ORGINAL ARTICLE

Protective effects of Fc-fused PD-L1 on two different animal models of colitis

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ABSTRACT
Objective Programmed death-ligand 1 (PD-L1) has been shown to negatively regulate immune responses via its interaction with PD-1 receptor. In this study, we investigated the effects of PD-L1-Fc treatment on intestinal inflammation using two murine models of inflammatory colitis induced by dextran sulfate sodium (DSS) and T-cell transfer.

Design The anti-colitis effect of adenovirus expressing Fc-conjugated PD-L1 (Ad/PD-L1-Fc) and recombinant PD-L1-Fc protein was evaluated in DSS-treated wild-type and Rag-1 knockout (KO) mice. We examined differentiation of T-helper cells, frequency of innate immune cells, and cytokine production by dendritic cells (DCs) in the colon from DSS-fed mice after PD-L1-Fc administration. In Rag-1 KO mice reconstituted with CD445RBhigh T cells, we assessed the treatment effect of PD-L1-Fc protein on the development of colitis.

Results Administration of Ad/PD-L1-Fc significantly ameliorated DSS-induced colitis, which was accompanied by diminished frequency of interleukin (IL)-17A-producing CD4 T cells and increased interferon-γ-producing CD4 T cells in the colon of DSS-fed mice. The anti-colitic effect of PD-L1-Fc treatment was also observed in DSS-treated Rag-1 KO mice, indicating lymphoid cell independency. PD-L1-Fc modulated cytokine production by colonic DCs and the effect was dependent on PD-1 expression. Furthermore, PD-L1-Fc protein could significantly reduce the severity of colitis in CD4 CD45RBhigh T-cell-transferred Rag-1 KO mice.

Conclusions Based on the protective effect of PD-L1-Fc against DSS-induced and T-cell-induced colitis, our results suggest that PD-1-mediated inhibitory signals have a crucial role in limiting the development of colonic inflammation. This implicates that PD-L1-Fc may provide a novel therapeutic approach to treat inflammatory bowel disease.

INTRODUCTION
Inflammatory bowel disease (IBD) is a chronic gastrointestinal disorder which is commonly classified into Crohn’s disease (CD) and ulcerative colitis (UC) which affects the whole gastrointestinal tract and the colon only, respectively. Although the precise aetiology of IBD is not fully understood, it is generally thought to result from complex interplay among genetic susceptibility, host immune system dysregulation, and environmental factors such as intestinal microbial flora.

Colitis induced by dextran sulfate sodium (DSS) is similar to human UC and is shown to respond to anti-colitic agents used for human UC, such as sulfasalazine, olsalazine and mesalazine. DSS administration leads to increased intestinal permeability and subsequent invasion of intestinal microflora through epithelial cell damage. Mice treated with 3% DSS for 6 days showed significant influx of innate cell populations into the colon (neutrophils from 1 to 8 days; DCs and macrophages from 5 to 12 days) followed by accumulation of T and B cells during late phage (from 8 to 25 days post DSS treatment). However, the role of lymphocytes in DSS colitis remains controversial. For instance, a previous study suggested that DSS administration in DSS-treated CD1 nu/nu and natural killer (NK) cell depleted SCID mice could generate qualitatively analogous colitis, as observed for wild-type (WT) mice treated with DSS. Conversely, another report


How might it impact on clinical practice in the foreseeable future?
These data suggest the use of PD-L1-Fc as a novel approach for treating human IBD.

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showed that Rag-1 knockout (KO) mice displayed less severe colitis than WT mice upon DSS administration, suggesting that lymphocytes play roles in exacerbating DSS-induced colitis. As another animal model of IBD, transfer of naïve CD4 CD45RB<sup>hi</sup> T cells into immunodeficient mice develops chronic colitis resembling human CD, in which interferon (IFN)-γ<sup>+</sup> T-helper (Th) 1 cells and interleukin (IL)-23/Th17 cells have been respectively implicated in induction and development of colitis. The T-cell transfer model of colitis has been extensively used to evaluate preventive and therapeutic effects of various IBD drug candidates.

