

Paradoxical role of programmed death-1 ligand 2 in Th2 immune responses *in vitro* and in a mouse asthma model *in vivo*

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Programmed death-1 ligand 2 (PD-L2) is a ligand for programmed death-1 (PD-1), a receptor that plays an inhibitory role in T cell activation. Since previous studies have shown up-regulation of PD-L2 expression by Th2 cytokines, and asthma is driven by a Th2 response, we hypothesized that PD-L2 might be involved in regulation of the immune response in this disease. We have found that lungs from asthmatic mice had sustained up-regulation of PD-1 and PD-L2, with PD-L2 primarily on dendritic cells. Although addition of PD-L2-Fc *in vitro* led to decreased T cell proliferation and cytokine production, administration of PD-L2-Fc *in vivo* in a mouse asthma model resulted in elevated serum IgE levels, increased eosinophilic and lymphocytic infiltration into bronchoalveolar lavage fluid, higher number of cells in the draining lymph nodes, and production of IL-5 and IL-13 from these cells. Although PD-1 was expressed on regulatory T cells, PD-L2-Fc did not affect regulatory T cell activity *in vitro*. This study provides *in vivo* evidence of an exacerbated inflammatory response following PD-L2-Fc administration and indicates a potential role for this molecule in Th2-mediated diseases such as asthma.

Key words: PD-1 / PD-L2 / Th2 cells / Dendritic cells / Regulatory T cells

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1 Introduction

B7/CD28 superfamily members play key roles in regulating T cell activation and tolerance, and are promising therapeutic targets [1]. These molecules may provide critical positive signals that augment and sustain T cell responses, or negative signals to attenuate or terminate immune responses [2–6].

Programmed death-1 (PD-1), a CD28 superfamily member, was cloned from T cells undergoing apoptosis [7]. It is a 50–55-kDa receptor with 23% identity to CTL-associated antigen-4 (CTLA-4) in its Ig domain [7], and is expressed on activated, but not resting, CD4⁺ and CD8⁺ T cells, B cells, and myeloid cells [8]. PD-1 has an immunoreceptor tyrosine-based inhibitory motif in its

cytoplasmic domain [9–11]. Depending on background strain, PD-1^{-/-} mice develop increased rates of glomerulonephritis, lupus-like arthritis [12], or autoimmune cardiomyopathy [13]. This provides direct evidence for PD-1 as a negative regulator of immune responses *in vivo* [13]. PD-1 mRNA is expressed at high levels in CD4⁺CD25⁺ regulatory T cells (Treg) and anergic T cells [14, 15], suggesting the possible involvement of PD-1 in regulating Treg function and T cell tolerance.

PD-1 ligand 1 (PD-L1) and PD-1 ligand 2 (PD-L2), members of the B7 superfamily, are ligands for PD-1 [16–19]. Mouse (m) PD-L1 and PD-L2 share 38% amino acid identity and have extracellular Ig-like domains [4]. When activated T cells are co-cultured with Chinese hamster ovary (CHO) cells expressing PD-1 ligands, T cell proliferation and cytokine production are markedly reduced [18]. The PD-1 ligands exert these effects by arresting the cell cycle in G₀/G₁, but not by inducing cell death [18]. When PD-L1-Ig or PD-L2-Ig fusion proteins were used, inhibition of T cell proliferation and cytokine production by both resting and previously activated CD4⁺

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Abbreviations: PD-1: Programmed death-1 PD-L1: PD-1 ligand 1 PD-L2: PD-1 ligand 2 Treg: Regulatory T cell BAL: Bronchoalveolar lavage BM-DC: Bone marrow-derived DC Alum: Aluminium hydroxide

and CD8⁺ T cells were observed. The proliferative responses of anti-CD3-activated wild-type, but not PD-1-deficient, T cells were inhibited by PD-L1-Ig, indicating that the inhibitory signal was transduced by PD-1 [17]. However, there is also evidence indicating that both PD-L1 and PD-L2 can costimulate T cell responses; *i.e.* in the presence of suboptimal TCR signals, PD-L1 or PD-L2 can stimulate increased proliferation and production of cytokines *in vitro* [16, 19, 20].

Since PD-L2 expression has been shown to be up-regulated by a Th2 response [21, 22], we chose to investigate whether PD-L2 plays a role in airway inflammation. Asthma is a chronic disease which can be characterized by airway hyper-responsiveness, mucus hypersecretion by goblet cells, infiltration of the airway wall with leukocytes, and elevation of serum IgE [23–24]. Studies in mouse models have established a critical role for Th2 cells, DC, Treg [24–28], and the Th2 type cytokines IL-4, IL-5, IL-9, and IL-13 in the asthmatic response [23, 26, 29–32]. The role of the PD-1/PD-L1/PD-L2 axis in asthma is currently unknown.

In this study we present the kinetics of PD-1 and PD-L2 expression in lungs of asthmatic animals, T cells, and DC, and demonstrate that Th2 cytokines can induce PD-L2 expression on DC. Moreover, we show that administration of PD-L2-Fc increased cellularity in lymph nodes, IL-5 and IL-13 production by draining lung lymph node cells, serum IgE levels, and infiltration of cells into bronchoalveolar lavage (BAL) fluid. Lastly, using *in vitro* analysis, we find that these PD-L2-Fc effects were not due to interference with Treg activity. This study indicates a potential role for PD-L2 in Th2-mediated diseases such as asthma.

