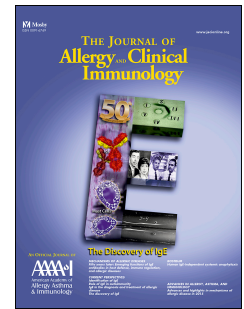


Accepted Manuscript

Early life innate immune signatures of persistent food allergy

Melanie R. Neeland, PhD, Jennifer J. Koplin, PhD, Thanh D. Dang, PhD, Shyamali C. Dharmage, MBBS, MSc, MD, PhD, Mimi L. Tang, MBBS, PhD, FRACP, FRCPA, FAAAAI, Susan L. Prescott, MD, PhD, FRACP, Richard Saffery, PhD, David J. Martino, PhD, Katrina J. Allen, MBBS, BMedSc, FRACP, FAAAAI, PhD



PII: S0091-6749(17)31754-2

DOI: [10.1016/j.jaci.2017.10.024](https://doi.org/10.1016/j.jaci.2017.10.024)

Reference: YMAI 13117

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 14 April 2017

Revised Date: 5 October 2017

Accepted Date: 10 October 2017

Please cite this article as: Neeland MR, Koplin JJ, Dang TD, Dharmage SC, Tang ML, Prescott SL, Saffery R, Martino DJ, Allen KJ, Early life innate immune signatures of persistent food allergy, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2017.10.024.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Early life innate immune signatures of persistent food allergy

Melanie R. Neeland PhD¹, Jennifer J. Koplin PhD¹, Thanh D. Dang PhD^{1,2}, Shyamali C. Dharmage MBBS, MSc, MD, PhD^{1,3}, Mimi L. Tang MBBS, PhD, FRACP, FRCPA, FAAAAI^{1,2,4}, Susan L. Prescott MD, PhD, FRACP⁵, Richard Saffery PhD^{1,2}, David J. Martino PhD^{1,2,†} and Katrina J. Allen MBBS, BMedSc, FRACP, FAAAAI, PhD^{1,2,4,†,*}

Affiliations:

¹Murdoch Childrens Research Institute, Parkville, VIC, Australia.

²Department of Paediatrics, University of Melbourne, Parkville, VIC, Australia.

³Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, University of Melbourne, VIC, Parkville Australia.

⁴Department of Allergy and Immunology, Royal Children's Hospital, Parkville, VIC, Australia

⁵Telethon Kids Institute, The University of Western Australia, Perth, WA, Australia.

[†] Contributed equally

*Corresponding author:

Professor Katrina J. Allen

Murdoch Childrens Research Institute

Department of Allergy and Immunology, The Royal Children's Hospital

Flemington Road, Parkville, Victoria 3052 AUS

katie.allen@rch.org.au

Phone: +613 9936 6585

Funding: The HealthNuts study is supported by funding from the National Health and Medical Research Council of Australia (NHMRC), the Ilhan Food Allergy Foundation, AnaphylaxiStop, the Charles and Sylvia Viertel Medical Research Foundation and the Victorian Government's Operational Infrastructure Support Program. The immunological work is supported by funding to MN from the Murdoch Childrens Research Institute and the NHMRC Centre for Food and Allergy Research.

Abstract

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 one year of age, 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, characterised by increased numbers of circulating monocytes and dendritic cells that produce more inflammatory cytokines both at baseline and following endotoxin exposure when compared to 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.

Conclusion: 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 immunomodulatory role in the development of natural tolerance.

Key Messages:

- Altered innate immune function in the first year of life is not only associated with active food allergy in infancy but may represent a key immunological driver and predictor of persistent food allergy outcomes in childhood
- Infants with persistent food allergy show elevated numbers of circulating monocytes and dendritic cells that uniquely produce more IL-1 β and IL-8 at baseline and more TNF- α and IL-8 following *in vitro* endotoxin exposure when compared to infants with transient allergy that resolves in childhood
- Increased serum vitamin D was associated with innate immune profiles during the natural resolution of food allergy

Capsule Summary: Identification of an early life innate immune signature that may predict persistent food allergy outcomes in childhood and the immunomodulatory role of vitamin D during the development of natural tolerance.

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

Abbreviations:

ANOVA: analysis of variance
 CBA: cytometric bead array
 CD: Cluster of Differentiation
 DCs: dendritic cells
 HLA: Human Leukocyte Antigen
 Ig: Immunoglobulin
 IL: Interleukin
 LPS: lipopolysaccharide
 MFI: Mean fluorescence intensity
 sIgE: specific IgE
 SPT: skin prick test
 Th: T helper
 TNF: Tumour Necrosis Factor
 TLR: toll like receptor

Introduction

The burden of food allergy is increasing globally, particularly during infancy and early childhood^{1, 2}. Using gold standard oral food challenge 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 4 years of age^{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 non-specific^{7, 8} and antigen specific⁹ 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 lipopolysaccharide (LPS)⁸. These inflammatory cytokines skewed naive T cells toward an allergic Th2 phenotype, suggesting a casual role for early life innate immune activation in predisposing to food allergy in infancy. However, it remained unclear if these responses persisted into postnatal life and if 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 characterised 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 phenotype¹⁰. In fact, infants with vitamin D insufficiency are 3 times more likely to have egg allergy and 11 times more likely to have peanut allergy¹¹, suggesting that early life vitamin D plays an important role in immune tolerance. However,

105 the mechanistic relationship between early life vitamin D and immune function in paediatric
106 food allergy is unclear

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

Materials and Methods

Subjects and study design

A total of 54 subjects from the HealthNuts cohort⁴ were used in this study (n=36 one year old infants who were egg allergic at one year of age; n=18 one year old infants with no food allergy at one year of age). Table 1 describes the demographics and clinical characteristics of the selected cohort. Egg allergic subjects (n=36) had challenge-confirmed egg allergy¹² at one year of age, follow up egg allergy status (age two-four years), and blood samples collected at both one year of age and at follow up for longitudinal analysis. All egg allergic subjects had negative SPT (<2mm) and sIgE (<0.35kUA/L) to both peanut and sesame at one year of age. 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 challenge¹² and a skin prick test (SPT) wheal size of 2mm or more or a specific IgE level of 0.35kUA/L or more were classified egg allergic. Selection criteria for non-food allergic control one year old infants (n=18) were SPT wheal sizes of <2mm and specific IgE of <0.35kUA/L to all foods at the clinic and a negative oral food challenge to egg or peanut at one year of age. Oral food challenges were performed as described previously¹², and serum-specific IgE was measured using the ImmunoCAP System FEIA (Phadia AB). Eczema at one year of age 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 n=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 bloods that were taken following OFC vs the bloods taken on a non-OFC day (Supplementary Figure 1). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient and cryopreserved using protocols previously described¹³. Serum vitamin D (25(OH)D₃) level was measured using liquid chromatography-tandem mass spectrometry at the Royal Melbourne Institute of Technology as previously described¹⁴. Vitamin D deficiency was defined as 25(OH)D₃ level of $\leq 50\text{nmol/L}$ ¹⁵.

