Early life innate immune signatures of persistent food allergy

Melanie R. Neeland, PhD,[a] Jennifer J. Koplin, PhD,[a] Thanh D. Dang, PhD,[a,b] Shyamali C. Dharmage, MBBS, MSc, MD, PhD,[a,c] Mimi L. Tang, MBBS, PhD, FRACP, FRCPA, FAAAAI,[a,b,d] Susan L. Prescott, MD, PhD, FRACP,[e] Richard Saffery, PhD,[a,b] David J. Martino, PhD,[a,b]* and Katrina J. Allen, MBBS, BMedSc, FRACP, FAAAI, PhD[a,b,d]* *Parkville and Perth, Australia*

Background: Food allergy naturally resolves in a proportion of food-allergic children without intervention; however the underlying mechanisms governing the persistence or resolution of food allergy in childhood are not understood.

Objectives: This study aimed to define the innate immune profiles associated with egg allergy at age 1 year, determine the phenotypic changes that occur with the development of natural tolerance in childhood, and explore the relationship between early life innate immune function and serum vitamin D.

Methods: This study used longitudinally collected PBMC samples from a population-based cohort of challenge-confirmed egg-allergic infants with either persistent or transient egg allergy outcomes in childhood to phenotype and quantify the functional innate immune response associated with clinical phenotypes of egg allergy.

Results: We show that infants with persistent egg allergy exhibit a unique innate immune signature, characterized by increased numbers of circulating monocytes and dendritic cells that produce more inflammatory cytokines both at baseline and following endotoxin exposure when compared with infants with transient egg allergy. Follow-up analysis revealed that this unique innate immune signature continues into childhood in those with persistent egg allergy and that increased serum vitamin D levels correlate with changes in innate immune profiles observed in children who developed natural tolerance to egg.

Conclusions: Early life innate immune dysfunction may represent a key immunological driver and predictor of persistent food allergy in childhood. Serum vitamin D may play an immune-modulatory role in the development of natural tolerance. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: *Food allergy, innate, monocytes, persistent, natural tolerance, endotoxin, cytokines, vitamin D, HealthNuts*

The burden of food allergy is increasing globally, particularly during infancy and early childhood.[1,2] Using criterion standard oral food challenge (OFC) outcomes, it was recently shown that 3% of 12-month-old infants were allergic to peanut and 9% to egg.[3,4] A proportion of these naturally outgrew their food allergy in childhood, with immunological tolerance developing in up to half of egg-allergic children by 2 years and 22% of peanut-allergic children by age 4 years.[5,6] The immunological mechanisms underlying the initial development of food allergy and the induction of natural tolerance in childhood are poorly understood.

Recent work suggests a role for the innate immune system in the development of allergic disease, including evidence that heightened inflammatory innate immune responses to both nonspecific[7,8] and antigen-specific[9] activation may be associated with the development of food allergy. Monocytes from cord blood of infants who go on to develop food allergy were recently shown to secrete higher levels of inflammatory cytokines in response to LPS.[8] These inflammatory cytokines skewed naive T cells toward an allergic T_H2 phenotype, suggesting a casual role for early life innate immune activation in predisposing to food allergy in infancy. However, it remained unclear whether these responses persisted into postnatal life and whether innate immune profiles were distinct between the clinical phenotypes of persistent and transient food allergy.

Furthermore, the development of natural tolerance to food allergens is poorly characterized in humans. Exposure to vitamin D has previously been strongly associated with tolerogenic immune function, acting through diverse immunological pathways to skew the immune response toward a regulatory phenotype.[10] In fact, infants with vitamin D insufficiency are 3 times more likely to have egg allergy and 11 times more likely to have peanut allergy,[11] suggesting that early life vitamin D plays an important role in immune tolerance. However, the mechanistic

From [a]Murdoch Childrens Research Institute, [b]the Department of Paediatrics, University of Melbourne, [c]the Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, University of Melbourne, and [d]the Department of Allergy and Immunology, Royal Children's Hospital, Parkville; and [e]Telethon Kids Institute, The University of Western Australia, Perth.

