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Cytokine/chemokine profile in J774 macrophage cells persistently infected with DA strain of Theiler's murine encephalomyelitis virus (TMEV)

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Theiler's murine encephalomyelitis virus (TMEV) is a picornavirus and persists in the spinal cords of mice, followed by inflammatory demyelinating disease. Viral persistence is a key determinant for the TMEV-induced demyelination. Macrophages are thought to serve as the site of TMEV persistence during the chronic demyelinating phase. We previously demonstrated that two nonstructural proteins of TMEV, L and L*, were important for virus growth in J774.1 macrophage cells. However, the key factors of macrophage cells related to virus persistence and demyelination remain poorly understood. The inflammatory response is heavily dependent on cytokine and chemokine production by cell of both the immune system and the central nervous system (CNS). In this study, we established the macrophage cells persistently infected with DA strain, and then analyzed the cytokine expression pattern in those cells. The present results are the first to demonstrate the up-regulation of B-lymphocyte chemoattractant (BLC) and granulocyte colony-stimulating factor (G-CSF) in the macrophage cells persistently infected with DA strain. Furthermore, up-regulation of interleukin (IL)-10 and down-regulation of interferon (IFN)-α4, IFN-β, and IFN-γ were shown in those cells. The data suggest that these cytokines/chemokines may contribute to the virus persistence and the acceleration of TMEV-induced demyelination. *Journal of NeuroVirology* (2010) **16,** 219–229.

Keywords: cytokine; demyelination; macrophage; persistent infection; TMEV

Introduction

Theiler's murine encephalomyelitis virus (TMEV) is a single-stranded RNA virus that belongs to the genus *Cardiovirus* of the family *Picornaviridae* and is divided into two subgroups on the basis of their different biological activities (Obuchi and Ohara, 1998; Oleszak *et al*, 2004; Roos, 2002). GDVII subgroup strains cause acute and fatal encephalomyelitis in mice. In the very few surviving mice, no virus persistence or demyelination is observed. In contrast, TO subgroup strains including DA and other viral strains induce an early, nonfatal polioencephalomyelitis of weanling mice, followed by virus persistence and chronic demyelination in the spinal cords (Obuchi and Ohara, 1998; Oleszak *et al*, 2004; Roos, 2002). This late chronic demyelinating disease serves as an experimental model for the human demyelinating disease, multiple sclerosis (MS) (Obuchi and Ohara, 1998; Oleszak *et al*, 2004; Roos, 2002). Viral persistence is essential for bystander demyelination (Drescher *et al*, 1997; Lipton *et al*, 2005; Monteyne *et al*, 1997). Studies suggest that macrophages serve as the site of TMEV persistence during the chronic demyelinating phase (Clatch *et al*, 1990; Lipton *et al*, 1995; Rossi *et al*, 1997).

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L^* protein of TMEV is out-of-frame with the viral polyprotein from an alternative AUG, 13 nucleotides downstream from the authentic polyprotein AUG (Obuchi and Ohara, 1998; Roos, 2002). L^* protein is only synthesized in TO subgroup strains, since the L^* AUG is present in TO subgroup strains, but not GDVII subgroup strains (Michiels *et al*, 1995; Obuchi and Ohara, 1998; Roos, 2002). L^* protein is shown to be essential for virus growth in J774.1 macrophage cells by both "loss of function" and "gain of function" experiments (Himeda *et al*, 2005; Obuchi *et al*, 1999; Takata *et al*, 1998), the major site of virus persistence.

Leader (L) protein of TMEV is a small 76 amino acid long protein located at the most N-terminal protein of the polyprotein. The homology of L between TO and GDVII subgroup strains is only 86% (Law and Brown, 1990; Ohara *et al*, 1998), although those of P1, P2, and P3 are 92%, 96%, and 98%, respectively. Therefore, the difference between the two subgroup Ls may account for TMEV subgroup-specific biological activities. L protein inhibits α/β interferon (IFN) production (van Pesch *et al*, 2001) at the level of IFN gene transcription by interfering with the nucleocytoplasmic shuttling of IFN regulatory factor-3 (Delhaye *et al*, 2004). On the other hand, L protein induces an intrinsic apoptosis in M1-D macrophage cells (Son *et al*, 2008, 2009; Fan *et al*, 2009). From these observations, it was unclear whether the role of L protein is beneficial or harmful for TMEV persistence in macrophage cells.

Recently, we demonstrated that L-deleted DA mutant virus cannot grow in J774.1 macrophage cells (Ichinose-Asakura *et al*, 2010). Therefore, it is thought that these two nonstructural proteins, L and L^*, play a crucial role for persistent infection of TMEV in macrophage cells. However, the key factors of macrophage cells related to persistent infection and demyelination remain poorly understood. Therefore, the establishment of the macrophage cells persistently infected with DA strain of TMEV (PDAJ774) is useful to investigate the key factors of macrophages, which are host cell for TMEV persistence during the chronic demyelinating phase.

In this study, we established PDAJ774 cells. Subsequently, we analyzed the cytokine/chemokine expression pattern in PDAJ774 cells, the J774 cells acutely infected with DA strain (DA/J774), and the J774 cells acutely infected with GDVII strain (GDVII/J774) by using anti-cytokine antibody array.

Results

Establishment of PDAJ774 cells

At 72 h post infection (p.i.), surviving cells were harvested and subcultured in fresh RPMI 1640 medium with 10% fetal bovine serum (FBS). In this culture, the cells that showed a continuous growth were handled as PDAJ774 cells. PDAJ774 cells required about 7 days to propagate from 2×10^6 cells to 1×10^7 cells.

