Induction of Anti-inflammatory Cytokines in CD11b- and Gr-1- Positive Cells after Neuropathogenic Coronaviral Infection

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Abstract

Mu-3 virus (Mu-3), a neuropathogenic strain of the mouse hepatitis virus, induces the apoptosis of pyramidal neurons in CA2 and CA3 subregions of the hippocampus at 4 days post-inoculation (dpi), without showing destructive changes or viral invasion there at 3 dpi. Since it has been considered that the apoptotic lesions occur through the indirect effects of infection, local expression of cytokines was examined in the brain in our previous study, revealing that the anti-inflammatory cytokine IL-10 is produced by pyramidal neurons of CA2 and CA3 after infection. However, infection of primary brain culture failed to elevate IL-10 production. Therefore, in order to examine whether Mu-3 infection elevates IL-10 production, CD11b-expressing Peritoneal Exudate Cells (PECs) were used, which showed an increased level of IL-10 production in the supernatant of PEC culture after infection. The finding that IL-10-producing cells expressed Lewis X (Le^x) supports our previous hypothesis that Le^x expression is involved in the immunosuppressive state induced by viral infection. In addition, in combination with an experiment involving *ex vivo* infection, it was indicated that Mu-3 elicits M2 macrophages or Gr-1$^+$CD11b$^+$ Myeloid-Derived Suppressor Cells (MDSCs) that produce IL-10 and TGF-β to circumvent the host immune response after infection.

Keywords: JHM; IL-10; Apoptosis; Hippocampus; Pyramidal neuron; Lewis X

Introduction

The Mutant virus Mu-3, isolated from the srr7 virus (srr7) [1,2], causes the apoptosis of pyramidal neurons in CA2 and CA3 subregions of the hippocampus after infection [3]. Apoptosis of the pyramidal neurons in the hippocampus is observed after infection with various kinds of viruses, as introduced in our previous report [3]. It has been considered that the apoptotic lesions after infection are not caused by the direct infection of neurons, but induced through indirect effects [3-5], triggered by immune responses that involve elevated levels of proinflammatory cytokines detected in the course of diseases. However, our study using the homogenized tissue or frozen sections obtained from Mu-3-infected mice failed to detect the production of proinflammatory cytokines in CA2 and CA3 subregions of the hippocampus [6]. Thereafter, we examined the involvement of anti-inflammatory cytokines, and found that IL-10 is expressed in pyramidal neurons in CA2 and CA3 subregions of the hippocampus [6]. TGF-β, which is associated with Theiler's Murine Encephalomyelitis Virus (TMEV) infection [4], was also detected in the hippocampus, although the expression level was low, and the expression was not limited to CA2 and CA3 subregions, but was also observed in a CA1 subregion. The involvement of anti-inflammatory cytokines in the development of the lesions observed in the hippocampus after infection with Mu-3 corresponds to our previous findings that Mu-3, srr7, and its maternal clone, JHM virus strain (JHMV) cl-2 virus (cl-2), induce an immunosuppressive state in the infected mice, which have a shrunk spleen with reduced cell population and the neuropathology showing very low-level inflammatory cell infiltration in the brain parenchyma in spite of a wide area of brain damage [2,7,8]. The leukocytes derived from the infected mice show unresponsiveness to the stimulation by Lipopolysaccharide (LPS) [9,10], mimicking the state in Endotoxin Tolerance (ET), or LPS tolerance, where a population of CD11b^{mid}Gr-1^{mid} suppressor Monocytes/Macrophages (Mo/Mas) plays a central role, secreting the anti-inflammatory cytokines IL-10 and TGF-β [11].

Although we observed IL-10 expression in pyramidal neurons in the hippocampus, we could not detect the induction of anti-inflammatory cytokines using brain tissue cultures after infection with MU-3, probably because of different population of neurons in the pyramidal layers *in vivo* and tissue cultures [6]. Therefore, in order to examine whether Mu-3 is capable of triggering the production of anti-inflammatory cytokines after infection, we used PECs, which are known to produce anti-inflammatory cytokines under the ET condition, and express the Lewis X (Le^x) carbohydrate structure after infection with srr7, accompanied by a shift of the Gr1$^+$CD11b$^+$ cell population [12]. In this report, we showed that IL-10 is produced in the PECs after infection with JHMV for the first time in the world.

