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Role of the Programmed Death-1 (PD-1) pathway in regulation of Theiler's murine encephalomyelitis virus-induced demyelinating disease

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ABSTRACT

Programmed death-1 (PD-1) belongs to the CD28 family of co-stimulatory and co-inhibitory molecules and regulates adaptive immunity. This molecule induces the development of regulatory T cells, T cell tolerance, or apoptosis. We examined the role of PD-1 pathway in Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease (TMEV-IDD) mice. Up-regulation of PD-1 and PD-1 ligand-1 (PD-L1) mRNA expression in bone marrow-derived dendritic cells were induced by TMEV infection in vitro. Furthermore, PD-1 and PD-L1 mRNA expression was increased in the spinal cords of the TMEV-infected mice in vivo. Treatment with a blocking monoclonal antibody (mAb) against PD-1, especially during the effector phase, resulted in significant deterioration of the TMEV-IDD both clinically and histologically. Flow cytometric analysis revealed a dramatically increase of $CD4^+$ T cells producing Th1 cytokines such as IFN-γ and TNF-α in the spinal cord of anti-PD-1 mAb-treated mice.
These results indicate that the PD-1 pathway plays a pivotal regulatory role in the development of TMEV-IDD.

1. Introduction

Multiple sclerosis (MS) is an immune-mediated chronic inflammatory demyelinating disease of the central nervous system (CNS) in humans (Wekerle, 1991). Although the etiology of MS is not clarified yet, epidemiological studies and investigations with experimental animal models have supported a potential role for viruses as the environmental trigger in disease induction (Allen and Brankin, 1993). Thus, a similar demyelinating disease induced by a virus could be one of the most attractive animal models in the study of the pathogenesis of MS. Theiler's murine encephalomyelitis viruses (TMEV), members of the genus Cardiovirus in the family of Picornaviridae, are natural enteric pathogens that cause CNS disease in mice (Pevear et al., 1987). Intracerebral (i.c.) injection of TMEV into susceptible strains of mice results in a chronic, progressive demyelinating disease characterized histologically by perivascular inflammatory cell infiltrates and primary demyelination of the CNS (Lipton and Dal Canto, 1976). The clinical signs of TMEV-induced demyelinating disease (TMEV-IDD) include a spastic waddling gait, extensor spasm, and incontinence (Lehrich et al., 1976). TMEV-IDD is considered an infectious mouse model for MS because the disease displays histopathologic, genetic, and clinical similarities to human MS (Dal Canto and Lipton, 1975; Nathanson and Miller, 1978; Kurtzke and Hyllested, 1986; Kappel et al., 1991). The demyelination is linked to persistent TMEV infection in the CNS (Lipton et al., 1991), and histological findings are characterized by perivascular inflammatory cell infiltrates and primary demyelination in CNS (Lehrich, Arnason, 1976; Lipton et al., 1984). TMEV persists in the white matter of the spinal cord, mainly in microglia/macrophages, but also in astrocytes and oligodendrocytes throughout the life of a mouse (Lipton et al., 1984; Lipton et al., 1995). Persistent CNS virus infection in susceptible mouse strains triggers clonal expansion and differentiation of TMEV-specific T cells that are poorly controlled by normal immunoregulatory mechanism. Activated Th1 cells release proinflammatory cytokines in the CNS, resulting in the subsequent recruitment and activation of mononuclear phagocytes that initiate myelin destruction via both direct and bystander mechanisms (Miller et al., 1997a; Kim et al., 1998). Furthermore, approximately 4 weeks after onset of clinical disease, i.e., 8 weeks post infection, T-cell responses to myelin epitopes arise in an ordered temporal progression (Miller et al., 1997b) consistent with a role for both virus- and myelin-specific responses in the chronic phase of the disease.

The immune system has the difficult challenge of discerning and defending against a diversity of microbial pathogens, while simultaneously avoiding self-reactivity. While central tolerance mechanisms result in deletion of the majority of self-reactive T lymphocytes, some T cells specific for self-antigens escape into the periphery (van Noort et al., 1993; Lohmann et al., 1996). To further control the development of autoimmunity, multiple mechanisms of peripheral tolerance have

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evolved, including T-cell anergy, deletion, and suppression by regulatory T cells (Tregs). Failure of any of these tolerance mechanisms can result in autoimmune disease. T-cell co-stimulatory pathways play critical roles in regulating the delicate balance between protective immunity and tolerance. The critical immunoregulatory function of T-cell co-stimulation has led to significant advances in the identification and characterization of T-cell co-stimulatory pathways (Sharpe, 2009). Now, it is well known that co-stimulatory pathways can provide positive second signals that promote T-cell activation as well as negative co-inhibitory signals that suppress T-cell responses, mediate T-cell tolerance, and prevent autoimmunity. In addition, co-stimulatory and co-inhibitory pathways not only regulate responses of naive T cells but can also control effector, memory, and Tregs. Thus, the functions of these pathways in regulating T-cell activation and tolerance have expanded. Co-inhibitory pathways can control effector T-cell responses and the development and function of Treg cells as well as the fate of naive T cells upon antigen encounter. The pathway consisting of programmed death-1 (PD-1) receptor (CD279) and its ligands, PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273), delivers inhibitory signals that regulate the balance among T-cell activation, tolerance, and immune-mediated tissue damage (Okazaki and Honjo, 2007; Sharpe et al., 2007; Nurieva et al., 2009). This pathway exerts critical inhibitory functions in the setting of persistent antigenic stimulation such as during encounter of self-antigens, chronic viral infections, and tumors (Sharpe, Wherry, 2007; Keir et al., 2008).

