Response of Respiratory Flour Allergics in an Ingested Flour Challenge May Involve Plasmacytoid Dendritic Cells, CD25+ and CD152+ T Cells

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Key Words

Basophils · N-methyl histamine · Occupational wheat allergy · DBPCFC · pDC · CD4+CD25+ T reg

Abstract

Background: A number of occupational respiratory allergens are food related, and little is known about the responses these allergens elicit in sensitized persons that ingest them. **Methods:** Nine respiratory flour-allergic volunteers were exposed in a double-blind placebo-controlled food challenge with flour. Responses were monitored by spirometry, acoustic rhinometry, determination of urinary methyl histamine and tryptase and flow cytometric evaluation of basophil, dendritic and T cell numbers and markers. **Results:** Significant increases in serum tryptase (compared with placebo post-exposure levels) and methyl histamine and a coordinated decrease in blood basophils and nasal volume after ingestion of allergen compared with placebo suggest an allergic response to ingested allergen. There was no change in forced expiratory volume in 1 s. The number of blood plasmacytoid dendritic cells (DC), but not of myeloid DC, decreased after exposure ($p = 0.001$). DC HLA DR was reduced after both exposures ($p < 0.001$). Expression of CXCR4 on DC was reduced after allergen ($p = 0.033$) but not after placebo exposure. CD4+ T cell expression of

CD25 was elevated after placebo ($p = 0.021$) but reduced after allergen provocation. The reduction in CD25 expression after allergen compared with placebo was significant ($p = 0.024$). CD152 was downregulated on these cells after allergen ($p = 0.039$) but less so after placebo exposure. **Conclusion:** Persons with respiratory allergy respond after ingestion of the relevant allergen. Response to this allergen challenge may selectively recruit plasmacytoid DC through CXCR4 and T cells expressing CD25 and CD152, which may be a regulatory phenotype.

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Introduction

Bakers' asthma covers allergy to wheat (prevalence 3.6%), rye and α -amylase (prevalence 1.0%) [1]. However, little is known about the responses raw ingested baker allergens elicit in sensitized persons, and bakers sensitized to raw bakery allergens can safely ingest cooked or baked allergens [2]. Gastrointestinal allergy to wheat may be underdiagnosed as it may be concealed by irritable bowel disease [3]. It is known that cross-reacting allergens found in fruit and nuts can elicit gastrointestinal and oral symptoms in persons allergic to pollen [4], and respiratory latex allergy is associated with reactions to several

Table 1. Demographic description of participants and challenge doses for allergen and placebo

Pa-tient	Age/sex	Time since WP exposure ¹ years	Smoking years	Diagnosis	PD20 methacholine µg	Sensitization, prick test or specific IgE	Medication	Wheat dose, g	Maize dose, g
1	41 M	4	none	asthma	>11,520	wheat, rye, barley, oat, α-amylase, grass, mugwort, <i>D. pteronyssimus</i> , <i>D. farinae</i> , cockroach, flour moth, <i>L. destructor</i> , <i>A. siro</i>	none	80.5	80.5
2	29 M	current	4	asthma/ rhinitis	641	wheat, barley, oat, grass, birch, dog, cat, <i>D. pteronyssimus</i> , <i>T. putrescentiae</i>	ICS, LABA, SABA prn	80.5	80.5
3	37 M	current	7	rhinitis	>11,520	wheat, rye, barley, birch, <i>T. putrescentiae</i>	none	80.5	80.5
4	38 F	2	none	rhinitis	>11,520	wheat, <i>T. putrescentiae</i>	none	80.5	55.5
5	33 F	none	none	rhinitis	179.90	wheat, rye, barley, oat, grass, birch, cat, <i>D. pteronyssimus</i> , <i>T. putrescentiae</i> , <i>L. destructor</i>	ICS, LABA, NCS, SABA prn (ulcerative colitis)	68.0	80.5
6	34 F	none	none	rhinitis	>11,520	wheat, grass, mugwort	mesalazin	68.0	43.0
7	38 F	3	none	asthma/ rhinitis	3,859	wheat, rye, barley, oat	ICS, LABA, SABA prn	80.5	80.5
8	26 M	current	7	rhinitis	>11,520	wheat, rye, cat, <i>D. pteronyssimus</i> , <i>D. farinae</i> , <i>Alternaria</i> , <i>Cladosporium</i>	oral AH prn	80.5	80.5
9	24 F	current	none	asthma/ rhinitis	196.89	wheat, rye, barley, oat, grass, birch, mugwort, <i>D. pteronyssimus</i> , <i>D. farinae</i>	ICS, LABA, NCS, SABA prn, oral AH prn	80.5	80.5