*These authors contributed equally to this work.

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Corresponding author: Katrina J. Allen, MBBS, BMedSc, FRACP, FAAAI, PhD, Murdoch Children's Research Institute, Department of Allergy and Immunology, The Royal Children's Hospital, Flemington Rd, Parkville, VIC 3052, Australia. E-mail: katie.allen@rch.org.au.

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Abbreviations used
CD: Cluster of differentiation
DC: Dendritic cell
FACS: Fluorescence-activated cell sorting
OFC: Oral food challenge
SPT: Skin prick test

relationship between early life vitamin D and immune function in pediatric food allergy is unclear.

Using novel longitudinally collected samples from a population-based cohort of challenge-confirmed egg-allergic 1-year-old infants with either persistent or transient egg allergy outcomes in childhood, we aimed to define the innate immune profiles associated with egg allergy at age 1 year, determine the phenotypic changes that occur with the development of natural tolerance in childhood, and explore the relationship between early life innate immune function and serum vitamin D.

METHODS

Subjects and study design

A total of 54 subjects from the HealthNuts cohort[4] were used in this study (n = 36 1-year-old infants who were egg-allergic at age 1 year; n = 18 1-year-old infants with no food allergy at age 1 year). Table I describes the demographic and clinical characteristics of the selected cohort. Egg-allergic subjects (n = 36) had challenge-confirmed egg allergy[12] at age 1 year, follow-up egg allergy status (age 2-4 years), and blood samples collected at both age 1 year and at follow-up for longitudinal analysis. All egg-allergic subjects had negative skin prick test (SPT) result (<2 mm) and specific IgE (<0.35 kUA/L) to both peanut and sesame at age 1 year. Persistent egg-allergic (n = 14) or transient egg-allergic (n = 22) subjects were classified according to egg allergy status at follow-up (positive at follow-up, persistent egg allergy; negative at follow-up, transient egg allergy). Only subjects with both a positive oral egg challenge[12] and an SPT wheal size of 2 mm or more or a specific IgE level of 0.35 kUA/L or more were classified as egg-allergic. Selection criteria for non–food-allergic control 1-year-old infants (n = 18) were SPT wheal sizes of less than 2 mm and specific IgE level of less than 0.35 kUA/L to all foods at the clinic and a negative OFC to egg or peanut at age 1 year. OFCs were performed as described previously,[12] and serum-specific IgE level was measured using the ImmunoCAP System FEIA (Phadia AB, Uppsala, Sweden). Eczema at age 1 year was defined as either a parent report of doctor-diagnosed eczema requiring topical steroid treatment or eczema observed by a trained nurse.

Blood collection and measurements of vitamin D

Blood was collected at clinic appointments 2 hours following egg OFC for all subjects, excluding a subset of 10 egg-allergic infants who had blood collected at a clinic appointment that did not include an OFC (non-OFC day) and were subsequently challenged to egg the following week. To determine whether collecting blood immediately following OFC influenced immune parameters investigated in this study, we compared results from blood samples of egg-allergic individuals taken following OFC (n = 26) with those taken on a non-OFC day (n = 10). There were no significant differences in any innate immune parameter between the blood samples that were taken following OFC and the blood samples taken on a non-OFC day (see Fig E1 in this article's Online Repository at www.jacionline.org). PBMCs were isolated by density gradient and cryopreserved using protocols previously described.[13] Serum vitamin D ($25(OH)D_3$) level was measured using liquid chromatography-tandem mass spectrometry at the Royal Melbourne Institute of Technology as previously described.[14] Vitamin D deficiency was defined as $25(OH)D_3$ level of 50 nmol/L or less.[15]

