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Differential regulation of PD-1 and its ligands in allergic asthma

Short title: PD-1 and its ligands in allergic asthma

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Abstract

BACKGROUND: Targeting PD-1/PD-1 ligand signalling is an established treatment option for cancer. The role of these molecules in allergic asthma has been investigated in several mouse studies yielding conflicting results. However, human studies investigating the expression and regulation of PD-1 and its ligands in allergic inflammation are lacking.

OBJECTIVE: To analyse the expression and regulation of PD-1 and its ligands in human allergic asthma.

METHODS: The well-established human asthma model of segmental allergen challenge (SAC) was used to analyse the regulation of PD-1 and its ligands PD-L1 and PD-L2 on T lymphocytes and dendritic cells by flow cytometry. The impact of immunoglobulin E (IgE)-mediated signalling on PD-L1 expression was analysed on isolated plasmacytoid dendritic cells (pDCs).

RESULTS: PD-1 expression by blood CD4⁺ T cells was negatively associated with total and specific (against the allergen used for provocation) IgE serum concentrations. 24 hours after SAC, a small decrease in endobronchial PD-1⁺CD4⁺ T cells was accompanied by an increase of PD-L1 expression on endobronchial myeloid dendritic cells (mDCs) and pDCs. The PD-L1 up-regulation on pDCs was not induced by IgE-mediated mechanisms. In contrast, PD-L2 was only detected on endobronchial mDCs and was significantly down-regulated 24 hours after SAC.

CONCLUSION AND CLINICAL RELEVANCE: This study shows, for the first time, an association of a low PD-1 expression by circulating CD4⁺ T cells with high total and specific (against the allergen used for provocation) IgE concentrations in allergic

asthma. In addition, we demonstrate a differential regulation of PD-1 ligands on endobronchial DCs after allergen challenge which may favour Th2-inflammation. Therefore, modulating PD-1 ligand-mediated pathways might be a promising target in allergic asthma.

Introduction

Allergic asthma is a chronic disease of the airways which is associated with airway hyperresponsiveness, variable airflow obstruction, airway remodelling and a characteristic airway inflammation [1]. Allergic asthma is associated with Th2 cells, eosinophils, basophils, and mast cells as well as an increase in cytokines such as IL-4, IL-5, and IL-13 [2, 3]. The activation of CD4 positive T cells requires two signals. The initial signal is specific and is mediated by the T cell receptor which recognises a specific MHC-peptide complex presented by dendritic cells [4]. To ensure complete activation of CD4 T cells a second, non-specific signal mediated by so-called co-stimulatory molecules expressed by dendritic cells is required [4]. In addition to the classical co-stimulatory molecules CD80 and CD86, a variety of other co-stimulatory molecules (with proinflammatory or antiinflammatory properties) such as PD-L1, PD-L2, CTLA-4, OX40L, and ICOSL have been identified [4, 5].

The two ligands of the T cell PD-1 receptor, PD-L1 and PD-L2, belong to the B7:CD28 family and are transmembrane glycoproteins composed of IgC and IgV-type extracellular domains [6 - 9]. Both molecules have only short cytoplasmic tails with no known signal transduction motif [4]. While PD-L1 is expressed on a wide

variety of resting cells such as dendritic cells, macrophages, T cells, and B cells, expression of PD-L2 appears to be restricted to activated dendritic cells and macrophages [10]. In addition, PD-L1 is expressed by several tumor cells and can protect these cells from T cell induced apoptosis [11]. Therefore, blocking the PD-1/PD-1 ligand-pathway with specific antibodies is an established treatment option in various malignant disorders [12].

The role of PD-1 and its ligands PD-L1 and PD-L2 in allergic inflammation was investigated in several experimental murine studies yielding conflicting results about protective and/or supporting effects for allergen-induced inflammation and airway hyperresponsiveness [13 - 16]. Unfortunately, human studies investigating the expression and regulation of PD-1 and its ligands in allergic inflammation are lacking. Therefore, we used a well-established human asthma model of segmental allergen challenge to analyse the expression and regulation of PD-1 on T cells as well as PD-L1 and PD-L2 on dendritic cell subsets before and after allergen challenge.

Methods

Patients

Eleven nonsmoking patients with mild allergic asthma (see table 1 for patient characteristics) were included in the study using the following inclusion criteria: 1. documented airway hyperresponsiveness using methacholine challenge ($PC_{20} < 16$ mg/ml), 2. positive allergen skin prick tests, 3. elevated total or specific (against the

allergen used for provocation, see table 1) immunoglobulin E (IgE) concentrations, 4. dual reaction after allergen inhalation (FEV₁ fall of > 20 % of baseline after 5 – 10 minutes and > 15 % after 4 – 6 hours). Inhaled allergen provocation and calculation of the individual provocation dose were performed as described [17]. Inhaled and segmental allergen challenges (SAC) were separated by at least four weeks. Cromoglycate, antihistamines and corticosteroids were withdrawn at least seven days before each allergen challenge.

For isolation of peripheral blood pDCs, 10 patients with mild allergic asthma (4 female, 6 male; age 24 ± 4 years; FEV₁ 101 ± 19 % predicted; total IgE: median 158 kU/l, range 22 -791 kU/l, with a documented airway hyperresponsiveness by methacholine challenge (PC₂₀ < 16 mg/ml)) who were at least 3 weeks off inhaled steroids were recruited. All patients had positive allergen skin prick tests and elevated total or specific IgE concentrations.

All patients gave their written informed consent and the study protocol was approved by the local ethics committee of Rostock (HV-2010-0021).

Segmental allergen challenge (SAC)

SAC was performed as described [17]. Briefly, 2.5 ml saline was instilled into the left S8 and S4 or S5 segment, and the left S8 was then lavaged using 100 ml of pre-warmed saline. Subsequently, allergen (diluted in 2.5 ml saline) was instilled into the right S8 and S4 or S5 segment, and the right S8 was lavaged by using 100 ml pre-warmed saline after 10 minutes. The second bronchoalveolar lavage was performed

in the left and right S4 or S5 segment 24 hours after challenge. Before each bronchoscopy, venous blood samples were obtained.

