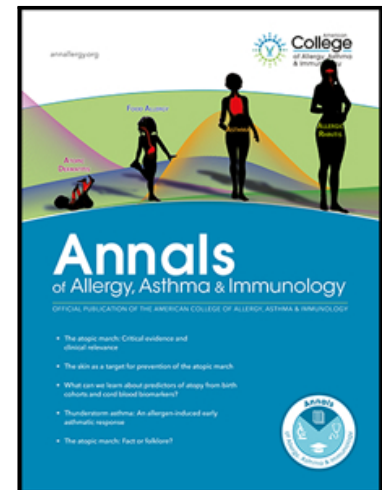


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Peripheral blood intermediate monocyte PAR-2 expression increases during asthma exacerbations and following inhalation allergen challenge

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Running Head: Monocytes and airway inflammation

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Key words: Monocytes, dendritic cells, asthma, asthma exacerbation, allergen challenge, inflammation.

Abbreviations/Acronyms:

ACQ6: Asthma control questionnaire

AQLQ: Asthma quality of life questionnaire

AUC: Area under the curve

CMo: Classical monocytes

DC: Dendritic cells

EAR: Early asthmatic response

ED: Emergency department

FcεRI: Fc Epsilon Receptor I

FEV₁: forced expiratory volume in 1 sec

GM-CSF: Granulocyte-macrophage colony stimulating factor

IMMo: Intermediate monocytes

IQR: Interquartile range

mDC: myeloid DC

MFI: Median fluorescence intensity

Mo: Monocytes

NCMo: Non-classical monocytes

PAR-2: Protease-activated receptor-2

PB: Peripheral blood

pDC: plasmacytoid DC

UAH: University of Alberta Hospital

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drafted the article or reviewed it critically for important intellectual content:

Shrestha Palikhe, Gandhi, Wu, Sinnatamby, Rowe, Mayers, Cameron, Vliagoftis

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Shrestha Palikhe, Gandhi, Wu, Sinnatamby, Rowe, Mayers, Cameron, Vliagoftis

Abstract

Background : Myeloid cells, especially dendritic cells and macrophages play important roles in asthma pathophysiology. Monocytes (Mo) and macrophages express PAR-2, a pro-inflammatory serine protease receptor implicated in the pathophysiology of allergic airway inflammation. We have shown that patients with severe asthma and those with a history of frequent asthma exacerbations exhibit increased PAR-2 expression on peripheral blood monocytes.

Objective : The study aims to determine PAR-2 expression on peripheral blood intermediate monocytes (IMMo) in subjects with increased airway inflammation, either as a result of an asthma exacerbation or following an inhalation allergen challenge. **Methods :** Sixteen adults presenting to the emergency department (ED) with asthma exacerbations were recruited after giving informed consent. Ten patients returned 2 weeks later for follow-up. Eleven mild asthmatics treated only with as needed bronchodilators were recruited and underwent inhalation allergen challenge after informed consent. Immune cell profiling was performed by whole blood flow cytometry in both groups of patients.

Results : PAR-2 expression in peripheral blood IMMo was increased in patients with an asthma exacerbation compared to patients with stable disease and this expression decreased following treatment of the asthma exacerbation. Subjects with mild asthma showed increase in percentages of IMMo expressing PAR-2 after allergen challenge. Patients presenting to the ED, had lower dendritic cell (DC) and DC subset numbers in peripheral blood during exacerbation compared to after treatment.

Conclusion : Increased PAR-2 expression on Mo during periods of increased airway inflammation may initiate a positive feedback loop leading to systemic inflammatory changes.

Introduction

Asthma is a chronic inflammatory disease of the airways that affects children and adults across the globe¹. Asthma is an important cause of impaired quality of life for patients and contributes to health care costs through clinic, emergency department (ED) hospital, and in-patient encounters². In particular ED visits for asthma exacerbations are an important contributor to high health care costs³, especially in children. Understanding the pathogenesis of asthma exacerbations and the role of airway inflammation during asthma exacerbations, may allow the development of novel therapeutic interventions.

Among myeloid cells, the role of macrophages and dendritic cell (DC) in asthma has been extensively studied and reviewed⁴; however, the role of monocytes (Mo) is still poorly understood. Monocytes circulate in the blood with a short half-life followed by migration into tissues, especially inflamed tissues, where they can maintain their monocytic characteristics and functions⁵, differentiate into macrophages, or give rise to DC⁶. Depletion of circulating Mo in mice suggests that these cells have a pro-inflammatory role in asthma, in contrast to alveolar macrophages that, in certain models, are anti-inflammatory⁷. In particular, depletion of circulating Mo decreases the number of eosinophils infiltrating into the lungs, the levels of Th1, Th2, Th17 cytokines^{7, 8} and airway hyperresponsiveness⁸. This effect of Mo could be mediated either through direct IL-5 secretion^{9, 10}, or through their ability to increase the capacity of T lymphocytes to produce IL-5¹¹. Alternatively, another important eosinophil survival factor, granulocyte-macrophage colony stimulating factor (GM-CSF) can also be released by Mo¹². The *in vivo* observations above, however, have not been validated with human Mo and/or in human asthma.

Protease-Activated Receptor-2 (PAR-2), a pro-inflammatory receptor for serine proteases, is expressed on multiple cells and its activation has been implicated in the pathophysiology of asthma¹³. Monocytes are the main cell expressing PAR-2 in peripheral blood. Human peripheral blood Mo are heterogeneous^{14, 15}. Mo are classified into CD14⁺⁺CD16⁻ (classical - CMo), CD14⁺⁺CD16⁺ (intermediate - IMMo), and CD14⁺CD16⁺ (non-classical – NCMo)¹⁶ subsets, which can be identified and characterized separately by flow cytometry¹⁷. Very little is known about the presence, functions, and activation status of Mo subsets in human asthma. The information presented above suggests that Mo may play important roles in asthma through various mechanisms including the release of pro-inflammatory mediators, facilitation of

recruitment and differentiation of lymphocytes and eosinophils in the airways and through their transformation into DC and/or macrophages in the airways.

