The role of T cell Immunoglobulin Mucin domain in Herpes simplex virus–induced Behçet’s Disease mouse model

by

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The T cell immunoglobulin mucin (TIM) proteins regulate T cell activation and tolerance. Individual TIM family members may serve as susceptibility markers for asthma, allergies and autoimmune diseases, as well as potential cell surface markers for T helper type (Th)1 and Th2 T cells. TIM-1 plays an important role in the regulation of immune responses and the development of autoimmune diseases. TIM-4 is a natural ligand of TIM-1, and interaction of TIM-1 and TIM-4 is involved in the regulation of Th cell responses and the modulation of the Th1/Th2 cytokines balance. TIM-4 expression was increased in patients with systemic lupus erythematosus (SLE). It has been also reported that TIM-3 expression was higher in patients with rheumatoid arthritis compared to controls. Further, Galectin-9 (Gal-9) has been identified as a TIM-3 ligand (L) and the TIM-3-TIM-3L interaction serves as a specific down-regulator of the Th1 immune response.

Behçet’s disease (BD) is a chronic, multisystemic inflammatory disorder with arthritic, gastrointestinal, mucocutaneous, ocular, vascular, and central nervous system involvement. In herpes simplex virus induced BD mouse model, the expression of Tim-1 and Gal-9 was lower levels compared to asymptomatic BD normal (BDN) mice. The expression of Tim-3 and Tim-4 was higher in BD mice than BDN mice. In addition, Tim-1 vector injected BD mice showed changes of BD-like symptoms and decreased the severity score.
Again, treatment with Tim-4 siRNA also improved the BD-like symptoms and decreased the severity score accompanied with up-regulation of regulatory T cells (Treg). Furthermore, administration of Gal-9 improved the BD-like symptoms, decreased the severity score, and increased Treg cells. In addition, Gal-9 induced improvement was associated with down-regulation of pro-inflammatory cytokines and induction of apoptosis.

In the present study, we showed that the regulation of Tim-1 or Tim-4 affected the BD-like symptoms and Tim-3-Tim-3L interaction improved the inflammatory symptoms in BD mice.

**Key words:** Tim-1, Tim-3, Tim-4, Galectin-9, Herpes simplex virus-induced systemic inflammation, Behçet’s Disease mouse model
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I. INTRODUCTION

The T cell immunoglobulin and mucin domain (TIM) family is located on chromosome 11B1.1 in mice and consists of several members (\(\text{Tim-1}~\text{8}\)). In human it is located on chromosome 5q33.2 and consists of three members (\(\text{TIM-1, 3 and 4}\)) (Kuchroo et al., 2003). Individual TIM family members may serve as susceptibility markers for asthma, allergies and autoimmune diseases, as well as potential cell surface markers for T helper type (Th) 1 and Th2 T cells (Kuchroo et al., 2003; Meyers et al., 2005b). Therefore, human TIM gene family is critical to Th1/Th2 mediated immunological regulation (Meyers et al., 2005b).

TIM-1 was first identified as a hepatitis A virus cellular receptor 1 (Kaplan et al., 1996; Feigelstock et al., 1998) and a kidney injury molecule, KIM-1 (Han et al., 2002; Ichimura et al., 1998). TIM-1 is expressed on CD4+ T cells after activation and its expression is sustained preferentially in Th2 but not Th1 cells (Kuchroo et al., 2003; Meyers et al., 2005a). TIM-1 plays an important role in the regulation of immune responses and the development of autoimmune disease. The high-avidity anti-Tim-1 antibody enhances the severity of experimental autoimmune encephalitis (EAE) by increasing autopathogenic Th1 and Th17 responses, whereas the low-avidity antibody inhibits autopathogenic Th1 and Th17 responses (Umestu et al., 2005).

TIM-4 is a natural ligand of TIM-1 (Meyers et al., 2005a) and exclusively expressed on antigen-presenting cells (APCs), including dendritic cells (DCs) and macrophages (Rodriguez-Manzanet et al., 2008; Mizui et al., 2008), where it mediates phagocytosis of apoptotic cells and plays an important role in maintaining tolerance.
(Kobayashi et al., 2007; Miyanishi et al., 2007). Interaction of TIM-1 and TIM-4 is involved in the regulation of Th cell responses and the modulation of the Th1/Th2 cytokines balance (Meyers et al., 2005a). DCs-derived TIM-4 maintains TIM-1 in Th2 cells in a stable status that plays a critical role in sustaining the Th2 polarization (Zhao et al., 2010). Recent report demonstrated that TIM-4 binding to TIM-1 has different effects on T cell proliferation. A higher dose of Tim-4-Ig consistently led to an increase in T cell proliferation upon TCR ligation, whereas a lower concentration of Tim-4-Ig inhibited T cell proliferation (Meyers et al., 2005a). Human TIM-1 is also associated with other types of immune dysfunction, such as atopic dermatitis, allergy, rheumatoid arthritis, asthma and systemic lupus erythematosus (SLE) (Chae et al., 2003a; Chae et al., 2003b; Chae et al., 2005; Page et al., 2006; Wang et al., 2008), suggesting that Tim-1 may regulate immune responses. In addition, in patients with SLE, TIM-4 expression in PBMC was also increased (Zhao et al., 2010).

Galectin-9 (Gal-9) is a ligand for TIM-3. TIM-3 was identified as a Th1-specific marker and has been found on cytotoxic CD8+ T cells, Th17, regulatory T (Treg) cells, monocytes, DCs, mast cells, and microglia (Nakae et al., 2007; Monney et al., 2002; Cua et al., 2003; Anderson et al., 2007; Wang et al., 2007). The TIM-3-TIM-3 ligand (TIM-3-TIM-3L) interaction serves as a specific down-regulator of the Th1 immune response (Meyers et al., 2005b). Gal-9 was previously shown to ameliorate collagen-induced arthritis (Seki et al., 2007) as well as EAE (Zhu et al., 2005) by inducing apoptosis in synoviocytes, suppressing generation of Th17 cells, and up-regulating the induction of Treg cells (Seki et al., 2008). The expression of TIM-3 by PBMC isolated from BD patients increased compared with healthy control (Lee et al., 2012).
Behçet’s Disease (BD) is a Th1-polarized (Ilhan et al., 2008), chronic, multi-systemic inflammatory disorder with arthritis, gastrointestinal, mucocutaneous, ocular, vascular, and central nervous system involvement. This disease takes on a chronic course with periodic exacerbations and progressive deterioration (Shimizu, 1979). The etiology of BD is unclear; however, viral infection has long been postulated as one of the main factors. Ever since Hulusi Behçet first proposed a viral etiology (Behçet, 1937), his hypothesis has been verified by detection of virus in saliva (Lee et al., 1996b), intestinal ulcers (Lee et al., 1993), and genital ulcers (Bang et al., 1997; Lee et al., 1996a) of patients with BD. Subsequent to these findings, herpes simplex virus (HSV) inoculation of the earlobes of ICR mice resulted in the development of BD-like symptoms (Sohn et al., 1998). Manifestations in mice inoculated with HSV include multiple symptoms such as oral ulcers, genital ulcers, skin ulcers, eye symptoms, gastrointestinal ulcers, arthritis, and neural involvement, as well as skin crusting. The frequencies of these symptoms are similar to those of patients with BD (Kim et al., 1988).

TIM in BD has not been studied much until today. Therefore, we investigated that the expression of Tim in BD mouse model with BD-like symptoms. The expression of Tim-1 and Tim-4 was analyzed in BD mice and the change of BD-like symptoms by regulating the expression of Tim-1 or Tim-4. Furthermore, the changes of cellular phenotypes and cytokine levels on immune cells were confirmed after up-regulation of Tim-1 or down-regulation of Tim-4 in BD mice. In addition, we demonstrated that administration of Gal-9 up-regulated apoptosis, down-regulated pro-inflammatory cytokines, and improved the BD-like symptoms in mice.
II. MATERIALS AND METHODS

A. Antibodies and Reagents

Mouse anti-CD4, anti-CD44, anti-CD107a, anti-Tim-1, anti-Tim-3, anti-Tim-4, anti-CD8a, anti-CD122, anti-CD34, anti-CD11b, anti-CD11c, anti-CD25, anti-CD86, anti-Gr-1 and anti-granzyme B antibodies, an anti-Foxp3 staining kit, and an Annexine V-FITC apoptosis detection kit were purchased from eBioscience (San Diego, CA). Anti-Gal-9 was purchased from R&D system (Minneapolis, MN).

