

Highly Neurovirulent Viruses Invade and Spread in the Brain Using the Host Reactions Triggered by Infection

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Abstract

Coronaviruses (CoVs) have relatively high mutation and RNA recombination rates and undergo rapid cross-species transmission events *in vitro* and *in vivo*. The viruses have evolved diverse strategies to evade, blocking host immune responses, and resulting in an emergence of highly virulent CoVs to cause fatal diseases in human, such as severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS). Among CoVs used for experimental studies, the cl-2 virus (cl-2), isolated from a neuropathogenic JHM viral strain (JHMV) of mouse hepatitis virus (MHV), shows extremely high virulence. All mice infected with cl-2 die within 3 days post-inoculation (dpi). Less virulent but still highly neurovirulent mutant viruses, soluble receptor resistant mutant virus (srr7) and Mu-3 virus (Mu-3), have been isolated from cl-2 and srr7, respectively. Although these viruses cause different pictures of neuropathologies with different distribution of viral antigens after infection, they use the same pathway to enter the brain before inducing lesions in the brain parenchyma. The pathway is formed as an astrocytic reticular network (ARN) after infection. The ARN functions as a conduit system in a similar way to a fibroblastic reticular network (FRN) in lymphoid organs. In addition, the viruses induce an immunosuppressive state in infected mice, mimicking endotoxin tolerance (ET), or lipopolysaccharide (LPS) tolerance. The immunosuppressive states are detected by; 1) pathological findings as 1a); a very low -level inflammatory response, 1b); the shrunken spleen, 1c); the refractory state to LPS challenge, and 1d); the local production of anti-inflammatory cytokines by neurons, and by 2) immunological studies, which showed 2a); the systemic production of anti-inflammatory cytokines by eliciting CD11b+Gra-1+ suppressor monocytes/macrophages (Mo/Mas). In addition, cultured splenic cells and Mo/Mas after infection exhibit 2b); low-level responses to stimulation of toll-like receptors (TLRs) 4 and 7, and 2c); the induction of Lewis X carbohydrate structure (Lex), which can trigger the C-type lectin-like receptor that initiates an immunosuppressive pathway, respectively.

Introduction

CoVs exhibit high mutation rate, leading to an emergence of life-threatening virulent viruses [1]. However, the mechanisms of the high-rate mutation and the emergence of highly virulent viruses have not been elucidated. In the experimental model, many types of CoVs, ranging from highly virulent to attenuated viral clones, have been isolated from mouse hepatitis virus (MHV), which would provide an opportunity to investigate the mechanisms more efficiently compared with those of human origin [2]. MHV is an enveloped virus with single-stranded, positive-sense genomic RNA that is about 30 kilobases long. The envelop of virion has projections of glycoprotein, Spike (S) protein, which is responsible for binding to a receptor and also for the cell entry mechanism of MHV [2]. Mutation frequently occurs in the MHV genes [3,4], and several mutant clones have been isolated from JHMV [2], such as cl-2 [5], srr7 [6], and Mu-3 [7,8]. Although many other highly virulent clones have been isolated from MHV, the immunological background of high virulence has been mainly explained that CoVs, in general, block interferon (IFN)-I signaling and counter the action of downstream effector molecules [8], until recent studies revealed that cl-2 and its progenies cause immunosuppressive states in the infected animals by triggering the production of anti-inflammatory cytokines after infection [9,10].

In addition to triggering the suppressive immune responses, the cl-2 exhibits extremely high neurovirulence by infecting neurons, which do

not express carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM 1), a major MHV receptor (MHVR), and the infected mice die within 3 days post-inoculation (dpi). The lesions caused by cl-2 infection in an MHVR-independent manner are mainly distributed in the grey matter of the brain, while srr7 infection induces lesions in the white matter of the brain without infecting neurons, and mice infected with srr7 survive more than 7 dpi up to 10 dpi [8,11,12]. These differences in the neuropathogenesis and dependency on MHVR between srr7 and cl-2 are attributed to a single mutation in the S2 gene [13] in the S protein-encoding region. The mutant virus isolated from srr7, Mu-3, induces apoptosis of the pyramidal neurons in the hippocampus, especially in CA2 and CA3 subregions after infection. This unique neuropathology is not observed after infection with srr7 or cl-2 [7]. Mu-3 has a single amino acid mutation at position 596 (Asn to Lys) of the S2 subunit of the S protein relative to srr7, and exhibits higher virulence than srr7 and lower virulence than cl-2. Mu-3 induces mixed neuropathological pictures induced by srr7 and cl-2 infection, causing the lesions in the brain distributed both in the white and grey matter [7].

