

Children with nut allergies have impaired gene expression of Toll-like receptors pathway

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Abstract

Background: Trends in food allergies prompted investigation into the underlying mechanisms. Genetic and epigenetic factors are of high interest, and, in particular, the interplay between genes relating to immune factors directly and indirectly involved in food allergy pathogenesis. We sought to determine potential links between gene expression and epigenetic factors relating to Toll-like receptor (TLR) pathways and childhood food allergies.

Methods: In a cross-sectional study, samples from 80 children with and without food allergies were analysed for gene expression, DNA methylation and a range of immune factors relating to TLR pathways. *TLR2*, *TLR4*, *CD14*, *IL5*, *IL13* and vitamin D were explored.

Results: The importance of these immune factors appeared to vary between the different types of food allergies. Expression of *TLR2* ($P < .001$), *TLR4* ($P = .014$) and *CD14* ($P = .028$) varied significantly between children with no food allergy, allergy to nuts and peanuts, and allergy to eggs. DNA methylation in the promoter regions of these genes had a significant association with gene expression. These trends persisted when subjects were stratified by nut allergy vs no nut allergy. Furthermore, *TLR2* ($P = .001$) and *CD14* ($P = .007$) expressions were significantly lower in children with food allergies when compared to those without.

Conclusion: Gene expression of TLR pathway genes was directly related to food allergy type, and DNA methylation had an indirect effect. TLR2 pathways are of significant interest in nut allergies.

KEYWORDS

DNA methylation, food allergy, gene expression, epigenetic factors, nut allergy, Toll-like receptor

Abbreviations: Ct, Cycle threshold; IL, Interleukin; OFC, Oral food challenge; PRR, Pattern recognition receptor; SPT, Skin prick test; TLR, Toll-like receptor.

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1 | INTRODUCTION

Food allergies are now among the most common chronic diseases in children, and it is believed the rates are increasing worldwide.¹ Anaphylaxis can be deadly and IgE-mediated food allergies account for around 30% of hospitalizations from anaphylaxis, with the main allergens being peanut, tree nut, shellfish and egg.²

The trends in food allergies have prompted urgency in understanding the specific biological mechanisms underlying the disease. IgE-mediated food allergies are largely mediated by Th2 pathways, and the activation of differentiation of naïve T cell (Th0) to Th2 cells leads to the release of several cytokines including IL4, IL5 and IL13.³ Upon ingestion of the allergenic food, dendritic cells capture and transport the antigen towards the lymph nodes, inducing Th2 cell differentiation. Currently, it is believed that the pattern recognition receptors (PRRs) involved in immunity, Toll-like receptor 2 (TLR2), Toll-like receptor 4 (TLR4) and cluster of differentiation-14 (CD14), play a role in the manifestation of an allergic reaction to a food; however, there are inconsistencies in the current literature of their level of significance.^{4,5} Development of food allergies may result from intrinsic genetic defects and dysfunctional DNA methylation in naïve T cells in early life.^{6,7} Further, early life dendritic cells and monocytes contribute to allergy profiles and vitamin D may be a modulator of these.⁸ Epidemiological and experimental evidence also suggests vitamin D has a broad but important role in food allergy pathogenesis; however, the relationship between vitamin D and specific immune factors is complex and the body of evidence is inconclusive.⁹

While many pathways are involved, there are many yet to be elucidated, and the level of significance of each individual immune factor remains unclear. The importance of gene expression of immunological factors, as well as the impact of epigenetic modulation, such as DNA methylation, is under investigation. Here, we attempted to elucidate relationships within a selection of genetic, epigenetic and immune factors in children with and without food allergies. These factors include TLR2, TLR4 and CD14 gene expression and DNA methylation as major outcomes of interest and vitamin D, IL5 and IL13 as minor.

