

ORIGINAL ARTICLE

Potential non-T cells source of interleukin-4 in food allergy

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Abstract

Background: Recently, a study from the Consortium of Food Allergy Research (CoFAR) showed that allergen-induced *IL-4* expression in $CD25^+$ mononuclear cells was increased in allergic patients. However, they did not find the expected increase in *GATA-3* expression, suggesting that allergen-induced *IL-4* might not be of T-cell origin. We sought to determine whether other cell types were responsible for the increased *IL-4* expression in the $CD25^+$ cell population.

Methods: Comparing six allergic patients and six healthy controls, we analyzed the $CD25^+$ isolated population from PBMC for the presence of potential *IL-4*-expressing non-T cells. We also compared spontaneous expression levels of surface markers (*CD203c*, *CD63*, *CD25*, and *HLA-DR*) on basophils from whole blood of 42 peanut-allergic patients and from 12 non-atopic controls. Expression of these markers was also evaluated following basophil activation in eight peanut-allergic patients selected from the previous cohort.

Results: In addition to $CD4^+$ T cells, a substantial proportion of non-T cells were found in the $CD25^+$ -isolated cell population: basophils, NK, and NK-T cells with a mean percentage $\pm$ s.e.m. of $5.24 \pm 0.63\%$, $6.65 \pm 1.01\%$, and $6.01 \pm 1.04\%$, respectively. The majority of these cells exhibited positive intracytoplasmic staining for *IL-4*. Expression of *CD63* and *CD25* was significantly higher in allergic patients compared with controls ($p < 0.05$). Interestingly, we found a significantly higher proportion of activated basophils expressing *HLA-DR*, compared with non-activated basophils ($p < 0.05$).

Conclusions: Our results support the suggested key role of non-T cells secreting *IL-4* in food allergy, particularly basophils, which may also play a central role in antigen presentation.

Food allergy is estimated to affect approximately 4%–6% of young children and appears to have increased in prevalence over the past decade (1, 2). Although our knowledge of the pathophysiology of allergic diseases has improved over the past several years, insights into the basic mechanisms responsible for the development of food allergy are lacking. IgE-mediated hypersensitivity responses are attributed to the generation of T helper (T_H)-2 cells that produce interleukins (*IL*)-4, *IL*-5, and *IL*-13 (3). While the consequences of T_H2 immunity and

inflammation in peripheral tissues are well characterized, the innate responses that promote T_H2 cell development, including the nature of the antigen-presenting cells (APC) involved in initiating and sustaining T_H2 cell differentiation, remain less well defined.

Recently, a study from the Consortium of Food Allergy Research (CoFAR) (4) investigated the basic mechanisms responsible for food allergy in a cohort of 512 infants with early evidence of food allergy. The results confirmed the expected importance of interleukin (*IL*)-4, by demonstrating that allergen-induced *IL-4* expression in peripheral blood mononuclear cells (PBMC) was associated with clinical allergy to milk and IgE sensitization to peanut. This is consistent with previous studies showing a T_H2 bias in food allergy, as well as our current understanding of the role of *IL-4* in inducing IgE class switching in activated B cells (5). The authors

Abbreviations

IL-4, Interleukin 4; T_H , T helper; APC, antigen-presenting cell; PBMC, peripheral blood mononuclear cells; *GATA*-3, *GATA*-binding protein 3; *TBET*, T-box transcription factor; NK, natural killer cells; NK-T, natural killer T cells; PBS, phosphate-buffered saline; s.e.m., standard error of the mean; MFI, mean fluorescence intensity; DCs, dendritic cells.

hypothesized that allergen-specific T cells activated in vitro would be predominantly T_H2 effector memory cells expressing a high *GATA3/TBET* ratio of transcripts detectable by means of RT-PCR. However, the increase in allergen-induced *IL-4* expression in CD25⁺ mononuclear cells was not associated with the expected increase in *GATA3* transcription factors, suggesting that allergen-induced *IL-4* might not be of T-cell origin.

The aim of this study was to determine the proportion of *IL-4*-expressing non-T cells (essentially basophils, NK-T, and NK cells) in the selected CD25⁺ population, comparing food-allergic patients and healthy controls. Our secondary objective was to investigate the expression of surface membrane antigens CD25, CD63, CD203c, and HLA-DR on basophils from patients with food allergy compared with non-atopic controls, and to evaluate the changes in their expression following activation.