Biological therapies for IBD have been developed to target pro-inflammatory and anti-inflammatory cytokines, leukocyte migration and T-cell signalling. Infliximab, an anti-tumour necrosis factor (TNF)-α agent, was the first biological agent to gain approval by the US Food and Drug Administration for treating CD and UC. Since then, infliximab has been extensively used for IBD therapy over the past decade. However, there have been concerns about lower response rates and decreased efficacy with repeated anti-TNF-α administration in patients with IBD. Other biological agents including CTLA-4-Ig, IL-10 and monoclonal antibodies (mAbs) against IL-12/IL-23 p40, IL-6, IL-2 receptor and integrins have also been evaluated in various clinical settings. However, these agents have raised safety issues, such as increased risk of infection, malignancy, and autoimmunity, suggesting the need for a novel therapeutic agent with enhanced safety and minimal toxicity.

Programmed death 1 (PD-1), which is expressed on T and B cells, macrophages and DCs upon their activation, has been shown to play a critical role in regulating peripheral tolerance and autoimmunity. Previous studies have shown that PD-1-deficient mice developed spontaneous autoimmune diseases, such as lupus-like glomerulonephritis, arthritis and cardiomyopathy. Engagement of PD-1 with its ligands, PD-L1 and PD-L2 delivers inhibitory signals to T cells in chronic infections and tumours. Moreover, PD-1 and its ligands negatively regulate Th17 cells that play pathogenic roles in the development of autoimmune diseases. Administration of soluble PD-L1-Ig protein has been reported to diminish the severity of collagen-induced arthritis in the mouse and inhibit cell proliferation and production of IL-17 and IL-23 by splenocytes. In a chronic colitis model, adoptive transfer of non-colitic CD4 cells with high PD-1 expression into immunodeficient mice resulted in significantly less production of IFN-γ, IL-17 and IL-10, and suppression of intestinal inflammation compared with mice transferred with colitic CD4 CD45RB<sup>hi</sup> cells. Also, the lack of PD-1 expression on splenic DCs or macrophages led to enhanced innate protection against bacterial infection, demonstrating that the PD-1 pathway inhibits innate immune responses by modulating DC and macrophage functions, and adaptive immune responses mediated by T cells.

Since the expression of PD-1 and PD-1 ligands is significantly upregulated in inflamed colon from humans with IBD and mice with chronic colitis, the PD-1-PD-Ls pathway may also be involved in the pathogenesis of intestinal mucosal inflammation. In this study, we found for the first time that administration of nonlytic Fc-conjugated PD-L1 (PD-L1-Fc), either in the form of recombinant protein or by utilising adenoviral vector as a delivery vehicle, could ameliorate DSS-induced and T-cell-induced colitis. The therapeutic effect of PD-L1-Fc in DSS-induced colitic mice was associated with decreased frequency of Th17 cells, increased production of IL-10-producing CD4 T cells, and modulation of cytokine secretion by DCs in the colon. Furthermore, we demonstrated that PD-L1-Fc has a role in impairing DC function through the interaction with PD-1 that is expressed upon various stimuli, including toll-like receptor (TLR) ligands such as lipopolysaccharide (LPS) and CpG.

**MATERIALS AND METHODS**

**Mice**

CS7BL/6 (B6) and Rag-1 KO mice of B6 backgrounds were purchased from Jackson Laboratory. PD-1 KO mice of B6 background were kindly provided by Professor Sang-Nae Cho (Yonsei University). Mice were bred and maintained at the Pohang University of Science and Technology animal facility.

**Recombinant adenoviruses and recombinant proteins**

The construction of adenoviruses (Ad) expressing enhanced green fluorescent protein (Ad/EGFP), mouse nonlytic Fc-fused mouse PD-L1 (amino acids (aa) 1–238) (Ad/PD-L1-Fc), and mouse nonlytic Fc-fused tissue plasminogen activator (tPA)-human CTLA-4 (aa 37–161) (Ad/CTLA-4-Fc) was performed as previously described. In vitro and in vivo expression of each adenovirus was confirmed by immunoblotting and ELISA, respectively.

Recombinant PD-L1-Fc protein was provided by Genexine Co. Recombinant human CTLA-4-Ig protein (Abatacept, Ocrelizumab) was purchased from Bristol-Myers Squibb.

**Induction and assessment of DSS-induced colitis**

DSS colitis was induced as previously described. Plaque-forming units (PFU) of Ad/EGFP, Ad/PD-L1-Fc or Ad/CTLA-4-Fc was administered intraperitoneally on days 0, 3 and 6. In other experiments, 30 μg PD-L1-Fc protein was injected intraperitoneally on day 2. The colon length was measured at day 9. H&E-stained sections (4 μm) of colon were obtained for histological analysis as previously described.