We have shown that IMMo of patients with severe asthma exhibit higher surface PAR-2 expression compared to those from subjects with mild/moderate disease¹⁸, while we found no difference in PAR-2 expression on CMo between patients with asthma of varying severity. That study raised the question whether increased PAR-2 expression was dependent on increased inflammation in patients with severe asthma or was associated with other determinant of disease severity.

To answer this question here we evaluated PAR-2 expression on peripheral blood Mo subsets in patients experiencing asthma exacerbations (increased inflammation and loss of asthma control independent of severity of the disease) or due to an inhalation allergen challenge (increased airway inflammation in a background of mild asthma).

Methods

Subjects

Patients >18 years presenting to the ED of University of Alberta Hospital (UAH) with an asthma exacerbation, according to ED attending physician diagnosis, were recruited by trained research nurses. Recruitment took place between March 2016- and Dec 2018. The study was approved by the University of Alberta institutional review board (Pro00052055) and all patients provided written informed consent.

Following consent, patients were interviewed, the charts were reviewed, and a blood sample was obtained, treated as per ED protocol (including at least a 5-day course of oral corticosteroids) without any study specific intervention and were asked to return to clinic 14 days after the index ED visit. In addition to the clinical data collected by ED, spirometry was obtained, and patients

completed the asthma control questionnaire (ACQ6) and the asthma quality of life questionnaire (AQLQ) during both the “exacerbation” (on presentation to the ED) and “treatment” (14 days later) visits. Details on clinical characteristics of these patients have been presented elsewhere¹⁹. Data were compared between the two visits. Data from the asthma exacerbation visit were also compared to data obtained from a cohort of patients with stable asthma we reported previously²⁰. Briefly, these stable asthmatics attended 4 clinic visits in 3-months intervals and had no significant worsening of their symptoms (exacerbation) in between the visits.

To study the effect of inhalation allergen challenge on peripheral blood myeloid cells, patients with mild asthma (i.e., treated only with as needed bronchodilators) were recruited between March 2016 and January 2019 and inhalation allergen challenges were performed in accordance with the University of Alberta protocol that is based on the description by O’Byrne et al²¹. This part of the study had been approved separately by the University of Alberta health review ethics board (Pro00069879). All recruited individuals provided written informed consent. Briefly, standard allergen extracts were prepared by study personnel and administered to patients by inhalation. A filter was positioned on the exhalation side of the valve to prevent the allergen from being nebulized or exhaled into the room. The starting concentration of allergen extract for inhalation during screening was up to four doubling concentrations below the concentrations that are predicted to cause a 20% decline in forced expiratory volume in 1 sec (FEV₁), estimated from the methacholine and allergen skin test results as described²². FEV₁ was measured 10, 20, 30, 45, 60, 90, and 120 minutes after allergen inhalation, then each hour until 7 hours after allergen inhalation.

The early asthmatic response (EAR) was defined as the largest decline in FEV₁ within 3 hours after allergen inhalation, and the late asthmatic response (LAR) as the largest decline in FEV₁

between 3 and 7 hours after allergen inhalation. For each subject, both EAR and LAR were recorded and the trapezoidal area under the curve (AUC) was determined for the early (0-3 hours) and late (3-7 hours) responses by calculating the area of the percent decline in FEV₁ versus time response.

Profiling of Peripheral Blood Mo and DC

Blood was collected in sodium heparin tubes (BD Bioscience, Mississauga, ON, Canada) and whole blood staining for flow cytometry was performed at room temperature. The following antibodies were used for cell identification: anti-CD14 (PerCy5.5, Clone 61D3; eBioscience), anti-CD16 (PE, Clone 3G8; BD Bioscience), biotinylated anti-PAR-2 (APC, SAM-11, Santa Cruz), Lin-1 (consisting of anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20 and anti-CD56) (FITC, BD Bioscience), anti-HLA-DR (PerCP Cy5.5, Clone G46-6; BD Bioscience), anti-CD11c (BV510, Clone B-ly6; BD Bioscience), anti-CD123 (BV421, Clone 6H6; eBioscience), anti-FcεR1 alpha (PE, Clone AER-37 [CRA1]; eBioscience).

Flow cytometry data were collected on a BD LSR Fortessa-II (BD Bioscience, CA, USA) using FACS Diva software and gates set in accordance with the profiles of the isotype control and/or negative control beads. Results were analyzed using FlowJo (TreeStar, Ashland OR, USA). Gating strategies to identify Mo and DC subsets are shown in supplemental Figures e1 and e2 respectively. Details of the flow cytometry protocol have been published previously²⁰.

Statistical Analysis

Values of continuous parameters are reported using median and interquartile range (IQR). Correlations between immune parameters were analyzed using Spearman's Rank correlation coefficient. Values between patients having asthma exacerbation and those with stable asthma (data presented in Table 1) were compared using two-way ANOVA and were adjusted for

current smokers. Change in immune parameters between the exacerbation and treatment visits were analyzed by non-parametric Wilcoxon rank test (data presented in Table 2).

For the allergen challenge study, the overall difference between data from 0h, 6hrs and 24hrs time points were calculated using Friedman test. Pairwise comparisons were then performed using a Nemenyi multiple comparisons between inter-individual groups (data presented in Table 4).

Statistical analyses were performed using SPSS (version 21.1, Chicago, IL, USA) and R (R Core Team, 2020).