Methods

Identification of *IL-4*-expressing non-T cells in the CD25⁺ selected population

To assess the presence of *IL-4*-expressing non-T cells in the CD25⁺ population, we selected six food-allergic subjects, diagnosed with peanut and/or milk allergy (male/female, 4:2; mean age 13.24 yr, range 0.67–34), and six non-atopic subjects (male/female, 2:4; mean age 29.83 yr, range 24–35). The study was approved by the Mount Sinai Institutional Review Board, and informed consent was obtained before enrollment.

To have the exact same conditions, we followed a similar protocol to that used in the previous study by Sicherer et al. (4). After isolation, PBMCs were cultured for 48 h, 4×10^6 cells per well with the following condition: (i) AIM-V serum-free media (Invitrogen, Carlsbad, CA, USA) with aqueous peanut extract (50 µg/ml), (ii) with purified α -, β -, and κ -caseins (each 50 µg/ml), (iii) with egg white protein (50 µg/ml), (iv) AIM-V medium alone, and (v) AIM-V medium plus anti-CD3/anti-CD28 beads (5 µl; DYNAL, Invitrogen) (4). Cells expressing CD25 were then positively selected using anti-CD25-coated paramagnetic beads according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were then stained for expression of several classic markers used to characterize basophils (i.e., CD3, CD123, and CD203c) and NK and NK-T cells (CD3, CD56, Nkp46, and CD16). Following this surface staining, cells were fixed/permeabilized and stained for intracytoplasmic *IL-4* as per the manufacturer's protocol (BD Biosciences, San Jose, CA, USA). Brefeldin A was added for the final 6 h of culture. Fluorescence data were acquired on a LSR II running Diva 3.0 software (BD Biosciences). The gating strategy is illustrated in Fig. 1.

Comparison of surface membrane antigens on basophils from peanut-allergic patients and non-atopic controls

A separate group of 42 peanut-allergic subjects (male/female, 24:18; mean age 12.59 yr, range 1.3–34) was prospectively recruited from the Jaffe Food Allergy Institute clinics at the

Mount Sinai Medical Center. We measured several surface membrane antigens on basophils (CD25, CD203c, CD63, and HLA-DR) from these allergic patients and compared these with the same molecules on basophils from 12 non-atopic donors (male/female, 6:6; mean age 31.08 yr, range 23–45). Briefly, heparinized peripheral blood was obtained during a period free of any symptoms. After washing with phosphate-buffered saline (PBS), cells were fixed with FACS Lysing Solution (BD Biosciences). Following centrifugation, 10% DMSO solution was added to the pellet and samples were frozen and stored at -80°C . Cells were later thawed, washed, and resuspended in PBS, followed by surface staining. Thereafter, acquisition and analysis were performed. To better characterize the basophils and to evaluate HLA-DR expression on their surface, a different gating strategy has been used to identify these cells: CD3⁺HLA-DR^{dim}CD123⁺CD203c⁺.

Basophil activation tests

For basophil stimulation assays, eight patients (male/female, 7:1; mean age 12.88 yr, range 6.6–22.3) were recruited from the previous cohort of peanut-allergic patients. Aliquots of fresh whole blood (250 µl) were incubated for 15 min at 37°C in three different conditions: (1) RPMI 1640 (BioWhittaker, Walkersville, MD, USA) alone, used as a negative control; (2) RPMI and rabbit anti-human IgE antibody (0.5 µg/ml) (Bethyl Laboratories, Montgomery, TX, USA), used as a positive control; or (3) RPMI and aqueous peanut extract (50 µg/ml). The reaction was stopped with 50 µl of cold PBS plus 20 mM EDTA. Following surface staining, samples were subjected to erythrocyte lysis with 3 ml of FACS Lysing Solution (BD Biosciences) for 15 min. Basophil activation was assessed by means of flow cytometry as described previously. To determine the change in expression of HLA-DR, a more specific gating strategy was used to identify basophils, particularly to exclude other cell types known to express HLA-DR: CD3⁺CD19⁺CD41a⁺CD14⁺ cells were selected to exclude platelets, monocytes, T and B cells, followed by a further gating of HLA-DR^{dim}CD123⁺CD203c⁺ cells.