B. Animal experiments

ICR male mice (4 to 5 weeks old) were infected with HSV type 1 (1x10⁶ pfu/mL, F strain) grown in Vero cells as previously described (Sohn et al., 1998). We used anesthetic composed of a mixture of Zoletil (Virbac Lab, Carros, France) and Rompun (Bayer, Seoul, Korea). The ratio of Zoletil and Rompun was 1:4, and it was administered at a dose of 40 μL per mouse (Tiletamine 10 mg/kg, zolazepam 10 mg/kg, and xylazine hydrochloride 36 mg/kg) via intramuscular injection. Virus inoculation was conducted twice at a 10 day intervals, after which the animals were observed for 16 weeks. Animals were handled in accordance with a protocol approved by the animal care committee of Ajou University School of Medicine.
C. BD-like symptoms

Manifestations in mice after HSV inoculation involved multiple symptoms. Of the total number of HSV-injected mice, 15% developed BD-like symptoms. Disappearance of symptoms or a higher than 20% decrease in lesion size were classified as effective. Determination of the severity score of BD was followed by determination of the value of the BD activity index, as outlined in the BD Activity Form (www.behcet.ws/pdf/BehcetsDiseaseActivity-Form.pdf). Among the symptoms exhibited by patients, mouth ulceration, genital ulceration, erythema, skin pustules, skin ulceration, joints-arthritis, diarrhea, red eye (right, left), reduced vision (right, left), loss of balance, discoloration, and swelling of the face were selected and analyzed in the BD mouse model. The score of each symptom was one, and after the score was added up, the total was used to determine the severity score of BD.

D. Tim-1 DNA constructs

*Tim-1* construct with an extracellular Flag epitope tag was generated (de Souza et al., 2005). A cDNA clone containing the entire coding sequence of murine *Tim-1* was provided from Prof. Lawrence P. Kane (University of Pittsburgh). Briefly, the ORF of *Tim-1* (excluding the start codon and signal sequence) was amplified from this clone by PCR and ligated into a pCDEF3 expression plasmid (Wu et al., 1999). All DNA constructs were verified by automated DNA sequencing. All plasmids used were purified by two passes through Endo-Free columns (Qiagen, Chatsworth, CA, USA) as described elsewhere (Ulker et al., 1990).
E. Preparation of Tim-4 small interfering RNA (siRNA)

Tim-4 siRNA (siTim-4) was synthesized from Genolution pharmaceuticals, Inc (Seoul, Korea). The synthesized sequences of Tim-4 siRNA was sense; 5’- CUA AAU CAC AUC AGA UCA ACA GCA GUU -3’, anti-sense; 5’- CUG CUG UUG AUC UGA UGU GAU UUA GUU -3’. For injection into mice, the Tim-4 siRNA with transfection reagent jetPEI (PolyPlus-transfection, Lilkirch, France) was used.

F. Tim-1 vector and Tim-4 siRNA administration to BD mice

For each mouse, 10 μg of Tim-1 vector was intraperitoneally injected 4 times at 2 day intervals in BD mice, followed by observation for 2 weeks. Control was used empty vector, PCDEF3. For the down-regulation of Tim-4, 5 μg of siRNA Tim-4 was intraperitoneally injected 3 times at 2 day intervals in BD mice, followed by observation for 2 weeks. And scramble siRNA was used as negative control (N.C) (Genolution pharmaceuticals, Inc, Seoul, Korea).

G. Gal-9 administration to BD mice

For each mouse, 100 μg of Gal-9 was injected intraperitoneally five times at 3 days interval, followed by observation for 2 weeks. PBS was used as a control. Over the next two weeks, the severity score was compared with that of the vehicle control.

H. Flow cytometry

The cells were surface-stained with anti-mouse antibodies (CD4, CD8, CD11b,
CD11c, CD25, CD34, CD44, CD122, Tim-1, Tim-3, Tim-4, Gr-1 and Gal-9) for 30 min at 4 °C in the dark. For the intracellular detection of Foxp3 and granzyme B, an anti-mouse Foxp3-staining buffer set was used according to the manufacturer's instructions. Briefly, cells were fixed using Fix/perm buffer after washing with 1X permeabilization buffer and then incubated with anti-mouse forkhead box p3 (Foxp3) and granzyme B antibodies. For staining of CD107a, brefeldin A (Sigma, st. Louis, MO, USA) was added to the incubation media for 5 h, after which the intracellular staining protocol was carried out according to the manufacturer's instructions. Stained cells were analyzed by a flow cytometer (FACS canto II; Becton Dickinson, Franklin Lakes, NJ, USA) with ≥10,000 gated lymphocytes.

I. Enzyme linked immunosorbent assay (ELISA)

Tim-1 vector, Tim-4 siRNA, and Gal-9 were injected into BD mice, and 14 days after the first administration, serum was collected. Serum was analyzed using commercial ELISA kits for the detection of mouse IL-6 (R&D system, Minneapolis, MN), TNF-α (R&D system), IL-17 (R&D system), IL-4 (R&D system), and IFN-γ (R&D system), according to the manufacturer’s instructions. The ELISA reader was Bio-Rad model 170-6850 microplate reader, and samples were read at a wavelength of 450 nm.

J. Transmission electron microscopy (TEM)

Gal-9 was injected into BD mice, and 14 days after first administration, peripheral blood was collected. After RBC lysis, blood cells were fixed with fixative solution [2% glutaraldehyde and 1% paraformaldehyde in 0.1M sodium cacodylate buffer (pH 7.4)] under
a fume hood and washed with 0.1M cacodylate buffer for 5 minutes at 3 times. Again the cells were fixed with 1% Osmium tetroxide for 30 min and washed with 0.1M cacodylate buffer for 5 min at 3 times. Then, cells were dehydrated by replacement of ascending series of ethanol (30, 40, 50, 60, 70, 80, 90, 95 and 100%) for 5 min in every step. During infiltration process, the cells were treated with a mixture of Epon and ethanol (1:2 for 30 min, 1:1 for 60 min, 2:1 for 90 min) and with Epon alone for overnight. To prepare “Epon block”, cells were incubated at 60 °C for 2 days. Further, the cells were sectioned (70-90 nm) with an ultramicrotome (Reichert ultracut S, Leica Microsystems, Wetzlar, Germany) and place on copper grids and double stained with uranyl acetate for 10 min and lead citrate for 2 min. Finally the cellular ultrastructural changes were observed using a Transmission electron microscope (Zeiss EM 902A, Zeiss, Oberkochen, Germany).

K. Statistical analysis

All data are expressed as the mean ± SD. Statistical differences between the experimental groups were determined using Student’s t test and Bonferroni correction. Statistical analysis was conducted using MedCalc® version 9.3.0.0.
III. RESULTS

A. The frequencies of Tim-1 and Tim-4 expressing cells in normal healthy, BDN and BD mice

The frequencies of Tim-1(+), CD4(+)Tim-1(+) and CD8(+)Tim-1(+) cells were analyzed in cells from lymph nodes (LN), spleen (SP) and peripheral blood mononuclear cells (PBMC) of normal healthy (Nor) and BD Normal (BDN; HSV-1 was inoculated but no symptoms) mice and compared with those in BD mice by FACS. In LN, the frequencies of Tim-1(+), CD4(+)Tim-1(+) and CD8(+)Tim-1(+) cells in BD mice were significantly lower than BDN mice [BDN vs. BD (%): Tim-1(+), 20.1±11.5 (n=12) vs. 11.1±6.7 (n=12), p=0.01; CD4(+)Tim-1(+), 3.4±1.9 (n=9) vs. 2.2±1.6 (n=9), p=0.09; CD8(+)Tim-1(+), 1.9±1.1 (n=9) vs. 1.1±0.6 (n=9), p=0.04]. (Fig 1A). In splenocytes, the BD mice showed significantly lower frequencies than Nor or BDN mice [Nor vs. BDN vs. BD (%): Tim-1(+), 21.3±6.5 (n=6) vs. 18.3±8.5 (n=10) vs. 12.2±5.9 (n=10), Nor vs. BD p=0.006, BDN vs. BD p=0.04; CD4(+)Tim-1(+), 3.1±1.6 (n=6) vs. 1.9±1.0 (n=8) vs. 1.7±0.5 (n=8), Nor vs. BD p=0.02, BDN vs. BD p=0.35]. CD8(+)Tim-1(+) cells showed similar frequencies among three groups (Fig 1B). In PBMC, the frequencies of BD mice were slightly lower than BDN, but not significant [BDN (n=9) vs BD (n=9) (%): Tim-1(+), 27.6±17.1 vs. 23.6±8.4 p=0.54; CD4(+)Tim-1(+), 8.3±5.9 vs. 5.7±4.4, p=0.31; CD8(+)Tim-1(+), 7.6±5.0 vs. 5.2±4.3, p=0.29]. BD mice were significantly higher than Nor mice (Fig 1C). These data indicated that the frequencies of Tim-1 expression cells in BD mice were down-regulated compare to
BDN mice.