Results

PAR-2 expression on peripheral blood IMMo during asthma exacerbations

To test whether loss of asthma control is associated with changes in PAR-2 expression on peripheral blood Mo we recruited patients presenting to the ED with an asthma exacerbation and evaluated PAR-2 expression on Mo by flow cytometry. The demographics of this population have been previously reported in detail¹⁹. Briefly, recruited subjects had a median age of 30 years (IQR: 25-40), body mass index (BMI) 29.4 (IQR: 23.8-32.2) and 50 % were female. Only 12.5% had a history of smoking >10 pack years whereas 37.5% of were current smokers. The median FEV₁ (percentages predicted) and the ratio of FEV₁/FVC were 67.2 (IQR:42.7-77.7) and 63.3 (IQR:52.6-71.6), respectively. The median ACQ score was 2.9 (IQR:2.2-4.4) whereas the median AQLQ was 4.0 (IQR:3.3-5.1).

We compared data from these patients presenting with an asthma exacerbation with data from a cohort of patients with stable asthma we recruited and reported previously¹⁹. The demographics of this stable asthma population have been previously reported in detail¹⁹. Briefly, the median age between the asthma exacerbation and stable asthma was significantly different although no

difference was observed in biological sex and median BMI. There was significantly a greater number of current smokers in asthma exacerbation compared to stable asthma. Further, patients with asthma exacerbation were more likely to have poor asthma control compared to those with stable asthma and had a lower FEV₁ (percentages predicted) and FEV₁/FVC ratio.

There was no difference in the percentages of Mo, percentages of IMMo or percentages of CMo in peripheral blood between the two populations after adjusting for current smoking (Table 1). We adjusted for smoking because the two populations had a significant difference in the numbers of current smokers included (see Table 4 of previously published paper¹⁹). However, the percentages of IMMo and CMo expressing PAR-2 was higher in patients presenting to the ED compared to those with stable asthma “(shown in Fig. 1A)”. Patients presenting to the ED were treated by the ED physician according to a standard protocol without any study-specific intervention. Of these subjects 10 returned 14 days later for repeat evaluation (treatment visit). We have published²⁰ that FEV₁ (percentages predicted) increased and ACQ6 decreased in this population between the exacerbation and treatment visit, indicating that treatment of the exacerbation improved both lung function and asthma symptoms. Comparisons of the numbers of Mo subsets and % of Mo subsets expressing PAR-2 between the two visits are shown in Table 2. The percentages of Mo in peripheral blood increased at the treatment visit compared to exacerbation visit, while percentages of IMMo expressing PAR-2 decreased “(shown in Fig. 2B)”, and percentages of CMo expressing PAR-2 showed a trend for decrease (Table 2). Changes in percentages IMMo and CMo expressing PAR-2 between the two visits did not correlate with changes seen in FEV₁ or ACQ6 (data not shown).

Peripheral blood DC during asthma exacerbations

We then evaluated DC subsets, myeloid DC (mDC, also referred as conventional DC) and plasmacytoid DC (pDC) in peripheral blood and their expression of HLA-DR and high affinity IgE receptor, also known as Fc epsilon RI (FcεRI). Our data indicate that peripheral blood DC of patients with asthma do not express PAR-2 (unpublished observation), and therefore we did not analyze PAR-2 expression on these cells. The percentages of mDC in peripheral blood, but not pDC or total DC in peripheral blood, were lower in subjects presenting with an asthma exacerbation compared to patients with stable asthma (Table 1). FcεRI expression on mDC (percentages of cells expressing FcεRI and median fluorescence intensity [MFI] of FcεRI on mDC) showed no difference between the two groups (Table 1). In contrast, the percentages of pDC expressing FcεRI was lower in patients with an asthma exacerbation, but MFI was not different (Table 1). In patients presenting to the ED, all DC populations increased during the treatment visit compared to the exacerbation visit (Table 2). There was also a trend towards increased FcεRI expression on pDC after treatment, although this difference did not reach statistical significance (Table 2). The MFI of HLA-DR expression on these cells did not change between exacerbation and treatment visits (data not shown).

PAR-2 expression on IMMo following inhalation allergen challenge

To further understand whether the changes in PAR-2 expression by Mo during an asthma exacerbation are dependent on increased airway inflammation, we used a human model characterized by acute induction of allergic airway inflammation. Eleven patients with mild asthma were recruited and underwent inhalation allergen challenge. Blood was obtained for analysis before the challenge (0h) and then again at 6 and 24hrs after the challenge. Demographics, clinical characteristics, and response to the allergen challenge for the 11 subjects included in our study are shown in Table 3. The recruited population had a median age of 23

(IQR:19-39) years, and median BMI of 23.6 (IQR: 21.8-27.9) and included 45% female subjects.

Seven of the subjects exhibited isolated EAR while 4 of them had dual response (DR).

The percentage of total Mo, IMMo or CMo in peripheral blood did not change after the challenge, but there was statistically significant increase in percentages of IMMo expressing PAR-2 after the allergen challenge, (Table 4). Post hoc analysis using Nemenyi multiple comparison test revealed statistically significant increase in IMMo expressing PAR-2 from 0h to 6hrs and 0h to 24hrs, but not from 6hrs to 24hrs “(shown in Fig. 2)”. In contrast, the percentages of CMo expressing PAR-2 did not change after allergen challenge.

Peripheral blood DC following inhalation allergen challenge

We also characterized mDC and pDC in the peripheral blood of subjects undergoing an inhalation allergen challenge. There were no differences in percentages of total DC and mDC in peripheral blood after allergen challenge and only a trend for decreased percentages of pDC at 6 hrs after allergen challenge returning to baseline by 24 hrs (Table 4). At baseline almost 88% of mDC expressed FcεRI and there was no change in this parameter, or in MFI of FcεRI expression on mDC following challenge. Approximately 85% of pDC expressed FcεRI at baseline. There was an overall difference in both pDC expressing FcεRI and MFI of FcεRI on pDC between the 3 time points using Friedman test, and also decreased MFI of FcεRI on pDC at 6hrs compared to 0hr. In addition, there was no change in the MFI of HLA-DR expression on any of the DC subsets after challenge (data not shown).

