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PD-L2 modulates asthma severity by directly decreasing dendritic cell IL-12 production

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Abstract

Studies examining the role of PD-L2/PD-1 in asthma have yielded conflicting results. To clarify its role, we examined PD-L2 expression in biopsies from human asthmatics and lungs of aeroallergen-treated mice. PD-L2 expression in bronchial biopsies correlated with the severity of asthma. In mice, allergen exposure increased PD-L2 expression on pulmonary myeloid dendritic cells, and PD-L2 blockade diminished allergen-induced airway hyperresponsiveness (AHR). In contrast, PD-1 blockade had no impact, suggesting that PD-L2 promotes AHR in a PD-1-independent manner. Decreased AHR was associated with enhanced serum IL-12 p40 and *in vitro* stimulation of DCs with allergen and PD-L2-Fc reduced IL-12 p70 production, suggesting that PD-L2 inhibits allergen-driven IL-12 production. In our model, IL-12 did not diminish Th2 responses, but rather directly antagonized IL-13-inducible gene expression, highlighting a novel role for IL-12 in regulation of IL-13 signaling. Thus, allergen-driven enhancement of PD-L2 signaling through a PD-1-independent mechanism limits IL-12 secretion, exacerbating AHR.

Introduction

Allergic asthma is an inflammatory lung disease whose prevalence continues to rise in developed nations. Although the origins of allergic asthma are complex, excessive activation of Th2 cells specific for normally innocuous environmental allergens drives disease pathology. Thus, in asthmatic individuals, allergen exposure triggers the development of allergen-specific Th2 cells producing IL-4, IL-5 and IL-13, which induce IgE, eosinophilia,

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mucus hypersecretion, and airway hyperresponsiveness (AHR)[1]. In contrast, non-asthmatic individuals develop protective responses mediated by IL-10 and TGF-β secreting Tregs[2-3]. Thus, the type of T cell response induced following allergen exposure is an important determinant of susceptibility to allergen-induced AHR.

In vivo, dendritic cells (DCs) represent the most potent professional antigen presenting cells in the lung, and they have been identified as key players in promoting both pathogenic or protective T cell responses[4]. In the lung, DCs lie immediately below the epithelial cell layer and extend their dendrites into the lumen, capture inhaled allergens, and then migrate to lung-draining lymph nodes (LNs) where they present captured allergens to naive T cells[5-6]. Many factors influence the ability of DCs to promote pathogenic versus protective T cell responses. Allergen presentation by myeloid DCs (mDCs) promotes both initiation of AHR in naive animals[4, 7] and exacerbations in previously sensitized animals[6], while allergen presentation by plasmacytoid DCs (pDCs) prevents the initiation of AHR[8] and limits allergic inflammation in previously sensitized hosts[9]. DCs also influence the development of allergen-induced AHR through production of T cell skewing cytokines. We have recently demonstrated that the production of high levels of IL-23 by pulmonary mDCs is associated with the development of a mixed Th2/Th17 cytokine profile that ultimately results in the development of severe AHR[4, 10]. In contrast, the development of weak AHR observed in C3H/HeJ mice is associated with the production of IL-12[11], and administration of exogenous IL-12 limits the severity of AHR in asthma-susceptible A/J animals[12]. Finally, the types of co-stimulatory molecules expressed by DCs also control T cell development such that expression of CD80, CD86 and OX40L all promote allergic disease[13-15], while ICOS-L expression induces the development of Tregs and promotes inhalational tolerance[16]. Thus, by virtue of the subsets of DCs involved in allergen presentation and the nature of the cytokines or co-stimulatory molecules produced, DCs regulate the development of pathogenic versus protective T cell responses.

Another way DCs regulate T cell responses is through expression of PD-1 family members. The PD-1 family consists of PD-L1 (found on DCs, T cells, B cells, and structural cells[17-18]), PD-L2 (expressed on DCs and macrophages[18]) and PD-1 (expressed on T cells, DCs and macrophages[19-21]). In general, stimulation of T cells through PD-1 or PD-L1 limits CD3-mediated activation of T cells. Indeed, PD-1 stimulation is responsible for the T cell "exhaustion" observed in models of chronic viral infection (LCMV, HIV)[22-23], contributes to allograft tolerance[24], and prevents autoimmune disease[17, 25]. Interestingly, despite the generally inhibitory role for PD-1 in regulating T cell activation, the role of the PD-1/PD-L axis in regulating allergic asthma is uncertain, with some reports suggesting a protective role for this pathway[9, 26-29], and others reporting a pro-asthmatic role for either PD-L1[27] or PD-L2[30].

Herein, we sought to clarify the role of PD-L2 in allergen-induced AHR. We demonstrate that in both mice and humans, increased PD-L2 expression is associated with more severe AHR. Supporting a role for a PD-L2 in modulating asthma severity, *in vivo* blockade of PD-L2 reduces the severity of allergen-driven AHR. Surprisingly, PD-L2 blockade does not alter the production of Th2 cytokines, the composition of inflammatory cells in the bronchoalveolar lavage, or synthesis of IgE, but rather, enhances the ability of DCs to

secrete IL-12 p70, and simultaneous blockade of both PD-L2 and IL-12 abrogate the protective effects observed upon blockade of PD-L2 alone. Strikingly, blockade of PD-1 had no impact on AHR, or systemic IL-12 levels, suggesting that PD-L2 regulates asthma severity in a PD-1 independent manner. Furthermore, in our model, increased IL-12 production is not associated with decreased Th2 cytokine production. However, we demonstrate that IL-12 directly antagonizes IL-13-driven gene induction and STAT6 phosphorylation, providing a mechanism whereby IL-12 may limit the severity of allergen-induced AHR. Collectively, these results demonstrate that PD-L2, through an unknown receptor, limits IL-12 secretion, thereby enhancing the magnitude of allergen-induced AHR.

Results

mDC expression of PD-L2 is associated with ongoing AHR

In this study, we sought to clarify the role of PD-L2 in asthma. To this end, we compared PD-L2 expression in the lung following sequences of allergen exposures designed to induce either robust AHR, or the development of tolerance. As such, we exposed mice to house dust mite extract (HDM) i.t. on days 0 and 14, a protocol previously shown to induce robust AHR in A/J mice[4]. As expected, HDM exposure induced robust AHR (Fig 1a) as measured by monitoring airway pressure changes over time (APTI), as described elsewhere[31]. We then examined PD-L2 mRNA expression in the lungs of mice at 2, 24 or 72 hours after the last i.t. challenge. PD-L2 expression was up-regulated by HDM at 2 hours and peaked 24 hours later (Fig 1b). To determine if PD-L2 induction was unique to HDM, A/J mice were sensitized i.p. with OVA in saline on day 0, and challenged i.t. with OVA on days 14 and 21. As previously reported[11], this treatment regimen induced AHR (Fig 1a). As with HDM, OVA exposure also induced a rapid increase in PD-L2 expression that peaked at 24 hours (Fig 1b). Interestingly, despite an identical number of allergen exposures, mice receiving OVA exclusively i.t., a regimen known to induce tolerance rather than AHR[8, 16], demonstrated no induction of AHR (Fig 1a) and failed to up-regulate PD-L2 expression significantly, suggesting that enhanced PD-L2 expression is associated with allergen-induced AHR (Fig 1b).

