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Pulmonary Allergic Responses Augment IL-13 Secretion by Circulating Basophils yet Suppress IFN- α from Plasmacytoid DCs

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Abstract

Background—Allergic inflammatory processes may have the capacity to propagate systemically through the actions of circulating leukocytes. Consequently, basophils from allergic individuals are often “primed”, as evidenced by their hyper-responsiveness *in vitro*. IFN- α , secreted predominately by plasmacytoid DCs, suppresses basophil priming for IL-13 production *in vitro*.

Objective—This study sought *in vivo* correlates, arising during experimental allergen challenge, that support an “axis-interplay” between basophils and pDCs.

Methods—Using segmental allergen challenge in the lung, the immune responses of both cell types from blood were investigated in volunteers (n=10) before and 24h after allergen exposure. These responses were then correlated with inflammatory parameters measured in bronchoalveolar lavage fluids.

Results—In blood, segmental allergen challenge significantly augmented IL-13 secretion by basophils induced by IL-3 (p=0.009) yet reduced IFN- α secreted by plasmacytoid dendritic cells stimulated with CpG (p=0.018). Both parameters were negatively correlated (p=0.0015), at least among those subjects secreting the latter. Circulating basophil IL-13 responses further correlated with post-segmental allergen challenge bronchoalveolar lavage parameters including IL-13 protein (p=0.04), basophil (p=0.051), eosinophil (p=0.0018) and total cell counts (p<0.003). Basophil and IL-13 levels in bronchoalveolar lavage likewise correlated (p=0.0002).

Conclusions—These results support a mechanism of immune regulation whereby allergen reduces innate immune responses and IFN- α production by plasmacytoid dendritic cells, resulting in enhanced inflammation and basophil cytokine production at sites of allergen exposure.

Keywords

allergen challenge; basophils; dendritic cells; cytokine; Toll-like receptor; lung; innate immunity; inflammation

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Introduction

It has become increasingly evident that respiratory allergic diseases manifest not only at the local level but also on a systemic level, upon allergen exposure. It is this latter response that is also thought to intensify the overall disease process. Nowhere is this more apparent than in the so-called “integrated airway hypothesis”, which suggests that allergic rhinitis and asthma are manifestations of one syndrome [1]. Sensorineural and physiological elements likely contribute to the mechanisms underlying this pathophysiology. However, there is mounting evidence that localized inflammatory processes are equally as important due to their capacity to “propagate” in the circulation thereby further intensifying local allergic responses. The exact cellular and/or humoral components that are involved in this paradigm remain unclear.

There has been substantial evidence that circulating basophils from allergic individuals show a “primed” phenotype, as indicated by their hyper-responsiveness *in vitro* to diverse stimuli. Support for this priming has been observed in our studies showing greater IL-13 secretion in response to IL-3 stimulation [2], and more recently in our report that IL-13 was spontaneously secreted by blood basophils of subjects undergoing repeated nasal allergen challenge [3]. In contrast, Type I IFNs (e.g. IFN- α/β), which are secreted predominately by plasmacytoid DCs (pDC) are the only cytokines reported to suppress this basophil response [4]. Moreover, we have described a mechanism whereby cross-linking the IgE receptor (Fc ϵ RI) α_2 variant found on pDC reduces their capacity to secrete IFN- α [5, 6]. A synthesis of these latter findings raises the possibility that allergen-dependent activation of pDC reduces specific innate immune responses in these dendritic cells that normally help to limit basophil priming.

In this study, we provide additional evidence that localized allergen exposure resulting from a segmental allergen challenge model in the lung induces systemic effects that augment IL-13 secretion by circulating basophils. Concurrently, we also monitor innate immune responses of pDCs to DNA containing unmethylated CpG motifs (CpG-DNA) and show that these cells have a reduced capacity to secrete IFN- α following experimental challenge. Overall, the findings further support the belief that systemic responses can arise from localized allergen exposure by propagating through circulating immune cells. Moreover, allergen exposure suppresses specific innate immune responses that would normally limit these priming events, resulting in an enhanced response at the site of exposure to allergen.