Correlation of immune parameters with allergen challenge-induced changes in FEV₁

To understand whether any immune parameters we studied are associated with the development of early bronchoconstriction after allergen challenge, we studied whether there was any correlation between the baseline immune parameters and the degree of the early drop in FEV₁

after allergen challenge. Baseline percentages of Mo, IMMo or CMo in peripheral blood and percentages of IMMo and CMo expressing PAR-2 did not correlate with the peak drop in FEV₁ during the early response or with AUC₀₋₃ (data not shown). Similarly, percentages of mDC and the expression of FcεRI by DC subsets did not correlate with these parameters (eTable 1). In contrast, percentages of DC and pDC in peripheral blood (eTable 1) and “(shown in Fig. 3)” at baseline positively correlated with the peak drop in FEV₁ during the early response, but not with AUC₀₋₃. Since only 4 subjects showed a late response, we did not analyze the correlation of immune parameters with drop in FEV₁ during the late response.

Discussion

Our previous results suggested that PAR-2 expression on Mo subsets may be a novel biomarker of asthma severity and/or asthma control^{18, 19} but provided no further insight. The studies presented here, address the relation between airway inflammation and PAR-2 expression by peripheral blood Mo subsets using two human models: airway inflammation associated with an asthma exacerbation or inflammation following an inhalation allergen challenge. The results suggest that airway inflammation is associated with changes in numbers and function of peripheral blood myeloid cells. We first showed that the percentages of IMMo and CMo expressing PAR-2 on their cell surface were higher in subjects experiencing an asthma exacerbation compared to patients with stable asthma. Following treatment of the exacerbation the percentages of IMMo expressing PAR-2 decreased significantly, while the percentages of CMo expressing PAR-2 showed only a trend for decrease. We also showed that percentages of IMMo expressing PAR-2 increase following inhalation allergen challenge.

We know little about the regulation of PAR-2 expression on Mo. A single publication suggests that freshly isolated Mo have low levels of PAR-2 surface expression that increases from

intracellular stores following mechanical stress and/or with differentiation towards DC or macrophages²³. However, there is no other information on the regulation of PAR-2 expression to explain our observation of increased PAR-2 expression on Mo subsets during asthma exacerbations or at times of increased airway inflammation. We hypothesize that a number of inflammatory mediators generated during allergic inflammation may be the cause of the upregulation. This is supported by the observation that PAR-2 expression on Mo also increases in various other inflammatory conditions²⁶⁻²⁸. The exact mediators, however, are not known at this point. It is interesting that IMMo from patients with chronic obstructive pulmonary disease show higher CCR5 expression compared to cells from healthy controls³⁰ and this increase is dependent on IL-6. We do not know whether the same CCR5 changes are present in patients with asthma, but IL-6 levels are increased in the serum of patients with asthma³¹, and correlate with frequency of exacerbations³². IMMo also exhibit selective surface expression of certain cytokine and chemokine receptors²⁹, and one or more of those may render IMMo more sensitive to allergic inflammatory signals. In particular, CCR5, a receptor for CCL5, is preferentially on IMMo and may mediate increased PAR-2 expression. In our two cohorts we did not see any correlation between CCL5 levels and PAR-2 expression on IMMo (data not shown). However, in a population of asthmatics of varying severity (n=36) (we have reported other results from that population¹⁸) we saw that serum CCL5 levels correlated with IMMo expressing PAR-2 (unpublished data). The lack of correlation in our current study may be due to low sample size of both cohorts.

Our observation that PAR-2 expression was increased on IMMo and CMo in patients with an asthma exacerbation compared to those with an independent cohort of stable asthma, but increased only on IMMo following allergen challenge, indicates differential regulation of PAR-2

expression by these two cell subsets. It is possible that the systemic component of allergic inflammation is able to upregulate PAR-2 expression on IMMo, but that different signals or a combination of signals present only during an asthma exacerbation are required to increase PAR-2 expression on CMo.

Our understanding of the physiological or pathophysiological role of PAR-2 surface expression on Mo or Mo subsets is also limited. PAR-2-mediated activation of peripheral blood Mo leads to release of inflammatory mediators, such as IL-6, IL-8 and IL-1 β ²³. PAR-2-mediated Mo activation may also have protective effects against bacterial and viral infections, by increasing phagocytosis²⁴, and enhancing IFN-activated pathways²⁵. Whether the changes in PAR-2 expression in peripheral blood Mo in subjects with increased airway inflammation, have significant effects on Mo function is not understood. In mice, PAR-2 expression is required for DC development *in vitro* and *in vivo*³³. If the same is true in humans, Mo differentiation to DC after recruitment to peripheral tissues, such as the lung, may be PAR-2-dependent. This may suggest that the increased PAR-2 expression we observed during asthma exacerbations or after an inhalation allergen challenge could support increased DC development in peripheral tissues. More detailed, *in vitro* studies with isolated Mo will allow us to determine the role of PAR-2 expression on Mo in asthma, but also in other inflammatory diseases.

DCs are pivotal in regulating allergic airway inflammation³⁴. For these reasons we also studied the presence and activation status of mDC and pDC subsets. We showed that mDC numbers, but not pDCs numbers, in peripheral blood are lower in subjects experiencing an asthma exacerbation compared to patients with stable asthma and these mDC numbers increase after treatment of the exacerbation. In contrast there was a trend of decrease of pDC, but not mDC, 6 hrs after an inhalation allergen challenge and the numbers returned to baseline by 24 hrs.