Characterization of PDAJ774 cells

In order to clarify the persistent infection, immunofluorescence microscopic analysis using a monoclonal antibody (DAmAb2) reacting with the capsid protein (VP1) (Nitayaphan *et al*, 1985) was performed in PDAJ774 cells cultured for 28 days (4 passages), 35 days (5 passages), and 70 days (10 passages). Viral antigen VP1 and β-tubulin were visualized using Alexa Fluor 594–conjugated secondary antibody (red) and fluorescein isothiocyanate (FITC)-conjugated secondary antibody (green), respectively (Figure 1). Nuclei were stained with Hoechst 33258 (blue) (Figure 1). Viral antigen was detected in PDAJ774 cells cultured for each period. The percentage of viral antigen–positive cells was calculated by dividing the number of viral antigen–positive cells by the total cell population (Table 1). Viral antigen–positive cells were usually maintained at about 40% throughout the cultivation period of PDAJ774 cells.

Some of PDAJ774 cells were enlarged. However, abnormal morphological cells were not matched with viral antigen–positive cells (data not shown). Furthermore, abnormal morphological cells decreased gradually during the culture. Cell viability was also maintained at 40% to 60% in PDAJ774 cells cultured for 28, 35, and 70 days (Table 1). PDAJ774 cells cultured for 28, 35, and 70 days produced infectious (cell-free) virus 24 h after the medium was changed at 1.2×10^6, 1.6×10^6 and 2.3×10^5 plaque-forming units (PFU)/ml, respectively (Table 1). The titer of cell-free virus from one PDAJ774 is calculated to be 3, 5, and 0.6 PFU at 28-, 35-, and 70-day cultivation, respectively. The titer of cell-free virus was maintained at 3.7×10^5 PFU/ml in PDAJ774 cells cultured for 140 days (20 passages).

Analysis of IFN-α/β transcripts in PDAJ774 cells

L protein produced by TMEV plays an important role in viral persistence (van Pesch *et al*, 2001). This protein was reported to inhibit α/β IFN production early after viral infection (Kong *et al*, 1994, van Pesch *et al*, 2001). Therefore, we first study the expression of IFN-α4 and IFN-β by reverse transcriptase–polymerase chain reaction (RT-PCR) using the mock-infected J774 cells (p.i. 24 h), DA/J774 cells (p.i. 6, 12, and 18 h), GDVII/J774 cells (p.i. 6, 12, and 16 h), and PDAJ774 cells (cultured for 70 [passage 10; P-10] and 140 [P-20] days). As shown in Figure 2A, the level of IFN-α4 transcripts in PDAJ774 cells was significantly restricted compared with that in DA/J774 cells (p.i. 12 and 18 h) and GDVII/J774 (p.i. 12 and 16 h) cells. It was not induced in both DA/J774 and GDVII/J774 cells at 6 h p.i. The level of IFN-β transcripts in PDAJ774 cells was lower than that in DA/J774 cells at 6, 12, and 18 h p.i.

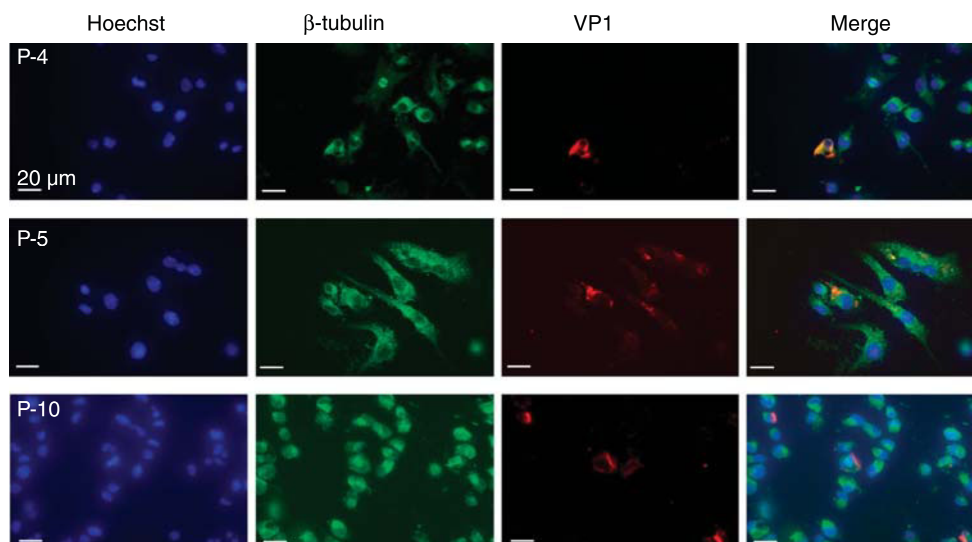

Figure 1 Immunofluorescent visualization of viral antigen in PDAJ774 cells. Viral antigen VP1 (*red*) was detected using anti-VP1 antibody (DAmAb2) and Alexa Fluor 594–conjugated secondary antibody. β-Tubulin (*green*) was detected using anti-β-tubulin antibody and FITC-conjugated secondary antibody. Nuclei (*blue*) were stained with Hoechst 33258. Magnification: ×400, Scale bar: 20 μm.

Table 1 Characteristics of the PDAJ774 cells

Period of cultivation	Cell viability	Total viral titer (PFU/ml)	Cell-free viral titer (PFU/ml)	The ratio of VP1-positive cells[a]
28 days (4 passages)	44%	3.2×10^6	1.2×10^6	41%
35 days (5 passages)	39%	2.5×10^6	1.6×10^6	46%
70 days (10 passages)	59%	4.3×10^5	2.3×10^5	35%

Note. The data were expressed as the mean of the triplicate or duplicate[a] experiments.