2 | METHODS

2.1 | Study group

The study population (n = 80) was selected from a set of samples from an ongoing study investigating the genetic and epigenetic markers of food allergies (Princess Margaret Hospital Human Research Ethics Committee, 2016046EP). Children (aged 1-18 years old) with suspected or diagnosed

Key Message

Children with nut allergies have lower expression of TLR genes than children with egg allergies or children without food allergies.

Methylation of TLR2 is associated with decreases in TLR gene expression in all children.

allergies to eggs, peanuts and/or tree nuts were recruited upon their visit to the outpatient immunology clinic at Princess Margaret Hospital (PMH). Non-food allergic children were recruited as control subjects. Those families who agreed to participate were asked to complete a short questionnaire and provide a sample of blood from their child via the onsite pathology services during their visit to the hospital. The questionnaire included questions asking the caregiver about duration of breastfeeding and the age, in months, that the child was introduced to solids, as best estimates. Written consent was obtained from all the children's caregivers.

2.2 | Clinical outcomes

Clinical diagnoses of food allergy were assessed from skin prick test (SPT) and oral food challenge (OFC) outcomes by a clinical doctor. Positive SPT (wheal size $\geq 3 \times 3$ mm) to any food allergen explored here, and/or failed OFC, confirmed the patient's status as food allergic. Allergens of interest in this study included eggs (whites, baked and/or cooked), and peanuts and tree nuts, collectively as nuts. A passed OFC and/or a negative SPT for any food allergens indicated the patient as a non-food allergic subject.

At the time of recruitment, 14 (17.5%) of the 80 participants did not have a diagnosed food allergy, 8 (10%) had an allergy to eggs only, and 58 (72.5%) had an allergy to at least nuts. The nut and mixed allergy group consisted of 18 allergic to peanuts only, 26 allergic to at least one type of nut (eg walnut, cashew) and 14 allergic to both eggs and nuts. Several participants in both allergic and non-allergic groups had self-reported eczema. Clinical diagnoses for environmental allergens were not sought for this study. The general characteristics of the study population including clinical outcomes can be found in Table 1.

2.3 | Blood collection and laboratory measurement

Venous blood was collected from the participants via venepuncture by phlebotomists at PathWest pathology services (PMH), with 1 mL

	Forward sequence	Reverse sequence	T_m (°C)
TLR2	TCATCATCAGCCTCTCCAAG	GTCAGTGTGCTAATGTAGGTG	53.0
TLR4	GTATTCAAGGTCTGGCTGGT	TCGAGGTAGTAGCTAAGTATGC	52.0
CD14	TTCATCGTCCAGCTCACAAGG	ACTTCCAGAGCCTGTCCGGA	57.2

TABLE 1 Primer sequences for TLR2, TLR4 and CD14 genes

collected in a serum clot tube and 4 mL in EDTA tube. Samples were transferred to the research facility for further processing, including the extraction of DNA, RNA and synthesis of cDNA from whole blood. 25(OH) vitamin D₂ and D₃ were measured in thawed human serum using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences). The interleukins IL5 and IL13 were also measured from serum using ELISA according to a protocol developed in-house. Briefly, samples were added to wells blocked with coating antibodies and incubated with detection antibodies and anti-species antibodies solubilized in a PBST and skim milk powder solution. The reaction was detectable with 3,3',5,5'-tetramethylbenzidine as the colour substrate. The gene expression of the genes *TLR2*, *TLR4* and *CD14* was determined using quantitative real-time polymerase chain reaction (qPCR) on the Thermo Fisher ViiA 7 Real-Time PCR System. DNA was prepared from 36 samples using the EpiTect II DNA Methylation Enzyme Kit (Qiagen), and methylation of *CD14* and *TLR2* was determined using qPCR and commercially available primers (Qiagen), with threshold set at 0.04 CT. The details are described in Appendix S1.