PD-L2 is chiefly expressed by antigen presenting cells[18], however, multiple pulmonary DC populations have been described to date. To determine if PD-L2 was differentially expressed on different DC subsets, mice were treated i.t. with HDM on day 0, and fluorescent HDM (AF405-HDM) on day 14 to facilitate the identification of allergen-bearing DCs by flow cytometry. 72 hours after HDM exposure 5 distinct populations of $CD11c^+$ cells could be identified by flow cytometry, including alveolar macrophages (AMs), plasmacytoid DCs (pDCs), neutrophils, inflammatory DCs, and myeloid DCs (mDCs) (Supplementary Fig 1a). While AMs demonstrated some allergen uptake, and AMs, neutrophils and inflammatory DCs all expressed some PD-L2 after allergen challenge, mDCs were the only population simultaneously positive for both AF405-HDM and PD-L2 (Supplementary Fig 1b). Thus, $HDM^+PD\text{-}L2^+$ mDCs represented up to ~15% of all mDCs found in the lung, while the increase in the frequency of other $HDM^+PD\text{-}L2^+$ DC populations was comparatively minor (≤ 3%) (Fig 1c). Moreover, the levels of PD-L2 expression (as measured by MFI) increased markedly on pulmonary mDCs after allergen exposures (Fig 1d). In contrast the MFI of PD-

L2 expression was not significantly increased on other pulmonary DC populations (Fig 1d). These data suggest that pulmonary mDCs are the principal PD-L2 expressing DC subset in the lung.

To determine if a similar association between PD-L2 expression and increased asthma severity is present in human populations, we compared PD-L2 expression in bronchial biopsies from patients with mild asthma, severe asthma, and healthy control subjects. PD-L2 expression was undetectable in healthy control subjects (Fig 1e). In comparison to healthy controls, the expression of PD-L2 was enhanced in patients with severe asthma ($p < 0.05$) (Fig 1e). Moreover, while not significant, there was a trend towards increased PD-L2 expression in individuals with mild asthma ($p = 0.07$). Thus, PD-L2 expression is significantly elevated in individuals with severe disease.

PD-L2 blockade limits the development of allergen-induced AHR

To directly assess the role of PD-L2 in AHR in mice, we examined the effect of a PD-L2 blocking mAb (clone TY25)[32] on the development of allergen-induced AHR. As expected, HDM increased AHR, which was not impacted by an isotype control mAb (Fig 2a). In contrast, administration of anti-PD-L2 significantly reduced the development of HDM-induced AHR, demonstrating that PD-L2 modulates asthma severity (Fig 2a). While PD-L2 is a ligand for PD-1[33], some reports suggest that PD-L2 can bind a receptor other than PD-1[34-35]. To determine if PD-L2 enhances asthma through interactions with PD-1, we examined the impact of PD-1 blockade on the development of AHR. However, treatment with a mAb known to block interactions between PD-1 and both of its known ligands (PD-L1 and PD-L2) (clone RMP1-14)[36] had no impact on the magnitude of HDM-induced AHR in A/J animals (Fig 2b). This suggests that PD-L2 regulates the severity of AHR through a PD-1 independent mechanism.

As airway inflammation is a hallmark of allergen-induced asthma, we also examined the cells present in the bronchoalveolar lavage (BAL) of mice following exposure to PBS, HDM, HDM + isotype control mAb, or HDM + anti-PD-L2. We harvested BAL at the time of sacrifice and performed differential cell counts. As expected, HDM exposure induced the recruitment of significant numbers of macrophages, eosinophils, neutrophils and lymphocytes (Fig 3a). However, the BAL cell profile was unaffected by either the presence of the control mAb, anti-PD-L2 (Fig 3a) or anti-PD-1 (data not shown). Moreover, analysis of BAL cytokine levels demonstrated that while HDM increased the amounts of IL-4, IL-5 and IL-13 found in the BAL fluid, the levels of these cytokines were not significantly impacted by administration of isotype control, or PD-L2 blocking mAbs (Fig 3b). To further characterize the immune response, we also examined HDM-stimulated cytokine production in lung cell cultures from mice treated *in vivo* with anti-PD-L2 or a control mAb. As previously shown, HDM-treated A/J mice display a mixed Th2/Th17 cytokine profile, with robust production of IL-4, IL-5, IL-13, and IL-17A (Fig 3c-f), but no IFNγ (data not shown). However, we observed no impact of *in vivo* anti-PD-L2 treatment on the production of any of the cytokines examined. Consistent with unaltered T cell cytokine production or BAL cell recruitment, the activation status of pulmonary myeloid DCs was also unaltered in mice treated with anti-PD-L2 (Supplementary Fig 2). Thus, in our model, endogenous expression

of PD-L2 appears to regulate the severity of allergen-induced AHR, but not by amplifying the intensity of the underlying Th2 or Th17 responses.

PD-L2 blockade significantly reduces airway mucus while enhancing IgG_{2a} production and systemic IL-12 p40 levels

As mucus hypersecretion is also a hallmark of allergic asthma, we assessed mucus production in the lungs of mice treated with PD-L2 blocking Ab. As expected, compared to mice treated with PBS, those treated with HDM + Isotype demonstrated markedly higher numbers of epithelial cells that stained with PAS (Fig 4a). In contrast, treatment of HDM-exposed mice with anti-PD-L2 significantly decreased the number of PAS+ epithelial cells (Fig 4a). To further characterize the immune response in HDM + anti-PD-L2-treated A/J mice, we assessed serum immunoglobulin levels. HDM exposure induced increased total IgE (Fig 4b) (as a surrogate for allergen-specific IgE activity[37]), HDM-specific IgG_1 (Fig 4c), and HDM specific-IgG_{2a} (Fig 4d), but treatment with control mAb had no impact on serum Ig levels. While PD-L2 blockade did not alter IgE or IgG_1 production, PD-L2 blockade nearly doubled the levels of HDM-specific IgG_{2a}. As IgG_{2a} production is driven by Th1-associated cytokines, we examined levels of IL-12 p40 in the serum (Fig 4e). IL-12 p40 levels in the serum were significantly enhanced in HDM + anti-PD-L2 treated mice, suggesting that PD-L2 can limit IL-12 p40 levels (Fig 4f). Interestingly, a similar increase was not observed in BAL, where IL-12 p40 levels were unaffected by anti-PD-L2 treatment (Supplementary Fig 3). As increases in systemic IL-12 levels limit the development of allergen-induced AHR in A/J mice[12], the increase in serum IL-12 p40 may explain the decreased AHR observed in anti-PD-L2 treated mice.

IL-12 blockade abrogates the protective effect of anti-PD-L2 treatment

To specifically test the hypothesis that increased IL-12 levels observed following PD-L2 blockade are responsible for decreased AHR, we treated mice with HDM in the presence of Isotype control mAbs, HDM + anti-PD-L2, HDM + anti-IL-12, or HDM + anti-PD-L2 + anti-IL-12. While treatment of A/J mice with anti-IL-12 had no impact on AHR, treatment of mice with anti-PD-L2 significantly diminished AHR (Fig 5a). However, treatment with anti-IL-12 abrogated the protective effect of PD-L2 blockade, as AHR in mice treated simultaneously with anti-PD-L2 and anti-IL-12 was indistinguishable from mice treated with isotype control mAbs alone (Fig 5a). Baseline AHR measurements (taken prior to injection of acetylcholine) demonstrated that while HDM increased basal AHR, this was not significantly different among treatment groups (Supplementary Fig 4).

Examination of HDM-restimulated cytokine production in lung cell cultures from anti-PD-L2, anti-IL-12 or anti-PD-L2 + anti-IL-12 treated mice revealed that while HDM induced production of both Th2 (IL-4, IL-5 and IL-13) (Fig 5 b-d) and Th17 (IL-17A) (Fig 5e) cytokines, there was no difference between treatment groups.