ICS = Inhaled corticosteroids; LABA = long-acting β_2 -agonists; SABA = short-acting β_2 -agonists; prn = pro re nata, as necessary; NCS = nasal corticosteroids; AH = antihistamines.

¹ Time since WP exposed indicates the time elapsed since bakers were exposed at their workplace. None means that the person is not a baker, current means that the person still works in a bakery.

fruit [2]. As the gastrointestinal response can be limited by immunotherapy against cross-reacting respiratory allergens, responses to allergens do occur in different organs [5].

As an allergic reaction is an adaptive response of the immune system, it is likely to be initiated by antigen-presenting cells and activated T cells. Plasmacytoid dendritic cells (pDC) have recently been implicated in initiation of the allergic response in the nose [6] and may be well suited to locally amplify the effector function of T cells. Additionally, activated and regulatory T cells expressing CD25 and CD152 [7] are thought to respond by leaving the circulation in response to ingestion of an allergen.

We exposed 9 wheat-allergic persons with respiratory allergy in an oral double-blind placebo-controlled food challenge (DBPCFC) with wheat. Symptoms as well as objective measurements – spirometry, acoustic rhinometry, N-methyl histamine (NMH), blood basophil numbers and serum tryptase – were recorded. In addition, activation of the adaptive immune system was monitored by flow cytometric surface phenotyping.

Methods

Wheat-allergic persons were selected from hospital files and recruited by mail. The Ethics committee of Århus County approved the project. Responders were screened by telephone and attended the clinic for up to three visits: visit 1 was to confirm the diagnosis, and visits 2 and 3 were components of a DBPCFC [8]. Inclusion criteria were age between 18 and 60 years, positive skin prick test or elevated specific IgE for wheat and documented asthma or rhinitis when exposed to inhaled relevant allergen. Nine persons were included. The demographic data are shown in table 1. The mean age was 33 ± 5.8 years and 4 participants were men. Seven participants had diagnosed rhinitis and 5 had diagnosed asthma. Three patients had both asthma and rhinitis.

Visits 2 and 3 were randomized exposures at least 2 weeks apart to either allergen (commercial wheat flour) or placebo (maize starch), given in blackcurrant juice. Four doses of allergen (0.5, 5, 25 and 50 g) were given at 30-min intervals. Immediately preceding ingestion of a dose (or 30 min after a dose that gave rise to symptoms), lung function, i.e. forced expiratory flow in 1 s (FEV₁) and forced vital capacity (FVC) [9], and acoustic rhinometry [10] were measured. Blood samples (serum and heparinized blood) were taken before and 30 min after ingestion of the last dose tolerated. Subjective symptoms were recorded. Spot urine of the last 8 participants was collected at baseline and a 24-hour urine sample after provocation.

Fig. 1. Markers associated with the allergic response significantly differentiate between allergen and placebo provocation. Significant changes are indicated in each figure. **a** Changes in the minimal cross-sectional nasal area assessed by rhinometry before each provocation. **b** Numbers of Lin1– HLA DR– CD123+ basophils per 250,000 events detected by flow cytometry.