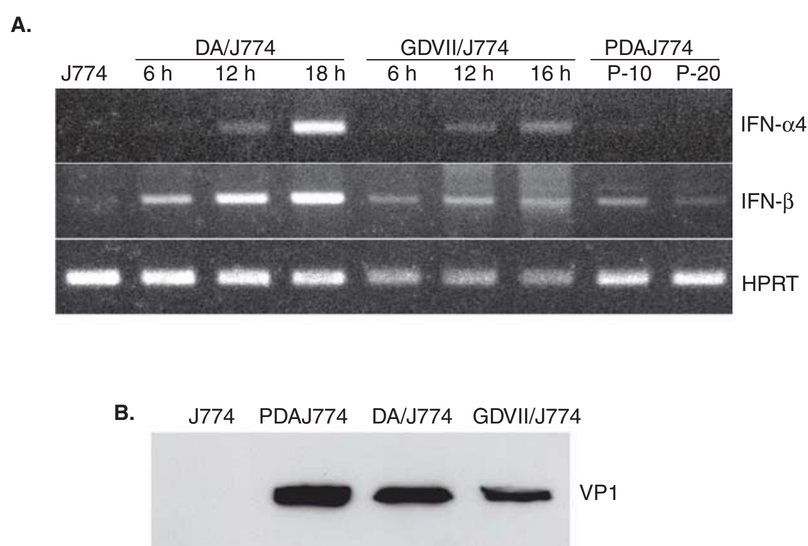

Figure 2 (**A**) RT-PCR analysis of IFN-α4 and IFN-β in mock-infected J774 (p.i. 24 h), DA/J774 (p.i. 6, 12, and 18 h), GDVII/J774 (p.i. 6, 12, and 16 h), and PDAJ774 (cultured for 70 (P-10) and 140 (P-20) days) cells. The sequences of the primers for IFN-α4, IFN-β, and HPRT were synthesized according to the published data (Hato *et al*, 2007). HPRT mRNA was used as a control. (**B**) Expression of viral antigen VP1 in PDAJ774 cells. To confirm the viral persistence, cell lysates of mock-infected J774, DA/J774, GDVII/J774, and PDAJ774 cells were analyzed by Western blotting to detect the viral antigen VP1.

Effect of anti-IFN-β antibody treatment in PDAJ774 cells

In order to clarify the role of IFN-β in PDAJ774 cells, PDAJ774 cells were treated with anti-IFN-β antibody (80 U/ml/48 h). After 8 days incubation, PDAJ774 cells treated with anti-IFN-β antibody died out. With this treatment, infectious viruses in the cultured medium of PDAJ774 cells treated with anti-IFN-β antibody increased more than 2 logs compared with untreated-PDAJ774 cells (Figure 3).

Analysis of cytokine secreted from the PDAJ774 cells

To further investigate the cytokine/chemokine signals related to persistent infection and demyelination, cytokine antibody array analysis was performed using PDAJ774 cells cultured for 70 days (10 passages). Mock-infected J774 cells (p.i. 24 h), DA/J774 cells (p.i. 18 h), and GDVII/J774 cells (p.i. 16 h) were also used as a control. Culture supernatants for cytokine antibody array analysis were prepared as described in Materials and Methods. Cells were used for detection of viral antigen VP1 by Western blotting to confirm the persistent infection. Viral antigen VP1 was detected in PDAJ774 cells, DA/J774 cells, and GDVII/J774 cells, although it was not detected in mock-infected J774 cells (Figure 2B).

Figures 4A and 5 and Table 2 represent experimental data and Table 3 summarizes cytokine/chemokine expression pattern in three types of cells. Eighteen cytokines (GM-CSF, sICAM-1, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-6, IL-10, IL-17, IL-23, IP-10, I-TAC, KC, M-CSF, MIG, RANTES, TARC, and TREM-1) were more than 2-fold up-regulated in DA/J774 cells compared with mock-infected J774 cells. In contrast, 16 cytokines (BLC, G-CSF, GM-CSF, IL-1α, IL-1β, IL-1ra, IL-6, IL-10, IL-17, IL-23, IP-10, KC, M-CSF, RANTES, TARC, and TREM-1) were more than 2-fold up-regulated in PDAJ774 cells compared with mock-infected J774 cells. sICAM, I-TAC, and MIG were not up-regulated in PDAJ774 cells, though they were up-regulated in DA/J774 cells. Interestingly, BLC and G-CSF were up-regulated only in PDAJ774 cells (Figure 4B and Table 3). Additionally, IL-10 was significantly up-regulated in PDAJ774 cells compared with that in DA/J774 cells (Figure 4B). Furthermore, IFN-γ was down-regulated in PDAJ774 cells, though it was up-regulated in DA/J774 cells (Figure 4B and Table 3). On the other hand, three cytokines (IL-1α, IP-10, and RANTES) were more than 2-fold up-regulated in GDVII/J774 cells compared with mock-infected J774 cells (Figures 4A and 5 and Table 2).

The expression of seven cytokines (MCP-1, MCP-5, MIP-1α, MIP-1β, MIP-2, TIMP-1, TNF-α) was neither up-regulated nor down-regulated by TMEV infection in J774.1 macrophage cells (Figures 4A and 5 and Table 2). And 13 cytokines (C5a, I-309, eotaxin, IL-2, IL-3, IL-4, IL-5, IL-7, IL-13, IL-12p70, IL-16, IL-27, SDF-1) were not detected in these cells (Figure 4A and Table 2).