Because the PD-1 pathway plays a crucial role in regulating autoimmunity by negatively controlling T cell activation as well as cytolytic $CD8^+$ T cell function, we have investigated the role of the PD-1 pathway in TMEV-IDD. Here, we describe for the first time that blockade of PD-1 pathway by a blocking monoclonal antibody (mAb) against PD-1 in the effector phase markedly exacerbated the disease progression of TMEV-IDD.

2. Materials and Methods

2.1. Mice

Female SJL/J mice, 6 weeks old, were purchased from the Charles River Laboratories Japan, Inc. (Ibaraki, Japan), housed and cared for in an approved facility, in accordance with the Shinshu University Guide for Laboratory. The animals were kept in aluminum cages containing pine chips, and given food and water ad libitum.

2.2. Virus

The BeAn strain of TMEV was expanded in Baby Hamster Kidney (BHK) cell monolayers in DMEM supplemented with 7% donor calf serum. Cell lysates with known Plaque Forming Unit (PFU) were used as viral stock for animal experiments. Partially purified virus was prepared following centrifugation through 30% sucrose as previously described and used for in vitro assays (Yauch et al., 1998). Viral titer was determined by the standard plaque assay on BHK cells.

2.3. Infection of mice with virus

Demyelinating disease was induced by injection of 1×10^6 PFU TMEV in 30 μl of PBS into the left cerebral hemisphere of female SJL/J mice after anesthetization. Intracerebral (i.c.) injection with 1×10^6 PFU consistently induces chronic gait abnormalities and neurologic signs in greater than 90% of SJL/J mice. Clinical symptoms of the disease were assessed alternate-day on the following grading scale: grade 0 = no clinical signs; grade 1 = mild waddling gait; grade 2 = moderate waddling gait and hindlimb paresis; grade 3 = severe hindlimb paralysis; grade 4 = severe hindlimb paralysis and loss of righting reflex; grade 5 = moribund.

2.4. Generation of bone marrow-derived dendritic cells (BMDCs)

Bone marrow (BM) cells were harvested from uninfected SJL/J mice, passed through nylon mesh and depleted of erythrocytes with RBC lysis buffer. 1×10^7 BM cells were seeded in 10-cm dish in 10 ml of RPMI 1640–based complete medium supplemented with 20 ng/ml GM-CSF (Wako, Oska, Japan). The cultures were usually fed every 2 days, aspirating 75% of the medium, and adding back fresh medium with GM-CSF. After 8 days, loosely adherent cells were collected and incubated with TMEV (MOI: multiplicity of infection = 10) for 24 h.

2.5. Treatment with anti-PD-1 mAb

Six weeks old female SJL/J mice were separated into groups (A-C; Table 1). TMEV was injected into SJL/J mice i.c. at day 0. Control rat IgG (Sigma Aldrich) anti-PD-1 mAb (RMP1-14) (Yamazaki et al., 2005) was injected intraperitoneally (i.p.) into mice on −3, 0, 3, 6, 9, 12 days after i.c. infection with TMEV at the induction phase or 17, 20, 23, 26, 29, 32 days after i.c. infection with TMEV at the effector phase with a dose of 250 μg in a volume of 100 μl/mouse per injection. Details of the experimental design are given in Table 1.

2.6. Isolation of infiltrating cells from spinal cord

Single-cell suspensions were prepared according to standard methods. Briefly, Percoll-NaCl solution was prepared by addition of 9 part (v/v) of Percoll (GE Healthcare) to 1 part (v/v) of 1.5 M NaCl. The spinal cord cell suspensions were centrifuged at 3000 g for 5 min and resuspended in 5 ml of 37% Percoll-NaCl solution diluted with culture medium and overlaid by 2.5 ml of 70% Percoll-NaCl solution diluted with culture medium. The gradient was centrifuged at 2000 g for 20 min and the mononuclear cells were harvested from the 37%–70% interface, washed, and counted.

2.7. Flow cytometry

For intracellular staining, cells were isolated from spleen and spinal cord. Cells were stimulated in culture medium with PMA (50 ng/ml; Wako pure chemical), Calcinomycin A23187 (1 μg/ml: Sigma), and Brefeldin (10 μg/ml; Sigma) for 6 h at 37 °C, in a humidified 5% CO_2 atmosphere. Cells were washed and triple-labeled with PerCP-conjugated anti-mouse CD4 mAb, PE-conjugated anti-mouse IL-4, IL-10 or IL-17 mAb, and FITC-conjugated anti-mouse IFN-γ or TNF-α mAb for 30 min at room temperature in dark (all antibodies were obtained from BD Biosciences, Japan). After two steps of wash, cells were analyzed on FACSCalibur and FACSCan flow cytometers (BD Biosciences).

2.8. Histology

In each experiment, mice were blindly selected from each group beforehand for histological examination and sacrificed on day 34 post i.c. infection. Spinal cords were removed and fixed in 4% paraformaldehyde in PBS, pH 7.4. Spinal cord sections from paraffin-embedded tissues were prepared at 3-μm thickness. Sections were stained with hematoxylin-eosin or Kluver-Barrera's staining.