2 Results

2.1 Expression of PD-L2 and PD-1 in lungs of asthmatic mice

To determine whether PD-1 and its ligands are expressed after allergen challenge in the lungs of mice with airway disease, mice were primed by *i.p.* injection of ovalbumin (OVA)/aluminium hydroxide (Alum) at days 1, 7, and 14, and aerosol-challenged with OVA at either day 21 only or days 21, 22, and 23. Lungs were excised at different time points and quantitative PCR analysis on cDNA from total lung tissue was performed. While the lungs from unprimed, unchallenged mice did not significantly express PD-L2 and PD-1, they did express PD-L1 (Fig. 1). Although exposure to allergen only transiently increased PD-L1 expression 4 h after the first challenge (Fig. 1B), PD-1 and PD-L2 expression levels in lung

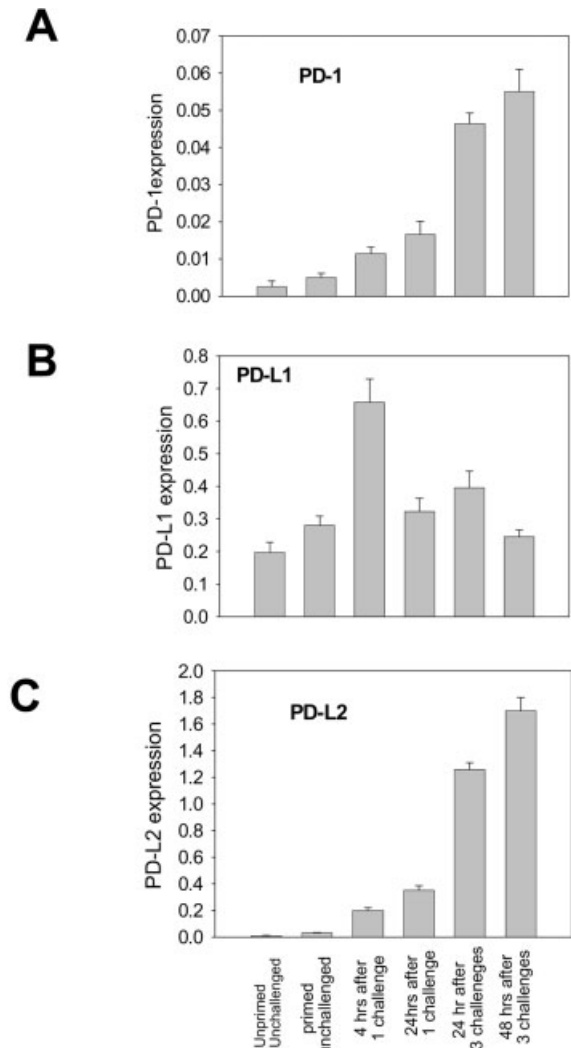


Fig. 1. Expression PD-1, PD-L1, and PD-L2 in lungs of animals sensitized and challenged with OVA. Taqman analysis of PD-1 (A), PD-L1 (B), and PD-L2 (C) shows mRNA expression in lungs of animals ($n=4$ mice/group) with or without OVA sensitization and challenge. Lungs were derived from either naive animals, or primed but unchallenged animals, or 4 h and 24 h after one challenge, or 24 h and 48 h after three challenges. Expression of target genes is normalized to HPRT housekeeping gene.

significantly increased with each additional allergen challenge (Fig. 1A, C).

2.2 PD-L2 and PD-1 are expressed on leukocytes isolated from asthmatic lungs

Since PD-1 and PD-L2 expression is increased in lungs of animals exposed to OVA, it was important to determine the contribution of leukocytes in the lung to these

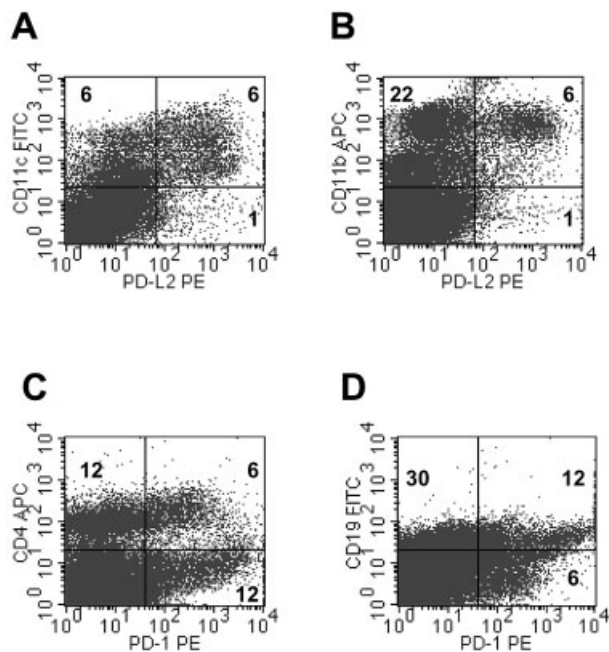


Fig. 2. PD-L2 and PD-1 are expressed on leukocytes isolated from asthmatic lungs. Flow cytometric analysis shows that PD-L2⁺ cells are CD11c⁺ (A) and CD11b⁺ (B) cells. PD-1 is expressed on CD4⁺ (C) and CD19⁺ (D) cells. These data represent two different experiments.

increases. In this experiment, mice were sensitized by i.p. injection of OVA/Alum at days 1, 7, and 14, then aerosol-challenged with OVA at days 21, 22, and 23, then lungs were excised at day 26. In cells derived from asthmatic lungs, PD-L2 expression was detected by flow cytometry on cells positive for CD11c (Fig. 2A) and CD11b (Fig. 2B). Back gating revealed this to be the same double-positive CD11c⁺CD11b⁺ subpopulation, a DC phenotype. Of the PD-1-expressing cells, one third were CD4⁺ T cells (Fig. 2C) with the remainder being CD19⁺ B cells (Fig. 2D).

2.3 Th2 cytokines induce PD-L2 but neither CD80 nor CD86 on dendritic cells

Since we found that PD-L2 was expressed on DC in asthmatic lungs, and asthma is a disease in which Th2 mediators play a critical role, we investigated whether Th2 cytokines can induce PD-L2 on DC. *In vitro* Flt3 ligand-generated bone marrow-derived DC (BM-DC) were incubated with various stimuli for 48 h. Unstimulated cells expressed very low levels of PD-L2. Treatment with CPG DNA, CD40L, GM-CSF, LPS, and IFN- γ led to increased expression of PD-L2 on the cell surface, but IL-4 and IL-13 induced the highest levels of PD-L2 compared to these other stimuli (Fig. 3). This up-

regulation was seen as early as 12 h, peaked at 48 h, and maintained as late as 72 h (data not shown). Interestingly, IL-4 and IL-13 strongly induced PD-L2, while expression of other B7 family members, such as CD80 and CD86, was virtually unaffected (Fig. 3). This indicates that expression of PD-L2 on DC may be specifically regulated by Th2 cytokines.