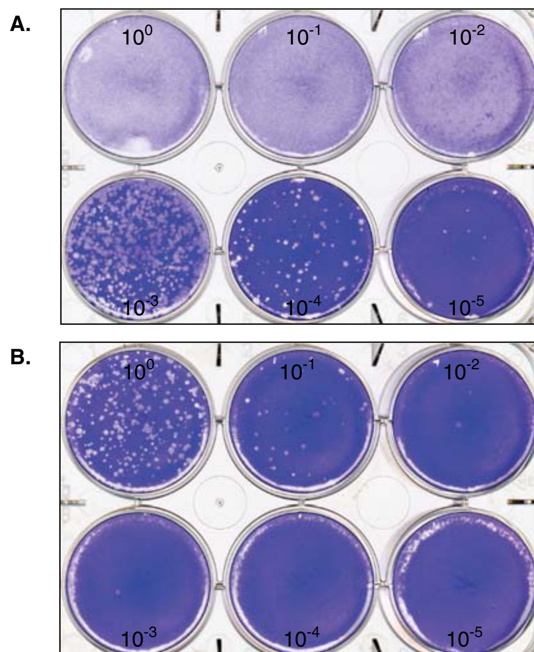

Figure 3 Detection of infectious virus produced from PDAJ774 cells treated with anti-IFN-β antibody by a standard plaque assay. (**A**) Infectious virus was harvested from the cultured medium of PDAJ774 cells treated with anti-IFN-β antibody after 8 days incubation. (**B**) As a control, infectious virus was harvested from the cultured medium of untreated-PDAJ774 cells. Virus solutions were prepared by the 10-fold dilutions.

Discussion

Macrophages were reported to be the site of TMEV persistence during the chronic demyelinating phase (Clatch *et al*, 1990; Lipton *et al*, 1995). The understanding of both viral and host cell factors are required for the elucidation of the mechanisms of TMEV persistence and demyelination. The inflammatory response is heavily dependent on cytokine and chemokine production by cell of both the immune system and the CNS (Chang *et al*, 2000). In this study, we analyzed the cytokine expression pattern in PDAJ774 cells.

Viral antigen was detected in PDAJ774 cells cultured for 28, 35, and 70 days (Figure 1). The percentage of viral antigen–positive cells was usually maintained at about 40% throughout the cultivation period of PDAJ774 cells (Table 1). In addition, PDAJ774 cells produced infectious (cell-free) virus at 0.6~5 PFU/cell 24 h after the medium was changed. Though the percentage of viral antigen–positive cells was a little higher, DA strain persists in macrophage cells as reported by Steurbaut *et al* (2006). In addition, cell viability was also maintained at 40% to 60% in PDAJ774 cells (Table 1). These results suggested

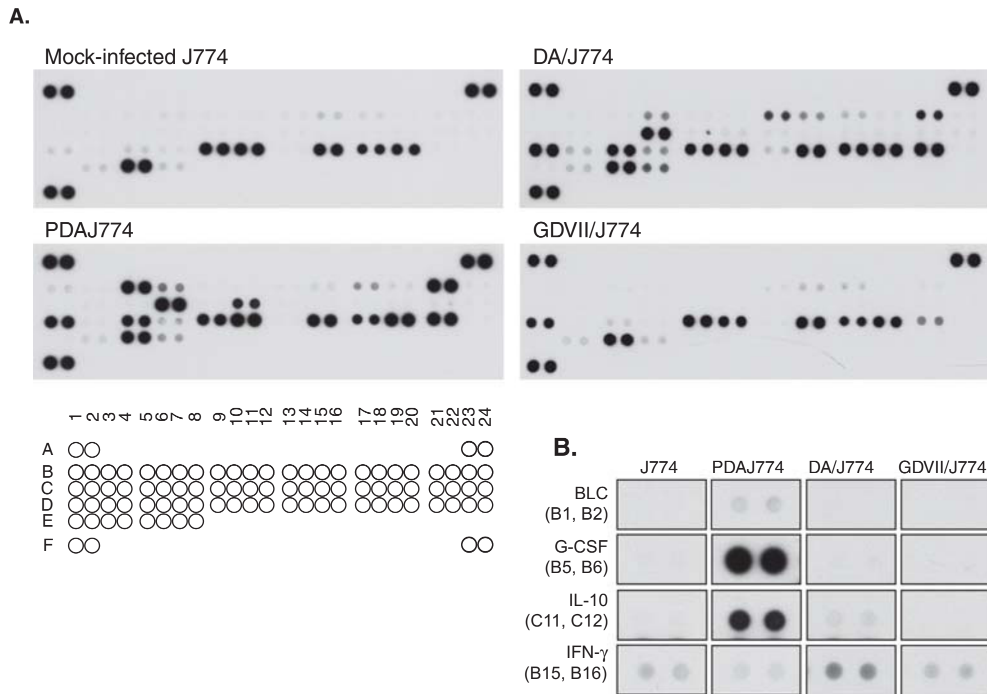

Figure 4 Images of cytokine antibody array analysis. (**A**) Antibody array analysis of cytokines secreted by mock-infected J774, PDAJ774, DA/J774, and GDVII/J774 cells. Antibodies have been spotted in duplicate on nitrocellulose membranes. The illustration of the bottom represented the array location of antibodies. For example, the duplicate spots of A1, A2 and F23, F24 expressed positive control and negative control, respectively. Details of array location were described in Table 2. (**B**) The spots of interest were highlighted. The parenthesis represented the array location.

that the balance of infected cells and non-infected cells is maintained appropriately in this persistent infection. The type of infection of PDAJ774 cells may belong to "chronic focal infection" according to the classification by Boldogh *et al* (1996).