In spite of these differences, the three viruses spread in the brain in the same manner during the early phase of infection. The viral antigens are initially detected in the CD11b-positive monocytes/macrophages (Mo/Mas) exuded in the meninges at 12 h post-inoculation (hpi) [8,11,12,4]. Before 48 hpi, infected Mo/Mas appear in the ventricle, and the viral antigens are detected in the ependymal cells and GFAP-

positive cells or nestin-positive progenitor cells that take up positions in the subventricular zone (SVZ) [12]. The infected GFAP-positive cells in the SVZ are found to project cell processes to the ependymal cell layer, which are referred to as type B cells [14]. Type B cells develop as the primary neuronal precursors besides contributing to the niche environment in the SVZ of adult mice [14]. The ventricular wall is also the target site during the initial phase of infection with other viruses, including herpes simplex virus [15] and lymphocytic choriomeningitis virus [16].

In addition to the viral spreading manner during the early phase of infection, the three viruses induce the same pathological state, that is an immunosuppressive state [12,4]. Although the viruses cause widespread pathological change in the brain after infection, the lesions in the brain parenchyma are accompanied by very low-level inflammatory cell infiltration. This type of host response after viral infection is typically observed in retroviral infection [17]. In addition, mice infected with the viruses show a shrunken spleen with reduced cell population [18,7]. Therefore, the host responses after viral infection that facilitate viral entry into the brain by forming a passage, and the immune responses that allow viral replication and spread have been investigated.

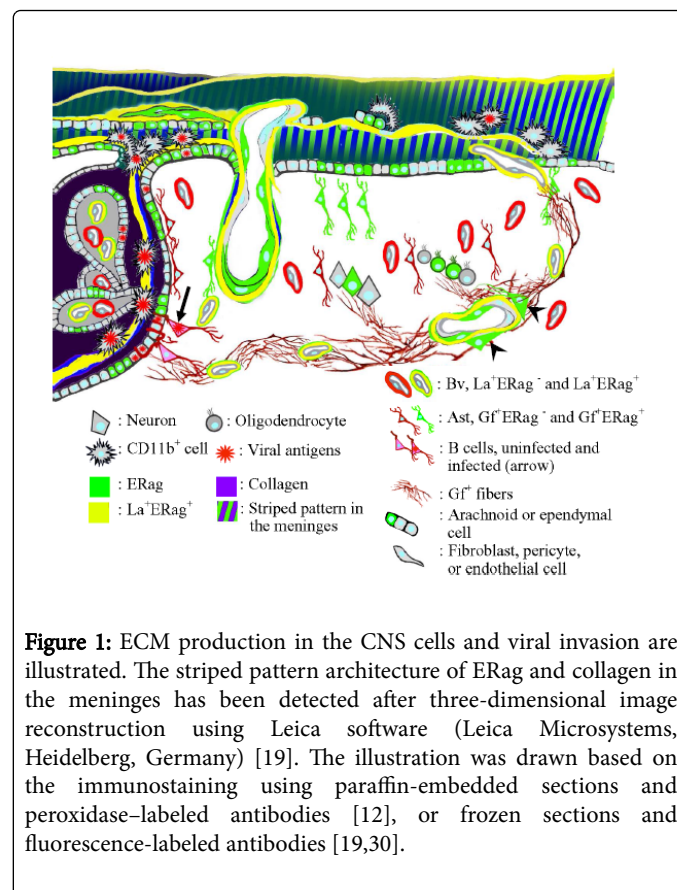
Astrocytic Reticular Network (ARN)

Fibroblastic reticular network and ARN

A unique passage used for viral entry between the meninges, the initial site of infection, and the ventricle is formed after infection with cl-2. The passage is found around the foramen of Luschka at the cerebellopontine angle [19]. In the normal state, the foramen of Luschka is used for cerebrospinal fluid (CSF) to flow from the ventricle into the , and there is a barrier called blood-CSF barrier, with a unique structure, the choroid plexus [20]. The passage is composed of fibrous extracellular matrix (ECM), which includes an antigen recognized by Erasmus University Rotterdam-thymic reticulum antibody 7 (ER-TR7) [21]. The antigen detected by ER-TR7 (ERag) in the passage is associated with collagen and laminin. The ERag-positive fibers (ERfibs) have been recognized as a hallmark of the fibroblastic reticular fibers that form the fibroblastic reticular network (FRN) in the lymph nodes [22,23] and spleen [24,25]. The FRN in lymphoid organs functions as a conduit, facilitating the transfer of foreign antigens or immuno-mediating cytokines to their proper sites, and guiding immuno-competent cells for their homing [25]. The fibers of FRN in the lymphoid organs contain collagen fibers as a core, which is wrapped by an ERag-positive ECM, and further by a basement membrane enriched with laminin [24]. The fibers are finally wrapped by the cytoplasm of fibroblastic reticular cells (FRC), which produce major components of the fibers and nurse the FRN in the lymphoid organs.