**Isolation of colonic lamina propria cells and flow cytometry analysis**

Lamina propria (LP) cells were obtained from colonic specimens using previously described protocols. For analysis of colonic CD4 Foxp3<sup>+</sup> regulatory T (T reg) cells, LP cells were stained with anti-CD4 and Foxp3 antibodies using Foxp3 staining buffer kit (eBioscience). For intracellular cytokine staining, LP cells were restimulated with 50 ng/mL PMA and 750 ng/mL ionomycin (all from Sigma) for 5 h and then stained for CD4, IFN-γ and IL-17A. Otherwise, LP cells were stained with anti-major histocompatibility complex (MHC) class II, CD11b, F4/80, CD11c, Ly-6G (BD Pharmingen) and PD-1 (BioLegend) antibodies. All antibodies were from eBioscience unless otherwise indicated. Samples were acquired on a Gallios (Beckman Coulter), and the data were analysed with FlowJo software (Tree Star).

**Cytokine ELISA**

CD4 TCR<sup>+</sup> LP cells and MHC II<sup>+</sup> CD11c<sup>+</sup> LP cells were sorted (>95% purity) by flow cytometry (MoFlo). CD4 T cells were restimulated with 5 μg/mL anti-CD3 and 2 μg/mL anti-CD28 mAbs (all from BD Pharmingen). CD11c<sup>+</sup> DCs from colon and spleen were isolated by magnetic separation using anti-CD11c microbeads (Miltenyi Biotec). Splenic DCs were restimulated with 1 μg CpG-ODN 1668 (InvivoGen). Splenic DCs were stimulated with 100 ng/mL LPS (Invitrogen). Cytokine concentrations in cell culture supernatants were measured by ELISA.
Induction of T-cell-induced colitis and clinical observation

T-cell transfer colitis was induced as previously described.28 From 3 weeks after T-cell transfer, Rag-1 KO mice were intraperitoneally treated with phosphate-buffered saline (PBS), 20 μg PD-L1-Fc protein at weekly intervals, or 200 μg CTLA-4-Ig protein three times per week for 4 weeks. Clinical scoring was performed as previously described.29

Statistical analysis

Data are typically expressed as the mean±SEM, and the differences between groups were analysed using either unpaired two-tailed Student’s t test or one-way ANOVA with Bonferroni correction. A value of p<0.05 was considered statistically significant.

RESULTS

Treatment with PD-L1-Fc ameliorates acute DSS-induced experimental colitis in mice

To investigate the effect of PD-L1-Fc on colitis, we generated recombinant adenovirus expressing mouse nonlytic Fc-fused mouse PD-L1 (Ad/PD-L1-Fc) and confirmed the expression of PD-L1-Fc in vitro and in vivo (see online supplementary figure S1A,B). Adenovirus-mediated gene transfer via intraperitoneal injection is known to induce gene expression in the intraperitoneal tissues, including liver, pancreas, spleen, and mesenterium.30 The concentration of PD-L1-Fc in the serum from B6 WT mice was 1.3±0.1 μg/mL on day 1 after the intraperitoneal treatment with Ad/PD-L1-Fc at 1×108 PFU and decreased after 7 days of treatment. Rag-1 KO mice sustained high level of serum PD-L1-Fc, reaching up to 3.1±0.2 μg/mL at day 21.

To evaluate whether PD-L1-Fc plays a protective role in DSS-induced colitis, 2% DSS-treated B6 mice were treated with Ad/PD-L1-Fc, negative control Ad/EGFP or PBS. As soluble CTLA-4 has been used in clinical trials for transplant rejection and autoimmune diseases,31 we used CTLA-4-Fc-expressing adenovirus as a positive control. As expected, DSS-induced colitic mice treated with PBS or Ad/EGFP showed progressive weight loss from day 5 to day 9 and exhibited shortened colon lengths (figure 1A,B). In contrast, the injection of Ad/PD-L1-Fc or Ad/CTLA-4-Fc during colitis induction significantly ameliorated body weight loss and colon shortening. Histological analysis of the colon from Ad/EGFP-treated colitic mice showed severe inflammation with loss of crypts and infiltration of inflammatory cells, whereas the administration of either Ad/PD-L1-Fc or Ad/CTLA-4-Fc alleviated histological colonic damage having no preferential effect on histological subcores for inflammatory infiltrate and epithelial damage in colon (figure 1C). It is worth noting that Ad/PD-L1-Fc-treated mice had statistically significant longer colon length and lower histologic injury compared with Ad/CTLA-4-Fc-treated mice, albeit similar in weight loss, suggesting that PD-L1-Fc is more potent than CTLA-4-Fc in mitigating acute DSS-induced colitis.