Heparinized blood was hemolyzed and labeled with titrated amounts of antibodies: Lin1 FITC (Cat. Nr. 340546, Becton-Dickinson, Brøndby, Denmark), which comprises antibodies against lineage markers CD3, CD14, CD16, CD19, CD20 and CD56, CD123 phycoerythrin (PE) (Cat. Nr. 340545, Becton-Dickinson), which binds the interleukin-3 receptor, anti-HLA DR PerCP (Cat. Nr. 347364, Becton-Dickinson), CD11a PE (Cat. Nr. 30425x, Becton-Dickinson), which binds an integrin used to differentiate MT1 and MT2 cells [11], CD62L FITC (Cat. Nr. 1231, Immunotech, Hialeah, Fla., USA), CD11c PE (Cat. Nr. 30425x, Becton-Dickinson), which binds an integrin expressed selectively on myeloid dendritic cells (mDC), CD45RA PE Cy5 (Cat. Nr. MCA1720, Serotec, Kidlington, UK), which differentiates between memory and naïve T cells, CD4 allophycocyanin (APC) (Cat. Nr. 30159x, Becton-Dickinson), which defines CD4 T cells, CD3 APC (Cat. Nr. 555335, Becton-Dickinson), which defines T cells, CD25 FITC (Cat. Nr. MCA1319F, Serotec), which defines the interleukin-2 receptor, CD152 PE (Cat. Nr. 34585x, Becton-Dickinson), which binds to CTLA4 [7], CD4 PE Cy5 (Cat. Nr. MCA1565C, Serotec), CD45RO APC (Cat. Nr. 340438, Becton-Dickinson), which differentiates between memory and naïve T cells, CXCR4 PE (Cat. Nr. FAB173P, R&D Systems, Abingdon, UK), which binds to the CXCR4 receptor, and CD14 APC (Cat. Nr. 555399, Becton-Dickinson), which binds to the lipopolysaccharide receptor and defines monocytes; then, they were washed and 250,000 events were acquired with a FACS Calibur (Becton-Dickinson). To evaluate cell numbers, the number of events in the basophil, pDC or mDC gates were related to all events collected (250,000). This was the level of evidence possible, as leukocyte counts were not available for all participants.

Data were analyzed offline in batch with FlowJo 6.2 (Treestar, Ashland, Oreg., USA). Tryptase was analyzed by fluorometry with UNICap (Pharmacia, Uppsala, Sweden), with a detection limit of 1 ng/l. NMH was analyzed by RIA (Pharmacia) [12].

Data were analyzed with the t test, given as mean $\pm$ SD and displayed as mean $\pm$ SD plots, or the Wilcoxon signed rank test, given as median and interquartile range (IQR) and displayed as box plots, depending on the type of distribution. Baseline was compared with exposure. Baseline values of allergen and placebo exposures were compared to confirm that values were similar, and to expose values to test for a challenge effect. Pearson's correlation coefficient was calculated to determine an association between the changes observed. The level of statistical significance was $p = 0.05$.

Results

Rhinometry, Blood Basophils, Serum Tryptase and Urinary NMH Differentiate between Ingested Allergen and Placebo

Four participants reported symptoms when challenged with allergen, whereas 5 participants reported symptoms when challenged with placebo. The difference was not significant (data not shown). As provocation was discontinued after symptoms were recorded, the total dose varied (table 1). Response of participants was evaluated by

Fig. 2. Flow cytometric identification of basophils, DC and evaluation of surface markers. **a** Identification of basophil granulocytes as Lin1⁻ HLA DR⁻ CD11c⁻ CD123^{bright} cells. **b** Identification of mDC and pDC as Lin1⁻ HLA DR⁺ CD11c^{bright} CD123^{dim} and

Lin1⁻ HLA DR⁺ CD11c^{dim} CD123^{bright} cells, respectively. **c** Identification of DC as CD14⁻ HLA DR⁺ cells and expression of CXCR4 (red) and isotype (blue). **d** Expression of CD25 and CD152 (red dots) on CD4⁺ T cells compared with isotype (blue).

Fig. 3. DC markers suggest that there is an activation of the adaptive immune system during provocation and that CXCR4 expression on DC is associated with the allergic response. **a** Number of mDC (Lin1[−] HLA DR⁺ CD11a^{bright} CD123^{dim}) and pDC (Lin1[−] HLA DR⁺ CD11a^{dim} CD123^{bright}) detected before and after exposure. **b** Decrease in HLA DR expression on CD14[−] HLA DR⁺ DC, a sign of tolerization. **c** Change in expression of the chemokine receptor CXCR4, associated with the allergic response, on CD14[−] HLA DR⁺ DC.

