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REVIEW

Critical roles of chemokines and cytokines in antiviral innate immune responses during rabies virus infection

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Abstract The innate immune response is the first line of defense against viral invasion and pro-inflammatory chemokines and cytokines have a critical function in the innate immune responses against virus infections. The ability of a rabies virus (RABV) to induce the expression of chemokines and cytokines can lead to viral clearance from the central nervous system (CNS), whereas the ability to evade such expression and activation contributes to virulence and pathogenicity. In this review, the crucial contribution of chemokines/cytokines to clearing RABV from the CNS is discussed, including recruiting leukocytes into the CNS, enhancement of blood brain barrier permeability and activation of various immune cells that are essential for viral clearance. In addition, recombinant RABV expressing cytokines and chemokines can induce elevated innate and adaptive immune responses which result in clearing an established wild-type RABV infection in the CNS.

Keywords antiviral, blood brain barrier, chemokines and cytokines, innate immunity, rabies virus

1 Introduction

Rabies is one of the oldest human diseases with the highest fatality rate of all infectious diseases, and it still presents a public health threat, causing more than 55000 human deaths globally each year[1]. Rabies occurs in more than 150 countries and territories, mostly in Asian and Africa where animal vaccination is not extensively deployed[2]. The causative agent of rabies, rabies virus (RABV), is a member of the *Lyssavirus* genus in the Rhabdoviridae family. Its genome is a non-segmented negative strand of RNA and encodes five structural proteins in a highly conserved order of nucleoprotein, phosphoprotein, matrix protein, glycoprotein and the RNA-dependent RNA polymerase (also known as the large protein)[3–5]. Among these five structural proteins, glycoprotein is the only viral protein exposed on the surface of the virion[6], which is responsible for binding to neurospecific receptors for invasion into the nervous system[7,8]. Moreover, glycoprotein is the only viral protein capable of inducing virus-neutralizing antibodies (VNA) that are protective against rabies[9–11].

Wild-type (wt) RABV usually infects hosts at peripheral sites and migrates from motor or sensory nerves to the central nervous system (CNS) via retrograde axonal transport[12]. Once the virus gets into nervous system, it employs a series of strategies to evade the host immune responses[13–17]. Preserving the integrity of infected neurons by limiting virus replication and subsequently reducing glycoprotein expression in the CNS is one of the mechanisms that contributes to wt RABV immune evasion[18–20]. The evidence of limited viral replication was obtained in mouse neuroblastoma cells (MNA) or BSR cells infected with wt (DRV) or laboratory-attenuated (CVS-B2c) RABVs, and 2- to 3-log units less DRV than CVS-B2c was produced in mouse neuroblasma cells or BSR cells at 72 h post infection. Furthermore, immunohistochemistry straining for viral nucleoprotein and glycoprotein also revealed that significantly less viral antigens were present in the brain of mice infected with wt RABVs than when infected with laboratory-attenuated RABVs. The minimal level of virus replication and glycoprotein expression enables wt RABV to evade early detection by the host innate immune system, including pro-inflammatory cytokines or chemokines production, and benefits their survive and spread within the CNS[21]. Wt

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RABV induces recruitment of fewer inflammatory cells in the CNS by limiting the expression of cytokines or chemokines and maintaining the blood brain barrier (BBB) integrity[22]. Furthermore, wt RABV does not activate dendritic cells (DCs), the most efficient antigen-presenting cells (APC), and thereby does not induce adaptive immune responses[23]. It has been known for a long time that more than 70% human rabies patients do not develop VNA in the periphery as well as in the cerebrospinal fluid (CSF) at any stage. The same phenomenon, that wt RABV fails to induce protective VNA, is also observed in other animal species, such as mice, dogs and skunks[24–26].

On the other hand, laboratory-attenuated RABV replicates rapidly and produces a large amount of the glycoprotein and induces strong innate and adaptive immune responses, such as extensive inflammation and neuronal apoptosis, expression of cytokines and chemokines, BBB permeability enhancement and DC activation as well as high level of VNA production[18,24,27–32]. Numerous comparative studies performed in laboratory animals have suggested that the wt RABV evades, while laboratory-attenuated the RABV actives, the host innate immune responses[16,17,23,24,26,33].

