infiltrating inflammatory cells, and this may enhance viral infection, replication, and/or cellular function. The combination of NK, CTL, and Th cells to some extent clears viral infection by removing virus-infected cells followed by resolution of inflammation. When, however, inadequate initial innate and virus-specific immune responses fail to clear viral infection in susceptible mice, a chronic immune-mediated demyelinating inflammatory response is established.

may amplify viral replication as well as the production of overlapping chemokines and cytokines in virus-infected cells. Although some of these cytokines and chemokines could play a protective role initially, the deficiencies in the initial NK/NKT responses, coupled with insufficient adaptive immune responses in susceptible mice, may fail to clear virus completely from the CNS. Chronic viral persistence and continued cytokine/chemokine production in susceptible mice may eventually lead to exacerbated inflammatory response, tissue damage, and immune-mediated demyelinating disease. However, in resistant mice, strong initial NK/NKT responses and subsequent adaptive immune responses may be capable of clearing virus, leading to the resolution of inflammatory responses preventing the onset of demyelinating disease.

Acknowledgments

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