Fig. 4. Lower levels of CD25 and CD152 on T cells are detected after allergen provocation. **a** Differential change in CD25 expression on CD4⁺ T cells. Placebo upregulates CD25 expression. There is significantly less upregulation after allergen provocation. **b** Change in CD152 expression on CD4⁺ T cells. CD152 expression is reduced further during allergen provocation than with placebo.

spirometry at baseline and before every ingested dose. There was no change in FEV₁ or in FEV₁/FVC throughout the provocations, even when considering only the 4 participants that had asthma (data not shown). Response of participants was evaluated by rhinometry at baseline and before every ingested dose. The minimum nasal cross-sectional area during the provocation test expanded slightly when provoked with placebo (fig. 1a). The first provocation with allergen resulted in a significant reduction in the minimum nasal cross-sectional area (mean $0.77 \pm 0.1 \text{ cm}^2$) compared with baseline (mean $0.78 \pm 0.2 \text{ cm}^2$; $p = 0.013$) and compared with the dilated nostrils after the first provocation with placebo (mean $0.93 \pm 0.2 \text{ cm}^2$; $p = 0.045$).

Blood basophils were identified as Lin1[−] HLA DR[−] CD123⁺ cells (fig. 2a) at baseline or 30 min after the final provocation dose (fig. 1b). After placebo provocation, blood basophil numbers remained constant at $2,152 \pm 890$ at baseline and at $2,164 \pm 1,000$ (n.s., $n = 8$) and decreased from $2,146 \pm 740$ to $1,705 \pm 790$ after the allergen challenge ($p = 0.034$, t test, $n = 8$). Serum tryptase was measured at baseline and 30 min after the highest dose. There was no change in serum tryptase from baseline to allergen exposure, but a significant decrease after exposure to placebo. The exposure/baseline ratio was significantly higher for allergen (1.05 ± 0.16) than for placebo (0.92 ± 0.09 ; $p = 0.021$, $n = 9$), and the difference in exposure/baseline was significantly higher for allergen (0.01 ± 0.7) than for placebo (-0.05 ± 0.67 ; $p = 0.011$). Urine NMH is an inflammatory marker used to objectively diagnose food allergy. The NMH content of urine increased significantly after allergen exposure ($10.37 \pm 3.4 \text{ } \mu\text{g MH/mmol creatinine}$ at baseline to $13.79 \pm 3.3 \text{ } \mu\text{g MH/mmol creatinine}$ after exposure; $p = 0.017$, $n = 8$), whereas the increase was not significant after placebo ingestion (11.81 ± 3.8 to $13.49 \pm 4.4 \text{ } \mu\text{g MH/mmol creatinine}$; n.s., $n = 8$).

Blood DC Tolerize in Response to Provocation and Express Less CXCR4 after Allergen Provocation

The number of blood DC2 identified as Lin1[−] HLA DR⁺ CD11a^{dim} CD123^{bright} cells (fig. 2b, 3a) decreased after exposure to allergen (at baseline 409 cells, IQR 363–761; after exposure 275 cells, IQR 195–469; $p = 0.001$, $n = 16$) and placebo (at baseline 442 cells, IQR 326–666; after exposure 260 cells, IQR 235–310), but there was no change in DC1 (Lin1[−] HLA DR⁺ CD11a^{bright} CD123^{dim}) numbers detected (at baseline 650 ± 244 cells; after exposure 683 ± 253 cells; $n = 16$). HLA DR expression (fig. 2b) on DC identified as CD14[−] HLA DR⁺ monocytic

cells (fig. 2c) was reduced after either exposure – baseline mean fluorescence intensity (MFI) 153, IQR 114–204, and exposure MFI 126, IQR 100–182 ($p = 0.001$, $n = 18$). There was no difference between allergen and placebo provocations. Expression of CXCR4 (fig. 2c) on DC identified as CD14[−] HLA DR⁺ monocytic cells was reduced significantly after allergen exposure (baseline MFI 288, IQR 232–321, and exposure MFI 246, IQR 192–268; $p = 0.05$, $n = 9$), but not after placebo exposure (baseline MFI 240, IQR 223–298, and exposure MFI 266, IQR 220–268). CCR5 could not be detected on CD14[−] HLA DR⁺ cells (data not shown).