The innate immune response is the first line of defense against viral invasion. Cells involved in the innate immune system utilize pattern recognition receptors to sense virus by engaging pathogen-associated molecular patterns[34]. This pattern recognition leads to the expression of pre-inflammatory cytokines/chemokines and costimulatory molecules that are the major contributors to virus elimination in absence of adaptive immunity[35].

2 Role of chemokines and cytokines in clearance of rabies virus from the central nervous system

The homeostasis between viral replication and host immune response predicts the clinical outcome of a viral infection. Immune recognition of viral antigens and its genome could initiate a rapid antiviral response mediated by chemokines and cytokines. The ability of a RABV to induce the expression of chemokines and cytokines can lead to virus clearance from the CNS, whereas the ability to evade their expression and activation contributes to virulence and pathogenicity[24,33,36,37]. Various studies utilizing laboratory-attenuate RABVs show that the induction of chemokines and cytokines has a crucial function in enhancing protective immunity and clearance of RABV from the CNS[38–45] (Table 1). The application of genomic array technology has shown that the laboratory-attenuated RABV induces upregulation of a variety of genes involved in the innate immune and antiviral responses, especially those related to interferon (IFN)-α/β signaling pathways, such as interferon regulatory factors (IRF-1, 2 and 7), genes involved in inflammatory pathways

Table 1 The functions of pro-inflammatory cytokines and chemokines involved in rabies virus infection

Cytokines/Chemokines	Function
CXCL10	Attracts activated T lymphocytes, activates other immune cells, enhances protective immunity
CXCL9	Recruits leukocytes
CCL5	Promotes leukocyte trafficking into the CNS, enhances the BBB permeability, enhances protective immunity and clearance of RABV from the CNS
CCL11	Selectively recruits eosinophils
MIP-1α	Enhances the BBB permeability, activates and recruits DCs and B cells, stimulates VNA production
MIP-1β	Promotes leukocyte trafficking into the CNS
MCP-1	Enhances BBB permeability
IFN-α	Enhances protective immunity and clearance of RABV from the CNS
IFN-γ	Inhibits viral replication, enhances BBB permeability, regulates other cytokines and chemokines expression and enhances protective immunity
IL-17	Enhances BBB permeability
IL-12	Promotes the development of Th1 responses
IL-13	Regulates eosinophilic inflammation
IL-7	Decreases local inflammation
IL-6	Enhances protective immunity and clearance of RABV from the CNS
IL-5	Activates eosinophils
CSF1-3	Activates dendritic cells
VEGF	Promotes vasculogenesis and angiogenesis
CXCL1-5	Attracts neutrophils

including toll-like receptors (TLR) 1-3, complement cascade genes and pro-inflammatory chemokines and cytokines, including macrophage inflammatory protein (MIP)-1α, chemokine RANTES, chemokine CXCL10, interleukin (IL)-6 and IFN-γ[24,37]. Similar study performed in raccoon (one of the primary hosts for rabies) found IFNs, IRF, TLR-3, tumor necrosis factor (TNF) receptor and IL-6 genes to be upregulated in RABV infection[46]. Further, studies conducted in mice using attenuated RABV, CVS-F3, showed that the differential upregulation of MIP-1β, TNF-α, IFN-γ, and intercellular adhesion molecule 1 in the cerebellum and the cerebral cortex is key to the clearance of apathogenic RABV from the CNS[42], INF-γ directly inhibits viral replication and regulates other chemokines that facilitate the loss of BBB integrity as well as immune cells invasion into CNS[42]. Thus, the induction of chemokines and cytokines and their role in orchestrating downstream immune events is a key feature in clearing RABV from the CNS. The crucial role of chemokines and cytokines in clearance of RABV from the CNS includes: (1) modulation of and leukocyte trafficking into the CNS, (2) enhancement of BBB permeability, (3) activation of various immune cells, which are essential for viral clearance and protection[36–40].