The frequencies of Tim-4(+) cells were also analyzed in LN, SP and PBMC of Nor, BDN and BD mice. In LN, the frequency of Tim-4(+) cells in BD mice was significantly higher than Nor and BDN mice [Nor vs. BDN vs. BD (%): 1.3±0.8 (n=11) vs. 3.2±0.9 (n=15) vs. 5.8±2.0 (n=15), Nor vs. BDN p=0.000005, Nor vs. BD p=0.0000001, BDN vs. BD p=0.00003](Fig 2A). In splenocytes, the frequency of Tim-4(+) cells in BD mice was also significantly higher than Nor mice [Nor vs BD (%): 3.9±2.8 (n=11) vs. 6.7±4.3 (n=17), p=0.035]. However, the difference between BDN and BD mice were not significant (Fig 2B). In PBMC, the frequencies of Tim-4(+) cells in BD mice were higher than Nor mice (Fig 2C). Moreover, in peritoneal macrophages, the frequencies of Tim-4(+) cells in BD mice were significantly higher than Nor and BDN mice [Nor vs. BDN vs. BD (%): 6.1±2.0 (n=5) vs. 8.6±4.0 (n=9) vs. 26.6±17.0 (n=5), Nor vs. BDN p=0.11, Nor vs. BD p=0.02, BDN vs. BD p=0.005]. There was not found any differences between Nor and BDN mice (Fig 2D). In addition, we also examined the frequencies of CD11b(+)Tim-4(+) and CD11c(+)Tim-4(+) cells in LN and spleen. In LN, both markers in BD mice were higher than Nor and BDN mice [Nor (n=5) vs. BDN (n=6) vs. BD (n=5) (%): CD11b(+)Tim-4(+), 1.1±0.3 vs. 1.7±0.7 vs. 3.0±1.4, Nor vs. BDN p=0.05, Nor vs. BD p=0.01, BDN vs. BD p=0.04; CD11c(+)Tim-4(+), 0.1±0.0 vs. 0.5±0.1 vs. 0.7±0.5, nor vs. BDN p=0.00003, Nor vs. BD p=0.01, BDN vs. BD p=0.14] (Fig 2E). In splenocytes, expression of CD11c(+)Tim-4(+) cells was found similar to the LN in Nor (0.3±0.1%, n=5), BDN (0.6±0.4%, n=6) and BD mice (1.0±0.6%, n=8) (Nor vs. BDN p=0.07, Nor vs. BD p=0.07, BDN vs. BD p=0.1). On the other hand, the frequencies of CD11b(+)Tim-4(+) cells in BD mice were significantly lower than Nor, but
higher than BDN mice (Fig 2F). These data suggested that the frequencies of Tim-4 expression cells in BD mice were up-regulated compare to BDN mice.
Figure 1. The frequencies of Tim-1(+) cell phenotypes in normal healthy, BDN and BD mice. The frequencies of Tim-1(+), CD4(+)Tim-1(+) and CD8(+)Tim-1(+) cells in BD mice were compared to Nor and BDN mice by FACS analysis in A. lymph nodes, B. spleen, and C. PBMC. (n=6–12) (*p < 0.1, ** p < 0.05, *** p < 0.01) Nor: normal healthy mice, BDN: BD normal mice
Figure 2. The frequencies of Tim-4(+) cell phenotypes in normal healthy, BDN and BD mice. The frequency of Tim-4(+) cells in BD mice was compared to Nor and BDN mice by FACS analysis in A. lymph nodes, B. spleen, C. PBMC, and D. peritoneal macrophage. The expression of CD11b(+)Tim-4(+) and CD11c(+)Tim-4(+) cells in BD mice were also compared to Nor and BDN mice in E. lymph nodes and F. spleen. (n=5~17) (*p < 0.05, ** p < 0.01, *** p < 0.001) Nor: normal healthy mice, BDN: BD normal mice
B. The expression of Tim-3 and Gal-9 in BD mice

The frequencies of Tim-3(+) cells in lymph node and Gal-9 in peritoneal macrophages of Nor and BDN mice were compared to those of BD mice by FACS analysis. The number of Tim-3(+) cells in BD mice (n=7) were significantly higher than those in normal (n=7) and BDN mice (n=6) (Nor 1.3±0.3% vs. BD 5.8±1.8%, p=0.00003; BDN 2.0±0.5% vs. BD 5.8±1.8%, p=0.005) (Fig 3A). On the other hand, the frequencies of Gal-9 in peritoneal macrophages from BD mice (n=7) were significantly lower than those in Nor (n=5) and BDN (n=6) mice (Nor 52.5±7.8% vs. BD 6.9±6.8%, p=0.000001, BDN 19.2±10.3% vs. BD 6.9±6.8%, p=0.027) (Fig 3B).
Figure 3. Expression of Tim-3 and Gal-9 in BD mice. The frequencies of (A) Tim-3(+) in lymph node and (B) Gal-9(+) in peritoneal macrophages were compared among Nor, BDN, and BD mice by FACS analysis. (n=5~7) (**p < 0.05, ***p < 0.01) Nor: normal healthy mice, BDN: BD normal mice.
C. Administration of Tim-1 vector up-regulates the frequency of Tim-1(+) cells in vivo lymph nodes

We investigated the change of BD-like symptoms according to the regulation of Tim-1 expression. To up-regulate the expression of Tim-1, Tim-1 expression vector was administered into BD mice and the frequencies of Tim-1(+) cells were down-regulated in BD mice compared to BDN mice (Fig 1). Tim-1 vector was injected intraperitoneally at 10, 30 and 50 μg per mouse into Nor mice for 2 times at 2 days interval. Then, next day of the last injection, the frequencies of Tim-1(+) cells from LN were compared to those of the control vector injected group. The expression of Tim-1(+) cells in 10 μg of Tim-1 vector injected group (19.1±5.1%, n=3~4) were significantly higher than control vector injected group (10.4±5.4%, n=4, p=0.03) (Fig 4). However, 30 and 50 μg of Tim-1 vector injected group showed lower expression than 10 μg injected group. After that, 10 μg of Tim-1 vector was used for the following study.
Figure 4. Administration of Tim-1 vector increased the frequency of Tim-1(+) cells. To observe the up-regulation of Tim-1 expression, Tim-1 vector was injected intraperitoneally at 10, 30 and 50 μg per mouse into Nor mice for 2 times at 2 days interval. Then, next day of the last injection, the frequency of Tim-1(+) cells in LN was compared to the control vector injected group by FACS analysis. Control was used empty vector, PCDEF3 (50 μg/mouse). (n=3–4) (**p < 0.05) Con: control vector injection to Nor mice, Tim-1: Tim-1 vector injection to Nor mice.
D. Administration of Tim-1 vector affected the BD-like symptoms

Ten μg of Tim-1 vector was injected intraperitoneally 4 times at 2 day intervals into BD mice, followed by the observation for 2 weeks. In this study, skin and genital ulcers were improved after Tim-1 vector administration when compared to the control vector injected group (Fig 5A). However, Tim-1 vector was not effective in eye involvement (Table 1). Additionally, in control vector group, the severity score was 2.83±0.41 before and 2.83±0.75 at one week and 2.67±1.21 at two week after first injection of control vector (p=0.8, n=6). In Tim-1 vector group, severity score was 3.17±0.75 before the injection, 1.50±1.22 at one week after injection (p=0.004), 1.33±1.51 at two weeks after injection in BD mice (p=0.03) (Fig 5B). After injection of Tim-1 vector, the severity score was decreased, whereas, control vector injection was not different in BD mice.