Inhibition of IFN production by TMEV L is thought to be critical for persistence of the DA *in vivo* (Delhaye *et al*, 2004; van Pesch *et al*, 2001). In the present results, the level of IFN-α4 transcripts was significantly restricted in PDAJ774 cells compared with DA/J774 and GDVII/J774 cells (Figure 2A). Furthermore, the level of IFN-β transcripts was restricted partly in PDAJ774 cells compared with DA/J774 cells (Figure 2A). Roos *et al* (1982) reported that treatment

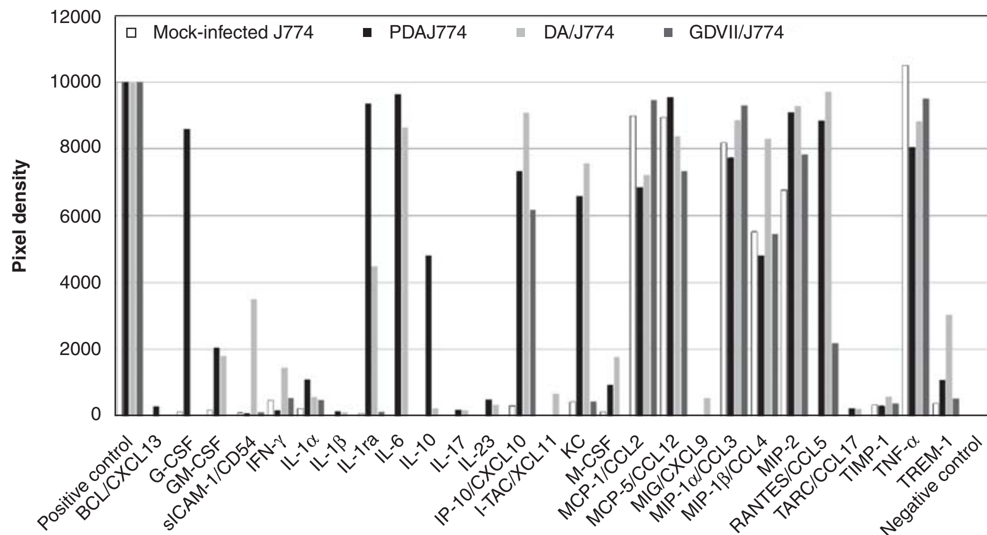

Figure 5 Cytokine expression pattern in TMEV-infected J774 cells. Array data on developed x-ray film were quantitated by scanning the film and analyzing the array image file using image analysis software Image J. The data were expressed as the mean of the duplicate spots. The values were corrected with the value of control (10,000). This graph showed only the data of cytokines that were detected.

Table 2 Profiles of cytokine/chemokine production in TMEV-infected J774 cells

Cytokine/chemokine	Array location	Pixel denisty			
		Mock-infected J774	PDAJ774	DA/J774	GDVII/J774
Positive control	A1, A2, A23 A24, F1, F2	10,000	10,000	10,000	10,000
BLC/CXCL13	B1, B2	n.d.	287	n.d.	n.d.
C5a	B3, B4	n.d.	n.d.	n.d.	n.d.
G-CSF	B5, B6	115	8587	n.d.	n.d.
GM-CSF	B7, B8	171	2045	1792	n.d.
I-309/CCL1	B9, B10	n.d.	n.d.	n.d.	n.d.
Eotaxin/CCL11	B11, B12	n.d.	n.d.	n.d.	n.d.
sICAM-1/CD54	B13, B14	94	77	3500	92
IFN-γ	B15, B16	479	166	1439	540
IL-1α	B17, B18	221	1083	547	471
IL-1β	B19, B20	n.d.	128	98	n.d.
IL-1ra	B21, B22	66	9353	4,480	114
IL-2	B23, B24	n.d.	n.d.	n.d.	n.d.
IL-3	C1, C2	n.d.	n.d.	n.d.	n.d.
IL-4	C3, C4	n.d.	n.d.	n.d.	n.d.
IL-5	C5, C6	n.d.	n.d.	n.d.	n.d.
IL-6	C7, C8	n.d.	9627	8624	n.d.
IL-7	C9, C10	n.d.	n.d.	n.d.	n.d.
IL-10	C11, C12	n.d.	4814	221	n.d.
IL-13	C13, C14	n.d.	n.d.	n.d.	n.d.
IL-12p70	C15, C16	n.d.	n.d.	n.d.	n.d.
IL-16	C17, C18	n.d.	n.d.	n.d.	n.d.
IL-17	C19, C20	n.d.	187	158	n.d.
IL-23	C21, C22	n.d.	485	317	n.d.
IL-27	C23, C24	n.d.	n.d.	n.d.	n.d.
IP-10/CXCL10	D1, D2	311	7315	9074	6170
I-TAC/CXCL11	D3, D4	n.d.	n.d.	652	n.d.
KC	D5, D6	431	6572	7546	437
M-CSF	D7, D8	126	916	1,756	n.d.
MCP-1/CCL2	D9, D10	8975	6838	7,191	9,469
MCP-5/CCL12	D11, D12	8933	9554	8370	7314
MIG/CXCL9	D13, D14	n.d.	n.d.	535	n.d.
MIP-1α/CCL3	D15, D16	8202	7742	8852	9304
MIP-1β/CCL4	D17, D18	5539	4805	8304	5450
MIP-2	D19, D20	6766	9082	9278	7828
RANTES/CCL5	D21, D22	n.d.	8838	9718	2182
SDF-1/CXCL12	D23, D24	n.d.	n.d.	n.d.	n.d.
TARC/CCL17	E1, E2	n.d.	212	193	n.d.
TIMP-1	E3, E4	341	294	574	355
TNF-α	E5, E6	10,508	8059	8815	9502
TREM-1	E7, E8	386	1077	3030	514
Negative control	F23, F24	n.d.	n.d.	n.d.	n.d.