2.4 PD-L2-Fc decreases T cell proliferation and cytokine production *in vitro*

To test the function of PD-L2, we prepared PD-L2-Fc and examined its effect *in vitro*. In this experiment, plates were coated overnight with various concentrations of anti-CD3 and with PD-L2-Fc or mouse IgG1. Different types of T cells were incubated in these plates for 48 h. In accordance with previous studies, our results showed that *in vitro* plate-bound PD-L2-Fc decreased the proliferation of pre-activated T cells (Fig. 4B), but not of resting T cells (Fig. 4A). Plate-bound PD-L2-Fc not only decreased the proliferation of Th2-polarized CD4⁺ T cells (Fig. 4C) but also resulted in decreased IL-4 (Fig. 4D), IL-5 (Fig. 4E), and IL-13 (Fig. 4F) production. This study demonstrates for the first time the inhibitory potential of PD-L2-Fc on pre-activated Th2-polarized CD4⁺ T cells *in vitro*.

2.5 PD-L2-Fc increases total cell number and cytokine secretion by cells from draining lymph nodes and total serum IgE level in an *in vivo* asthma model

To examine a potential role for PD-L2 *in vivo*, mice were sensitized by i.p. injection of OVA/Alum at days 1, 7, and 14, followed by aerosol challenge at days 21, 22, and 23. Either mouse IgG or PD-L2-Fc was administered i.p. 1 day before each sensitization with OVA/Alum and 30 min before each challenge with OVA (days 0, 6, 13, 21, 22, and 23). Lungs from asthmatic animals were excised at day 26 and draining lymph nodes were examined. Surprisingly, the number of total cells in the draining lymph nodes of the PD-L2-Fc-treated group increased about twofold compared to the control group (Fig. 5A).

To examine cytokine production by these cells, equal numbers of draining lymph node cells were incubated with different doses of OVA. Analysis of supernatants in the PD-L2-Fc-treated group yielded increased IL-5 (Fig. 5B) and IL-13 (Fig. 5C) levels compared to control over a broad range of OVA concentrations, but the IL-4 levels were similar (data not shown). PD-L2-Fc treatment also resulted in significantly ($p=0.026$) increased serum

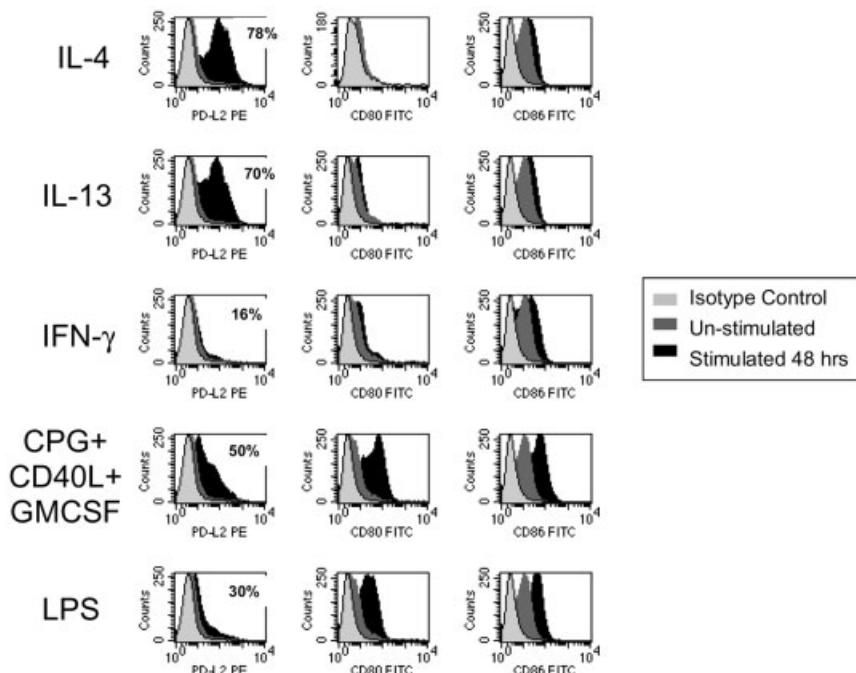


Fig. 3. Th2 cytokines induce PD-L2, but neither CD80 nor CD86, on DC. Flow cytometric analysis of Flt3 ligand-generated BM-DC for PD-L2, CD80, and CD86 after various stimuli, including IL-4 (20 ng/ml), IL-13 (20 ng/ml), IFN- γ (20 ng/ml), CpG DNA (1 μ g/ml) + CD40L (5 μ g/ml) + GM-CSF (20 ng/ml) or LPS (0.5 μ g/ml) are shown in black histograms. Dark gray histograms represent unstimulated DC. Isotype control stainings are represented by light gray histograms. Cells were gated on the CD11c⁺CD11b⁺ cell populations. This figure represents four experiments.