Material and Methods

Subjects

The study was reviewed and approved by the Institutional Review Board of the Johns Hopkins Medical Institutions; all subjects participating had given informed consent. Participants (n=10) included atopic allergic subjects with some being classified as mildly asthmatic, as determined by clinical history, pulmonary function testing, and bronchial provocation with methacholine using previously established criteria. All subjects had positive skin-test reactions to either ragweed or dust mite.

Segmental Allergen Challenge (SAC) and Bronchoalveolar lavage (BAL)

Bronchoscopy was performed as described according to National Institutes of Health recommended guidelines. Off-label use of ragweed and dust mite allergen for SAC was covered under Investigational New Drug (IND) number 11311 from the Food and Drug Administration. Subjects were pre-medicated with 0.6 mg intravenous atropine and 0.1-0.2 mg fentanyl. After inhalation of nebulized 4% lidocaine, a fiberoptic bronchoscope was inserted into the lower airways, supplementing local anesthesia with 2% lidocaine. At the

initial bronchoscopy, BAL was performed in one airway segment; challenge was performed with 5 ml normal saline in a second airway segment; and challenge with allergen performed in a third airway segment in the opposite lung. After 24h, BAL was performed in airway segments challenged with normal saline and allergen. BAL was standardized using 5-20ml aliquots of pre-warmed (37°C) normal saline with immediate aspiration and low suction following instillation of each aliquot.

SAC was conducted with either ragweed (*Ambrosia artemisiifolia*) or dust mite (*Dermatophagoides pteronyssinus*) (Greer Laboratories, Lenoir, NC) by instilling doses of the allergen in 5 ml of normal saline solution, depending on skin test sensitivity determined by intradermal skin test titration. Subjects received a test dose of instilled allergen that was 10x higher the allergen concentration producing a 2⁺ skin reaction (i.e. wheal of 5-10mm, erythema of 20-30 mm). If no airway reaction was observed after 5 minutes, then a challenge dose was instilled that was 100x the allergen concentration of the skin-test endpoint. Recovered BAL fluids were pooled for each airway segment with aliquots removed for cell counts and cytopsin slides (Shandon II, Pittsburgh, PA). The remaining BAL fluids were centrifuged at 150xg for 10 minutes, with supernatants kept for cytokine and mediator content. Cell pellets were resuspended in buffer to assess both the total cell count and the number of cells staining with Alcian blue (i.e. basophils). To assess the percentages of the major leukocytes, differential cell counts were also made by counting 500 cells on the cytopsin preparations stained using Diff-Quik (Harleco, Kansas City, MO). BAL basophil frequencies were determined as previously described [7] and involved total cell counts averaging 9094±3184 (mean±SD, range=2457-13,575).

Cell purification and culture

Venipuncture was performed immediately before SAC and once again 24h later, or immediately before the BAL was performed. Basophil-enriched cell (BEC) suspensions were immediately prepared using density gradient centrifugation, as previously described [5]. This involved first the preparation of buffy-coat suspensions generated from the centrifugation (300xg) of blood anti-coagulated with EDTA (10 mM). These were then diluted with an equal volume of Pipes/Albumin/Glucose (PAG)-EDTA buffer, layered onto double-Percoll (55%/61%, d = ~1.075/1.091 g/ml) gradients that were then centrifuged at 700xg for 20 minutes at room temperature (~23°C). This centrifugation resulted in both a basophil-depleted cell (BDC) interface that was recovered from atop the 55% interface and a basophil-enriched cell (BEC) interface recovered from the 61% Percoll. BEC suspensions were washed twice with PAG-EDTA and once with PAG before staining with Alcian blue, which facilitated basophil and total cell counts using a Spiers-Levy hemocytometer.

The BDC suspensions that floated on the lesser (55%) Percoll interface contained the majority of the mononuclear cells (~70 to 80%) recovered between the 2 interfaces and from which pDCs were prepared. This first involved washing away platelets by performing 4 low-speed centrifugations (each 100xg, 10 min.) all done using PAG-EDTA buffer. PDCs were then isolated using BDCA-4 selection (Miltenyi Biotec). Cells recovered from LS columns (Miltenyi Biotec) were counted using a Spiers-Levy chamber.