Note. The data are expressed as the mean of the duplicate spots. The values were corrected with the value of control (10,000). n.d.: not detected.

of L929 cells persistently infected with the DA strain with anti-mouse L cell interferon caused a significant increase in infectious virus production. On the other hand, Steurbaut *et al* (2007) reported that IFN-α and IFN-γ contribute to the antiviral response of RAW macrophages against TMEV, but it is not the case of IFN-β. To investigate the role of the low level expression of IFN-β in PDAJ774 cells, PDAJ774 cells were treated with anti-IFN-β antibody. In our present results, infectious virus produced from PDAJ774 cells treated with anti-IFN-β antibody significantly increased (Figure 3), and PDAJ774 cells died out. The reason of this phenomenon is thought to be that the balance of chronic focal infection was collapsed by treatment with anti-IFN-β antibody. It is suggested that the level of IFN-β expressed in PDAJ774 cells is critical for maintaining the persistent infection, by preventing infectious virus from spreading cell to cell.

In order to further study the cytokine/chemokine signals related to TMEV persistence and demyelination, we analyzed the cytokine expression pattern in PDAJ774, DA/J774, and GDVII/J774 cells by using cytokine antibody array (Figure 4A). IL-1α, IP-10, and RANTES were up-regulated similarly in all the three types of cells (Table 3), suggesting it may be due to the inflammatory response induced by TMEV infection. Other investigators have reported that the mRNAs of IP-10 and RANTES increase in primary astrocytes infected with BeAn strain of TMEV (Palma and Kim, 2004) and in spinal cord of PLJ mice infected DA strain of TMEV (Ransohoff *et al*, 2002). Furthermore, Steurbaut *et al* (2008) reported that

Table 3 Summary of cytokine/chemokine expression profile in the three types of infected cells

Up-regulated in GDVII/J774	Up-regulated in DA/J774	Up-regulated in PDAJ774	Down-regulated in PDAJ774
		BLC/CXCL13	
		G-CSF	
	GM-CSF	GM-CSF	
	sICAM-1/CD54		
	IFN-γ		**IFN-γ**
IL-1α	IL-1α	IL-1α	
	IL-1β	IL-1β	
	IL-1ra	IL-1ra	
	IL-6	IL-6	
	IL-10	**IL-10** (*significantly*)	
	IL-17	IL-17	
	IL-23	IL-23	
IP-10/CXCL10	IP-10/CXCL10	IP-10/CXCL10	
	I-TAC/CXCL11		
	KC	KC	
	M-CSF	M-CSF	
	MIG/CXCL9		
RANTES/CCL5	RANTES/CCL5	RANTES/CCL5	
	TARC/CCL17	TARC/CCL17	
	TREM-1	TREM-1	

The summary of cytokine/chemokine expression pattern in three types of cells. Bold letters represented the cytokine/chemokine of interest in PDAJ774 cells.

RANTES was up-regulated in RAW264.7 macrophage cells persistently infected with DA strain. Therefore, it is suggested that these chemokines are important for the initiation of an inflammatory response against the TMEV infection. Fifteen cytokines/chemokines were up-regulated in DA/J774, although those are neither up-regulated nor down-regulated in GDVII/J774 cells (Table 3). Therefore, those cytokines may play important role(s) to regulate TMEV biological activities, especially in the acute phase of the disease, though further studies are required.

Three interesting issues are noted (Figure 4B and Table 3): First, BLC and G-CSF were up-regulated only in PDAJ774 cells. Second, in PDAJ774 cells, IL-10 was more up-regulated than in DA/J774 cells. Third, IFN-γ was down-regulated in PDAJ774 cells, though it was up-regulated in DA/J774 cells.

IL-10 is well known as an anti-inflammatory cytokine. It has been reported to inhibit cytokine expression by Th1 and natural killer (NK) cells (Fiorentino *et al*, 1991), monocyte-dependent T-cell proliferation (Taga and Tosato, 1992), monocyte class II major histocompatibility complex (MHC) expression (de Waal-Malefyt *et al*, 1991), and allo-reactive cytotoxic lymphocyte (CTL) generation *in vivo* (Wang *et al*, 1994). Furthermore, Chang *et al* (2000) reported that significantly higher level of IL-10 transcripts was found in the spinal cord of DA-infected SJL mice with chronic demyelinating disease. Therefore, the up-regulation of IL-10 in PDAJ774 may contribute to the virus persistence through down-regulation of the pro-inflammatory response *in vivo*.

