

ORIGINAL ARTICLE

Reduced membrane bound CD14 expression in the cord blood of infants with a family history of allergic disease

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Summary

Background Infants at increased risk of allergic disease have altered immune function at birth, but the specific immune changes described differ between studies and their precise nature is not well defined. Changes affecting innate immune responses may be particularly important in allergic disease pathogenesis.

Objective We investigated whether inherited risk of allergic disease is associated with altered markers of innate immunity, T cell regulation or dendritic cell (DC) percentage in human newborns.

Methods Cord blood was collected from infants at low risk (no parent affected by allergic disease, $n = 14$), intermediate risk (one affected parent, $n = 54$) or high risk (two affected parents, $n = 25$) of developing allergic disease. Cord blood mononuclear cells were cultured with ovalbumin (OVA), lipopolysaccharide, lipoteichoic acid (LTA), heat-killed lactobacillus, α -CD3 or medium alone. Cells were analysed by flow cytometry for expression of CD14, FoxP3 and DC percentage, and by real-time RT-PCR for TLR2 and TLR4 expression in infants at intermediate or high risk of allergic disease.

Results Infants at high risk of allergic disease had reduced percentage of CD14⁺ monocytes ($P = 0.01$) and reduced CD14 mean fluorescence intensity ($P = 0.01$) in uncultured mononuclear cells. They also had decreased DC percentage in mononuclear cells cultured with OVA ($P = 0.04$), lipopolysaccharide ($P = 0.01$), LTA ($P = 0.02$) and α -CD3 ($P = 0.03$) as compared with infants at intermediate or low risk of allergic disease. No relationship was seen between risk of allergic disease and TLR2 or TLR4 expression, or FoxP3 expression in cultured cells.

Conclusions Infants with a biparental history of allergic disease have altered markers of innate immunity at birth, with reduced expression of membrane bound CD14 and consequently reduced *in vitro* development of DCs. Further work is needed to understand the role that these alterations play in the pathogenesis of allergic disease, and whether interventions to up-regulate fetal CD14 expression can prevent allergic disease.

Keywords allergy, CD14, cord blood, dendritic cell, regulatory T cell

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Introduction

The prevalence of allergic diseases such as asthma, eczema, allergic rhinitis and food allergy has increased in recent decades [1, 2]. Although susceptibility genes for the different allergic diseases have been identified, the rapidity with which prevalence has increased suggests that environmental exposures are also important in disease pathogenesis [3, 4]. Several studies suggest that early life environmental exposures are important, making early

identification of high-risk infants important for future prevention strategies [5–10]. Moreover, an increased understanding of the early pathogenesis of allergic disease is likely to help in the development of such strategies [11–13]. Previous studies from our laboratory and others have identified a range of immune alterations in cord blood from infants with a family history of allergic disease, or from infants who subsequently develop allergic disease. These include defects in IFN- γ secretion, reduced TLR2, TLR4 and soluble CD14 expression, reduced

IL-10 and FoxP3 responses to TLR2 ligation, and low level monocyte human leucocyte antigen (HLA)-DR expression [14–20]. Studies examining innate immunity in such infants have yielded conflicting results. Krauss-Etschmann *et al.* [21] found reduced TLR2 and TLR4 mRNA expression in cord blood from infants born to allergic vs. non-allergic mothers. Jones *et al* [22] found that amniotic fluid CD14 levels were reduced in those who subsequently developed allergic sensitization, and were also reduced in breast milk from mothers of infants who developed eczema. A smaller study of 18 infants failed to find a relationship between maternal history of allergic disease and infant CD14 expression, but may not have had sufficient statistical power to do so [18]. Support for the importance of innate immunity in the pathogenesis of allergic disease comes from functional studies of subjects with established allergic disease, where there is evidence for deficient TLR2 and TLR4 signalling [23, 24]. Genetic association studies have found some inconsistent associations between polymorphisms in the TLR2 gene and allergic disease, while murine models suggest that TLR2 and TLR4 agonists may prevent allergic sensitization [25–30]. Innate immune defects may be particularly important modifiable factors influencing allergic disease development, because recent epidemiological studies of allergic disease have identified a relationship between TLR2, TLR4 and CD14 expression and protective early life exposures [31].

The aim of this study was to investigate whether infants at increased risk of developing allergic disease have early immune abnormalities affecting components of the innate immune response or regulatory T cell markers, focussing on the expression of pattern recognition receptors (PRR) and FoxP3 [31]. We investigated these parameters in a group of infants stratified for risk of allergic disease according to family history [32].