Statistical analysis

The Wilcoxon matched pairs signed-rank test for comparing changes from the same individuals and the *t*-test for unpaired variances (different populations) were applied. The *p* Value presented is for a two-tailed test, and a value of <0.05 was considered to indicate statistical significance.

Results

Identification of *IL-4*-expressing non-T cells in the CD25⁺ preparation

We determined the proportion of basophils, NK, and NK-T cells in the CD25⁺ preparations from six food-allergic patients and six non-atopic controls. In medium alone culture wells, we found a substantial proportion of basophils, NK cells, and NK-T cells, with a mean percentage $\pm$ standard error of the

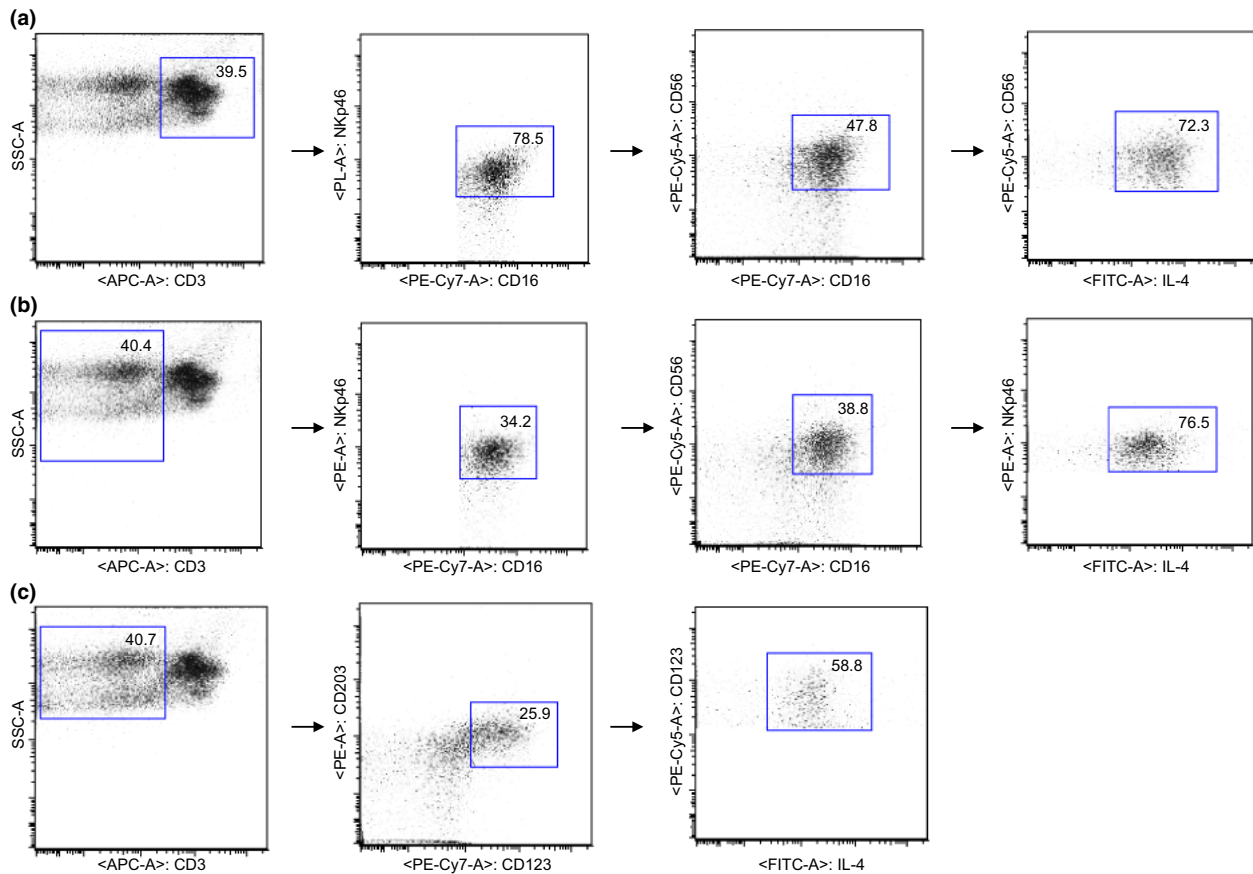


Figure 1 Gating strategy for the identification of different interleukin (IL)-4-expressing non-T cells in the CD25 preparation. (a). Putative natural killer T (NK-T) cells were gated as CD3⁺CD16⁺CD56⁺ NKp46⁺ cells. (b). Natural killer (NK) cells were characterized as CD3⁻CD16⁺CD56⁺ NKp46⁺ cells. (c). Basophils were identified as CD3⁻CD123⁺CD203⁺ cells.

