DR. JENNIFER KOPLIN (Orcid ID : 0000-0002-7576-5142)

PROF. KATRINA J ALLEN (Orcid ID : 0000-0002-1921-4493)

Article type : Letter to the Editor

B cell phenotype and function in infants with egg allergy

To the Editor,

IgE-mediated food allergy is a major public health burden, affecting up to 10% of infants (1). B cells play a crucial role in the development of food allergy, primarily through allergen-specific IgE (sIgE) production that mediates allergic immune responses. B cells also modulate other immunological processes, including the production of inflammatory and regulatory cytokines. Increasing evidence suggests a role for these cytokines in human disease, however, their role in the development or resolution of IgE-mediated food allergy remains largely unknown. In the present study, we aimed to phenotype and quantify circulating B cell subsets in infants with food allergy and investigate the contribution of B cell-derived cytokines in the development of food allergy and the acquisition of natural tolerance in childhood.

A subset of 59 infants were selected from the HealthNuts cohort for this study (n=38 egg allergic one-year-old infants and n=21 non-sensitised, non-food allergic one-year-old infants). Oral food challenges (OFC) were performed according to standardised protocols (2). Egg allergic infants (n=38) had a positive SPT ≥ 2mm and an sIgE level of ≥0.35kUA/L to egg and an unequivocal objective allergic reaction during egg OFC at age one year. Healthy control infants (n=21) were non-sensitised and non-allergic by OFC. All infants with

challenge-confirmed egg allergy at one year of age undertook repeat raw egg OFC, SPT and sIgE tests to determine their egg allergy status at age four years. Of the 38 raw egg allergic participants selected for this study, 22 acquired tolerance to egg by age four (designated transient egg allergic) while 16 remained egg allergic (persistent egg allergic). Supplementary Table 1 describes the demographics of the selected cohort. Blood was collected at one year of age and cryopreserved peripheral blood mononuclear cells (PBMCs) used for flow cytometry as previously reported (3, 4). B cell subpopulations (naïve, switched memory, non-switched memory and double negative) were quantified using the strategies outlined in Supplementary Figure 1. For assessment of B cell cytokine production, purified $CD3^{-}CD19^{+}$ B cells (BD Influx Cell Sorter) were stimulated for 72h at 37°C with 300µg/ml of endotoxin cleaned egg white extract (0.3EU/ml) or 1µM of CpG-2006. Supernatants were then harvested and frozen at -80°C for later quantification of cytokines by cytometric bead array (CBA). For detailed methods, please see online supplement.

We report that egg allergic one-year-old infants show lower numbers of total circulating B cells relative to non-allergic infants ($2.0x10^5$ cells/ml vs $2.9x10^5$ cells/ml, p=0.031) (Figure 1A). We have additionally observed that the reduction in circulating B cell number in egg allergic infants was evident in the naïve ($1.3x10^5$ cells/ml vs $1.9x10^5$ cells/ml, p=0.046), non-switched memory ($0.5x10^4$ cells/ml vs $1.3x10^4$ cells/ml, p<0.0001) and switched-memory ($2.8x10^4$ cells/ml vs $6.0x10^4$ cells/ml, p=0.0041) B cell sub-populations (Figures 1B-E). When B cell subsets were expressed as frequencies of the total B cell pool, this reduction was most prominent in the switched and non-switched memory B cell subsets (Supplementary Figure 2A-D).

A decrease in the frequency of total B cells in children with eczema has recently been reported (5). However, the authors observed no differences between the major B cell subsets in children with eczema relative to healthy controls. Given that infants with egg allergy often have concomitant eczema, we sought to determine whether the B cell subset decreases observed in our food allergic infants were also associated with the presence of eczema. We found that the decrease in the number of memory B cell subsets in egg allergic infants was observed independent of eczema status, and we report no significant differences in B cell subset numbers between infants with eczema alone (without egg allergy) relative to non-egg allergic, no-eczema controls (Supplementary Figure 3).

IL-10 producing B cells have been shown to have a protective effect in human allergic diseases by supressing effector T cells and inducing regulatory T cell responses (6). One study of milk allergy found greater proliferation of IL-10^{+} B cells following allergen stimulation in non-allergic patients when compared to those with milk allergy (7). We found greater production of B cell-derived IL-10 (13pg/ml vs 7.7pg/ml, p=0.04), IL-6 (54pg/ml vs 30pg/ml, p=0.03) and CCL3 (75pg/ml vs 14pg/ml, p=0.009) in non-allergic infants relative to egg allergic infants following non-specific stimulation with the TLR-9 ligand CpG (Figure 1F-H). Interestingly, both egg and CpG stimulation increased B cell-derived IL-8 production from egg allergic infants relative to non- allergic infants (1.6-fold and 3.7-fold increase, respectively; Figure 1I).