Inflammatory cells recovered during BAL were subjected to density centrifugation on Ficoll to remove high-density cells (e.g. eosinophils, neutrophils). Cells recovered from the Ficoll interface then underwent basophil isolation as described for isolating these cells from blood. Purity was determined using Alcian blue staining.

All cultures were performed in conditioned Iscove's Modified Dulbecco's Medium (IMDM) or C-IMDM (IMDM supplemented with 5% heat-inactivated FCS, 1x non-essential amino acids, 10 µg/ml gentamicin, pH 7.2 -7.4). Basophil responses before and after SAC were

analyzed using BEC suspensions cultured using protocols previously described in detail [3]. Importantly, the same numbers of basophils/cultures were used pre and post SAC and ranged between $0.5\text{--}1.0 \times 10^5$ basophils depending on the subject. In brief, cells were added to microtiter wells (96-well flat-bottom plates, Costar, Corning, NY) in volumes of 0.125 ml. After equilibrating to 37°C, 5% CO₂ for 15 min., an equal volume of 2x stimulus (also pre-equilibrated) was added giving a final culture volume of 0.250 ml. Supernatants harvested after 3h were assayed for histamine and IL-4, while those harvested after 18h were assayed for IL-13. Automated fluorimetry was used in determining histamine release. IL-4 and IL-13 protein levels were determined by ELISA (eBioscience, San Diego, CA). Stimuli included recombinant IL-3 (Biosource), goat polyclonal anti-IgE (in-house) [3], and peptidoglycan (Fluka).

Functional studies investigating IFN- α secretion by pDCs were done using $2.0\text{--}5.0 \times 10^4$ cells cultured in microtiter plate wells (96-well flat-bottom plates). Cells were added in 0.125 ml volumes, pre-equilibrated as done for the BEC suspensions above, and then stimulated by adding an equal volume of 2x CpG oligodeoxynucleotide (ODN-2216, Invitrogen). Supernatants were harvested after 24h and 72h and analyzed for IFN- α protein, as previously described [5].

Statistics

Statistical analysis was performed with Prism4 software (San Diego, CA), using non-parametric tests unless otherwise stated. P values less than 0.05 were considered significant.

Results

Late phase responses (LPR) in the lung of subjects undergoing SAC

Subject profiles, doses of allergen used for SAC, and BAL cell numbers recovered in both the allergen- and saline- challenged sites of the lung are summarized in Table 1. A total of 10 subjects with a mean age of 31 ± 3 were evaluated: 5 were classified as allergic asthmatic and 5 as allergic rhinitic without asthma, all having positive skin tests to either ragweed or dust mite used for SAC. As expected, an increase in the total number of BAL cells recovered from the allergen- ($86.5 \pm 24.7 \times 10^6$) compared to the saline -challenged site ($22.7 \pm 3.5 \times 10^6$) was highly significant ($p < 0.007$) and was evident in all but one subject (#8). Likewise, a significant ($p < 0.006$) increase in the percentage of eosinophils among the total cells recovered was also observed between these challenge sites ($35 \pm 7.3\%$ vs. $1.6 \pm 0.9\%$, respectively). Total BAL cell numbers recovered in the allergen-challenged site ranged from 11.9 to 270×10^6 cells, with the percentage of eosinophils also having a wide range (1.4 to 62.8%). Despite these varied cellular infiltrations, no significant differences were observed between the asthmatic and rhinitic subjects.

Circulating basophil numbers recovered pre- vs. post- SAC

No significant differences were observed in the percentage of basophils among the basophil-enriched cell (BEC) suspensions prepared on the Percoll gradients pre and post SAC ($6.5 \pm 0.5\%$ vs. $6.5 \pm 0.5\%$, respectively). Likewise, the total cell numbers recovered in the basophil-depleted cell (BDC) suspensions did not significantly change after SAC. Finally, there was no evidence for increased numbers of hypodense basophils in the BDC suspensions recovered from the 55% Percoll following SAC. Estimations based on the few numbers of Alcian blue positive cells that were seen indicated fewer than 0.1% basophils, which did not differ significantly from pre-SAC counts (data not shown).

