

Differential control of CD4⁺ T-cell subsets by the PD-1/PD-L1 axis in a mouse model of allergic asthma

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Studies examining the role of PD-1 family members in allergic asthma have yielded conflicting results. Using a mouse model of allergic asthma, we demonstrate that blockade of PD-1/PD-L1 has distinct influences on different CD4⁺ T-cell subsets. PD-1/PD-L1 blockade enhances airway hyperreactivity (AHR), not by altering the magnitude of the underlying Th2-type immune response, but by allowing the development of a concomitant Th17-type immune response. Supporting differential CD4⁺ T-cell responsiveness to PD-1-mediated inhibition, naïve PD-1^{-/-} mice displayed elevated Th1 and Th17 levels, but diminished Th2 cytokine levels, and ligation of PD-1 in WT cells limited cytokine production by in vitro polarized Th1 and Th17 cells, but slightly enhanced cytokine production by in vitro polarized Th2 cells. Furthermore, PD-1 ligation enhanced Th2 cytokine production by naïve T cells cultured under nonpolarizing conditions. These data demonstrate that different CD4⁺ T-cell subsets respond differentially to PD-1 ligation and may explain some of the variable results observed in control of allergic asthma by the PD-1 family members. As the PD-1/PD-L1 axis limits asthma severity by constraining Th17 cell activity, this suggests that severe allergic asthma may be associated with a defective PD-1/PD-L1 regulatory axis in some individuals.

Keywords: Airway hyperreactivity · Asthma · Cytokines · PD-1/PD-L1 · Th1 response · Th2 response · Th17 response



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Introduction

The prevalence of allergic asthma continues to rise in developed nations. As overproduction of Th2 cytokines such as IL-4, IL-5, and

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IL-13 following allergen exposure is sufficient to explain many of the changes associated with allergic asthma (elevated IgE production, eosinophil-dominated inflammation, airway hyperreactivity (AHR)), the Th2 cell is considered central to disease pathology [1]. However, other T-cell subsets can markedly influence the course of disease. For example, regulatory T (Treg) cells have been ascribed a protective role in both mouse models of allergic asthma and human disease [2–4]. In contrast, recent evidence suggests that the production of Th17-associated cytokines may drive the development of more severe disease [5–7]. Thus, factors that subtly alter the balance of T-cell effector function serve as important regulators of susceptibility to allergen-induced AHR.

As professional APCs, dendritic cells (DCs) within the lung integrate a multitude of environmental signals that drive the development of pathogenic or protective T-cell responses. As such, DCs play an important role in regulating the development of allergen-induced AHR. One method of conveying these integrated signals to developing T cells is through the expression of the PD-1 family of co-stimulatory molecules. PD-1 (CD279) is expressed primarily by activated CD8⁺ T cells, CD4⁺ T cells, and B cells [8]. It binds two unique ligands, PD-L1 (CD274), expressed on both nonhematopoietic and hematopoietic including DCs, and PD-L2 (CD273) whose expression is restricted to activated APCs [9, 10]. However, despite expression on both CD4⁺ and CD8⁺ T cells, the actions of PD-1 in vivo are best described in CD8⁺ T cells where high PD-1 drives "exhaustion" of virus-specific CD8⁺ T cells observed following chronic infection [11, 12] and restrains CD8⁺ memory development after acute lower respiratory tract infections with human metapneumovirus, respiratory syncytial virus, or influenza virus [13]. In contrast to its well-defined role in controlling CD8⁺ responses, the importance of PD-1 in regulating different effector CD4⁺ T-cell responses is poorly studied. In CD4⁺ T cells, PD-1 is best known as a promoter of CD4⁺CD25⁺ Treg-cell activity [14] and the capacity of pulmonary plasmacytoid DCs (pDCs) to induce Treg cells and limit asthma development has been linked to their elevated expression of PD-L1 [15]. Interestingly, despite the characterization of PD-1 as a driver of Treg-cell development, 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 [15–19], and others reporting a pro-asthmatic role for either PD-L1 [17] or PD-L2 [20, 21]. Collectively, these data suggest that the ability of PD-1 family members to control CD4⁺ T-cell responses may be more complicated than previously appreciated.

Herein we sought to clarify the role of the PD-1/PD-L1 axis in control of allergic asthma. In contrast to our previous observations of limited impact of PD-1 blockade on allergen-induced AHR in A/J mice, in which allergen uptake is primarily by pulmonary myeloid DCs, we found that PD-L1 and PD-1 blockade in C3H mice (a model of mild AHR in which allergen uptake is primarily by pDCs) significantly enhanced the severity of allergen-induced AHR. Surprisingly, the enhanced AHR was associated with a shift in the pulmonary inflammatory cell profile toward increased pulmonary neutrophil infiltration and increased production of Th17-associated cytokines while Th2 cytokine responses were unaltered. These findings suggest that the PD-1/PD-L1 axis

may variably regulate the activity of different effector CD4⁺ T-cell subsets, and may have differential capacity to inhibit T-cell responses in different strains of mice. Consistent with these findings, we observed increased Th1- and Th17-associated cytokine production, concomitant with decreased Th2-associated cytokine production in naïve *Pdcd1*^{−/−} mice. Additionally, PD-1 ligation (with rPD-L1-Fc) during in vitro Th1/Th17 differentiation or restimulation significantly decreased Th1/Th17 cytokine production, while stimulation of PD-1 on Th2 cells via either PD-L1 or PD-L2 enhanced Th2 cytokine production. The ability of PD-1 signaling to enhance Th2 differentiation was even noted in cultures of naïve CD4⁺ T cells stimulated in the absence of any additional T-cell polarizing cytokines, wherein PD-1 stimulation was associated with increased expression of the Th2-associated transcription factor GATA3. Collectively, these data suggest that overall regulation of CD4⁺ T-cell responses by PD-1 is more complicated than previously anticipated, and varies between different subsets and strains of mice. It seems likely that differences in cell type and strain responsiveness may have contributed to some of the conflicting data regarding the role of PD-1 family members in allergic asthma.

Results

PD-1 blockade enhances Th17, but not Th2 responses, in a mouse model of allergic asthma

After intratracheal allergen exposure, A/J mice develop allergen-induced AHR associated with allergen uptake by pulmonary myeloid DCs, and a mixed Th2/Th17 response [7, 22]. In A/J mice, PD-L2 blockade enhanced DC-derived IL-12 production and diminished AHR, while PD-1 blockade had no impact on AHR severity [20]. In contrast to A/J mice, C3H mice develop mild AHR associated with allergen uptake by pulmonary pDCs and Th2 differentiation [7, 22]. As pDCs exert an antiasthmatic influence through PD-L1 expression [15], we hypothesized the PD-1/PD-L1 axis may play a greater role in C3H mice. To determine the roles of the PD-1 family members in regulating asthma development in a more asthma-resistant strain, we treated C3H/HeJ mice with house dust mite (HDM) extract in the presence of an isotype control mAb (IgG_{2a}), or blocking mAbs to PD-L2 (clone TY25) [23], PD-L1 (clone MIH-6) [9], or PD-1 (clone RMP1-14) [24]. Consistent with our previous results [20], blockade of PD-L2 in C3H mice decreased AHR, and increased circulating IL-12 levels (Supporting Information Fig. 1). However, in contrast to the results reported in A/J animals, blockade of either PD-1 or PD-L1 in HDM-exposed C3H animals resulted in a significant increase in the severity of AHR (Fig. 1A). Consistent with our previous results, these findings suggest that PD-L2 acts in a PD-1-independent manner [20]. However, these data also demonstrate a protective role for the PD-1/PD-L1 axis selectively in an asthma-resistant strain.

To dissect the mechanisms responsible for increased AHR observed following PD-1 and PD-L1 blockade, we assessed cellular recruitment to the airways. HDM treatment induced significant

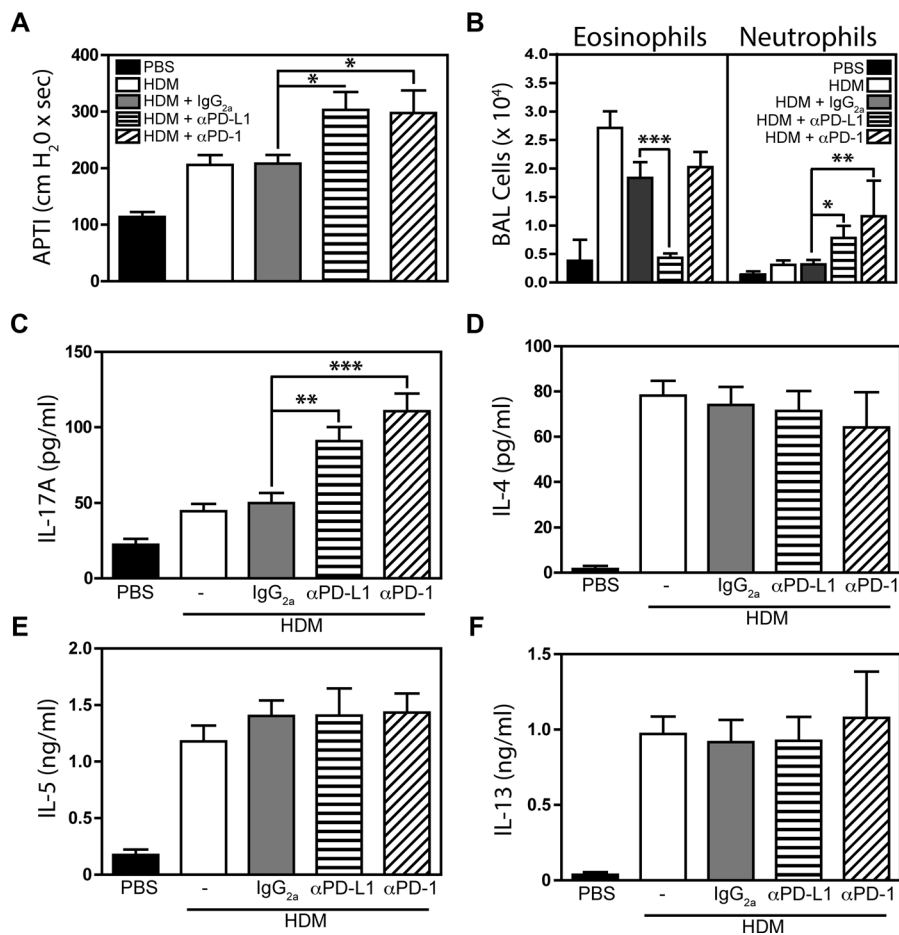


Figure 1. PD-1 blockade enhances Th17, but not Th2 responses, in a mouse model of allergic asthma. Experimental asthma was induced, and PD-1 or PD-L1 was blocked as described in *Materials and Methods*. (A, B) Mice were sacrificed and (A) AHR was evaluated by assessing changes in tracheal pressure following administration of a cholinergic agonist over time and expressed as airway pressure time index. (B) BAL cellularity was evaluated by cytocentrifugation of BALF, staining with Diff-Quik and counting using morphologic criteria. (C–F) Lung cells isolated from mice in which asthma had been induced were restimulated *in vitro* with HDM and the production of (C) IL-17A, (D) IL-4, (E) IL-5, and (F) IL-13 was evaluated by ELISA. Data are shown as mean + SEM of $n = 14$ –20 mice pooled from four independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ between groups (ANOVA).

pulmonary eosinophilia that was not impacted by isotype or anti-PD-1 treatment (Fig. 1B). Surprisingly, anti-PD-L1 completely abrogated eosinophil recruitment (Fig. 1B). The reason for the decreased eosinophilia in anti-PD-L1-treated mice is unclear, however, flow cytometric analysis revealed that both BM-derived, and pulmonary eosinophils recruited to the lung after HDM exposure were PD-L1 positive (Supporting Information Fig. 2) suggesting direct interactions between anti-PD-L1 and eosinophils may contribute. Only a small increase in the number of neutrophils was observed in HDM, or HDM + isotype animals, but treatment of mice with either anti-PD-1 or anti-PD-L1 significantly enhanced pulmonary neutrophilia (Fig. 1B). As neutrophil recruitment is associated with Th17 responses, we assessed cytokine production in HDM-stimulated lung cell cultures. Consistent with an enhanced Th17 response, mice receiving anti-PD-1 or anti-PD-L1 produced significantly greater levels of IL-17A than those receiving HDM or HDM + isotype (Fig. 1C). In contrast, production of the Th2 cytokines IL-4, IL-5, IL-13 (Fig. 1D–F), and IL-10, associated with a Treg-cell response (Supporting Information Fig. 3), was not significantly altered in cells from anti-PD-1- or PD-L1-treated mice. IFN- γ was not detectable in any HDM-restimulated cultures.