PD-L1-Fc affects the differentiation of colonic T-helper cells in DSS-induced colitis

IL-17A is thought to play a major role in the pathogenesis of IBD as its expression is increased in the serum of patients with CD and UC, and acute murine models of DSS colitis.32 Direct evidence for the role of IL-17A in IBD was provided in a previous study that showed the attenuation of DSS-induced colitis in mice lacking the IL-17A gene.33 Thus, we investigated whether Ad/PD-L1-Fc treatment could modulate the relative production of IL-17A, IFN-γ and IL-10 by colonic T cells and the frequency of Treg cells in the colon of DSS-treated mice. In contrast to Ad/EGFP injection, the administration of Ad/PD-L1-Fc markedly reduced the percentage of CD4 T cells producing IL-17A in the colonic LP cells derived from DSS-treated mice following stimulation with PMA plus ionomycin, while the percentage of IFN-γ-producing CD4 T cells was enhanced (figure 2A). This result suggests that PD-L1-Fc may inhibit Th17 responses but promote Th1 responses during development of DSS-induced colitis. However, the colonic LP CD4 Foxp3+ Treg population that expresses CD2527 remained unaffected by Ad/PD-L1-Fc treatment, presumably due to the suppression of acute DSS colitis.34 Also, sorted colonic CD4 T cells from Ad/PD-L1-Fc-treated mice secreted a low level of IL-17A and high levels of IFN-γ and IL-10 upon restimulation with anti-CD3 and anti-CD28 mAbs (figure 2B). These results agree with a previous report that the engagement of PD-L1 with PD-1 expressed on T cells inhibits the development of Th17 cells in a Foxp3-independent manner and ameliorates Th17 cell-mediated autoimmune encephalomyelitis.35

PD-L1-Fc diminishes the frequency of innate immune cells in the colon of DSS-treated Rag-1 KO mice

When the effect of PD-L1-Fc was examined in DSS-treated Rag-1 KO mice lacking B and T cells, similar therapeutic efficacy was observed in terms of percentage body weight, colon length and histological injury compared with WT mice (figure 3A,B, left panel; figure 3C, upper panel), implicating that non-lymphoid cells may play a key role in PD-L1-Fc-mediated amelioration of DSS-induced colitis. Therapeutic efficacy of PD-L1-Fc was also confirmed by using recombinant protein (figure 3A,B, right panel; figure 3C, bottom panel). Therefore, PD-L1-Fc can exert a protective effect on DSS-induced mouse colitis in a delivery method independent manner.

Since DSS colitis is characterised by the infiltration of inflammatory cells into the colon, we analysed the effect of Ad/PD-L1-Fc treatment on the recruitment of innate immune cells, including macrophages, neutrophils and DCs in Rag-1 KO mice treated with DSS. In colitic Rag-1 KO mice injected with Ad/EGFR, the percentage of DCs and neutrophils was significantly increased while the frequency of macrophages was decreased in the colonic LP on day 5 compared with those of DSS-untreated mice (figure 4). Interestingly, treatment of Ad/PD-L1-Fc resulted in a decrease in all of the above-mentioned innate immune cells, especially in DCs, in the diseased colons of Rag-1 KO mice. These results suggest that PD-L1-Fc could inhibit the recruitment of innate immune cells into the colon during DSS-induced colitis.

PD-L1-Fc modulates the production of cytokines by colonic DCs expressing PD-1

Since PD-1 plays a role in attenuating DC function in protection against bacterial infection,32 we investigated whether Ad/PD-L1-Fc treatment could also alter the functions of colonic DCs during the development of DSS-induced colitis. When we measured the capacity of colonic DCs to secrete various cytokines, the production of cytokines, including IL-1β, IL-6, TNF-α, IL-10, IL-12p40, IL-12p70 and IL-23, by sorted CD11c+ colonic DCs from DSS-treated mice was significantly elevated by CpG restimulation (figure 5), which is consistent with a previous report that showed the dependence of acute DSS colitis development on DC activation.36 Interestingly, the administration of Ad/PD-L1-Fc to DSS colitis mice led to a profound decrease in the levels of IL-1β, IL-6, TNF-α and IL-10, while those of IL-12p40 and IL-12p70 were markedly increased
and that of IL-23 remained unaffected. These results indicate that PD-L1-Fc has a role in modulating cytokine production by DCs at the sites of colonic inflammation.