Preparation of PBMCs for cell sorting

PBMCs collected at age one 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 x 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 $1 \times 10^6/\text{ml}$ in PBS and 0.5 μl of fixable viability stain 510 (BD Biosciences) was added per ml of cell suspension. Cells were incubated at room temperature for 15 minutes protected from light, washed twice in FACS buffer (2% FCS, 2mM EDTA in PBS) and resuspended in 50 μl FACS buffer for antibody staining. 50 μl of antibody cocktails made up at 2X (Supplementary Table 1) were 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, 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 lipopolysaccharide (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) were added to stained cells prior to 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 analysed 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 ($HLADR^+ CD14^+ CD16^-$), non-classical 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 Supplementary Table 1.

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 (1ng/ml) for 24h at 37°C, 5% CO₂. Cell culture supernatants were harvested and frozen at -80°C for later quantification of IL-12p70, TNF, IL-6, IL-1 β , IL-8 and IL-10 by cytometric bead array (CBA). Cell culture supernatants were thawed and prepared for CBA analysis using the Human Inflammatory Cytokine bead array Kit (BD Biosciences) according to manufacturer's instructions. CBA data was acquired on an LSR II X-20 Fortessa (BD Biosciences) and analysed using LEGENDplex software (BioLegend).

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 $\pm$ SEM. For cross-sectional analysis, a Mann-Whitney U-test (two tailed) was performed to compare between two groups and a Kruskal-Wallis test with Dunn's multiple comparisons test was performed to compare between more than two groups. For all cytokine and longitudinal analyses, differences between groups and within each group over time were calculated with a two-way repeated-measured ANOVA using a Holm-Sidak post-test to correct for multiple comparisons. For correlations, a two-tailed Spearman test was performed. To determine significance between proportions in two populations, a 2-proportion z test was used. Significance was determined as $p < 0.05$. All statistical analyses were performed using GraphPad Prism, version 6.01.

Ethics

Approval to conduct the HealthNuts study was obtained from the Victorian State Government Office for Children (reference no. CDF/07/492), the Victorian State Government Department of Human Services (reference no. 10/07), and the Royal Children's Hospital Human Research Ethics Committee (reference no. 27047).

Results

Characterising the innate immune response of egg allergy in infancy

To determine the innate response of egg allergic and healthy one year old infants, multiplex flow cytometry was performed to quantify monocyte (classical, intermediate and non-classical) and dendritic cell (DC; myeloid and plasmacytoid) subsets (Figure 1A) in PBMCs from all one year old infants. Multiplex cytometric bead arrays were also used to quantify the production of TNF, IL-6, IL-1 β , IL-8, IL-12p70 and IL-10 in CD3-depleted mononuclear cells following 24h resting in culture or stimulated with 1ng/ml of LPS.

Cross-sectional analysis revealed a significant increase in the numbers of circulating classical monocytes (7.9×10^4 cells/ml vs 2.9×10^4 cells/ml, $p=0.0015$), intermediate monocytes (5.6×10^3 cells/ml vs 2×10^3 cells/ml, $p=0.035$) and myeloid DCs (1.2×10^4 cells/ml vs 9.4×10^3 cells/ml, $p=0.048$) in egg allergic one year old infants compared with non-allergic controls (Figure 1B_{I-IV}). Significantly increased expression of HLA-DR was also observed on monocytes from egg allergic infants when compared with non-allergic controls (Figure 1C). Mean fluorescence intensity (MFI) of HLA-DR was assessed in classical monocytes (6.4×10^3 vs 3.7×10^3 , $p=0.04$), non-classical monocytes (6.7×10^3 vs 3.1×10^3 , $p=0.019$) and intermediate monocytes (13×10^3 vs 5×10^3 , $p=0.012$) from egg allergic infants compared with non-allergic controls. HLA-DR expression on myeloid DCs was comparable between groups (10.8×10^3 vs 9.4×10^3 , $p=0.33$) (Figure 1C).

Egg allergy was also characterised by significantly higher baseline production of the inflammatory cytokine IL-8 and significantly lower baseline production of Th1 associated (IL-12p70) and regulatory (IL-10) cytokines in unstimulated CD3-depleted cells relative to non-allergic infants (Figure 2). LPS-stimulated CD3-depleted cells from egg allergic one year

old infants produced significantly more TNF- α , IL-6, IL-1 β , IL-8 and IL-10 when compared with non-allergic controls (Figure 2).

Persistent egg allergy is associated with a distinct innate immune signature in infancy and at follow up

We next sought to determine if the altered innate signatures observed in egg allergic one year old infants were associated with allergy outcomes in later childhood. To do this, we stratified the egg allergic infants by their subsequent egg allergy status at follow up and performed cross-sectional analysis comparing immune responses at one year of age. Infants with subsequent persistence of egg allergy exhibited significantly higher numbers of classical monocytes ($p=0.0004$), non-classical monocytes ($p=0.044$), intermediate monocytes ($p=0.035$) and myeloid DCs ($p=0.021$) relative to non-allergic one year old controls (Figure 3A_{I-IV}). In contrast, infants with subsequent transient egg allergy (clinical tolerance to egg at follow up) showed significantly higher numbers of circulating classical monocytes only (not other monocyte subsets or myeloid DCs) relative to non-allergic controls at one year of age ($p=0.027$) (Figure 3A_I).

Reduced baseline production of IL-12p70 by unstimulated CD3-depleted cells at one year of age was a common feature of both persistent and transient egg allergy when compared to healthy controls ($p<0.01$), however higher baseline levels of IL-8 and IL-1 β were unique to infants who go on to develop persistent egg allergy ($p<0.05$) (Figure 3B-C). Following LPS stimulation, CD3-depleted cells from infants with both persistent and transient egg allergy produce significantly more IL-6 and IL-1 β when compared with healthy controls (Figure 3B). However, increased production of TNF- α and IL-8 were unique to infants who go on to develop persistent egg allergy when compared to both non-allergic controls ($p<0.001$ and $p<0.001$, respectively) and infants with transient allergy ($p<0.001$, and $p<0.05$, respectively)

(Figure 3B). Both phenotypes of egg allergy were characterised by reduced production of IL-12p70 following LPS stimulation, however increased production of LPS-induced IL-10 was unique to persistent egg allergy when compared to non-allergic controls ($p < 0.01$) (Figure 3C).