PD-L2 stimulation inhibits allergen-induced IL-12 production

As IL-12 p40 is a component of both bioactive IL-12 p70 and IL-23, which may have opposing effects on the development of allergen-induced AHR[4, 11], we next determined whether changes in IL-12 p40 observed following PD-L2 blockade were due to specific

effects on IL-12 p70 or IL-23. To this end we cultured bone marrow-derived mDCs (BMDCs) with HDM on plates coated with PD-L2-Fc, PD-L1-Fc or a control IgG-Fc. HDM induced IL-12 p70 production from BMDCs, and this production was unaltered by IgG-Fc (Fig 6a). DCs cultured on PD-L2-Fc-coated plates produced significantly less IL-12 p70 (Fig 6a) suggesting that stimulation with PD-L2-Fc can limit HDM-induced IL-12 p70 production. In contrast, stimulation of DCs with HDM in the presence of PD-L1-Fc, an alternative ligand for PD-1, did not impair IL-12 p70 production, suggesting PD-1 signaling is not regulating IL-12 production. The inhibition was specific for IL-12, as stimulation with PD-L2-Fc did not alter HDM-induced secretion of other DC-derived cytokines IL-23, IL-6, TNFα (Fig 6b-d) or IL-10 (data not shown). To directly test whether the capacity of PD-L2 signaling to limit bioactive IL-12 production was independent of PD-1, we cultured BMDCs on plates coated with either rIgG-Fc, or PD-L2-Fc and stimulated these cells in the presence of anti-PD-1. As shown in Figure 6e, HDM-stimulated BMDCs plated on PD-L2 coated plates produced less IL-12 p70, and their overall production of IL-12 p70 was not affected by the presence of PD-1 blocking mAb. Similar results were observed when cells were stimulated with LPS (Fig 6f). Thus stimulation with PD-L2 appears to specifically limit the capacity of DCs to secrete IL-12 p70 through a PD-1 independent mechanism.

IL-12 directly antagonizes IL-13-induced gene expression

Our observation that enhanced IL-12 production was associated with diminished AHR and mucus secretion, but unaltered production of IL-4, IL-5, IL-13 and IL-17A suggests that IL-12 may be regulating the severity of AHR by directly antagonizing signaling initiated by Th2 cytokines. As IL-13 plays a major role in both allergen-induced AHR and mucus secretion[38], we tested the hypothesis that IL-12 was antagonizing IL-13 signaling. To this end, BMDCs were cultured with medium, IL-12, IL-13 or IL-12 + IL-13 and phosphorylation of STAT6, a signaling intermediate directly activated as a result of IL-13R engagement, was measured by western blot. As shown in Figure 7a, no detectable STAT6 phosphorylation was observed in medium, or IL-12 stimulated BMDC cultures, while IL-13 induced robust STAT6 phosphorylation. In the presence of IL-12, IL-13 induced STAT6 phosphorylation was reduced, but not completely abrogated (Fig 7a). To confirm that the reduced STAT6 phosphorylation observed in IL-12 + IL-13 stimulated BMDC cultures was biologically significant, we subsequently examined the impact of IL-12 exposure on the expression of IL-13-driven genes by RT-PCR. While IL-13 induced the expression of FIZZ1, Arg1, CD206 in BMDCs (Fig 7b) the induction of these genes was reduced following co-culture with IL-12 (Fig 7b). As reported elsewhere[28], IL-13 also induced the expression of PD-L2 on BMDCs. Interestingly, IL-13-driven PD-L2 expression was similarly inhibited in the presence of IL-12, suggesting that IL-12 may support its own production by antagonizing IL-13-driven PD-L2 expression. Not all IL-13 driven genes were inhibited to the same extent, as IL-12 completely abrogated CD206 expression, while effects on other IL-13 driven genes (Arg1, Fizz1, PD-L2) were only partially abrogated, consistent with reduced, but not abrogated, phosphorylation of STAT6 observed in Fig 6a. In contrast, while IL-12 increased IFNγ expression in A/J BMDCs, this was not antagonized by IL-13, suggesting that IL-13 may not antagonize IL-12 signaling (Supplementary Fig 6a). Moreover, other Th1 cytokines can have a similar impact, as co-culture of BMDCs with IFNγ and IL-13 also abrogated IL-13-driven gene expression (Supplementary Fig 6b). In

contrast to the inability of IL-13 to inhibit IL-12-driven genes, IL-13 strongly inhibited the induction of an IFNγ-driven gene (PD-L1) (Supplementary Fig 6b).

As IL-12 is widely known as an inducer of IFNγ, we also determined if the inhibition of IL-13 signaling by IL-12 was mediated by IFNγ, through the blockade of IFNγ in cultures of BMDCs treated with IL-12 and IL-13. Inhibition of IL-13-induced Fizz1 expression by IL-12 was completely reversed by addition of anti-IFNγ (Supplementary Fig 6c). However it is unlikely that the effects of IL-12 on IL-13 signaling were entirely dependent upon induction of IFNγ, a s I L-12-mediated inhibition of STAT6 phosphorylation was observed as little as 15 minutes after IL-13 stimulation (too rapid to be due to *de novo* IFNγ synthesis) (Fig 7a), and IFNγ blockade did not restore expression of Arg1 or CD206 to levels seen in the absence of IL-12 (Supplementary Fig 6c).

As epithelial cell responsiveness to IL-13 is sufficient to observe IL-13 induced alterations in AHR, mucous cell hyperplasia and cell recruitment[39], it is also important to assess the ability of IL-12 to inhibit IL-13 signaling in airway structural cells. To this end, primary, murine tracheal epithelial cells were stimulated with IL-12, IL-13, or IL-12 + IL-13 and expression of IL-13-driven genes was assessed by RT-PCR. As shown in Figure 7c, IL-13 induced upregulation IL-33, FIZZ1, AMCase, and SPRR2b. Consistent with the effects observed on BMDCs, IL-12 abrogated IL-13-driven induction of these genes in epithelial cells as well. Thus, IL-12 appears to directly inhibit IL-13-induced gene expression in both hematopoietic and structural cells through both IFNγ-dependent, and independent mechanisms. Collectively these data demonstrate that IL-12 may limit AHR by directly antagonizing signaling of Th2-associated cytokines.

Discussion

In this study, we have examined the role of the co-stimulatory molecule, PD-L2, in the development of allergen-induced AHR. We find that PD-L2 is upregulated in the lungs of individuals with severe (but not mild) asthma and in allergen-exposed mice. In contrast to current dogma, which would suggest an inhibitory role for PD-L2 based on interactions with PD-1, *in vivo* PD-L2 blockade reduces allergen-induced AHR. While PD-L2 blockade reduced AHR, no significant impact on eosinophilia, IgE synthesis, or pulmonary dendritic cell activation was observed, suggesting that PD-L2 regulates AHR without impacting other features of allergic asthma. Collectively these data suggest that PD-L2 expression regulates the overall magnitude of AHR in allergic asthma.

Our observation that PD-L2 signaling exacerbates asthma severity is in agreement with the previous report that PD-L2-Fc treatment enhances pulmonary inflammation in OVA (alum) sensitized BALB/c mice[30]. However, in contrast to our results, it has also been reported that blockade of PD-L2 (but not PD-1) in OVA (alum) sensitized BALB/c mice triggers increased AHR, a phenotype that was blocked with co-administration of IFNγ-blocking antibodies[26]. Similarly, AHR induced by exposure of BALB/c mice to OVA (alum) or αGalCer was increased in PD-L2 deficient mice due to increased IL-4 production by iNKT cells[27]. While these reports appear to conflict with our own, both are consistent with an increase in IL-12 production in the absence of PD-L2 signaling. In the study by Matsumoto

et al, while IL-12 levels were not assessed, it is likely that the IFNγ driving increased AHR following PD-L2 blockade was driven by enhanced IL-12 production triggered by the lack of PD-L2 signaling. Moreover, IL-12 has been shown to drive IL-4 production in iNKT cells[40-41]. Thus as PD-L2 −/− mice lack a negative regulator of IL-12 production, iNKT cells from these mice display increased production of IL-4[27]. Thus, collectively these studies support an important role for PD-L2 in the direct regulation of IL-12 synthesis.