To further characterize the allergic response in mice treated with anti-PD-1/PD-L1, we also assessed serum immunoglobulin levels, and mucus production. HDM exposure induced a marked

upregulation in both total IgE (Fig. 2A) (a surrogate for antigen specific IgE [25]) and HDM-specific IgG₁ (Fig. 2B). However, levels of both IgE and IgG₁ remained unaffected by anti-PD-1 or anti-PD-L1 (Fig. 2A and B). Similarly, while HDM + isotype treated mice displayed a marked increase in the frequency of periodic acid-Schiff (PAS) positive mucus cells, this was not impacted by PD-1 or PD-L1 blockade (Fig. 2C). Thus, in a model of allergen-induced AHR, the PD-1:PD-L1 axis has a limited role in inhibiting Th2 immune responses, but plays an important role in constraining Th17 responses. The differential effect of PD-1:PD-L1 blockade on Th2 and Th17 responses suggest that different effector CD4⁺ T-cell populations may be differentially impacted by PD-1.

Increased Th1 and Th17, and reduced Th2 cytokine production in *Pdcd1*^{−/−} mice

To clarify whether PD-1 differentially regulates the activity of CD4⁺ T-cell subsets, we assessed T-cell cytokine production using cells from PD-1-deficient mice. To this end, we isolated cells from the spleens of naïve C57Bl/6 and *Pdcd1*^{−/−} animals. To gauge the relative levels of Th1, Th2, and Th17 activity, cells were stimulated with a variety of polyclonal stimuli (ConA, PMA + Ionomycin,

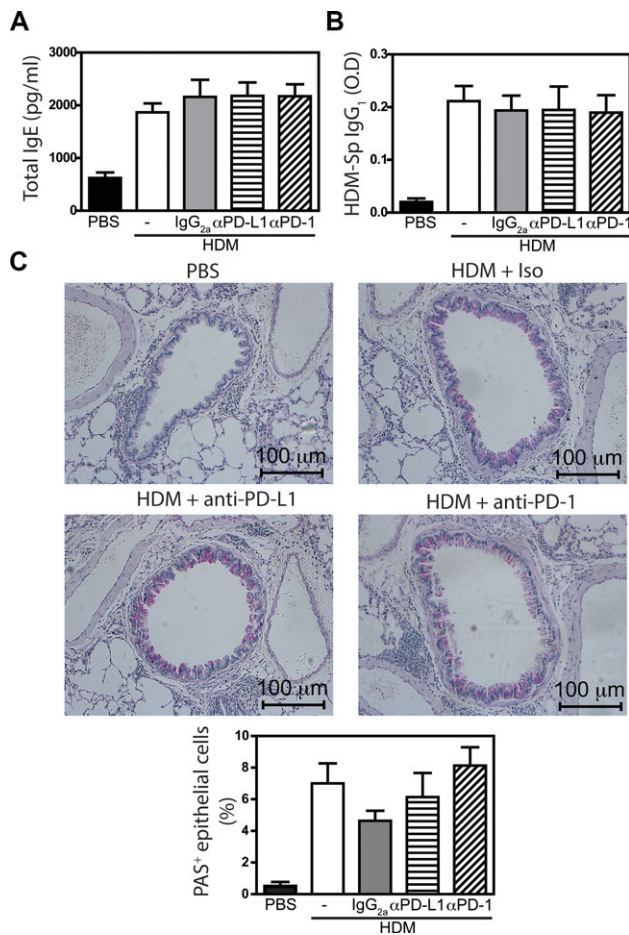


Figure 2. PD-1/PD-L1 blockade does not enhance immunoglobulin or mucus production in a mouse model of allergic asthma. Experimental asthma was induced, and PD-1 or PD-L1 was blocked as described in *Materials and Methods*. (A, B) Peripheral serum was collected on day 17 and the levels of (A) total IgE, or (B) HDM-specific IgG₁ were determined by ELISA. Data are shown as mean + SEM of $n = 14$ –20 mice pooled from four independent experiments. (C) Mucus-secreting cells present in medium-sized airways were identified by PAS staining. PAS⁺ cell counts from $n = 7$ –10 total mice per group are shown as mean + SEM from a single experiment representative of three independent experiments.

or α CD3 + α CD28), and overall cytokine production was assessed by ELISA. Consistent with the reported inhibitory role for PD-1, spleen cells from *Pdcd1*^{-/-} mice produced more IFN- γ and IL-17A following stimulation when compared to cells from wild-type (WT) mice (Fig. 3A and B). In contrast, cells from *Pdcd1*^{-/-} mice produced significantly less IL-4, IL-5, and IL-13 (Fig. 3C–E). Similar results were observed when T cells were isolated from the lungs (Supporting Information Fig. 4). Thus, regardless of the method of T-cell stimulation, or the source of cells, PD-1 limits Th1- and Th17-associated cytokine production, while potentially stimulating Th2-associated cytokine production.

To determine if PD-1 signaling impacted the frequency of Th1, Th2, or Th17 cells in vivo, the frequency of cytokine-secreting CD4⁺ T cells was determined in WT and *Pdcd1*^{-/-} mice by flow

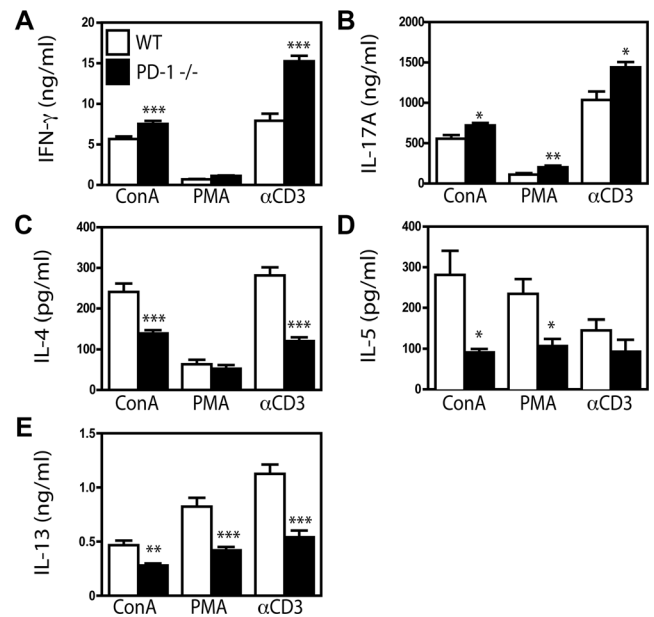


Figure 3. Spleen cells from PD-1^{-/-} mice produce increased levels of Th1 and Th17 cytokines, reduced levels of Th2 cytokines. Spleen cells from naïve WT C57Bl/6 (open bars) and PD-1^{-/-} (closed bars) mice were stimulated with Concanavalin A (ConA), PMA + Ionomycin (PMA), or α CD3 + α CD28 (α CD3) for 24 h. Cell culture supernatants were collected and assayed for (A) IFN- γ , (B) IL-17A, (C) IL-4, (D) IL-5, and (E) IL-13 by ELISA. Data are shown as mean $\pm$ SEM of $n = 5$ WT and 4 PD-1^{-/-} mice from one experiment representative of three performed. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ between strains (Student's *t*-test).

cytometry (gating strategy depicted in Supporting Information Fig. 5). Consistent with earlier data, a significantly greater frequency of pulmonary and splenic CD4⁺IFN- γ ⁺ and CD4⁺IL-17A⁺ cells was observed in *Pdcd1*^{-/-} mice, while the frequency of CD4⁺IL-13⁺ cells did not differ in *Pdcd1*^{-/-} and WT mice (Fig. 4A). The increased frequency of CD4⁺IFN- γ ⁺ and CD4⁺IL-17A⁺ cells observed in *Pdcd1*^{-/-} mice also correlated with higher levels of circulating IFN- γ and IL-17A (Fig. 4B) as determined using an in vivo cytokine capture assay (IVCCA) [26]. Paradoxically, while no change in the frequency of Th2-cells was observed, a small but statistically significant decrease in circulating IL-4 and IL-13 levels was noted (Fig. 4B). Collectively, these data suggest that PD-1 selectively restricts Th1 and Th17 responses, but supports basal Th2 responses in naïve hosts.