Blood T Cells Express Less CD25 and CD152 (CTLA4) after Allergen Provocation than after Placebo Exposure

CD4⁺ T cell expression of CD25 (fig. 2d, 3a) was elevated after placebo exposure (baseline MFI 4.9, IQR 4.3–5.6, and exposure MFI 6.1, IQR 5.7–6.5; $p = 0.021$, $n = 9$) but only marginally after allergen provocation (baseline MFI 4.8, IQR 3.7–7.8, and exposure MFI 5.4, IQR 3.5–6.4; n.s., $n = 9$). The decrease of CD25 expression after allergen exposure compared with placebo was significant (MFI of -0.50 ± 1 and 1.77 ± 2.3 for allergen and placebo, respectively; $p = 0.024$, $n = 9$). CD152 (fig. 3b) measured on the same cells (CD4⁺) was downregulated after allergen provocation (baseline MFI 140 ± 82 , exposure MFI 102 ± 70 ; $p = 0.039$, $n = 9$), but less after placebo exposure (baseline MFI 116 ± 92 , exposure MFI 104 ± 55 ; n.s., $n = 9$).

The expression of HLA DR as activation marker and CD27, thought to be a Th1 marker [13], did not vary between baseline and exposure, or between allergen and placebo (data not shown). The ratio of MT1/MT2 cells (MT1 is CD62L⁺ CD11a^{dim}, MT2 is CD62L[−] CD11a^{bright}) on CD4⁺ CD45RA[−] memory T cells [11] did not vary between baseline and exposure, or between allergen and placebo (data not shown).

Discussion

We have exposed 9 persons sensitized to inhaled flour and diagnosed with asthma or rhinitis to their respiratory allergen in a DBPCFC to investigate whether ingestion of flour allergen results in clinical symptoms in the respiratory tract. Early parameters of the cellular immune response were measured to investigate the processes by which the allergen was detected by the immune system and which reactions are critical.

There were as many symptoms with placebo as with allergen. The exposure did not result in severe symptoms or side effects. The significant increase in urinary NMH and serum tryptase (compared with levels after exposure) and the decrease in blood basophils and nasal volume after ingestion of allergen compared with placebo suggest a specific response to ingested allergen. Rhinitis is among the frequent clinically detectable responses to a food challenge [14]. The challenge utilized a wide range of amounts of allergen (0.5–50 g) compared with regimes where the maximal challenge dose was 8 g protein [15]. However, in accordance with the conclusions in the previous study, reduction in the nasal volume was significant at the lowest concentration of wheat. Responses to provocation with food allergens tend to occur at the lowest dose given, rather than as a dose response. Severity did not increase as the amount of challenge food increased [15]. This suggests that there is a cross-reactivity between the organ systems, as is well documented by the cross-reactivity in allergy to birch pollen and apple [4, 5] and that of latex and bananas, kiwis and other fruit [2]. The only clinical response to ingested allergen was a decrease in nasal volume after the first dose of allergen. This may be because most participants (7/9) had been diagnosed with rhinitis, or because the nasopharyngeal region is the first part of the respiratory tract to come into contact with a typically airborne allergen and is populated with immune sentries (resulting in edema) when a large dose of allergen is delivered to the gastrointestinal tract. Rhinitis has previously been shown to be the most frequent respiratory symptom in DBPCFC [14]. The response in the nose was supported by (1) the detection of the metabolite of histamine, NMH in urine and a clinical test used to monitor gastrointestinal allergy [16], which was elevated at baseline ($10 \pm 4 \mu\text{g}/\text{mM}$ creatinine) compared with reference values of a normal population ($5.2 \pm 2.4 \mu\text{g}/\text{mM}$ creatinine) [12] and increased significantly after exposure to allergen, and (2) decreased blood basophil numbers after allergen exposure. Part of the increase in NMH may be due to histamine released in the stomach to digest the large dose ingested. Reduction in basophil numbers may be due to recruitment of basophils at the site of allergen exposure, which has been documented during challenges in the nose [17]. During the same time, serum tryptase decreased significantly only after placebo exposure, but remained unaltered after allergen exposure. This and elevated NMH suggest that there is an ongoing subclinical allergic response in flour-allergic persons that are constantly exposed to baked and raw allergen as part of their diet (anecdote: 'I can tell whether something is baked

properly') and that they are under constant oral immunotherapy.