3 The role of chemokines/cytokines in triggering leukocytes infiltration and central nervous system inflammation

In neurotropic virus infection, mononuclear leukocytes, monocytes and macrophages from the periphery can be recruited into the CNS once they are activated[47]. Laboratory-attenuated RABV was observed to induce numerous inflammatory cell infiltration into the CNS, including T cells, B cells, macrophages, and neutrophils[18,24,48], and this extensive infiltration is related to the high expression of chemokines and cytokines in the CNS. On the other hand, wt RABV infection fails to trigger sufficient pro-inflammatory chemokines and cytokines production, resulting in less cell infiltration and neuronal inflammation in the CNS[24,28,49].

Laboratory-attenuated RABV infection has been found to upregulate a variety of pro-inflammatory chemokines and cytokines in the CNS, such as CXCL10, CXCL9, CCL5, MIP-1α, IL-17, IL-6 and IFN-γ[50–52]. Among these, the highly expressed chemokine, CXCL10, initially induced by infected neuron cells, is the most prominently expressed chemokine in the CNS during laboratory-attenuated RABV infection[22,53]. Recently, Chai et al. showed the mechanism by which CXCL10 triggers T cells migration in RABV infection[53]. CXCL10 binds to its receptor, CXCR3, expressed on activated $CD4^+$ Th1 cells to attract $CXCR3^+CD4^+$ T cells infiltrating the CNS along the chemokine gradient and subsequently differentiating to IL-17-producing Th17 and IFN-γ-producing Th1 cells. IFN-γ secreted by Th1 cells further promotes the positive-feedback loop and amplifies CXCL10 production and $CXCR3^+CD4$ T cell infiltration[53]. Blocking CXCL10 with an anti-CXCL10 antibody dramatically reduces the Th17 infiltration numbers and decreases the IFN-γ production[49,53]. CCL5 is another highly expressed chemokine and promotes leukocytes and macrophage migration into the CNS by binding its receptor CCR5 in laboratory-attenuated RABV infection, while blocking CCL5 with the CCL5 antagonist, Met-CCL5, dramatically reduced $CD3^+$ T cell and macrophage infiltration in the CNS[54]. Although the mechanisms by which other inflammatory cells migrate into the CNS are not well understood in RABV infection, recent studies on recombinant RABVs expressing cytokines or chemokines (GM-CSF, MIP-1α or CCL5) suggest that the upregulation of chemokines and cytokines contributes to these inflammatory cell infiltration in the CNS[40,55,56].

4 The role of chemokines/cytokines in enhancing the blood brain barrier permeability

BBB is a dynamic barrier that regulates the movement of nutritional and toxic substances in and out of the CNS, and is crucial in maintaining a stable environment for the CNS[57]. Many viral infections can disrupt BBB integrity, such as herpes simplex virus 1, Japanese encephalitis virus, T cell leukemia virus, lymphocytic choriomeningitis virus, West Nile virus and mouse adenovirus type 1[58–60]. While wt RABV infection does not alter the BBB permeability but finally leads to a lethal outcome[15,61]. The role of transient BBB enhancement and RABV clearance is evident from the study of Roy et al., where silver-haired bat RABV (SHBRV) infection in mice was lethal due to an intact BBB, although the functional immune responses (i.e., the production VNA and $CD4^+$ and $CD8^+$ T cells infiltration) to SHBRV develops in the periphery[61]. However, the lethal outcome of this SHBRV infection can be prevented by opening the BBB to facilitate the immune effectors infiltrating the CNS to clear RABV, thereby facilitating the survival of SHBRV infection[62]. Moreover, VNA administered in the periphery was unable to enter the CNS to clear wt RABV and failed to protect mice from rabies infection[63], while increasing the BBB permeability by administration of MCP-1 after VNA treatment improved the survival rate up to 80%, resulting in VNA entering into the CNS to clear RABV[63]. Together, these studies suggest that changes in BBB permeability are critical for surviving RABV infection.