2.4 | Statistical analyses

Experimental data were transformed into ranked values. Expression of *TLR2*, *TLR4* and *CD14* was further combined as "total TLR expression" with a principal component analysis. The rank transformation achieved a normal approximation. General characteristics such as age, duration of breastfeeding and age of introduction of solids were normally distributed. Pearson's coefficient was used to determine correlations between each of the ranked variables, and partial correlation was used to adjust these results for any age and sex differences.

The subjects were initially grouped into the type of food allergy they had—egg allergy ($n = 8$), nut and mixed allergy (both egg and at least one nut allergy; $n = 58$) and no allergy ($n = 14$). The group without food allergies either had never had an allergic reaction to a food or had outgrown their food allergies. Differences in gene expression and methylation were evaluated in these groups by one-way ANOVA. The subjects were then simplified into food allergy vs no food allergy ($n = 66$ and $n = 14$) and nut allergy vs no nut allergy ($n = 58$ and $n = 22$), and differences in these groups were determined using independent sample *t* test.

All analyses were performed using SPSS version 25 (IBM) and Excel 2016 (Microsoft), with statistical significance considered at $P < .05$.

3 | RESULTS

Analysis of general characteristics found children with egg allergies were younger than those in the other allergy groups ($P = .040$), but there were no significant differences in the sex of the participants in each group ($P > .05$). Season of birth, season that the blood sample was taken, duration of breastfeeding and the age that solid foods were introduced were not significantly different when each of the groups were compared ($P > .05$).

Prior to transformation into ranked values, the mean values of all participants for *TLR2*, *TLR4* and *CD14* expression were 6.2 ± 16.8 , 3.8 ± 11.8 and 1.7 ± 4.4 , respectively, fold change relative to 18S RNA, and values for DNA methylation (percentage methylated) of *TLR2* and *CD14* were $16.6 \pm 18.2\%$ and $40.4 \pm 19.5\%$. Further, the net optical density values for the immune factors IL5, IL13 and vitamin D were 0.27 ± 0.10 , 0.23 ± 0.16 and 0.48 ± 0.09 . Ranked values were then determined and stratified by allergy type, and significant differences were observed in gene expression (Table 2). Additionally, "total TLR expression"—the sum of each ranked value for *TLR2*, *TLR4* and *CD14* for each participant, was determined and was found to be significantly different between the groups (Table 2). There were no relationships observed for DNA methylation or the interleukins, IL5 and IL13, regarding type of allergy. Despite not reaching statistical significance, the data showed trends of lower levels of methylation of the *TLR2* and *CD14* genes in participants without a food allergy.

Serum vitamin D had no relationship overall with allergy status or on expression or methylation of any gene, except for *TLR2* expression (Table 3). This effect is only weakly positive. Strong positive correlations were observed between expression of the three genes, and moderate negative correlations between *TLR2* methylation and expression of *TLR2* and *CD14* (Table 4).

The large differences between the nut/mixed allergies and the no food allergy and egg allergy groups, particularly with total TLR expression, prompted further investigation into these relationships. We separated the population into nut allergies and no nut allergies, to observe the effects of a nut allergy endotype. Nut allergy endotypes were associated with a lower mean ranked expression of each of the PRR's individually, and total TLR expression (Figure 1); *TLR2* expression with the strongest effect ($P < .001$). However, there were no direct effects from DNA methylation, relative vitamin D levels or the interleukins. Further, significant differences were observed for both *TLR2* ($P = .001$) and *CD14* ($P = .007$, Figure 2) gene expression, and total TLR ($P = .005$) gene expression, when the participants were stratified by case (food allergy) and control (no food allergy), but there were no significant findings with the other factors analysed.