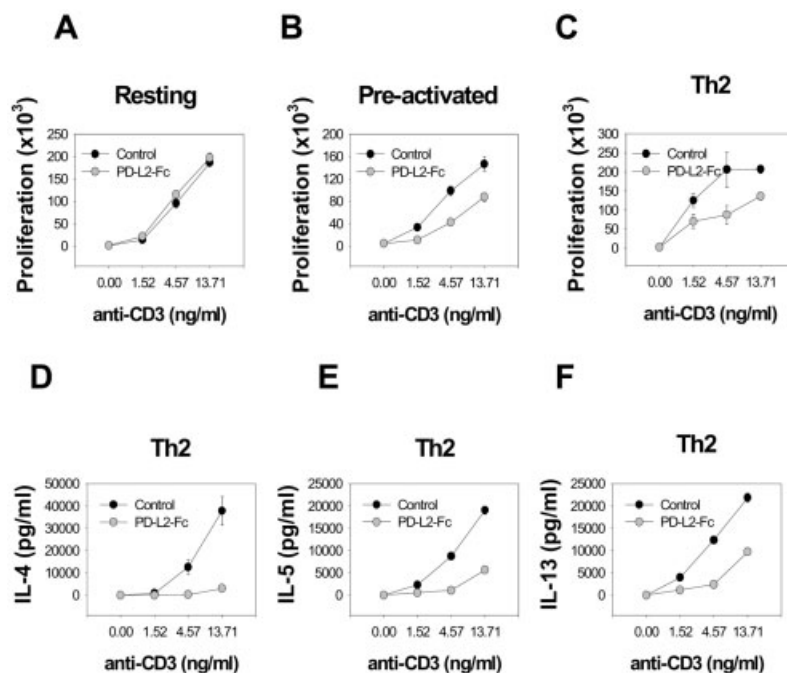


Fig. 4. Effect of PD-L2-Fc on T cells *in vitro*. Different doses of anti-CD3 Ab were pre-coated overnight in the presence of either mouse IgG or PD-L2-Fc (5 μ g/ml). (A–C) Negatively selected resting CD3⁺ T cells (A) or anti-CD3-pre-activated T cells (B) or Th2-polarized CD4⁺ T cells (C) were incubated for 48 h at 37°C, 10% CO₂, and their proliferation measured by a ³H assay. (D–F) Supernatants from Th2-polarized CD4⁺ T cells were analyzed by ELISA for IL-4 (D), IL-5 (E), and IL-13 (F) production. Similar data were obtained in two experiments.

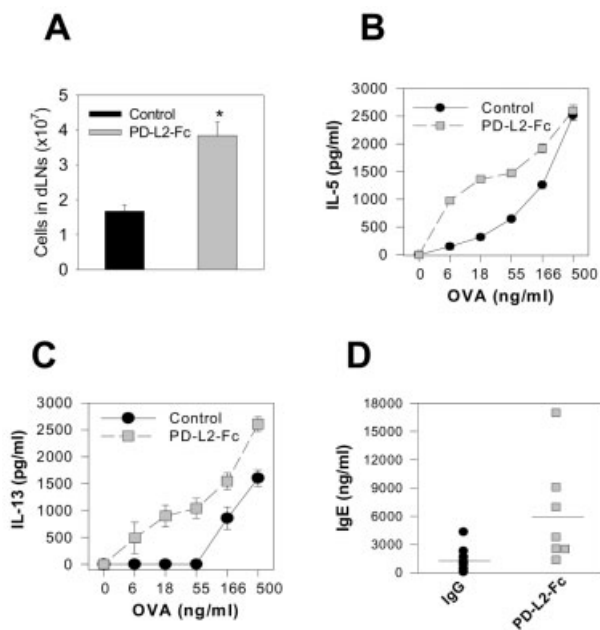


Fig. 5. Effect of PD-L2-Fc on immune cells *in vivo*. Both the PD-L2-Fc-treated groups and the mouse IgG-treated groups were sensitized on 3 consecutive weeks (days 0, 7, and 14) i.p. with 10 μ g of OVA emulsified in Alum, and then received aerosolized OVA (5% in saline) for 20 min on 3 consecutive days. Draining lymph nodes were collected 48 h after the last challenge and cells were counted. (A) Total cell numbers from draining lymph nodes. (B, C) Cells were incubated with a threefold dose titration of OVA for 48 h. Supernatants were analyzed by ELISA for IL-5 (B) and IL-13 (C) production. (D) Sera from animals were collected 48 h after the last challenge and analyzed by ELISA for total serum IgE. These data are representative of two experiments with groups of seven mice.

IgE levels compared to the control group (Fig. 5D). In contrast to our *in vitro* findings with PD-L2-Fc, this study showed an exacerbated T cell and B cell response following *in vivo* PD-L2-Fc administration.

2.6 PD-L2-Fc increases cell infiltration to bronchoalveolar lavage fluid following antigen challenge *in vivo*

To evaluate the role of PD-L2-Fc administration on the inflammatory response in lungs, we investigated the cell infiltration to airways by examining the BAL fluids obtained from lungs 48 h after the last challenge (Fig. 6). There was a significant increase in leukocyte recruitment, including eosinophils ($p=0.001$) and lymphocytes ($p=0.0004$), but not macrophages ($p=0.2$), into the airways in the PD-L2-Fc-administered group compared to mouse IgG-treated group. This provides

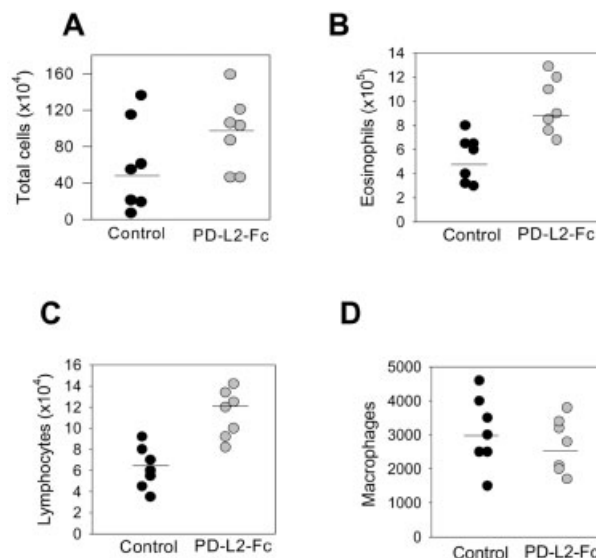


Fig. 6. PD-L2-Fc increases cell infiltration to BAL following antigen challenge. Both the PD-L2-Fc-treated and the mouse IgG-treated animals were sensitized for 3 consecutive weeks (days 0, 7, and 14) i.p. with 10 μ g of OVA emulsified in Alum and received aerosolized OVA (5% in saline) for 20 min over 3 consecutive days. Lavage was performed 48 h later and airway inflammatory cells were distinguished by morphological criteria and enumerated: (A) total inflammatory cells, (B) eosinophils, (C) lymphocytes, and (D) macrophages. These data are representative of two independent experiments with groups of seven mice.

additional evidence for an exacerbated inflammatory response in the lung following *in vivo* PD-L2-Fc administration.