Two weeks after the first administration of Tim-1 vector to BD mice, isolated lymph node and PBMC were analyzed for Tim-1(+) cells by FACS. In LN, the frequency of Tim-1(+) cells in Tim-1 vector injected group was slightly high compared to control vector injected group [Con vs. Tim-1 (%): 10.3±1.7 (n=4) vs. 11.9±3.2 (n=5), p=0.19]. However, CD4(+)Tim-1(+) and CD8(+)Tim-1(+) cells were similar to control vector injected group (Fig 5C). The frequencies of Tim-4(+) cells were also not different (Fig 5D). In PBMC, Tim-1(+) cells was slightly lower in Tim-1 vector injected group. CD4(+)Tim-1(+), CD8(+)Tim-1(+) and Tim-4(+) cells were not different (Fig 5C-D). Interestingly, CD4(+) T cells in Tim-1 vector injected group were higher than control vector injected group [Con vs. Tim-1 (%): 21.3±10.7 (n=8) vs. 27.6±13.7 (n=8), p=0.36, Fig 5C]. One week after administration of Tim-1 vector, the frequencies of those marker expressing cells were similar compared with
two weeks after injection (data not shown). Up-regulated Tim-1 expression improved BD-like symptoms in BD mice.
<table>
<thead>
<tr>
<th>A</th>
<th>Control (empty vector)</th>
<th>Tim-1 vector</th>
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</thead>
<tbody>
<tr>
<td>Before</td>
<td>![Control Before Image]</td>
<td>![Tim-1 Before Image]</td>
</tr>
<tr>
<td>After 1 week</td>
<td>![Control After 1 Week Image]</td>
<td>![Tim-1 After 1 Week Image]</td>
</tr>
<tr>
<td>After 2 weeks</td>
<td>![Control After 2 Weeks Image]</td>
<td>![Tim-1 After 2 Weeks Image]</td>
</tr>
</tbody>
</table>

B

**Severity Score**

- **before**
- **1wk**
- **2wks**

**Comparison:**
- **con**
- **Tim-1**

![Graph showing severity score comparison](image-url)
Figure 5. Administration of Tim-1 improved the BD-like symptoms, but the frequencies of Tim-1 expressing cells were not changed. Ten μg per mouse of Tim-1 vector was injected intraperitoneally 4 times at 2 days interval into BD mice, followed by observation for 2 weeks. A. photographs of mice were taken before and at one and two weeks after first treatment of Tim-1 vector and control vector injected group. B. The severity score was compared before and at one and two weeks after treatment of them. C. Two weeks after first Tim-1 vector injection, the frequencies of Tim-1(+), CD4(+)Tim-1(+), CD8(+)Tim-1(+) and CD4(+) T cells, and D. Tim-4(+) cells in LN and PBMC by FACS analysis. (n=5~6) (*p < 0.1) Con: control vector injection to BD mice, Tim-1: Tim-1 vector injection to BD mice
Table 1. The change of BD-like symptoms after *Tim-1* and control vector administration

<table>
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<th>2 wks</th>
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<td>++</td>
<td>++</td>
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<tr>
<td></td>
<td>Left eyeball inflammation</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
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<td>-</td>
</tr>
<tr>
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<td>++</td>
<td>++</td>
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<tr>
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<td>+</td>
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<td>Back skin crust</td>
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<tr>
<td></td>
<td>Left earlobe erythema</td>
<td>+</td>
<td>+</td>
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BD symptom: +, deteriorated: ++, improved: -
E. *Tim-1* vector administration affects the regulatory cellular phenotypes

After *Tim-1* vector administration, several cellular phenotypes in LN and PBMC were analyzed in BD mice. The frequencies of CD4(+)CD25(+), CD4(+)Foxp3(+) and CD4(+)CD25(+)Foxp3(+) (regulatory T, Treg) cells in LN were not significantly changed in *Tim-1* vector compared to control vector injected group [Con (n=5) vs. Tim-1 (n=6) (%): CD4(+)CD25(+), 6.5±1.6 vs. 6.1±2.3 p=0.35; CD4(+)Foxp3(+), 5.3±1.5 vs. 4.7±1.7, p=0.29; Treg, 5.2±1.4 vs 4.7±1.7, p=0.3] (Fig 6A). But, in PBMC, CD4(+)CD25(+), CD4(+)Foxp3(+) and Treg cells in *Tim-1* vector injected group were significantly higher than control vector injected group [Con (n=8) vs. Tim-1 (n=8) (%): CD4(+)CD25(+), 0.31±0.18 vs. 0.61±0.21 p=0.01; CD4(+)Foxp3(+), 0.10±0.10 vs. 0.31±0.12, p=0.004 ; Treg, 0.04±0.08 vs 0.16±0.10, p=0.03] (Fig 6A).

CD8(+)CD122(+) T cells are newly identified and regarded as Treg cells (Saitoh et al., 2007) and reported the effect as an anti-inflammatory responses (Rifa’i et al., 2008). Another type of Treg cells, CD8(+)CD122(+) T cells were also analyzed in LN of *Tim-1* vector injected group. [Con (n=5) vs. Tim-1 (n=6) (%): 1.3±0.3 vs. 1.6±0.7 p=0.25] (Fig 6B).

In PBMC, CD122(+) and CD8(+)CD122(+)T cells in *Tim-1* vector injected group were also higher than control vector injected group [Con (n=8) vs. Tim-1 (n=8) (%): CD122(+), 1.2±1.0 vs. 9.8±7.9 p=0.04; CD8(+)CD122(+), 1.9±1.5 vs. 2.4±2.1 p=0.58).

Our study indicated that *Tim-1* vector up-regulated Treg cells and CD8(+)CD122(+) Treg cells in BD mice. Up-regulation of these cellular phenotypes may be involved in the improvement of BD-like symptoms after injection of Tim-1 vector.
Figure 6. The frequencies of Treg cells in PBMC were affected in Tim-1 vector injected BD mice. Two types of Treg cells and apoptotic cell phenotypes were confirmed in LN and PBMC after Tim-1 administration to BD mice. A. The frequencies of CD4(+)CD25(+), CD4(+)Foxp3(+) and Treg cells were compared between control and Tim-1 vector injected group. B. CD8(+)CD122(+) T cells were up-regulated compared to control group but not significant. CD122(+) cells was significantly increased in Tim-1 vector injected group. (n=5~8) (**p < 0.05, ***p < 0.01) Con: control vector injection to BD mice, Tim-1: Tim-1 vector injection to BD mice
F. Pro-inflammatory cytokines were down-regulated by Tim-1 vector administration in BD mice

To determine the level of cytokines, two weeks after Tim-1 vector injection into BD mice, sera were analyzed by ELISA. The IL-17 level in Tim-1 vector injected group was significantly lower than control group [Con (n=7) vs. Tim-1 (n=7) (pg/ml): 11.53±4.45 vs. 6.84±2.17, p=0.03]. TNF-α was also significantly decreased in the Tim-1 vector injected group compared to control group [Con (n=11) vs. Tim-1 (n=11) (pg/ml):16.5±13.8 vs. 6.9±5.1, p=0.04]. The IL-6 level in Tim-1 vector injected group was decreased [Con (n=8) vs. Tim-1 (n=8) (pg/ml):145.7±176.9 vs.72.2±38.8, p=0.27]. In contrast, IL-4 was slightly increased in Tim-1 vector injected group [Con (n=8) vs. Tim-1 (n=9) (pg/ml):9.3±5.4 vs. 10.8±5.0, p=0.53]. But IFN-γ did not differ between the Tim-1 and control vector injected group (Fig 7). Consequently, these results indicated that Tim-1 vector might be served as down-regulator for pro-inflammatory cytokines in BD mice.
Figure 7. *Tim-1* vector administration decreased pro-inflammatory cytokines in BD mice. Two weeks after first injection of *Tim-1* vector isolated sera in blood. The levels of IL-17, TNF-α, IL-6, IFN-γ and IL-4 were analyzed in the sera of *Tim-1* and control vector injected BD mice by ELISA. (*n*=7~11) (**p < 0.05) Con: control vector injection to BD mice, Tim-1: *Tim-1* vector injection to BD mice
G. Tim-4 siRNA treatment down-regulated the expression of Tim-4 in normal healthy mice

In BD mice, the frequencies of Tim-4(+) cells were higher than Nor and BDN mice in LN cells and peritoneal macrophages (Fig 2). For down-regulation of Tim-4(+) cells, siTim-4 was injected into Nor mice intraperitoneally and the frequencies of Tim-4(+) cells were analyzed in peritoneal macrophages by FACS. Tim-4 siRNA (siTim-4) at 2, 5 or 10 μg per mouse or negative control (N.C.) scramble siRNA (2 and 5 μg per mouse) were injected [N.C - 5 μg vs. siTim-4 - 2, 5 and 10 μg (n=3~4): 8.9±1.4% vs. 6.6±3.4% (p=0.42), 3.7±0.9% (p=0.013), 2.8±2.2% (p=0.04)] (Fig 8A). siTim-4 down-regulated Tim-4(+) macrophages by dose dependent manner and showed statistical significance at 5 μg and 10 μg administered groups. Therefore, 5 μg injection was used for the following experiment. To see the time dependent efficacy of Tim-4 siRNA, 5 μg of siTim-4 was injected, and after 24, 48, and 72 hours, the frequencies of Tim-4(+) macrophages were analyzed by FACS. Until 72 hours, siTim-4 significantly down-regulated Tim-4(+) macrophages compared to N.C. The frequencies of Tim-4(+) at 48 hours were lowest [N.C vs. siTim-4 (n=2): 24h, 7.9±0.6% vs. 4.9±0.2%, p=0.02; 48h, 8.9±1.4% vs. 2.9±0.4%, p=0.03; 72h, 15.4±0.1% vs. 4.2±3.0%, p=0.03] (Fig 8B).
Figure 8. The expression of Tim-4(+) cells was down-regulated after siTim-4 treatment.