To further elucidate whether the effect of PD-L1-Fc on DC responses is mediated by PD-1 expression on DCs, CD11c + colonic DCs and CD11c + splenic DCs were isolated from naive WT or PD-1 KO mice and then stimulated in vitro with TLR ligands in the presence or absence of recombinant PD-L1-Fc protein. For the analysis of cytokine production by DCs, colonic and splenic DCs were respectively stimulated with CpG and LPS, owing to their differential expression levels of TLRs. We found that PD-L1-Fc protein exhibited reduced release of cytokines such as IL-6 and TNF-α in response to CpG stimulation compared with untreated WT colonic DCs (figure 6A). However, these effects were not

Figure 1  Effects of Fc-conjugated programmed death ligand 1 (PD-L1-Fc) treatment on the development of dextran sulfate sodium (DSS)-induced acute colitis in B6 mice. C57BL/6 mice received either 2% DSS-containing (DSS-positive) or regular drinking water alone (DSS-negative) for 9 days. DSS-treated mice were injected intraperitoneally with either phosphate-buffered saline (PBS) or recombinant adenovirus expressing enhanced green fluorescent protein (Ad/EGFP), PD-L1-Fc (Ad/PD-L1-Fc) or CTLA-4-Fc (Ad/CTLA-4-Fc) at a dose of 1×10⁸ plaque-forming units (PFU) on days 0, 3 and 6. (A) Change in weight over time is expressed as the percentage of the initial body weight. Student’s t test, *p<0.05; **p<0.01 compared with DSS(+)+Ad/EGFP group at day 9. (B) The colon length was measured at day 9. (C) 9 days after DSS treatment, representative H&E-stained colon sections are shown (original magnification×100) and histological score was calculated. *p<0.05; **p<0.01 in a one-way analysis of variance followed by Bonferroni correction. Results are representative of the three experiments performed.
observed in PD-1 KO colonic DCs. Moreover, a similar pattern of results was observed in splenic DCs stimulated with LPS, suggesting that cell surface PD-1 receptor expression is a crucial factor for PD-L1-Fc-mediated suppression of DC activation (figure 6B). Collectively, our findings indicate that PD-1-mediated signalling plays a major role in the modulation of cytokine production by DCs regardless of their source and the type of stimulus.
Figure 3  Effects of Fc-conjugated programmed death ligand 1 (PD-L1-Fc) treatment on the development of dextran sulfate sodium (DSS)-induced acute colitis in lymphocyte-deficient Rag-1 knockout (KO) mice. Rag-1 KO mice received either 2% DSS-containing or regular drinking water alone for 9 days. (A and B, left panel; C, upper panel) adenovirus expressing enhanced green fluorescent protein (Ad/EGFP) or Ad/PD-L1-Fc was injected intraperitoneally into DSS-treated mice at a dose of 1×10^8 plaque-forming units on days 0, 3 and 6. (A and B, right panel; C, lower panel) DSS-treated mice were injected intraperitoneally on day 2 with phosphate-buffered saline (PBS) or with 30 μg of PD-L1-Fc protein. (A) Weight change of individual mice is expressed as the percentage change from day 0. Student’s t test, **p<0.01 at day 9. (B) On day 9, colons were removed, and their length was measured. (C) 9 days after DSS treatment, colons were stained with H&E and histologically scored. Original magnification×100. **p<0.01 in a one-way analysis of variance followed by Bonferroni correction. The data are representative of three independent experiments.
Administration of recombinant PD-L1-Fc protein attenuates the severity of T-cell-induced colitis