Processing of bronchoalveolar lavage fluid (BALF)

BALF was filtered and centrifuged at 4 °C and 400 g for 10 minutes. Supernatants were removed and stored at -80 °C until measured. Cells were resuspended in PBS. A fraction of the suspension was used for cell counts (using a Neubauer chamber) and for cytopins. Cytopins were stained with May-Grünwald-Giemsa solution, and differential cell counts were determined by using standard morphologic criteria.

Flow cytometry

The following antibodies were used for flow cytometry: anti-CD3 allophycocyanin (APC) (clone MEM-57, ImmunoTools, Friesoythe, Germany), anti-CD4 peridinin chlorophyll protein (PerCP) (clone SK3, BD Biosciences, Heidelberg, Germany), anti-CD8 fluorescein isothiocyanate (FITC) (clone HIT8a, Bio Legend, Fell, Germany), lineage cocktail 1 FITC (BD Biosciences), anti-HLA-DR PerCP (clone L243, BD Biosciences), anti-CD11c APC (clone S-HCL-3, BD Biosciences), anti-CD123 APC (clone 6H6, Bio Legend), anti-PD-1 phycoerythrin (PE) (clone EH12.2H7, Bio Legend), anti-PD-L1 PE (clone 29E.2A3, Bio Legend), anti-PD-L2 PE (clone 24F.10C12, Bio Legend). Flow cytometric detection of surface antigens was performed as described [18]. Briefly, freshly collected EDTA blood or BALF cells were incubated with the respective antibodies for 20 min. Afterwards, FACS lysing solution (BD Biosciences, Heidelberg, Germany) was added for another 10 min.

Cells were centrifuged for 5 min (400 g) and washed with phosphate-buffered saline (PBS) containing 2 % fetal calf serum. Finally, cells were resuspended in PBS and analysed on a FACS Calibur using Cell Quest Pro software (both BD Biosciences). PDCs were identified as lineage-negative/dim HLA-DR⁺ CD123⁺ and mDCs as lineage-negative/dim HLA-DR⁺ CD11c⁺. T cell subsets were detected as CD4⁺CD8⁻ CD3⁺ (CD4⁺ T cells) and CD4⁻CD8⁺CD3⁺ (CD8⁺ T cells), respectively (see figure S1 for the gating strategy).

Detection of soluble PD-1, PD-L1, and PD-L2

Soluble PD-1 and PD-L2 were analysed in serum and BALF using specific ELISA systems (R&D Systems, Wiesbaden, Germany) with a detection limit of 156 pg/ml. Soluble PD-L1 was analysed using a specific ELISA with a detection limit of 15.6 pg/ml (MyBioSource, San Diego, CA, USA).

In vitro stimulation of isolated peripheral blood pDCs

PDCs were isolated using a commercial Plasmacytoid Dendritic Cell Isolation Kit (Miltenyi, Bergisch Gladbach, Germany), reaching a purity of > 90 % pDCs in all cases. Then, pDCs (but not control pDCs) were loaded with human IgE (1 µg/ml, AbD Serotec, Puchheim, Germany) for one hour and washed two times with RPMI 1640 medium (Sigma-Aldrich, München, Germany). Afterwards, pDCs were incubated at 10⁶ cells/ml with goat anti-human IgE or goat IgG isotype control (control pDCs) (both AbD Serotec) for 48 hours in RPMI 1640 medium supplemented with 10% fetal calf serum (Invitrogen, Karlsruhe, Germany) and interleukin-3 (10 ng/ml, ImmunoTools, Friesoythe, Germany). Finally, cells were

harvested and PD-L1 expression was analysed by flow cytometry. Because anti-PD-L1 staining on isolated and stimulated pDCs shows a fluorescence shift in the whole population the net median fluorescence intensity (MFI) values of all pDCs were calculated as follows: MFI anti-PD-L1 – MFI isotyp control antibody.

Statistical analysis

Data were analysed using SPSS 22 (IBM, Chicago, IL, USA). Correlation analysis was performed using the Spearman's correlation coefficient. The comparison of BALF parameters between allergen and saline challenged control segments and blood parameters before and after SAC was performed using the Wilcoxon signed rank test. Probability values of $p < 0.05$ were regarded as significant.

Results

Patient characteristics and allergen-induced airway inflammation

Patient characteristics are detailed in **Table 1**. Allergen-induced changes in endobronchial and blood leukocyte numbers are shown in **Table 2 and S1**, respectively. 24 hours after SAC, a significant increase in eosinophil numbers (control segment: median 1.1×10^3 , range $0 - 4.6 \times 10^3$, allergen challenged segment: median 34.6×10^3 , range $3.0 - 271.2 \times 10^3$ eosinophils/ml BALF; $p = 0.003$), pDCs (control segment: median 0.08×10^3 , range $0.01 - 2.28 \times 10^3$; allergen challenged segment: median 0.55×10^3 , range $0.10 - 5.25 \times 10^3$ pDCs/ml BALF; $p = 0.021$), and mDCs (control segment: median 0.91×10^3 , range $0.10 - 4.50 \times 10^3$; allergen challenged segment: median 3.16×10^3 , range $0.32 - 18.04 \times 10^3$ mDCs/ml

BALF; $p = 0.041$) were detected in BALF indicating a robust allergen-induced inflammatory response. In addition, leucocyte counts as well as percentages of neutrophils and eosinophils were significantly elevated in peripheral blood 24 hours after SAC (**Table S1**).

No correlations between the allergen and/or the allergen dose used for challenge and any of the measured parameters were found.

PD-1 expression on circulating CD4⁺ T cells at baseline

PD-1 expression on peripheral blood CD4⁺ T cells was negatively correlated with total IgE ($r_s = -0.827$; $p = 0.002$) and specific IgE (against the allergen used for provocation, see table 1) ($r_s = -0.836$; $p = 0.001$) values in serum samples (**Figure 1**). In addition, a trend for a positive correlation with FEV₁ values was observed ($r_s = 0.548$; $p = 0.081$) (**Figure 1**). In contrast, no association of PD-1 expression on circulating CD8⁺ T cells with total and specific IgE as well as FEV₁ values was detected.

