Expression and significance of molecular profiles on eosinophils of children with food allergy

Junqiong Ding, Jia Hou, Danru Liu, Ying Wang, Xiaoming Wang, and Xiaochuan Wang

Abstract
Peripheral blood eosinophils may increase in food allergy (FA). However, the correlation between activation status of blood eosinophils and features of FA are unknown. We collected 25 cases of FA out-patients with increased number of eosinophils and 20 healthy children. Eosinophil surface markers were analyzed by flow cytometry. We found that CD23, CD44, CD54, and CRTH2 were positive on different eosinophils and that their expressions were increased in FA patients compared to the control individuals. There was a positive correlation with strong protein–protein interactions between the four eosinophil surface markers which were functionally involved in regulation of cell killing, interferon-gamma-mediated signaling pathway, and Epstein–Barr virus infection pathway. Thus, blood eosinophils change their phenotype during FA and induce significant gene regulation changes that may cause FA with eosinophilia. Detection of the expression of CD23, CD44, CD54, and CRTH2 on eosinophils can be used as indicators of FA with eosinophilia.

Keywords
children, eosinophils, food allergy, surface markers

Date received: 4 May 2019; accepted: 18 July 2019

Introduction
Food allergy (FA) is a typically chronic allergic inflammation, which involves multiple pathways and cellular components (CCs) in its pathogenesis. Previous studies have shown that more than 170 foods can cause IgE-mediated FA, but more than 90% of FA are caused by peanuts, nuts, eggs, milk, fish, crustaceans, wheat, and soybeans. FA can occur in individuals of all ages. A latest survey shows that the total incidence and the incidence of FA in children are 6.56% and 3.49%, respectively, and this seriously affects children’s normal growth and quality of life and causes huge economic losses. At present, IgE-mediated FA is relatively well understood, and the pathogenesis is relatively clear. However, the mechanism of non-IgE-mediated FA, particularly the role played by circulating eosinophils is unknown.

Eosinophils may participate in non-IgE-mediated FA, but their role in the pathogenesis of FA is unclear. The clinical features of FA are possibly influenced by surface molecules expressed in eosinophils. Eosinophils surface proteins, including CCR3 (CD193), CD44, CD23, intercellular adhesion molecule-1 (ICAM-1 or CD54), and CRTH2, have been reported to correlate with...
allergy disorders and are suggested to be the biomarkers of activation. Previous findings reported the expression of CD23, CD44, CD54, and CRTH2 on eosinophils, thus suggesting their involvement in the complex immunopathogenesis of allergy.

Herein, we had assessed the expression levels of the surface molecules on eosinophils in children with ongoing FA. The aim was to detect activation and changes in eosinophil surface markers to reflect different aspects of eosinophil activation in FA and to analyze the association between eosinophil surface molecules and eosinophilia in children with FA.

**Methods and materials**

The Allergy and Immunology Specialist Outpatient Service of Pediatric Hospital of Fudan University was the referral center for pediatric allergic disorders. The patients in this study were selected among children referred to this unit for evaluation of non-IgE-mediated FA between 1 July 2017 and 1 February 2018. We included children with a clinical history of FA following diagnosis, examination, and questionnaire analysis. Children without allergic disorders served as controls.

Twenty-five FA patients (median age: 11 months; range: 6 months–7 years; 68% males) with eosinophilia, and 20 control children (median age: 9 months; range: 5 months–9 years; 65% male) without allergic diseases were recruited. All FA patients had a peak count of 0.5 × 10⁹/L eosinophils, their absolute eosinophil count (AEC) ranged from (0.5–17.8) × 10⁹/L, the level of IgE ranged from (3.5–5890) KU/L in Table 1. There was no statistically significant difference in the age and gender of the subjects in each group. Written consent was obtained from the parents of all participants. Exclusion criteria were as follows: (1) patients with a primary diagnosis of allergic inflammatory diseases, (2) identification of an infectious disease, (3) systemic diseases associated with eosinophilia, and (4) all the control subjects having taken the glucocorticoid or anticoagulant drugs and immunosuppressants 2 weeks prior to the study.