Materials and methods

Subject recruitment, treatment and classification

We enrolled 250 pregnant women into the pilot phase of a planned birth cohort study between September 2003 and March 2004. Women were excluded if they planned to leave the area before delivery or had a significant illness. In a separate study, 250 pregnant women were enrolled between February 2006 and February 2008 in a randomized-controlled trial (RCT) of prenatal *Lactobacillus rhamnosus* GG (LGG) supplementation for the prevention of eczema in their infant (Probiotic Eczema Prevention Study registered with Cochrane Skin Group <http://www.nottingham.ac.uk/ongoingskintrials> Trial No. 36). Women were recruited into the RCT if they, their partner or a previous child had a history of doctor diagnosed asthma, allergic rhinitis, food allergy or atopic eczema. Both studies were approved by the Human Research Ethics Committees of the

Mercy Hospital for Women, Melbourne. Written informed consent was obtained from all participants. In both studies women and their partners were classified as allergic if they had a history of doctor diagnosed asthma, atopic eczema, allergic rhinitis or food allergy. Food allergy or allergic rhinitis were included if supported by a history of positive skin prick test or supervised challenge. All couples were interviewed by an allergist (R. J. B.) to ascertain the presence or absence of allergic disease, classified before undertaking statistical analyses.

Cord blood collection

Cord blood was collected within 30 min of delivery into polypropylene tubes with preservative-free heparin [17]. Up to 10 mL cord blood was collected for the pilot cohort study, and ≤ 30 mL for the intervention trial. Cell numbers were only sufficient for real-time PCR analyses in the larger volume samples from the intervention trial. For this investigation we used all available cord blood samples which were transported to the laboratory within 12 h of birth, and contained $\geq 10 \times 10^6$ mononuclear cells. From the birth cohort study 156 samples were successfully taken, 56 cryopreserved within 12 h, and of these 20 had mononuclear cell counts $\geq 10 \times 10^6$ and were used for these studies. From the RCT 77 samples were successfully taken and cryopreserved within 12 h – of these 73 had cell counts $\geq 10 \times 10^6$ and were used for these studies.

Preparation of blood samples and cell culture

Cord blood mononuclear cells (CBMC) were separated by density centrifugation and cryopreserved at $10\text{--}20 \times 10^6/\text{mL}$ in RPMI with 42.5% fetal bovine serum (FBS) and 7.5% DMSO (Gibco, Invitrogen, Carlsbad, CA, USA) [17]. Median CBMC viability after subsequent thawing was 83.0% [inter-quartile range (IQR) 80.0–87.0]. CBMC were incubated for 48 h at 37 °C in 5% CO₂ in AIM-V serum-free medium supplemented with 2-mercaptoethanol (4×10^{-5} M; Gibco, Invitrogen) for cultures stimulated with ovalbumin (OVA), anti-CD3 or heat-killed LGG (HKL), or RPMI supplemented with 10% FBS for 48 h at 37 °C in 5% CO₂ for cultures stimulated with lipoteichoic acid (LTA) or lipopolysaccharide (LPS), all at 10^6 cells/mL in 1 mL volumes in 5 mL culture tubes (BD Bioscience, San Jose, CA, USA). LTA of *Staphylococcus aureus* origin ($10 \mu\text{g/mL}$) is a pathogen-associated molecular pattern (PAMP) that signals through TLR2, and LPS of *Escherichia coli* O111:B4 origin (10ng/mL) signals through TLR4 (Sigma, St Louis, MO, USA). HKL was prepared by washing 3×10^9 colony forming units (CFU) *Lactobacillus* GG in phosphate buffered saline (PBS) three times and incubating at 75 °C for 45 min (Dicofarm SpA, Rome, Italy). HKL was stored at -80 °C and the same batch was used throughout the study. An aliquot was cultured for 72 h to confirm sterility. Monoclonal anti-CD3 antibody

(α -CD3) was a kind gift of Dr Stuart Mannering (Walter and Elisa Hall Institute, Parkville, Australia). Optimal concentrations of LTA, LPS, HKL and α -CD3 were determined by preliminary titration experiments. Grade V Chicken ovalbumin (OVA; Sigma) was used in cell culture at 100 µg/mL as described previously [33]. All reagents were confirmed to be endotoxin free before use in cell culture, using the Limulus Amebocyte Lysate assay (Cape Cod Associates, E. Falmouth, MA, USA). The assay limit of detection was 0.03 Endotoxin U/mL. Detectable endotoxin levels were found in OVA, and successfully removed using polymyxin B columns before use (Detoxi-Gel, Pierce, Rockford, IL, USA).