PD-1 expression on T cells after SAC

PD-1 expression on blood CD4⁺ T cells was significantly upregulated 24 hours after SAC (before SAC: median 25.3 %, 24 hours after SAC: median 29.8%, $p = 0.025$). In contrast, the trend to increased CD8⁺ T cell PD-1 expression was not significant (before SAC: median 12.1 %, 24 hours after SAC: median 15.8 %) (**Figure 2**). BALF CD4⁺ T cell PD-1 expression was unchanged 10 minutes after SAC (control segment after 10 min: median 81.0 %; allergen challenged segment after 10 min: 81.6 %).

However, 24 hours after SAC a significant decrease in PD-1 expression on BALF CD4⁺ T cell was detected (control segment after 24 hours: median 81.4 %; allergen challenged segment after 24 hours: 76.7 %; $p = 0.008$) (**Figure 2**). In contrast, PD-1 expression on BALF CD8⁺ T cells was not significantly changed 10 minutes and 24 hours after SAC (**Figure 2**).

PD-L1 and PD-L2 expression on dendritic cells after SAC

While PD-L1 could be detected on circulating as well as on BALF pDCs and mDCs, PD-L2 expression was only observed on BALF mDCs. Ten minutes after SAC, no differences in PD-L2 expression were observed on BALF mDCs (control segment after 10 min: median 14.7 %; allergen challenged segment after 10 min: 13.4 %). In contrast, 24 hours after SAC, a significant decrease in PD-L2 expression on BALF mDCs was detected (control segment after 24 hours: median 15.8 %; allergen challenged segment after 24 hours: 6.5 %; $p = 0.003$) (**Figure 3**).

PD-L1 expression on blood mDCs (before SAC: median 4.0 %; 24 hours after SAC: median 9.3 %) and pDCs (before SAC: median 19.9 %; 24 hours after SAC: median 21.5 %) was not significantly affected by SAC (**Figure 3**). Ten minutes after SAC, no differences in PD-L1 expression were observed on BALF mDCs (control segment after 10 min: median 29.7 %; allergen challenged segment after 10 min: median 27.2 %). In BALF from control and allergen challenged lung segments after 10 minutes, appropriate amounts of pDCs for a valid analysis of surface molecules (> 100 pDCs per sample) could not be obtained in most of the patients. However, 24 hours after SAC, a significant increase in PD-L1 expression was detected on BALF mDCs (control segment after 24 hours: median 30.6 %; allergen challenged segment after

24 hours: median 49.1 %; $p = 0.026$) and pDCs (control segment after 24 hours: median 35.0 %; allergen challenged segment after 24 hours: median 50.6 %; $p = 0.007$) compared to the respective control segments (**Figure 3**).

Soluble PD-1, PD-L1 and PD-L2 after SAC

There was a significant decrease of soluble PD-L2 in serum samples after SAC (**Figure 3**). Soluble PD-1 and PD-L1 could only be detected in single serum samples (data not shown). In BALF, soluble PD-1, PD-L1, and PD-L2 were below the detection limit in all samples.

PD-L1 expression on pDCs after crosslinking of the high affinity IgE receptor

A significant association of PD-L1 on BALF pDCs 24 hours after SAC with total ($r_s = 0.736$; $p = 0.010$) and specific (against the allergen used for provocation, see table 1) ($r_s = 0.673$; $p = 0.023$) serum IgE values was observed (**Figure 4**). The pDCs express the high affinity IgE receptor and this expression is increased in patients with allergic asthma [19]. Therefore, it could be speculated that PD-L1 expression by pDCs is directly affected by crosslinking of the IgE receptor. Therefore, blood pDCs from ten patients with allergic asthma were isolated and loaded with human IgE. Crosslinking of the IgE receptor was induced by anti-human IgE antibodies. Surprisingly, a significant down-regulation of PD-L1 (in comparison to control pDCs) was observed (**Figure 4**). No correlation between this phenomenon and clinical parameters (total IgE, specific IgE, FEV1) was observed.

Discussion

This is the first mechanistic study describing the regulation of PD-1 and its ligands in patients with allergic asthma following allergen challenge. We demonstrate a negative association between PD-1 expression by circulating CD4⁺ T cells and serum IgE concentrations in allergic asthma. In addition, we demonstrate a differential regulation of PD-1 ligands on endobronchial DCs after allergen challenge.

Our data suggest a possible protective effect of PD-1 expression on circulating CD4⁺ T cells for some aspects of allergic asthma, as evidenced by the negative correlation between PD-1 expression by circulating CD4⁺ T cells and IgE concentrations in serum. It is, however, unclear whether elevated IgE concentrations could be promoted by a defect in upregulation of PD-1 expression, e.g. on Th2 effector cells, or the reduction in a PD-1⁺ CD4⁺T cell population. Both have been described in the literature: the expression of PD-1 on regulatory T cells [20] and the expression of PD-1 by Th2 effector cells [21]. Furthermore, in a murine model of asthma it has been reported that inducible regulatory T cells suppress airway hyperresponsiveness and inflammation in a PD-1-dependent manner [22].

In addition, the present study shows a moderate but significant increase in the percentage of blood PD-1⁺CD4⁺ T cells 24 hours after SAC, accompanied by a moderate decrease of these CD4⁺ T cells in BALF. It is therefore reasonable to speculate that this might be caused by migration of PD-1-negative CD4⁺ T cells from peripheral blood into the lung following allergen challenge. Indeed, there was a trend

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towards a decrease in total CD4⁺ T cells in peripheral blood and an increase of these cells in BALF 24 hours after SAC. PD-1 is not expressed on naïve CD4 T cells but promptly upregulated after classical CD4 T cell activation [23, 24]. Alternatively, it has been shown that freshly activated murine Th2 cells do not respond to stimulation by PD-1 ligands, while re-stimulation renders them sensitive [25]. In addition, in a murine model of schistosomiasis, PD-1⁺CD4⁺ T cells increase over time and peak at nine weeks after infection [26]. In a murine model of helminth infection, PD-1 expression on Th2 cells increases over time and is associated with a loss of functionality [21]. Together, these studies suggest that Th2 cells need repeated stimulation to upregulate PD-1 expression. In our study, PD-1-negative and positive CD4⁺ T cells were not further characterised. Therefore, it remains unclear whether the allergen-induced decrease in endobronchial PD-1⁺CD4⁺ T cells results from an influx of naïve or freshly activated Th2 cells or from a defect in PD-1 upregulation by some CD4⁺ T cells.