There is little information on changes in DC numbers and activation status in patients with asthma and comes almost exclusively from allergen challenge studies. Numbers of pDC and mDC increase in the sputum of subjects with mild asthma after an inhalation allergen challenge^{35, 36}, but the situation for peripheral blood DC is not clear; one study showed no changes³⁶, while two other studies showed decreased numbers^{37, 38}. The lack of a significant effect in our challenge study may be the result of having studied a slightly different population, since in our study, the majority of subjects exhibited isolated early responses, while other studies included only patients with dual responses. Decreased pDC numbers after allergen challenge may be the result of cells migrating to the airways, since pDC are increased in induced sputum during asthma exacerbations³⁹, and in mice they drive the development of airway inflammation in allergic models³⁹. Although we do not have direct data, decreased pDC numbers in subjects with an asthma exacerbation may indicate a viral cause for the exacerbations, as has also been shown in children during RSV-induced asthma exacerbations⁴⁰. However, we have published that our subjects with an asthma exacerbation also had lower peripheral blood lymphocyte numbers¹⁹, something that can be seen during viral infections^{41,42}.

DC also express Fc Epsilon Receptor I (FcεRI) in humans³⁴. FcεRI expression increases in patients with asthma^{34, 43} and FcεRI-positive DC increase within the airway epithelium of subjects with Th2 high asthma⁴⁴. We observed high level of FcεRI expression on the surface of DCs in all patients in both models. We saw a decrease of FcεRI surface expression on pDC during the exacerbation visit and also an early decrease of FcεRI surface expression on these cells following inhalation challenge. The reason for this decrease is not clear. TLR ligation can decrease FcεRI expression on human pDC⁴⁵, but we do not know if pattern recognition receptor ligation is the cause in our case. It is also possible that pDC expressing high levels of FcεRI are

preferentially recruited to the lungs during episodes of increased airway inflammation leading to decreased presence of these cells in the periphery. Further studies will be needed to clear this question.

It is interesting that in our study the percentages of DC and pDC in peripheral blood at baseline correlated with the degree of early bronchoconstriction after allergen challenge. The role of pDC in asthma is not very well understood. Studies in mouse models indicate that they may have a dual role, since they may prevent the development of allergic sensitization⁴⁶, but in the case of established disease are recruited to the lung and drive Th2 inflammation³⁹. The presence of higher numbers of pDC in the blood followed by recruitment of pDC to the lungs/airways after allergen challenge and the subsequent pDC-induced airway inflammation may be the link between the abundance of pDC in peripheral blood and the degree of bronchoconstriction after allergen challenge, we show in our study. Larger studies to validate this observation would be required to better understand the role of pDC in asthma.

Our study has several limitations that need to be considered. First, both components of the current study have small sample sizes, which may limit our ability to identify small differences in cell numbers and/or cell surface markers. We did not measure monocyte specific cytokines such as TNF, IL-10 and IL-6, partly because our sample size is not big enough to achieve adequate power. As for our allergen challenge study we included both patients with isolated EAR and patients with dual responses. This again may have limited our ability to identify changes that would be restricted to one of these two groups. Finally, since we conducted a number of statistical comparisons, there is a possibility that certain of the significant differences we identified may be a chance occurrence due to the multiple tests we conducted.

Notwithstanding these concerns, this study provides first in human evidence indicating that allergic airway inflammation and possibly also other systemic changes during asthma exacerbations affect the characteristics of peripheral blood Mo. *In vitro* studies to elucidate the mechanisms of PAR-2 regulation on Mo and individual Mo subsets and describe the effects of PAR-2-mediated Mo activation will allow us to understand the biological significance of increased PAR-2 expression on Mo during allergic inflammation. Further, a comparison between changes in monocyte populations in the blood and induced sputum following an allergen challenge will provide greater insight into the processes we described here.

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Table 1. Characteristics of Mo and DC subsets between patients presenting with an asthma exacerbation and stable asthma

Parameters	Exacerbation (N=16)	Stable (N=18)	<i>p</i>
Mo subsets			
% of Mo in PB	4.68 (4.05-11.04)	7.4 (5.25-10.5)	0.728
% of IMMo in PB	1.13 (0.47-1.39)	1.11 (0.48-2.21)	0.542
% of IMMo expressing PAR-2	15.45 (10.09-26.82)	10.35 (4.25-16.4)	0.023
% of CMo in PB	0.81 (0.63-1.34)	0.85 (0.33-1.37)	0.324
% of CMo expressing PAR-2	6.36 (3.50-12.5)	3.07 (1.48-4.67)	0.016
DC subsets			
% of DC in PB	0.16 (0.10-0.32)	0.47 (0.26-0.87)	0.088
% of mDC in PB	0.08 (0.03-0.21)	0.24 (0.12-0.63)	0.019
% of pDC in PB	0.05 (0.01-0.17)	0.08 (0.05-0.12)	0.247
% of mDC expressing FcεRI	65.1 (50.0-75.0)	84.2 (37.7-90.9)	0.162
% of pDC expressing FcεRI	63.3 (36.7-80.5)	78.7 (74.3-89.8)	0.009
MFI of FcεRI on mDC	39.5 (9.0-243.2)	48.6 (3.7-115.8)	0.303
MFI of FcεRI on pDC	20.9 (12.7-57.3)	17.6 (8.6-55.3)	0.311

CMo, classical monocytes; DC, Dendritic cells; FcεRI, Fc Epsilon Receptor I; IMMo, intermediate monocytes; PB, Peripheral blood; mDC, myeloid dendritic cells; Mo, monocytes; pDC, plasmacytoid dendritic cells

Data are presented as median (interquartile range)

Values between patients having acute exacerbation and those with stable asthma were compared using two-way ANOVA and were adjusted for current smokers.