While IL-12 is classically regarded as a shaper of adaptive immunity, we find that the enhancement of IL-12 production observed following PD-L2 blockade does not regulate the balance of Th1 and Th2 associated cytokines in our model. It should be noted that while we observe robust PD-L2 expression after allergen challenge, we observe very few PD-L2+ mDCs immediately after a single allergen inhalation (data not shown). This suggests that the elevated IL-12 observed following anti-PD-L2 treatment is likely only present during the effector phase, after allergen challenge. Thus, by inhibiting IL-12 levels exclusively during the effector phase, we find that PD-L2 directly promotes IL-13-driven sequelae (AHR, mucus hypersecretion) in allergic asthma independently of its effects on T cell differentiation. While it is conceivable that blockade of PD-L2 induces other changes which may contribute to the reduced AHR observed, simultaneous blockade of PD-L2 and IL-12 abrogates the protective effects of PD-L2 blockade, suggesting that the increased IL-12 observed is the dominant influence on the reduced AHR observed following PD-L2 blockade.

While the precise gene products induced by IL-13 that promote the development of AHR are not clear, we have demonstrated that IL-12 can inhibit a number of IL-13-induced products with described roles in allergic asthma. In bone marrow-derived dendritic cells IL-12 inhibits IL-13-induced expression of *Fizz1* and *Arg1*, products associated with alternatively activated macrophages which have been shown to play a pathogenic role in allergic asthma[42-43]. Additionally, IL-12 also antagonized the IL-13-induced expression of CD206 (a receptor shown to promote the phagocytosis of Derp1[44]) in BMDCs. While we do not observe an increase in overall HDM uptake by pulmonary DCs following blockade of PD-L2 *in vivo*, it is possible that PD-L2 blockade may subtly regulate the ability of DCs to take up or present specific allergenic peptides. In primary murine tracheal epithelial cells, IL-12 also inhibited the IL-13-induced expression of the genes encoding IL-33, (a Th2-inducing cytokine[45-46]), AMCase (capable of enhancing allergen-driven IL-13 production[47-48]), and Fizz1 (capable of inducing airway eosinophilia and airway remodeling[49]). We also see reduced IL-13-driven expression of Sprr2b following IL-12 treatment. While little is known about the role of Sprr2b in the development of allergic asthma, it is induced by IL-13, but not IL-4[50]. This is of particular interest as IL-13 is considerably more important than IL-4 in the induction of AHR[38], suggesting that gene products induced by IL-13, but not IL-4 may have an important role in promoting AHR. Finally, we also demonstrate that IL-12 can directly antagonize STAT6 phosphorylation triggered by IL-13. It is likely that this effect may directly contribute to the reduced AHR observed in mice treated with anti-PD-L2 as STAT6 activation is critically required to trigger IL-13-induced AHR[39, 51-52]. Thus, despite unaltered levels of overall IL-13 production, IL-12 may impact the overall bioactivity of IL-13 through a number of different mechanisms.

That IL-12 may directly antagonize IL-13 signaling is also consistent with our previous observation that IL-12 blockade in A/J mice enhanced basal acetylcholine-induced airway constriction, suggesting that IL-12 can regulate airway tone in the absence of airway inflammation[11] and with reports showing IL-12-deficient mice can display enhanced AHR, with minimal impact on Th2 cytokine production[11, 53-54]. We also find elevated PD-L2 expression in individuals with severe asthma, but not those suffering from milder forms of disease, suggesting that PD-L2 expression may also be correlated with asthma severity in humans as well. Although we did not examine IL-12 levels in individuals with severe versus mild asthma, Morahan *et al*. have shown that a combination of SNPs in the human IL-12 p40 promoter resulting in reduced LPS-stimulated IL-12 p40 secretion by PBMCs is associated with more severe asthma, regardless of the atopic status of the individual[55]. This suggests that impaired IL-12 production in humans also correlates with asthma severity, but not T cell skewing leading to initial sensitization (i.e. atopic status). Thus, our study highlights a previously underappreciated role of IL-12 in directly limiting asthma severity.

PD-L2 is typically thought to signal through PD-1 providing an inhibitory signal, limiting proliferation, and cytokine production of both T cells[30, 56-58] and APCs[20-21, 59]. However interactions between PD-L2 and a receptor other than PD-1 were observed in a number of *in vitro* studies where it was demonstrated that such interactions enhance T cell activity[33-35, 60]. Multiple lines of evidence suggest that, in our model, the dominant signals provided by PD-L2 are mediated by a non-PD-1 receptor including: 1) *in vivo* blockade of PD-L2 and PD-1 have differential impacts on HDM-induced AHR, 2) stimulation with PD-L2, but not PD-L1, limits HDM- and LPS-induced IL-12 production *in vitro*, and 3) the ability of PD-L2 to inhibit HDM or LPS-driven IL-12 p70 production in BMDCs is not abrogated in the presence of antibodies known to block the interactions between PD-L2 and PD-1. As stimulation of DCs with PD-L2, but not PD-L1, limits HDM-induced IL-12 production, we speculate that DCs also express the unidentified PD-L2 receptor, and that signaling through this receptor in DCs directly regulates IL-12 p70 production. Intriguingly, as PD-L2 is primarily expressed by mDCs, the ability of PD-L2 to downregulate DC-derived IL-12 production suggests that autocrine or paracrine interactions between DCs are capable of regulating IL-12 production. Thus, PD-L2-mediated regulation of immune responses is complex, being regulated via interactions between PD-L2 and at least two distinct receptors, both of which are expressed on DCs and T cells, and which have distinct effects based on the types of cells studied. Such complexities suggest that PD-L2 (and other family members) acts as a rheostat, modulating the responsiveness to immune mediators such as IL-13, rather than providing an “on/off” switch for one particular type of response. However, our observation of differential expression levels of PD-L2 in human tissues taken from individuals with mild versus severe asthma, suggest that these subtle influences can have a substantial impact on disease severity.

Collectively these data demonstrate that PD-L2 expression modulates the overall magnitude of AHR in allergic asthma. However, rather than directly regulating T cell activity through interactions with its previously described receptor, PD-1, the dominant function of PD-L2 *in vivo* is inhibition of APC-derived IL-12 through interactions with an unknown receptor. Moreover, PD-L2 stimulation of purified DCs directly inhibits IL-12 production,

demonstrating that the unknown receptor is also expressed by DCs and suggesting that DC:DC interactions, either autocrine or paracrine, modulate IL-12 synthesis *in vivo*. Finally, these data also demonstrate that IL-12 can directly antagonize IL-13-driven gene transcription, highlighting a previously unappreciated role for IL-12 in modulating the activity of Th2 effector cytokines. Thus, in asthmatics, increased PD-L2 expression in the inflamed lung downregulates IL-12 secretion, facilitating greater IL-13 responsiveness and allowing the development of more severe airway hyperresponsiveness.

Materials and Methods

Mice

Male A/J mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 4 weeks of age and housed in a specific pathogen-free facility at Cincinnati Children's Hospital Medical Center. Experiments were initiated at 6 weeks of age. Animal experiments were approved by the CCHMC Institutional Animal Care and Use Committee.

Treatment protocols

Mice were treated with 40 μl PBS, HDM (100 μg; Greer Laboratories, Lenoir, NC), or AlexaFluor405-labeled (Invitrogen, Carlsbad, CA) HDM (AF405-HDM) i.t. on days 0 and 14. Where indicated mice were treated with low-endotoxin OVA (Worthington Biochemicals, Lakewood, NJ) to induce AHR (10 μg i.p. on day 0, and 100 μg i.t. on days 14 and 21) or tolerance (100 μg i.t. on days 0, 14 and 21). Mice were treated intraperitoneally with 250 μg anti-PD-L2 (TY25[18, 26]), anti-PD-1 (RMP1-14[36]) or control rat IgG_{2a} (GL117) 6 hours before, and 48 hours following each allergen exposure. Where indicated mice were also treated with 1 mg of anti-IL-12 (clone C17.8, a gift of Dr. Fred Finkelman, University of Cincinnati) or control rat IgG_{2a} (GL117) on days −2 and 12.