On the other hand, IFN-γ is well known as a type of cytokine that has anti-proliferating and immune-modulatory effects. Recently, Yonekura *et al* (2003) and Oba *et al* (2008) reported that IFN-γ down-regulates heat shock protein (Hsp27) expression and suppresses the inhibition of apoptosis by Hsp27. In the present results, IFN-γ was down-regulated in PDAJ774 cells, though it was up-regulated in DA/J774 cells. This fluctuation was similar with that of IFN-α4 and IFN-β. Therefore, it is thought that the down-regulation of IFN-γ in PDAJ774 cells may contribute to the viral persistence through the suppression of host cell apoptosis and antiviral response. Furthermore, Fiette *et al* (1995) reported that IFN-γ gene is involved in the resistance/susceptibity of inbred strains of mice to persistent infection. Additionally, Rodriguez *et al* (2003) reported that IFN-γ plays a critical role in protecting spinal cord neurons from persistent infection and death. From these observations, the following scenario is addressed: DA-infected macrophages express IFN-γ to protect spinal cord neurons from persistent infection and death in acute phase *in vivo*. Thereafter, DA-infected macrophages die by virus (DA L)-induced apoptosis, overexpressing IFN-γ-induced apoptosis and CTL response, etc. However, a part of DA-infected macrophages survive because cell death as described above is suppressed by antiapoptotic effect of DA L*, down-regulation of IFN-γ, and up-regulation of IL-10, etc.

Krumbholz *et al* (2005) reported that B-lymphocyte chemoattractant (BLC/CXCL13) was produced in actively demyelinating MS lesions, and the intracerebral BLC production strongly correlated with intrathecal immunoglobulin (Ig) production, as well as the number of B cells, plasma blasts, and T cells in cerebrospinal fluid. Additionally, Vegaeva *et al* (2006) reported that BLC was up-regulated in the CNS during experimental autoimmune encephalomyelitis (EAE) following the adoptive transfer of myelin-specific $CD4^+$ T cells, as well as following

active immunization with myelin peptides. Furthermore, they demonstrated that anti-BLC treatment significantly ameliorated clinical disease of adoptively transferred EAE. From these reports, it is thought that the up-regulation of BLC in PDAJ774 cells may contribute to the acceleration of demyelination. Granulocyte colony-stimulating factor (G-CSF) was identified initially as a growth factor for neutrophils (Demetri and Griffin, 1991). It is, however, recently reported that the induction of hematopoietic stem cell mobilization in MS patients for transplantation has resulted in significant worsening of neurologic deficiencies in numerous patients following G-CSF administration (Blanco *et al*, 2005; Openshaw *et al*, 2000). Snir *et al* (2006) reported that G-CSF enhances MS autoreactive T-cell adhesion to the extracellular matrix (ECM) components collagen IV and fibronectin as effectively as the pro-inflammatory IFN-γ and tumor necrosis factor (TNF)-α, known to exacerbate MS symptoms. Therefore, the up-regulation of G-CSF in PDAJ774 cells may also enhance myelin reactive T-cell adhesion to the ECM components and accelerate demyelination. Further studies were required to support these possibilities, since the cytokine/chemokine profile was examined at one point in time and this is a potential limitation of this study.

In conclusion, the pattern of cytokine expression was significantly different in PDAJ774, DA/J774, and GDVII/J774 cells. The present study first demonstrates the up-regulation of BLC and G-CSF in macrophage cells persistently infected with DA. Furthermore, the up-regulation of IL-10 and the down-regulation of IFN-α4, INF-β, and IFN-γ were shown in PDAJ774 cells. These data suggest that the up-regulation of IL-10 and the down-regulation of IFN-α4, INF-β, and IFN-γ may contribute to the TMEV persistence, and the up-regulation of BLC and G-CSF may contribute to the acceleration of TMEV-induced demyelination. Therefore, the inhibition of BLC and/or G-CSF may be a promising novel therapeutic approach for not only TMEV-induced demyelinating disease, but also MS. Further studies using anti-BLC and/or anti-G-CSF antibodies are required to confirm this promising possibility.

Materials and methods

Cell culture

J774.1 cells, an $H\text{-}2^d$ macrophage-like cell line derived from a tumor of a female BALB/c mouse (Ralph *et al*, 1975), were obtained from the Cancer Cell Repository, Tohoku University, Sendai, Japan. The cells were maintained in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% FBS (Invitrogen, Carlsbad, CA) containing 50 U/ml of penicillin and 50 μg/ml of streptomycin. PDAJ774 cells were maintained in the complete medium described above. BHK-21 cells, a baby hamster kidney-derived fibroblast cell line permissive for TMEV infection, were maintained in Eagle's minimum essential medium (Nissui, Tokyo, Japan) supplemented with 0.03% L-glutamine and 5% newborn calf serum (Invitrogen, Carlsbad, CA) containing 60 mg/ml of kanamycin.

Virus infection

The seed viruses of DA and GDVII strains of TMEV were propagated in BHK-21 cells. In all experiments, after virus adsorption at a multiplicity of infection (MOI) of 10 PFU per cell for 60 min at 37°C, cells were washed twice with phosphate-buffered saline (PBS) and incubated in RPMI 1640 medium containing 10% FBS at 37°C for the times indicated.

Establishment of PDAJ774 cells

The J774.1 cells were seeded at a density of 1.0×10^6 cells in a 35-mm dish. After 24 h, the cells were infected with DA virus at an MOI of 10 PFU per cell. After virus adsorption at 37°C for 60 min, the cells were washed twice with PBS, and incubated at 37°C in RPMI 1640 medium with 1% FBS. After 72 h, surviving cells were washed twice by PBS and continued the culture in fresh RPMI 1640 medium with 10% FBS. Culture medium of PDAJ774 cells was changed every 2 days.

Determination of cell viability

The cell survival was analyzed by trypan blue dye exclusion test. PDAJ774 cells were seeded at a density of 4×10^5 cells in a 35-mm dish. After 24 h, cells were washed twice with PBS, and incubated at 37°C in fresh RPMI 1640 medium with 10% FBS. After 24-h incubation, cells were harvested and resuspended in the medium. Then the cell suspension was mixed with the equal volume of 0.5% trypan blue dye solution for 1 min, followed by the counting of cells in a hemocytometer. The percentage of viability was calculated from the number of cells that did not exclude the dye.