2.9. Gene expression measurements

Total RNA was extracted from spleen and spinal cord using TRIzol (Invitrogen) following the manufacturer's instructions. Prior to the reverse transcription, potentially contaminating residual genomic DNA was eliminated with DNase I (Roche). RNA was reverse transcribed using SuperScript™ III. Messenger RNA (mRNA) was quantified by real-time RT-PCR using a TaqMan Fast Universal Master Mix (Applied Biosystems) in an Applied Biosystems StepOnePlus™ real-time PCR System (Applied Biosystems) with the following probe, sense primer,

Table 1
Summary of effects of anti-PD-1 mAb administration on TMEV-IDD.

Group	Treatment	Number of mice	Date of Treatment	MDO[a] + SD (day)	MMS[b] ± SD	MCS[c] ± SD
A	Control rat IgG	15	Day 17,20,23,26,29,32 (effector phase)	23.7 ± 5.0	2.4 ± 1.4	10.8 ± 8.4
B	anti-PD-1 mAb	15	Day −3,0,3,6,9,12 (induction phase)	22.8 ± 2.2	2.2 ± 1.2	10.1 ± 6.6
C	anti-PD-1 mAb	18	Day 17,20,23,26,29,32 (effector phase)	20.9 ± 3.1*	3.3 ± 1.4*	19.2 ± 9.2*

'Significant difference between Group A and B ($p < 0.05$) MDO: Mean Day of Onset.
MMS: Mean maximum clinical score.
MCS: Mean cumulative clinical score.

and anti-sense primer: TMEV (5′-CGCGCGCCCAAAAGCAAGC-TAMRA-3′, and 5′-TTGAGCTCTCTGAGGGTGAACA-3′ and 5′-CTAGAACCTTCCCG CCTCCTT-3′). The primers for PD-1 (Mm00435532_m1), PD-L1 (Mm00452054_m1) and β-actin (Mm01205647_g1) were obtained from Applied Biosystems. Amplification was conducted in a total volume of 10 μl for 50 cycles of 1 sec at 95 °C and 20 sec at 60 °C. mRNA levels were determined after normalization of mRNA concentration against β-actin.

2.10. Statistical analysis

Statistical comparison among groups was performed with Mann–Whitney U-test using JMP software (SAS institute Inc, Tokyo, Japan). Data was considered statistically significant when the p-value was <0.05.

3. Results

3.1. TMEV induces PD-1 and PD-L1 in BMDCs

The identification of altered expression of PD-1 and PD-L1 during TMEV infection provides initial data for justifying their role during disease. To determine the levels of PD-1 and PD-L1 expression upon TMEV infection, freshly grown and isolated BMDCs were infected with TMEV (MOI = 10) and assessed for PD-1 and PD-L1 mRNA expression by real-time RT-PCR analyses (Fig. 1). The data indicated that, compared with uninfected DCs, TMEV infection significantly induced the up-regulation of both PD-1 and PD-L1 (*$p < 0.05$).

3.2. Expression of PD-1 and PD-L1 in spinal cord

We further analyzed the expression of PD-1 and PD-L1 in the spinal cord at different time points after infection with TMEV by real-time RT-PCR. The expression of PD-1 and PD-L1 significantly increased at day 10, 20, 30, and 40 compared to day 0 post infection (Fig. 2). This suggested that intracerebral infection with TMEV induced persistent up-regulation of the PD-1 pathway signaling.

3.3. Treatment with anti-PD-1 mAb exacerbates the development of TMEV-IDD

The experimental results of anti-PD-1 mAb treatment are summarized in Table 1 and Fig. 3. Control IgG-treated animals showed the typical disease course of TMEV-IDD. Mean maximum clinical score (MMS) of the control group A was 2.4. MMS of the mice (group B) treated with anti-PD-1-mAb at the induction phase was 2.2. In contrast, MMS of the mice (group C) treated with anti-PD-1 mAb at the effector phase was significantly increased to 3.3 (*$p < 0.05$). Similarly, mean cumulative clinical score (MCS) of the control group was 10.8. MCS of the mice in group B was 10.1. In contrast, clinical symptoms were markedly elevated in animals treated with anti-PD-1 mAb at the effector phase (group C) (*$p < 0.05$) of which MCS was 19.2. Therefore, the PD-1-mediated signals appear to play an important role in preventing the development of TMEV-IDD particularly during the effector phase. Hereafter, we performed comparative study between the mice treated with anti-PD-1-mAb and control IgG.

3.4. Histologic findings

At day 34, the disease severity reached nearly maximum (grade 4 or 5) in some mice. Therefore, representative mice were blindly selected from each group at day 34 for histological examination. Mild perivascular mononuclear cell (MNC) infiltration and demyelination were observed in the white matter of spinal cord from the control IgG-treated mice and the mice treated with anti-PD-1 mAb at induction phase (Fig. 4A, B, D, E) which are the characteristics of TMEV-IDD. In contrast, severe inflammatory MNCs and demyelination were observed in the white matter of spinal cord from the mice treated with anti-PD-1 mAb at effector phase (Fig. 4C, F).