On the other hand, laboratory-attenuated RABV infection has been demonstrated to enhance the permeability of the BBB[15,22,63]. One of the key mechanisms of BBB enhancement is the reduction of tight junction (TJ) proteins in brain microvascular endothelial cells (BMECs)[49,57].

One study showed that laboratory-attenuated RABV infection dramatically decreased the TJ protein expression, including claudin-5 and ZO-1, and enhanced the BBB permeability[64]. The BBB permeability enhancement corresponded to the reduction of TJ proteins[65]. However, the reduction in TJ protein expression was not directly due to infection with laboratory-attenuated RABV since neither laboratory-attenuated nor wt RABV are able to infect BMECs *in vitro*[49]. The brain extracts from mice infected with laboratory-attenuated RABV induce the disruption of TJ proteins[49], indicating that this is an induced rather than direct effect of the virus. Another study showed that laboratory-attenuated RABV-induced chemokines expression, such as CXCL10, CCL5 and MIP-1α, in parallel to changes in BBB permeability, suggesting cytokines and chemokines are important in BBB permeability enhancement in animals infected with laboratory-attenuated RABV[22].

The expression profile of chemokines/cytokines in laboratory-attenuated RABV infected CNS, determined using a cytokine/chemokine magnetic bead panel, showed that 25 out of 30 chemokines are highly upregulated, including CXCL10, CXCL9, CCL11, CXCL2, CXCL1-5, IFN-α, IFN-γ, IL-1α, IL-17, IL-13, IL-12, IL-7, IL-6, IL-5, VEGF, CSF3, CSF2, CSF1, and LIF[22,49]. In addition, the ingenuity pathway analysis based on these molecules reveals IFN-γ is in the center of the signaling network and directly links with many other chemokines/cytokines, such as CXCL10, CXCL9, CCL5, IL-17, IL-12, IL-6 and VEGF[49]. Moreover, silencing the IFN-γ decreased the BBB permeability and increased the TJ protein expression[15], suggesting that the mechanism of BBB permeability enhancement is modulated by these cytokines/chemokines in an IFN-γ dependent signaling pathway.

Also, CXCL10, the most highly upregulated chemokine after laboratory-attenuated RABV infection, was found to initiate the change of the BBB permeability (Fig. 1)[53]. CXCL10 is initially secreted by infected neurons, which can be detected as early as three days after laboratory-attenuated RABV infection, and then neuronal CXCL10 initiates the cascade to activate other CNS resident cells, such as microglia or astrocytes, to express more CXCL10 and other chemokines/cytokines[53]. CXCL10 functions to recruit the $CXCR3^+CD4^+$ T cells trafficking into the CNS and subsequently differentiating into Th17 and Th1 cells[49,53]. Th17 cells produce IL-17 in the CNS that initiates the alteration of TJ proteins, resulting in the BBB permeability enhancement. Meanwhile, Th1 cells produce IFN-γ that promotes the positive feedback to amplify CXCL10 production, which further boosts the TJ protein breakdown and subsequently enhances the BBB permeability[49,53]. Neutralizing CXCL10 in RABV infected mice using anti-CXCL10 antibody led to downregulation of IFN-γ production, amelioration of TJ protein breakdown, and reduction of BBB permeability enhancement[53].