PD-1 stimulation limits cytokine production of Th1 and Th17 cells, and enhances Th2 cytokines

Because PD-1 signaling supports Treg-cell differentiation and activity [14], it is possible that alterations in effector cytokine production observed in *Pdcd1*^{-/-} and anti-PD-1/PD-L1-treated C3H/HeJ mice may be secondary to altered Treg-cell activity. Therefore, to limit the influence of PD-1 to a single effector T cell subset, we moved to an in vitro system. We isolated naïve

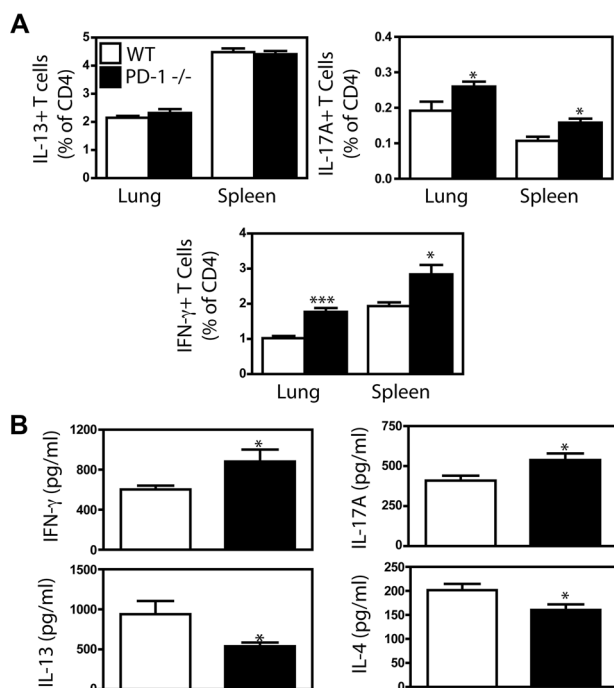


Figure 4. PD-1^{-/-} mice have increased Th1 and Th17, and decreased Th2, cytokine production in vivo. (A) Spleen and lung cells were isolated from naïve WT C57Bl/6 (open bars) and PD-1^{-/-} (closed bars). Cells were stimulated with PMA/Ionomycin overnight, and cultured in the presence of brefeldin A and monensin for 4 h. Cells were stained with mAbs to CD4 plus IFN-γ, IL-13, or IL-17A for analysis of the frequency of cytokine-positive CD4⁺ cells by flow cytometry (gating strategy in Supporting Information Fig. T 5). (B) Circulating levels of IFN-γ, IL-17A, IL-4, and IL-13 were assessed by in vivo cytokine capture assay. Data are shown as mean + SEM of nT = T 5 WT and 4 PD-1^{-/-} mice, from one experiment representative of three performed. *pT < T 0.05, **pT < T 0.01, and ***pT < T 0.001 between strains (Student's t-test).

(CD4⁺CD62L⁺CD25^{neg}) T cells from the LNs of naïve C3H/HeJ mice, and cultured them under Th1-, Th2-, or Th17-polarizing conditions. As expected, Th1-polarized T cells expressed abundant message for *Tbx21* and *Ifng*, Th2-polarized cells expressed *Gata3*, *Il4*, and *Il13*, and Th17-polarized cells expressed *Rorc*, *Il17a*, and *Il17f* (Table 1). We restimulated identical numbers of in vitro Th1-, Th2-, or Th17-polarized T cells on plates coated with αCD3 (5 μg/mL) plus either IgG-Fc or PD-L1-Ig. Compared to Th1 or Th17 cells restimulated in the presence of IgG-Fc, Th1 and Th17 cells restimulated in the presence of PD-L1-Ig showed marked inhibition of either IFN-γ (~66% inhibition; Fig. 5A) or IL-17A and IL-17F (~45% inhibition; Fig. 5B) production. Interestingly, the Th1 cells restimulated in the presence of 1 μg/mL of rPD-L1 demonstrated more substantial inhibition (66%) than those restimulated in the presence of 5 μg/mL of rPD-L1 (40%) (Fig. 5A). In contrast, the magnitude of IL-17A and IL-17F inhibition was dependent upon the amount of PD-L1-Ig present, as 5 μg/mL PD-L1-Ig resulted in greater inhibition than 1 μg/mL PD-L1-Ig (Fig. 5B). Consistent with our previous observations that Th2 cells are poorly regulated by the PD-1/PD-L1 axis in *Pdcd1*^{-/-} and anti-PD-1/PD-L1-treated C3H/HeJ mice, PD-1 ligation on Th2 cells had no effect on IL-4 or IL-13 production, and only marginally inhibited

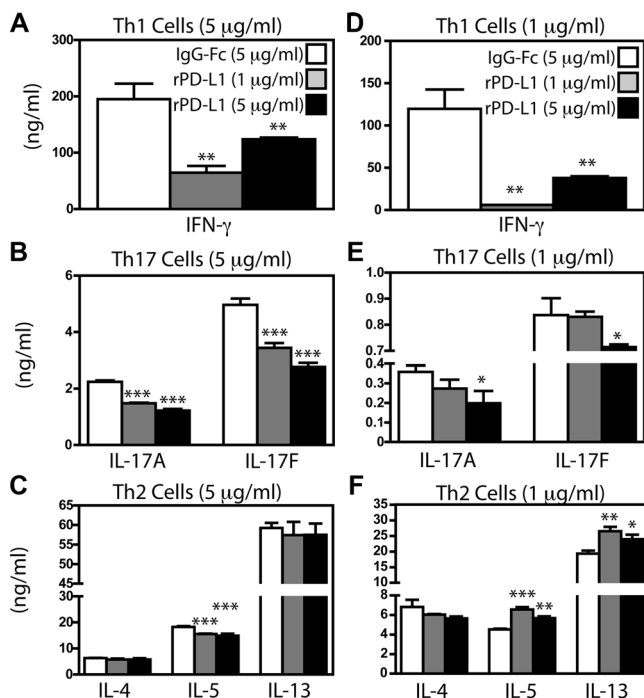


Figure 5. Cytokine production by restimulated in vitro differentiated T-cell subsets. (A–F) Naïve C3H/HeJ CD4⁺CD62L⁺CD25^{neg} T cells were polarized toward Th1, Th2, and Th17 cells as described in Materials and Methods. After differentiating without PD-L1, cells were rested for 24 h, and 5×10^4 were stimulated with αCD3 at (A–C) 5 μg/mL or (D–F) 1 μg/mL in the presence of IgG-Fc (5 μg/mL, open bars), or rPD-L1-Fc (1 μg/mL, gray bars or 5 μg/mL, black bars). After 72 h, tissue culture supernatant was collected for analysis of (A, D) IFN-γ, (B, E) IL-17A and IL-17F, and (C, F) IL-4, IL-5, and IL-13 by ELISA. Data are shown as mean + SEM of n = 4 individual cultures per condition, from one experiment representative of two performed. *p < 0.05, **p < 0.01, and ***p < 0.001 (ANOVA).

IL-5 production (~10%) (Fig. 5C). Restimulation of polarized Th2 cells on plates coated with αCD3 (5 μg/mL) and PD-L2-Ig similarly enhanced cytokine production (Supporting Information Fig. 6). Furthermore, alteration of cytokine production in cultures of skewed Th1, Th2, or Th17 cells was not a result of increased cell death or induction of Treg cells (assessed by flow cytometry—

Table 1. Gene expression by Th1-, Th2-, and Th17-polarized T cells^{a)}

Gene	Th1	Th2	Th17
Rorc	5785 ± 725	3667 ± 549	121 156 ± 10 373
IL-17A	0	11 ± 11	34 675 ± 2081
IL-17F	84 ± 11	33 ± 13	175 785 ± 24 214
GATA3	6506 ± 877	88 314 ± 10 545	20 792 ± 485
IL-13	24 ± 9	3450 ± 278	21 ± 21
IL-4	126 ± 11	3853 ± 154	59 ± 38
Tbet	12 429 ± 929	91 ± 25	163 ± 28
IFN-γ	56 152 ± 1282	365 ± 60	225 ± 183

^{a)}CD4⁺CD62L⁺CD25^{neg} T cells were cultured in Th1-, Th2-, and Th17-polarizing conditions as described in Materials and Methods. Total mRNA was isolated after 96 h in polarizing conditions and expression of the indicated genes was evaluated by quantitative real-time PCR. Data are shown as mean ± SEM of three replicates from one experiment representative of three performed.

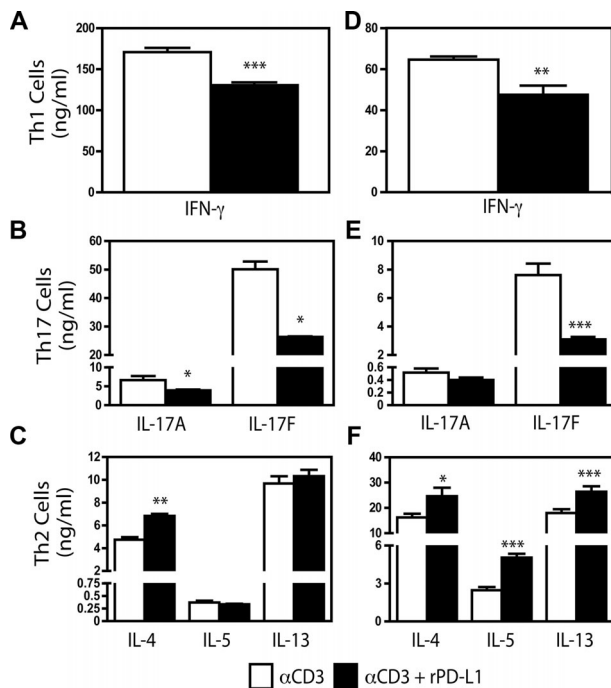


Figure 6. Cytokine production by T cells differentiated in the presence of PD-1 stimulation. Naïve C3H/HeJ CD4⁺CD62L⁺CD25^{neg} T cells were polarized toward Th1, Th2, and Th17 cells as described in *Materials and Methods*. (A–C) T cells were differentiated in the presence of plate-bound IgG-Fc (5 μg/mL, white bars) or PD-L1-Ig (5 μg/mL, black bars). Production of (A) IFN-γ, (B) IL-17A, and IL-17F and (C) IL-4, IL-5, and IL-13 was measured by ELISA. (D–F) After differentiating as in (A–C), cells were rested overnight, and 5×10^4 cells were stimulated with plate-bound αCD3 (5 μg/mL) and soluble αCD28 (5 μg/mL). Production of (D) IFN-γ, (E) IL-17A and IL-17F, and (F) IL-4, IL-5, and IL-13 was measured by ELISA. Data are shown as mean + SEM of $n = 4$ individual cultures per condition, from one experiment representative of two performed. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ between strains (Student's *t* test).

gating strategy depicted in Supporting Information Fig. 7A)—as neither the frequency of dead cells (Supporting Information Fig. 7B) nor the frequency of Foxp3⁺ T cells (Supporting Information Fig. 7C) was consistently altered in cultures restimulated in the presence of PD-L1-Ig or PD-L2-Ig. These suggest that stimulation of PD-1 on Th2 cells with either PD-1 ligand is sufficient to increase their cytokine production.

PD-1 recruits phosphatases to the TCR complex, resulting in dephosphorylation of signaling molecules downstream of TCR activation [27]. Thus, the inability of PD-1 to limit cytokine production by established Th2 cells may be due to excessively strong activation signals overwhelming the capacity of PD-1-activated phosphatases to limit T-cell activation. Thus, we assessed the capacity of PD-1 to inhibit Th1, Th2, and Th17 cells stimulated with a lower dose of αCD3 (1 μg/mL). Stimulation with 1 μg/mL αCD3 resulted in a 50–85% reduction in overall cytokine production (Fig. 5D–F) compared to cultures restimulated with 5 μg/mL αCD3 (Fig. 5A–C). However, even with reduced TCR stimulation, restimulation of Th1 or Th17 cells in the presence rPD-L1 significantly decreased IFN-γ (Fig. 5D), or IL-17A and IL-17F production (Fig. 5E). In contrast, Th2 cells stimulated with 1 μg/mL of αCD3 in the presence of rPD-L1 produced significantly more (~30%)

IL-5 and IL-13 (Fig. 5F). Again, restimulation of skewed Th2 cells in the presence of PD-L2-Ig also enhanced cytokine production (Supporting Information Fig. 8), and neither PD-L1-Ig nor PD-L2-Ig consistently altered cell viability (Supporting Information Fig. 9A and C) or the frequency of Foxp3⁺ cells (Supporting Information Fig. 9B and D). Thus, while PD-1 signaling significantly inhibits the activity of Th17 cells, PD-1 can provide a co-stimulatory signal for Th2 cells under suboptimal stimulation conditions.