3.5. Treatment with anti-PD-1 mAb has no significant effect on viral persistence

To determine the effect of anti-PD-1 mAb on TMEV replication in spinal cords, viral RNA levels in spinal cords of mice at 34 days post-

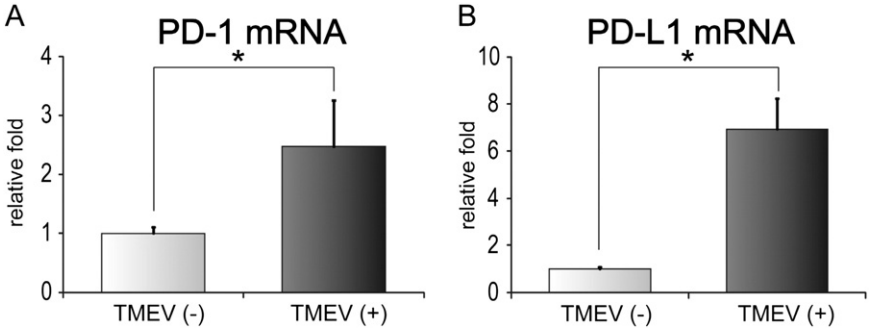


Fig. 1. TMEV induces PD-1 and PD-L1 in BMDCs. BM-derived DCs from SJL/J mice were infected of TMEV for 24 h. Then, total RNA was harvested and expression of PD-1 and PD-L1 mRNA was measured by real-time PCR. Relative fold in PD-1 and PD-L1 expression was determined by comparison with the uninfected culture. Expression of PD-1 and PD-L1 mRNA in BMDCs infected with TMEV was significantly increased compared with the uninfected culture (*$p < 0.05$).

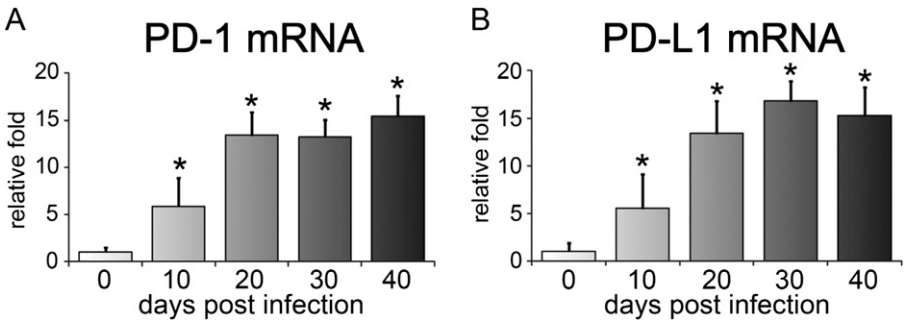


Fig. 2. Expression of mRNA for PD-1 and PD-L1 in the spinal cord at different time points (days 0, 10, 20, 30, and 40) after TMEV infection determined by real-time PCR. The expression of PD-1 in the spinal cords was shown in (A), and that of PD-L1 was shown in (B). The expression of PD-1 and PD-L1 was increased in the spinal cords at day 10, 20, 30, and 40 compared to day 0. (*$p < 0.05$).

infection were assessed using real-time RT-PCR. TMEV RNA levels were not significantly different between anti-PD-1 mAb-treated mice and control IgG-treated mice (Fig. 5). These results suggest that the blockade of PD-1-mediated signals inhibited the development of TMEV-IDD without significantly hindering TMEV persistence.

3.6. Effect of anti-PD-1 mAb on the production of cytokines

To examine the effect of anti-PD-1 mAb on the production of immune cytokines, we assessed the production of IFN-γ, TNF-α, IL-4, IL-10, IL-17 by spleen cells and cells from spinal cords at day 34 post-infection. Numbers of cytokine-producing CD4 positive cells were analyzed by flow cytometry. The proportions of IFN-γ or TNF-α producing cells were significantly increased in spleen and spinal cord from the mice treated with anti-PD-1 mAb at effector phase (Figs. 6 and 7). The percentage of IFN-γ producing cells and IL-17 producing cells are very small around 1% in the spleen of mice with TMEV-IDD as published before (Takeichi et al., 2010; Yanagisawa et al., 2010; Tsugane et al., 2012). The percentage of IFN-γ producing cells and IL-17 producing cells were significantly increased in the spleen of TMEV-infected and anti-PD-1 mAb treated mice from 0.51% to 1.77% ($p < 0.05$) and from 1.18% to 1.54% ($p < 0.05$) respectively. These results suggest that the cells producing inflammatory cytokines were increased in the mice treated with anti-PD-1 mAb at effector phase, which might be responsible for the exacerbated TMEV-IDD.