Flow cytometry

Cell cultures were harvested at 48 h, or analysed before culture. Cell pellets were stained with fluorochrome-conjugated monoclonal antibodies in 50 µL staining volumes. Antibodies used were from E-Bioscience (San Diego, CA, USA – CD25-FITC and FoxP3-PE), and BD Bioscience (all others). Lineage cocktail-FITC (anti-CD3, 14, 16, 19, 20 and 56), HLA-DR-peridinin chlorophyll protein (PerCP; clone L243), CD123-PE (clone 9F5) and CD11c-allophycocyanin (APC; clone S-HCL-3) were used to identify dendritic cell (DC) phenotypes as CD11c^{hi}CD123^{lo} myeloid DC (mDC), CD123^{hi}CD11c^{lo} plasmacytoid DC (pDC) and CD11c^{lo}CD123^{lo} immature DC (iDC). CD3-APC (clone SK7), CD4-PerCP (clone SK3), CD25-PE-Cy7 (clone 2A3), CD25-FITC (clone BC96) and FoxP3-PE (clone PCH101) were used to identify CD3⁺CD4⁺CD25^{hi}FoxP3^{hi} T cell populations. CD14-FITC (clone MφP9) and HLA-DR-PerCP (clone L243) were used to identify CD14⁺ monocytes, CD14^{lo} HLA-DR^{hi} B cells and mean fluorescence intensity (MFI) of CD14 and HLA-DR expression on different cell populations. Aminostilbamidine methanesulphonate was used as a viability dye (Molecular Probes, Eugene, OR, USA). CBMC were incubated with labelled antibodies or isotype controls for 30 min at room temperature. For surface staining, cells were washed once with PBS and stored at 4 °C until analysed the same day. For intracellular staining, cells were permeabilized, fixed and stained with FoxP3-PE antibody or isotype control according to the manufacturer's instructions. All flow cytometric data were acquired on a 4-laser LSR II (BD Bioscience) and analysed with FACSDiva v4.1 software using well-defined gating strategies.

Real-time reverse transcriptase-polymerase chain reaction

RNA was extracted from cord blood samples in the RCT using the RNAeasy Mini Kit (Qiagen, Hilden, Germany), and reverse transcribed to cDNA using the Superscript First Strand Synthesis System and oligo(dT) primers (Invitrogen). All reactions included a 'RT minus' control with no

reverse transcriptase to control for the possibility of contaminating DNA. TLR2 and TLR4 mRNA were quantified by real-time PCR using FAM-labelled Taqman Gene Expression Assays on an ABI Prism 7300HT system (Applied Biosystems, Foster City, CA, USA). Eukaryotic translation elongation factor 1 alpha (EEF1A1) was used as a reference gene [34]. The mean level of gene expression in cDNA samples was expressed as a ratio to mean EEF1A1 expression.

Statistics

Prior analyses showed no differences in immunological outcomes between placebo and probiotic supplementation groups, and no evidence that the relationship between risk status and immunological outcomes differed according to randomization group [35]. Data from the two groups were therefore combined for this study. Infants were categorized into three groups according to the number of parents with allergic disease: (1) low risk (no parent affected), (2) intermediate risk (one parent affected) and (3) high risk (both parents affected). Inter-group differences were further explored by analysing according to maternal or paternal history of allergic disease. Data are presented as arithmetic mean and standard error of the mean (SEM), or median with IQR or 95% confidence intervals (CI). One-way ANOVA or unpaired *t*-test were used for data that approximated to a normal distribution and Kruskal–Wallis *H*-statistic or Mann–Whitney *U*-test for data that did not. Categorical data were analysed using Pearson χ^2 test. Interpretation of all analyses took account of the number of comparisons made, but formal correction for multiple comparisons such as Bonferroni correction was not used [36]. Moreover the majority of the immune outcomes evaluated in these studies were positively correlated, reducing the negative impact of multiple comparisons. Statistical analyses were performed using SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Demographic characteristics of infants with differing risk of allergic disease

Table 1 shows the characteristics of infants in the three risk strata, including variables which are known to influence CBMC immune markers – namely parity, mode of delivery, smoking and gestational age [8, 37–39]. There was little evidence of difference between the three groups for any of these variables.