2.7 PD-L2-Fc does not interfere with the suppressor activity of regulatory T cells *in vitro*

Treg have been shown to play a role in suppressing airway inflammation [28]. One possible explanation for the discrepancy of the *in vitro* vs. *in vivo* effects of PD-L2-Fc is that PD-L2-Fc might inhibit Treg function. To examine this possibility, we tested the role of PD-L2-Fc on proliferation and cytokine production by Th2 cells in the presence of Treg using the system described by George et al. [33]. By flow cytometric analysis, PD-1 was expressed on the majority of CD4⁺CD25⁺ Treg (Fig. 7A) and on some Th2-polarized CD4⁺ T cells (Fig. 7B). When Th2-polarized CD4⁺ T cells were incubated with DC, Treg, and various doses of OVA in the presence of 5 μ g/ml soluble PD-L2-Fc or mouse IgG1 control, we found that PD-L2-Fc did not significantly affect the suppressor

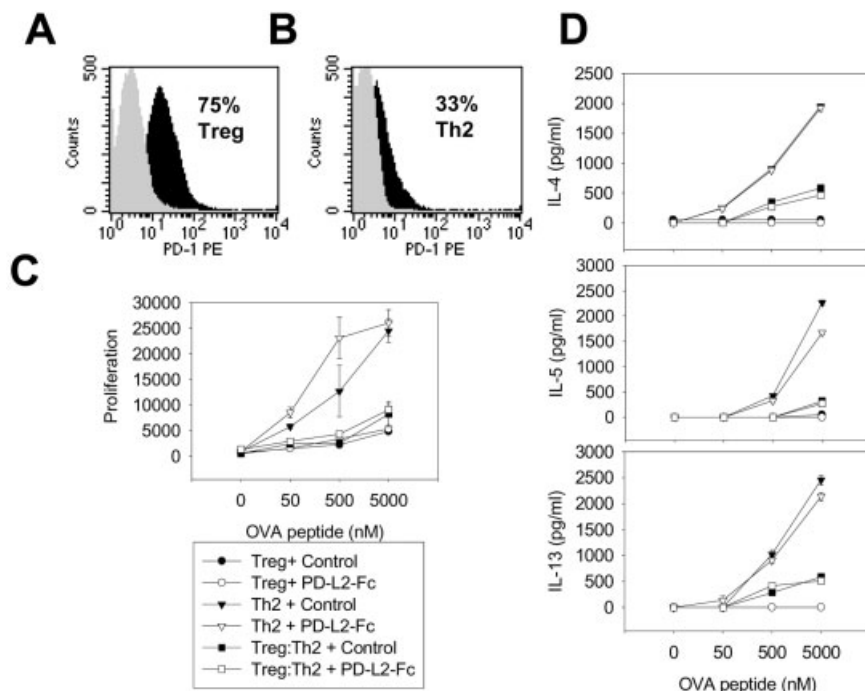


Fig. 7. PD-L2-Fc does not inhibit Treg-mediated suppression of Th2 cells. (A, B) Flow cytometric analysis of PD-1 expression on (A) Treg isolated and expanded *in vitro* in the presence of plate-bound anti-CD3 (10 μ g/ml) and IL-2 (20 ng/ml) and (B) Th2-polarized CD4⁺ T cells, are shown in black histograms. Isotype controls are represented by light gray histograms. (C–F) Th2 cells (2.5×10^4), Treg (at a 1:3 ratio to Th), BM-DC (10:1 to Th), and OVA peptide 323–339 (50–5,000 nM OVA) were co-incubated for 48 h in the presence of 5 μ g/ml PD-L2-Fc or mouse IgG1 isotype control. (C) Proliferation was measured by a ³H assay. (D–F) Supernatants from these cultures were analyzed by ELISA for IL-4 (D), IL-5 (E), and IL-13 (F) production. These data are representative of two experiments.

activity of Treg as determined by proliferation (Fig. 7C) as well as IL-4, IL-5, and IL-13 production (Fig. 7D).

Functional inactivity of PD-L2-Fc on Treg in this setting was confirmed using 1:1, 1:3, or 1:6 Treg/Th2 ratios, and using an alternative setting with plate-bound PD-L2-Fc and a similar range of Treg/Th2 ratios (data not shown). This suggests that the exacerbating effects of PD-L2-Fc *in vivo*, whether mediated by soluble or aggregated forms of PD-L2-Fc, might not be due to interference with the activity of Treg.

3 Discussion

This study was designed to investigate whether PD-L2 is involved in the Th2-mediated immune response and pathogenesis of a mouse model of asthma. We showed that lung PD-L2 and PD-1 expression levels significantly increased with allergen challenge, with expression of PD-L2 mostly on DC while PD-1 was mostly on T and B cells. We also demonstrated that Th2 cytokines play an important role in the induction of PD-L2 on DC. Although the presence of PD-L2-Fc resulted in decreased T cell

proliferation and cytokine production *in vitro*, administration of PD-L2-Fc *in vivo* exacerbated the immune and inflammatory response. Using *in vitro* analysis we found that these paradoxical PD-L2-Fc effects were not due to interference with Treg activity. Collectively, these findings support a potential but complex role for the PD-1/PD-L1/PD-L2 receptor-ligand system in Th2 diseases such as asthma.