Effects of SAC on circulating basophil responses

In a previous report we observed that blood basophil suspensions spontaneously secreted IL-13 following a protocol of repeated nasal allergen challenge [3]. We therefore investigated whether a single challenge in the lower airways would produce similar responses. Fig 1 shows the median and interquartile range (IQR) of IL-13 secreted by BEC suspensions before and after SAC. Unlike our previous findings using the repeated allergen challenge in the upper airways, we did not observe consistent evidence that IL-13 was spontaneously secreted post- vs. pre- challenge. However, marked increases in IL-13 were detected in post-challenge BEC suspensions cultured 18h in both low (1 ng/ml) and high (10 ng/ml) IL-3 – a response that is specific for basophils among mixed leukocyte suspensions [8]. In particular, the median level of IL-13 secreted with 1 ng/ml IL-3 was 368 pg/10⁶ basophils (IQR=264-490 pg/10⁶ basophils) pre-SAC, which increased significantly to 635 pg/10⁶ basophils (IQR= 336-1073 pg/10⁶ basophils) in post-SAC cultures (p=0.009). As expected, greater IL-13 levels were also detected using the 10 ng/ml IL-3 concentration, with the pre-SAC median value being 488 pg/10⁶ basophils (IQR=378-840 pg/10⁶ basophils) vs. 1050 pg/10⁶ basophils (IQR=430-1404 pg/10⁶ basophils) post-SAC (p=0.013). In contrast, there were no significant differences in the IL-13 secreted by BEC suspensions pre vs. post SAC when stimulating with peptidoglycan, a TLR2 agonist (median=147 pg/10⁶ [IQR=70-374 pg/10⁶ basophils] vs. 168 pg/10⁶ basophils [IQR=115-294 pg/10⁶ basophils], p=0.72).

Histamine and IL-4 responses to IgE-dependent activation were also monitored in blood BEC suspensions before and after SAC in the lung. As shown in Fig 2, there was no evidence that SAC induced a shift in the anti-IgE dose-response curves for either histamine or IL-4. In fact, percent histamine release followed a predictable dose response to this stimulus, with minimal, intermediate, and maximum release seen at 1, 10, and 100 ng/ml anti-IgE. More importantly, these histamine responses were not significantly increased in the BEC suspensions prepared post- vs. pre- SAC. The data actually indicated that slightly less histamine was released in post-SAC BEC suspensions challenged with 10 ng/ml anti-IgE concentration, but this finding did not reach statistical significance (p=0.08). The levels of IL-4 secreted in these same culture supernatants were also unaltered in post-SAC BEC specimens. This was not only true for cultures stimulated with the 10 ng/ml dose of anti-IgE which induced the highest levels of IL-4, but was also observed at both the sub- (1 ng/ml) and super- (100 ng/ml) optimal concentrations.

Basophil IL-13 responses post-SAC correlate with LPR lung eosinophilia

The intensity of late phase responses to allergen challenge, whether they are in the skin, nose, or lung is often measured by the influx of cells, particularly eosinophils. As a result, we sought a measure of the basophil IL-13 response post-SAC by investigating its relationship to the percentage of eosinophils recovered in the BAL. As shown in Fig 3, simple regression analyses showed that blood basophil IL-13 responses significantly correlated with total BAL cell counts ($r^2 = 0.654$, $p < 0.003$), with a greater correlation to the percentage of eosinophils among the cells entering the lung ($r^2 = 0.875$, $p < 0.0001$). This latter relationship was also significant when delta values were plotted (i.e. post-SAC – pre SAC blood basophil IL-13 vs. BAL cells in allergen – saline challenged sites). The estimated number of eosinophils infiltrating the lung and recovered in the BAL also correlated with basophil IL-13 induced by IL-3 at either the 1 ng/ml ($r^2 = 0.723$, $p = 0.0018$) or 10 ng/ml ($r^2 = 0.37$, $p = 0.0628$) concentrations (data not shown). There were no significant correlations observed between basophil IL-13 secretion and any other cell type (i.e. macrophages, lymphocytes, or neutrophils) infiltrating the lung during LPRs (data not shown).