Table 2. Characteristics of Mo and DC subsets between the exacerbation and treatment visits for patients recruited during an asthma exacerbation

Parameters	Exacerbation	Treatment	<i>p</i>
Mo subsets			
% of Mo in PB	4.61 (4.10-7.47)	8.79 (6.11-10.31)	0.028
% of IMMo in PB	0.88 (0.40-1.33)	1.12 (0.40-2.52)	0.139
% of IMMo expressing PAR-2	15.45 (10.43-35.35)	8.52 (5.03-13.22)	0.013
% of CMo in PB	0.77 (0.58-1.17)	1.43 (0.86-2.24)	0.114
% of CMo expressing PAR-2	7.29 (2.84-22.95)	3.0 (1.87-6.98)	0.074
DC subsets			
% of DC in PB	0.16 (0.10-0.31)	0.35 (0.19-0.46)	0.012
% of mDC in PB	0.07 (0.03-0.17)	0.25 (0.07-0.32)	0.014
% of pDC in PB	0.04 (0.01-0.08)	0.10 (0.02-0.19)	0.041
% of mDC expressing FcεRI	57.1 (38.8-72.1)	90.1 (41.4-92.7)	0.314
% of pDC expressing FcεRI	69.1 (43.6-86.8)	81.6 (70.2-100)	0.123
MFI of FcεRI on mDC	12.2 (6.3-115.8)	53.4 (8.8-380.6)	0.575
MFI of FcεRI on pDC	19.2 (10.3-66.1)	51.0 (19.2-100.8)	0.333

CMo, classical monocytes; DC, Dendritic cells; FcεRI, Fc Epsilon Receptor I; IMMo, intermediate monocytes; PB, Peripheral blood; mDC, myeloid dendritic cells; Mo, monocytes; pDC, plasmacytoid dendritic cells

Data are presented as median (interquartile range).

Change in immune parameters between the exacerbation and treatment visits were analyzed by non-parametric Wilcoxon rank test.

Table 3. Demographics and clinical characteristics of subjects who underwent allergen challenge

No	Age	Sex	BMI (kg/m ²)	Allergen	Response	FEV ₁ (%) predicted	FEV ₁ / FVC	EAR (% peak drop in FEV ₁)	LAR (% peak drop in FEV ₁)
1	50	F	20.20	Cat	DR	80	63	20.6	31.6
2	19	F	23.60	Birch	DR	98	98	39.4	25.6
3	39	M	27.90	Cat	DR	84	55	31	25
4	37	F	28.00	Cat	DR	96	81	26	43
5	24	M	23.60	Birch	EAR	96	81	19.61	NA
6	21	F	20.80	HDM	EAR	107	82	36.4	5.16
7	18	M	25.70	Birch	EAR	104	85	38	11
8	21	M	21.80	Birch	EAR	111	84	35	5.4
9	19	M	23.00	HDM	EAR	97	73	45.8	11.9
10	63	M	26.30	Birch	EAR	93	119	40	8.7
11	23	F	30.50	Birch	EAR	80	68	29	10.4

BMI, Body mass index; DR, Dual response; EAR, Early asthmatic response; F, Female; FEV₁, Forced expiratory volume in 1 sec; FVC, Forced vital capacity; HDM, House Dust Mite; LAR, Late asthmatic response; M, Male

Table 4. Characteristics of Mo and DC before and after inhalation allergen challenge

Parameters	0hr	6hrs	24hrs
Mo subsets			
% of Mo in PB	7.55 (5.41-11.29)	5.37 (3.37-12.07)	6.57 (4.71-12.34)
% of IMMo in PB	0.91 (0.34-2.35)	0.48 (0.27-1.65)	0.72 (0.33-2.15)
#% of IMMo expressing PAR-2	6.15 (3.78-13.3)	10.6 (4.85-16.4)	15.4 (9.61-26.71)
% of CMo in PB	1.35 (0.57-2.0)	0.79 (0.22-1.88)	0.69 (0.49-2.47)
% of CMo expressing PAR-2	5.65 (0.81-12.5)	2.86 (0.00-10.70)	4.49 (0.69-11.1)
DC subsets			
% of DC in PB	0.49 (0.41-0.80)	0.45 (0.34-0.78)	0.68 (0.41-0.82)
% of mDC in PB	0.19 (0.14-0.26)	0.19 (0.12-0.30)	0.24 (0.16-0.28)
&% of pDC in PB	0.22 (0.09-0.32)	0.10 (0.05-0.18)	0.20 (0.14-0.39)
% of mDC expressing FcεRI	88.2 (83.0-92.1)	87.2 (72.9-93.0)	86.7 (77.1-91.9)
*% of pDC expressing FcεRI	85.2 (69.7-90.9)	80.7 (48.4-90.0)	84.0 (68.4-93.5)
MFI of FcεRI on mDC	209 (82-352)	90.4 (65.1-147.0)	115.7 (72.6-198.4)
**MFI of FcεRI on pDC	65.3 (18.7-85.1)	26.6 (10.7-49.3)	27.4 (16.4-58.8)

CMo, classical monocytes; DC, Dendritic cells; FcεRI, Fc Epsilon Receptor I; IMMo, intermediate monocytes; PB, Peripheral blood; mDC, myeloid dendritic cells; Mo, monocytes; pDC, plasmacytoid dendritic cells

Data are presented as median (interquartile range)

Overall differences at three-time point was calculated by Friedman test

#Overall $p=0.003$, For inter individual comparison between 0hr vs 6hrs vs 24hrs see figure 2.

&Overall $p=0.052$

*Overall $p=0.043$

**Overall $p=0.017$, 0 vs 6hrs, $p=0.021$

Figure Legends

Figure 1. A, % of IMMo cells expressing PAR-2 in patients with stable asthma (stable, n=18) and patients with an asthma exacerbation (exacerbation, n=16). B, % of IMMo cells expressing PAR-2 in patients with an asthma exacerbation during the first (exacerbation, n=10) and the second (treatment, n=10) visits, where line (—) indicates median value in each group.

Figure 2. % of IMMo expressing PAR-2 in patients before (0 h) and 6 hrs and 24 hrs after inhalation allergen challenge, (n=11), where line (—) indicates median value in each group.

Figure 3. A, Correlation between peak drop in FEV₁ during the EAR and baseline (0 h) % DC B, % pDC in peripheral blood of patients undergoing inhalation allergen challenge, (n=11).