Assessment of allergen-induced airway responsiveness

Airway hyperresponsiveness (AHR) was measured by assessing changes in tracheal pressure following administration of a cholinergic agonist over time and expressed as the airway pressure time index (APTI), and is described in detail elsewhere[31]. Importantly, this parameter has been shown to provide data similar to Flexivent-derived data generated using a full dose response of inhaled methacholine[61] To evaluate AHR, mice were anaesthetized, intubated and respirated at a rate of 120 breaths per minute with a constant tidal volume (0.2 ml) and paralyzed with decamethonium bromide (25 mg/kg) 72 hours after final allergen challenge. Acetylcholine (50 μg/kg) was injected into the inferior vena cava and dynamic airway pressure (cmH_20xSec) followed for 5 minutes. Blood was collected to measure total IgE and HDM-specific IgG_1 and IgG_{2a}. To collect BALF, lungs were lavaged with HBSS (Invitrogen, Carlsbad, CA). Cells were recovered by centrifugation and total cells counted. Slides were prepared by cytocentrifugation and stained with Diff-Quik (Dade Behring, Düdingen, Switzerland) for differential cell counts.

Lung cell isolation

After AHR measurements, lungs were removed, minced and placed in 6 ml of RPMI 1640 containing Liberase CI (0.5 mg/ml)(Roche Diagnostics) and DNase I (0.5 mg/ml)(Sigma) at

37°C for 45 minutes. The remaining tissue was forced through a 70-micron cell strainer, and red blood cells were lysed with ACK lysis buffer (Invitrogen). Cells were washed with RPMI containing 10% FBS, viable cells were counted via trypan blue exclusion. Where indicated, lung cells were cultured at 350,000 cells per well of a 96-well plate (250 μl final volume) with HDM (30 μg/ml). Tissue culture supernatants were harvested at 72 hours.

Flow Cytometry

Staining reactions were performed at 4°C following incubation with FcBlock (mAb 2.4G2) for 30 minutes. Alveolar Macrophages (AM - $CD11c^{bright}$, $CD11b^{neg}$, $CD317^{neg}$), plasmacytoid DCs (pDCs - $CD11c^{dim}CD11b^{neg}CD317^{+}$), neutrophils (PMNs - $CD11c^{bright}CD11b^{bright}Gr1^{bright}CD103^{neg}$), inflammatory DCs (Inf DCs - $CD11c^{bright}CD11b^{bright}Gr1^{dim}CD103^{neg}$) and myeloid DCs ($CD11c^{bright}CD11b^{bright}Gr1^{neg}CD103^{neg}$) were quantified using anti-CD11c-PerCPCy5.5 (HL3), anti-CD11b-PE-Cy7 (M1/70), anti-Gr1-APC-Cy7 (RB6-8C5), anti-CD103-FITC (2E7), and anti-CD317-AF647 (eBio129c). Co-stimulatory molecule expression was examined using PE-conjugated mAbs to MHC Class II (14-4-4s) and PD-L2 (TY25). mAbs were purchased from eBioscience. Antigen uptake was measured in the Pacific Blue channel following administrated of AF405-conjugated HDM. Dead cells were excluded using Live/Dead Blue (Invitrogen). Data were acquired with an LSRII flow cytometer equipped with lasers tuned to 355 nm, 405nm, 488nm, 562nm, and 640nm, and digital DiVa Software. FlowJo software (Treestar Inc., Ashland, OR) used to compensate spectral overlap and analyze data.

Subject recruitment

Subjects with no asthma, or well-defined mild and severe asthma were recruited from the Montreal Chest Institute and Sacré-Coeur Hospital (Montreal, Quebec, Canada) based on the criteria of the ATS workshop for Refractory Asthma[62]. As described elsewhere[63], mild asthmatics had FEV1 readings greater than or equal to 70% postbronchodilator and required infrequent use of inhaled short-acting β_2-agonist medication alone. Severe asthmatics had at least one of (1) treatment with daily oral steroids for more than 50% of the previous 12 months, or (2) treatment with high-dose inhaled steroids and at least one other add-on therapy continuously over the past 12 months. In addition, 2 or more of the following were required: (1) daily use of short-acting β-agonist; (2) persistent airflow obstruction as documented by prebronchodilator FEV_1 less than 70% and FEV_1/FVC ratio less than 80% predicted; (3) at least 1 urgent care visit in the previous 12 months; (4) ≥3 steroid bursts in the previous 12 months; (5) prompt deterioration with less than 25% dose reduction of steroids; or (6) a near-fatal asthma event in the last 3 years. Detailed definition of the inclusion and exclusion criteria for the enrollment of asthmatic subjects were reported previously[63]. The protocols for the study were reviewed and approved by the ethics committees of both hospitals, and informed consent was obtained from all participating subjects. RNA was extracted from lung biopsies using the RNeasy columns, reverse transcribed with oligo(dT) (Amersham) and Superscript II (Invitrogen), in the presence of RNAguard (Amersham).

Quantitative real-time PCR

PCR primer pairs for mPD-L2, mFIZZ1, mArg1, mSPRR2b, mCD206, mC3, mS14, mIL-5, mIL-13, mAMCase, mIL-33, mIFNγ, mPD-L1, hPD-L2 and hS9 were obtained from the mouse or human primer depot[64] and designed to span an intronic region to avoid co-amplification of genomic DNA. Gene expression in mouse cells was analysed by real-time PCR using the iCycler system (BioRad) and SYBR green, while gene expression in human bronchial biopsies was done by real-time PCR using the StepOnePlus PCR system (Applied Biosystems) and SYBR green. Gene expression analysis was performed using the ΔΔCt method described elsewhere [65].

Generation and stimulation of bone marrow-derived DCs (BMDCs)

Bone marrow cells (3×10^5 cells/ml) were cultured in complete RPMI 1640 supplemented with GM-CSF (10 ng/ml, Peprotech, Rocky Hill, NJ, USA). Medium was changed on day 3 and harvested on day 6. 250,000 cells were plated on wells coated with 5 μg/ml PD-L1-Fc, PD-L2-Fc, or control IgG-Fc (R&D Systems) and stimulated with 30 μg/ml HDM or 100 ng/ml LPS for 24 hours. Culture supernatants were harvested to assay IL-12 p70, IL-6, TNF-α, IL-1β (Pharmingen) and IL-23 (R&D Systems) by ELISA. Where indicated BMDCs were stimulated with IL-12, IL-13, or IL-12 + IL-13 (all 10 ng/ml) for 24 hours. RNA was harvested for PCR analysis. Where indicated cultures also included anti-PD-1 (clone RMP1-14) (eBioscience) at 5 μg/ml.

Mouse tracheal epithelial cells (mTEC) isolation

Tracheas from 4-6-week old mice were removed from the thyroid cartilage to the level of bifurcation and incubated in Pronase (1 mg/ml; Roche Applied Science, Indianapolis, IN) and incubated (18 h × 4°C while rocking). The next day, 10% FBS and 1 mg/ml DNase (Sigma-Aldrich, St. Louis, MO) were added, and the tube inverted multiple times. The trachea was discarded; cells were washed and plated onto a cell culture plate with Primaria surface treatment (BD Biosciences, Bedford MA) for 4 h to remove fibroblasts. Non-attached cells were washed, counted and plated in DMEM/F12 (50/50) containing L-glutamine (2 mM), penicillin (100 U/ml)/streptomycin (100 μg/ml) $NaHCO_3$ (3.6 mM), FBS (5%), Cholera toxin (0.1 μg/ml), mouse EGF (0.5 ng/ml), amphotericin B (0.25 μg/ml), bovine pituitary extract (50 μg/ml), insulin-transferrin-selenium media supplement (Sigma Chemical Corp, St. Louis MO), and retinoic acid (0.1 ng/ml). mTECs were grown on collagen-coated 6 well tissue culture plates until confluent. mTECs were stimulated with nothing, IL-13 (10 ng/ml) or IL-12 + IL-13 (both at 10 ng/ml) for 24 hours. Cells were lysed with Trizol, total RNA was isolated, and cDNA synthesized to measure gene expression by RT-PCR.