Immune cell profiles and cytokine production were also longitudinally analysed in matched samples at one year of age and at follow up from children with persistent or transient egg allergy (Figure 4). We observed that children with transient egg allergy showed a significant increase in the numbers of myeloid DCs from baseline to follow up (1.5×10^4 cells/ml vs 2.2×10^4 cells/ml $p = 0.033$) but this was not seen in those with persistent egg allergy (Figure 4A_{IV}). At follow up, children with persistent egg allergy continued to show significantly more IL-6, IL-1 β and IL-8 from unstimulated CD3⁻depleted cells compared to children who developed tolerance to egg at follow up ($p = 0.002$, $p = 0.006$, and $p = 0.026$, respectively), whilst no difference was seen in TNF, IL-12p70 and IL-10 (Figure 4B-C).

Serum vitamin D levels are uniquely associated with innate cell profiles in children with transient egg allergy

Given that vitamin D has previously been shown to modulate the allergic immune response^{16, 17}, and is a key regulator of innate immune cells¹⁰, we next sought to investigate the relationship between serum vitamin D concentration, innate responses and the clinical phenotypes of egg allergy. In Table 1, we show that children with persistent egg allergy demonstrate higher rates of vitamin D deficiency (≤ 50 nmol/L) at follow up compared to children with transient egg allergy (54% vs 13%, $p = 0.027$). Serum vitamin D levels at follow up inversely correlated with proportions of classical monocytes ($r = -0.709$, $p = 0.027$) and positively correlated with proportions of myeloid DCs ($r = 0.806$, $p = 0.007$) in children with transient egg allergy; however, these associations were not observed in children with

274 persistent egg allergy (Figure 5A-B). No significant correlations were observed between
275 serum vitamin D and cytokine production at baseline or following LPS stimulation at either
276 time point (data not shown).

277 *Innate signatures of egg allergy are not driven by eczema at one year of age*

278 Given early eczema is a strong risk factor for the development of egg allergy, we sought to
279 determine if the immune profiles observed in egg allergic one year old infants were
280 influenced by the presence of eczema. We found that 47% of infants with persistent egg
281 allergy, 40% of infants with transient egg allergy and 25% of non-allergic infants had eczema
282 at one year of age (Table 1). Significant increases in circulating innate cell numbers were
283 observed only in infants with egg allergy without eczema and not observed in infants with
284 both egg allergy and eczema (Supplementary Figure 2). Infants with persistent egg allergy
285 without eczema had elevated numbers of classical ($p=0.0003$), non-classical monocytes
286 ($p=0.02$) and intermediate monocytes ($p=0.027$), and myeloid DCs ($p=0.002$) relative to no
287 egg allergy no eczema controls. This was also observed in infants with transient egg allergy
288 without eczema who had elevated numbers of classical monocytes when compared to no egg
289 allergy no eczema controls ($p=0.02$) (Figure 5A). No significant differences in numbers of
290 monocyte and dendritic cell subsets were observed in one year old infants with eczema alone
291 (without egg allergy) compared with non-egg allergic, no eczema controls (Supplementary
292 Figure 2).

Discussion

This is the first study to investigate the innate immune response of infants and children with carefully defined clinical phenotypes of egg allergy. We show that altered innate immune function in the first year of life is not only associated with active food allergy in infancy but may represent a key immunological driver and predictor of a persistent food allergy outcome in childhood. Infants with persistent food allergy showed heightened inflammatory cytokine responses both at baseline and following *in vitro* endotoxin (LPS) exposure when compared to infants with transient egg allergy. Follow up analysis revealed that elevated baseline production of inflammatory cytokines continues into childhood in those with persistent food allergy and is not observed in children whose allergy had resolved by this time. We also showed that innate immune profiles observed in children who resolve their food allergy significantly correlate with their serum vitamin D levels, revealing a relationship between serum vitamin D and the development of natural tolerance in childhood.

Previous work has revealed that an inflammatory cord blood environment is associated with the development of food allergy in the first year of life⁸. Here, we extend these findings to show that innate immune dysfunction is also observed in postnatal life of one-year-old infants with egg allergy, characterised by increased numbers of circulating monocytes that express higher levels of HLA-DR and elevated production of TNF- α , IL-6, IL-1 β and IL-8 following *in vitro* endotoxin exposure relative to non-allergic healthy infants. To our knowledge we are the first to report these findings in the context of challenge-confirmed paediatric food allergy, however a similar monocyte-driven innate profile was recently observed in older asthmatic children. A study comparing the innate profiles of children from two genetically similar U.S. farming populations – the Amish and the Hutterites – showed that monocytes from Hutterite children, who show strikingly higher rates of asthma, exhibited a pro-inflammatory

phenotype that included increased HLA-DR expression, reduced expression of the regulatory receptor ILT-3 and heightened cytokine production following LPS stimulation¹⁸.

We additionally show that early life innate immune function differs between the clinical phenotypes of food allergy, with infants whose allergy persists into childhood exhibiting significantly elevated number of monocytes and myeloid DCs at one year of age, that uniquely produce more IL-1 β and IL-8 at baseline and more TNF- α and IL-8 following *in vitro* endotoxin exposure when compared to infants with transient allergy that resolves in childhood. A recent study showed that genes associated with monocyte and toll-like receptor 4 (TLR-4) activation were upregulated in children who are raw egg allergic and also reactive to baked egg⁹. Interestingly, we have previously shown that infants with both raw and baked egg allergy are less likely to resolve their allergy by 2 years of age compared with infants who are raw egg allergic but tolerant to the baked form⁵. Our current findings add to the small but growing body of evidence implicating monocyte function, TLR-4 activation and heightened inflammatory responses in the development and maintenance of childhood allergic disease, including food allergy.

The strengths of this study include the longitudinally collected samples from a population-based cohort of clearly phenotyped persistent and transient egg allergic children, with food allergy outcomes confirmed via both SPT and oral food challenge at age one and follow up. It should be taken into account, however, that a positive food challenge could influence some of the innate immune parameters investigated in this study. Indeed, IgE cross-linking on monocytes enhances monocyte proinflammatory processes, including increased CD14 expression and secretion of TNF- α and IL-6¹⁹. Here, we showed no differences in innate activation or cytokine production in egg allergic infants that had bloods taken on a non-challenge day vs an active challenge day, consistent with a previous report by our group showing no changes in plasma TNF- α and IL-6 levels pre or post oral food challenge²⁰. In

addition, the similar levels of TNF- α and IL-6 in unstimulated cell culture conditions between egg allergic and non-allergic infants argues against IgE-dependent monocyte activation post challenge as an explanation for our study findings. Due to limited cell numbers, we could not perform allergen-specific co-culture experiments to determine if activated monocytes and/or DCs from our egg allergic one year old infants are directly responsible for the Th2 allergic immune skew associated with food allergy. These are important experiments that will be required to determine if these cells can be targeted to reduce inflammation for the prevention of allergic disease.