PD-1 ligation during T-cell differentiation inhibits Th1 and Th17 activity, enhances Th2 activity

To determine whether PD-1 ligation during effector differentiation had an impact on cytokine producing capacity, naïve T cells were cultured under Th1-, Th2-, or Th17-polarizing conditions in the presence of IgG-Fc or rPD-L1. As with Th1- and Th17-polarized cells restimulated in the presence of rPD-L1, the presence of PD-L1-Ig during polarization, decreased cytokine production by both Th1 (IFN-γ; Fig. 6A) and Th17 cells (IL-17A, IL-17F; Fig. 6B). In contrast, the presence of rPD-L1 during Th2 polarization did not significantly impact IL-5 and IL-13 production, but enhanced production of IL-4 by ~40% (Fig. 6C). However, as ligation of PD-1 limits T-cell proliferation [28], it is conceivable that the observed differences in cytokine production were due to differences in the number of T cells present in the culture, rather than an overall decreased capacity for cytokine production. To determine if overall cytokine producing capacity was altered by polarization in the presence of PD-L1, T cells polarized in the presence of IgG-Fc or rPD-L1-Ig were rested overnight, and equivalent numbers of T cells were restimulated for 24 h with αCD3/αCD28 (in the absence of either IgG-Fc or PD-L1-Ig). Th1 and Th17 cells polarized in the presence of rPD-L1 produced significantly lower IFN-γ or IL-17A and IL-17F (Fig. 6D and E), respectively, while rPD-L1-polarized Th2 cells produced significantly greater levels of IL-4, IL-5, and IL-13 (Fig. 6F). Th2 cells polarized in the presence of PD-L2-Ig displayed elevated IL-4 production, and comparable IL-5 and IL-13 production in the skewing cultures, but significantly increased levels of cytokines were produced upon restimulation (Supporting Information Fig. 10). Differentiating in the presence of PD-L1-Ig or PD-L2-Ig did not alter cell death, or induce the differentiation of Treg cells (Supporting Information Fig. 11). Thus, PD-1 ligation during T-cell differentiation selectively restricts Th1- and Th17-cell cytokine production, even following restimulation in the absence of further PD-1 ligation. In contrast, PD-1 enhances Th2-derived cytokine production.

PD-1 signaling during T-cell differentiation promotes a Th2-skewing environment

As the presence of rPD-L1-Ig and rPD-L2-Ig during polarization enhanced the production of Th2 cytokines, and GATA3 is a master regulator of Th2 identity, we also assessed the frequency of

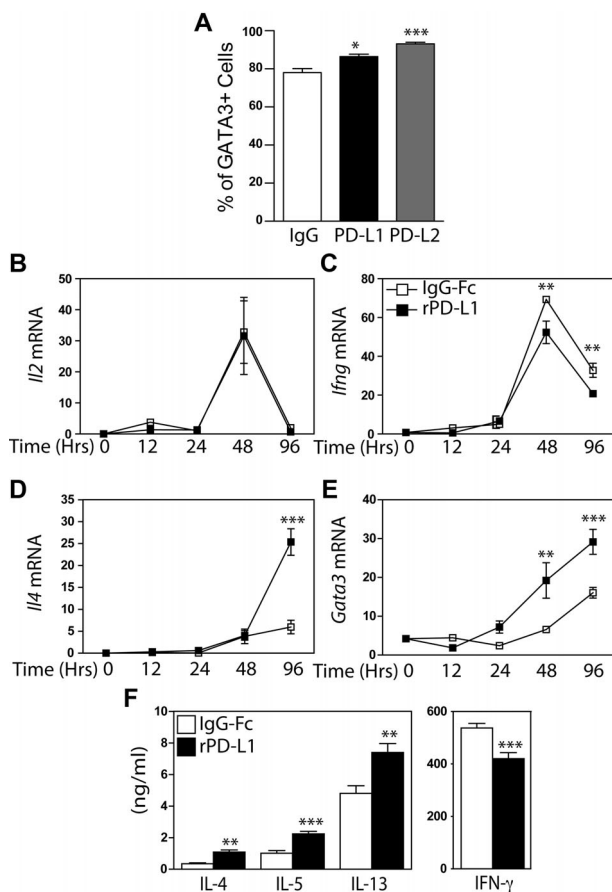


Figure 7. PD-1 supports Th2 differentiation in the absence of strong polarizing cytokines. (A) Naïve C3H/HeJ CD4⁺CD62L⁺CD25^{neg} T cells were skewed toward Th2 cells as described in *Materials and Methods*, and the frequency of viable CD3⁺CD4⁺GATA3⁺ T cells was assessed by flow cytometry (gating strategy in Supporting Information Fig. 5). (B–E) Naïve C3H/HeJ CD4⁺CD62L⁺CD25^{neg} T cells were stimulated with α CD3 and α CD28 in the presence of IgG-Fc (open squares) or PD-L1-Fc (closed squares). mRNA levels of (B) IFN- γ , (C) IL-2, (D) IL-4, and (E) GATA3 were assessed at the indicated time points by RT-PCR. (F) Secretion of Th2 (IL-4, IL-5, IL-13) and Th1 (IFN- γ) cytokines was measured at times of maximal transcription (Th2, 96 h; IFN- γ , 48 h). Data are shown as mean $\pm$ SEM of three to five replicate wells per condition, from one experiment representative of two performed. ** p < 0.01, and *** p < 0.001 (ANOVA (A), and Student's t test (B–F)).

GATA3⁺ cells in cells cultured under Th2-polarizing conditions in the presence and absence of PD-1 stimulation. As shown in Figure 7A, polarizing T cells in the presence of PD-L1-Ig and PD-L2-Ig enhanced the frequency of GATA3⁺ cells, suggesting that PD-1 signaling can support Th2 differentiation. To determine if PD-1 signaling may be sufficient to drive Th2 differentiation directly, naïve T cells were isolated from C3H/HeJ mice, stimulated with α CD3 and α CD28 in the presence of IgG-Fc, or PD-L1-Ig, and the production of cytokines was assessed over time by RT-PCR. Under these conditions, a spike in IL-2 mRNA was observed at 48 h, but, surprisingly, the magnitude of this induction was not impacted by PD-L1 (Fig. 7B). In contrast, while expression of *Ifng* followed similar kinetics as IL-2, synthesis of *Ifng* mRNA was significantly reduced by rPD-L1-Ig (Fig. 7C). Significant induction of message

for IL-17A or IL-17F was not seen under these conditions. Consistent with our previous observations of enhanced Th2 cytokine production in the presence of PD-L1, there was a significant (~fivefold) increase in the levels of *Il4* mRNA in cultures stimulated in the presence of PD-L1 (Fig. 7D). Similar patterns were seen with expression of *Il5* and *Il13* (data not shown). As GATA3 expression is an important regulator of Th2-cell differentiation, we also assessed the levels of *Gata3* mRNA. Consistent with the rise in expression of mRNA for multiple Th2 cytokines, we also observed a significant enhancement of *Gata3* mRNA in PD-L1-stimulated cultures (Fig. 7E). Finally, changes in mRNA expression resulted in significantly increased production of IL-4, IL-5, and IL-13, and significantly decreased production of IFN- γ (Fig. 7F). Collectively, these data show that PD-L1 can provide a co-stimulatory signal that selectively enhances Th2-cell differentiation.

Discussion

Our observation of reduced Th1/Th17 cytokine production following PD-1 ligation is consistent with previous reports of PD-1-mediated inhibition of cytokine production in nonpolarized CD4⁺ T cells [13, 29–35]. While inhibition of Th2 cytokine production by PD-1 has been reported (in nonpolarized T cells) [30, 34, 36–38], we find that PD-1 consistently enhances Th2 cytokine production. Our findings are consistent with the observation of reduced Th2 cytokine production in PD-L1^{−/−} mice following *Leishmania mexicana* infection [39], and in the placenta in a model of allogeneic fetomaternal tolerance [31]. Similarly, PD-1 blockade decreases Th2 cytokines in superantigen-stimulated PBMCs from cancer patients [29], suggesting that PD-1 can stimulate Th2 responses in human CD4⁺ T cells. While the reasons for the discrepancies regarding an inhibitory/stimulatory role for PD-1 on Th2 cytokine production are unclear, our findings suggest that PD-1 enhances Th2 responses in conditions of suboptimal TCR stimulation. Thus, in conditions where PD-1 plays an inhibitory role, overall TCR signaling may be very robust, while in conditions where PD-1 plays a stimulatory role, TCR signaling may be comparatively weaker. However, as our study is the first to examine the effect of PD-1 on purified Th2 cells, it is also plausible that inhibition of Th2 cytokine production reported elsewhere [30, 34, 36–38] reflects reduced availability of T-cell growth factors (e.g. IL-2) resulting from inhibition of non-Th2 cells, rather than direct effects on Th2 cells.

The reasons for the differential effects of PD-1 ligation in Th2 and Th1/Th17 cells also remain unclear. However, we speculate that this is due to intrinsic differences that govern metabolic activity of Th2 cells and Th1/Th17 cells. Different mTOR complexes are utilized in different CD4⁺ T-cell subsets; while Th1 and Th17 differentiation is supported by mTOR complex 1 (mTORC1), Th2 differentiation requires mTORC2 [40, 41]. Activation of mTORC1 in T cells follows the classical mTOR activation pathway and is dependent upon the PI3K/AKT pathway [42]. While the mechanisms of mTORC2 activation in Th2 cells is not entirely clear, it may be independent of the CD28/PI3K/Akt pathway [42]. As

PD-1 inhibits the activation of the PI3K/Akt pathway in CD4⁺ T cells [14], it is likely that PD-1 directly limits mTORC1, while mTORC2 may be insensitive to inhibition via PD-1. This is an area of ongoing investigation in our laboratory.

We observe that PD-1 ligation enhances both GATA3 expression, and production of IL-4. As GATA3 expression is both downstream of IL-4 signaling [43] and can promote IL-4 expression [44, 45], it is unclear whether PD-1 influences IL-4 production, which indirectly enhances *Gata3* expression, or vice versa. However, it is interesting to note that when PD-1 is ligated on naïve T cells under nonpolarizing conditions, increased *Gata3* expression is observed at 24 h, a time point that precedes a significant induction of *Il4* mRNA (96 h). Thus, PD-1 may influence Th2 differentiation by directly enhancing GATA3 expression. This finding also argues that the ability of PD-1 to influence Th2 differentiation may be most evident in conditions in which there are no other powerful GATA3-inducing factors present (i.e. in the absence of IL-4). Such results may help reconcile our potentially confounding observations of increased Th2 cytokine production in naïve *Pdcd1*^{-/-} mice and the failure of anti-PD-1 treatment of C3H/HeJ to substantially alter Th2 cytokine production in a model of allergic asthma.