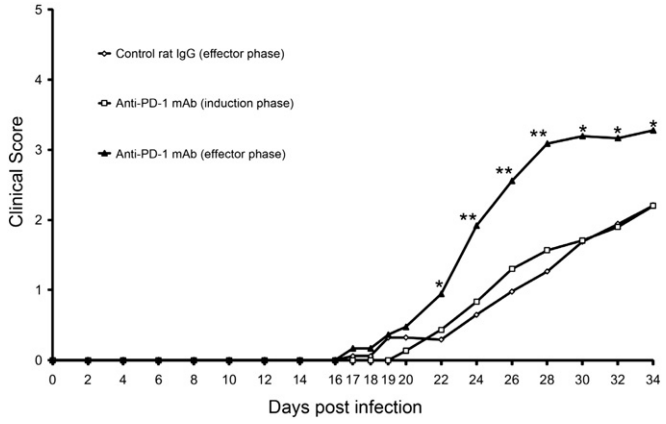


Fig. 3. Effect of anti-PD-1 mAb treatment on the clinical course of TMEV-IDD. This figure shows the summary of the clinical course of anti-PD-1 mAb treatment experiments. All mice were clinically observed from day 0 to day 34 post infection. Control rat IgG or anti-PD-1 mAb were injected i.p. on days −3, 0, 3, 6, 9, and 12 in the induction phase or days 17, 20, 23, 26, 29, and 32 in the effector phase after i.c. infection of TMEV. Treatment design is given in Table 1. Clinical signs of demyelinating disease were significantly exacerbated (*$p < 0.05$; **$p < 0.01$) in mice treated with anti-PD-1 mAb in the effector phase from day 22 to day 34 post infection, as compared with control mice.

4. Discussion

In this study, we examined the role of PD-1 pathway in the development of TMEV-IDD. We showed that administration of blocking mAb to PD-1 exacerbated the clinical signs of TMEV-IDD. After i.c. inoculation with TMEV, an initial viremia is followed by a persistent low level of CNS infection (Lipton et al., 1984). TMEV is known to infect neurons, glial cells, and macrophages in the spinal cord (Brahic et al., 1981; Clatch et al., 1990). Antigen presentation is mediated by either macrophages or glial cells, which contain TMEV antigens. As a consequence of the immunological process, major histocompatibility complex (MHC) class II restricted TMEV-specific delayed-type hypersensitivity (DTH)-mediating $CD4^+$ T cells are activated, which in turn participate in immune-mediated inflammatory responses. It has been proposed that virus-specific DTH results in the recruitment and accumulation of macrophages in the CNS, producing demyelination via a nonspecific "bystander" mechanism (Clatch et al., 1986). Myelin damage induced following TMEV infection is an immune-mediated process. In susceptible mice, the persistence of viral antigens within the CNS may result in continuous activation of T cells that can mediate pathogenesis through locally produced cytokines or direct cytolytic mechanisms. The role of T lymphocytes in immune-mediated demyelination has been confirmed by the fact that thymectomized, irradiated, and bone marrow-restored SJL/J mice fail to develop any clinical signs of TMEV-IDD (Gerety et al., 1994). It has been shown that treatment of susceptible mice with blocking Abs against CD4 or MHC class II molecules can significantly suppress the development of inflammation and demyelination after infection with TMEV (Rodriguez et al., 1986; Friedmann et al., 1987; Welsh et al., 1987; Gerety, Rundell, 1994). Demyelination correlates well with the development of a chronic, virus-specific DTH response (Clatch, Lipton, 1986). In addition, TMEV-IDD is inhibited after induction of virus-specific tolerance, which downregulates Th1-type responses (Peterson et al., 1993; Karpus et al., 1995), and Th1-type cells specific for $VP1_{233–250}$ and $VP2_{74–86}$ were further shown to be involved in the pathogenesis of viral demyelination induced by TMEV (Yauch, Palma, 1998). These studies strongly suggest that virus-specific Th1 cells are the major component in myelin destruction by TMEV infection. The activation and differentiation of T cells require both antigen/MHC recognition and costimulatory signals. The costimulatory signals are provided by binding of specific receptors on T cells to their ligands on antigen-presenting cells (APCs) and are necessary for functional T cell activation (Williams and Unanue, 1990).

PD-1 has been described as a negative regulator of immunity that limits T- and B-cell function. PD-1 is a cell surface molecule expressed

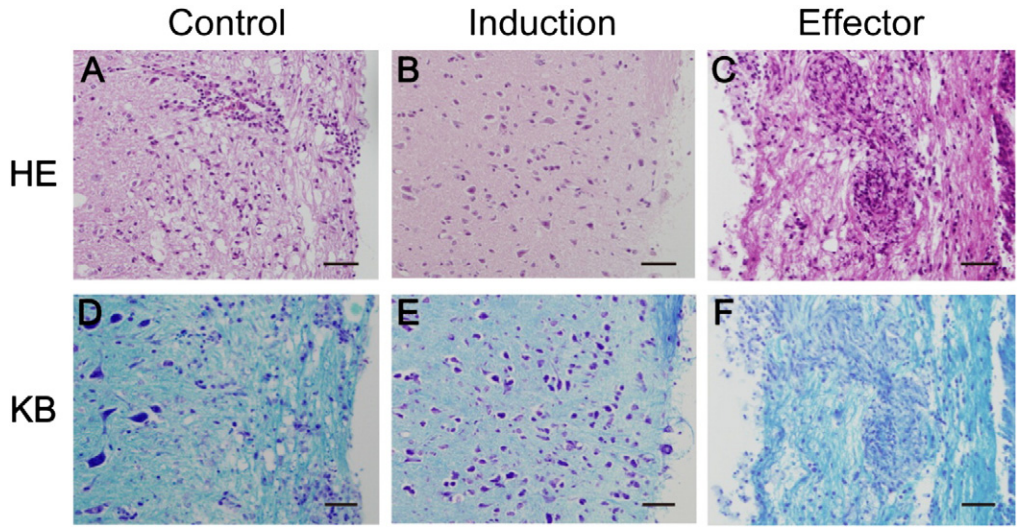


Fig. 4. Histological findings. Spinal cords of infected mice at days 34 post infection were stained with hematoxylin–eosin (A, B, C) and kluver-Barrera (D, E, F). Spinal cords of control IgG-treated mice (A, D) and the mice treated with anti-PD-1 mAb in induction phase (B, E) showed mild perivascular and parenchymal mononuclear cell infiltration, and mild demyelination in white matter. In contrast, spinal cord of the mice treated with anti-PD-1 mAb in effector phase (C, F) showed severe perivascular and parenchymal mononuclear cell infiltration and extensive demyelinative lesion.