Materials and Methods

Viruses and animals

A neuropathogenic MHV strain, Mu-3, was used in this study. This virus was propagated and titrated in DBT cells, as previously described [3]. Specific pathogen-free inbred BALB/c mice purchased from Charles River (Tokyo, Japan) were housed in a specific pathogen-free animal facility, and maintained according to the guidelines with

respect for the Helsinki Declaration set by the ethical committee of our university. For the experiment, mice were transferred to a Biosafety Level 3 (BSL-3) laboratory after obtaining permission from the committee of our university. Six-seven-week-old mice were used for all experiments. Mice were inoculated with 1×10^2 Plaque-Forming Unit (PFU)/50 µL of Mu-3 virus into the right frontal lobe under deep anesthesia using ethyl ether (SHOWA-ETHER, Tokyo, Japan).

Preparation of peritoneal exudate cells

Thioglycolate Medium (TM) (BD Diagnostics, Loveton Circle Sparks, MD, USA) was prepared as previously described [12]. Four days after the injection of 2 ml of TM into the peritoneal cavity, Peritoneal Exudate Cells (PECs) were collected. To obtain adherent PECs, the cells were seeded at 2×10^5 per well in eight-well glass-bottomed chamber slides (Nalge Nunc International), and cultured as previously described [12]. After overnight culture, the adherent cells were inoculated with 1.0×10^1 PFU (plaque-forming units) of Mu-3 virus per well and incubated for the specified time period.

Immunofluorescence

Fixed cultured cells on slide-glasses were processed for immunostaining using the relevant antibodies (Table 1), followed by nuclear counterstaining using Hoechst 33342 (Invitrogen, Carlsbad, Calif., USA), as previously described [12]. Some cells were stained with hematoxylin (Sigma) to observe nuclear structures. Stained cell cultures were mounted with gold antifade reagent (Invitrogen) and examined using a confocal laser scanning microscope (Leica Microsystems, Knowlhill, UK) or fluorescence microscope (KEYENCE, Osaka, Japan).

Detection of cytokines in culture supernatant

Cytokines obtained from the culture supernatant were assayed using the Luminex microbead-based multiplexed assay (Luminex Corp., Austin, TX, USA) and commercially available kits according to the manufacturer's protocol (Affimetrix, Santa Clara, CA, USA). Cytokines analysed by the array include Interferon (IFN)-α, IFN-β, Interleukin (IL)-1β, IL6, IL-10, Tumor Necrosis Factor (TNF)-α, Monocyte Chemoattractant Protein 1 (MCP-1), and Transforming Growth Factor (TGF)-β1.

Flow cytometry

PECs infected with 1.3×10^5 PFU of Mu-3 virus *in vitro* in suspension culture [12], or infected with 1.0×10^5 PFU of Mu-3 virus *in vivo* in the peritoneal cavity for 24 h, were collected and processed for cytometry using a FACSCalibur (BD Biosciences) after staining with fluorochrome-conjugated antibodies (Table 1) as previously described [12]. Appropriate isotype controls were used in all cases. Data were analysed using FlowJo software (Tree Star, Ashland, OR, USA).