Tim-4 siRNA was applied intraperitoneally and the frequencies of Tim-4(+) cells were analyzed in peritoneal macrophages in Nor mice by FACS. A. siTim-4 2, 5 and 10 μg per mouse or negative control (N.C.) siRNA (2 and 5 μg per mouse) were injected intraperitoneally into Nor mice. B. Five μg of siTim-4 was injected, and after 24, 48, and 72 hours, the frequencies of Tim-4(+) peritoneal macrophages were analyzed. Scramble siRNA was used as negative control (n=3~4) (**p < 0.05). N.C: negative control injection to normal mouse, siTim-4: siRNA Tim-4 injection to normal mouse, Mac: macrophage.
H. Administration of siTim-4 changed the BD-like symptoms

Five μg of siTim-4 was injected intraperitoneally 3 times at 2 days interval into BD mice and followed by observation for 2 weeks (Fig 9A). After administration of siTim-4, BD-like symptoms, such as skin ulcer and genital ulcer were compared to the control group. However, siTim-4 was not effective in eye involvement (Table 2). In negative control group, the severity score was 2.25±0.46 before and 2.00±1.07 at one week (p=0.35), and 1.88±1.13 at two weeks after first injection of BD mice (p=0.28, n=8). In siTim-4 treated group, score was 2.63±0.52 before and 1.25±0.89 at one week (p=0.001), 1.25±0.89 at two weeks after injection into BD mice (p=0.001, n=8) (Fig 9B). At one and two weeks after first administration of siTim-4 into BD mice, macrophages were isolated from peritoneal cavity and analyzed for Tim-4 by FACS. The frequencies of Tim-4(+) cells at one and two weeks after siTim-4 treated group were lower than negative control group even though not significant (1 week: 18.6±4.8% vs. 15.7±3.7%, p=0.31, 2 weeks: 20.7±6.5% vs. 18.8±3.3%, p=0.42) (Fig 9B). However in LN, Tim-4(+), CD11b(+)Tim-4(+), CD11c(+)Tim-4(+), Tim-1(+), CD4(+)Tim-1(+) and CD8(+)Tim-1(+) cells of siTim-4 treated group were similar to control treated group (Fig 9C~D). At one week after first injection of siTim-4, those markers assessed but not different to two weeks (date not shown). In these results, BD-like symptoms were improved with siTim-4 treatment and decreased severity score but the changes of Tim-4 related cellular phenotypes could not be found in this study.
A  

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<td>![Image 15]</td>
<td>![Image 16]</td>
</tr>
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</table>

B  

**severity score**

- N.C.
- siTim-4

![Graph showing severity score over time]

**Tim-4**

<table>
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<th>siTim-4</th>
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C  

**Tim-4/LN**

![Graph showing Tim-4/LN comparison]

**CD11bTim-4/LN**

![Graph showing CD11bTim-4/LN comparison]

**CD11cTim-4/LN**

![Graph showing CD11cTim-4/LN comparison]

D  

**Tim-1/LN**

![Graph showing Tim-1/LN comparison]

**CD4Tim-1/LN**

![Graph showing CD4Tim-1/LN comparison]

**CD8Tim-1/LN**

![Graph showing CD8Tim-1/LN comparison]
Figure 9. BD-like symptoms were changed after siTim-4 administration. Five μg per mouse of Tim-4 siRNA was injected intraperitoneally 3 times at 2 days interval into BD mice and observed for 2 weeks. A. Photographs of mice taken before and at one and two weeks after treatment with siTim-4 and N.C treated group. B. The severity score was compared before and at one and two weeks after treatment between the siTim-4 and N.C treated group. The frequencies of Tim-4(+) cells in peritoneal cavity were compared between siTim-4 and N.C treated groups. C–D. The frequencies of Tim-4(+), CD11b(+)Tim-4(+), CD11c(+)Tim-4(+), Tim-1(+), CD4(+)Tim-1(+) and CD8(+)Tim-1(+) cells in LN were compared to siTim-4 and negative control treated group. (n=7~8) (**p < 0.05) N.C: negative control injection to BD mice, siTim-4: siRNA Tim-4 injection to BD mice, LN: lymph node
Table 2. The change of BD-like symptoms in siTim-4 administered BD mice

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<td>Face and neck crust</td>
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<td>Right ear inflammation and earlobe ulcer</td>
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<td>Left ear inflammation</td>
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<td>4</td>
<td>Right ear inflammation</td>
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<td>Back skin ulcer</td>
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<td>Peri-ocular skin crust</td>
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<td>7</td>
<td>Left hind leg arthritis</td>
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<td>Right eyeball inflammation</td>
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BD symptom: +, deteriorated: ++, improved: -
I. Treg cells were up-regulated in siTim-4 treated BD mice

The frequencies of CD4(+)CD25(+), CD4(+)Foxp3(+) and Treg [CD4(+)CD25(+)] Foxp3(+) cells were also analyzed by FACS at one and two weeks after first treatment with siTim-4 into BD mice. After one week, Treg cells were slightly increased in siTim-4 treated BD mice compared with N.C treated group [N.C vs. siTim-4: CD4(+)CD25(+), 5.82±2.01% vs. 6.84±2.03%, p=0.45, n=5; CD4(+)Foxp3(+), 4.64±1.9% vs. 5.82±2.4%, p=0.41, n=5; Treg, 3.28±1.57% vs. 4.08±1.73%, p=0.47, n=5]. After two weeks, Treg cells were significantly higher in siTim-4 treated BD mice than N.C treated group [N.C vs. siTim-4: CD4(+)CD25(+), 4.8±0.6% vs. 5.9±1.2%, p=0.03, n=6; CD4(+)Foxp3(+), 4.1±0.7% vs. 5.2±1.2%, p=0.03, n=7; Treg, 2.7±0.5% vs. 3.5±0.9%, p=0.04, n=7] From this result, the increases of Treg cells are associated with knock down of Tim-4 in BD mice.
Figure 10. The frequencies of Treg cells were up-regulated after treatment with siTim-4 in BD mice. Treg cells and granzyme B(+) cells were confirmed in LN of BD mice after treatment with siTim-4. At one and two weeks after first treatment with siTim-4, the frequency of CD4(+)CD25(+), CD4(+)Foxp3(+) and Treg [CD4(+)CD25(+)Foxp3(+)] cells were compared between siTim-4 and control treated group. (n=5~7) (**p < 0.05) N.C: negative control injection to BD mice, siTim-4: siRNA Tim-4 injection to BD mice, LN: lymph node.
J. Treatment with siTim-4 decreased the serum level of IL-17 in BD mice

After administration of siTim-4 into BD mice, serum level of IL-17 was analyzed by ELISA and compared with N.C siRNA treated BD mice. The level of IL-17 was decreased in siTim-4 treated group compared to N.C treated group, but not significant [N.C vs. siTim-4 (n=8): 19.4±11.5 pg/ml vs. 15.6±8.1 pg/ml, p=0.25] (Fig 11).
Figure 11. Administration of siTim-4 decreased the serum level of IL-17 in BD mice.