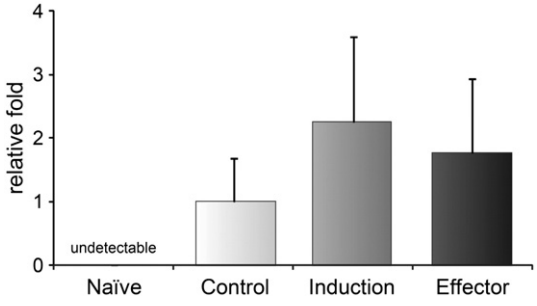


Fig. 5. Semi-quantitative real-time PCR to measure the mRNA expression of TMEV in whole spinal cord. TMEV mRNA expression in the mice treated with anti-PD-1 mAb in either induction or effector phase were comparable to the control IgG-treated mice.

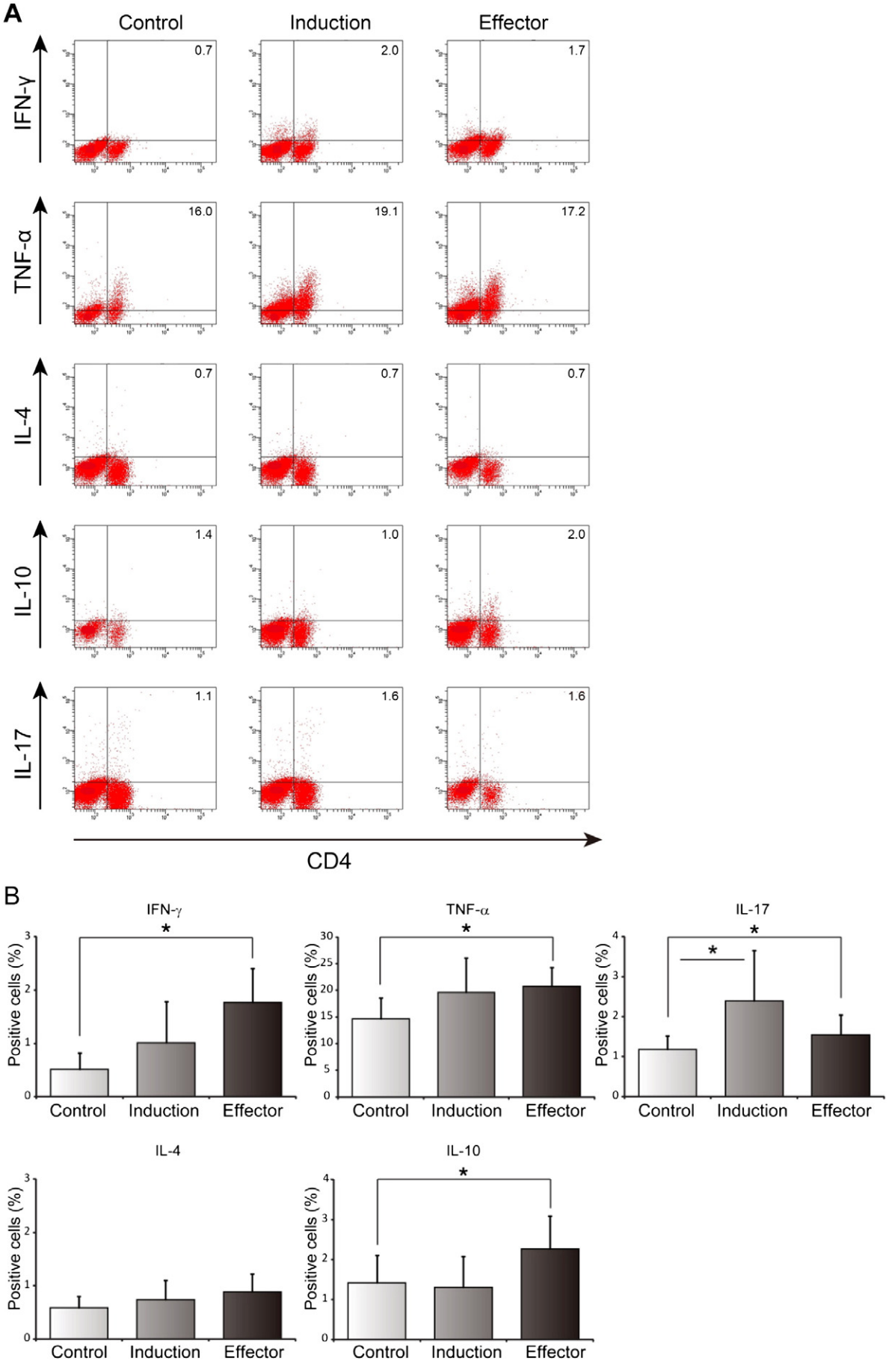
on activated T and B cells (Greenwald et al., 2005). PD-1 interacts with at least two ligands: PD-L1 and another closely related molecule, PD-L2 (Freeman et al., 2000; Latchman et al., 2001; Ishida et al., 2002). The expression patterns of PD-L1 and PD-L2 are largely different. PD-L1 is broadly expressed on leukocytes, nonhematopoietic cells, and in non-lymphoid tissues including islets (Freeman, Long, 2000; Yamazaki et al., 2002; Brown et al., 2003), while PD-L2 is expressed exclusively on DCs and monocytes (Freeman, Long, 2000; Ishida, Iwai, 2002; Ansari et al., 2003; Loke and Allison, 2003). Signaling through PD-1 limits T-cell functions including IFN-γ production, proliferation, and increased T-cell apoptosis (Baecher-Allan et al., 2001; Sandner et al., 2005). Notably, negative signals through PD-1 on T cells control autoimmunity. Disruption of the gene encoding PD-1 in BALB/c mice resulted in autoimmune cardiomyopathy (Nishimura et al., 2001), while C57BL/6 PD-1 knockout (KO) mice developed progressive arthritis and lupus like glomerulonephritis disease (Nishimura et al., 1999), and NOD PD-1 KO mice developed accelerated autoimmune diabetes (Wang et al., 2005). The role of PD-1 and its ligands has been further investigated in autoimmune models such as diabetes and multiple sclerosis using blocking reagents. Administration of anti-PD-L1 mAb to young NOD mice resulted in rapid onset of diabetes (Ansari, Salama, 2003). Moreover, PD-1 plays a critical role in murine experimental autoimmune encephalomyelitis (EAE), a mouse model for MS. In EAE, PD-1 blockade resulted in accelerated and more severe clinical disease (Salama et al., 2003).