Western Blot

For western blot analysis of STAT6 phosphorylation, BMDCs from BABL/c mice were generated as described above. After harvest, 2.5 million BMDCs were plated in complete RPMI culture medium (containing 10% fetal bovine serum (FBS)) in single wells of a 24 well plate, and allowed to adhere for 12 hours. After 12 hours, the culture medium was removed, and BMDCs were starved in RPMI containing 0.1% FBS overnight. The following

morning cells were stimulated with medium alone (containing 0.1% FBS), IL-12, IL-13 or IL-12 + IL-13 (all at 10 ng/ml) for 15 minutes. Cells were immediately placed on ice, washed with ice-cold PBS and lysed with a lysis buffer containing protease inhibitors (Roche) and phosphatase inhibitor cocktails (CalBiochem). Lysates were stored at −80° until required. Total proteins (10 μg) were separated by electrophoresis on an SDS polyacrylamide gel, and transferred onto PVDF membranes and blocked with 5% BSA for 1 hr at RT. Afterwards, the membranes were incubated overnight with primary antibodies (in 5% BSA in TBS) as follows: rabbit anti-human/mouse phospho-STAT6 (Tyr761) (1:10000, Cell Signaling Technology, Beverly, MA), rabbit anti-mouse STAT6 (1:10000, Cell Signaling Technology), or anti-α-tubulin (1:10000, Cell Signaling Technology). Membranes were then washed and incubated for 1 h at RT with an HRP-conjugated goat anti-rabbit secondary (in 5% BSA in TBS at 1:10000, Cell Signaling Technology), blots were developed using an ECL Prime (GE Healthcare, Pittsburgh, PA)

Statistical Analysis

To determine differences between multiple groups, analysis of variance (ANOVA) was used with *post hoc* comparisons using Tukey's method. For comparison between two groups, a Student's t-test was performed. In human samples, ANOVA was used with *post hoc* comparisons using the Kruskal-Wallis test. Significance was assumed at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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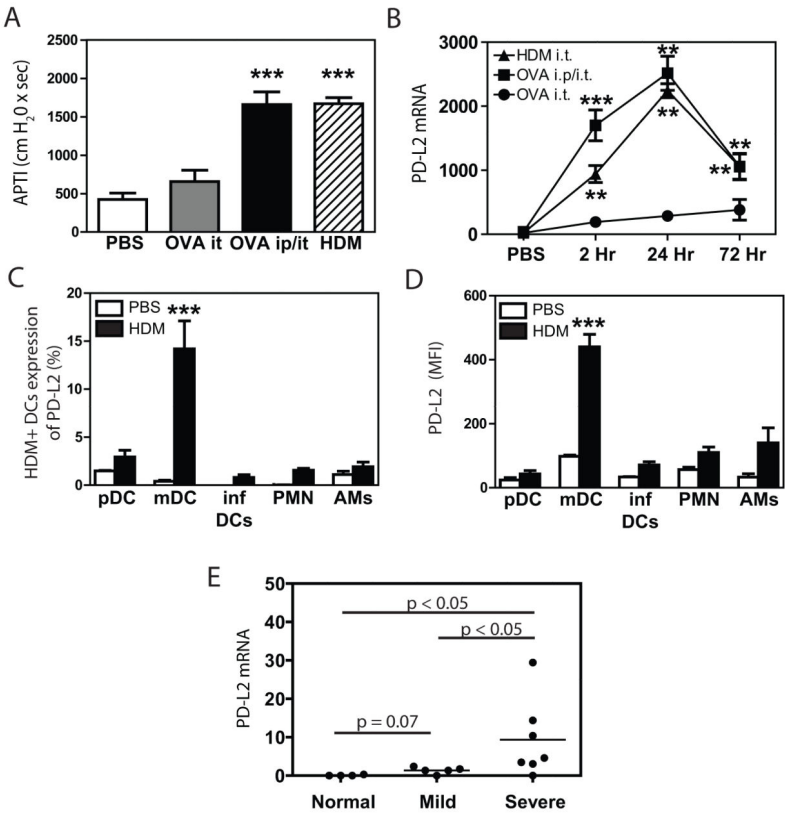

Figure 1. PD-L2 expression is enhanced in the airways of asthmatic individuals and allergen-exposed mice

Mice were treated with PBS (PBS - intratracheally on days 0, and 14), intratracheal OVA (OVA i.t. - 100 μg on days 0, 14 and 21), intraperitoneal/intratracheal OVA (OVA i.p./i.t. - 10 μg OVA i.p. on day 0 followed by 100 μg i.t. on days 14 and 21), or intratracheal HDM (HDM - 100 μg on days 0 and 14). (A) Mice were sacrificed 72 hours after final allergen exposure to measure AHR via the airway pressure time index (APTI) method. (B) PD-L2 expression (normalized to mouse ribosomal protein s14) was measured by RT-PCR. n = 4 mice per group. 1 representative experiment of 2 shown. Mean + SEM shown. *** and ** indicate $p < 0.001$, and $p < 0.01$ versus PBS-treated mice respectively. The frequency of HDM+ DCs of the indicated subsets expressing PD-L2 (C) and the MFI of PD-L2 expression on the total DC population (regardless of allergen uptake) (D) was assessed by flow cytometry. n = 4 mice per group. Mean + SEM shown. *** indicates $p < 0.001$ versus PBS-treated mice respectively. 1 representative experiment of 2 shown. (E) PD-L2 expression (normalized to human ribosomal protein s9) was assessed by PCR in samples taken from normal controls, or individuals categorized as mild or severe asthmatics. Each point represents a single individual.

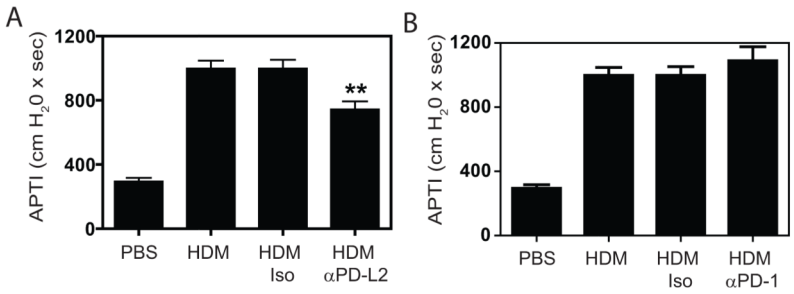

Figure 2. *In vivo* PD-L2 blockade, but not PD-1 blockade, limits the severity of allergen-induced AHR

A/J mice were treated with PBS or HDM intratracheally on days 0 and 14, 250 μg of rat IgG_{2a} control (Iso) or anti-PD-L2 intraperitoneally (αPD-L2) on days 0, 2, 14 and 16, and AHR was assessed was assessed via the APTI method on day 17 (A). To determine PD-1 dependence, A/J mice were treated with PBS or HDM intratracheally on days 0 and 14, and 250 μg of rat IgG_{2a} control (Iso) or anti-PD-1 (αPD-1) intraperitoneally on days 0, 2, 14 and 16, and AHR was assessed via the APTI method on day 17 (B). n = 30 mice pooled from 5 independent experiments. Mean + SEM shown. ** indicates $p < 0.01$ versus Iso