Serum obtained at two weeks after first injection of siTim-4 and N.C in BD mice. The level of IL-17 was analyzed by ELISA. (n=8) N.C: negative control injection to BD mice, siTim-4: siRNA Tim-4 injection to BD mice.
K. Gal-9 treatment up-regulated the expression of Gal-9 in vitro and in vivo

We checked the expression of Gal-9 after Gal-9 treatment in in vitro and in vivo normal healthy mice. Lymph node cells and splenocytes from Nor mice were cultured with or without Gal-9, including anti-CD3 and anti-CD28 antibodies, for 48 h in vitro. In LN cells, the frequencies of Gal-9(+) cells were significantly higher in the Gal-9-treated group than in the non-treated or IgG1-treated control group (non-treated 1.4±0.4% vs. Gal-9-treated 46.7±1.5, p=0.000001; IgG1-treated 1.5±1.2 vs. Gal-9-treated 46.7±1.5, p=0.000002). The frequencies of Gal-9(+) cells in splenocytes were significantly higher in the Gal-9 treated group than in the non-treated group or IgG1-treated group (non-treated 1.3±0.4% vs. Gal-9-treated 64.6±6.2%, p=0.00003; IgG1-treated 1.7±0.8% vs. Gal-9-treated 64.6±6.2%, p=0.00006) (Fig 12A). For in vivo administration, 100 μg per mouse of Gal-9 was injected intraperitoneally once a day for 3 days. One day after the last injection, the frequency of Gal-9(+) cells was compared to those of the PBS-injected control group. The expression of Gal-9(+) cells was higher in lymph node cells of Gal-9 treated mice (45.5±7.4%) compared to control mice (35.5±7.6%) (p= 0.069) (Fig12B). We confirmed that treated with Gal-9 can increase Gal-9 expression in in vitro and in vivo Nor mice.
Figure 12. Gal-9 treatment increased the expression of Gal-9 in vitro and in vivo. Lymph node cells and splenocytes isolated from Nor mice were cultured with anti-CD3 and anti-CD28 antibodies with or without Gal-9 for 48 h. A. The frequencies of Gal-9(+) cells were higher in the Gal-9-treated group than in the non-treated or IgG1-treated group. B. For in vivo treatment, 100 μg of Gal-9 was injected 3 times once a day. One day after the last injection, the frequencies of Gal-9 were higher in the Gal-9 treated group than in the control (PBS) group. (n=5) (*p < 0.1, ***p < 0.01)
**L. Gal-9 administration improved BD-like symptoms**

Gal-9 (100 μg per mouse) was injected intraperitoneally into BD mice 5 times at 3 days interval, followed by observation for 2 weeks. Changes in symptoms are shown in Figure 13A. The BD-like symptoms, such as skin ulcers and arthritis, improved after Gal-9 administration as compared to the control group. The severity score was significantly different between the control (2.4±0.6 to 2.7±0.8) and Gal-9 treated groups (2.8±0.8 to 1.2±0.5) (p=0.016, n=5) (Fig 13B). Two weeks after administration of Gal-9 into BD mice, lymph node cells were isolated and analyzed for Tim-3 by FACS. The frequencies of Tim-3(+) cells were 3.7±0.4% in the Gal-9 treated group and 5.9±1.1% in the control group (p=0.003, n=5) (Fig 13C). In this study, administration of Gal-9 was significantly decreased the frequencies of Tim-3(+) cells in the lymph nodes. These results suggest that the down-regulation of Tim-3(+) cells may be related to the improvement of BD-like symptoms.
Figure 13. Gal-9 treatment improved BD-like symptoms. For each mouse, 100 μg of Gal-9 was injected intraperitoneally five times at 3 days interval, followed by observation for 2 weeks. A. Photographs of mice were taken before and after treatment. B. The severity score was compared before and after treatment between the Gal-9 and control treated groups. C. The frequency of Tim-3(+) cells in Gal-9 treated group was compared with control group in LN by FACS analysis. (n=5) (**p < 0.05, ***p < 0.01) Con: PBS injection to BD mice, Gal-9: Gal-9 injection to BD mice
M. Gal-9 induced the expression of cell death-related molecules in BD mice

To confirm the apoptotic cell death in Gal-9 administered BD mice, lymph node cells were analyzed by flow cytometry after staining with Annexin V and propidium iodide (PI). The frequencies of Annexin V(+) and PI(-) apoptotic cells were 12.0±2.9% in the Gal-9-treated group (n=6) and 6.9±2.4% in the PBS treated control group (n=7) (p=0.006) (Fig 14A). In this study, Gal-9 administration induced apoptosis in lymph node cells in BD mice. Granzyme B induces apoptosis (Saito et al., 2008) and is expressed in resting and activated plasmacytoid dendritic cells, monocytes, resting T-cells, B-cells, activated granulocytes, and activated monocyte-derived dendritic cells (Rissoan et al., 2002). Granzyme B(+) cells were also significantly higher in the Gal-9 treated group (11.0±6.3%) compared to the control group (5.6±3.6%) (p=0.04, n=9) (Fig 14B). CD107a is a marker of functional NK cells (Alter G et al., 2004) and CD8 T cells (Betts et al., 2003). CD107a is a transmembrane glycoprotein found in lysosomal and cell membranes and a marker of degranulation on lymphocytes (Betts et al., 2003). The frequencies of CD107a(+) cells were significantly increased after Gal-9 administration (10.9±4.9%) when compared to the PBS-administered control BD mice (0.4±0.2%) (p=0.001, n=5) (Fig 14C).

For confirmation of apoptosis induced by Gal-9, lymph node cells were cultured for 24 h with Gal-9, after which the frequencies of Tim-3(+) and Gal-9(+) among the apoptotic cells were analyzed by FACS. In apoptotic cells, the Gal-9 treated group showed 42.5±3.5% frequencies of Gal-9(+) cells compared to 17.9±0.9% in the control group (Fig 14D). In the Gal-9-treated group, the frequencies of Tim-3(+) cells were also up-regulated to 1.7±1.1% compared to the control group (0.6±0.5%) (Fig 14D). Further, Gal-9 treatment for 48 h also...
up-regulated the frequencies of CD11b(+)Gal-9(+)CD107a(+) cells lymph node (1.80±0.3\%) compared to the control group (0.20±0.1\%) (Fig 14E). The frequencies of CD11c(+)Gal-9(+)CD107a(+) cells were also higher in the Gal-9 treated group (1.2±0.1\%) compared to the control group (0.2±0.1\%). Gal-9 treatment increased the expression of CD107a(+) in Gal-9(+) macrophages (CD11b) and Gal-9(+) dendritic cells (CD11c). Furthermore, we applied CD11b(+) or Gr-1(+) staining as a neutrophil marker to lymph node cells and splenocytes for FACS analysis (Fig 14F). The lymph node cells and splenocytes isolated from BD mice were treated with Gal-9 (10 μg/ml) for 16 h. Then, anti-CD11b and anti-Gr-1 staining were performed on Annexin V(+) and PI(-) cells in order to measure the frequencies of apoptotic neutrophils. The frequencies of CD11b(+) cells among apoptotic lymph node cells were 11.5±1.2\% in the Gal-9 treated group compared to the control group (8.4±2.4\%). In splenocytes, the frequencies of CD11b(+) cells were 44.5±20.7\% in the Gal-9 treated group compared to the control group (23.3±0.8\%). The frequencies of Gr-1(+) cells among the apoptotic cells were also higher in the Gal-9 treated group (7.7%±2.1%) than in the control group (5.9±0.4\%) among lymph node cells. In splenocytes, the frequencies of Gr-1(+) cells among the apoptotic cells were 29.3±22.1\% in the Gal-9 treated group compared to the control group (23.1±1.3\%) (Fig 14F). The frequencies of Gr-1(+) apoptotic cells were also higher in the Gal-9 treated group than in the control group.

After Gal-9 treatment to BD mice, to determine which cell populations were regulated, additionally, PBMC were isolated from Gal-9-treated BD mice and subjected to transmission electron microscopy (TEM). Gal-9-treated BD mice showed rare neutrophils compared to the control group under TEM (Fig. 14G).
Figure 14. Gal-9 treatment increased the expression of cell death-related molecules in BD mice. A. Annexin V(+) and PI(−) apoptotic cells were higher in the Gal-9 treated group. B. Frequencies of granzyme B(+) cells were also higher in the Gal-9 treated group than in the control group. C. CD107a(+) cells were increased in the Gal-9 treated group compared to the control group. (A-C, n=7–9) D–F. Lymph nodes and spleen were isolated from BD mice, and isolated cells were incubated with 10 μg/mL of Gal-9 for 16–48 h, then applied to FACS. D. Annexin V(+) and PI(−) apoptotic cells were analyzed after staining with anti-Tim-3 or anti-Gal-9 antibody to determine the frequencies of Tim-3(+) or Gal-9(+) cells among the apoptotic cells, respectively. E. In LN, CD11b(+)Gal-9(+)CD107a(+) cells and CD11c(+)Gal-9(+)CD107a(+) cells were up-regulated after Gal-9 treatment. F. CD11b(+) or Gr-1(+) neutrophils in Annexin V(+) and PI(−) apoptotic cells were higher in Gal-9-treated apoptotic cells than in the control group. G. After Gal-9 treatment, PBMC were isolated from the Gal-9-treated group and control group and subjected to transmission electron microscopy (TEM). In Gal-9-treated group, neutrophils were rarely found compared to the control group (×4000) (bar = 2.5 μm). Arrow indicates neutrophil in the control group. (**p < 0.05, ***p < 0.01) Con: PBS injection to BD mice, Gal-9: Gal-9 injection to BD mice
N. Gal-9 modulated the cell population in BD mice