Effects of SAC on IFN- α responses by circulating pDCs

We have previously shown that IFN- α inhibits IL-13 secretion by basophils stimulated with IL-3 [4]. In contrast, the findings in Fig 1 indicate that SAC augments this IL-13 response in basophils. Therefore, we tested whether SAC affects the capacity of circulating pDC to secrete IFN- α , hypothesizing that it would reduce production of this Type I IFN. As shown in Fig 4, pDCs isolated from 7 of the 10 subjects before undergoing SAC produced IFN- α in response to CpG after a 3d culture that included IL-3 (10 ng/ml) to augment the CpG effect. As expected, the levels of IFN- α secreted varied among subjects with a median level of 794 pg/10⁶ pDC (IQR= <15-3207 pg/10⁶ pDC). However, pDCs isolated from the blood of these subjects post-SAC showed a significant ($p=0.018$) reduction in the IFN- α response to CpG, with a median level of 164 pg/10⁶ pDC (IQR= <15 to 1648 pg/10⁶ pDC).

We performed an analysis to determine whether a potential relationship exists between pDC IFN- α and basophil IL-13 secretion. Using pre- and post- SAC values for the first 5 subjects (i.e. 10 comparisons in all), a logarithmic regression analysis of the cytokine levels secreted by these cells *in vitro* produced an adjusted r^2 value of 0.704 ($p=0.0015$). However, this correlation did not remain significant for values obtained from all 10 subjects, since pDCs from 3 individuals did not produce measurable IFN- α in response to CpG.

Basophils selectively recruited to the lung during allergic inflammation secrete IL-13

Basophils are among the leukocytes selectively infiltrating the lung during late phase responses arising from experimental allergen challenge and have been reported to be a source of IL-4 in these reactions [9, 10]. To investigate their capacity to secrete IL-13 and thus provide proof-of-concept that basophils also constitute a source of this cytokine at sites of allergic inflammation, we isolated them from among the leukocytes recovered in the BAL. Fig 5 shows results from two BAL preparations where we were able to enrich for basophils, achieving purities of 60 and 90% respectively as determined by Alcian blue staining. As predicted, BAL basophils showed signs of *in vivo* activation evidenced by partial to complete degranulation when viewed microscopically using Wright's stain (data not shown). Both BAL basophil suspensions secreted comparable levels of IL-13 when stimulated with IL-3 (Fig 5A and B). Spontaneous secretion of this cytokine was also detected in one of these preparations (Fig 5A).

Finally, regression analyses shown in Fig 6 revealed that IL-13 protein levels in the BAL correlated not only with the frequency of basophils recovered in the cellular fraction of these fluids ($r^2 = 0.832$, $p=0.0002$, panel A), but also with the levels of this cytokine secreted by circulating basophils following SAC ($r^2 = 0.523$, $p=0.04$, panel B). Likewise, a weak correlation was detected between BAL basophil numbers and the IL-13 secreted by circulating basophils ($r^2 = 0.400$, $p=0.051$, panel C). In contrast, BAL IL-13 did not correlate with either the frequency of eosinophils or the number of mononuclear cells recovered from the lung post-SAC (data not shown).

Discussion

The findings presented here using an *in vivo* allergen challenge model support: 1) our *in vitro* work of a basophil/pDC axis, and 2) an emerging theory that localized allergic reactions propagate through the blood by affecting the responses of circulating leukocytes. To date, most studies addressing this **later** hypothesis have focused on changes in circulating eosinophil numbers and how specific phenotypic markers on these granulocytes are altered following an experimental allergen challenge [1, 11]. Our interest in basophils and pDCs stems from three interrelated lines of evidence that support the existence of an axis-interplay between these seemingly very different immune cells. Foremost, is our