Target	Species	Clone or designation	Conjugate	Reference/supplier
Primary antibodies for immunofluorescence staining				
Gr-1	Rat	RB6-8C5	FITC	eBioscience, San Diego, CA, USA
Gr-1	Rat	RB6-8C5	Biotin	BioLegend, San Diego, CA, USA
CD11b	Rat	M1/70	Biotin	BD Pharmingen, San Diego, CA, USA
JHMV	Mouse	Monoclonal	Purified	-12
IL-10	Rat	JES5-2A5	Purified	Abcam, Tokyo, Japan
Le^X	Mouse	MC480	Purified	Developmental Studies Hybridoma Bank, University of Iowa, Ames, IA
IFN-b	Rabbit	Polyclonal	Purified	PBL Interferon Source, Piscataway, NJ, USA
Primary antibodies for flow cytometry				
Gr-1	Rat	RB6-8C5	FITC	eBioscience
CD11b	Rat	M1/70	PE	eBioscience
Secondary antibodies for immunofluorescence staining				
Rabbit IgG	Donkey	Polyclonal	Alexa568	Invitrogen, Carlsbad, CA, USA
Rat IgG	Donkey	Polyclonal	Alexa488	Molecular Probe, Carlsbad, CA, USA
Rat IgG	Donkey	Polyclonal	Alexa568	Abcam
Mouse IgG	Goat	Polyclonal	FITC	Jacson ImmunoReseach, Baltimore Pike, PA, USA
Streptavidin			Alexa568	Invitrogen
Reagent to stain nuclei				

Hoechst33342			Invitrogen

Table 1: Antibodies and reagents used in this study.

Results

The production of cytokines detected by *in vitro* assay

Because an examination using primary brain cultures failed to detect the production of anti-inflammatory cytokines [6], the *in vitro* inducibility of cytokines by Mu-3 infection was studied using PECs, which produce measurable IL-10 under the ET state [13], in order to examine whether Mu-3 infection is able to trigger the production of anti-inflammatory cytokines. At 24 h post inoculation (hpi), the infection spread to all areas of the culture accompanied by the detachment of many cells from the bottom, showing variable levels of cytokines, usually low-levels, in the culture supernatant (data not shown). Therefore, the levels of cytokine production were examined, comparing with those of mock infection (Mock), during eight to 16 hours after *in vitro* inoculation with Mu-3. The production of proinflammatory cytokines was variable. A slight increase in the level was observed in IL-6 production, and a marked one was detected in that of TNF-α and MCP-1. The IFN-β production was completely suppressed after infection (Figure 1), as previously reported [14]. In contrast, the elevated levels of anti-inflammatory cytokines were detected after infection. In the supernatant of PEC culture, the level of IL-10 increased after *in vitro* inoculation with Mu-3, reaching its peak at 12 hpi, and declined afterwards (Figure 1). The production of TGF-β showed more rapid elevation after infection than that of IL-10. The levels of TGF-β reached their peak at 8 hpi.
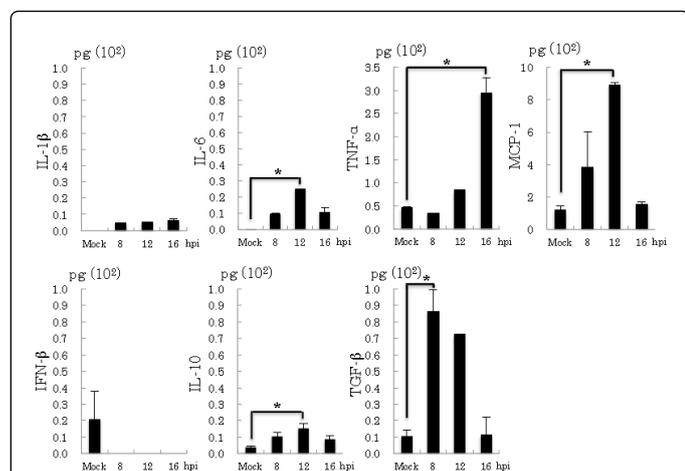

Figure 1: Twenty-four hours after seeding eight-well chamber slides with PECs, the cultured cells were inoculated with 10 PFU of Mu-3 per well, or left uninfected (Mock). Production levels of cytokines in the culture supernatant were measured by the multiplex bead-based immunoassay; pico-gram (pg) per one ml of the supernatant is shown. The results shown are representative of three independent experiments. Vertical lines indicate SD. *indicates the p-value (p) calculated by Student's t-test at <0.05 levels of significance.