4 | DISCUSSION

We found there were substantially less children with exclusive egg allergies than those with nut allergies or with both egg and other allergies. It was expected that children with egg allergies would be less populous than groups defined by nut allergies and no food allergies, as many children outgrow egg allergies at a young age, whereas nut allergies tend to persist into adulthood.¹⁰ Furthermore, egg allergy is known to be phenotypically heterogenous, leading to the likelihood of having other food allergies or growing out of the disease.¹¹

We hypothesized that positive interactions between the Toll-like receptors and CD14 protein would be associated with the absence of food allergy. These pattern recognition receptors are integral to the innate immune system, have relevance in allergen detection and are known to closely interact, notably, with CD14

TABLE 2 General characteristics

	All n = 80	No food allergy n = 14	Food allergic ^a		P
			Egg allergy n = 8	Nut and mixed allergy n = 58	
Age (y ± SD)	8.2 ± 4.5	8.8 ± 5.1	4.4 ± 3.1	8.6 ± 4.3	.040
Sex					
Male (n)	48	9	7	32	.203
Female (n)	32	5	1	26	
Season of birth					
Summer (n)	25	6	2	17	.845
Autumn (n)	17	2	2	13	
Winter (n)	17	4	2	11	
Spring (n)	21	2	2	17	
Season of blood draw					
Summer (n)	35	3	4	28	.140
Autumn (n)	26	8	3	15	
Winter (n)	6	2	1	3	
Spring (n)	13	1	0	12	
Duration of breastfeeding (M ± SD)	13.0 ± 9.8	10.0 ± 6.8	11.8 ± 8.6	14 ± 10.6	.440
Age of introduction of solids (M ± SD)	5.9 ± 1.5	5.8 ± 2.4	5.8 ± 0.4	5.9 ± 1.3	.967

^aThe food allergic group includes children that were clinically allergic to eggs, nuts or both at the time of recruitment.

Bold value indicates statistically significant difference with a *P*-value less than 0.05.

TABLE 3 Gene expression, DNA methylation and immune markers in the three groups of children

	No food allergy	Food allergic		P ^a
		Egg allergy	Nut and mixed allergy	
TLR2 expression	60.5 [48.8, 72.2]	55.1 [41.2, 68.9]	35.0 [29.0, 41.0]	<.001
TLR4 expression	50.7 [36.2, 65.3]	57.5 [43.3, 71.8]	35.9 [29.9, 41.9]	.014
CD14 expression	56.4 [44.0, 68.8]	37.2 [11.0, 63.4]	37.9 [32.1, 43.8]	.028
Total TLR expression ^b	165.5 [128.6, 202.4]	140.2 [95.9, 184.6]	108.9 [93.0, 124.8]	.008
TLR2 methylation	13.1 [4.4, 21.8]	19.7 [−2.3, 41.8]	18.8 [14.9, 22.8]	.424
CD14 methylation	12.0 [3.3, 20.6]	17.0 [−1.9, 35.9]	20.2 [15.9, 24.4]	.220
Serum 25(OH)D OD	39.9 [24.2, 54.5]	56.7 [44.3, 69.1]	38.5 [32.5, 44.5]	.112
IL5 OD	37.8 [23.6, 52.1]	48.0 [25.2, 70.7]	41.4 [35.4, 47.4]	.628
IL13 OD	38.7 [26.7, 50.8]	47.3 [29.9, 64.8]	40.3 [33.9, 46.9]	.691

Note: Values expressed as mean rank [95% CI].

Abbreviation: OD, Optical density.

^a*P* values were calculated using analysis of variance (ANOVA).

^bTotal TLR is the sum of *TLR2*, *TLR4* and *CD14* ranked expression.