The role of PD-1 family members in allergic asthma is complex. Some reports suggest a protective role for the PD-1 family [15–17, 46] and others describe a pro-asthmatic role for either PD-L1 (resulting from decreased iNKT cell derived IFN- γ production [17]) or PD-L2 (resulting from a PD-1-independent reduction in DC-derived IL-12 production [20, 21]). Moreover, in contrast to our observation that PD-1/PD-L1 blockade enhances asthma severity in C3H/HeJ mice described herein, we previously found that PD-1/PD-L1 blockade has no impact on asthma severity in A/J animals [20], suggesting that the capacity of PD-1 ligation to regulate CD4⁺ T-cell cytokine production may vary by strain. In support of this, stimulation of C3H/HeJ, or BALB/c-derived (data not shown) Th1 or Th17 cells in the presence of rPD-L1 significantly inhibits Th1 and Th17 cytokine production, while similar stimulation of A/J-derived Th1 or Th17 cells fails to alter cytokine production (data not shown), suggesting that PD-1 responsiveness may be limited in A/J mice. As we observe increased pDC activity in C3H mice [20], and pDCs have been shown to limit the development of AHR through a PD-1-dependent mechanism [15], it is plausible that the selective effect of PD-1/PD-L1 blockade in C3H mice may be indicative of a major regulatory role for pDCs. However, given the wide expression pattern of PD-L1 on both hematopoietic and nonhematopoietic cells [9, 10], it seems likely that the global PD-1 unresponsiveness present in A/J mice plays a much larger role in the development of Th17 cells and severe asthma than a decreased frequency of activated pulmonary pDCs. It is also possible that natural variation in PD-1-responsiveness among common laboratory mouse strains may contribute to some of the confusion regarding the role of PD-1/PD-L1/PD-L2 in control of allergic asthma.

While asthma is widely regarded as the result of a Th2-dominated immune response, there is increasing evidence in both humans [5] and animal models [7] that a mixed Th2/Th17

response drives the development of more severe AHR. However, while IL-17A enhances IL-13-driven AHR, IL-17A does not exacerbate all aspects of asthma, inasmuch as IL-17A neutralization has no impact on IgE synthesis or mucus cell metaplasia in HDM-sensitized A/J mice [7]. The failure of PD-1 to stimulate Th2 responses in this model may be related to the Th2-dominant immune response, because PD-1 does not stimulate Th2 cytokine production under conditions of high TCR stimulation (Fig. 4C). Rather, as only the Th17 arm of the immune system is constrained by PD-1/PD-L1 in our model of allergic asthma, these data suggest that the Th17 cell, rather than the Th2 cell, is the target of PD-1/PD-L1 blockade. Thus, our observation that in vivo PD-1/PD-L1 blockade enhances AHR, pulmonary neutrophilia, and IL-17A production, without impact on Th2 cytokine synthesis, IgE levels, or mucus cell metaplasia, is wholly consistent with a pathogenic role for IL-17A.

From a clinical perspective, these data suggest that the PD-1/PD-L1 axis does exert an inhibitory influence in asthma, but is only involved in regulating the severity of disease, not disease initiation. Our observation that the PD-1/PD-L1 axis constrains the development of severe allergic asthma by targeting Th17-cell activity suggests that in certain individuals, more severe allergic asthma may be associated with a defective PD-1/PD-L1 regulatory axis. Thus, these findings highlight the complexity of immune regulation by PD-1 and suggest that the variability seen in studies examining the role of PD-1 family members in control of allergic asthma may be due the differential capacity to regulate specific effector CD4⁺ T-cell subsets. As Th17 cells appear to be strongly inhibited by the PD-1/PD-L1 axis, and excessive Th17 responses are associated with the development of more severe allergic asthma, stimulating the PD-1/PD-L1 regulatory axis may represent a novel therapeutic approach for the treatment of severe allergic asthma in certain individuals.

Materials and methods

Mice

Male C3H/HeJ, C57Bl/6J (Jackson Laboratories), and *Pdcd1*^{-/-} mice on a C57Bl/6 background (a gift of Tasuku Honjo) [47] were housed in a specific pathogen-free facility at Cincinnati Children's Hospital Medical Center (CCHMC). All experiments were approved by the CCHMC Institutional Animal Care and Use Committee.

Assessment of asthmatic phenotype

Mice were treated intratracheally with 40 μ L PBS, or 100 μ g HDM (Greer Laboratories, Lenoir, NC, USA) on days 0 and 14 and i.p. with 250 μ g anti-PD-L1 (MIH6), anti-PD-1 (RMP1-14), or rat IgG_{2a} (GL117) on days 0, 2, 14, and 16 followed by sacrifice on day 17. AHR was evaluated by assessing changes in tracheal pressure following administration of acetylcholine over time and expressed

as airway pressure time index as previously described [20]. BALF was collected, total cells counted and cytocentrifuged onto slides, and stained with Diff-Quik (Dade Behring). Differential cell counts of ≥ 500 cells/slide were determined morphologically. Whole lung cells were restimulated with HDM for 72 h, and cytokine production was assessed by ELISA. Mucous-producing airway epithelial cells were counted from at least seven medium-sized airways, and the percentage of PAS⁺ cells was calculated per individual mouse.

Polarization and stimulation of T-cell subsets

Naïve CD4⁺CD25^{neg}CD62L⁺ T cells were isolated using CD4⁺ T-cell isolation kit II, CD25 microbead kit, and CD62L microbeads (Miltenyi Biotec, Auburn, CA, USA). Cells were cultured for 5 days in flasks coated with α CD3 (5 μ g/mL) in the presence of α CD28 (5 μ g/mL) and Th1-polarizing (10 ng/mL IL-12, 1 μ g/mL α IL-4), Th2-polarizing (10 ng/mL IL-4, μ g/mL α IFN- γ), or Th17-polarizing (20 ng/mL IL-6, 5 ng/mL TGF β 1, 10 ng/mL IL-23, 1 μ g/mL α IL-4, 1 μ g/mL α IFN- γ) cytokines in the presence of plate-bound IgG-Fc or PD-L1-Ig (5 μ g/mL) (R&D Systems, Minneapolis, MN, USA). Cells were rested overnight in media and restimulated with various concentrations of plate-bound α CD3, IgG-Fc, and PD-L1-Ig. Cytokine production was assessed by ELISA.

Spleen and lung cell cultures

Single-cell suspensions were made with total cells isolated from whole spleens or lungs. Cells were cultured in the presence of ConA (5 μ g/mL), PMA/Ionomycin (50/500 pg/mL), or α CD3/ α CD28 (1 μ g/mL of each) for 24 and 96 h. Supernatants were harvested and cytokine levels were assessed by ELISA.

Quantitative real-time PCR

PCR primer pairs for *S14*, *Pdcd1*, *Tbx21*, *Ifng*, *Gata3*, *Il4*, *Il13*, *Rorc*, *Il17a*, and *Il17f* were obtained from the mouse primer depot [48] and designed to span an intronic region to avoid co-amplification of genomic DNA. Gene expression was analyzed by real-time PCR using the iCycler system (Bio-Rad) and SYBR green.

IVCCA and ELISA

In vivo IL-4 production was measured by an IVCCA kit purchased from Becton-Dickinson. In vivo IL-13, IFN- γ , and IL-17A production was assessed as previously described [26] with biotin-labeled eBio1316H (IL-13), R46A2 (IFN- γ), or IL-17A (eBio17B7) and using plates coated with eBio13A (IL-13), XMG1.2 (IFN- γ), or eBio17CK15A5 (IL-17A) (eBioscience). Serum or cell culture levels of IL-4, IL-5, IL-10, IL-13, IL-17A, IL-17F IFN- γ (antibodies from eBioscience) and IgE, IgG₁ (antibodies from Becton-Dickinson) were measured by ELISA.

Flow cytometry

Staining and analysis were performed as previously described [20] using the following antibodies FcBlock (mAB 2.4G2), CD4 (RM4-5), CD3 (145-2C11; all from eBioscience), and CCR3 (83101) from R&D Systems.

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. Significance was assumed at *p* < 0.05.

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Differential control of CD4⁺ T-cell subsets by the PD-1/PD-L1 axis in a mouse model of allergic asthma

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Handling Executive Committee member: Prof. Francesco Annunziato

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision – 18 June 2014

Dear Dr. Lewkowich,

Please accept my sincere apologies for the prolonged delay in processing the review of your manuscript ID eji.201444778 entitled "Differential control of CD4 T cell subsets by the PD-1/PD-L1 axis in allergic asthma" which you submitted to the European Journal of Immunology. One of the referee reports was severely delayed and we therefore sought a third opinion. We have now received all reports and the comments of the referees are included at the bottom of this letter. You should know that this is not the usual turnaround time for peer review at the Journal.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. You will see that referee 2 had only two concerns to be addressed but we felt that the first concern (that of chemical structures) falls out of the scope of the Journal. We therefore felt that, with this referee's limited comments, a third opinion providing more constructive criticism would be

more helpful - hence the third review and delay. We therefore invite you to address the comments of referee 1 and 3.

You should also pay close attention to the editorial comments included below. *In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.*

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Karen Chu

On behalf of Prof. Francesco Annunziato

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Reviewer: 1

Comments to the Author

In the manuscript "Differential control of CD4 T cell subsets by the PD-1/PD-L1 axis in allergic asthma" Lajoie et al. show that the in C3H, differently from A/J, mice the PD1/PDL1 axis inhibits Th17 and promotes Th2 responses. Authors conclude that a defect in the PD1/PDL1 axis may be associated to severe allergic asthma and that its stimulation could represent a possible therapeutic target in this condition.

Peer review correspondence

Experiments are well designed and performed, but the interpretation of the data is arguable, particularly for what concerns the possible translation to humans.

Specific points

The most important criticism to this paper concerns the interpretation of the data. The results are variable in different mice strains, and this renders difficult a translation to humans.

The second important point is that the role of PD-L2 is under evaluated in the paper.

It is clear to the reviewer that both PD-L1 and PD-L2 axis are blocked in the system of PD1^{-/-} mice and in the experiments in which anti-PD mAbs are used, but in the whole paper only PD-L1 has been evaluated for its ability to interfere with cytokine production.

The third criticism concerns the lack of any evaluation of CD4⁺CD25⁺ regulatory cells, despite the authors themselves state in the introduction that PD-1 is a promoter of their activity.

In figure 1, both anti-PD-L1 and anti-PD, are able to enhance the HDM- induced AHR, with increased numbers of neutrophils and decreased numbers of eosinophils in BAL. Since IL-5 is not affected, which are the mechanisms for eosinophils recruitment in BAL? Which would be the effect of anti-PD-L2 in this system?

In figure 2, why don't the authors evaluate HDM-specific IgE? And, again, which would be the effect of anti-PD-L2 in this system?

In figure 5, 6, and 7, the effects of adding rPDL2 should be evaluated

Most of these points must be addressed in order to render the manuscript suitable for publication in the European journal of Immunology.

Reviewer: 2

Comments to the Author

Lajoie et al. present a paper on differential control of CD4 T cells by PD-1/PD1-L1 axis in allergic asthma. The authors use a HDM model in different genetic engineered mice.