We next sought to determine if the altered B cell responses were associated with the natural history of egg allergy in early childhood. To do this, we stratified egg allergic infants by their subsequent egg allergy status at follow up and compared immune responses at one year of age in infants with persistent or transient egg allergy. Both persistent and transient egg allergic infants showed reduced numbers of non-switched memory B cells at age one relative to non-allergic controls ($0.3x10^4$cells/ml vs $1.2x10^4$cells/ml, p=0.0002, and $0.5x10^4$cells/ml vs $1.2x10^4$cells/ml, p=0.01, respectively) however only infants with persistent egg allergy outcomes demonstrated a decrease in the switched-memory subset ($2.6x10^4$cells/ml vs $6x10^4$cells/ml, p=0.012) (Figure 2A-D). Whether the observed suppression of circulating memory B cell numbers in food allergic infants is due to an innate deficit in these subsets or is due to T cell-induced immune suppression is unclear. However, the observation that the deficit is primarily observed in the memory subsets, both of which have been shown to demonstrate characteristics of antigen selection, may suggest the latter. Future work investigating the phenotype and function of these B cells in prior to allergy onset (within the first 6 months of life) will help answer these questions.

Purified B cells from infants with transient egg allergy produced less IL-6 and IL-10 following CpG stimulation relative to infants with persistent egg allergy or healthy controls (Figure 2E-G, all $p<0.05$). This suggests that reduced early life B cell capacity following toll like receptor engagement may be associated with the development of tolerance in childhood. Future investigation of the role of regulatory B cell subsets in this response will provide further insight. In all stimulation conditions, B cell IL-8 production was significantly elevated in infants with persistent egg allergy relative to infants with transient egg allergy (Figure 2H, all $p<0.05$). We have previously shown that monocytes from infants with persistent egg

allergy produce more inflammatory cytokines (IL-1β, TNF-α and IL-8) at baseline and following *in vitro* endotoxin exposure when compared to infants with transient egg allergy (3). We now extend these findings to report that egg allergy in the first year of life is also associated with elevated production of inflammatory IL-8 from activated B cells, and that this effect was most significant in infants with persistent egg allergy. Whilst we are the first to report these findings in the context of food allergy, a positive correlation between B cell IL-8 production and disease severity has been observed in other diseases of the gut, including Crohn's disease and ulcerative colitis (8, 9).

In summary, we have shown that egg allergic infants have reduced numbers of circulating B cells that produce altered cytokine responses at baseline and following TLR stimulation. Our results provide new insights into the underlying biology that drive the clinical phenotypes of egg allergy, highlighting the possibility that increased inflammatory activation of B cells in the first year of life may contribute to the persistence of allergic immune responses in childhood and that reduced B cell capacity following innate stimulation may be associated with the induction of natural tolerance.

References

1. Osborne NJ, Koplin JJ, Martin PE, Gurrin LC, Lowe AJ, Matheson MC, et al. Prevalence of challenge-proven IgE-mediated food allergy using population-based sampling and predetermined challenge criteria in infants. *J Allergy Clin Immunol* 2011;**127**(3):668-676 e661-662.
2. Koplin JJ, Tang ML, Martin PE, Osborne NJ, Lowe AJ, Ponsonby AL, et al. Predetermined challenge eligibility and cessation criteria for oral food challenges in the HealthNuts population-based study of infants. *J Allergy Clin Immunol* 2012;**129**(4):1145-1147.
3. Neeland MR, Koplin JJ, Dang TD, Dharmage SC, Tang ML, Prescott SL, et al. Early life innate immune signatures of persistent food allergy. *J Allergy Clin Immunol* 2017.
4. Dang TD, Allen KJ, Martino D, Koplin JJ, Licciardi PV, Tang ML. Food allergic infants have impaired regulatory T cell responses following in vivo allergen exposure. *Pediatr Allergy Immunol* 2016;**27**(1):35-43.
5. Czarnowicki T, Esaki H, Gonzalez J, Renert-Yuval Y, Brunner P, Oliva M, et al. Alterations in B-cell subsets in pediatric patients with early atopic dermatitis. *J Allergy Clin Immunol* 2017;**140**(1):134-144 e139.

6. van de Veen W, Stanic B, Wirz OF, Jansen K, Globinska A, Akdis M. Role of regulatory B cells in immune tolerance to allergens and beyond. *J Allergy Clin Immunol* 2016;**138**(3):654-665.
7. Noh J, Lee JH, Noh G, Bang SY, Kim HS, Choi WS, et al. Characterisation of allergen-specific responses of IL-10-producing regulatory B cells (Br1) in Cow Milk Allergy. *Cell Immunol* 2010;**264**(2):143-149.
8. Nikolajczyk BS. B cells as under-appreciated mediators of non-auto-immune inflammatory disease. *Cytokine* 2010;**50**(3):234-242.
9. Noronha AM, Liang Y, Hetzel JT, Hasturk H, Kantarci A, Stucchi A, et al. Hyperactivated B cells in human inflammatory bowel disease. *J Leukoc Biol* 2009;**86**(4):1007-1016.