Immunofluorescence staining

Cells were seeded onto cover glasses coated by poly-L-lysine in 12-well plate at a density of 1.0×10^5 cells and incubated. After 48 h, cells were washed with PBS and then fixed in 10% formalin for 20 min at 4°C. After three washes with PBS, cells were permeabilized with 0.25% triton X-100 in PBS for 20 min at room temperature and blocked with 5% bovine serum albumin in PBS for 60 min at room temperature. Cells were incubated with mouse anti-VP1 antibody (DAmAb2) detectable to VP1 protein (Nitayaphan *et al*, 1985) and rabbit anti-β-tubulin antibody (Thermo Scientific, Fremont, CA) at 4°C overnight. After five washes with PBS, cells were incubated with Alexa Fluor 594-conjugated anti-mouse IgG (Molecular Probes, Invitrogen, Carlsbad, CA) and FITC-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 60 min at room temperature. Cell nuclei were stained with

Hoechst 33258 (Molecular Probes, Invitrogen). Photomicrographs were obtained at room temperature with a microscope equipped with a digital camera (Axiovision, Carl Zeiss).

Quantitation of viruses produced from PDAJ774 cells
PDAJ774 cells were seeded at a density of 4×10^5 cells in a 35-mm dish. After 24 h, cells were washed twice with PBS, and incubated at 37°C in fresh RPMI 1640 medium with 10% FBS. Cell-free viruses were harvested by collecting the supernatants after 24-h incubation. Cells were used for determination of cell viability. Cell-free and cell-associated viruses were harvested by three cycles of freeze-thawing from cells/supernatants mixture after 24-h incubation. The amount of viruses produced from PDAJ774 cells was determined by standard plaque assay on BHK-21 cells.

RT-PCR
Total RNA was extracted from mock-infected J774 (p.i. 24 h), DA/J774 (p.i. 6, 12, and 18 h), GDVII/J774 (p.i. 6, 12, and 16 h), and PDAJ774 (cultured for 70 and 140 days) cells using RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Total RNA (300 ng) was reverse transcribed using oligo dT (20) primers and ReverTraAce reverse transcriptase (TOYOBO). First-strand cDNA (2 μl) was amplified by PCR (25 cycles) using KOD plus DNA polymerase (TOYOBO). The sequences of the primers for IFN-α4, IFN-β, and hypoxanthine guanine phosphoribosyltransferase (HPRT) were synthesized according to the published data (Hato *et al*, 2007).

Western blotting
The proteins were extracted from mock-infected J774 (p.i. 24 h), DA/J774 (p.i. 18 h), GDVII/J774 (p.i. 16 h), and PDAJ774 (cultured for 70 days) cells with the lysis buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0], 2.5 % sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 10% glycerol, and 0.005% bromophenol blue [BPB]), separated by SDS–10% polyacrylamide gel electrophoresis, and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was blocked with 5% skim milk in PBS-T (PBS containing 0.05 % Tween 20) for 60 min and incubated at 4°C for overnight with DAmAb2, followed by incubation with horseradish peroxidase–conjugated anti-mouse IgG for 1 h. Signals were detected using enhanced chemiluminescence (ECL) plus Western blotting detection reagents (GE Healthcare Bio-Science) according to the manufacturer's instructions.

Treatment of anti-IFN-β antibody of PDAJ774 cells
PDAJ774 cells were seeded at a density of 50% confluent in T25 flask. Cells were incubated in RPMI 1640 medium supplemented with 10% FBS containing rabbit anti-mouse IFN-β antibody (80 U/ml; PBL Biomedical Laboratories). Media were changed every 2 days. Cell-free viruses were harvested after 8 days incubation. The infectious viruses produced from PDAJ774 cells treated with anti-IFN-β antibody were detected by a standard plaque assay on BHK-21 cells.

Cytokine antibody array analysis
PDAJ774 cells were seeded at a density of 5×10^5 cells in a 35-mm dish. After 24 h, cells were washed twice with PBS, and incubated at 37°C in 1 ml of fresh RPMI 1640 medium with 10% FBS. After 24 h, conditioned media were harvested by collecting the supernatants. The J774.1 cells were seeded at a density of 5×10^5 cells in a 35-mm dish. After 24 h, the cells were infected with mock, DA, or GDVII virus at an MOI of 10 PFU per cell. After virus adsorption at 37°C for 60 min, the cells were washed twice with PBS, and incubated at 37°C in RPMI 1640 medium with 10% FBS.

Culture supernatant of DA/J774 cells was harvested at 18 h post infection when cytopathic effect (CPE) appeared at about 50% cells, and culture supernatant of GDVII/J774 cells was harvested at 16 h post infection when CPE appeared at about 80% cells. Culture supernatant of mock-infected cells was harvested at 24 h. These culture supernatants were used for Proteome Profiler Mouse Cytokine Array Kit, Panel A (R&D Systems, Minneapolis, MN) according to the manufacurer's instructions. Briefly, each culture supernatant was incubated with detection antibody cocktail for 60 min at room temperature. Array membranes blocked with supplied blocking buffer were incubated with sample/antibody mix at 4°C overnight, followed by incubation with horseradish peroxidase–conjugated streptavidin for 30 min. Signals were detected using ECL plus (GE Healthcare Bio-Science). Array data on developed x-ray film were quantitated by scanning the film and analyzing the array image file using image analysis software Image J (National Institute of Health).

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