**Blood samples**

Fresh peripheral venous blood samples were collected in ethylenediaminetetraacetic acid (EDTA) anticoagulation tube for diagnostic analysis of eosinophils before the starting of the treatment. The surface markers on eosinophils were analyzed using flow cytometry within 24 h after blood collection.

**Eosinophil isolation**

Eosinophils were isolated and purified according to a previous method. Briefly, most of the erythrocytes were removed from blood using dextran sedimentation, followed by centrifugation on a Ficoll gradient to isolate granulocytes. Then, eosinophils were isolated from granulocytes by immunomagnetic depletion of leukocytes expressing CD3, CD14, CD16, and CD19.

**Flow cytometry analysis**

Eosinophils were incubated with Vivaglobin (CSL Behring, PA, USA) for 20 min in the dark at 4°C. Subsequently, after staining with fluorescence of surface markers on eosinophils, anti-CD16 (BV510), anti-CCR3 (CD193, BV421), anti-CD23 (PE-Cy7), anti-CD44 (APC/Cy7), anti-CD54 (APC), and anti-CRTH2 (Percp-Cy5.5) antibodies (BD Bioscience, CA, USA), samples were washed twice with phosphate-buffered saline (PBS). A total of 10,000 events were acquired on the FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, and USA) and results were analyzed using FlowJo v10 software (Tree Star, OR, USA). Background staining was monitored using an isotype control antibody and a “fluorescence minus one” control. Eosinophils were identified with anti-human CCR3 and anti-human CD16 antibodies, with low or no CD16 expression and high CCR3 expression. Data were

| Characteristic FA | Control P-value |
|-------------------|-----------------|
| Number | 25 | 20 |
| Age (months) | 11 (6–84) | 9 (5–108) |
| Male | 17/25 (68%) | 14/20 (65%) |
| AEC (× 10⁹/L) | 8.4 (0.5–17.8) | 0.18 (0–0.4) |
| IgE (KU/L) | 3.5–5890 | 4.6–380 |
| CD23 (mFI) | 8129 ± 1971 | 3067 ± 267.1 |
| CD44 (mFI) | 9084 ± 528.1 | 4859 ± 343.3 |
| CD54 (mFI) | 9864 ± 1853 | 4724 ± 390.2 |
| CRTH2 (mFI) | 7973 ± 481.1 | 7264 ± 286.5 |
| EOS (CCR3⁺/CD16⁻)% | 31.55 ± 3.952 | 5.605 ± 0.623 |

Statistically significant differences between the groups were determined using the non-paired t test. FA: food allergy; NS: no significant difference; range: minimum to maximum; AEC: absolute eosinophil count; mFI: median fluorescence intensity. **** P < 0.0001.
expressed as median fluorescence intensity (mFI) and percentage (%).

**Quantitative reverse transcription PCR (RT-qPCR)**

Total RNA was extracted from peripheral blood using Trizol (TRI reagent, Ambion, Inc., Austin, TX) according to the manufacturer’s protocols and reverse transcribed to cDNA. The reaction was performed in the Thermal Cycler Dice Real Time System TP800 3.00 D system, and the Ct value of each template was measured. A sample with a small Ct value was selected as a standard to prepare a standard curve. The relative expression levels of CD23, CD44, CD54, and CRTH2 were determined by double-label curve method. β-actin was used as housekeeping gene. The primer sequences are as follows: CD23 forward: 5′-CTGCTTAAACCTGTCTCTACG-3′, reverse: 5′-GCTTGGATTCTCCCGATGATG-3′; CD44 forward: 5′-CCCACGGGATCTGAAACAGTG-3′, reverse: 5′-TTCTGGAAATTTGAGGTCTCCGTAT-3′; CD54 forward: 5′-ACTGTGTGCCCTATTCCAGGC-3′, reverse: 5′-GGCTCACTACAGACAGCACAT-3′; CRTH2 forward: 5′-CCCTGCTGCCCAGAGCCCCACGATGTCG-3′, reverse: 5′-CACGGCCGAAGAATGAGTGAAAGAAG-3′; β-actin forward: 5′-TGACGTGGACATCCGCAAAG-3′, reverse: 5′-CTGGAAGTGGAAGGCTGAGG-3′.