Various cellular phenotypes were regulated after treatment with Gal-9 in BD mice. The frequencies of Treg cells in the Gal-9 treated group were 2.4±0.6% compared to the control group (1.8±0.4%) (p=0.048, n=5) (Fig 15A). The frequencies of plasmacytoid dendritic cells were lower in BD patients than in the healthy control group (Pay S et al., 2007). CD11c(+) dendritic cells in the lymph node were significantly higher in the Gal-9-treated group (1.8±0.4%) compared to the control group (1.2±0.3%) (p=0.033, n=4) (Fig 15B). CD34 is a cell surface glycoprotein that functions as a cell-cell adhesion factor (Satterthwaite et al., 1992). The frequencies of CD34 in the Gal-9-treated group (2.5±1.0%) were significantly lower than the control group (5.6±3.7%) (p=0.025, n=7) (Fig 15C). Gal-9 led to the down-regulation of CD34, resulting in decreased cell adhesion in the inflammatory cells and therefore, changes in the symptoms.
Figure 15. Gal-9 treatment affected the expression of several cell types in BD mice. A. The frequencies of Treg [CD4(+)CD25(+)Foxp3(+)] cells in the Gal-9-treated group were higher than in the control group. B and C. Expression levels of dendritic cells (CD11c) and myeloid suppressor cells (CD34) were compared between the control and Gal-9 treated groups. (n=4–9) (**p < 0.05) Con: PBS injection to BD mice, Gal-9: Gal-9 injection to BD mice.
O. Gal-9 regulated cytokine expression in BD mice

To determine the level of cytokines after Gal-9 treatment in BD mice, sera were analyzed by ELISA. The TNF-α level was significantly lower in the Gal-9 treated group (153.9±33.1 pg/ml) than the control group (206.1±28.7 pg/ml) (p=0.025, n=4). IL-6 was also decreased in the Gal-9 treated group (133.7±65.6 pg/ml, n=12) compared to the control group (219.3±147.00 pg/ml, n=10) (p=0.04). In addition, IL-17 was significantly lower in the Gal-9 treated group (263.5±179.0 pg/ml, n=12) than in the control (500.8±325.1 pg/ml, n=10) (p=0.02). IFN-γ and IL-4 levels did not differ significantly between the Gal-9 and control treated groups (Fig 16).
Figure 16. Gal-9 treatment regulated pro-inflammatory cytokines in BD mice. Pro-inflammatory cytokines were analyzed in the sera of Gal-9 treated BD mice by ELISA. The levels of TNF-α, IL-6, and IL-17 significantly decreased in the Gal-9 treated group compared to the control group. IFN-γ and IL-4 levels were not significantly different between the Gal-9 and control groups. (n=4~12) (**p < 0.05) Con: PBS injection to BD mice, Gal-9: Gal-9 injection to BD mice
Ⅳ. DISCUSSION

According to our study, BD mice display down-regulation of Tim-1 levels and up-regulation of Tim-4 levels, in LN, spleen, PBMC or peritoneal macrophages compared to BDN mice. *Tim-1* vector administration up-regulated Tim-1(+) cells and Tim-4 siRNA down-regulated Tim-4(+) cells. *Tim-1* vector administration improved the BD-like symptoms, such as genital ulcer and skin ulcer, and decreased the severity score. Recently, in active SLE patients, the expression of TIM-1 was lower compared to inactive patients (Wang Y et al., 2008). Human TIM-1 is associated with immune dysfunction, such as atopic dermatitis, allergy, rheumatoid arthritis, asthma and SLE (Chae et al., 2003b; Chae et al., 2003a; Chae et al., 2005; Page et al., 2006; Wang et al., 2008). Our data indicated that after *Tim-1* vector administration, Tim-1 related T cells phenotype did not change. Interestingly, the frequency of CD4(+) T cells in PBMC was increased in *Tim-1* vector injected group compared to control vector injected group. Actually, TIM-1 is expressed on CD4+ T cells after activation and its expression is sustained preferentially on Th2 but not Th1 cells (Kuchroo et al., 2003; Meyers et al., 2005a). Recently, reported that Tim-1-Fc triggered a significantly increase in the frequency of CD4(+) T cells (Xiao et al., 2011). In our study, Treg cells were up-regulated in *Tim-1* vector injected BD group. Another Treg cells, CD8(+)CD122(+) T cells were higher in *Tim-1* vector injected group compared to control group in PBMC and LN. CD8(+)CD122(+) T cells are newly identified and regarded as Treg cells (Saitoh et al., 2007) and reported the effect in anti-inflammatory responses (Rifa‘i et al., 2008). Our study
suggest that the function of Tim-1 influenced on BD-like symptoms, associated with Treg cells and CD8(+)CD122(+) T cells.

Recent studies suggested that IL-17 may play a dominant role in provoking chronic autoimmune inflammation, and it is considered to be essential for T cell-mediated colitis and promotion of inflammation (Cua et al., 2003; Langrish et al., 2005; Yen et al., 2006). Pro-inflammatory cytokine TNF-α over-expression has been implicated in acute and chronic inflammatory diseases, such as septic shock, bowel disease, Crohn’s disease, rheumatoid arthritis, atopic dermatitis, psoriasis, and BD (Edwards, 2004). Overproduction of IL-6 has been shown to play a role in rheumatoid arthritis, juvenile idiopathic arthritis (Nishimoto and Kishimoto, 2008), inflammatory bowel disease (Mudter and Neurath, 2007), and SLE (Chun et al., 2007). Recently reported that anti-Tim-1 antibody (high avidity/agonistic) was determined as a down-regulator of pro-inflammatory Th-17 cells. TIM-1-TIM-4 interaction is involved in the regulation of Th cell responses and the modulation of the Th1/Th2 cytokines balance (Meyers et al., 2005a). Our data also indicate that Tim-1 vector administration decreased the pro-inflammatory cytokines, such as IL-17, TNF-α and IL-6. And Th2 type cytokine, IL-4 was up-regulated in Tim-1 vector injected group.

BD mice display markedly increased Tim-4 levels in LN and peritoneal macrophages compared to Nor and BDN mice. In Tim-4 siRNA administration to Nor mice, the frequencies of Tim-4(+) cells were down-regulated compared to N.C group. siTim-4 administration to BD mice decreased Tim-4(+) cells in peritoneal macrophages even though not significant. siTim-4 administered BD mice displayed improved symptoms such as skin ulcer, genital ulcer and arthritis and also decreased the severity score. But Tim-4(+) cells
related T cell phenotypes were not changed after siTim-4 treatment. TIM-4 is a ligand for TIM-1 (Meyers et al., 2005a) and it is not expressed in T cells but is exclusively expressed in APC, including DCs and macrophages (Rodriguez-Manzanet et al., 2008; Mizui et al., 2008). Recently two groups reported that Tim-4 deficient mice on the 129 or b6 background developed little or no autoimmunity (Wong et al., 2010; Rodriquez-manzanet et al., 2010). According to the improvement of symptoms, Treg cells in siTim-4 treated group were significantly up-regulated compared to control group and IL-17 level was decreased. In our previous study, adoptive transfer of Treg cells into BD mice also improved the BD-like symptoms (Shim et al., 2011). In addition, the frequencies of Treg cells were lower in patients with autoimmune and inflammatory diseases, such as Crohn’s disease (Ricciardelli et al., 2008), multiple sclerosis (Huan et al., 2005), and SLE (Lee et al., 2008), than in healthy controls and inactive patients. TIM-1-TIM-4 interaction is important in regulating the proliferation of T cells and the modulation of the Th1/Th2 cytokines balance (Meyers et al., 2005a). In our data suggested that up-regulated Treg cells induced by siTim-4 might contribute to the improvement of BD-like symptoms.