mean (s.e.m.) of $5.24 \pm 0.63\%$, $6.65 \pm 1.01\%$, and $6.01 \pm 1.04\%$, respectively (Fig. 2). No difference in the mean percentage of basophils, NK, and NK-T cells was observed between allergic patients and non-atopic controls. Stimulation with CD3/CD28 beads lead to a significant increase in the proportion of basophils, NK-T, and NK cells in the CD25⁺ population, both in allergic and non-atopic subjects ($p < 0.05$). Also, we found a significant increase in the proportion of basophils and NK cells in CD25⁺ preparations after stimulation with peanut and/or casein in patients with peanut and/or milk allergy, respectively ($p < 0.05$). A high proportion of basophils, NK, and NK-T cells were positive for IL-4 intracytoplasmic staining (Fig. 2). Stimulation with either CD3/CD28 beads or food allergen did not alter the percentage of basophils, NK, and NK-T cells exhibiting positive staining for IL-4.

Comparison of basophil surface markers between peanut-allergic patients and non-atopic controls

Expression of different basophils surface markers was evaluated in a cohort of 42 peanut-allergic patients and compared with 12 non-atopic controls. As expected, the mean percentage

of basophils in whole blood was similar in both groups. As shown in Fig. 3, expression of various basophil surface markers differed significantly between the two groups. The level of spontaneous CD25 expression was higher on basophils from allergic patients than from non-atopic subjects, with a mean fluorescence intensity (MFI) ($\pm$ s.e.m.) of 710.5 ± 41.24 and 493 ± 59.53 , respectively ($p < 0.05$). Moreover, basophils from allergic patients showed significantly higher levels of CD63 expression than those from controls (MFI ($\pm$ s.e.m.) of 29402 ± 2097 and 18576 ± 1766 , respectively ($p < 0.05$)). In addition, a higher percentage of basophils expressing CD63 was found in allergic patients compared with controls, $84.95\% \pm 2.5\%$ and $76.5\% \pm 4.7\%$, respectively ($p < 0.05$). Although we found a trend toward a higher level of expression of CD203c and HLA-DR on basophils from allergic patients compared with non-atopic controls, this difference did not reach statistical significance (Fig. 3). Of note, the mean age of the patients was significantly higher in non-atopic controls compared with allergic patients ($p < 0.05$). While some basophils' properties have been related to the age of the patient (6, 7), basophils' responses after allergen activation have been shown to be similar in children and adults (8, 9). Although not formerly excluded, the difference between the