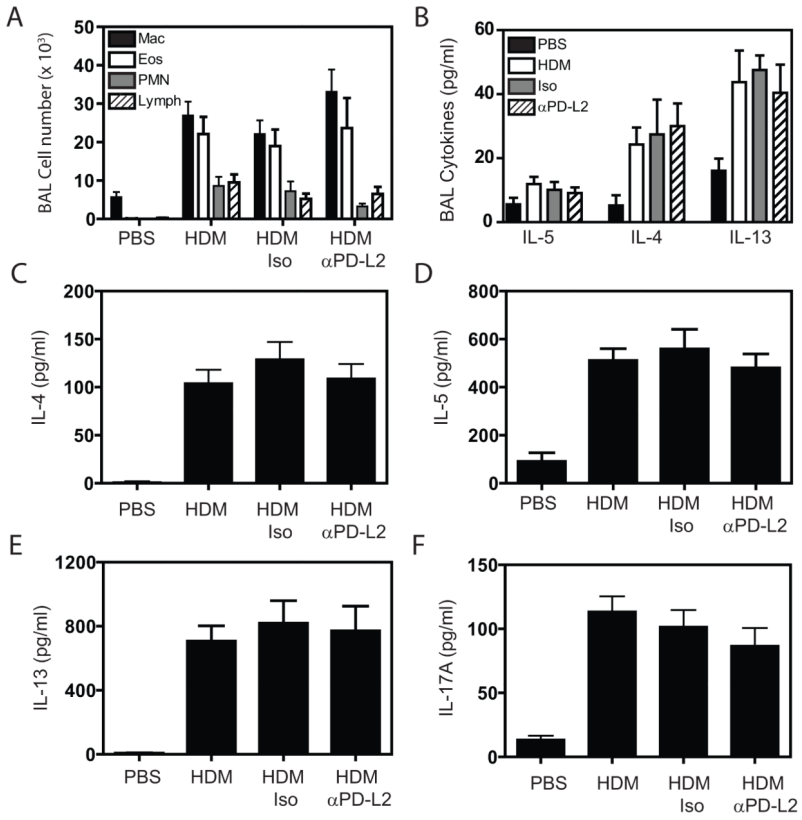

Figure 3. ***In vivo*** **PD-L2 blockade does not alter T cell cytokine production**

A/J mice were treated with PBS, HDM, HDM + rat IgG_{2a} control (Iso) or HDM + anti-PD-L2 (αPD-L2) as described in Materials and Methods. (A) Cellular profile and (B) cytokine levels present in BAL fluid taken at time of sacrifice. Lung cells were re-stimulated *in vitro* with HDM and levels of IL-4 (C), IL-5 (D), IL-13 (E) and IL-17A (F) were assessed by ELISA. n = 30 mice from 5 independent experiments, or n = 10 mice from 2 independent experiments for BAL cytokine assessment. Mean + SEM shown.

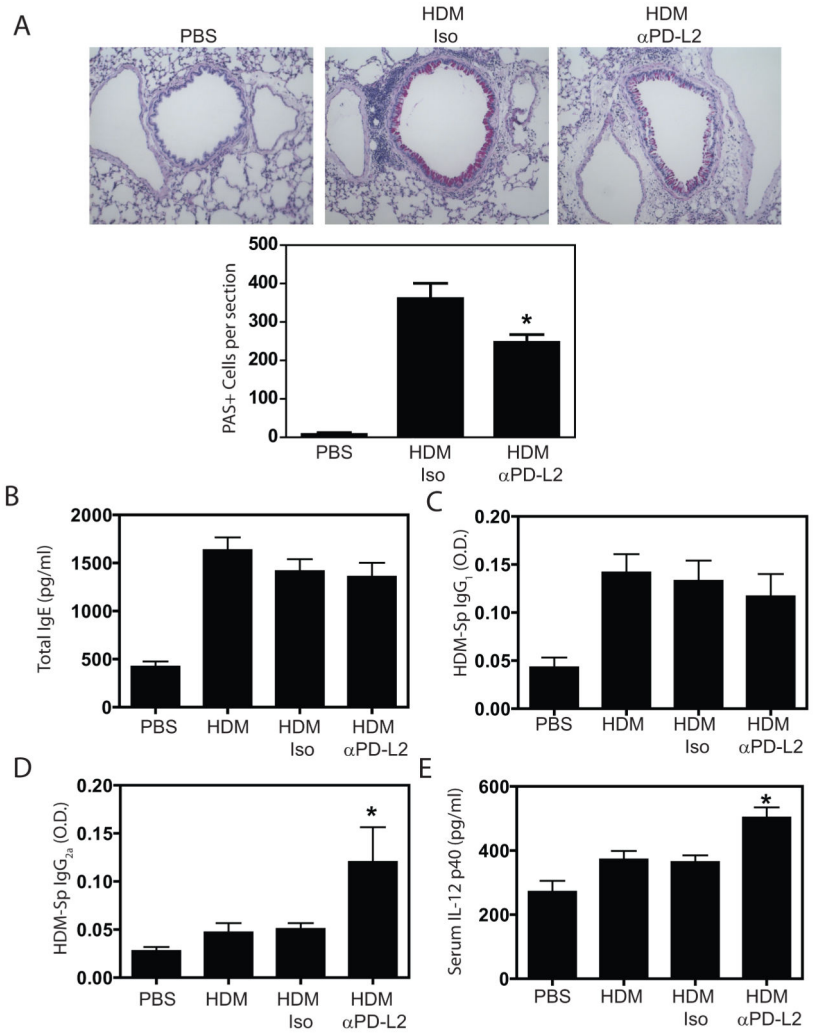

Figure 4. ***In vivo*** **PD-L2 blockade enhances allergen-specific IgG_{2a} production and levels of systemic IL-12 p40**

A/J mice were treated with PBS, HDM, HDM + rat IgG_{2a} control (Iso) or HDM + anti-PD-L2 (αPD-L2) as described in Materials and Methods. Airway sections were stained with PAS to quantify mucus secreting cells, and the number of PAS+ epithelial cells in 4 separate sections from four individual animals from each treatment group were determined (A). Serum collected at the time of harvest was assayed for levels of total IgE (B), HDM-specific IgG_1 (C) HDM-specific IgG_{2a} (C) and IL-12 p40 (D) by ELISA. n = 30 mice from 5 independent experiments. Mean + SEM shown. * indicates $p < 0.05$ versus Iso.

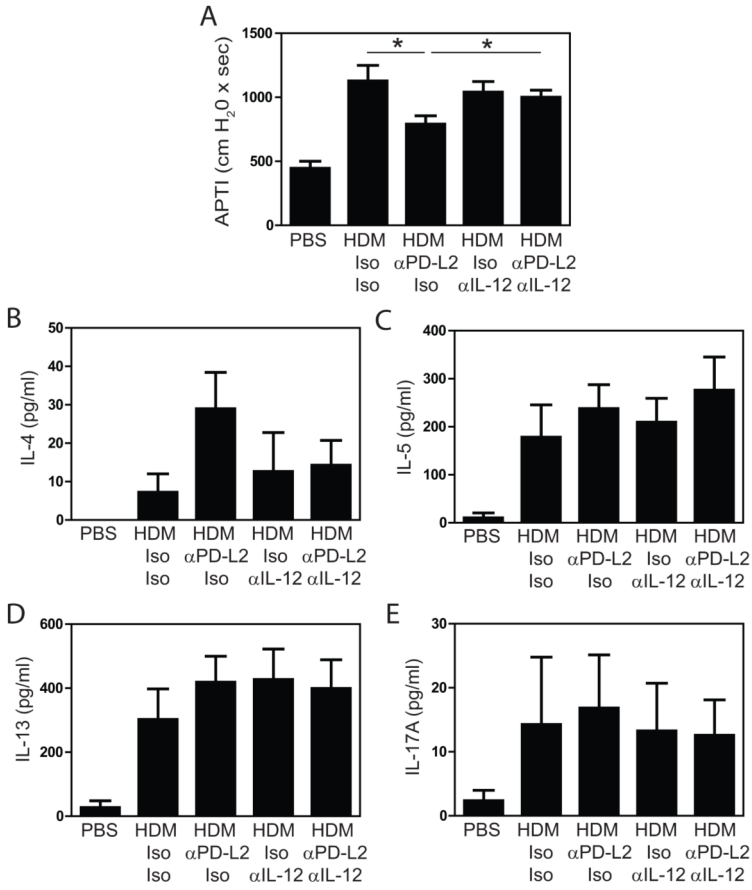

Figure 5. Simultaneous neutralization of endogenous IL-12 reverses decreased AHR observed following PD-L2 blockade

A/J mice were treated with PBS or HDM intratracheally on days 0 and 14. Mice were given 250 μg of rat IgG_{2a} control (Iso) or anti-PD-L2 (αPD-L2) intraperitoneally on days 0, 2, 14 and 16 and/or 1 mg of rat IgG2a control (Iso) or anti-IL-12 (αIL-12) intraperitoneally on days −2 and 12. Mice were sacrificed on day 17 for assessment of AHR by the APTI method (A). Lung cells were re-stimulated *in vitro* with HDM and levels of IL-4 (B), IL-5 (C), IL-13 (D) and IL-17A (E) were assessed by ELISA. n = 10 mice from 2 independent experiments. Mean + SEM shown. * indicates $p < 0.05$ compared to anti-PD-L2/Iso treated mice.