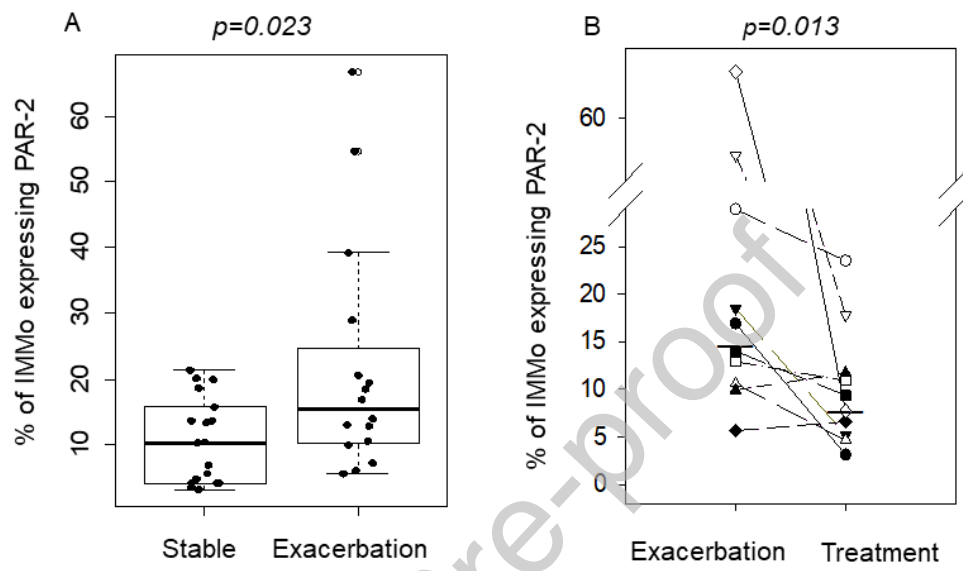


Figure 1

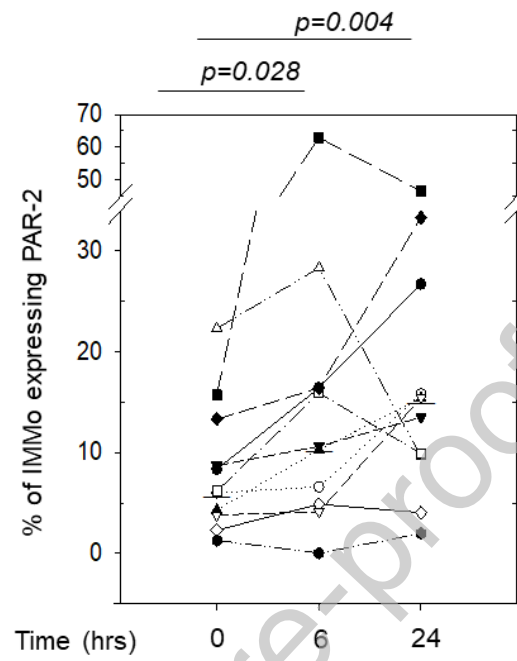


Figure 2

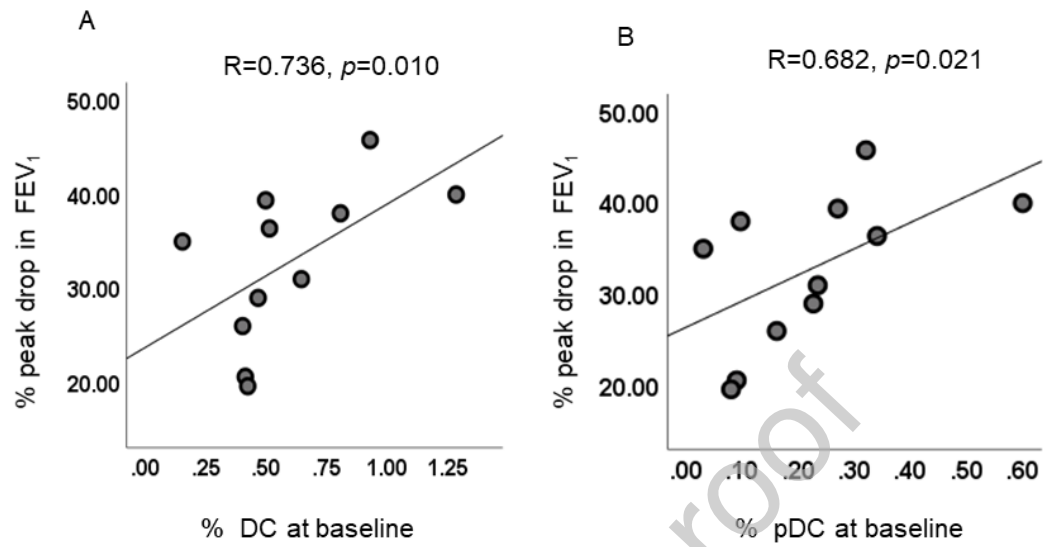
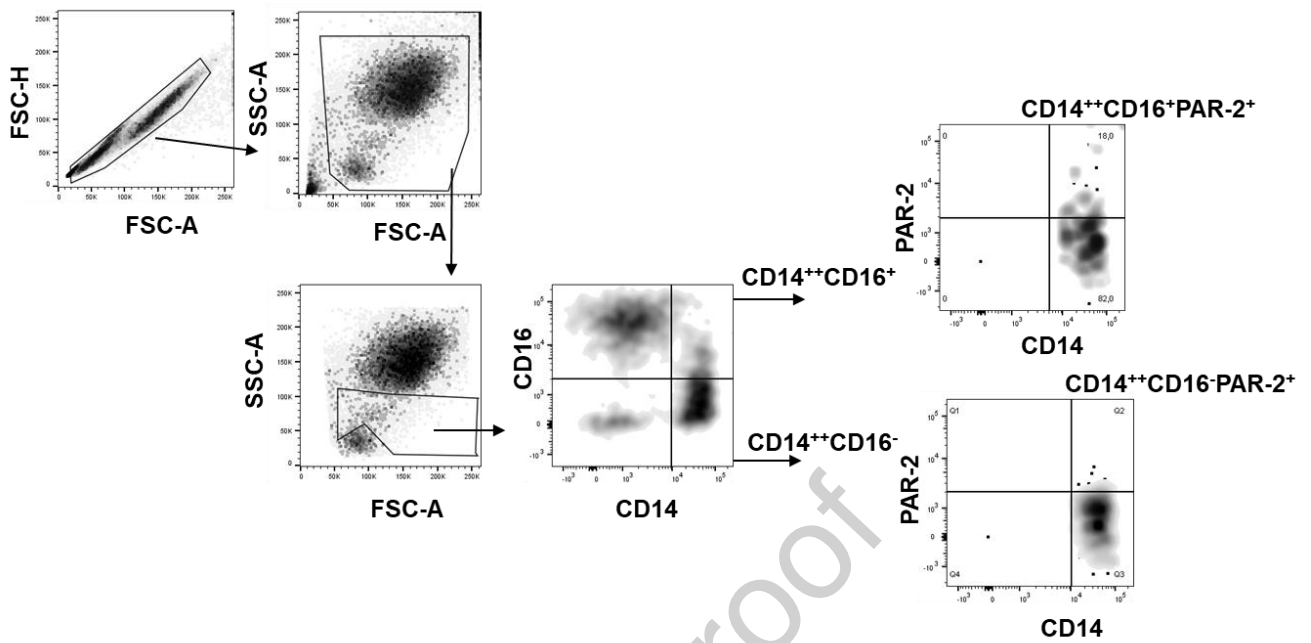