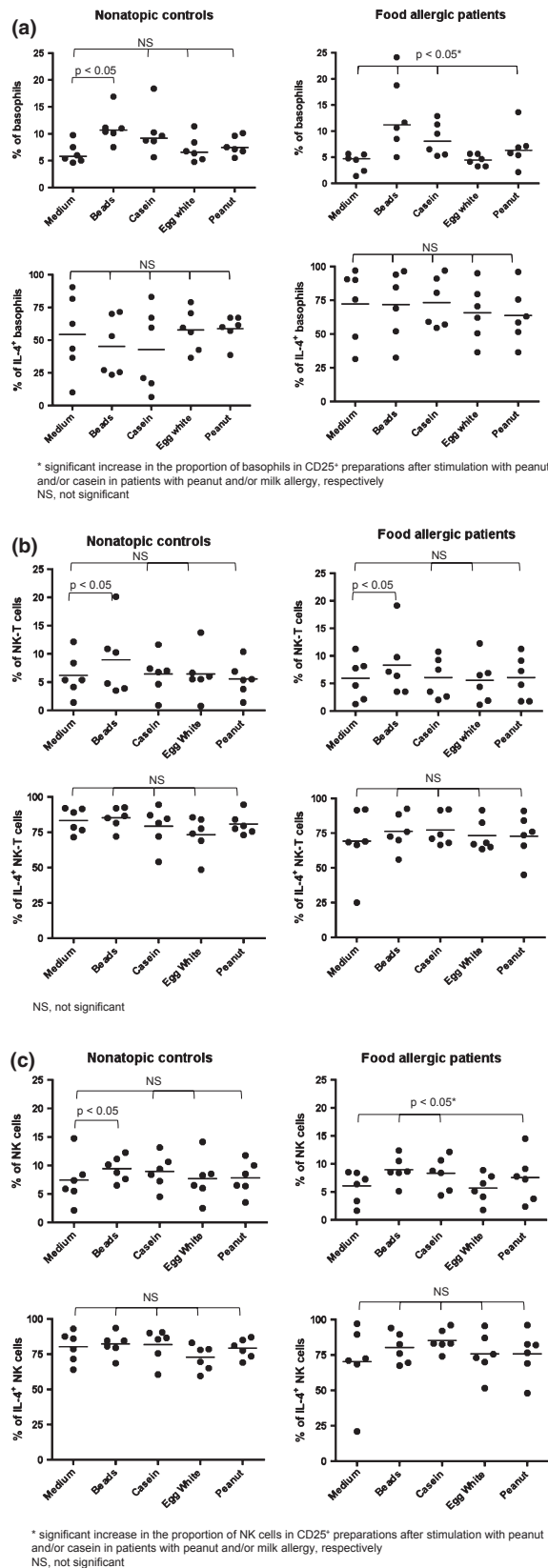


Figure 2 Percentage of different non-T cells found in the CD25⁺ preparation, as well as the percentage of these cells exhibiting a positive intracytoplasmic staining for IL-4. These data compare six food-allergic patients and six non-atopic controls. (a). Basophils (CD3⁺HLA-DR^{dim}CD123⁺CD203⁺) (b). Natural killer T (NK-T) cells (CD3⁺CD16⁺CD56⁺ NKp46⁺). (c). Natural killer (NK) cells (CD3⁺CD16⁺CD56⁺ NKp46⁺).

two groups is unlikely to be only related to the age differences between the groups.

Basophil stimulation assays

Based on eight patients selected from the previous cohort of peanut-allergic subjects, we evaluated expression of surface markers following basophil activation. After selecting CD3⁺CD19⁺CD41a⁺CD14⁺HLA-DR^{dim} cells, activated basophils were identified by flow cytometry as CD123⁺CD25⁺CD203c⁺CD63⁺ cells. As expected, exposure of the basophils to anti-IgE antibodies or specific allergen resulted in a significant up-regulation of classical activation markers, CD203c and CD63 ($p < 0.05$). Interestingly, we found a significantly higher proportion of activated basophils expressing HLA-DR, compared with non-activated basophils ($p < 0.05$) (Fig. 4a). Expression (measured as MFI) of HLA-DR and CD25 was also significantly increased on basophils after incubation with anti-IgE or peanut extract for 15 min ($p < 0.05$) (Fig. 4b and c).

Discussion

Allergic diseases are characterized by a skewing of the T_H1/T_H2 balance toward T_H2 (10). Although the function of T_H2 cells and requirements of cytokines and transcription factors for their differentiation have been extensively studied in vitro, the factors that initiate and amplify T_H2 cell responses in vivo have only recently begun to be clarified in animal models (11). Our results support the potential role of IL-4-expressing non-T cells (basophils, NK, and NK-T cells) in the development of food allergy in humans, particularly in the induction of T_H2 responses.

Traditionally, IL-4 has been viewed as the keystone of a T_H2 cell response. In addition to being an important T_H2 effector cytokine, IL-4 has a crucial role in the differentiation of T_H2 cells in vitro (12). Recently, attention has focused on identifying the cells that provide the early innate source of IL-4 (11). Proposed sources of IL-4 have included classically naïve CD4⁺ T cells, basophils, mast cells, NK, and NK-T cells (13, 14). In a recent study from the CoFAR (4), the expected importance of IL-4 was confirmed by showing that allergen-induced IL-4 expression in CD25⁺ mononuclear cells was significantly increased in infants with food allergy. However, an associated increase in *GATA-3* transcripts was not detected, a finding that raised questions as to the initiating role of T cells in the early development of allergy.