Peer review correspondence

The first question goes to the "allergen" the authors use. HDM extracts were used. The question is which extracts - whole body extract, faeces extracts or even a mix of everything - was used. The chemical characterization of the extracts would be of interest in order to understand the effects of allergens, adjuvants or irritants in this model.

Second, the authors use a model of "two time" administration of HDM-extracts intranasally after ip sensitization. Here the question arises what happens at what time at the different challenge time points in the different KO-Mice. The question of kinetics of cell influx depending on the absence or presence of PD1 is of great importance.

Reviewer: 3

Comments to the Author

This paper from Lajoie et al shows that the PD-L1/PD1 axis is important in constraining Th2 immune responses by limiting of Th1/Th17 cytokine production. The data are convincing but I have a few issues with the paper.

What is not clear from the paper is which cell population is affected by the anti-PDL1 treatment and changes the type of immune response. The authors refer to pDCs being suppressive in their C3H mice, but does it mean that after anti-PD-L1 injection, these cells are able to induce Th2 responses? A sort of the different DC populations after anti-PD-L1 treatment followed by a coculture with naive T cells would help addressing this point.

There are no data showing what the treatment does to regulatory T cells (no Foxp3 or IL-10 determination). This would be nice and easy to look at.

In the ex vivo/in vitro experiments shown in figure 6, the authors start the stimulation using a similar number of T cells. Did they look at whether the reduced Th1/Th17 cytokine production could be due to the Th1/Th17 cells being more susceptible to cell death after stimulation?

What was confusing to me is that the paper starts with a model of allergic asthma where PD1/PDL1 blockade is accompanied with increased Th2 responses. But from Figure 3 onwards, the authors do not use any allergen anymore to stimulate cells but rely on a polyclonal stimulation. For me, it is very difficult to reconcile what they see using polyclonal stimuli in vitro with what is seen in vivo using HDM. The authors should find a way to do one key experiment using irradiated cells and the allergen as the stimulus, and the authors should block PD1/PDL1 and look at whether Th1/Th17 responses would be limited in such a model as well.

Peer review correspondence

First revision – authors' response – 12 December 2014

We have made a point-by-point response to the comments of reviewers 1 and 3, as well as the editorial comments (at the editors request, we did not respond to comments by reviewer 2). These are detailed below.

COMMENTS FROM REVIEWER 1

Comments to the Author

In the manuscript "Differential control of CD4 T cell subsets by the PD-1/PD-L1 axis in allergic asthma" Lajoie et al. show that in C3H, differently from A/J, mice the PD1/PDL1 axis inhibits Th17 and promotes Th2 responses. Authors conclude that a defect in the PD1/PDL1 axis may be associated to severe allergic asthma and that its stimulation could represent a possible therapeutic target in this condition.

Experiments are well designed and performed, but the interpretation of the data is arguable, particularly for what concerns the possible translation to humans.

Specific points

Comment: The most important criticism to this paper concerns the interpretation of the data. The results are variable in different mice strains, and this renders difficult a translation to humans.

Response: The reviewer is correct to observe that the impact of PD-1 signaling is different in different mouse strains, and we agree that this does make *generalizations* about the role of PD-1/PD-L1 interactions in human disease necessary for simple therapeutic interventions difficult. However, we take the view that if two different mouse strains (which represent single individuals) respond differently, then similar variability likely occurs across the human population as well. We do feel that it is unlikely that PD-1/PD-L1 targeting therapy will be effective in every severely asthmatic individual. Rather, an assessment of the involvement of these molecules in human disease may need to occur on a case-by-case basis to identify individuals in which such targeted therapy may be appropriate. However, we will also point out that a number of recently published, high profile "asthma therapeutic" studies or reviews demonstrate that different disease endotypes are driven by different immunological processes, and thus "personalized treatment" for asthma may be needed to match effective therapeutic approaches to the right target populations. (Corren et al, *N Engl J Med*, 2011, 365 (12): 1088; Tantisira et al, *N Engl J Med*. 2011, 265 (13): 1173; Fajt et al, *J Asthma*, 2014, 51 (7): 669; Poon et al, *Expert Rev Clin Immunol*, 2012, 8 (7): 595). As such, we feel that the observation that different mouse strains respond differently does not in anyway diminish the novelty, or impact of our studies.

However, in light of the reviewer's comments (and implicit concerns) and after careful consideration of the manuscript, we have identified a number of instances in the text wherein our description regarding the potential impact of these findings could have been interpreted as "over-reaching". As such, where appropriate we have added the qualifying statement "in certain individuals" where appropriate in the text (Abstract – lines 44-46, Conclusion – line 376, Conclusion 384). We hope that this more accurately reflects our belief that only in select individuals might such mechanisms be involved.

Comment: The second important point is that the role of PD-L2 is under evaluated in the paper.

It is clear to the reviewer that both PD-L1 and PD-L2 axis are blocked in the system of PD1-/- mice and in the experiments in which anti-PD mAbs are used, but in the whole paper only PD-L1 has been evaluated for its ability to interfere with cytokine production.

Response: The reviewer is correct, that the focus of the current paper was on the PD-1/PD-L1 axis, and the role of PD-L2 was evaluated in only limited scope. The principal reason for this is that we previously demonstrated the effects of PD-L2 blockade in an *in vivo* model asthma model using A/J mice (Lewkowich et al, *Mucosal Immunol.* 2013, 6 (4): 728). In our prior publication we demonstrate that, in contrast to the enhanced AHR observed following PD-1/PD-L1 blockade observed herein, blockade of PD-L2 diminished AHR and reduced airway mucus production. This was associated with no observable effect on airway inflammation, or Th2 or Th17 cytokine production in HDM-restimulated lung cell cultures. Rather, decreased AHR was associated with increased circulating IL-12 p40 levels, and simultaneous blockade of IL-12 and PD-L2 reversed the protective effects of PD-L2 blockade, suggesting that increased IL-12 production was central to decreased AHR observed following PD-L2 blockade. We subsequently demonstrated that exposure of bone marrow derived DCs to rPD-L2 inhibited HDM and LPS-induced IL-12 p70 production, and that PD-L2 seemed to be inhibiting IL-12 p70 production through a PD-1 independent manner. In an effort to streamline the current manuscript, and prevent duplication of previously published data we chose to focus our *in vivo* studies on the PD-1/PD-L1 axis, rather than recapitulate the entire PD-L2 phenotype in C3H mice. However, to link the current report to our previously published results, we do demonstrate that PD-L2 blockade has a similar impact in C3H/HeJ mice, enhancing IL-12 production and diminishing AHR (see Supplementary Figure 1).

We would also like to point out that our observations that PD-L2 may work through PD-1 independent mechanisms are supported by the recent publication demonstrating that PD-L2 can interact with RGMb, a co-factor in the BMP signaling cascade (Xiao et al, *J Exp Med.* 2014, 211 (5): 943). In the paper by Xiao et al, they actually report a tolerogenic role for PD-L2 in contrast to the pro-asthmatic role for PD-L2 observed in our study. However, the previous publication of an inhibitory role for RGMb on macrophage cytokine production (Xia et al, *J Immunol*, 2011, 186 (3): 1369) is wholly consistent with our observations that stimulation with PD-L2 inhibited IL-12 production by DCs through a PD-1-independent mechanism,

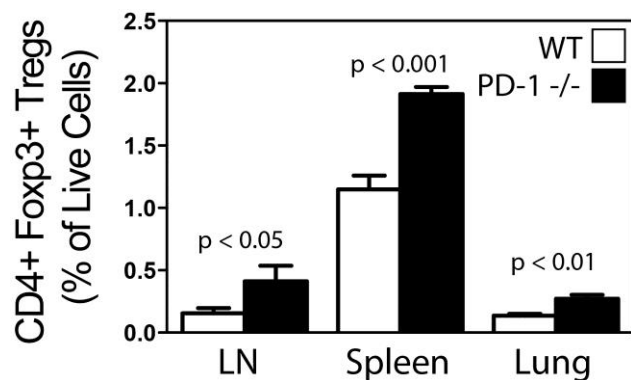
leading us to speculate that our pro-asthmatic effect of PD-L2 may be mediated by the PD-L2:RGMb interaction, rather than the “classical” PD-L2:PD-1 interaction. The reasons for the dominance of the PD-L2:RGMb pathway remain unclear, but may be due to the delayed kinetics of PD-L2 expression (we find it is only expressed on pulmonary DCs during the “challenge” phase in our model), and the comparatively limited expression pattern of PD-L2 compared to PD-L1.

I hope the reviewer will appreciate that this appears to be a very complex regulatory mechanism, and thus to streamline the discussion, and to focus on the data in the current manuscript, we did not include a discussion regarding the differential roles of PD-L2 and PD-L1:PD-1 in the current manuscript. However, if the reviewer and/or editor wish we can add a paragraph to the discussion section highlighting our thoughts on the differences between the roles of PD-L1:PD-1 and PD-L2.

Comment: The third criticism concerns the lack of any evaluation of CD4+CD25+ regulatory cells, despite the authors themselves state in the introduction that PD-1 is a promoter of their activity.

Response: Both reviewers raise the excellent point regarding the role of PD-1 in the regulation of CD4+CD25+ regulatory T cells. While we were not able to assess the frequency of Tregs in anti-PD-1 or anti-PD-L1 treated animals (neither mRNA nor cells were available from previous experiments), we did examine HDM-restimulated IL-10 production in existing supernatants taken from cultures of lung cells of control, HDM, HDM + Iso, HDM + α PD-1, or HDM + α PD-L1 treated C3H/HeJ mice as a “proxy” for Treg activity. As shown in new Supplementary Figure 3, we observed no alteration in IL-10 production in α PD-1 or α PD-L1 treated C3H/HeJ mice, arguing against any alterations in Treg frequency following short term PD-1/PD-L1 blockade. This data is discussed on lines 146 – 148 of the revised manuscript.

We have also assessed the frequency of Tregs in *Pdcd1*^{-/-} mice used in Figures 3 and 4. Surprisingly despite the previously reported Treg enhancing role for PD-1 (Francisco et al, *J Exp Med.* 2009, 206 (13):3015), we find that there are an *increased* number of Tregs in the LNs, spleen and lungs of PD-1-deficient mice, suggesting that PD-1 is actually suppressing the development of Tregs at baseline (see figure below).



We are confident that these results reflect the true quantity of Tregs in the *Pdcd1*^{-/-} mice as they have been observed in multiple organs, multiple experiments, and have been confirmed in collaborator labs. We would point out that these findings do *not* explain the increased Th1 and Th17 cytokine production that is observed in the *Pdcd1*^{-/-} mice (Figure 3 and 4) and even if the increased Treg numbers were responsible for the decreased Th2 cytokine production (Figure 3 and Figure 4), it is unclear why only the Th2 cells would be impacted by the increased numbers of Tregs. Thus while we present this data to the reviewers and editors to generate a complete response to this inquiry, we hesitate to include it in the published manuscript as we believe that it would not add much to our story. Moreover, we feel that these data would require, at the very minimum lengthy discussions regarding the implications of these findings, or at the very worst, confuse the reader to the point where our main message might be lost. However, if deemed important by the reviewer and/or editor, we can include the data in the published manuscript as an additional Supplemental Figure.