Reduced vitamin D in early life has also been associated with increased risk of eczema²¹, asthma²² and food allergy^{11, 14}. However, due to limited studies that collect well-defined clinical measures of allergy, serum vitamin D measurements and detailed immunological responses, the relationship between early life vitamin D and immune function in paediatric food allergy is unclear. We found that increased serum vitamin D was associated with circulating numbers of monocytes and myeloid DCs during the natural resolution of egg allergy, suggesting that these innate cell types may be targets of early life vitamin D immune-modulation. A previous study has shown that infants at high risk of allergy with increased serum vitamin D at birth produced less inflammatory cytokines following *in vitro* allergen stimulation¹⁶. However, follow up studies are required to determine if vitamin D can directly act on innate cells to reduce inflammatory cytokine production in egg allergic infants. Randomised controlled trials of postnatal vitamin D supplementation are also required to confirm if vitamin D plays a causal role in the onset or resolution of early life food allergy²³.

In conclusion, we show that infants with persistent food allergy demonstrate a unique innate immune signature in the first year of life, characterised by increased numbers of circulating monocytes and dendritic cells that produce more inflammatory cytokines both at baseline and following endotoxin exposure when compared to infants with transient egg allergy. This

could have important implications for current patient care, assisting in the early identification of high risk infants whose food allergy is likely to persist into childhood. Our results also reveal a novel relationship between the early life circulating innate immune profile, serum vitamin D and the natural resolution of food allergy.

Acknowledgements: We thank the children and parents who participated in the HealthNuts study. We also thank current and past staff for recruiting and maintaining the cohort and processing the data and biospecimens.

Author contributions: MN, RS, DM and KA were involved in the conception and design of the study, MN performed the experiments, MN, JK, TD, RS, DM, and KA analysed and interpreted the results; all authors drafted and provided intellectual input into the manuscript.

379 **References**

- 380 1. Prescott S, Allen KJ. Food allergy: riding the second wave of the allergy epidemic.
381 *Pediatr Allergy Immunol* 2011; 22:155-60.
- 382 2. Tang ML, Osborne N, Allen K. Epidemiology of anaphylaxis. *Curr Opin Allergy Clin*
383 *Immunol* 2009; 9:351-6.
- 384 3. Osborne NJ, Koplin JJ, Martin PE, Gurrin LC, Lowe AJ, Matheson MC, et al.
385 Prevalence of challenge-proven IgE-mediated food allergy using population-based
386 sampling and predetermined challenge criteria in infants. *J Allergy Clin Immunol*
387 2011; 127:668-76 e1-2.
- 388 4. Koplin JJ, Wake M, Dharmage SC, Matheson M, Tang ML, Gurrin LC, et al. Cohort
389 Profile: The HealthNuts Study: Population prevalence and environmental/genetic
390 predictors of food allergy. *Int J Epidemiol* 2015; 44:1161-71.
- 391 5. Peters RL, Dharmage SC, Gurrin LC, Koplin JJ, Ponsonby AL, Lowe AJ, et al. The
392 natural history and clinical predictors of egg allergy in the first 2 years of life: a
393 prospective, population-based cohort study. *J Allergy Clin Immunol* 2014; 133:485-
394 91.
- 395 6. Peters RL, Allen KJ, Dharmage SC, Koplin JJ, Dang T, Tilbrook KP, et al. Natural
396 history of peanut allergy and predictors of resolution in the first 4 years of life: A
397 population-based assessment. *J Allergy Clin Immunol* 2015; 135:1257-66 e1-2.
- 398 7. Tulic MK, Hodder M, Forsberg A, McCarthy S, Richman T, D'Vaz N, et al.
399 Differences in innate immune function between allergic and nonallergic children: new
400 insights into immune ontogeny. *J Allergy Clin Immunol* 2011; 127:470-8 e1.
- 401 8. Zhang Y, Collier F, Naselli G, Saffery R, Tang ML, Allen KJ, et al. Cord blood
402 monocyte-derived inflammatory cytokines suppress IL-2 and induce nonclassic
403 "T(H)2-type" immunity associated with development of food allergy. *Sci Transl Med*
404 2016; 8:321ra8.
- 405 9. Kosoy R, Agashe C, Grishin A, Leung DY, Wood RA, Sicherer SH, et al.
406 Transcriptional Profiling of Egg Allergy and Relationship to Disease Phenotype.
407 *PLoS One* 2016; 11:e0163831.
- 408 10. Suaini NH, Zhang Y, Vuillermine PJ, Allen KJ, Harrison LC. Immune Modulation by
409 Vitamin D and Its Relevance to Food Allergy. *Nutrients* 2015; 7:6088-108.
- 410 11. Allen KJ, Koplin JJ, Ponsonby AL, Gurrin LC, Wake M, Vuillermine P, et al. Vitamin
411 D insufficiency is associated with challenge-proven food allergy in infants. *Journal of*
412 *Allergy and Clinical Immunology* 2013; 131:1109-+.
- 413 12. Koplin JJ, Tang ML, Martin PE, Osborne NJ, Lowe AJ, Ponsonby AL, et al.
414 Predetermined challenge eligibility and cessation criteria for oral food challenges in
415 the HealthNuts population-based study of infants. *J Allergy Clin Immunol* 2012;
416 129:1145-7.
- 417 13. Dang TD, Allen KJ, Martino D, Koplin JJ, Licciardi PV, Tang ML. Food allergic
418 infants have impaired regulatory T cell responses following in vivo allergen exposure.
419 *Pediatr Allergy Immunol* 2016; 27:35-43.
- 420 14. Koplin JJ, Suaini NH, Vuillermine P, Ellis JA, Panjari M, Ponsonby AL, et al.
421 Polymorphisms affecting vitamin D-binding protein modify the relationship between
422 serum vitamin D (25[OH]D3) and food allergy. *J Allergy Clin Immunol* 2016;
423 137:500-6 e4.
- 424 15. Paxton GA, Teale GR, Nowson CA, Mason RS, McGrath JJ, Thompson MJ, et al.
425 Vitamin D and health in pregnancy, infants, children and adolescents in Australia and
426 New Zealand: a position statement. *Med J Aust* 2013; 198:142-3.