We have confirmed the presence of non-T cells in CD25-enriched PBMC (Fig. 2). Stimulation of PBMC with

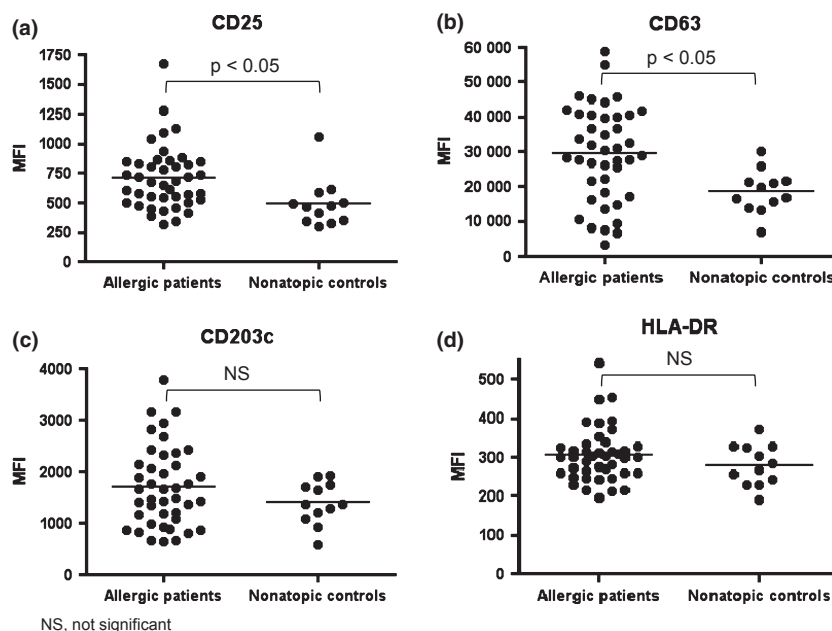


Figure 3 Expressions of different basophils surface markers (CD25, CD63, CD203c, HLA-DR) were evaluated in a cohort of 42 peanut-allergic patients and compared with 12 non-atopic controls. The results are expressed by mean fluorescence intensity (MFI) related to the entire basophil population.

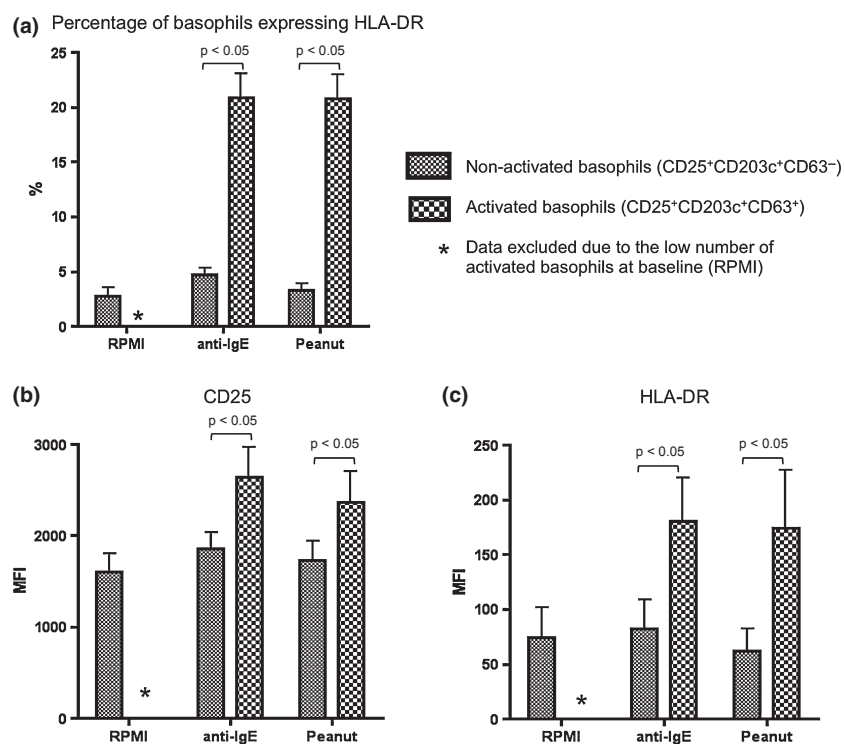


Figure 4 Effect of basophil activation by anti-IgE and specific allergen on surface antigens expression. (a). Percentage of activated (CD25⁺CD203c⁺CD63⁺) and non-activated basophils (CD25⁺CD203c⁺CD63⁻) expressing HLA-DR. (b) and (c). Expression (as measured by mean fluorescence intensity (MFI)) of CD25 and HLA-DR on activated and non-activated basophils.