We here demonstrated that the expression of PD-1 and PD-L1 on APCs is up-regulated in BMDCs following TMEV infection (Fig. 1). Furthermore, in the spinal cord, the expression of PD-1 and PD-L1 was significantly increased at day 10 post infection. This indicated that TMEV infection induced up-regulation of PD-1 pathway, and this up-regulation might contribute to the establishment of persistent infection of TMEV in the CNS. However, in the induction phase, blockade of PD-1 pathway with anti-PD-1 mAb did not affect viral persistence or development of demyelinating disease (Figs. 2, 3, and 4). This suggests that PD-1 pathway alone is not sufficient to establish TMEV persistence in the CNS, and thus, other mechanisms are likely involved.

We have also demonstrated that the expression of PD-1 and PD-L1 was up-regulated at days 20, 30, and 40 post infection when the mice infected with TMEV showed clinical signs and demyelination in the CNS. In this study, we also administrated anti-PD-1 mAb into mice infected with TMEV to block the PD-1 pathway during the effector phase. In consequence, the production of IFN-γ and TNF-α by Th1 cells was dramatically increased in the spinal cord and spleen (Figs. 6 and 7). The clinical course of disease was significantly exacerbated by the blockade of PD-1 pathway. Furthermore, IL-17-producing Th17 cells were significantly increased in the spleen of mice treated with anti-PD-1 mAb compared to control mice. Previous studies have shown that IL-17-producing cells are also involved in inflammatory tissue damage, leading to the pathogenesis of various autoimmune diseases (Ouyang et al., 2008) and TMEV-IDD (Hou et al., 2009). Our data suggests that up-regulation of IL-17-producing Th17 cells after blocking the PD-1 pathway might also contribute to the deterioration of TMEV-IDD.

We have examined viral persistence levels in the spinal cord by real-time RT-PCR. Viral persistence in the spinal cord of mice treated with anti-PD-1 mAb was comparable to control mice (Fig. 5). This shows that administration of anti-PD-1 mAb did not affect the viral load in

Fig. 6. Anti-PD-1 mAb treatment in the effector phase increased IFN-γ-, TNF-α-, or IL-17-producing cells in the spleen. Cells were isolated from spleen at day 34 post TMEV infection. After stimulation with PMA and calcinomycin for 4 h, cells were triple stained for CD4/IL-4/IFN-γ or CD4/IL-10/TNF-α or double stained for CD4/IL-17. (A) Representative FACS plots of splenocytes. (B) Summary of FACS plots data of $CD4^+$ cells in the spleen from total of 6 mice per group. IFN-γ-, TNF-α-, IL-10, or IL-17-producing cells were significantly increased in the mice treated with anti-PD-1 mAb in effector phase as compared to the control IgG-treated mice (*$p < 0.05$).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1.6
1.6
CD4
B
IFN-γ
TNF-α
IL-17
IL-4
IL-10
Positive cells (%)
Control
Induction
Effector
*