We further explored whether treatment of PD-L1-Fc could inhibit chronic colitis induced by transfer of CD4 CD45RB<sup>high</sup> (naive) T cells into lymphocyte-deficient mice. Three weeks after T-cell reconstitution, recombinant PD-L1-Fc protein was administered at 1-week intervals at a dose of 20 μg into Rag-1 KO mice for 4 weeks. Since CTLA-4-Ig, when used in intervention mode, was previously known to suppress T-cell transfer colitis in SCID mice, 7 another group of mice was treated three times a week with 200 μg CTLA-4-Ig protein over the same time period. At 7 weeks post cell transfer, PD-L1-Fc treatment led to marked amelioration of weight loss, and the degree of protection was similar to CTLA-4-Ig (figure 7A). Notably, significant attenuation of clinical symptoms and histological damage in colon was seen in PD-L1-Fc-treated Rag-1 KO mice (figure 7B,C). In this T-cell transfer model of colitis, PD-L1-Fc exerted a more profound inhibitory effect on the production of IFN-γ, IL-17A and IL-10 by CD3/CD28-restimulated colonic LP cells than CTLA-4-Ig (figure 7D). Taken together, our results indicate that PD-L1-Fc exerts a protective effect in various murine models of IBD, including acute DSS colitis and chronic T-cell colitis.

DISCUSSION

The PD-1:PD-Ls pathway has been reported to be crucial for the induction of immune tolerance and the establishment of
therapeutic strategy for autoimmune diseases. Engagement of PD-L1 or PD-L2 with their receptor, PD-1, on T cells leads to downregulation of T-cell responses by mediating programmed cell death and inhibiting cytokine secretion.38 In this study, we demonstrated for the first time that PD-L1-Fc is even more effective than CTLA-4-Fc in treating DSS-induced colitis and CD4 CD45RBhigh T-cell-mediated colitis. Superior potency of PD-L1-Fc may be attributed to the presence of two binding partners, PD-1 and B7-1, as CTLA-4-Fc can only interact with B7-1 to deliver inhibitory signals. Although the affinity of interaction between B7-1 and PD-L1 (dissociation constant (KD) of 1.7 μM) is lower than that of B7-1 for CTLA-4 (KD of 0.2 μM), the additional interaction between PD-1 and PD-L1 (KD of 0.5 μM) may compensate for this lower affinity.39 The importance of PD-1 in the context of the negative feedback mechanism for T-cell responses may also be reflected by the induction of PD-1 expression on activated T cells. PD-1-mediated signals in activated T cells are known to result in decreased proliferation, survival and IL-2 production.13 PD-1 is also expressed on activated innate immune cells, such as DCs and macrophages, and inhibits cytokine production. In support of this observation, our study shows that cytokine (IL-1β, IL-6, TNF-α and IL-10) production from colonic DCs was impaired by PD-L1-Fc and its inhibitory effect was dependent on PD-1 expression in DCs. In addition, B7-1 expression is also induced in activated T cells, and its interaction with PD-L1 is shown to suppress IFN-γ secretion by these T cells.39 B7-1 is also expressed constitutively on DCs and macrophages and is upregulated upon the activation of these cells. In previous reports, treatment of CTLA-4-Ig generated tolerogenic DCs through the ligation of CTLA-4 by B7-1 expressed on DCs and induced indoleamine-pyrrole 2, 3-dioxygenase production in these DCs.40 41 Although there is no direct evidence for the role of B7-1:PD-L1 interaction in DC modulation, judging from its previously known inhibitory functions in T cells,42 we can speculate that B7-1 expressed on DCs may also transduce inhibitory signals via interaction with PD-L1.

It has been shown that PD-L1:PD-1 interaction inhibits the differentiation of Th17 cells. Engagement of PD-L1 expressed on mesenchymal stem cells (MSCs) and IL-27 primed CD4 T cells with PD-1 exerts selective suppressive effects on Th17 cell