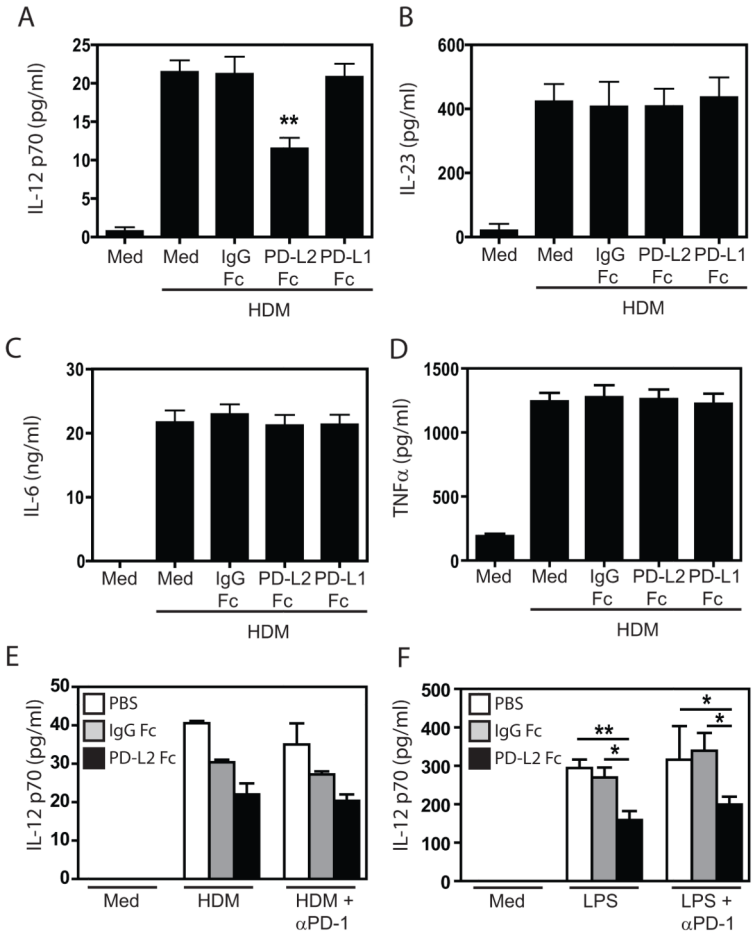

Figure 6. Stimulation of BMDCs with PD-L2-Fc limits HDM-induced IL-12 p70, but not IL-23, IL-6 or TNFα production

BMDCs were grown as described in Materials and Methods. Cells were cultured in the presence of medium alone or HDM on plates coated with IgG-Fc, PD-L2-Fc, or PD-L1-Fc. After 18 hours, supernatants were harvested, and the levels of IL-12 p70 (A), IL-23 (B) IL-6 (C) and TNFα (D) were determined by ELISA. n = 17 individual wells, over 3 independent experiments. Mean + SEM shown. ** indicates $p < 0.01$ versus HDM + IgG-Fc stimulated wells. BMDCs were cultured on plates coated with PBS (open bars), IgG-Fc (grey bars), or PD-L2-Fc (black bars) in the presence of HDM (E), or LPS (F) in the presence or absence of anti-PD-1. After 18 hours, supernatants were harvested and the levels of IL-12 p70 were determined by ELISA. n = 4 wells from 1 of 2 experiments performed. Mean + SEM shown. * and ** indicate $p < 0.05$ and $p < 0.01$ respectively.

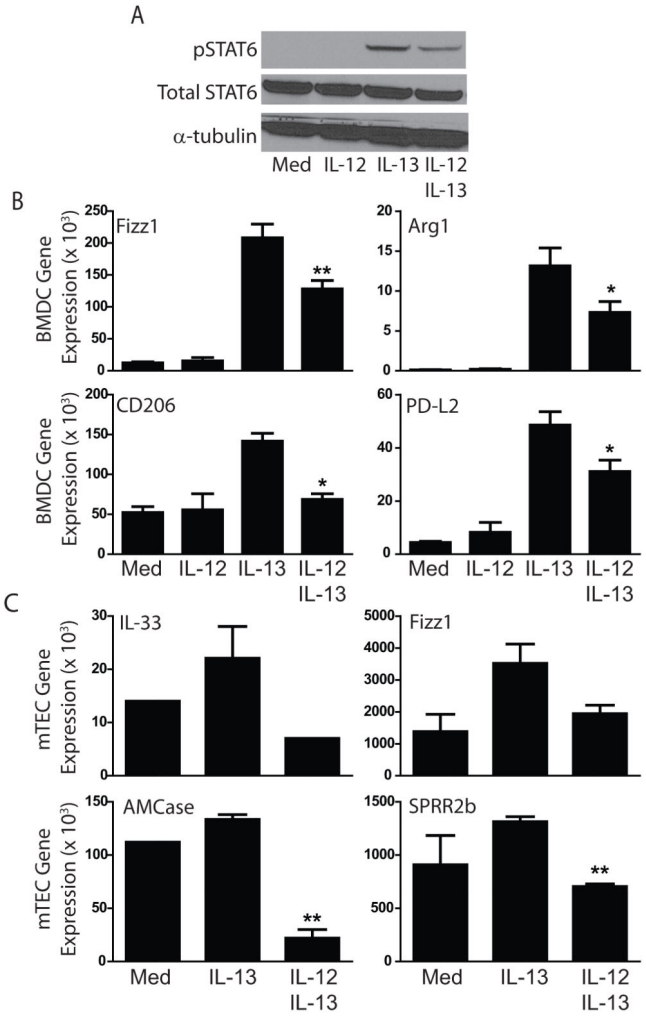

Figure 7. IL-12 directly antagonizes IL-13-induced STAT6 phosphorylation and gene expression

BMDCs were stimulated with medium alone, IL-12 (10 ng/ml), IL-13 (10 ng/ml) or a combination of IL-12 + IL-13 for 15 minutes. Proteins were separated on an SDS gel and transferred to a PVDF membrane for detection of pSTAT6, total STAT6, and as a loading control, α-tubulin (A). BMDCs were stimulated with medium alone, IL-12 (10 ng/ml), IL-13 (10 ng/ml) or a combination of IL-12 + IL-13 for 18 hours. mRNA was harvested from the cell and expression of IL-13 driven genes was examined by RT-PCR. (B) Primary murine tracheal epithelial cells (mTECs) from C57Bl/6 mice were treated with medium alone, IL-13 (10 ng/ml) or IL-12 + IL-13 (both at 10 ng/ml) for 24 hours. RNA was isolated and expression of IL-13-driven genes was examined by RT-PCR (C). Mean + SEM shown. * and ** indicate $p < 0.05$, and $p < 0.01$ versus IL-13-treated cultures respectively. n = 4 replicates for each condition tested.