previous finding that Type I IFNs (i.e. IFN- α/β made predominately by pDCs in humans), inhibit *in vitro* basophil IL-13 secretion induced by IL-3 yet do not affect IgE-dependent responses (histamine release and cytokine secretion) [4]. There are currently no reports of other cytokines or factors that mediate this same inhibition. Interestingly, IL-4, IL-13, and histamine (all basophil products) are reported to suppress IFN- α secretion by pDCs [12, 13]. A second set of findings supporting a basophil/pDC axis links to clinical observations. For instance, basophils isolated from allergic subjects secrete greater levels of IL-13 in response to IL-3 (and to nerve growth factor, NGF) than do basophils prepared from non-allergic subjects [2]. In contrast, others have shown (using PBMC cultures) that IFN- α production in response to virus, or to CpG, is impaired using cells prepared from allergic asthmatics (both in adults and children) [14, 15] and allergic rhinitics [16]. In fact, the data shown here using purified pDC suspensions are consistent with these observations. For example, we regularly detect IFN- α within 24h following CpG activation of pDCs isolated from non-allergic subjects (data not shown). However, 72h incubation was required in order to consistently detect this cytokine using pDCs from the allergic individuals examined in this study. It is not surprising that clinical symptoms are reported to improve in steroid resistant allergic asthmatics undergoing recombinant IFN- α therapy, with PBMCs from these subjects showing Th1-like responses when activated *in vitro* [17, 18].

The molecular mechanisms potentially underlying adaptive vs. innate immune responses in pDCs set the stage for a third set of observations. For example, the $\alpha\gamma_2$ variant of Fc ϵ RI, reportedly expressed on many human DC subtypes and seemingly regulated by serum IgE levels [19-21], has long been implicated in allergen presentation [22]. However, stimuli that crosslink IgE have been shown to suppress IFN- α secretion by pDCs activated by CpG [5, 23]. This is dependent in part on autocrine TNF- α down-regulating TLR9 expression [6]. This latter finding would imply that allergen binding to specific IgE on pDC functions to suppress innate immune responses (e.g. CpG or virus binding to TLR9) in these cells that would normally induce IFN- α and promote Th1 polarization. Naturally, basophils respond very differently to IgE crosslinking, releasing histamine, LTC4 and producing IL-4, IL-13 all of which are more likely to drive Th2 responses [24].

Assuming the *in vivo* existence of molecular mechanisms whereby TLR- and Fc ϵ RI-dependent responses oppose **one** another, it is then also intriguing to speculate that increased viral infections, especially those commonly linked to asthma exacerbations, may actually result from impaired Type I interferon production by pDCs. In this respect, allergen exposure may actually make an atopic asthmatic more susceptible than usual to subsequent viral infections.

In light of the aforementioned studies and hypotheses, we sought to investigate whether an experimental *in vivo* allergen challenge would alter the immune responses of circulating basophils and pDCs in a manner consistent with an immune modulating relationship between these cell types. As the results indicate, this was the case. In particular, IL-13 secretion by blood basophil-enriched cell (BEC) suspensions in response to IL-3 nearly doubled when analyzed 24h following a single segmental allergen challenge (SAC) in the lung (Fig 1). In fact, the findings bear remarkable similarity to our previous results of increased IL-13 secreted by basophil suspensions prepared from subjects who had undergone repeated nasal allergen challenge [3]. However, an important difference between the two studies is that IL-13 was spontaneously secreted by BEC suspensions following the repeated allergen challenge in the nose but this response was not apparent following a single challenge in the lung (Fig 1). While the protocols are not equal in comparison, one could reason that the single allergen challenge in the lung is qualitatively less provoking than 3-day challenges in the nose. Thus, while there was no consistent evidence for IL-13 spontaneously secreted by basophil suspensions post-SAC in the lung, physiological

differences between pre and post challenge BEC responses were uncovered using IL-3 stimulation. This would suggest that depending on the mode of allergen provocation, those that produce only subtle changes in circulating leukocytes may be revealed only under specific conditions. Indeed, using the Ca^{2+} -ATPase blocker, thapsigargin, Lie, et al. reported evidence that circulating basophils analyzed post bronchial allergen challenge secreted more histamine in response to IgE-dependent stimulation, and that this “priming” phenomenon was not apparent without use of the blocker [25].