Enumeration of pDC and mDC has shown that people with atopic disease and controls have fewer pDC than mDC [18–21]. Previous work has shown that exposure to the relevant allergen in the lung of asthmatics recruits mDC from the circulation [22] and that after allergen provocation, pDC [6] and mDC [23] accumulate in the nose and lung, respectively. Only the latter study sought to identify both mDC and pDC. The papers detecting mDC leaving the circulation [22] and showing an accumulation of mDC, but not of pDC [23], complement each other to suggest that mDC are recruited to the lung during local/inhalative allergen exposure. We have enumerated mDC and pDC at baseline and after a DBPCFC and found that only pDC were recruited from the circulation after provocation in the gastrointestinal tract, which is thought to be very tolerogenic [24, 25]. The depletion was associated with a decrease in DC HLA DR expression characteristic for tolerizing DC [26]. The suggestion that pDC recruited during the allergen exposure have a tolerogenic function is supported by the observation that participants in the study reported similar symptoms with allergen as they did with placebo. In a murine model of asthma, pDC have been shown to be essential for preventing asthmatic reactions to harmless inhaled antigen [27]. The chemokine receptor profile of mDC and pDC is similar and included CXCR4 [28]; however, only pDC migrate in response to stromal-derived factor 1 (the ligand for CXCR4) in vitro. The present finding that mDC numbers remain constant while pDC numbers and the level of expression of CXCR4 on DC decrease during an allergic stimulus is consistent with the mechanism in which pDC are selectively recruited from the circulation by the allergic stimulus involving stromal-derived factor 1. Our observations support previous reports [22, 29] that DC are recruited from the circulation in response to appropriate allergic signals from the environment and expand on it by showing that also pDC can selectively be recruited from the circulation by exposure to a relevant antigen where few clinical symptoms are reported. Thus, whilst mDC may be crucial for the generation of an allergic response [30], our results are consistent with the observation that pDC may be even more important in maintaining tolerance to harmless antigens [27], and the balance between the two subsets could determine how the immune response develops.

There appeared to be a general activation of T cells by placebo, as documented by the increase in expression of CD25 on CD4+ T cells. In contrast, expression of CD25

on T cells was reduced after allergen compared with placebo exposure. This is consistent with a model in which CD25⁺ T cells, together with tolerized pDC, are recruited to the site of allergic exposure. The observation that the expression of CD152/CTLA-4 is reduced is consistent with a model in which these potentially regulatory T cells [7] leave the circulation after allergen stimulation (a strong, specific stimulus that could be mediated through IgE/FcεRI expressed on, amongst others, pDC) [31] and less so after placebo stimulation (a diffuse stimulus). Blocking CD152 on regulatory T cells has inhibited their regulatory activity [7]. The time frame for expression of CD27 HLA DR on T cells and MT1 and MT2 markers [11] may have been suboptimal in this study.

Allergic persons with rhinitis, asthma or both respond to relevant ingested allergen. Exposure to allergen could be differentiated from placebo exposure by rhinometry, blood basophil, serum tryptase and urine NMH content. In addition, the blood concentration of pDC as well as the expression of HLA DR on DC decreased after each challenge, supporting a drain from the circulation as well as tolerization of DC. Expression of CXCR4 on DC was

reduced only after allergen challenge. Change in T cell expression of CD25 and CD152 was consistent with recruitment of activated and regulatory T cells from the circulation to the site of allergen exposure. The reduction in circulation of pDC and T cells with tolerizing phenotype may explain why there was no allergic response to the provocation beyond the clinical response after the first dose. In contrast to the cross-reacting allergens that frequently elicit gastrointestinal and oral responses, wheat was both respiratory and the gastrointestinal allergen. Amongst the markers measured, blood basophil numbers are candidates for objective measurements of the response in a DBPCFC.

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