Bold value indicates statistically significant difference with a *P*-value less than 0.05.

mediating TLR4 signalling. Cytokine responses to TLRs have been observed to be greater in infants with sensitization, food allergy and atopic dermatitis.¹² Infants at high risk for developing atopic disease had low expression of cell *TLR2*, *TLR4* and *TLR9*.¹² Most

recently, intestinal TLR4 has been implicated in the induction of tolerance to oral allergens in mice.¹³ *CD14* has been observed to regulate and promote the signalling of *TLR4*.¹⁴ Subjects with food allergies had significant variations in PRR expression when

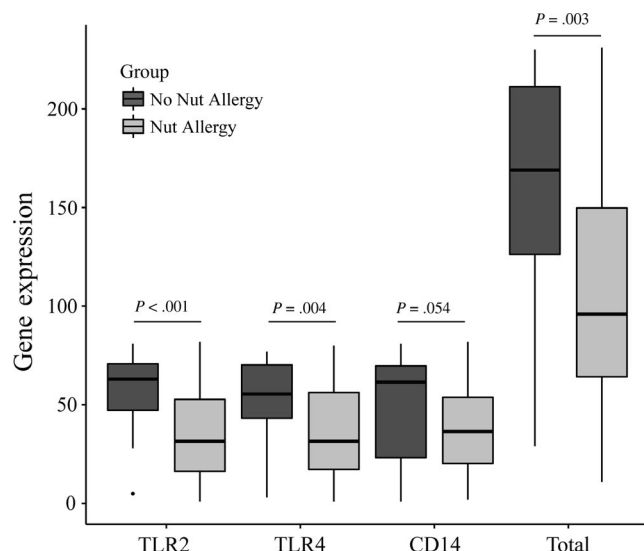


FIGURE 1 Ranked expression of *TLR2*, *TLR4* and *CD14* in children with nut allergies and children without nut allergies. Total gene expression is the sum of each above gene for each participant

demonstrated to be associated with a decrease in IgE in atopic children.²⁸ *CD14* polymorphisms are associated with both atopic and non-atopic asthma, and food allergy.^{29,30} We did not explore single-nucleotide polymorphisms in the present study, considering the sample size. Future investigation of *FoxP3* expression and methylation, and single-nucleotide polymorphisms of immune genes, in conjunction with the immune factors explored here, may enhance the findings of the current study, providing the missing link between *TLR2* and tolerance or intolerance to food allergens.

In this study, we focussed on *TLR*-related pathways. There is evidence that food allergies are characterized by a domination of Th2 cells, and the development of oral tolerance via immunotherapy is accompanied by a return to Th0/Th1 domination.²² There remains little research into the mechanisms behind re-establishing dominance of Th0/Th1 pathways.³¹ However, concentrations of interleukins additional to the ratio of Th2 to Th0/Th1 cells have been shown to vary considerably during development of oral tolerance via immunotherapy.³¹ Additionally, IL5 and IL13 have been previously implicated in peanut and egg allergies via allergen-specific CD4+ T-cell responses, when compared to other atopic subjects (^{32,33}). Here, we observed no significant differences between the interleukins IL5 and IL13 in subjects with and without food allergies, or with and without nut allergies.

For the purposes of this study, we did not proceed to determining vitamin D sufficiency or deficiency and are unable compare our data to external studies for vitamin D concentration. Our interest was making comparisons within the study population, yet our results for this vitamin were not comparable to other studies. Further, we were unable to confirm our gene expression results with protein levels and did not correct for cell composition in our use of whole blood.

However, our data show promising results regarding differences in gene expression between different food allergy endotypes and controls. Significant variation and associations persisted when stratifying the groups by nut allergies vs no nut allergy, indicating