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**Figure 5** Effects of Fc-conjugated programmed death ligand 1 (PD-L1-Fc) on cytokine production from colonic dendritic cells (DCs). On day 5 after dextran sulfate sodium (DSS) administration, major histocompatibility complex (MHC) class II+ CD11c+ were sorted from colonic lamina propria (LP) cells of normal or adenovirus expressing enhanced green fluorescent protein (Ad/EGFP) or Ad/PD-L1-Fc received colitic Rag-1 knockout (KO) mice. Sorted LP-DCs (8×10⁴) were restimulated with 1 μM CpG for 24 h. Interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)-α, IL-10, IL-12p40, IL-12p70 and IL-23 concentrations in culture supernatants were measured by ELISA. Data represent the mean±SEM of four mice per group. Student’s t test, *p<0.05; **p<0.01 versus Ad/EGFP-treated colitis group. Three independent experiments showed similar results.
induction. PD-L1 blockade with anti-PD-L1 neutralising antibody reversed the inhibition of mature Th17 cells by MSCs, while soluble PD-L1 fusion protein suppressed IL-17 production by CD4 T cells under Th17 cell-polarizing conditions. This is in accordance with our observation that treatment of DSS colitic mice with PD-L1-Fc led to a preferential suppression of pathogenic Th17 cells in the colon. The pathogenicity of Th17 cells in the development of IBD is supported by the observation that IL-17 expression is elevated in patients with CD or UC. Additionally, it was reported that the deficiency of IL-17 resulted in reduced disease severity in the DSS-induced colitis model. Unlike colonic Th17 cells, IFN-γ-producing colonic CD4 T cells were increased by PD-L1-Fc in our DSS-induced colitis model. These findings are in line with those of our previous study about the anti-colitic effects of IL-12p40 that was associated with decreased frequency of Th17 cells and increased frequency of Th1 cells. A possible explanation for the increase in colonic Th1 cells by PD-L1-Fc treatment might be related to counter-reaction of Th1 and Th17 cells, as shown in a previous study of patients with rheumatoid arthritis in which IL-17 suppressed development of Th1 cells by reducing IL-12Rβ2 expression in peripheral blood mononucleated cells. Accordingly, we speculate that preferential suppression of Th17 cells by PD-L1-Fc may promote the development of colonic Th1 cells. Moreover, our results are in line with a previous study showing that IL-23/IL-17 elicited tissue-specific function in the pathogenesis of intestinal inflammation whereas IL-12/IFN-γ played a role in the induction of systemic inflammatory responses, as PD-L1-Fc treatment exerted greater effects on the protection of colonic damage than it did on the prevention of weight loss during development of DSS-induced colitis. PD-L1-Fc treatment in a T-cell-induced colitis model showed

![Figure 6](image_url)

**Figure 6** Effects of Fc-conjugated programmed death ligand 1 (PD-L1-Fc) through interaction with PD-1 expressed on dendritic cells (DCs). (A) Colonic CD11c+ DCs (1x10⁵) were isolated from wild-type (WT) and PD-1 knockout (KO) mice and cultured with medium alone or 1 μM CpG ±10 μg/mL PD-L1-Fc protein for 24 h. (B) Splenic CD11c+ DCs (1x10⁵) derived from WT or PD-1 KO mice were cultured alone or with 100 ng/mL lipopolysaccharide (LPS) ±10 μg/mL PD-L1-Fc protein for 36 h. Cell culture supernatants were analysed for interleukin (IL)-6 and tumour necrosis factor (TNF)-α production by using ELISA. *p<0.05; **p<0.01 in a one-way analysis of variance followed by Bonferroni correction. Results are representative of three independent experiments.

Figure 7  Effects of Fc-conjugated programmed death ligand 1 (PD-L1-Fc) or CTLA-4-Ig treatment on the development of colitis induced by adoptive transfer of CD4 CD25<sup>−</sup> CD45RB<sup>high</sup> T cells into Rag-1 knockout (KO) mice. Rag-1 KO mice were injected intraperitoneally with CD4 CD25<sup>−</sup> CD45RB<sup>high</sup> T cells (5×10<sup>5</sup>) or phosphate-buffered saline (PBS). 20 μg of PD-L1-Fc protein was treated intraperitoneally once a week or 200 μg of CTLA-4-Ig protein was given intraperitoneally three times a week for 4 weeks from 3 weeks after T-cell transfer. (A) Body weight was monitored during the whole experiment. Data represent the percentage of body weights relative to initial body weights on day 0. Student’s t test, *p<0.05 compared with CD45RB<sup>high</sup>+ PBS group at week 7. (B) Clinical scores were determined at 7 weeks after T-cell transfer as the sum of three parameters: hunching and wasting, stool consistency, and colon thickness. (C) Seven weeks after T-cell transfer, colon sections were stained with H&E and scored for histological injury. Original magnification×100. (D) At 7 weeks after T-cell transfer, colonic lamina propria (LP) cells (4×10<sup>5</sup>) were restimulated with anti-CD3/CD28 monoclonal antibodies (mAbs) for 72 h. Cytokine levels in the supernatants were measured by ELISA. *p<0.05, **p<0.01 in a one-way analysis of variance followed by Bonferroni correction. Experiments were repeated twice with similar results.
protective effects on both systemic and mucosal inflammation, presumably due to downregulation of IFN-γ and IL-17 production. In both models of colitis, PD-L1-Fc displayed protective effects with disparity of suppressive effects on IFN-γ production, and the ability of IFN-γ suppression by PD-L1-Fc correlated with the degree of suppression of the weight loss or systemic inflammation in either model. The reason for the differential effects of PD-L1-Fc on IFN-γ production is not clear, although one possible explanation is that PD-L1-Fc might exert protective effect by targeting different types of immune cells driving inflammation, such as myeloid cells in the DSS model and CD4 Th cells in the T-cell transfer model, respectively.