**Protein–protein interaction network**

The protein–protein interaction network was performed online using the STRING database (https://string-db.org/). The generated network was downloaded and used for visualization in Cytoscape software (http://www.cytoscape.org/).

**GO and KEGG enrichment analysis**

The functional enrichment analysis was performed using the clusterProfiler package (http://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html) in R programming software. GO enrichment analysis was performed to analyze the main functions of the eosinophil surface markers (CD23, CD44, CD54, and CRTH2) according to biological process (BP), CC, and molecular function (MF). KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis was used to identify some significant pathways in which are involved the eosinophil surface markers. Pathway annotation information of the entire array genes were downloaded from the KEGG database (http://www.genome.jp/kegg/). In both GO and KEGG enrichment analysis, Fisher’s exact test was used to identify the significant enrichment pathways and reported pathway categories with false discovery rate (FDR) < 0.05. As the degree of enrichment increases, the corresponding functions are more specific, which helps to identify more important pathways.

**Statistical analysis**

We used the GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA) for statistical analysis. To determine statistical significance, we used the one-way analysis of variance nonparametric t test. Pearson’s correlation analysis was performed using the Hmisc package (http://cran.r-project.org/web/packages/Hmisc/index.html) and the correlation heatmap was generated using the pheatmap package (http://cran.r-project.org/web/packages/pheatmap/index.html) in R software. A probability of 0.05 or less was considered statistically significant.

**Results**

**Clinical features of children**

The clinical features of children with non-IgE-mediated FA and healthy children who participated in this study are summarized in Table 1. The results showed that children with FA had higher AEC (8.4 (0.5–17.8) × 10⁹/L) and IgE than healthy children (0.18 (0–0.4) × 10⁹/L), with significant differences (P < 0.0001). The IgE content of children with FA (3.5–5890 KU/L) was significantly higher than that of healthy children (4.6–380 KU/L) (P < 0.0001).

**Increased expression of the eosinophil molecular markers in FA with eosinophilia**

The levels of the protein markers expressed by eosinophils in peripheral blood were measured using flow cytometry. Four surface marker molecules, CD23, CD44, CD54, and CRTH2, were studied. Figure 1(a) shows CD23, CD44, CD54, and CRTH2 expressions on eosinophils with their isotype control before being analyzed by flow cytometry. Histograms show fluorescence intensity. The results showed that, compared to the
control children, children with FA had a higher fraction of eosinophils that expressed CD23, CD44, CD54, and CRTH2 (Figure 1(b)). The frequency of CD23 (9.456% ± 0.43%), CD44 (95.96% ± 0.66%), CD54 (12.26% ± 0.84%), and CRTH2 (97.29% ± 0.51%) on the eosinophils (CD16−CCR3+) surface were increased in children with FA. As compared with control children, a higher frequency of eosinophils (31.55% ± 3.952%) that expressed a higher level of CD23, CD44, CD54, and CRTH2 (P < 0.0001) in children with FA was recorded (Table 1). As shown in Table 1, the mFI of CD54 (9864 ± 1853) was significantly higher than control group (4724 ± 390.2; P = 0.0188). The mFIs of CD23 and CD44 in children with FA were higher than control children (P < 0.0001). The mFI of CRTH2 was 7973 ± 481.1, which was not significantly different