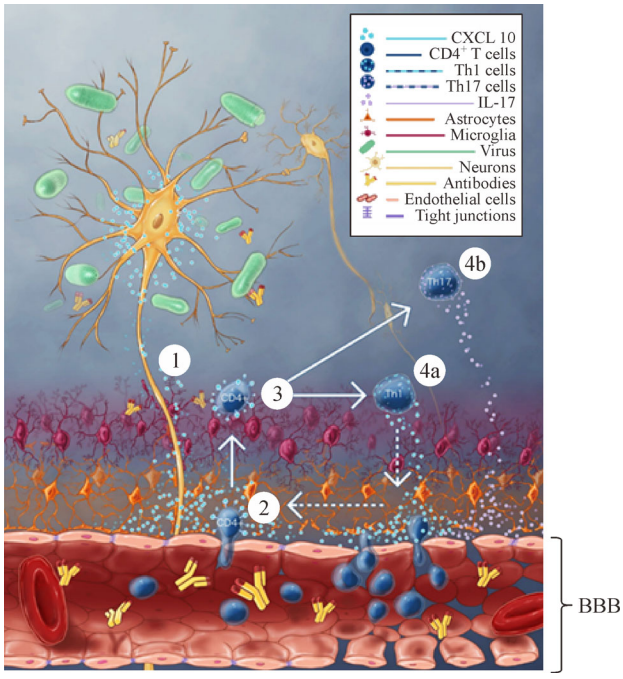

Fig. 1 The mechanism of blood brain barrier (BBB) enhancement by neural expression of CXCL10 induced by laboratory-attenuated rabies virus (RABV). 1, Laboratory-attenuated RABV-infected neurons secrete chemokine, CXCL10; 2, CXCL10 mediates the recruitment of $CD4^+$ T cells into the CNS; 3, CXCL10 mediates the differentiation of $CD4^+$ T cells into Th1 and Th 17 cells; 4a, IFN-γ secreting Th1 cells could further boost the induction of CXCL10 through positive feedback; 4b, IL-17 secreting Th17 cells alters the TJ (tight junction) proteins resulting in BBB breakdown. This figure was adapted from reference[66].

5 Evidence that rabies virus expression of chemokine/cytokine elicits superior immune response capable of clearing the virus from the central nervous system

The crucial role of chemokines or cytokines in wt-RABV clearance from CNS has been further demonstrated by recombinant RABVs expressing MIP-1α, GM-CSF or IFN-γ[36]. RABV expressing MIP-1α induces an early, transient expression of MIP-1α at the inoculation site in mice and enhances the recruitment of antigen presenting DCs and antibody producing B cells in the lymph nodes, and infiltration of inflammatory cells into the CNS. MIP-1α (major chemoattractant) expressed by the recombinant RABV recruits and activates DCs and B cells in the draining lymph nodes and at the peripheral blood, resulting in the production of a high level of VNA and protection from lethal challenge[38].

Then cytokine GM-CSF regulates the production and functional activation of hemopoietic cells, such as monocyte/macrophages and APCs. Recombinant RABV expressing GM-CSF stimulates the activation of DCs, and B and T cells in the periphery, leading to robust production

of VNA and strong protection against challenge with virulent RABV in mice and dogs. Furthermore, these recombinant RABVs administered intracerebrally were able to clear an established infection with wt RABV from the CNS and prevent mice from developing rabies[39,40]. The recombinant RABVs induced high levels of chemokine/cytokine expressions (MIP-1α, RANTES, CXCL10, MCP-1, IL-6 and IFN-γ) in the CNS, infiltration of inflammatory and immune cells (such as neutrophils, activated microglia/macrophages and T cells) into the CNS, and enhancement of BBB permeability. Similarly, a recombinant RABV expressing dog GM-CSF was found to be efficient in recruitment and activation of DCs and B cells, induction of a high level of VNA production and protection against lethal challenge in dogs[67].

Although chemokines are crucial for RABV attenuation, various studies have shown the importance of interferons, particularly IFN-γ, in RABV clearance from the CNS[41–43]. To study the role of IFN-γ in RABV attenuation and clearance, murine IFN-γ gene have been cloned in the RABV genome. This incorporation of IFN-γ led to attenuation of pathogenic RABV. Further investigation using knockout mice unable to signal through the type I IFN receptor indicated that the recombinant RABV expressing IFN-γ strongly attenuates the pathogenicity via induction of type I IFN[68]. A subsequent study found that the incorporation of the murine IFN-γ gene in a highly attenuated GAS backbone can enhance safety and immunogenicity[69].