CD3/CD28 beads lead to a significant increase in the proportion of basophils, NK-T, and NK cells in the CD25⁺ population, both in allergic and non-atopic subjects ($p < 0.05$). As it is unlikely that these cells would expand within the 48 h, this could reflect activation of these cells, as CD25 is a well-known activation marker of NK/NK-T cells and possibly of basophils (Fig. 4). Although the exact mechanism

is not known, secretion of soluble cytokines by activated T cells might induce CD25 on NK/NK-T cells and/or basophils in addition to rescuing these cells from apoptosis in culture. These data support our hypothesis that IL-4-expressing non-T cells, that is, basophils, NK, and NK-T cells, could explain the increased level of *IL-4* expression without a concomitant increase in *GATA-3* transcripts found in infants developing

food allergy (15, 16). Although the exact role of NK and NK-T cells in allergy is controversial (16–22) and remains to be elucidated, our results raise a question about the contribution of these cells in the early development of food allergy and require further investigation. In addition, basophils have recently been implicated to play an important role in priming and enhancing memory T_H2 responses (23, 24). Based on these data, we decided to focus on the potential role of basophils in the development of food allergy.

Expressions of different basophils surface markers were evaluated in a cohort of 42 peanut-allergic patients and compared with 12 non-atopic controls. We found a significantly higher expression of CD25 and CD63 on basophils from allergic patients compared with those of non-atopic controls ($p < 0.05$) (Fig. 3a and b). Although we found a trend toward a higher level of expression of CD203c and HLA-DR on basophils from allergic patients compared with non-atopic controls (Fig. 3c, d), this difference did not reach statistical significance. The up-regulation of CD25 and CD63 on basophils from allergic patients could reflect persistent activation of these cells after exposure to a specific allergen. It has long been observed that a substantial percentage of food-allergic patients have basophils that release histamine spontaneously without in vitro provocation (25–27). Interestingly, these patients reportedly do not express high baseline levels of CD63 (28). However, our data showed a high percentage of CD63⁺ basophils at baseline ($84.95\% \pm 2.5\%$ in allergic subjects and $76.5\% \pm 4.7\%$ in non-atopic controls). Similar results were found in a recent study of patients with hymenoptera allergy, showing a persistent activation, up to 6 months, after the initial sting (29). This could also reflect a higher sensitivity to degranulation of basophils from allergic patients, in response to non-specific stimuli during experimental processing (i.e., fixation prior to freezing cells). As stimulated basophils have been shown to secrete IL-4 (30–32), a higher reactivity or persistent activation could thus play a central role in the development of food allergy.

The lack of understanding of the induction of T_H2 immune responses reflects to a large extent our lack of understanding of the mechanisms of innate immune recognition of 'type 2 pathogens'. Three different groups identified MHC class II-producing basophils as the 'professional' APCs that were

necessary and sufficient for the generation of type 2 immunity (28, 33, 34). These findings were unexpected, as basophils have been thought to be MHC class II negative; however, these authors showed very convincingly that some activated basophils express MHC class II. In addition, Sokol et al. have shown that basophils are able to endocytose, process, and present an allergen (28). However, this point is still a matter of debate as all of these studies cited have been performed in mice and questions have been raised about the identity of mouse basophils (35). In addition, two recent studies including one with human subjects did not confirm a role for basophils as APCs for aeroallergens (36, 37). Our results demonstrated an increased expression of HLA-DR after 15 min in activated human basophils (CD25⁺CD203c⁺CD63⁺ cells) (Fig. 4c). The expression of HLA-DR antigens on the surface of immune cells is crucial for appropriate antigen presentation, suggesting that human activated basophils are capable of presenting antigens in food allergy.

In conclusion, our data support a key role for IL-4-expressing non-T cells in the early development of food allergy. Particularly, our current results suggest that basophils could direct T_H2 responses in humans by secreting IL-4 and presenting antigens. IL-4 is responsible for the MHC II expression on mast cells, and the same effect is likely for basophils as well. Basophils may function as accessory cells for DC-mediated T_H2 differentiation (38–40). This has to be confirmed by more specific studies. It also needs to be confirmed whether the early IL-4 response shown in allergic patients (be it basophils, NK cells or NK-T cells or any combination of the above) is important for priming clinical allergy. This would corroborate findings in animal models and suggest new targets for primary prevention of allergic disease.

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Conflict of interests

None declared.

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