Figure 1. Univariate analyses of eosinophil molecules that distinguish children with FA from healthy children: (a) CD54, CD23, CRTH2, and CD44 expression on eosinophils with their isotype matched control before being analyzed by using flow cytometry. Histograms show fluorescence intensity. (b) The fractions of blood eosinophils that express CD23, CD44, CD54, and CRTH2 on eosinophils derived from children with FA (n = 25) and healthy children (n = 20). (c) The expression levels of CD23, CD44, CD54, and CRTH2 were detected using RT-qPCR. Each study person is indicated by a symbol and the group medians are represented by horizontal bars. Statistically significant differences between the groups were determined using the non-paired t test. **P < 0.01, ****P < 0.0001 versus the control group.
compared to control group (7264 ± 286.5; \( P = 0.2412 \)). Furthermore, we verified the expression of CD23, CD44, CD54, and CRTH2 by RT-qPCR experiment. The results showed that the expression levels of CD23, CD44, CD54, and CRTH2 were significantly increased in FA compared with the control group (\( P < 0.01 \), Figure 1(c)). These results suggested that CD23, CD44, CRTH2, and CD54 are constitutively expressed in eosinophils surface, and their expressions are significantly up-regulated in children with FA.

**Evaluation of the correlation between the eosinophil surface markers from FA patients**

We used bioinformatics to further investigate the correlation between eosinophil surface markers (CD23, CD44, CD54, and CRTH2). The results indicated that eosinophil surface markers were not associated with the age of FA patients (\( P > 0.05 \), Figure 2(a)). Further protein–protein network analysis and Pearson’s correlation analysis revealed that there was a positive correlation between the...
Figure 3. GO and KEGG enrichment analysis of eosinophil surface markers. (a)–(c) GO enrichment analysis of four eosinophil surface markers. (d) KEGG pathway enrichment analysis of four eosinophil surface markers.

BP: biological process; CC: cellular component; MF: molecular function.
four eosinophil surface markers which strongly interact with each other (Figure 2(b) and (c)). There was a greatest correlation between CD44 and CRTH2 while the correlation between CD54 and CD23, and CRTH2 and CD44 was minimal.

**GO and KEGG enrichment analysis of eosinophil surface markers**

Since the four eosinophil surface markers (CD23, CD44, CD54, and CRTH2) were up-regulated in FA patients through flow cytometry and RT-qPCR experiments, we further performed the GO functional and KEGG pathway enrichment analysis of these surface markers. For GO enrichment analysis, the results indicated that regulation of cell killing and interferon-gamma-mediated signaling pathway were significantly enriched as BPs (Figure 3(a)). External side of plasma membrane, focal adhesion, cell-substrate adherens junction and cell-substrate junction were significantly enriched as CC (Figure 3(b)). In addition, integrin binding, prostanoid receptor activity, and icosanoid receptor activity were significantly enriched as MFs (Figure 3(c)). For the KEGG pathway enrichment analysis, as shown in Figure 3(d), Epstein–Barr virus infection was the most significant enriched pathway regulated by the activated molecular markers. Furthermore, we discovered, for the first time, that regulation of cell killing and interferon-gamma-mediated signaling pathways were involved in the progression of FA. As a limitation of the present study, the number of FA cases was limited. In addition, we only tested the eosinophilia in FA and the healthy individuals as control group. It remains unclear at present how valuable it is to measure activation markers of eosinophils in FA and we assume that it will be beneficial to further elucidate the pathophysiology of FA and therapeutic strategies for eosinophil-mediated food allergy.