16. Jones AP, D'Vaz N, Meldrum S, Palmer DJ, Zhang G, Prescott SL. 25-hydroxyvitamin D3 status is associated with developing adaptive and innate immune responses in the first 6 months of life. *Clin Exp Allergy* 2015; 45:220-31.
17. Hartmann B, Heine G, Babina M, Steinmeyer A, Zugel U, Radbruch A, et al. Targeting the vitamin D receptor inhibits the B cell-dependent allergic immune response. *Allergy* 2011; 66:540-8.
18. Stein MM, Hrusch CL, Gozdz J, Igartua C, Pivniouk V, Murray SE, et al. Innate Immunity and Asthma Risk in Amish and Hutterite Farm Children. *N Engl J Med* 2016; 375:411-21.
19. Pyle DM, Yang VS, Gruchalla RS, Farrar JD, Gill MA. IgE cross-linking critically impairs human monocyte function by blocking phagocytosis. *J Allergy Clin Immunol* 2013; 131:491-500 e1-5.
20. Dang TD, Tang ML, Koplin JJ, Licciardi PV, Eckert JK, Tan T, et al. Characterization of plasma cytokines in an infant population cohort of challenge-proven food allergy. *Allergy* 2013; 68:1233-40.
21. Baiz N, Dargent-Molina P, Wark JD, Souberbielle JC, Annesi-Maesano I, Group EM-CCS. Cord serum 25-hydroxyvitamin D and risk of early childhood transient wheezing and atopic dermatitis. *J Allergy Clin Immunol* 2014; 133:147-53.
22. Hollams EM, Teo SM, Kusel M, Holt BJ, Holt KE, Inouye M, et al. Vitamin D over the first decade and susceptibility to childhood allergy and asthma. *J Allergy Clin Immunol* 2016.
23. Allen KJ, Panjari M, Koplin JJ, Ponsonby AL, Vuillermine P, Gurrin LC, et al. VITALITY trial: protocol for a randomised controlled trial to establish the role of postnatal vitamin D supplementation in infant immune health. *BMJ Open* 2015; 5:e009377.

Table 1. Demographics and clinical characteristics of study cohort

	Non-Allergic	Persistent Egg Allergic	Transient Egg Allergic	p-value
Total number	n=18	n=14	n=22	
Sex: male (%)	42	52	46	ns
Both parents born in Australia (%)	76.2	70.5	60.7	ns
Any siblings (%)	60	41.2	46.4	ns
Family history of allergy* (%)	57.1	82.4	96.4	^a ns ^b 0.0008 ^c ns
Sensitised to peanut or sesame (%)	0	0	0	
Eczema at age one year[†] (%)	25	47	40	ns
Egg SPT (mm) (mean ± SD)				^a <0.0001
Age one year	0.1 ± 0.3	5.2 ± 2.2	3.9 ± 2.3	^b <0.0001
Follow up		3.1 ± 2.4	0.5 ± 1.0	^c ns ^d <0.0001
Egg sIgE (kUA/L) (mean ± SD)				^a <0.0001
Age one year	0.1 ± 0.1	8.8 ± 13.9	1.2 ± 1.2	^b <0.0001
Follow up		5.2 ± 14.9	0.2 ± 0.39	^c 0.0471 ^d 0.0002
Serum vitamin D (nmol/L) (mean ± SD)				
Age one year	77.4 ± 26.2	63.5 ± 29.9	67.3 ± 20.6	ns
Follow up		53.26 ± 18.67	61.71 ± 14.47	
Vitamin D deficient (≤50nmol/L) (%)				^a ns
Age one year	19	29.4	21.4	^b ns
Follow up		54	13	^c ns ^d 0.0276

* asthma, allergic rhinitis, eczema or food allergy

[†] doctor diagnosed eczema requiring topical steroid treatment or eczema observed by a trained nurse

ns; not significant

^a p value (age one persistent vs non allergic), ^b p value (age one transient vs non allergic), ^c p value (age one persistent vs transient), ^d p value (follow up persistent vs transient)

Figure 1. Circulating innate immune profiles in one year old egg allergic and healthy infants. **(A)** Representative flow cytometry gating strategies, where CD14 and CD16 were used to identify the three circulating monocyte subsets (NC; non-classical, Int; intermediate and C; classical), and CD11c and CD123 used to identify myeloid (mDC) and plasmacytoid (pDC) dendritic cell populations. **(B_{I-IV})** Classical monocytes, non-classical monocytes, intermediate monocytes, and myeloid DCs expressed as cell number per ml in egg allergic (n=36) and non-allergic (n=18) one year old infants. **(C)** Representative flow cytometry histogram showing HLA-DR expression on classical monocytes, non-classical monocytes, intermediate monocytes and myeloid DCs in egg allergic (red) and non-allergic (grey) infants. *p<0.05, **p<0.01.

Figure 2. Innate cytokine production from FACS-sorted CD3⁺ cells in egg allergic and healthy one year old infants. CD3⁺ cells from egg allergic (n=11) and non-allergic (n=13) infants were cultured for 24h in the presence of media alone or LPS. Following culture, supernatants were harvested and production **(A)** TNF- α , **(B)** IL-6, **(C)** IL-1 β , **(D)** IL-8, **(E)** IL-12p70 and **(F)** IL-10 was assessed by cytometric bead array, *denotes significance between groups, #denotes significance from baseline (media), *p<0.05, **p<0.01, ***p<0.001, ##p<0.01, ###p<0.001.

Figure 3. Circulating innate immune phenotype and function in one year old infants when stratified by their persistent or transient food allergy outcome at follow up. **(A_{I-IV})** Classical monocytes, non-classical monocytes, intermediate monocytes, and myeloid DCs expressed as cell number per ml in egg allergic one year old infants, stratified by their persistent (n=14) or transient (n=22) outcome at follow up compared to non-allergic control infants (n=18). **(B_{I-IV})** FACS sorted CD3⁺ cells from infants with persistent (n=5) or transient (n=6) allergy and non-allergic healthy infants (n=13) were cultured for 24h in the presence of media alone or LPS. Following culture, supernatants were harvested and production of TNF- α , IL-6, IL-1 β and IL-

8, along with (**C_I**) IL-12p70 and (**C_{II}**) IL-10 were assessed by cytometric bead array, *denotes significance between groups, #denotes significance from baseline (media), *p<0.05, **p<0.01, ***p<0.001, #p<0.05, ##p<0.01, ###p<0.001.