Immunofluorescent studies using adherent PECs revealed that, as reported previously [12], PECs expressed CD11b and Gr-1 (Figure 2a). The shape of nuclei was that of classical macrophages, that is small and round or rod-shaped (Figures 2a and 2b). The morphology of PECs did not show marked changes after *in vitro* infection, except for syncytium formation, and this is markedly different from those infected *in vivo* as described below. Although, in the pictures, the numbers of the cells expressing the markers look to have increased after infection, this is due to stained syncytial cells, and the numbers did not show marked differences after infection when marker-expressing cells were counted (data not shown), as we previously reported [12].
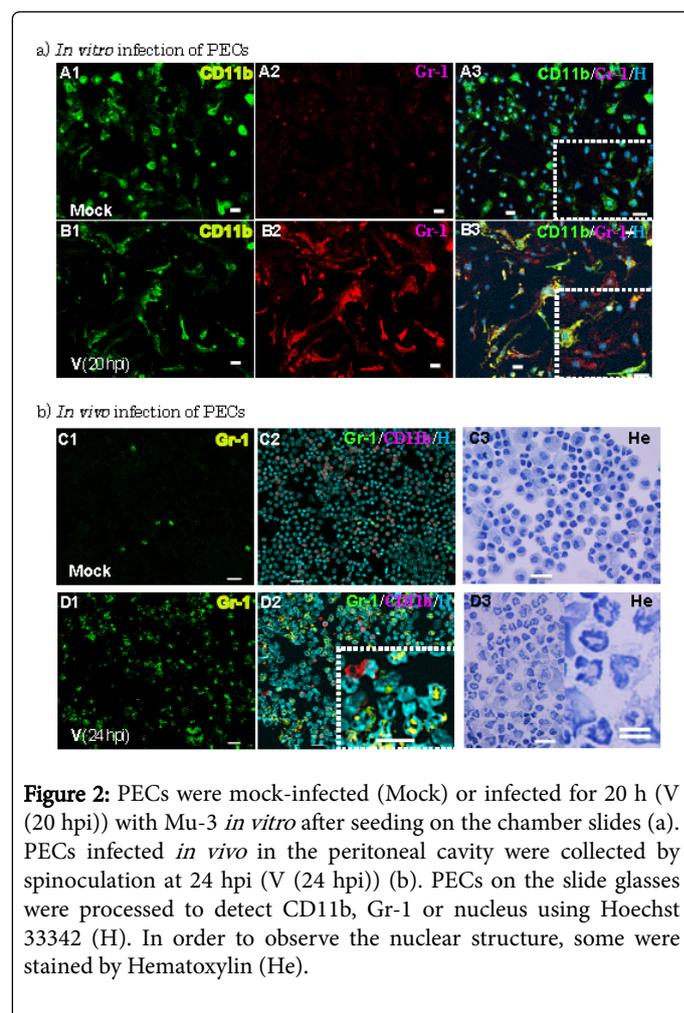

Figure 2: PECs were mock-infected (Mock) or infected for 20 h (V (20 hpi)) with Mu-3 *in vitro* after seeding on the chamber slides (a). PECs infected *in vivo* in the peritoneal cavity were collected by spinoculation at 24 hpi (V (24 hpi)) (b). PECs on the slide glasses were processed to detect CD11b, Gr-1 or nucleus using Hoechst 33342 (H). In order to observe the nuclear structure, some were stained by Hematoxylin (He).

Next, we examined the production of anti-inflammatory cytokines in the cultured adherent PECs. IL-10 was produced by Gr-1- or CD11b-positive cells, especially those near syncytia (Figures 3A and 3B). Many CD11b-positive (CD11b+) cells near the syncytia have been reported to extend their foot processes to connect syncytia [12]. Cells near syncytia and those already involved in the syncytia, but located at the periphery showed the marked expression of IL-10 (Figure 3B). However, in the area with the marked expression of viral antigens,