The *in vivo* functions of PD-1, PD-L1, and PD-L2 have been of interest because PD-1^{-/-} mice progressively develop autoimmune diseases [12, 13]. Since previous studies have shown that PD-L2 expression may be regulated in some cell types by Th2 cytokines or Th2 cells [21], and because asthma is believed to be a Th2-driven disease [23–32], it was of interest to examine whether PD-L2 can play a role in immune regulation in this context.

We found that PD-L2 and PD-1 are expressed in the lungs of animals exposed to OVA allergen, and levels of both molecules significantly increased during challenges with OVA, with PD-L2 predominantly expressed on CD11c⁺CD11b⁺ DC (Fig. 1, 2). A previous study showed

that IL-4 induces PD-L2 expression on macrophages in an IL-4R α - and STAT6-dependent fashion [21]. We found that IL-4 and IL-13 specifically induced PD-L2 expression on BM-DC while other B7 family members such as CD80 and CD86 were not affected (Fig. 3). The fact that PD-L2 induction is specific to Th2-polarized immune responses suggests a role in disease models of asthma.

Consistent with the data of Latchman et al. [18] we found that *in vitro* plate-bound PD-L2-Fc decreased the proliferation of pre-activated T cells. We saw a similar PD-L2-Fc-mediated inhibition of proliferation and cytokine production by activated, Th2-polarized CD4⁺ T cells (Fig. 4), suggesting that in this culture system, immobilized PD-L2-Fc is cross-linking an inhibitory receptor, most probably PD-1. However, our *in vivo* studies yielded contrasting results. We found that PD-L2-Fc administration exacerbated immune and inflammatory responses *in vivo* (Fig. 5, 6). While paradoxical, this novel finding may be consistent with other *in vitro* studies showing that PD-L2 can increase naive T cell proliferation and production of cytokines [19]. The contrasting *in vitro* and *in vivo* results might reflect the importance of the activation status of the cells at the time of exposure to PD-L2, or the previous stimulation history of PD-L2-restimulated cells. In our current protocol PD-L2-Fc was administered to mice 1 day before each sensitization and 1 h before each challenge to antigen, which might suggest that the timing of administration of PD-L2-Fc in relation to exposure to the antigen could be critical.

An explanation for the *in vivo* results might be that PD-L2-Fc targets a Treg population involved in suppression of the activated T cell response. We explored this possibility via a series of *in vitro* studies using co-culture of DC, DO11.10-derived, Th2-polarized T cells, and Treg. We found that although Treg expressed PD-1, addition of PD-L2-Fc did not block the suppressor activity of Treg *in vitro*. Similarly, studies by Baecher-Allan et al. [34] have shown that human Treg can still suppress proliferation in the absence of PD-1/PD-L1.

There could be several possible mechanisms to explain why PD-L2-Fc can enhance an immune response *in vivo*. Perhaps PD-L2-Fc blocks the inhibitory interaction of cell-associated PD-L2 or PD-L1 with PD-1. However, this would not be consistent with our *in vitro* studies using DC and DO11.10-derived, Th2-polarized CD4⁺T cells, where PD-L2-Fc did not significantly affect PD-1⁺ T cell proliferation, IL-4, IL-5, or IL-13 production (Fig. 7). Since PD-L1 and PD-L2 bind to different sites on PD-1 [35], this would rule out the possibility of PD-L2-Fc blocking the interaction between PD-L1 and PD-1.

A second possibility might be that PD-L2-Fc interacts with a second receptor and enhances T cell activation via a PD-1-independent mechanism. Recent studies have shown that PD-L2-Fc can still bind and costimulate naive CD4⁺ T cells from PD-1-deficient mice [36, 37]. This putative activating receptor for PD-L2 may dominate the inhibitory PD-1 receptor *in vivo*. Such dominance might be mediated by a higher affinity for PD-L2, a higher receptor number than of PD-1, earlier expression than PD-1, or stronger signaling mechanisms than of PD-1. Until the identity of the second receptor is established these mechanisms remain speculative.

4 Materials and methods

4.1 Mice

Female, 8–12-week-old BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were housed at Amgen (Seattle, WA) under specific pathogen-free conditions and according to federal guidelines.

4.2 Flow cytometry

Cells were blocked at 4°C for 20 min in FACS buffer containing PBS, 2% FBS, 1% normal rat serum, 1% normal hamster serum, 10 μ g/ml CD16/CD32 (2.4G2) rat anti-FcR mAb (BD PharMingen), and 0.02% sodium azide. Cells were then stained with mAb at 4°C for 30 min. All of the following mAb were purchased from BD PharMingen (San Diego, CA): FITC-anti-mouse CD3, allophycocyanin (APC)-anti-mouse CD4, APC-anti-mouse CD8a, FITC-anti-mouse CD11b, APC-anti-mouse CD11c, FITC-anti-mouse CD19, CyC-anti-mouse B220, FITC-anti-mouse I-Ad, PE-anti-mouse CD40, and PE-anti-mouse CD80. PE-anti-mouse B7-DC and PE-anti-mPD-1 were purchased from Biolegend (San Diego, CA). Cells were washed twice with PBS and analyzed by flow cytometry with FACSCalibur cytometer and CellQuest Software (both from BD Biosciences, Mountain View, CA). Number of events was 40,000.

4.3 Dendritic cell cultures

BM cells were flushed from femurs of BALB/c mice with PBS supplemented with 2% heat-inactivated FBS (Life Technologies, Grand Island, NY), then resuspended in culture medium consisting of McCoy's medium supplemented with essential and nonessential amino acids, 1 mmol/l sodium pyruvate, 2.5 mmol/l Hepes buffer (pH 7.4), vitamins, 5.5×10^{-5} mol/l 2-ME, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.3 mg/ml L-glutamine, and 10% FBS (all from Life Technologies). BM cells were cultured for the time indicated in the figures at 1×10^6 cells/ml, in tissue culture flasks

(Falcon; BD Biosciences) or in 24-well plates (Costar Corning, Cambridge, MA) in culture medium supplemented with 200 ng/ml recombinant human Flt3 ligand (Amgen, Seattle, WA). Cultures were incubated at 37°C in a humidified atmosphere containing 10% CO₂. Cells were harvested from the cultures by vigorous pipetting and removal of non-adherent cells. To recover adherent cells, Versene 1:5,000 (Life Technologies) was added after two washes and cells were incubated for 10 min at 37°C. The cells were then pooled with the nonadherent fraction.