Figure 4. Longitudinal analysis of circulating innate immune profiles in matched samples collected at age one and at follow up in children with persistent or transient egg allergy. (**A_{I-IV}**) Classical monocytes, non-classical monocytes, intermediate monocytes and myeloid DCs expressed as cell number per ml in matched samples at age one and follow up from children with persistent (n=14) and transient (n=22) egg allergy. (**B**) FACS sorted CD3⁺ cells were cultured for 24h in the presence of media alone or LPS and production of inflammatory cytokines TNF, IL-6, IL-1 β and IL-8, (**C_I**) Th1 inducing cytokine IL-12p70 and (**C_{II}**) regulatory IL-10 were assessed in matched samples at age one and follow up from children with persistent (n=5) and transient (n=6) egg allergy. *denotes significance between groups, #denotes significance from baseline (media), *p<0.05, **p<0.01, ***p<0.001, #p<0.05, ##p<0.01, ###p<0.001.

Figure 5. Correlations between serum vitamin D level and immune profiles at follow up in children with persistent and transient egg allergy. (**A-B**) Classical monocytes and myeloid DCs at follow up in persistent (n=7) and transient (n=10) two-year-old children. A Spearman test with two-tailed p-value was performed, *p<0.05, **p<0.01.

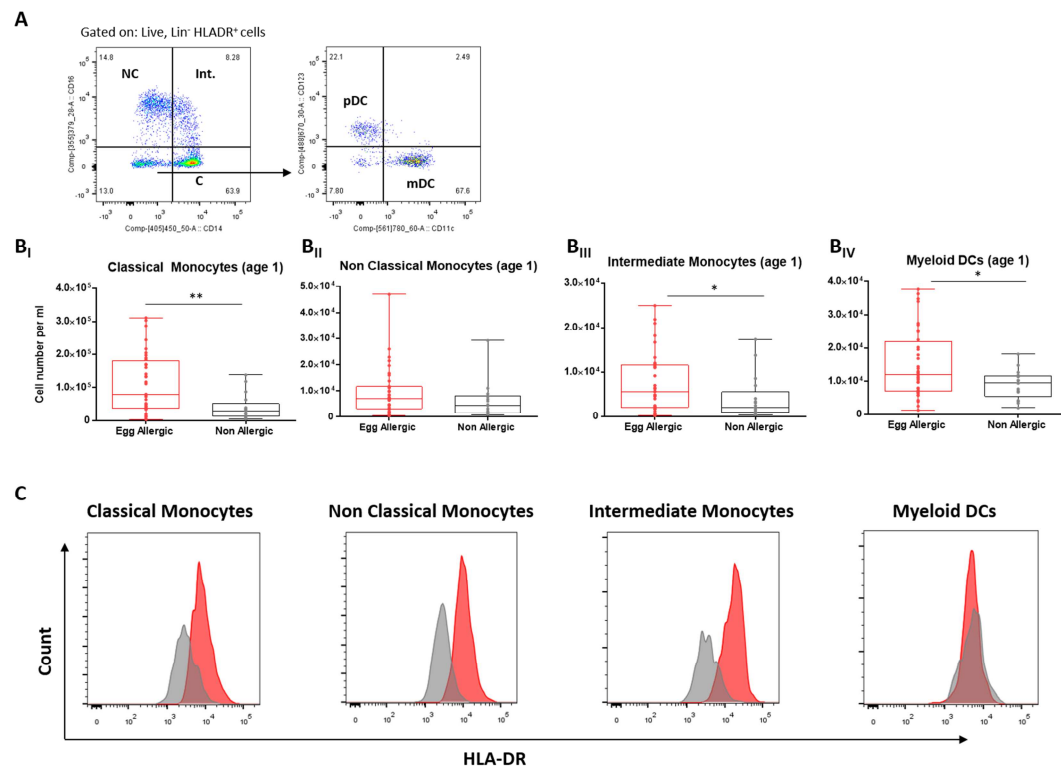


Figure 1

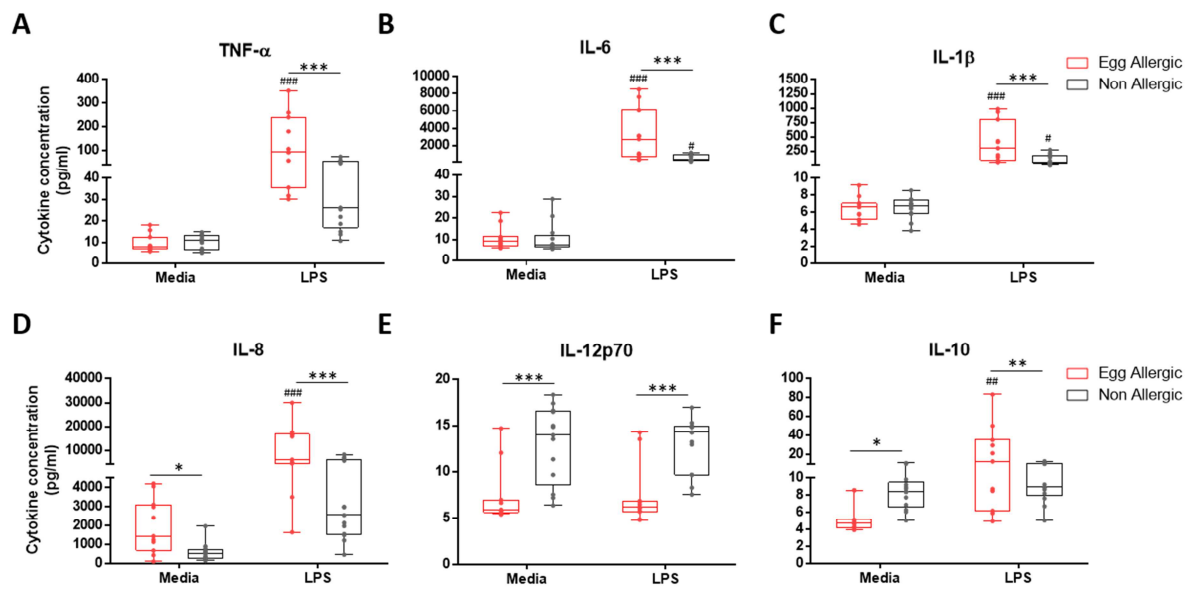


Figure 2

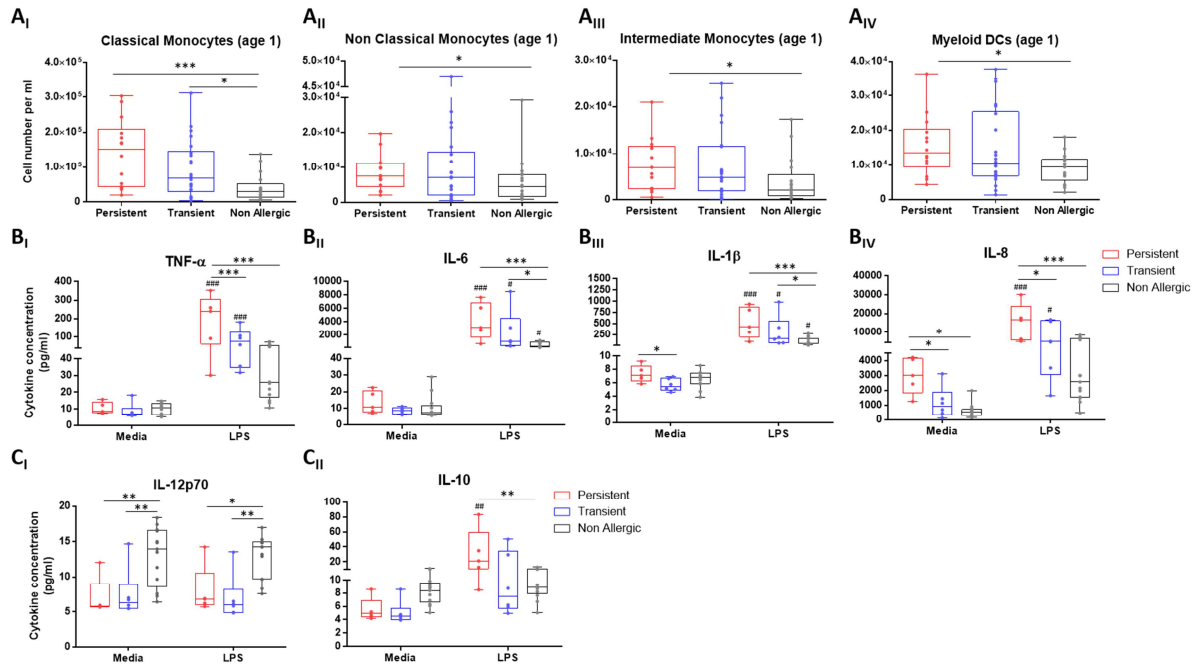


Figure 3

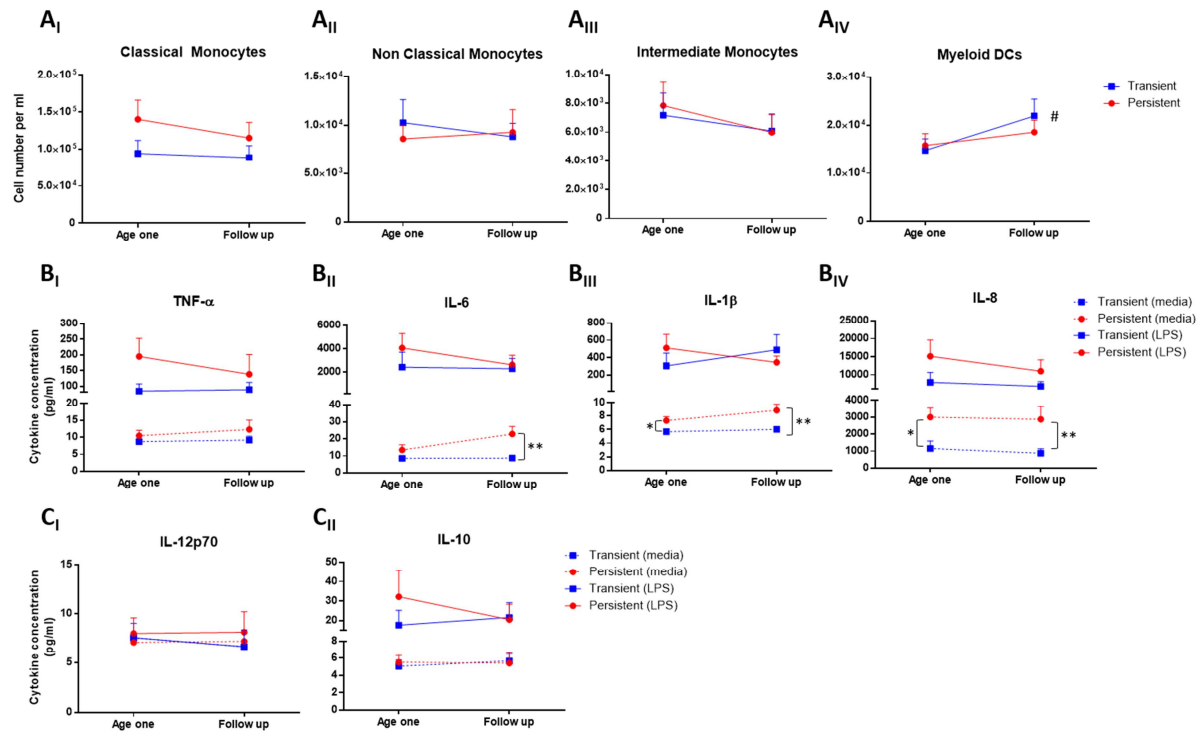


Figure 4

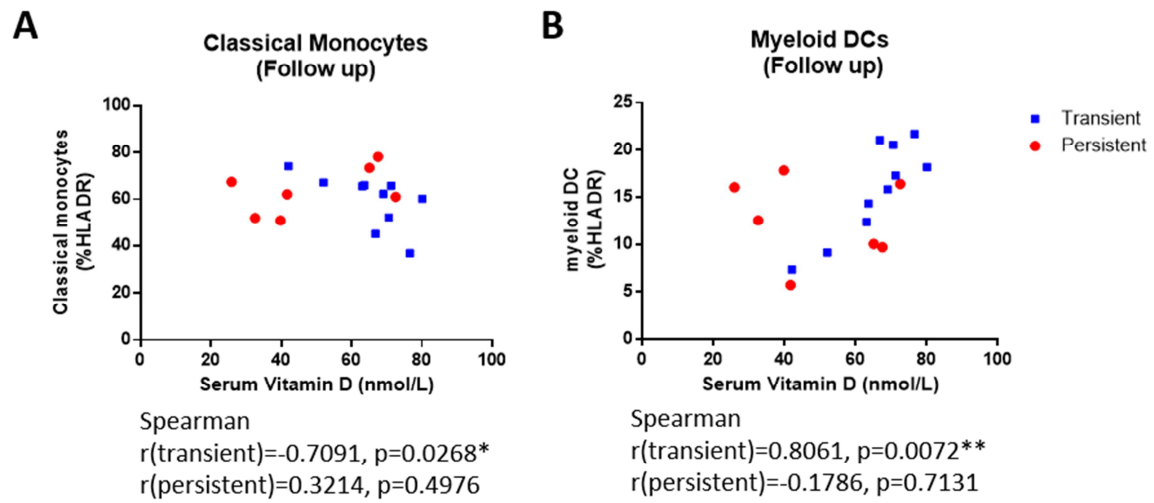
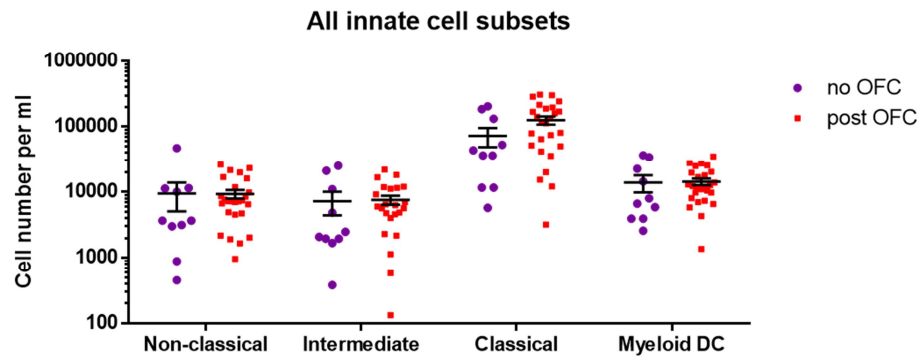


Figure 5

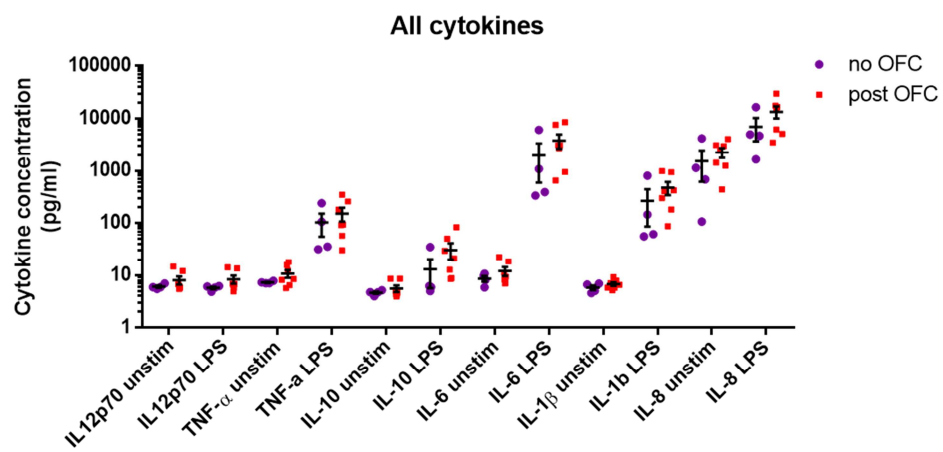
Supplementary Figure 1. Comparison of innate parameters in egg allergic infants that had blood collected on a non-OFC day with egg allergic infants that had blood collected following OFC. No statistical difference was observed between (A) circulating innate cell subsets and (B) cytokine production between the post OFC blood collection and no OFC blood collection.