To further address the reviewers' comments, we have performed extensive additional experiments to determine whether restimulation of skewed Th1, Th2, or Th17 cells in the presence of PD-L1, or whether skewing of T cells under Th1, Th2 or Th17-inducing conditions in the presence of PD-L1 has any impact on Treg numbers. These data, presented in new Supplementary Figures 7, 9 and 11, demonstrate that:

- 1) Restimulation of Th1, Th2 or Th17 cells in the presence of PD-L1 has no impact on the frequency of Foxp3-expressing cells following strong stimulation (5 µg αCD3)(Supplementary Figure 7).
- 2) Restimulation of Th1, Th2 or Th17 cells in the presence of PD-L1 has no impact on the frequency of Foxp3-expression cells following weak stimulation (1 µg αCD3)(Supplementary Figure 9).
- 3) Under Th2 and Th17 conditions, the presence of PD-L1 during differentiation had no impact on the frequency of Foxp3⁺ cells, but slightly decreases the frequency of Foxp3⁺ Th1 cells. Upon restimulation of Th1 and Th2 cells differentiated in the presence of PD-L1, there is no observable effect on the frequency of Tregs, but upon restimulation of Th17 cells differentiated in the presence of PD-L1, a significant decreased in the frequency of Foxp3⁺ cells is observed (Supplementary Figure 11).

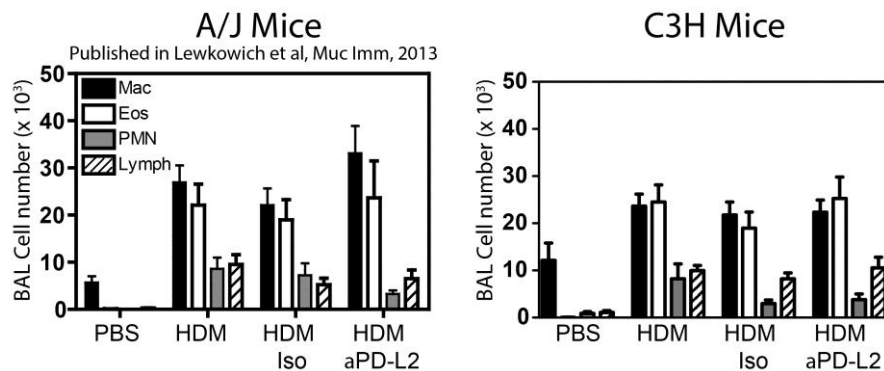
These new data are discussed in the manuscript on lines 212 – 220, 232 – 235, and 257 – 261.

Comment: In figure 1, both anti-PD-L1 and anti-PD, are able to enhance the HDM- induced AHR, with increased numbers of neutrophils and decreased numbers of eosinophils in BAL. Since IL-5 is not affected, which are the mechanisms for eosinophils recruitment in BAL? Which would be the effect of anti-PD-L2 in this system?

Response: The reviewer keys on an interesting dichotomy between anti-PD-1 and anti-PD-L1 treated

animals. The former display no change in eosinophil recruitment while the latter demonstrate a profound defect in eosinophil recruitment. As stated in the text, the reasons for this profound defect on eosinophil recruitment remain unclear. We present evidence in the manuscript that eosinophils express PD-L1 but not PD-1 (Supplementary Figure 2) suggesting that a direct cytotoxic effect may be at play. We have performed preliminary experiments to determine if anti-PD-L1 influences eosinophil maturation using an in vitro culture system. However, while we see a very slight enhancement of eosinophil death (1 to 2%) in some experiments when eosinophils are incubated in the presence of anti-PD-L1, this is unlikely to explain the rather remarkable drop in the levels of eosinophils seen in the anti-PD-L1 treated animals.

Regarding the impact of PD-L2 blockade on eosinophils, the data from A/J mice has been published in the previously cited manuscript from our lab (Lewkowich et al, *Mucosal Immunol.* 2013, 6 (4): 728). However, the data for the C3H mice has not been published and was not included in the current manuscript to avoid replication of very similar, previously published data. However, for completeness the relevant data is included as a figure here:



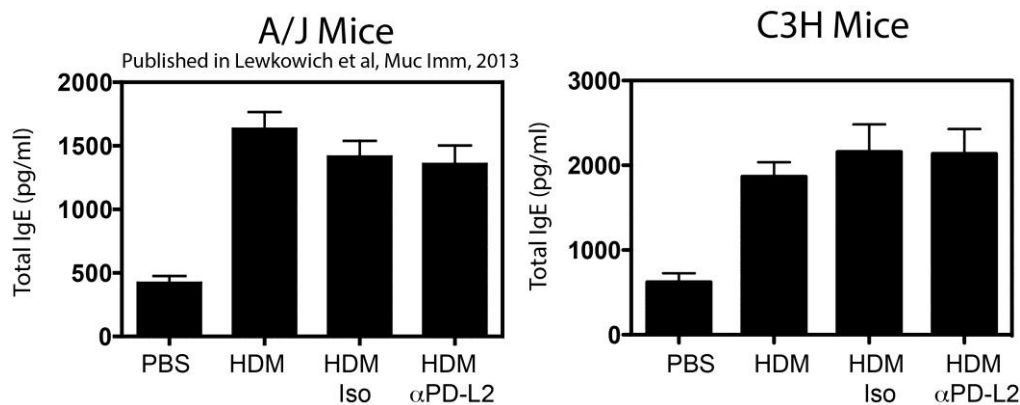
The data clearly show that in C3H mice, as in A/J mice, that PD-L2 blockade has no impact on eosinophilia in C3H or A/J mice. This is consistent with our hypothesis that the anti-PD-L1 antibody is having a direct effect on eosinophils, as we can detect no expression of PD-L2 on eosinophils (data not shown). While we did not include it in the revised version of the manuscript, we would be happy to include the data on eosinophilia in the anti-PD-L2 treated C3H/HeJ mice within Supplementary Figure 1 if the reviewer/editor feels it sufficiently important.

Comment: In figure 2, why don't the authors evaluate HDM-specific IgE? And, again, which would be the effect of anti-PD-L2 in this system?

Response: Given that the levels of HDM-specific IgG1 and IgG2a are several thousand-fold higher than the levels of HDM-specific IgE present in the serum, even a small amount of cross reactivity between commercially available IgE "specific" reagents and IgG will result in a significant misrepresentation of HDM-specific IgE present in the serum. Extensive comparison in many different mouse strains, under

many different immunization conditions has revealed that total IgE levels following allergen sensitization are strongly correlated with the levels of IgE "activity" present in the serum (as measured by passive cutaneous anaphylaxis assays) (Lewkowich et al, *J Immunol Methods*, 2004; 286:123). As such we feel that total IgE can be used as a reliable surrogate for allergen-specific IgE activity in the serum.

Regarding IgE levels in PD-L2 treated animals, the data for the A/J mice has been published in the previously cited manuscript from our lab (Lewkowich et al, *Mucosal Immunol.* 2013, 6 (4): 728). However, the data for the C3H mice has not been published here and was not included to avoid replication of very similar, previously published data. However, for completeness the relevant data is included as a figure here:



The data clearly show that PD-L2 blockade has no impact in either the A/J mice (as previously published) or in the C3H mice. While this data further supports our hypothesis that the PD-1/PD-L1 axis is independent of PD-L2 in this model, we did not include the data as it was very similar to our previously published data. However, if the reviewer and/or editor feel this is an important piece of data to include, we can include it in supplementary Figure 1.

Comment: In figure 5, 6, and 7, the effects of adding rPDL2 should be evaluated.

Response: While we have previously published the impact of PD-L2 blockade in our in vivo model, the reviewer correctly raises the concern that the role of PD-L2 blockade in our in vitro experiments is under explored. As such, we have performed additional extensive experiments to determine if PD-L2 stimulation alters cell viability or the development of Tregs, and similarly supports the growth or differentiation of Th2 cells. These data are shown in Supplementary Figures 6, 7, 8, 9, 10 and 11, and in Figure 7A. These figures demonstrate that:

- 1) rPD-L2 enhances IL-4 and IL-13 production in Th2 cells cultured on plates coated with 5μg αCD3 (Supplementary Figure 6)

- 2) rPD-L2 does not substantially alter the viability, or the frequency of CD4⁺ Foxp3⁺ cells in cultures of previously polarized Th1, Th2 or Th17 cells restimulated in plates coated with 5µg αCD3 (Supplementary Figure 7)
- 3) rPD-L2 enhances IL-4 and IL-13 production in Th2 cells cultured on plates coated with 1µg αCD3 (Supplementary Figure 8)
- 4) rPD-L2 does not substantially alter the viability, or the frequency of CD4⁺ Foxp3⁺ cells in cultures of previously polarized Th1, Th2 or Th17 cells restimulated in plates coated with 1µg αCD3 (Supplementary Figure 9)
- 5) Th2 cells differentiated in the presence of PD-L2 produced similar levels of Th2 cytokines in the initial polarizing cultures, but produced higher levels of IL-4, IL-5, and IL-13 when restimulated in the absence of any additional PD-1 ligands (Supplementary Figure 10)
- 6) Under Th1 and Th2 conditions, the presence of PD-L2 during differentiation slightly decreased the frequency of Foxp3⁺, while slightly increasing the frequency of Foxp3⁺ cells when included during Th17-differentiating conditions. Upon restimulation of Th1 and Th2 cells differentiated in the presence of PD-L2, there is no observable effect on the frequency of Tregs, but upon restimulation of Th17 cells differentiated in the presence of PD-L2, a significant decrease in the frequency of Foxp3⁺ cells is observed (Supplementary Figure 11).
- 7) Stimulation of developing TH2 cells with both PD-L1 and PD-L2 increased the frequency of CD4 cells expressing the central regulator of Th2 activity, GATA3, as measured by flow cytometry (Figure 7A).

These new data are discussed in the manuscript on lines 99, 212 – 220, 232 – 235, 257 – 261 and lines 268 – 276.

COMMENTS FROM REVIEWER 3

Comment: This paper from Lajoie et al show that the PD-L1/PD1 axis is important in constraining Th2 immune responses by limiting of Th1/Th17 cytokine production. The data are convincing but I have a few issues with the paper.

Response: We are happy that the reviewer has found our submitted data convincing, but would like to stress the point that our data from the *in vivo* model really demonstrate that there is fact no evidence for a role for the PD-1/PD-L1 axis in constraining the development of a Th2 response in a model of allergic asthma. In fact, our *in vitro* work suggests that the PD-1/PD-L1 axis may actually *support* the development of Th2 cells. Rather, we feel that our data suggest that in our model of allergic airway disease, the PD-1/PD-L1 axis is preventing the development of Th17 cells. As we (Lajoie et al, Nat Immunol, 2010, 11: 928), and others (Al-Ramli et al, J Allergy Clin Immunol, 2009, 123: 1185; Bullens et al, Respir Res, 2006, 7: 135), have shown that IL-17A production is associated with more severe asthma, we feel this may be a Th2-independent mechanism through which the PD-1:PD-L1 axis may regulate asthma severity.