IL-10 was not colocalized (Figure 3D). In the mock-infected culture, IL-10 expression was not prominent. Colocalization of Le^x and IL-10 was detected at the periphery of syncytia as well as in solitary cells near the syncytia, supporting our previous hypothesis that Le^x expression is involved in the syncytium formation and immunosuppressive state induced by viral infection. The Le^x expression was prominent in mock infected cells (data not shown) as we previously reported [12]. In order to compare the sensitivity to detect cytokines between the multiplex bead-based immunoassay and immunostaining, IFN-β, whose production was totally inhibited after infection by the former (Figure 1), was immunostained, and the latter was proven to be more sensitive than the other (Figures 3A-3F), as we detected IFN-β in the *in vivo* assay, with immunostaining showing IFN-β-producing cells both near and at a distant from infected sites deep in the brain parenchyma during the early phase of infection, but not in the infected cells [15].
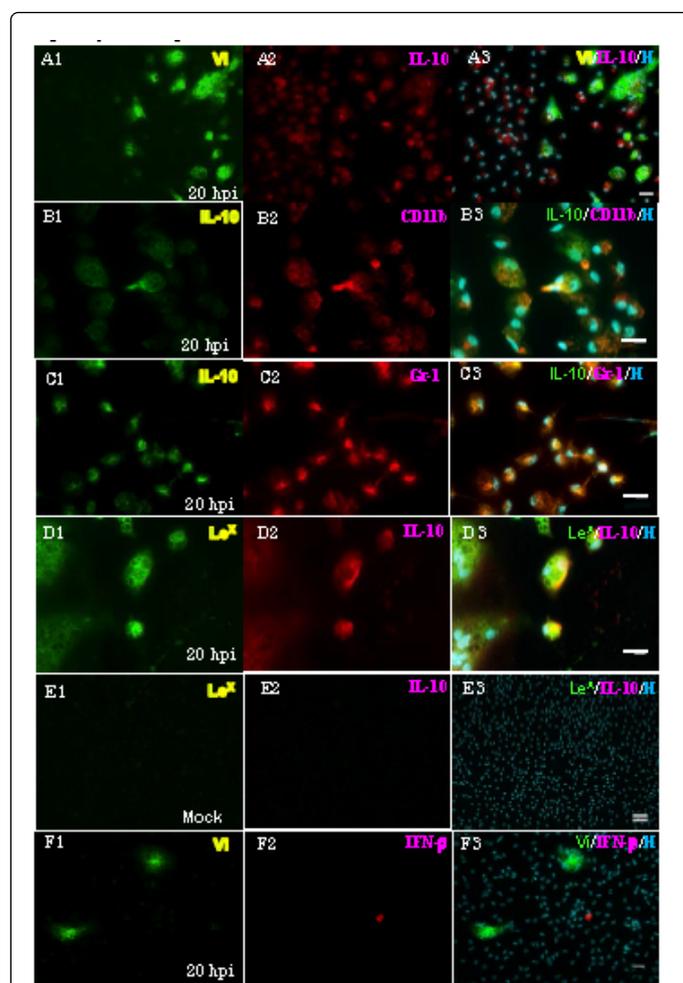

Figure 3: PECs mock-infected (Mock) or inoculated with Mu-3 *in vitro* after seeding on the chamber slides were processed to detect IL-10, JHMV (V), CD11b, Gr-1, and Le^x (LeX) antigens or nucleus using Hoechst 33342 (H) at 20 hpi. Note that neither Le^x nor IL-10 is expressed by mock infection (E). Single and double bars indicate 20 and 10 μm, respectively. White bars indicate 20 μm.