According to our results, BD mice display markedly elevated Tim-3 levels in lymph node cells compared to BDN mice. In Gal-9-treated Nor mice, the frequencies of Gal-9 were up-regulated. In addition, Gal-9 administration reduced the frequencies of Tim-3(+) cells in BD mice. In Gal-9-treated BD mice, the serum levels of TNF-α, IL-6, and IL-17 were lower than in PBS-injected BD mice. In addition, the severity score of BD mice was lower after Gal-9 injection compared to that of PBS injected group. Therefore, most impressively, Gal-9-administered BD mice improved the BD-like symptoms, such as skin ulcer, oral ulcer, and
arthritis. Overproduction of Tim-3 has also been shown to play a role in rheumatoid arthritis (Liu et al., 2010) and inflammatory bowel disease (Li et al., 2010). Gal-9 administration has beneficial effects on rheumatoid arthritis (Seki et al., 2007), psoriasis (Niwa et al., 2009), lung inflammation (Arikawa et al., 2010), diabetes (Chou et al., 2009), asthma (Katoh S et al., 2007), and in an EAE model (Zhu et al., 2005). And TIM-3 expression cells in PBMC with BD patients increased healthy control (Lee et al., 2012). Gal-9 induces the termination of Th1 immune responses through binding to Tim-3. Moreover, Gal-9 plays a role as a mediator, contributing to the development of allergic airway inflammation. Gal-9 may serve as a recruiter of eosinophil granulocytes and as a promoter of Th2 dominance (Sziksz et al., 2010). Therefore, balance expression of the Tim-3 and Gal-9 may play important role in homeostasis of inflammatory reactions and amelioration of BD inflammation.

A strong Th1 immune response occurs in active BD and contributes to the prolonged inflammatory condition of BD (Frassanito et al., 1999; Sohn et al., 2001). Tim-3 was originally identified as a mouse Th1-specific cell surface protein that was expressed after several rounds of in vitro Th1 differentiation (Monney et al., 2002; Sánchez-Fueyo et al., 2003). It was later shown to also be expressed on Th17 cells (Hastings et al., 2009). Previously, we have also shown that highly expressed Th17 in inflammation of BD mice (Shim et al., 2009). In this study, we confirmed that Tim-3 expression was also correlated to up-regulation of IL-17 in BD mice.

It has been reported that Gal-9 has a beneficial effect on rheumatoid arthritis by inducing apoptosis in synovial fibroblasts (Seki et al., 2007) and Th1 cells in experimental autoimmune encephalomyelitis (Zhu et al., 2005). In our experiment, Gal-9 treated BD mice
also showed higher frequencies of apoptosis in lymph node cells. Granzyme B is an important effector molecule in the granule-mediated killing pathway of cytotoxic T lymphocytes and NK cells (Lord et al., 2003; Trapani et al., 2003). Gal-9 treated BD mice showed increased frequencies of granzyme B(+) cells in lymph nodes when compared to control. CD107a is known as a lysosomal-associated membrane protein (LAMP-1) and has been shown to be a marker of degranulation on lymphocytes such as CD8(+) and NK cells (Chang et al., 2002; Esin et al., 2009). Gal-9 treated BD mice significantly up-regulated CD107a(+) cells in lymph nodes. Treg cells have been found to play a role in maintaining immunological self-tolerance, which leads to a decrease in the spontaneous development of various autoimmune diseases (Takahashi et al., 1998). It has also been reported that the frequencies of CD4(+)CD25(+) Treg cells were lower in patients with autoimmune and inflammatory diseases, such as Crohn’s disease (Ricciardelli et al., 2008), multiple sclerosis (Huan et al., 2005), and SLE (Lee et al., 2008), than in healthy controls and inactive patients. In this study, Gal-9 administration up-regulated the frequencies of Treg cells in the lymph nodes of BD mice compared to the PBS-administered group. Recently, it was reported that adoptive transfer of CD4(+)CD25(+) Treg cells ameliorated the symptoms of BD mice (Shim et al., 2011). Gal-9 increased the number of DCs (Nagahara et al., 2008) and induced maturation of monocyte-derived DCs (Dai et al., 2005). Gal-9 increased CD11c, a marker of dendritic cells (O’Keeffe et al., 2003), in the lymph nodes of BD mice. CD34 is a marker of cell-cell adhesion and was found on hematopoietic cells (da Silva et al., 2010). CD34 also interacts with L-selectin (CD62L), an important factor in inflammation (Koenig et al., 1999), and is localized by endothelial cells of microvessels in rheumatoid and osteoarthritic synovia.
and Crohn’s disease colon (Middleton et al., 2005). In BD patients, CD34 was highly expressed in oral aphthous lesions (Yalçın et al., 2006). In this study, the frequencies of CD34(+) cells were significantly down-regulated after treatment with Gal-9 in BD mice.

The improvement of inflammatory symptoms was reported in BD mice after TNF-α inhibition through TNF-α siRNA treatment (Choi et al., 2008). Concordant to this, the Gal-9-treated group had a significantly lower serum level of TNF-α than the control group and showed improvement of symptoms. IL-6 levels in cerebrospinal fluid were previously found to be dramatically decreased in all methotrexate-treated patients. Low-dose methotrexate therapy has a beneficial effect in the treatment of progressive neuropsychiatric BD (Hirohata et al., 1998). IL-6 down-regulation with IL-6 siRNA also shows a therapeutic effect in this BD mouse model (Shim et al., 2009). The serum level of IL-6 was also significantly down-regulated in Gal-9-injected BD mice. IL-6 participates in the generation of Th17 cells from naïve T cells, and TNF-α in combination with TGF-β expands the Th17 cell pool. In BD mice, lower serum IL-17 and lower mRNA levels of IL-17A and IL-17F were accompanied by improved symptoms. Furthermore, Gal-9 administration significantly down-regulated the serum level of IL-17 in BD mice.
V. CONCLUSION

In conclusion, the frequencies of Tim-1(+) cells in BD mice were down-regulated compared to BDN mice. *Tim-1* vector administration to BD mice improved the BD-like symptoms and decreased the severity score through up-regulation of Treg cells and down-regulation of pro-inflammatory cytokines. In addition, treatment with siTim-4 up-regulated Treg cells, and improved the BD-like symptoms, which were related to the expression of Tim-1 and Tim-4 with Tim-1-Tim-4 interaction. Furthermore, Tim-4 siRNA down-regulated the level of IL-17, might be involved to the improvement of BD-like symptoms. Consequently, regulation of Tim-1 and Tim-4 was effective to improve the BD-like symptoms in mice.

The frequencies of Tim-3 in BD mice were significantly higher than normal and BDN mice. On the other hand, the frequencies of Gal-9 in BD mice were lower than in normal and BDN mice. After treatment with Gal-9, BD-like symptoms were improved, and the disease severity score was decreased. Gal-9 induced expression of cell death-related molecules, such as Annexin V(+) PI(-) apoptotic marker, granzyme B, and CD107a were increased in BD mice. In addition, Gal-9 increased Treg cells, and CD11c(+) DCs, but decreased cell-cell adhesion factor CD34(+) cells. Additionally, Gal-9 also decreased pro-inflammatory cytokines such as TNF-α, IL-6, and IL-17 in BD mice. Taken together, these findings indicate that Gal-9 could be a potential therapeutic agent for chronic inflammatory diseases, including BD.
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단순포진 바이러스로 유도한 베체트병 마우스 모델에서 T cell immunoglobulin mucin domain (Tim)의 역할

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T cell immunoglobulin mucin domain (TIM) 단백질은 T 세포의 활성과 tolerance를 조절한다. TIM family는 천식, 알레르기, 자가면역질환에 대한 감수성이 높은 표식자로 알려져 있다. TIM-1은 면역반응 조절과 자가면역질환을 일으키는데 중요한 역할을 하며, TIM-4는 TIM-1의 ligand로 알려져 있다. Tim-1과 Tim-4의 상호작용은 Th 세포 반응의 조절과 Th1/Th2 사이토카인의 균형을 조절한다고 보고되었다. TIM-3와 TIM-3 ligand인 galectin-9 (Gal-9)의 상호작용은 Th1 면역반응을 특이적으로 저해한다고 알려져 있다.

베체트병은 만성 염증질환으로 재발성 구강궤양, 외음부궤양, 피부 및 안증상 등이 특징적인 질병이다. 베체트병은 여러증상들이 동시에 발병하거나 초기 피부 점막 증상이 호전과 악화를 거듭하다 관절 증상, 안 증상, 신경계 증상까지 발전할 수 있다. 특히 안 증상은 대표적인 베체트병 주 증상으로 설명이라는 심각한 후유증을 초래할 수 있다. 이러한 증상을 조절할 수 있는 방법으로, 본

본 연구에서는, Tim-1과 Tim-4 발현 수준을 조절하여 베체트병 마우스 모델의 증상을 호전시키고, Tim-3L 증가에 의한 Tim-3-Tim-3L 상호작용이 베체트병 마우스모델의 염증 증상의 호전에도 영향을 주는 것을 확인하였다.

**Key words:** Tim-1, Tim-3, Tim-4, Galectin-9, 베체트병 마우스 모델, 단순포진바이러스