In vitro activation of DC was achieved by adding different stimulus mixtures that included 1 µg/ml phosphorothioate-modified oligodeoxynucleotides containing CPG motifs 1826 (TCCATGACGTTTCCTGACGTT; Sigma, The Woodlands, TX), 5 µg/ml CD40L trimer (Amgen), 20 ng/ml recombinant mouse GM-CSF (R&D Systems, Minneapolis, MN), 0.5 µg/ml LPS (Sigma), 20 ng/ml IL-4 (BD-PharMingen), 20 ng/ml IL-13 (R&D Systems), and/or 20 ng/ml IFN-γ (BD-PharMingen) into cultures for 24 h.

4.4 Generation of CD4⁺ Th2 cells *in vitro*

Single-cell suspensions from spleens of OVA-specific TCR-transgenic DO11.10 mice were resuspended in Tris-NH₄Cl at room temperature for 1 min to lyse red blood cells (RBC). To generate Th2-skewed cells, 20 ng/ml of IL-4, 10 µg/ml of anti-mouse IFN-γ, 5 µg/ml of anti-mouse IL-12, and 0.3 mM of OVA peptide were added to the cultures. After 2 days, these skewed cells were split and cultured in 1 ng/ml IL-2 (R&D Systems) for 2 more days. CD4⁺ Th2 cells were enriched from splenocytes by immunomagnetic cell separation using negative selection with the StemSep Enrichment mixture kits for CD4⁺ T cells (StemCell Technologies, Vancouver, Canada). The percentage of enriched T cells expressing the transgenic TCR was determined by flow cytometry (>90%) using APC-anti-CD4 and PE-KJ1-26 mAb.

4.5 Generation of regulatory T cells

Single-cell suspensions of mesenteric lymph nodes (mLN) and RBC-lysed (Sigma) splenocytes from Flt3 ligand-injected DO11.10 mice were washed twice in Iscove's modified Dulbecco's medium (IMDM; Invitrogen, Carlsbad, CA) containing 5% FCS, 5.5 × 10⁻⁵ M 2-ME (Invitrogen), 1 mM sodium pyruvate, 100 mM nonessential amino acids, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (cIMDM). Spleen and mLN suspensions were then combined and enriched for CD4⁺ T cells by negative immunomagnetic selection using a CD4 enrichment cocktail (StemCell Technologies). Labeled cells were passed over a 0.6-inch pump-feed column in a magnetic field (Blue magnet; StemCell Technologies). CD4-enriched cells were stained with PerCP-Cy5.5-anti-CD4 and PE-anti-CD25, and sepa-

rated into CD4⁺CD25⁺ (Treg) population using a FACS Vantage (Becton Dickinson) or MoFlo (Cytomation, Fort Collins, CO) cell sorter. Later cells were expanded on plate-bound anti-CD3 (10 µg/ml) and 1 ng/ml of IL-2 (R&D Systems) for 5 days.

4.6 Analysis of proliferation and cytokine production

Different doses of anti-CD3 mAb were pre-coated overnight in the presence of either mouse IgG or PD-L2-Fc (5 µg/ml). Negatively selected resting CD3⁺ T cells (2.5 × 10⁴) or pre-activated T cells (2.5 × 10⁴) were incubated in 200 µl/well for 48 h at 37°C and their proliferation measured by a ³H assay. Th2 cells (2.5 × 10⁴), Treg (at a 1:1 or 1:3 ratio to Th), BM-DC (10:1 to Th), and OVA peptide class II 323–339 (50 nM–5,000 nM OVA) in cIMDM were added to 96-well U-bottom plates (Costar Corning) for a total of 200 µl/well. Some cultures were incubated with 5 µg/ml PD-L2-Fc or mouse IgG1 isotype control. Cultures were maintained at 37°C, 5% CO₂ for the indicated time periods.

For analysis of [³H]thymidine incorporation, plates were incubated with 0.5 mCi of [³H]thymidine (Amersham Biosciences, Piscataway, NJ) per well for the last 16 h of the 72-h culture period. Contents of each well were transferred to Filtermat A glass fiber filters (Wallac, Turku, Finland) using the Brandel harvester (Brandel, Gaithersburg, MD) and read on a TriLux 1450 MicroBeta counter. Cytokine levels in tissue culture supernatant were measured by ELISA. IL-4, IL-5 (BD PharMingen), and IL-13 (R&D Systems) ELISA were performed according to manufacturers' directions. The limits of detection were 62.5 pg/ml for IL-4, 156 pg/ml for IL-5, and 100 pg/ml for IL-13.

4.7 Expression and purification of mPD-L2-Fc

The mPD-L2-Fc fusion protein consists of the extracellular domain of mPD-L2 (amino acids 1–221) fused to the constant region of mouse IgG1 [18]. The fusion protein was produced as a secreted protein from CHO cells using standard procedures [38]. CHO cell conditioned medium was concentrated ~20× using a 30-kDa MWCO membrane (Millipore), then diluted with an equal volume of 3 M KCl. The pH was adjusted to 8.70 using Tris-HCl and bound to protein A-Sepharose (Amersham Biosciences) at 4°C. The protein A-Sepharose was washed with five volumes of 25 mM Tris-HCl, 1.5 M KCl, pH 8.70 before elution of bound mPD-L2-Fc with IgG elution buffer (Pierce) followed by adjustment to pH 7.5 using Tris-HCl. The eluted fusion protein was dialyzed vs. PBS, then filtered through a 0.2-µm filter. Protein concentration was determined by absorbance at 280 nm, using an extinction coefficient of 1.38. The endotoxin level of the purified protein was typically less than 0.54 EU/ml.