Mo/Mas detected by *ex vitro* assay

In order to assess *in vivo* responses to infection, we performed an *ex vivo* assay. Three days after the injection of Thioglycollate Medium (TM), Mu-3 was injected into the peritoneal cavity. The PECs collected at 24 hpi were seeded in glass-bottomed chamber slides. However, almost no cells adhered to the bottom (data not shown). Therefore, we employed cytospin procedure [2]. The morphology of PECs with *in vivo* infection markedly differed from that of those with *in vitro* infection, with ring-shaped nuclei in more than 80% of the PECs (Figure 2b). The ring-shaped nucleus is a hallmark of differentiated Gr-1$^+$CD11b$^+$ Myeloid-Derived Suppressor Cells (MDSCs) (16). The flow cytometric analysis revealed that only a small population of PECs in suspension culture shifted to the CD11b^{mid}Gra-1^{mid} population after *in vitro* infection (Figure 4a), which corresponds to the observation in the adherent PECs (Figure 2a), and our previous report [12]. However, a large CD11b$^+$Gra-1$^+$ population appeared after *in vivo* infection (Figure 4b). The inflow of suppressor cells, namely differentiated Gr-1$^+$CD11b$^+$ MDSCs, into the peritoneal cavity should have occurred after *in vivo* infection.
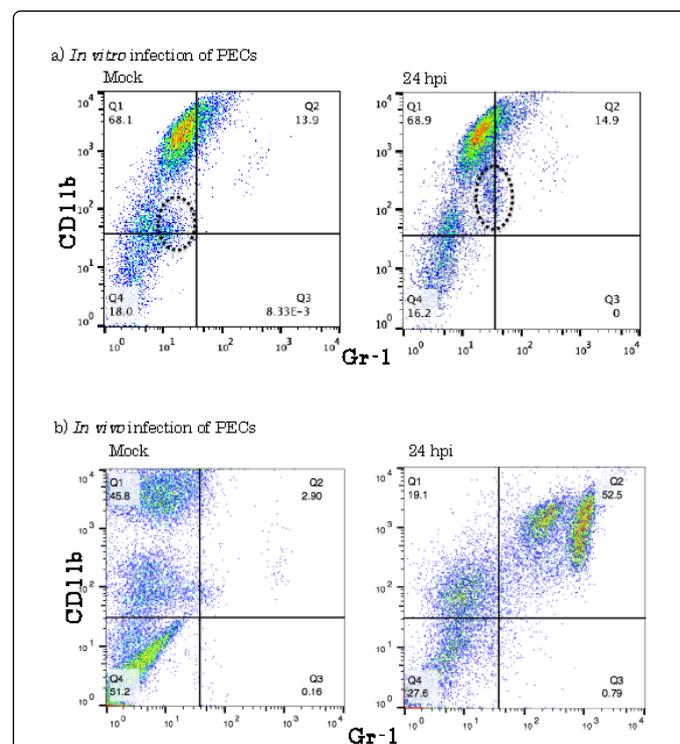

Figure 4. a) PECs after suspension culture for 24 h with *in vitro* infection (24 hpi) or without (Mock), and b) PECs directly collected from the peritoneal cavity with *in vivo* infection (24 hpi) or without (Mock) were studied by flow cytometric analysis to detect populations of CD11b-, Gr-1-positive cells. The populations indicated by dotted circles in a) show the shift of population after infection. b) Three days after the injection of Thioglycollate Medium (TM), Mu-3 was injected into the peritoneal cavity. The PECs were collected at 24 hpi.

Discussion

The studies on the immunological pathogenesis of MHV including neuropathogenic JHMV strains have been mainly focused on a robust innate immune response in the fulminant and acute inflammation induced by virulent viral strains, or T cell-mediated immune responses in chronic inflammation induced by attenuated types of viruses in animal models for studying human demyelinating diseases [16]. Therefore, the interests have been mainly focused on immuno-stimulating proinflammatory cytokines, and we are the first to report that one of the JHMV strains induces the production of anti-inflammatory cytokines after infection. There are two types of inflammatory responses caused by viral infection. One is a marked one [16], and the other is a very mild one, typically observed in the neuropathogenic retroviral infection [17]. The viruses cl-2 [18], its attenuated mutant virus srr7 [19], and Mu-3 [3] derived from srr7 as a mutant clone, cause the latter type of response after infection, showing the neuropathologies with very mild inflammation in the brain parenchyma in spite of marked brain injury. A marked inflammation is observed only in the meninges during the early phase of infection with the viruses. The population of exudate cells in the meninges are rich in Mo/Mas, which are the initial target of infection [3,7,8].