In the brain, ERag already appear at 12 hpi in the astrocytes facing the border of peripheral nerve and the central nervous system (CNS) in the root of trigeminal nerve and those facing the arachnoid membrane, as well as in the fibrous structure in the meninges [19]. The finding of ERag-positive fibrous structure in the meninges is obtained through two-dimensional observation, a usual microscopic finding. However, three-dimensional reconstruction of the ERag-positive structure in the meninges revealed that the appearance of ERfibs are the sections of a stack of thin sheets with a striped pattern architecture of ERag and collagen (Figure 1). Each of the sheets was approximately 0.7-1 μm in thickness, which corresponds to the diameter of a ERag-

positive reticular fiber serving as a unit of the conduit system in the lymphoid organs [26]. Furthermore, the cells exuded in the meninges, which are initial targets of infection, are put between the sheets [19]. The meningo-brain barrier (MBB) has been considered to be constructed by the thin arachnoid membrane and astrocytes facing the membrane. However, these observations indicate that the MBB is formed immediately after infection by producing a pile of ERag-positive membranous sheets, and the production of ERag is one of the host defense mechanisms against infection.



After 24 hpi, ERfibs or ERag become apparent in the ventricles, ependymal cells, and ventricular wall including SVZ [19]. Along with ERfibs formed between the meninges and ventricle, infected Mo/Mas enter the ventricle from the meninges. Usually, extracellularly released viral particles are not detectable by immunofluorescence, but concentrated viral antigens in the ERag-positive fibrous structure could be observed. Such colocalization of viral antigens or viral particles with ECM is detectable after infection with lymphocytic choriomeningitis virus (LCMV) in the spleen [27]. Similar phenomena have been reported on infection with extremely virulent viruses, such as Ebola [28], Marburg, and Lassa [29] viruses in the lymphoid organs. It has been considered that the intimate interaction of the viral particles with ECM facilitate the spread of the viruses. Because close contact of cl-2 to ERfibs is observed, the viral antigens expressed on the surface of infected MoMas possibly interact with newly formed ERfibs, which guide the cells to enter the ventricle.

ERag-producing cells in the CNS

By *in vivo* studies using immunofluorescence, ERag is detected at 48 hpi in almost all cell types that constitute the CNS, including arachnoid cells, ependymal cells, pericytes, oligodendroglia, astrocytes, and neurons. However, CD11b-positive cells including microglia do not produce ERag. Using the brain primary culture, most of the observation on ERag-producing cells were confirmed, except for neurons [30]. Reexamination of the *in vivo* brain infected with an attenuated viral clone, Mu-3, using serial sections raised the possibility that ERag-positive satellite cells attached to neurons (Figure 2) show a falls-positive appearance of ERag-positive neurons. Satellitosis has been recognized as a response of the brain to a variety of infections, and it is considered to be an increase in oligodendrocytes as satellites to neurons [31]. Therefore, we should wait for the results of electron microscopic studies to see whether neurons produce ERag after infection.

Although many cell types in the CNS produce ERag after infection, the most prominent ERag-producing cells in the brain parenchyma are astrocytes, which start producing ERag at 12 hpi. Furthermore, astrocytes in *in vivo* and *in vitro* produce ECM other than ERag after infection, such as collagen type I, type III, and laminin [30], which are main components of ERag-positive reticular fibers in the lymphoid organs. Finding of close interaction of GFAP-positive astrocytic fibers with fine ERfibs indicates that astrocytes play a role in producing ERag-positive reticular network in the brain parenchyma in a way FRCs do in the lymphoid organs.