The rationale for using BEC suspensions in this current study is straightforward—they are more rapidly prepared than are pure basophil suspensions, and are less likely to be manipulated in a way that alters their function. This is particularly important if only subtle physiological changes are predicted. Nonetheless, we acknowledge that the use of BEC suspensions could raise concern that the IL-13 responses measured in the current study could be in part attributed to cells other than basophils. It has been our experience from studies performed during the past decade, however, that the IL-13 secreted in these cultures and under the conditions tested, is of basophil origin. First, there is a remarkable correlation between the presence of basophils and the levels of IL-13 secreted in mixed leukocyte suspensions, particularly during the incubation times (i.e. 18-20h) used in this study [8]. Moreover, this was evident whether the stimuli tested were basophil-specific (i.e. IL-3, anti-IgE) or those that typically activate additional cell types (i.e. ionomycin or PMA). Likewise, we demonstrated in our previous study using repeated allergen challenge in the nose that leukocyte suspensions depleted of basophils did not secrete IL-13, but that this cytokine was only produced by the BEC suspensions [3]. Finally, the IL-13 secreted in the present study was in response to IL-3—a mode of stimulation that is specific for basophils. There is currently no other cell type in blood that secretes IL-13 in response to IL-3. We therefore conclude with a high level of confidence that the IL-13 secreted by the blood BEC suspensions, including the increased levels observed post SAC, is basophil-derived.

The exact mechanism(s) underlying the physiological effects observed in circulating basophils and pDCs following the SAC in the lung remain unknown. However, with regard to basophils, we have recently shown that they have the ability to secrete IL-3 following IgE-dependent stimulation *in vitro*. Moreover, this response has the capacity to then prime these cells through autocrine activity to increase production of cytokines [26]. Therefore, it seems possible that the increased IL-13 response observed in the present study could be related to the autocrine activity of IL-3 produced by basophils. Although this possibility was not investigated here, it is certainly one hypothesis requiring attention in future studies. Of course, this hypothesis also implies that allergen is somehow reaching circulating basophils to initiate IL-3 production. If so, then exactly how this is happening is also of great importance.

Finally, it is generally acknowledged that with all allergen challenge protocols, including the lung model utilized here, late phase responses are not always provoked and those that are can vary both in the total number of cells and eosinophils infiltrating the reaction site (Table 1). This premise allowed us to look for correlations between post-SAC blood basophil and pDC responses and the intensity of the LPR, as determined by cells infiltrating the lung and recovered in the BAL. These analyses were important since our study design did not include non-allergic subjects who underwent SAC in the lung—an issue that could raise concern regarding whether the increased basophil IL-13 responses post-challenge are indeed allergen specific. However, the data clearly demonstrate a strong relationship between the levels of IL-13 secreted by post-SAC blood basophil suspensions and the percentage of eosinophils among lung infiltrates ($p < 0.0001$). In contrast, an inverse relationship post-SAC was observed between the IL-13 secreted by basophils and the capacity of pDCs to secrete IFN- α in response to CpG. Equally as important was the striking relationship observed between

BAL IL-13 levels and the presence of basophils in the lung as well as with the IL-13 responses seen in circulating basophils post-SAC. These observations not only implicate the basophil as an important source of IL-13 during allergic pulmonary inflammation, but also substantiate this cell's relationship to systemic responses and the intensity of inflammation in localized sites. Collectively, these findings help to validate the conclusion that local allergen exposure can mediate systemic effects in the blood by augmenting pro-allergic activity not only in basophils, but likely in pDCs as well, where this pro-allergic activity (if confirmed) is seemingly through suppression of inherently pro-Th1 innate immune functions.

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Nonstandard abbreviations

pDC	plasmacytoid DC
mDC	monocytoid DC
FcεRI	high affinity IgE receptor
PAG	Pipes/Albumin/Glucose
C-IMDM	conditioned Iscove's Modified Dulbecco's Medium
CpG-DNA	DNA containing unmethylated CpG motifs
ODN	oligodeoxynucleotide
BDCA	blood dendritic cell antigen
SAC	segmental allergen challenge
BAL	bronchoalveolar lavage
PNU	protein nitrogen units
BDC	basophil-depleted cell
BEC	basophil-enriched cell
LPR	late phase response
IQR	interquartile range

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Fig 1. IL-3-dependent IL-13 secretion by blood basophils is augmented following SAC in the lung

BEC suspensions were prepared before (Pre) and 24h following (Post) SAC and stimulated 18h as indicated with recombinant IL-3, peptidoglycan (PGN, 10 μ g/mL), or medium alone. IL-13 protein was assayed by ELISA. Shown are median values with the IQR and lower/upper extreme levels, n=10.