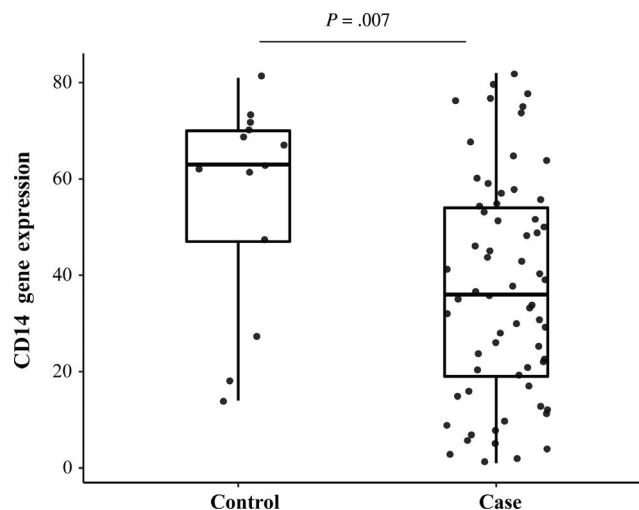


FIGURE 2 Ranked *CD14* gene expression in children with and without food allergy

individuality in genetic and epigenetic pathways in differing types of food allergies that warrant further investigation. A key strength of our study is that the use of whole blood is more indicative of overall immune function. We found a significant correlation between *TLR2* methylation and *TLR2* and *CD14* expression, and in children with nut allergies, the resulting change in gene expression was strongest. Our findings indicate methylation of the promoter region has a role in overall regulation of expression of these genes.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

AP and GZ: study design. AP: experiments and data analysis with assistance from Y.S, JG and KHL JG and YS: primer and laboratory protocol design. GZ, MO, JM, HB, BM and RL: project supervision. AP: manuscript writing. GZ: intellectual inputs and work supervision.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Study group

The criteria of the recruitment for the study included: 1) Patients that presented to the immunology outpatient clinic at Princess Margaret Hospital (PMH) for evaluation of a suspected or diagnosed allergy to egg, peanut and/or tree nut; 2) Patients that were not allergic to any foods; 3) Those above must be between the ages of 1 and 18 years.

At the commencement of the present study, a total of 105 patients had been recruited into the genetic and epigenetic study. Twenty-five participants were excluded from the present study for reasons including: no blood sample obtained, insufficient biological sample volumes and/or low-quality RNA.

Clinical outcomes

Medical records were screened approximately 3 months following recruitment of these patients. These tests were performed at PMH, often at multiple times throughout the child's life. Only the most recent SPT and/or OFC results were used. OFC results override SPT findings, where available.

Blood collection and processing

RNA was isolated from cell pellet using the RNeasy Mini Kit (Qiagen, Germany) as per the kit instructions. cDNA was prepared from 1 ug general RNA using the commercially available QuantiTect Reverse Transcription Kit to a final volume of 20 ul, according to the manufacturer's instructions.

Measurement of vitamin D and interleukins

The kit can detect vitamin D as 25(OH)D₂ and 25(OH)D₃ as total 25(OH)D in both serum and plasma. The experiment was performed according to the kit protocol. Each variable was reported as optical density (OD).

Measurement of gene expression

Relative expression levels of the genes *TLR2*, *TLR4*, and *CD14* were determined using quantitative real-time polymerase chain reaction (qPCR) and cDNA synthesised from RNA. The Thermo Fisher (USA) ViiA 7 Real-Time PCR System was used, with SYBR® Green as the nucleic acid stain. 18s rRNA was measured as the reference gene for qPCR.

Primers for *TLR2*, *TLR4* and *CD14* for gene expression were developed in-house using BioEdit v7.0.5 (Table S1) and were purchased from GeneWorks (Australia).

Measurement of DNA methylation

The qPCR was conducted using SYBR® Green and the same instruments as in the gene expression experiments. DNA methylation percentages were calculated using the equations described in the kit manual. *TLR4* primers were not commercially available at the time, and so assay of *TLR4* DNA methylation was not performed in this study.

Table S1. Primer sequences for *TLR2*, *TLR4* and *CD14* genes

	Forward sequence	Reverse sequence	T _m (C°)
<i>TLR2</i>	TCATCATCAGCCTCTCCAAG	GTCAGTGTGCTAATGTAGGTG	53.0
<i>TLR4</i>	GTATTCAAGGTCTGGCTGGT	TCGAGGTAGTAGTCTAAGTATGC	52.0
<i>CD14</i>	TTCATCGTCCAGCTCACAAGG	ACTTCCAGAGCCTGTCCGGA	57.2