Supplementary Figure 2. Circulating innate immune profiles in one year old egg allergic and non-egg allergic infants when stratified by eczema status at one year of age. (A) Classical monocytes, (B) non-classical monocytes, (C) intermediate monocytes, and (D) myeloid DCs expressed as cell number per ml in persistent egg allergic one year old infants (n=8 with eczema, n=6 no eczema), transient egg allergic one year old infants (n=11 with eczema, n=10 no eczema), and non-egg allergic infants (n=5 with eczema, n=12 no eczema).

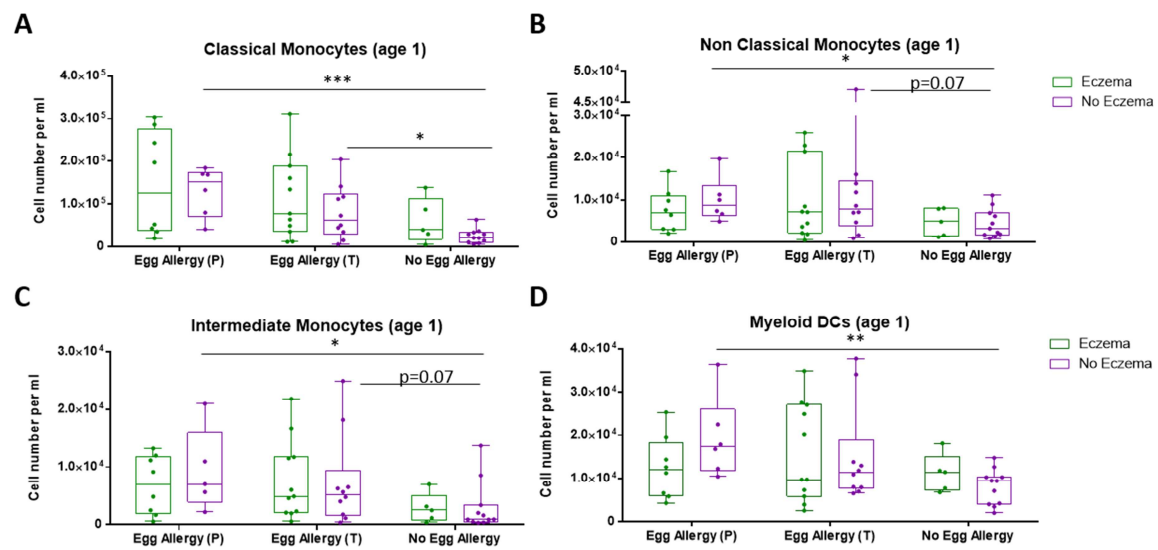
A.



B.



Supplementary Figure 1



Supplementary Table 1. Flow cytometry antibody cocktail and dilutions.

Antibody cocktail for phenotyping monocyte and DC subsets	
	Dilution (2X)
HLADR BB515	1:50
CD123 PerCPCy5.5	1:20
CD11c PE-Cy7	1:200
Lineage APC-H7	1:100
CD14 V450	1:200
CD16 BUV395	1:200
Live/dead V500	