This is the first study to show a differential regulation of PD-L1 and PD-L2 on human dendritic cells following allergen challenge. Interestingly, *in vitro* crosslinking of the IgE receptor on pDCs resulted in a strong down-regulation of PD-L1, whereas there was a significant up-regulation of PD-L1 on pDCs and mDCs following SAC *in vivo*. These findings might indicate that allergen-mediated IgE receptor crosslinking is either not responsible for PD-L1 upregulation on pDCs or that the cytokine microenvironment in the airways can overcome the IgE receptor mediated mechanisms *in vivo*. Our contrasting *in vivo* and *in vitro* findings reflect the contrasting findings for the role of PD-1 ligands in murine asthma models described in the literature. Kool et al. showed a PD-1/PD-L1-mediated anti-inflammatory role of pDCs in allergic airway

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inflammation [13] while another study showed significantly reduced airway hyperresponsiveness in PD-L1 (-/-) mice which seems to be mediated by enhanced IFN- γ production [14]. A third study reported that different T cell subsets respond differently to PD-L1-mediated PD-1 ligation: Th2 cells, but not Th1 or Th17 cells, showed an enhanced cytokine secretion after ligation [25].

In agreement with a previous study, we found a significant expression of PD-L2 on lung cells of asthmatic patients [27]. However, in contrast to murine models where PD-L2 is upregulated by lung mDCs after allergen challenge [16, 27], we found a significant allergen-induced down-regulation on endobronchial mDCs in human asthma which was accompanied by a downregulation of soluble PD-L2 in serum. For systemic sclerosis it was suggested that elevated soluble PD-L2 might reflect its elevated cellular expression [28]. In agreement with this study, it could be speculated that in our study allergen-induced reduced soluble PD-L2 in serum might reflect its reduced expression on mDCs. It is currently unclear whether this discrepancy between mice and humans is due to species-specific differences or differences in the duration of the disease or the mode of sensitisation between the models. Of note, there are conflicting reports on the role of PD-L2 in murine models of asthma. PD-L2 (-/-) mice showed an increased airway inflammation and hyperresponsiveness after allergen challenge [14] and addition of blocking antibodies against PD-L2 during allergen challenge enhanced the production of Th2 cytokines and airway hyperresponsiveness [15]. Accordingly, a significant role for PD-L2 in tolerance induction has been postulated [29, 30]. In contrast, Oflazoglu et al. described an increased inflammatory response in a murine asthma model following PD-L2-Fc

administration [16]. In patients with asthma sensitised to flour or latex antigens, an *in vitro* up-regulation of PD-L2 by mDCs after allergen-pulsing was observed, which increased the expression of IL-5 and IL-13 by CD4⁺ T cells [31].

Taken together, most studies show a suppressive effect for PD-L1 on Th1 and Th17 immune responses while PD-L2 limits Th2 responses suggesting that the upregulation of PD-L1 and downregulation of PD-L2 on endobronchial DC subsets favour a Th2 inflammation in our human asthma model. Therefore, modulating PD-1 ligand-mediated pathways (e.g. blocking PD-L1 or activating PD-L2 signalling) might be a promising target in allergic asthma. However, due to conflicting results obtained from different asthma models, further studies are needed to clarify the role of PD-1 and its ligands in human allergic asthma.

In conclusion, we report a differential allergen-induced regulation of PD-1 ligands on DC subsets in patients with allergic asthma which may favour a Th2-type inflammation. In addition, we demonstrate an inverse association between the expression of PD-1 by circulating CD4⁺ T cells and IgE serum concentrations in allergic asthma.

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Author contribution

KB designed the study, analysed the data and wrote the manuscript, LF, FN and KaG performed experiments, KrG planned and performed segmental allergen challenge, ML wrote the manuscript, JCV designed the study, performed segmental allergen challenge and wrote the manuscript.

Conflict of interest

LF, FN, KaG, KrG, ML have no interest of conflict to declare. KB received funding for research from Deutsche Forschungsgemeinschaft (DFG). JCV has lectured for and received honoraria from: ALK-Abello, Allergopharma, AstraZeneca, Avontec, Bayer, Bencard, Bionorica, Boehringer-Ingelheim, Cipla, Chiesi, Essex/Schering-Plough, GSK, Janssen-Cilag, Leti, MEDA, Merck/MSD, Mundipharma, Novartis, Nycomed/Altana, Pfizer, Revotar, Sandoz-Hexal, Stallergens, TEVA, UCB/Schwarz-Pharma, Zydus/Cadila and possibly others, has participated in Advisory Boards of ALK-Abello, Allergopharma, Avontec, Boehringer-Ingelheim, Cipla, Chiesi, Essex/Schering-Plough, GSK, Janssen-Cilag, Leti, MEDA, Merck/MSD, Mundipharma, Novartis, Regeneron, Revotar, Roche, Sanofi-Aventis, Sandoz-Hexal TEVA, UCB/Schwarz-Pharma and possibly others, and has received funding for research from Deutsche Forschungsgemeinschaft, Land Mecklenburg-Vorpommern, GSK, MSD/Merck.

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Tables

Table 1. Patient characteristics

sex	age	Duration of asthma	Total IgE (kU/l)	Allergen	Specific IgE (kU/l)	Allergen dose (AU)	FEV1 (l)	FEV1 (%predicted)	medication
f	27	10	298	DP	79.6	5600	2.8	89	BA, OAH
f	55	6	51	DP	7.9	2740	2.4	101	BA, OAH
f	25	20	215	Rye	1.2	8000	3.2	98	BA, OAH
m	25	15	53	Birch	10.0	1530	4.1	87	-
m	25	22	29	DP	9.1	79	3.9	87	-
m	42	23	33	Rye	7.2	1500	4.3	112	BA, IC
f	21	20	759	Birch	96.9	450	3.1	93	BA
f	21	20	900	DP	89.2	1500	3.0	90	BA, OAH
f	22	19	1143	Birch	>100	3030	3.0	90	BA, IC, OAH
m	26	23	678	DP	75.6	380	4.3	84	BA, IC
f	22	19	30	DP	8.0	590	3.2	92	CR

The table displays the sex, age, and duration of asthma of the patients, the prebronchodilator forced expiratory volume in the first second (FEV1), the medication prior to the study (BA: inhaled beta-2-agonist, CR: cromoglycate, IC: inhaled corticosteroid, OAH: oral anti-histamine), serum levels of total (normal range: < 100 kU/l) and allergen-specific (normal range: < 0.7 kU/l) IgE, and the allergen (DP, *Dermatophagoides pteronyssinus*) and the dose (AU: allergen units) used for segmental allergen challenge.