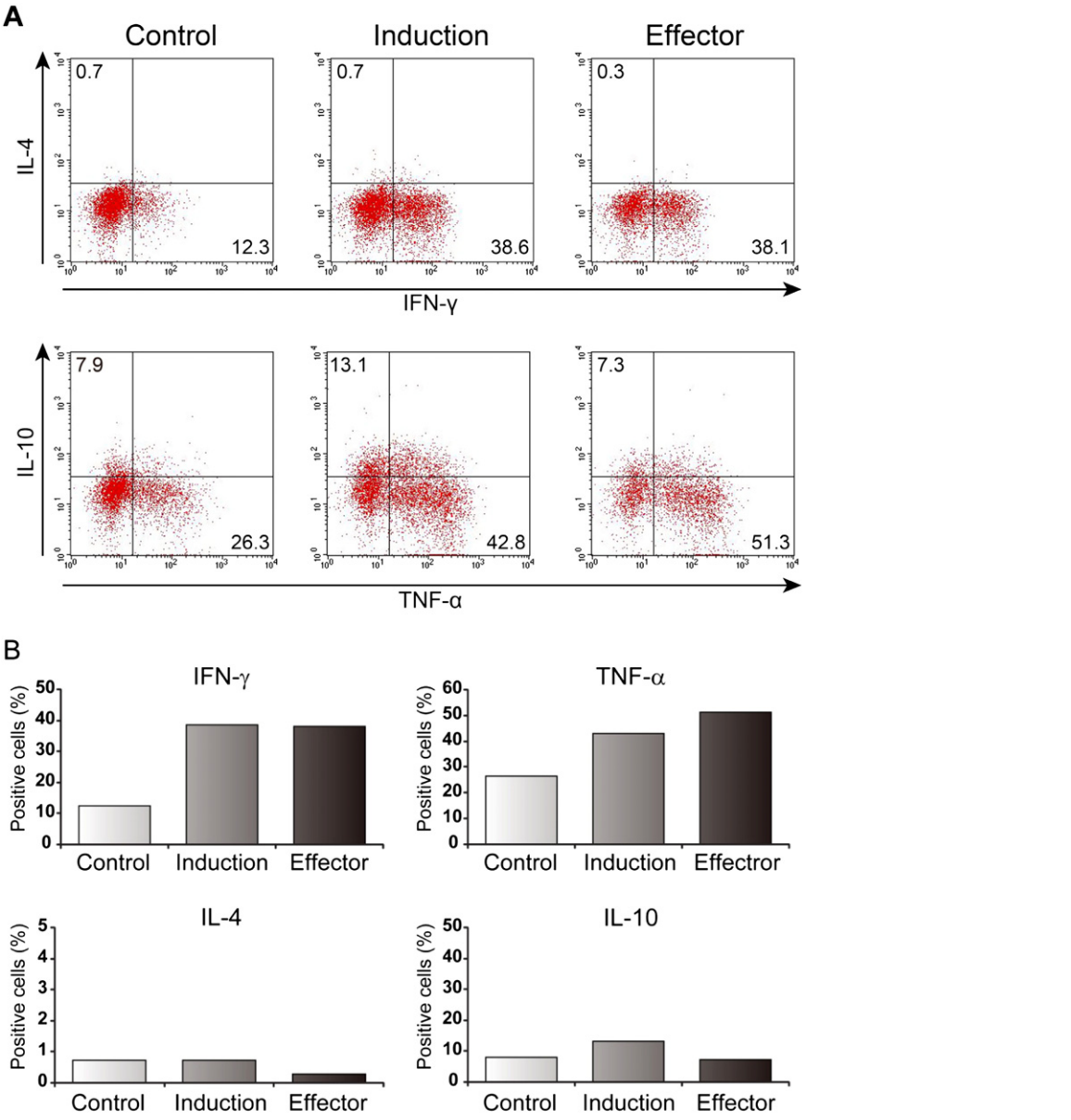

Fig. 7. Cytokine-producing cells in the spinal cords at day 34. Infiltrating cells were isolated from the spinal cords of three mice at day 34 and pooled. After stimulation with PMA and calcinomycin for 4 h, cells were triple stained for CD4/IL-4/IFN-γ or CD4/IL-10/TNF-α. (A) Representative FACS plots of $CD4^+$ cells in the spinal cords. (B) Summary of FACS plots data of $CD4^+$ cells in the spinal cords. IFN-γ- or TNF-α-producing cells were increased in the mice treated with anti-PD-1 mAb compared to the control IgG-treated mice.

the CNS, despite the deteriorated disease development. There are increasing evidences that viral persistence levels are not associated with the development of TMEV-IDD (Myoung et al., 2008; Jin et al., 2009). Viral persistence alone may not be sufficient to induce the disease and that the level of T cell immunity to viral capsid epitopes may be critical for the development of demyelinating disease in SJL mice (Myoung, Bahk, 2008).

In summary, administration of anti-PD-1 mAb during effector phase exacerbated TMEV-IDD. Blockade of PD-1 pathway affected the development of IFN-γ-producing Th1 cells. TMEV-IDD is considered to be a Th1 disease, and Th1 is also involved in human MS. Therefore, our data suggests the PD-1 pathway may play a critical role in regulating disease onset and progress of virus-induced demyelinating diseases. There is a report that interactions between PD-1/PD-L1, but not PD-1/PDL-2, are crucial in attenuating T cell responses in EAE (Carter et al., 2007). Therefore, we speculate that blockade of PD-1/PD-L1 pathway is more critical for development of TMEV-IDD than PD-1/PD-L2 pathway. Taken together, PD-1 pathway could be a novel target in the treatment of MS.

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