Immunosuppression

Induction of immunosuppressive Mo/Mas

Mice infected with *srr7* show a low response to bacterial endotoxin/lipopolysaccharide (LPS) stimulation [32], mimicking the state in endotoxin tolerance (ET), or LPS tolerance, which has been thought to be a protective system to regulate the over-production of proinflammatory cytokines. Experimentally, ET is induced by prior exposure of Mo/Mas to LPS, which causes them to be refractory to further LPS stimulation [33]. Activated macrophages that play a key role in ET have been recognized as M2 macrophages or CD11b⁺Gra-1⁺ suppressor Mo/Mas, an immunosuppressive subset of macrophages, which secrete the anti-inflammatory cytokines IL-10 and TGF- β , appearing in the blood stream [34] and lymphoid organs [35]. M2 macrophages also appear in local inflammatory sites as exuded cells, such as peritoneal exudate cells (PECs) in the ET state [36]. Mu-3-infection in the peritoneal cavity induces a large CD11b⁺Gra-1⁺ population among PECs. Furthermore, cultured PECs secrete IL-10 and TGF- β after infection with Mu-3 [10]. In the brain, the pyramidal neurons in the hippocampus produce IL-10 and TGF- β after infection with Mu-3 [9]. Thus, Mu-3-infection causes systemic and local immunosuppressive states.

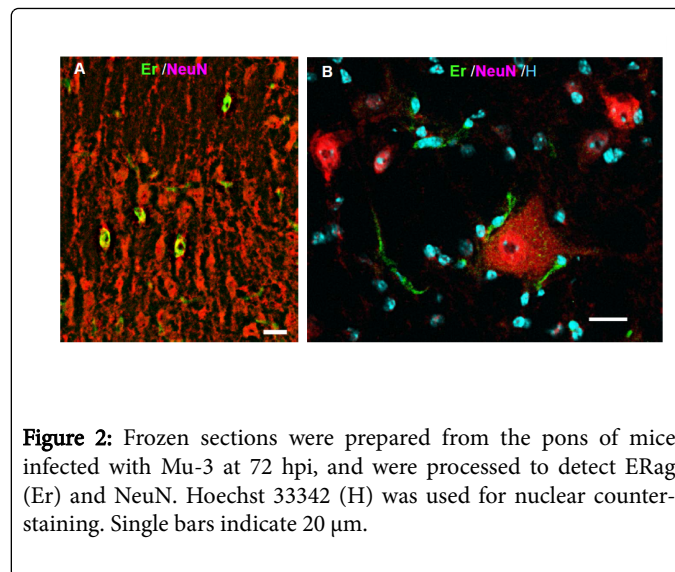


Figure 2: Frozen sections were prepared from the pons of mice infected with Mu-3 at 72 hpi, and were processed to detect ERag (Er) and NeuN. Hoechst 33342 (H) was used for nuclear counter-staining. Single bars indicate 20 μ m.

Involvement of Lewis X carbohydrate structure

Using *Fut9*^{-/-} mice [37], which lack α 1,3-fucosyltransferase 9 and are unable to synthesize the Lewis X carbohydrate structure (Lex). It has been shown that the expression of Lex is involved in the immunosuppressive state induced by infection with cl-2 and its progeny. The ET-mimicking immunosuppression observed in wild-type mice after infection does not occur in *Fut9*^{-/-} mice, which respond to LPS stimulation equally with or without infection [32]. Furthermore, stimulation of toll-like receptor (TLR) 4, TLR7, and TLR9 with ligands LPS, single-stranded RNA (ssRNA), and cytosine-phosphodiester-guanine oligonucleotides (CpG) revealed that the splenic cells after infection with *srr7* failed to trigger not only TLR4-mediated cascade by LPS but also TLR7-mediated cascade by ssRNA, as summarized in (Figure 3). The splenic cells derived from both wild-type and *Fut9*^{-/-} mice failed to produce interferon (IFN)- β after infection by stimulation of TLRs. It occurred because coronaviral infection causes direct interference in the cascades to produce IFN- β [1].

The interaction between carbohydrate structures and their binding partners, glycan-binding proteins, or lectins play varied roles in immunological responses [38]. Lex is a natural ligand of dendritic cell-specific intercellular-adhesion-molecule-grabbing nonintegrin (DC-SIGN), a C-type lectin-like receptor. DC-SIGN initiates an immunosuppressive pathway after interaction with Lex [39].

In the normal state, the expression of Lex is very low level and undetectable by immunofluorescence. Infection of cultured PECs with *srr7* or Mu-3 induces detectable level of Lex expression in the PECs by immunofluorescence [40,10]. IL-10-producing PECs observed after infection are found to express Lex [10]. Thus, highly neurovirulent viruses proliferate and spread under the immunosuppressive condition induced in the host animals.

These studies would provide an opportunity to investigate the mechanisms of emergent coronaviruses that cause lethal diseases in humans, such as SARS and MARS, where neither ECM production that facilitate viral spread nor immunosuppressive states induced after infection have been studied.