Table 2. BALF parameters

	BALF 10 min		BALF 24h	
	control	allergen	control	allergen
Recovery (ml)	61 (25 – 76)	48 (17 – 59)*	60 (43 – 75)	54 (26 – 68)
macrophages (10 ³ /ml BALF)	62.4 (2.3 – 109.8)	46.0 (0 – 140.2)	76.3 (25.0 – 186.6)	41.9 (3.8 – 143.5)
lymphocytes (10 ³ /ml BALF)	11.8 (0.6 – 23.0)	5.2 (0 – 43.6)	11.0 (2.9 – 86.5)	9.1 (2.1 – 73.8)
neutrophils (10 ³ /ml BALF)	0.8 (0 – 7.3)	0.8 (0 – 6.5)	9.8 (0.4 – 177.5)	24.3 (1.1 – 49.7)
eosinophils (10 ³ /ml BALF)	0 (0 – 2.3)	0.2 (0 – 4.4)	1.1 (0 – 4.6)	34.6 (3.0 – 271.2)*
CD4 ⁺ T cells (10 ³ /ml BALF)	4.2 (1.3 – 17.4)	2.7 (0 – 10.6)	4.2 (1.2 – 30.3)	5.7 (1.0 – 41.3)
CD8 ⁺ T cells (10 ³ /ml BALF)	2.8 (0.3 – 36.6)	2.0 (0 – 25.7)	1.9 (0.5 – 24.3)	1.9 (0.3 – 6.4)
pDCs (10 ³ /ml BALF)	0.06 (0.00 – 0.15)	0.05 (0.00 – 0.18)	0.08 (0.01 – 2.28)	0.55 (0.10 – 5.25)*
mDCs (10 ³ /ml BALF)	0.56 (0.02 – 0.97)	0.53 (0.34 – 1.55)	0.91 (0.10 – 4.50)	3.16 (0.32 – 18.04)*

The table displays the BALF recovery and number of cells in in BALF after saline and allergen challenge, respectively. All parameters are shown as median values (range). Asterisks mark significant differences ($P < 0.05$) between allergen challenged and saline challenged lung segments.

Figure Legends

Figure 1. PD-1 expression on circulating CD4⁺ T cells is associated with clinical parameters in allergic asthma. Specific IgE values refer to the allergen used for provocation in each patient (see table 1). P-values < 0.05 were regarded as significant.

Figure 2. Expression of PD-1 on CD4⁺ T cells but not CD8⁺ T cells is altered by allergen challenge. (A) CD4⁺ and CD8⁺ T cells were identified as described. Histogram plots show specific PD-1 staining (red line) compared with the respective isotype control (grey). Percentages of positive cells are presented from one patient. (B) Box plots show the median (line within the box), interquartile range (edges of the box), and range of values less distant than 1.5 interquartile ranges from the upper or lower quartile (vertical lines). Outliers are shown as single points. * P-values < 0.05 were regarded as significant. A, allergen challenged lung segment; BAL, bronchoalveolar lavage; C, saline challenged control segment; PE, phycoerythrin.

Figure 3. Differential allergen-induced regulation of PD-1 ligands on endobronchial dendritic cells. (A) PDCs and mDCs were identified as described. Histogram plots show specific PD-L1 and PD-L2 staining (red line) compared with the respective isotype control (grey). Percentages of positive cells are presented from one patient. (B) Box plots show the median (line within the box), interquartile range (edges of the box), and range of values less distant than 1.5 interquartile ranges from the upper or lower quartile (vertical lines). (C) Single point presentation of soluble PD-L2 in serum

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samples obtained before and 24 hours after SAC. * P-values < 0.05 were regarded as significant. A, allergen challenged lung segment; BAL, bronchoalveolar lavage; C, saline challenged control segment; PE, phycoerythrin.

Figure 4. PD-L1 expression on pDCs and IgE. (A) Scatter plots show the association of PD-L1 expression on endobronchial pDCs 24 hours after allergen challenge with total and specific (against the allergen used for provocation, see table 1) IgE concentrations in serum. (B) IgE-mediated crosslinking of the high-affinity IgE receptor on isolated peripheral blood pDCs downregulates PD-L1 expression. * P-values < 0.05 were regarded as significant. MFI, median fluorescence intensity; SAC, segmental allergen challenge.