6 Conclusions

The immune responses to infection differ between wt and laboratory-attenuated RABV. Comparative studies indicate that wt RABV evades, while laboratory-attenuated RABV activates innate immune responses. Chemokines and cytokines produced in RABV infection are crucial for RAVB clearance from the CNS through modulation of leukocyte recruitment into the CNS, enhancement of BBB permeability and activation of various immune cells that are essential for viral clearance and protection (Fig. 2). In RABV infection, chemokine CXCL10 initially produced by infected neurons activates other CNS resident cells, such as microglia and astrocytes to produce CXCL10 and other chemokines and cytokines, initiates infiltration by inflammatory cells and enhances the BBB permeability. Moreover, recombinant RABV expressing chemokines or cytokines induces strong innate and adaptive immune responses compared with their parental virus, resulting in increased cytokines and chemokines expression, further BBB permeability enhancement as well as higher VNA production to clear wt RABV rapidly from infected CNS.

7 Prospectives

Rabies is almost fatal once the virus entered into CNS, and no effective treatment is available once the clinical symptoms show. Maintaining the BBB integrity and failure of VNA production are two major reasons contributing to RABV fatality. Chemokines and cytokines are the initiators of the BBB permeability in RNBV infection, and using chemokines will be an effective clinical treatment for rabies to enhance the BBB permeability and let the immune effectors, especially VNA produced or administrated in periphery, enter the CNS for RABV clearance. In addition, recombinant RABVs

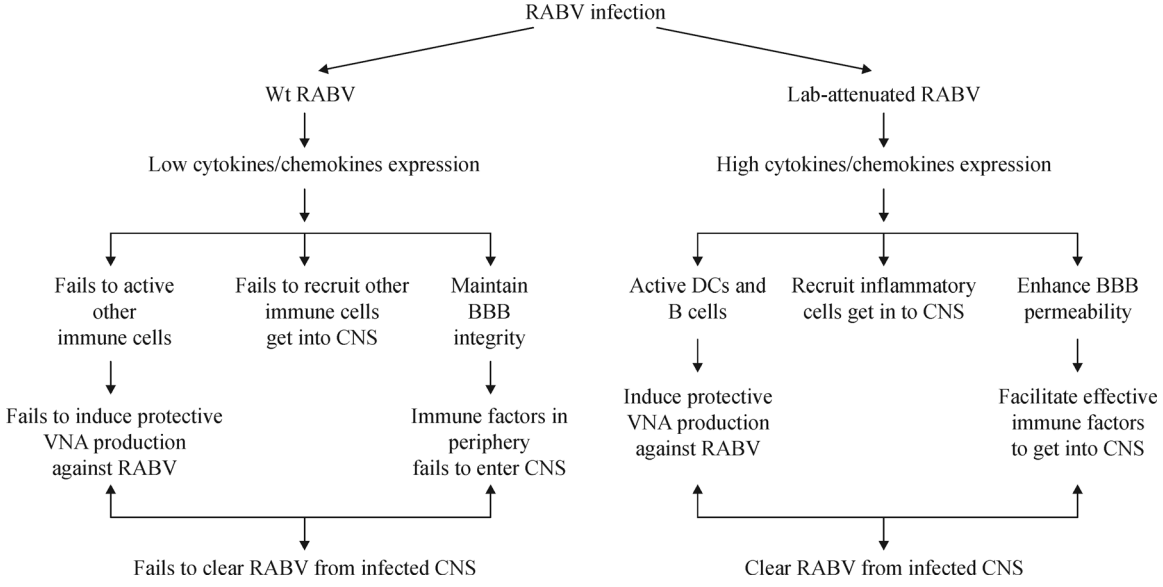

Fig. 2 The functions of cytokines and chemokines in rabies virus infection

expressing chemokines or cytokines will also be more efficacious rabies vaccines for prevention as well as for therapy.

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Compliance with ethics guidelines Ying Huang, Clement Wesley Gnanadurai, and Zhenfang Fu declare that they have no conflict of interest or financial conflicts to disclose.

This article is a review and does not contain any studies with human or animal subjects performed by any of the authors.

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