Preparation of PBMCs for cell sorting

PBMCs collected at age 1 year and at follow-up for each subject were randomly selected to avoid batch effects and thawed using warm RPMI (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS (HyClone), centrifuged at 500*g*, and washed twice before viability count. Mean PBMC viability after thawing as determined by trypan blue exclusion was 87%. Cell pellets were resuspended at 1×10^6/mL in PBS and 0.5 μL of fixable viability stain 510 (BD Biosciences, North Ryde, Australia) was added per milliliter of cell suspension. Cells were incubated at room temperature for 15 minutes protected from light, washed twice in fluorescence-activated cell sorting (FACS) buffer (2% FCS, 2 mM EDTA in PBS), and resuspended in 50 μL FACS buffer for antibody staining. Fifty microliter of antibody cocktails made up at 2× (see Table E1 in this article's Online Repository at www.jacionline.org) was added 1:1 to the resuspended cells and incubated on ice for 30 minutes. Cells were washed and resuspended in 300 μL of FACS buffer for cell sorting. Live, cluster of differentiation 3 (CD3)-negative cells were sorted using an Influx Cell Sorter (BD Biosciences).

Flow cytometry analysis and cell culture

The CD3-negative cell fraction was then used to (1) quantify and phenotype innate cell populations in peripheral blood and (2) quantify the resting and stimulated cytokine responses to LPS in cell culture supernatant. To assess innate cell populations, 6×10^5 CD3-negative cells were washed in FACS buffer and prepared for flow cytometry as described above. Count bright beads (Thermo Fisher Scientific, Scoresby, Australia) were added to stained cells before sample acquisition on an LSR II X-20 Fortessa (BD Biosciences) to allow enumeration of absolute cell numbers. For all flow cytometry experiments, compensation was performed at the time of sample acquisition using compensation beads (BD Biosciences) and all results analyzed using FlowJo Version 10 software (FlowJo LLC).

For innate cell phenotyping, lineage-negative ($CD3^-$ $CD19^-$ $CD20^-$ $CD56^-$) cells were first excluded by appropriate gating analysis. Classical monocytes ($HLADR^+CD14^+CD16^-$), nonclassical monocytes ($HLADR^+CD14^{low}CD16^+$), intermediate monocytes ($HLADR^+CD14^+$ $CD16^+$), myeloid dendritic cells (DCs) ($HLADR^+CD14^-CD16^-CD11c^+CD123^-$), and plasmacytoid DCs ($HLADR^+CD14^-CD16^-CD11c^-CD123^+$) were quantified using the innate cell antibody cocktail described in Table E1.

Where cell number permitted, remaining freshly sorted CD3-negative cells were resuspended at 5×10^5/mL in RPMI supplemented with 10% FCS. Cells were then cultured in duplicate in 96-well plates with media alone or LPS (1 ng/mL) for 24 hours at 37°C, 5% CO_2. Cell culture supernatants were harvested and frozen at −80°C for later quantification of IL-12p70, TNF, IL-6, IL-1β, IL-8, and IL-10 by cytometric bead array. Cell culture supernatants were thawed and prepared for cytometric bead array analysis using the Human Inflammatory Cytokine bead array Kit (BD Biosciences) according to manufacturer's instructions. Cytometric bead array data were acquired on an LSR II X-20 Fortessa (BD Biosciences) and analyzed using LEGENDplex software (BioLegend, San Diego, Calif).

Statistical analysis

Results are presented as box and whisker plots (where the box represents the 25th to 75th percentiles, the line in the box represents the median, and the whiskers extend to minimum and maximum values) or mean ± SEM. For cross-sectional analysis, a Mann-Whitney *U* test (2-tailed) was performed to compare between 2 groups and a Kruskal-Wallis test with Dunn's multiple comparisons test was performed to compare between more than 2 groups. For all cytokine and longitudinal analyses, differences between groups and within each group over time were calculated with a 2-way repeated-measured ANOVA using a Holm-Sidak posttest to correct for multiple comparisons. For correlations, a 2-tailed Spearmen test was performed. To determine significance between proportions in 2 populations, a 2-proportion *z* test was used. Significance was determined as a *P* value of less than .05. All statistical analyses were performed using GraphPad Prism, version 6.01 (GraphPad Software, Inc, La Jolla, Calif).