Authors:

Melanie R. Neeland[1,2], David J. Martino[1, 2], Thanh D. Dang[1, 2], Jennifer J. Koplin[1,3], Rachel L. Peters[1, 2], Alexander Grishin[4], Shyamali C. Dharmage [1, 3], Mimi L. Tang [1, 2,5], Hugh A. Sampson[4], Richard Saffery[1, 2] and Katrina J. Allen [1, 2, 5*]

Affiliations:

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

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

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

[4]Pediatric Allergy and Immunology, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

[5]Department of Allergy and Immunology, Royal Children's Hospital, Parkville, VIC, Australia

*Corresponding author:
Professor Katrina J. Allen
Murdoch Children's 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, the NHMRC Centre for Food and Allergy Research and The Thrasher Research Fund.

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

The authors declare no conflict of interest in relation to this work.

Figure 1. Egg allergic infants show reduced numbers of circulating B cells and altered B cell-derived cytokine profiles at one year of age. (A-E) Total B cells, naïve, switched memory, non-switched memory and double negative B cells expressed as cell number per ml in egg allergic (n=38) and non-egg allergic (n=21) one year old infants. (F-I) FACS-sorted B cells from egg allergic (n=10) and non-allergic (n=10) infants were cultured for 72h in the presence of media alone, egg allergen or CpG. Following culture, supernatants were harvested and production of IL-6, CCL3, IL-8, IL-10 and L-8 was assessed by cytometric bead array, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

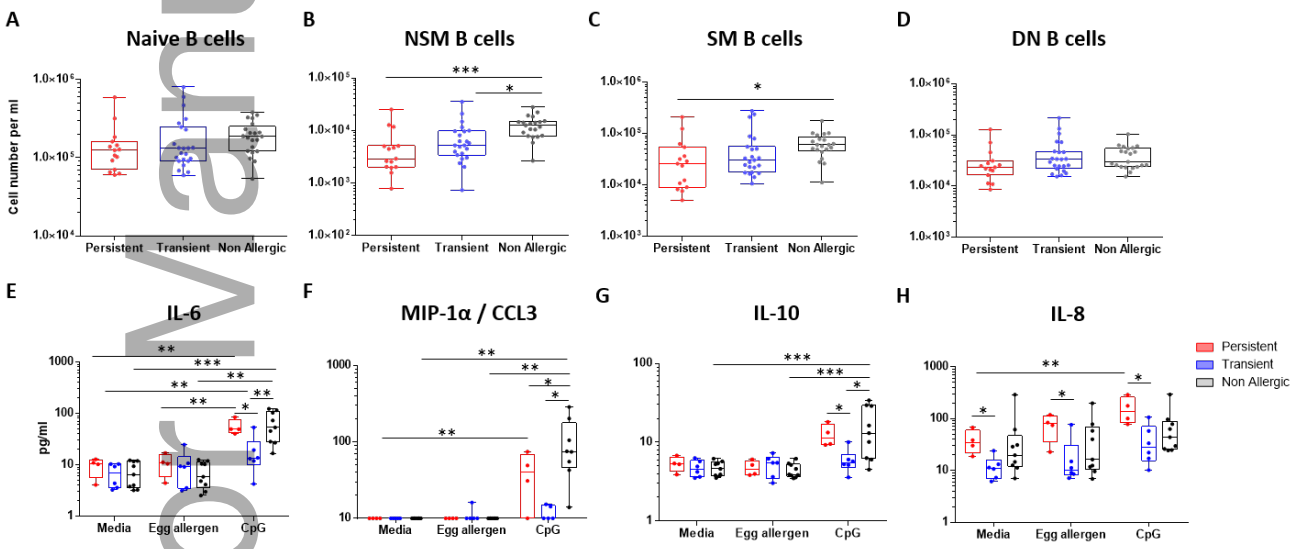

Figure 2. Distinct B cell profiles at one year of age in persistent and transient egg allergy (A-D). Naïve B cells, switched memory B cells, non-switched memory and double negative B cells expressed as cell number per ml in egg allergic one year old infants, stratified by their persistent (n=15) or transient (n=23) outcome at follow up compared with non-allergic control infants (n=21). (E-H) FACS-sorted B cells from infants with persistent (n=5) or transient (n=5) allergy and non-allergic healthy infants (n=10) were cultured for 72h in the presence of media alone, egg allergen or CpG. Following culture, supernatants were harvested and production of IL-6, CCL3, IL-8, IL-10 and L-8 was assessed by cytometric bead array, *p<0.05, **p<0.01, ***p<0.001.

Author/s:
Neeland, MR; Martino, DJ; Dang, TD; Koplin, JJ; Peters, RL; Grishin, A; Dharmage, SC; Tang, ML; Sampson, HA; Saffery, R; Allen, KJ

Title:
B-cell phenotype and function in infants with egg allergy

Date:
2019-05-01

Citation:
Neeland, M. R., Martino, D. J., Dang, T. D., Koplin, J. J., Peters, R. L., Grishin, A., Dharmage, S. C., Tang, M. L., Sampson, H. A., Saffery, R. & Allen, K. J. (2019). B-cell phenotype and function in infants with egg allergy. ALLERGY, 74 (5), pp.1022-1025. https://doi.org/10.1111/all.13707.