Figure 3

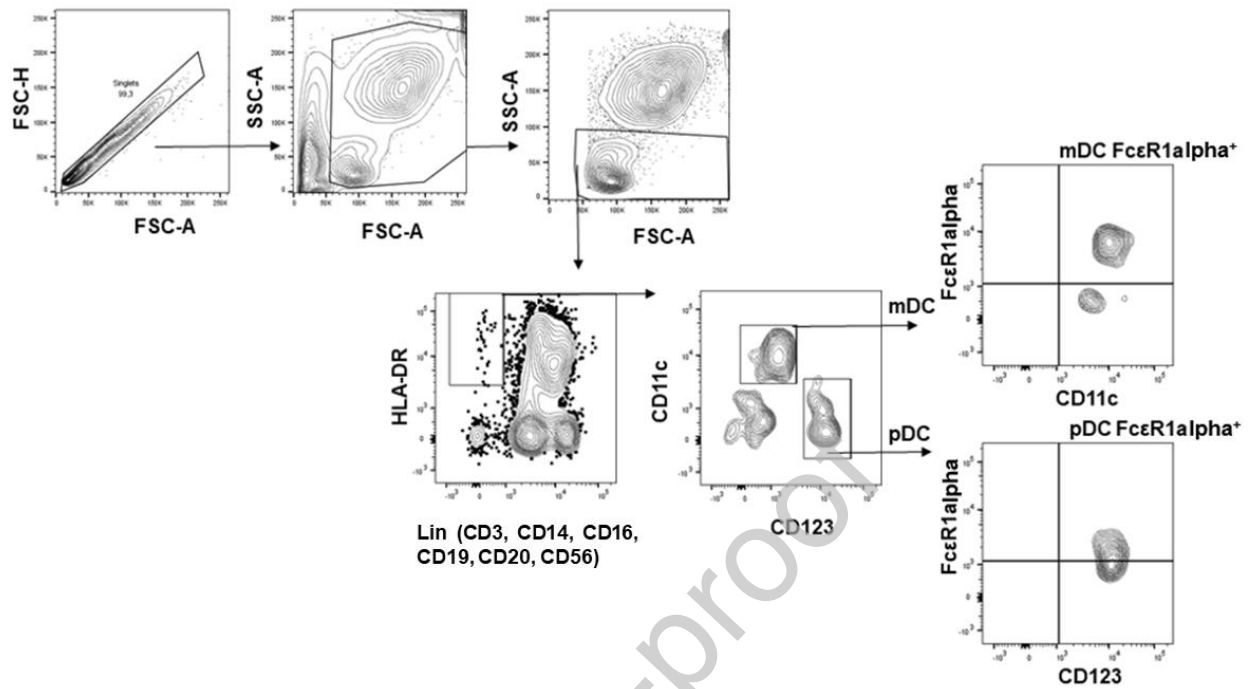
eTable 1. Correlation between baseline immune parameters and EAR following inhalation allergen challenge

Parameters	% peak drop in FEV ₁	AUC _{0-3hr}
DC subsets		
% of DC in PB	R= 0.736 <i>p</i> = 0.010	R= 0.282 <i>p</i> = 0.401
% of mDC in PB	R= 0.082 <i>p</i> = 0.811	R= -0.191 <i>p</i> = 0.574
% of pDC in PB	R= 0.682 <i>p</i> = 0.021	R= 0.191 <i>p</i> = 0.574
% of mDC expressing FcεRI	R= 0.545 <i>p</i> = 0.083	R= -0.400 <i>p</i> = 0.223
% of pDC expressing FcεRI	R= 0.000 <i>p</i> = 1.000	R= 0.041 <i>p</i> = 0.905
MFI of FcεRI on mDC	R= 0.118 <i>p</i> = 0.729	R= 0.200 <i>p</i> = 0.555
MFI of FcεRI on pDC	R= 0.018 <i>p</i> = 0.958	R= 0.009 <i>p</i> = 0.979

DC, Dendritic cells; FcεRI, Fc Epsilon Receptor I; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; Correlations between parameters were analyzed using Spearman's Rank correlation coefficient.



eFigure 1. Gating Strategy for flow cytometry analysis of monocyte subsets



eFigure 2. Gating Strategy for flow cytometry analysis of dendritic cell subsets