The finding, presented in this report that Mo/Mas produce anti-inflammatory cytokines after infection, only partially explains the neuropathology with mild inflammation in the brain parenchyma, because the production of IL-10 was observed in the pyramidal neurons in the CA2 and CA3 subregions of the hippocampus and not in the CD11b-positive Mo/Mas [6] in the area where brain injury occurs without prominent inflammation [3]. However, we could prove that Mu-3 infection triggered the production of anti-inflammatory cytokines, which explains the low response to LPS stimulation after infection with srr7, mimicking the state of ET [9,10].

ET is a classic form of protective mechanisms whereby cells receiving low doses of endotoxin, enter a transient state of hypo-responsiveness to the next endotoxin challenges [11]. Activated macrophages that play a key role in ET have been recognized as M2 macrophages, an immunosuppressive subset of macrophages, which appear in both the blood stream [20] and lymphoid organs [21], as well as in local inflammatory sites as exuded cells, such as PECs [13]. The term M2-like Gr-1+CD11b+ myeloid suppressor cell is also employed to identify immunosuppressive macrophages that appear in ET [11]. Gr-1+CD11b+ myeloid suppressor cells, or Gr-1+CD11b+ Myeloid-Derived Suppressor Cells (MDSCs), the name of which is often used to identify suppressor cells that appear around tumors, comprise a heterogeneous population [22], and are not a distinctly different population of M2 macrophages, as the term M2-like Gr1+CD11b+ myeloid suppressor cell indicates. For example, M2 macrophages among MDSCs can be differentiated into the M1 type [23]. Both M2 macrophages and MDSCs mediate immunosuppression by secreting IL-10, which is used as one of the markers to identify M2 macrophages [24].

Thus, we also showed that Mu-3 infection induced the appearance of MDSCs or M2 macrophages, which were Gr-1-, CD11b-, and IL-10-positive cells with ring-shaped nucleus. The observation by an immunostaining and a flow cytometry that the population shifted to those immunosuppressive Mo/Mas, which consisted of only a small population among PECs after *in vitro* infection, explains the slight increase in the levels of some proinflammatory cytokines after infection. In contrast, the production of IFN-β measured in the culture supernatant was totally abolished, which has been considered to occur through direct interference in the cascades to produce IFN-β by coronaviral infection [14]. However, at eight hpi, less than 0.1% of the PECs should be infected, because they were inoculated at a Multiplicity of Infection (M.O.I.) of 0.5×10^{-4}. The influence of anti-inflammatory cytokines should be considered to explain the down-regulation of IFN-β production after MHV infection. In contrast to *in vitro* infection, the *in vivo* infection of PECs induced a large population of Gr-1+CD11b+ cells among PECs, which should have occurred after the migration of Gr-1+CD11b+ myeloid cells into the peritoneal cavity [13]. This finding indicates that *in vivo* infection causes overwhelming immunosuppressive state throughout the body accompanied by accelerated anti-inflammatory cytokine production compared with that observed on the *in vitro* infection.

The appearance of IL-10- and Le^x-double positive cells supported our previous study, using gene manipulated mice that do not express Le^x, revealing that Le^x expression is involved in the immunosuppressive state caused by infection with srr7 [9]. Many complex sugar structures including LPS have tolerogenic properties [25]. Furthermore, their binding partners, glycan-binding proteins or lectins play as varied roles in immunological responses. Le^x is a natural ligand of dendritic cell-specific intercellular-adhesion-molecule-grabbing non-integrin (DC-SIGN), a C-type lectin-like receptor. DC-SIGN initiates a Raf-1-indipendent pathway after interaction with fucose, which is located at the end of the Le^x carbohydrate structure, resulting in the induction of anti-inflammatory cytokines [26].

Our series of studies has shown that highly neurovirulent viruses invade and spread in the brain using the host reactions triggered by infection, which includes not only immune responses and induction of Le^x expression, but also the production of unique extracellular matrices, which form an Astrocytic Reticular Network (ARN) after infection [15], and are used as a pathway for the viruses to enter into the brain parenchyma from the meninges, the initial site of infection [10,27].

Acknowledgments

Conflict of Interest

None

References

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