Activation of DCs is known to be a prerequisite for the development of DSS-induced colitis.66 Based on our results showing the anti-colitis effect of PD-L1-Fc treatment in DSS-treated Rag-1 KO mice and in DSS-treated WT mice, it is likely that PD-L1-Fc may act directly on colonic activated DCs, via upregulated PD-1, to modulate cytokine production. In T cells, PD-1 suppresses nuclear factor xB (NF-xB)-mediated production of IL-2 by inhibiting phosphatidylinositol-3-kinase activity and downstream activation of Akt.47 A previous study noted that PD-1-mediated signalling in tumour-associated DCs suppressed phosphorylation of p65 NF-xB and resulted in less production of cytokines such as IL-6, IL-10, IL-12 and TNF-α,48 suggesting PD-1-driven inhibition of DCs. It has been also reported that PD-1-deficient splenic DCs were more resistant to apoptosis triggered by TLR ligand, LPS, which was associated with upregulation of mitogen-activated protein kinase and CD40 ligand-dependent signalling.49 These observations are consistent with our data in which PD-L1-Fc treatment suppressed LPS (TLR4)-mediated and CpG ODN (TLR9)-mediated cytokine production by splenic DCs and colonic DCs, respectively. Therefore, our study proposes that during development of DSS colitis, PD-L1-Fc treatment may activate the PD-L1:PD-1 signalling pathway primarily in DCs and then lead to the dysfunction of DC responses, thereby attenuating the severity of colonic inflammation. It is worth noting that colonic DCs from PD-1 KO mice secreted lower levels of IL-6 and TNF-α with or without TLR stimulation than those from WT mice. Thus, further studies are required to clearly elucidate the colonspecific impairment of DC functionality in PD-1 KO mice. However, in Rag-1 KO mice with anti-CD40-induced colitis, we did not observe the protective effect by PD-L1-Fc treatment in contrast to anti-TNF-α mAb treatment as positive control50 (see online supplementary figure S3). Since significant and rapid weight loss (loss of > 20% of body weight at 3 days) was caused by anti-CD40 antibody injection, this murine experimental colitis model may be inappropriate for the evaluation of the anti-colitogenic effects induced by PD-L1-Fc engagement with PD-1 expressed on activated DCs.

As PD-1 is also expressed on macrophages, the attenuation of DSS-induced colitis with PD-L1-Fc treatment could be mediated by the interaction with PD-1-expressing macrophages and subsequent regulation of macrophage responses. However, it was previously found that human intestinal macrophages do not produce pro-inflammatory cytokines in response to an array of inflammatory stimuli.50 Furthermore, macrophages were shown to play a stimulatory and inhibitory role in DSS-induced colonic inflammation.51 Therefore, based on the results obtained from previous studies, it is reasonable to speculate that macrophages have minor pathogenic function in the development of colitis, but rather contribute to protection against DSS-induced colitis.

In conclusion, we found that treatment of PD-L1-Fc during colitis development exerted a protective effect on intestinal inflammation by promoting the regulation of colonic CD4 T cells and the modulation of DC activation, to a greater degree than CTLA-4-Fc. It is worth noting that abatacept (CTLA-4-Ig) has failed to achieve remission in clinical trials for CD and UC.52 Moreover, PD-L1-Fc effectively ameliorated not only DSS-induced acute colitis but also T-cell-induced chronic colitis, suggesting that PD-L1-Fc is a potential therapeutic agent for treating inflammatory IBD. Therefore, PD-L1-Fc treatment may feasibly be a novel and promising biological strategy to inhibit inflammatory symptoms associated with IBD.

Correction notice Figure legends have been updated since published Online First.

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