4.8 Analysis of PD-L2-Fc effect *in vivo*

Eight-to-twelve-week-old mice were sensitized by i.p. injection of 10 µg of OVA (grade V; Sigma) emulsified in 2.25 mg of Alum (AlumImject; Pierce, Rockford, IL) in a total volume of 200 µl on days 0, 7, and 14. Mice were aerosol-challenged (20 min) with OVA (5% in saline) for 3 days (days 21, 22, and 23) using ultrasonic nebulization (Par). mPD-L2-mFc fusion protein was administered by i.p. injection (0.5 mg/mouse) 1 h prior to each sensitization and challenge. As a control, same amount of mouse IgG (Sigma) was administered in the same fashion.

4.9 Lymph node cultures and measurement of cytokines

Lung draining lymph nodes were harvested 3 days after last challenge and single-cell suspension was prepared. Cells were incubated with media alone, with pre-coated anti-CD3 (10 µg/ml), or with increasing concentration of OVA. Cytokine levels in tissue culture supernatant were measured by ELISA as described above.

4.10 Measurement of serum IgE levels

Total serum IgE was measured by ELISA. Briefly, 96-well plates were coated with purified anti-IgE capture Ab (Serotec). After addition of serum samples and IgE standard, a biotinylated anti-IgE Ab was used as detecting Ab, and the reaction was amplified with streptavidin-horseradish peroxidase (Sigma-Aldrich). Levels were calculated by comparison with known mouse IgE standards. The limit of detection was 31.25 ng/ml.

4.11 Bronchoalveolar lavage

At day 26, mice were intubated via the trachea and lungs were lavaged with Hanks' balanced salt solution (HBSS, 2×0.5 ml). Total leukocytes were counted. Differential cell counts were performed by counting 200 cells on cytocentrifuged preparations (Cytospin 2; Cytospin, Runcorn, UK), stained with hematoxylin and eosin and differentiated by standard hematological procedures.

4.12 Lung tissue digestion protocol

Mice were injected with 200 U heparin sodium (American Pharmaceutical Partners Inc., Los Angeles, CA) 30 min prior to sacrifice and lungs collected in HBSS. Lungs were transferred to 6-well tissue culture plates containing 3 ml tissue digestion media (HBSS; Gibco-BRL, Rockville, MD) with 2% FBS (Hyclone Laboratories, Logan, UT), 3 mM CaCl₂, 200 µg/ml collagenase type II (Gibco-BRL), 50 U/ml DNase (Sigma-BRL, St. Louis, MO), 1 U/ml Dispase (Gibco-

BRL), and 300 U/ml type IV-S hyaluronidase (Sigma-BRL), and minced with scissors.

Minced tissue was transferred to 50-ml conical tubes along with 2 ml of digestion medium used to wash remaining cells off the Petri plate. Tissue was rotated from a 37°C water bath to a shaker plate every 5 min for approximately 1 h, with an additional 5 ml digestion medium added after 30 min. Gravity separated the remaining larger tissue fragments, and cells in solution were transferred to a new 50-ml conical tube and spun at 1,000 rpm for 5 min. The pelleted cells were reconstituted with 5 ml of Cell Dissociation Solution (Sigma, St. Louis, MO) and vortexed frequently for 10 min. Five milliliters of Cell Dissociation Solution was then added to the larger tissue fragments, with frequent vortexing for 10 min. Cells and tissue fragments from individual lungs were filtered into a new 50-ml conical tube to prepare a single-cell suspension. Cells were spun at 1,000 rpm for 5 min and reconstituted with FACS buffer [PBS, 2% FBS (Life Technologies), sodium azide (ICN, Costa Mesa, CA)] and kept on ice.

4.13 Taqman analysis

Commercially available (Ambion, Austin, TX, and Clontech Laboratories, Palo Alto, CA) and in-house prepared RNA were DNase-treated (Ambion), and reverse-transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions using random hexamers. Samples were distributed on plates at 20 ng per well and run in triplicate. TaqMan primer/probe sets were designed using Primer Express software version 2.0 (Applied Biosystems). Forward and reverse primer concentrations for each set were optimized, FAM/MGB-labeled probes (Applied Biosystems) were used at 200 nM. An oligo set for hypoxanthine-guanine phosphoribosyl transferase (HPRT) housekeeping gene was optimized and the probe was labeled with VIC/TAMRA (Applied Biosystems). Multiplex TaqMan PCR reactions were set up in 25-µl volumes with TaqMan Universal PCR Master Mix (Applied Biosystems) on an Applied Biosystems Prism 7700 Sequence Detection System. Cycle threshold values (C_T) were determined using Sequence Detector software version 1.7a (Applied Biosystems) and transformed to 2^{-ΔC_T} for relative expression comparison of genes to HPRT.

TaqMan oligo sets were: for PD-1, forward 5'-CCTAGTGGG-TATCCCTGTATTGCT-3', probe 5'-CGTCCTTGCTTCCAGC-3', reverse 5'-CTAGGGACAGGTGCTGCTGAA-3'; for PD-L1, forward 5'-ATGATGTTTTCTACTGTACGTTTTGGA-3', probe 5'-CAAAACCACACAGCGGAG-3', reverse 5'-GTGAGTCCT-GTTCTGTGGAGGAT-3'; for PD-L2, forward 5'-GCTCCTGC-TGCCGATACTG-3', probe 5'-AGCTTACAACCTCATCCTG-3', reverse 5'-GGCTGTCACGGTGAATAAAGC-3'; for HPRT, forward 5'-GACCGGTCCCCTCATGC-3', probe 5'-ACCCG-

CAGTCCCAGCGTCGT-3', reverse 5'-CATAACCTGGTTCAT-CATCGCTAA-3'. Oligos were selected using ABI Primer Express software.

4.14 Statistical analysis

Results are expressed as means of $\pm$ standard error of the mean. Student's *t*-tests were used for analysis of significance among the groups.

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