Figure 1

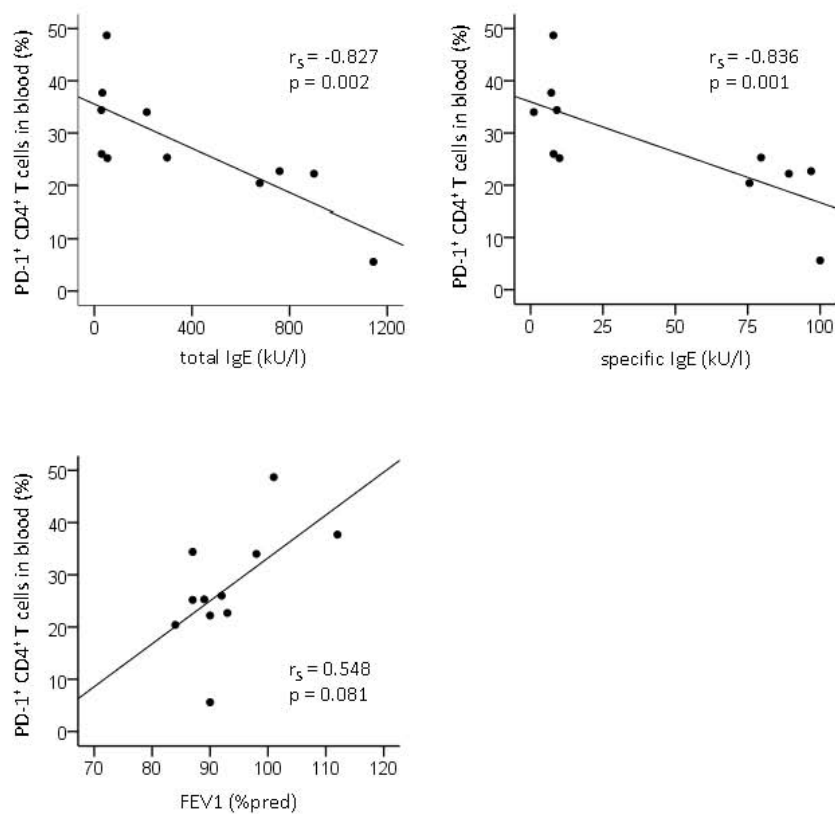
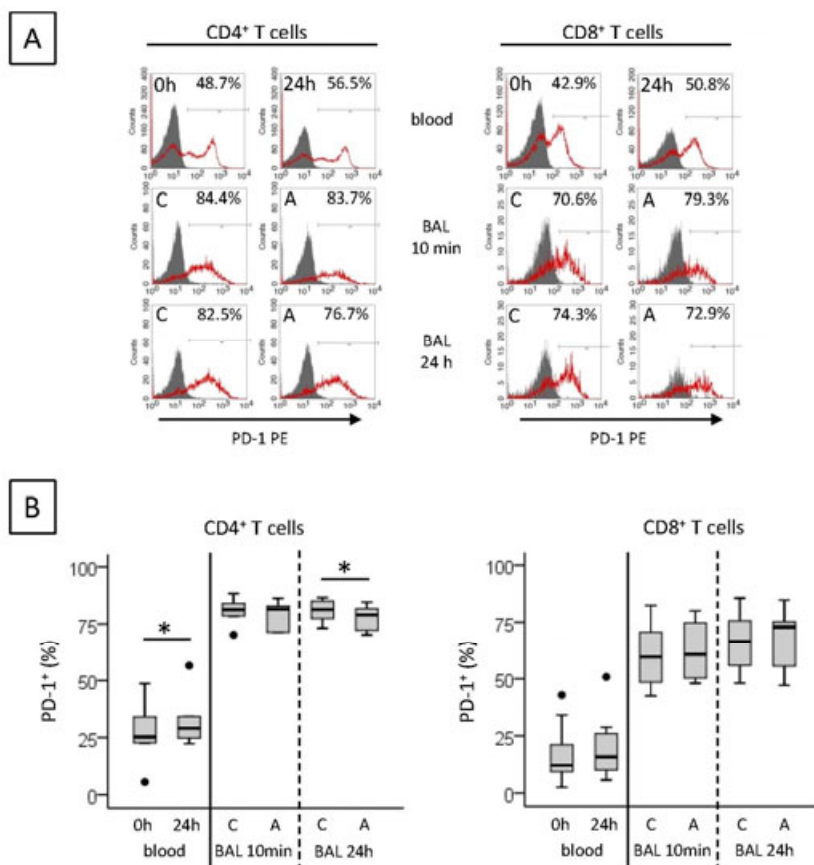