**Discussion**

In this study, we found that the expression levels of CD23, CD44, CD54, and CRTH2 were significantly up-regulated in FA with eosinophilia, which indicated the activation status of eosinophils in FA. This was in corroboration with a previous study showing that eosinophils in patients with eosinophilic esophagitis have high expression of CD23, CD54, and CRTH2 compared to healthy controls. Furthermore, we found that the number of circulating eosinophils was lower in the control group compared to the FA group and was correlated with the expression levels of surface markers. This positive correlation suggested that the number of circulating eosinophils may influence the expression levels of eosinophil surface markers and their activation as previously reported. In addition, we found that eosinophil surface markers were not associated with the age of FA patients, while there was a positive correlation between the four eosinophil surface markers (CD23, CD44, CD54, and CRTH2) suggesting a coordinated molecular mechanism driven by their surface markers in the pathogenesis of FA. Early research found that Epstein–Barr virus infection has nothing to do with the pathogenesis of early childhood allergic diseases. However, recent studies have shown that Epstein–Barr virus viremia was one of the risk factors for the de novo FA. In this study, we observed that Epstein–Barr virus infection pathway was the most significant enriched pathway regulated by the activated molecular markers. Furthermore, we discovered, for the first time, that regulation of cell killing and interferon-gamma-mediated signaling pathways were involved in the progression of FA.

**Acknowledgements**

We thank all other members of the Laboratory of Clinical Immunology of Children’s Hospital of Fudan University.

**Declaration of conflicting interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**

The author(s) received no financial support for the research, authorship, and/or publication of this article.

**ORCID iD**

Xiaoming Wang https://orcid.org/0000-0002-1270-449X

**References**

1. Renz H, Brandzaeg P and Hornef M (2012) The impact of perinatal immune development on mucosal homeostasis and chronic inflammation. *Nature Reviews Immunology* 12(1): 9–23.

2. Valenta R, Hochwallner H, Linhart B et al. (2015) Food allergies: the basics. *Gastroenterology* 148(6): 1120–1131.e4.
3. Boyce JA, Assa’ad A, Burks AW et al. (2011) Guidelines for the diagnosis and management of food allergy in the United States: Summary of the NAID-sponsored expert panel report. *Nutrition Research* 31(1): 61–75.

4. McGowan EC and Keet CA (2013) Prevalence of self-reported food allergy in the national health and nutrition examination survey (NHANES) 2007-2010. *The Journal of Allergy and Clinical Immunology* 132(5): 1216–1219.e5.

5. Gupta R, Holdford D, Bilaver L et al. (2013) The economic impact of childhood food allergy in the United States. *JAMA Pediatrics* 167(11): 1026–1031.

6. Chinthrajah RS, Hernandez JD, Boyd SD et al. (2016) Molecular and cellular mechanisms of food allergy and food tolerance. *The Journal of Allergy and Clinical Immunology* 137(4): 984–997.

7. Na HJ, Hamilton RG, Klion AD et al. (2012) Biomarkers of eosinophil involvement in allergic and eosinophilic diseases: Review of phenotypic and serum markers including a novel assay to quantify levels of soluble Siglec-8. *Journal of Immunological Methods* 383(1–2): 39–46.

8. Johansson MW (2014) Activation states of blood eosinophils in asthma. *Clinical and Experimental Allergy: Journal of the British Society for Allergy* 44(4): 482–498.

9. Andersson J, Cromvik J, Ingelsten M et al. (2014) Eosinophils from hematopoietic stem cell recipients suppress allogeneic T cell proliferation. *Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation* 20(12): 1891–1898.

10. Johnsson M, Bove M, Bergquist H et al. (2011) Distinctive blood eosinophilic phenotypes and cytokine patterns in eosinophilic esophagitis, inflammatory bowel disease and airway allergy. *Journal of Innate Immunity* 3(6): 594–604.

11. Sidorchuk A, Lagarde F, Pershagen G et al. (2003) Epstein-Barr virus infection is not associated with development of allergy in children. *The Pediatric Infectious Disease Journal* 22(7): 642–647.

12. Sinitkul R, Manuyakorn W, Kamchaisatian W et al. (2018) De novo food allergy in pediatric liver transplantation recipients. *Asian Pacific Journal of Allergy and Immunology* 36(3): 166–174.