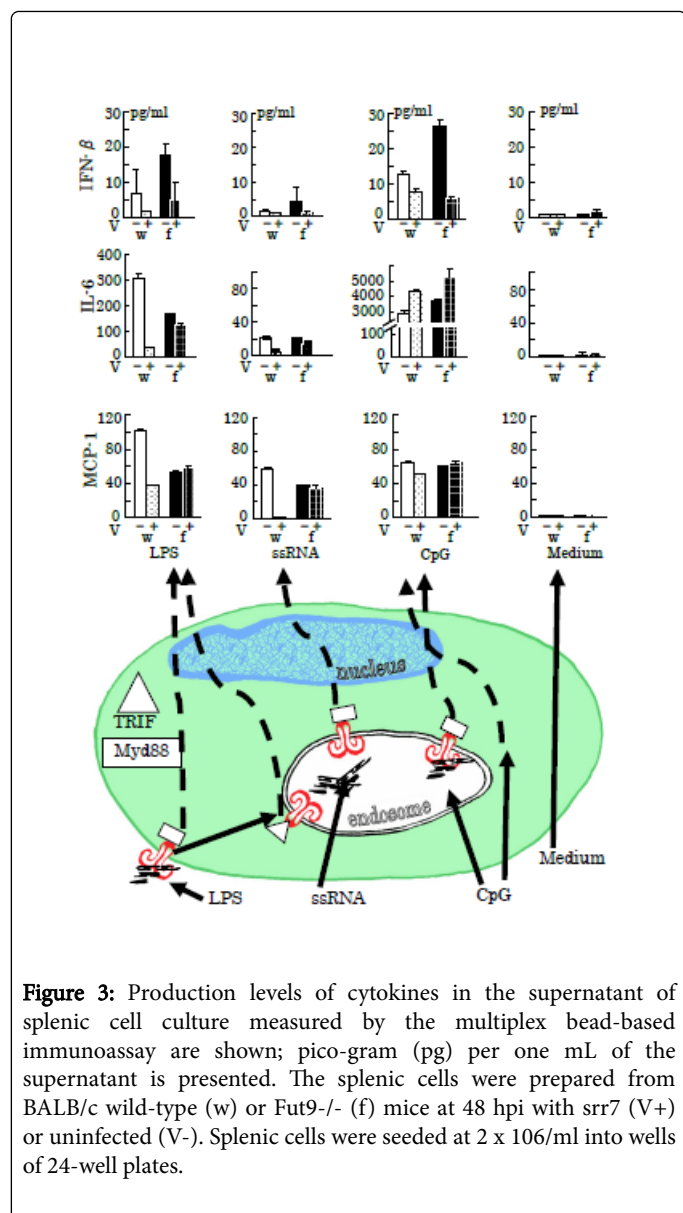


Figure 3: Production levels of cytokines in the supernatant of splenic cell culture measured by the multiplex bead-based immunoassay are shown; pico-gram (pg) per one mL of the supernatant is presented. The splenic cells were prepared from BALB/c wild-type (w) or Fut9^{-/-} (f) mice at 48 hpi with srr7 (V+) or uninfected (V-). Splenic cells were seeded at 2 x 10⁶/ml into wells of 24-well plates.

Representative and simplified cascades for cytokine production after stimulation for 24 h of TLRs by LPS (5 µg/ml), ssRNA (1 µg/ml), and CpG (5 µM), with respect to adaptor protein myeloid differentiation factor 88 (MyD88) (square)-dependent and -independent signaling [41], are shown in the lower picture. The triangle indicates the adaptor protein Toll-interleukin 1 receptor (TIR) homology domain-containing adaptor-inducing interferon-β (TRIF). Vertical lines indicate SD.

The production levels of MCP-1 and IL-6 are shown because the most marked differences between infected wild-type and Fut9^{-/-} mice were observed in the production of these two cytokines among several cytokines examined. The production level of INF-β, which is down-regulated through direct interference in the cascades by coronaviral infection [1], is reduced after infection showing no difference between wild-type and Fut9^{-/-} mice.

The LPS initiates intracellular cascades *via* TLR4 in a MyD88- or TRIF-dependent manner using them as cytoplasmic signaling adaptor

proteins, while ssRNA binds to TLR7 with MyD88-dependency to cause signal transduction [41]. The TLR9 also shows MyD88-dependency in signal transduction, but CpG induced similar levels of MCP-1 and IL-6 production in the culture of SpCs derived from infected and mock infected mice regardless of the mouse strains. CpG can initiate proinflammatory responses through a TLR9- and Myd88-independent mechanism [42].

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