Figure 2



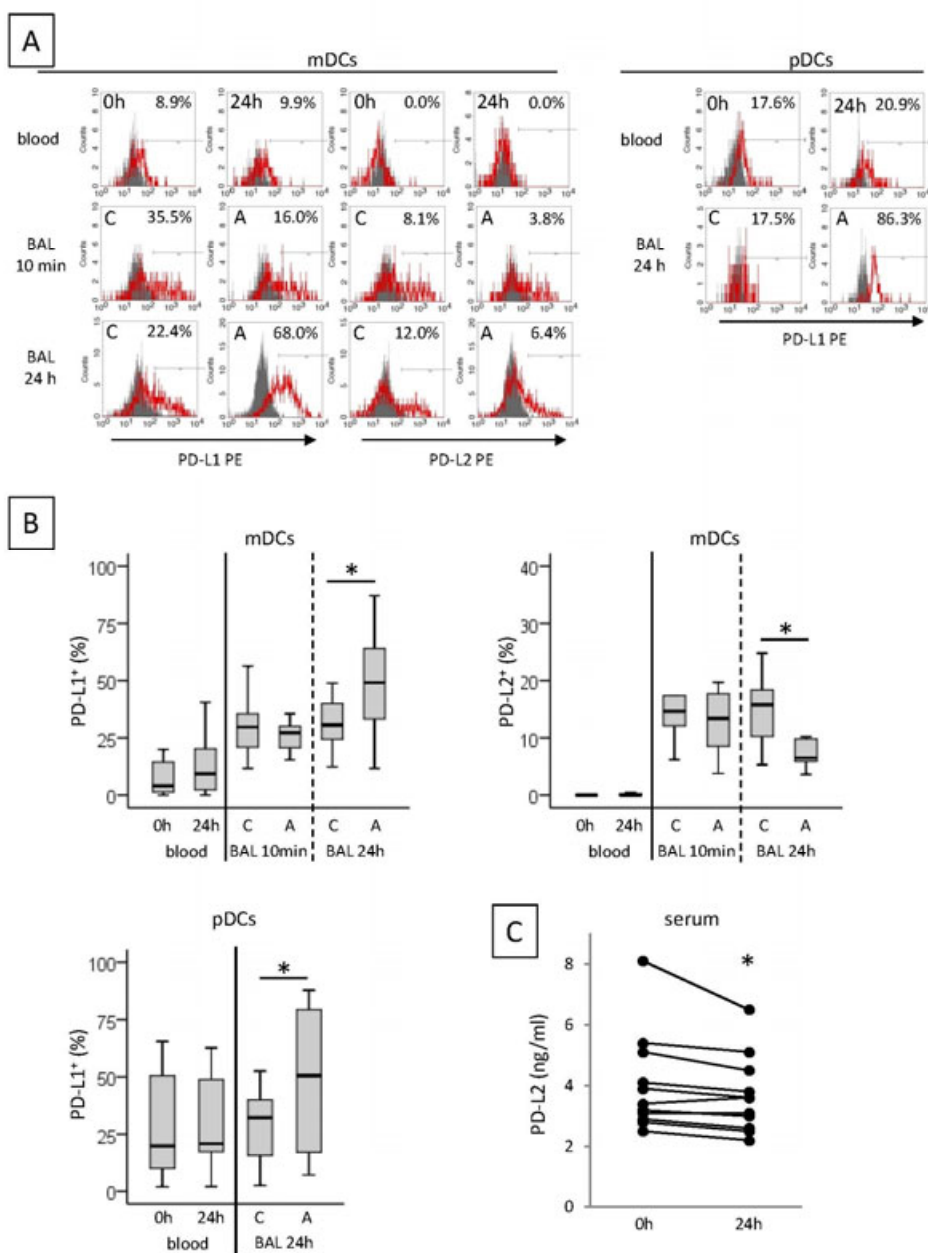


Figure 3

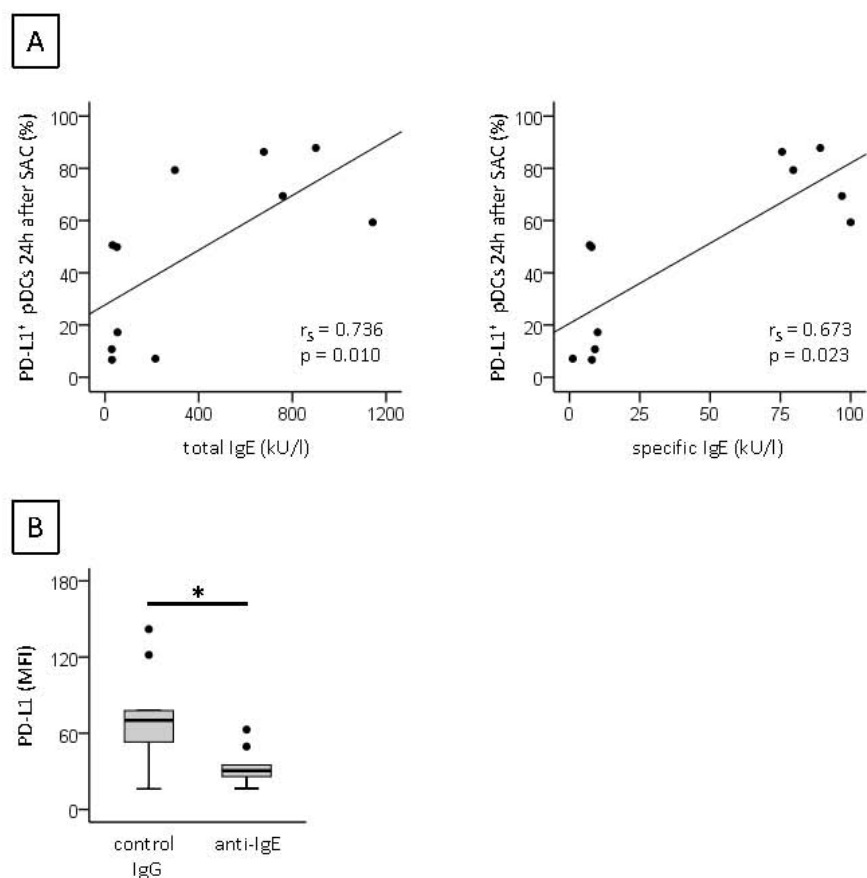


Figure 4

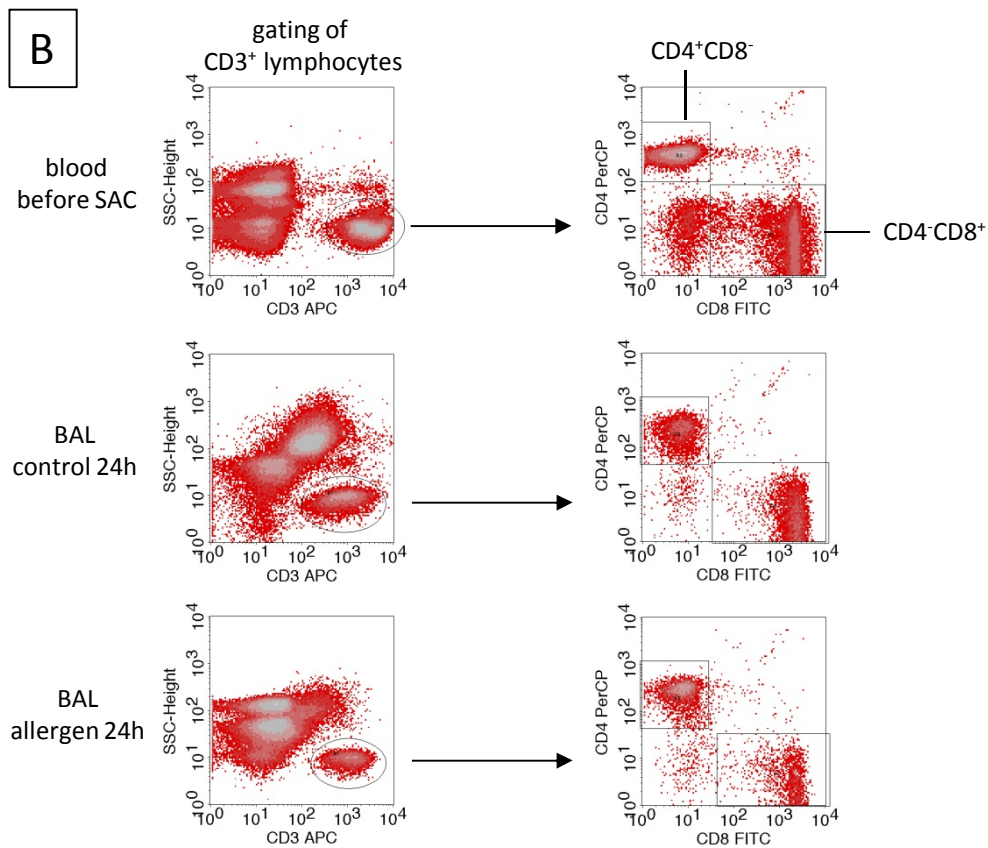
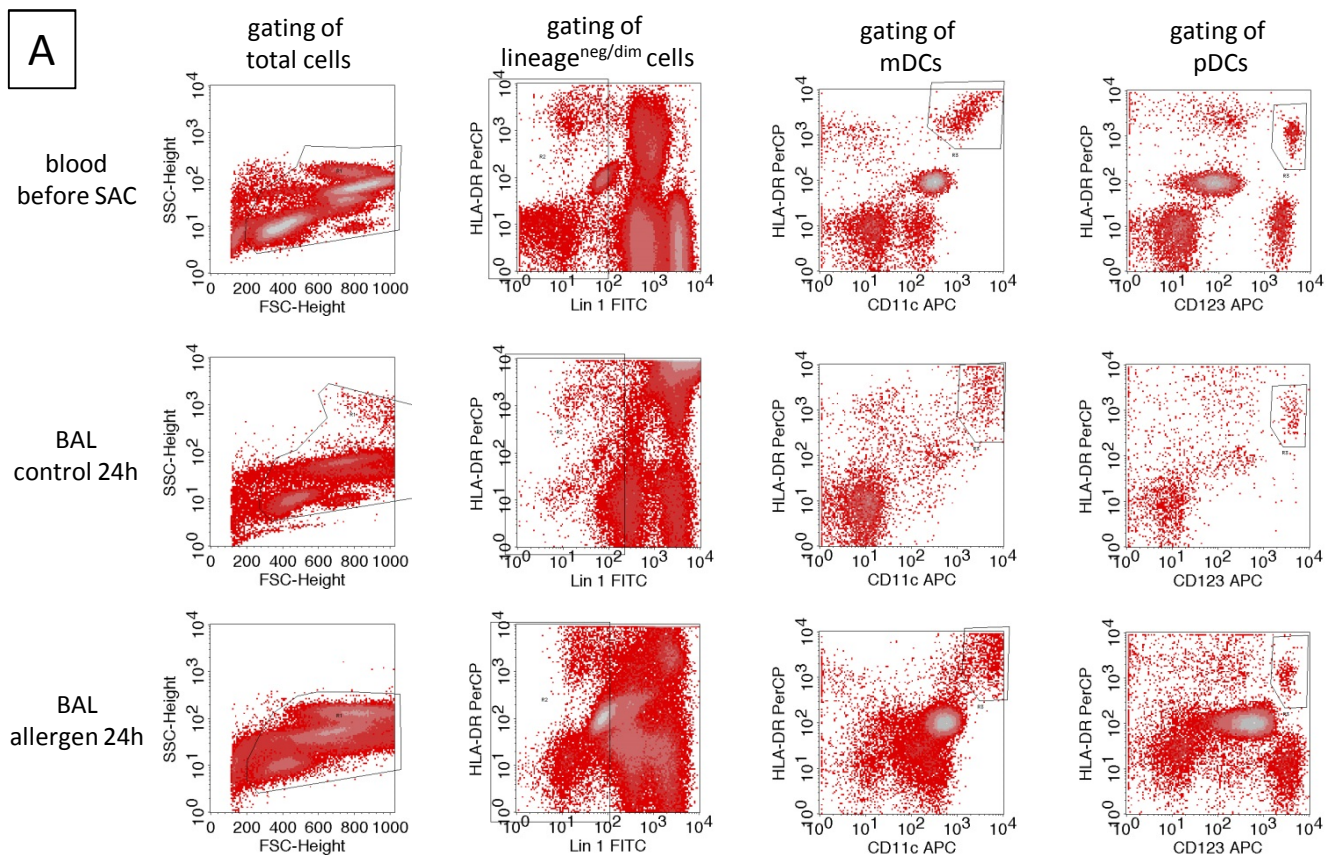


Figure S1

Supporting information

The PD-1/PD-L1/PD-L2-axis in the human asthma model of segmental allergen challenge

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Table S1. Blood parameters

	before SAC	24h after SAC
haemoglobin (mmol/l)	8.4 (7.6 – 9.5)	8.2 (7.5 – 9.1)
erythrocytes ($10^{12}/l$)	4.6 (4.2 – 5.3)	4.7 (4.2 – 5.3)
platelets ($10^9/l$)	245 (145 – 412)	252 (142 – 346)
leucocytes ($10^9/l$)	5.8 (3.3 – 8.3)	6.8 (4.9 – 13.6)*
neutrophils (%)	43 (28 – 55)	56 (47 – 69)*
lymphocytes (%)	43 (28 – 64)	32 (17 – 42)*
monocytes (%)	9 (4 – 12)	8 (5 – 10)
eosinophils (%)	4.2 (0 – 8.6)	6.3 (1.6 – 11.1)*
basophils (%)	0.6 (0.3 – 1.1)	0.4 (0.1 – 1.1)*
CD4 ⁺ T cells ($10^5/ml$)	10.3 (7.3 – 18.5)	9.3 (6.1 – 14.7)
CD8 ⁺ T cells ($10^5/ml$)	6.0 (3.1 – 10.6)	5.5 (2.7 – 7.9)
pDCs ($10^3/ml$)	10.6 (4.7 – 20.1)	7.1 (4.9 – 16.2)
mDCs ($10^3/ml$)	17.0 (7.7 – 29.1)	13.3 (3.8 – 26.7)

The table displays blood parameters before and 24 hours after segmental allergen challenge. All parameters are shown as median values (range). Asterisks mark significant differences ($P < 0.05$).

Figure S1. Gating strategy. (A) Identification of dendritic cell subsets in blood and BALF. Total cells were identified in a forward scatter (FSC)/side scatter (SSC) plot (first row). Then lineage negative/dim cells were gated (second row) and among them myeloid dendritic cells (mDCs) were identified as HLA-DR⁺CD11c⁺ (third row) and plasmacytoid dendritic cells (pDCs) as HLA-DR⁺CD123⁺ (fourth row). (B) Identification of T cell subsets in blood and BALF. Total T cells were identified as CD3⁺ cells with low side scatter (SSC) characteristics (first row). Among these cells CD4⁺ T cells were gated as CD4⁺CD8⁻ cells and CD8⁺ T cells as CD8⁺CD4⁻ cells (second row).