Fig 2. SAC does not alter IgE-dependent responses by peripheral blood basophil suspensions BEC suspensions were prepared before (Pre) and 24h after (Post) SAC and stimulated as indicated. Supernatants were harvested after 3h incubation for histamine release (A) and IL-4 protein (B) by automated fluorimetry and ELISA, respectively. Shown are median values with the IQR and lower/upper extreme levels, n=10.

Fig 3. Blood basophil IL-13 responses post-SAC correlate with lung inflammation

Left panels (A and B) reflect correlations between basophil IL-13 produced in response to IL-3 (10 ng/ml) vs. BAL cell counts. Right panels (C and D) are correlations using delta values (i.e. post-SAC – pre SAC blood basophil IL-13 vs. BAL cells in allergen –saline challenged sites).

Fig 4. IFN- α secretion by blood pDCs is impaired following SAC

pDCs were prepared from each subject pre- and post- SAC. Cells were stimulated with the combination of oligodeoxynucleotide (ODN)-2216 (100 nM) and IL-3 (10 ng/ml). Cells were incubated for 72h before harvesting the supernatants for determination of IFN-alpha protein by ELISA. Shown are median values with the IQR and lower/upper extreme levels, n=10.

Fig 5. IL-13 secretion by BAL basophils

BAL basophils were isolated following SAC as, described in *Material and Methods*. *A* and *B*, two experiments (Exp) where IL-13 was secreted by BAL basophil suspensions (59 and 90% purity, respectively) following 18h incubation as indicated. No IL-13 was detected in BAL mononuclear cell (MNC) cultures depleted of basophils (*B*, Exp 2).

Fig 6. Regression plots for

A, IL-13 protein in BAL fluids vs. basophil frequencies in the lung and **B**, with the levels of IL-13 secreted by blood basophils 24h following SAC. **C**, The relationship between BAL basophil frequencies and IL-13 secretion by circulating basophils analyzed 24h following SAC.

Table 1

Subject profile, SAC Antigen (Ag) doses, BAL cell counts^A

Subj	DX	Age	Ag	Ag dose (mlxunits/ml)	BAL Cell Counts					
					Total Cells (×10 ⁶)		% eos		% baso	
					NS site	Ag site	NS site	Ag site	NS site	Ag site
1	AA	19	RW	5×10	22.7	131.3	0.3	62.8	4.10	
2	AR	26	RW	5×10	8.5	27.6	1.6	58.4	0.32	
3	AR	41	RW	5×1	41.9	115.5	0.4	38.8	0.94	
4	AR	25	RW	5×100	23.9	53.2	1.2	19.0	1.65	
5	AR	43	RW	5×10	13.2	43.3	0.6	18.6	0.01	
6	AR	43	DP	5×10	16.4	36.2	0.4	56.0	0.25	
7	AA	27	DP	5×10	41.8	130.4	0.2	2.0	0.10	
8	AA	33	DP	5×0.1	22.5	11.9	0	1.4	0.03	
9	AA	18	RW	5×10	18.8	270	9.6	52.4	0.83	
10	AA	32	RW	5×10	16.8	45.3	1.4	40.4	0.91	
mean±SEM					22.7±3.5	86.5±24.7	1.6±0.9	35.0±7.3	0.91±0.39	
					p<0.007					
					p<0.006					

^A Subj, subject; DX, diagnosis; AA, allergic asthmatic; AR, allergic rhinitic; Ag, antigen; RW, ragweed, DP, Dermatophagoides pteronyssinus; NS, normal saline; eos, eosinophil; baso, basophil; p values are relative to NS site.