Based on these comments from the reviewer we feel that we have not made this aspect of the work sufficiently clear in the current manuscript, and have thus made changes attempting to highlight this aspect of our conclusions. These changes are located in the abstract (lines 34 – 37), in the results (line 157-159) and in the discussion (line 366 – 368)

Comment: What is not clear from the paper is which cell population is affected by the anti-PDL1 treatment and changes the type of immune response. The authors refer to pDCs being suppressive in their C3H mice, but does it mean that after anti-PD-L1 injection, these cells are able to induce Th2 responses? A sort of the different DC populations after anti-PD-L1 treatment followed by a coculture with naive T cells would help addressing this point.

Response: The reviewer raises an interesting question regarding which cells are the specific targets in our *in vivo* PD-1/PD-L1 blockade experiments. However, we would again stress that we are not really changing the nature of the immune response per se, as the pro-asthmatic Th2 response that is needed to drive allergen-induced AHR is not impacted by either antibody. What our data show is the expansion of Th17 cells *in addition* to the normally induced Th2 cell response. Thus, we would argue that it is specifically the Th17 cell that is targeted by blockade of either PD-1 or PD-L1. We feel that there is ample evidence that products produced by the expanded population of Th17 cells enhance the response to the Th2 cytokines that are already present, and drive the development of more severe AHR.

While we do report that C3H mice display a greater frequency of allergen-bearing pDCs, and use the

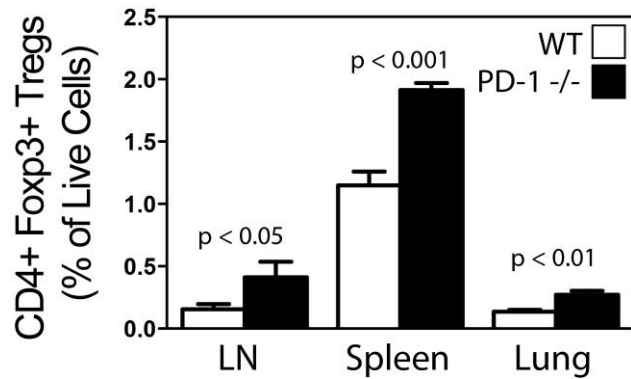
observation that pDCs have been shown to exert their suppressive influence through the PD-1/PD-L1 axis as a rationale for exploring the role of PD-1/PD-L1 axis in C3H mice, a number of other cell types express PD-L1 and have been shown to limit the development of immune responses as well. These include (but are not limited to) myeloid derived suppressor cells (Noman et al, *J Exp Med*, 2014, 211: 781), myeloid dendritic cells (Xu et al *J Immunol*, 2010, 185:7340), epithelial cells (Kim et al, *Am J Respir Cell Mol Biol* 2005, 33: 280), fibroblasts (Beswick et al, *J Immunol*, 2014, 193:2218). As such, we feel it unlikely that pDCs would be the only cell type that would be involved in selective inhibition of Th17 responses in our model of allergen-induced AHR. Rather, we expect that the apparent lack of a functional PD-1/PD-L1 axis in the A/J mice, rather than the lack of allergen uptake by pulmonary pDCs, is more likely to be responsible for the outgrowth of Th17 cells in these animals.

Statements describing these concepts have been added to the discussion and can be found at lines 348 – 357, and 366 - 368 of the revised manuscript.

Comment: There are no data showing what the treatment does to regulatory T cells (no Foxp3 or IL-10 determination). This would be nice and easy to look at.

Response: Both reviewers raise the excellent point regarding the role of PD-1 in the regulation of CD4+CD25+ regulatory T cells. While we were not able to assess the frequency of Tregs in anti-PD-1 or anti-PD-L1 treated animals (neither mRNA nor cells were available from previous experiments), we did examine HDM-restimulated IL-10 production in existing supernatants taken from cultures of lung cells of control, HDM, HDM + Iso, HDM + α PD-1, or HDM + α PD-L1 treated C3H/HeJ mice as a “proxy” for Treg activity. As shown in new Supplementary Figure 3, we observed no alteration in IL-10 production in α PD-1 or α PD-L1 treated C3H/HeJ mice, arguing against any alterations in Treg frequency following short term PD-1/PD-L1 blockade. This data is discussed on lines 146 – 148 of the revised manuscript.

We have also assessed the frequency of Tregs in *Pdcd1*^{-/-} mice used in Figures 3 and 4. Surprisingly, despite the previously reported Treg enhancing role for PD-1 (Francisco et al, *J Exp Med*. 2009, 206 (13):3015), we find that there are an *increased* number of Tregs in the LNs, spleen and lungs of PD-1-deficient mice, suggesting that PD-1 is actually suppressing the development of Tregs at baseline (see figure below).



We are confident that these results reflect the true quantity of Tregs in the *Pdcd1*^{-/-} mice as they have been observed in multiple organs, multiple experiments, and have been confirmed in collaborator labs. We would point out that these findings do *not* explain the increased Th1 and Th17 cytokine production that is observed in the *Pdcd1*^{-/-} mice (Figure 3 and 4) and even if the increased Treg numbers were responsible for the decreased Th2 cytokine production (Figure 3 and Figure 4), it is unclear why only the Th2 cells would be impacted by the increased numbers of Tregs. Thus, while we present this data to the reviewers and editors for completeness of this response, we hesitate to include it in the published manuscript as we believe that it would not add much to our story. Moreover, we feel that these data would require, at the very minimum, lengthy discussions regarding the implications of these findings, or at the very worst, confuse the reader to the point where our main message might be lost. However, if deemed important by the reviewer and/or editor, we can include the data in the published manuscript as an additional Supplemental Figure.

To further address the reviewers' comments, we have performed extensive additional experiments to determine whether restimulation of skewed Th1, Th2, or Th17 cells in the presence of PD-L1, or whether skewing of T cells under Th1, Th2 or Th17-inducing conditions in the presence of PD-L1 has any impact on Treg numbers. These data, presented in new Supplementary Figures 7, 9 and 11, demonstrate that:

- 1) Restimulation of Th1, Th2 or Th17 cells in the presence of PD-L1 has no impact on the frequency of Foxp3-expressing cells following strong stimulation (5 μ g α CD3)(Supplementary Figure 7).
- 2) Restimulation of Th1, Th2 or Th17 cells in the presence of PD-L1 has no impact on the frequency of Foxp3-expression cells following weak stimulation (1 μ g α CD3)(Supplementary Figure 9).
- 3) Under Th2 and Th17 conditions, the presence of PD-L1 during differentiation had no impact on the frequency of Foxp3+ cells, but slightly decreases the frequency of Foxp3+ Th1 cells. Upon restimulation of Th1 and Th2 cells differentiated in the presence of PD-L1, there is no observable effect on the frequency of Tregs, but upon restimulation of Th17 cells differentiated in the presence of PD-L1, a significant decreased in the frequency of Foxp3+ cells is observed (Supplementary Figure 11).

These new data are discussed in the manuscript on lines 212 – 220, 232 – 235, and 257 – 261.

Comment: In the ex vivo/in vitro experiments shown in figure 6, the authors start the stimulation using a similar number of T cells. Did they look at whether the reduced Th1/Th17 cytokine production could be due to the Th1/Th17 cells being more susceptible to cell death after stimulation?

Response: The reviewer raises the excellent point that change in cell viability may explain the reduced cytokine production observed in PD-L1-stimulated Th1 and Th17 cultures. Thus, we have performed additional in vitro experiments to assess viability in our in vitro T cell culture systems. These results, presented in Supplementary Figures 6, 8 and 10, show that:

- 1) Restimulation of differentiated Th2 and Th17 cells in the presence of a strong TCR-activating signal (5 μ g α CD3) and PD-L1 do not demonstrate any substantial change in viability compared to those stimulated without PD-L1 (as assessed by flow cytometry). While there is a slight increase in dead cells in Th1 cells restimulated in the presence of 5 μ g α CD3 and PD-L1 (~70%), compared to culture stimulated with α CD3 alone (~75%), we feel it is unlikely that this slight increase in the frequency of dead CD4+ T cells can account for the 50-70% reduction in cytokine production observed under these circumstances. This data is shown in Supplementary Figure 7.
- 2) Restimulation of differentiated Th1, Th2 or Th17 cells in the presence of a weak TCR-activating signal (1 μ g α CD3) and PD-L1 does not demonstrate any substantial change in viability compared to those stimulated without PD-L1 (as assessed by flow cytometry). This data is shown in Supplementary Figure 9.
- 3) Polarization of T cells in the presence of PD-L1-Ig did not significantly increase the frequency of dead cells in the initial polarizing cultures, nor following restimulation in the absence of additional PD-1 ligand stimulation. In fact, Th17 cells differentiated in the presence of PD-L1 actually showed a *decreased* frequency of dead cells upon restimulation. These data are presented in Supplementary Figure 11.

Collectively, we feel that these data support the conclusion that we state in the revised manuscript – that the inhibitory effects of PD-1 ligation on Th1 and Th17 cytokine production is not related to increased cell death. These data are described on lines 212 – 220, 232 – 235, and 257 – 261 of the revised manuscript.

Comment: What was confusing to me is that the paper starts with a model of allergic asthma where PD1/PDL1 blockade is accompanied with increased Th2 responses. But from Figure 3 onwards, the authors do not use any allergen anymore to stimulate cells but rely on a polyclonal stimulations. For me, it is very difficult to reconcile what they see using polyclonal stimuli in vitro with what is seen in vivo using HDM. The authors should find a way to do one key experiment using irradiated cells and the allergen as the stimulus, and the authors should block PD1/PDL1 and look at whether Th1/Th17 responses would be

limited in such a model as well.

Response: We appreciate the reviewer's sentiment that demonstration of an impact on the development/function of antigen specific T cells using a whole DC:T cell co-culture system would be useful. Indeed, in the initial conception of the project we did consider this approach. However, dendritic cells express many molecules that can influence T cell responses (including, but not limited to, MHC, CD80, CD86, ICOS-L, ICAM-1, OX40L, 4-1BBL). We were afraid that any subtle effects of PD-1/PD-L1 blockade in a DC:T cell co-culture system would be masked (or even enhanced) by additional stimulatory signals beyond our control. Thus, we concluded that in order to truly determine if altering PD-1 signaling alone was sufficient to induce the kinds of changes we were seeing in vivo, we would need to ultimately take a more "reductionist" approach and simply set up a system whereby T cells were activated under a limited, and well defined set of stimuli. Indeed, we feel that this approach was vindicated with our observation that under weak stimulatory conditions (restimulation of Th2 cells with 1 μ g of α CD3, stimulation of naïve T cells under non-skewing conditions), PD-L1 can actively support Th2 cell differentiation, whereas this Th2-polarizing potential is much less evident under more robustly Th2-polarizing conditions.

Second Editorial Decision – 12 January 2015

Dear Dr. Lewkowich,

It is a pleasure to provisionally accept your manuscript entitled "Differential control of CD4 T cell subsets by the PD-1/PD-L1 axis in allergic asthma" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1521-4141/accepted](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted)). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Karen Chu

Peer review correspondence

on behalf of Prof. Francesco Annunziato

Dr. Karen Chu

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