Antigen presenting cell phenotypes in uncultured cord blood mononuclear cell in relation to risk of allergic disease

Antigen presenting cell (APC) phenotypes were analysed in freshly thawed CBMC of infants at differing risk of

Table 1. Characteristics of infants according to number of parents affected by allergic disease

Characteristic	No parent	One parent	Two parents	<i>P</i> -value
<i>N</i>	14	54	25	
Mean (95% CI) maternal age	30 (27–33)	33 (32–34)	33 (31–35)	0.1*
Primiparous (%)	38.5	35.2	32.0	0.7 [†]
Smoker in pregnancy (%)	15.4	16.7	4.0	0.2 [†]
Median (IQR) gestation (week)	40 (39–41)	40 (39–41)	40 (39–41)	0.5 [‡]
Caesarean (%)	21.4	24.5	36.0	0.5 [†]
Female (%)	50.0	32.7	40.0	0.5 [†]
Mean birth weight (g) (95% CI)	3604 (3333–3876)	3588 (3427–3749)	3659 (3506–3811)	0.7*

Pregnancy characteristics of infants with no parent affected by allergic disease (12 infants from cohort study, two from RCT), one parent affected (36 mothers, 18 fathers affected; six infants from cohort study, 48 from RCT) and two parents affected (two infants from cohort study, 23 from RCT).

*One-way ANOVA.

[†]Pearson χ^2 statistic.

[‡]Kruskal–Wallis *H*-statistic.

CI, confidence interval; RCT, randomized-controlled trial; IQR, inter-quartile range.

developing allergic disease, based upon parental history of such disease. There was a significant association between risk of allergic disease and CD14 expression, when measured as MFI of CD14 expression on the total viable mononuclear cell population (median MFI 4372 no parents with allergic disease; 3439 one parent and 2166 in both parents; $P = 0.01$ by Kruskal–Wallis *H*-statistic) or as percentage of CD14⁺ monocytes (mean 15.0% no parents with allergic disease; 10.5% one parent and 7.2% both parents; $P = 0.01$ by one-way ANOVA). These data are summarized in Figs 1 and 2. The relationship between risk of allergic disease and low expression of membrane bound CD14 was also present if data were analysed according to maternal history of allergic disease. Those with a maternal history of allergic disease had reduced MFI of CD14 expression on mononuclear cells (median 3914 maternal allergic disease absent; 2419 present; $P = 0.01$; Mann–Whitney *U*-test) and reduced percentage of CD14⁺ monocytes (mean 12.84% if maternal allergic disease is absent; 8.77% if present; $P = 0.01$; unpaired *t*-test). When data were analysed according to paternal history, these differences were less evident – a positive paternal history of allergic disease did not have significantly different CD14 MFI ($P = 0.2$; Mann–Whitney *U*-test), but there was weak evidence of reduced total CD14⁺ monocyte percentage ($P = 0.05$; unpaired *t*-test). We found no evidence of difference between groups in HLA-DR expression on CD14⁺ monocytes ($P > 0.9$) or on B cells ($P = 0.9$). There

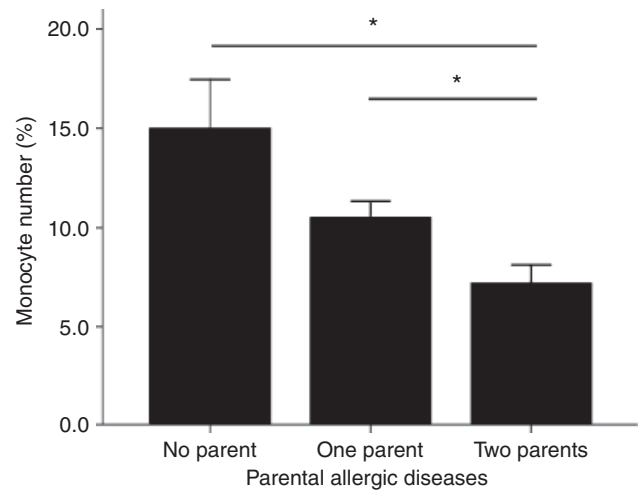


Fig. 1. Monocyte percentage in infants at different risk of allergic disease. CD14⁺ monocyte percentage in total viable mononuclear cells in uncultured cord blood mononuclear cells from infants at low risk (no parent affected by allergic disease; $n = 13$), intermediate risk (one parent affected; $n = 53$) or high risk (two parents affected; $n = 25$) of developing allergic disease – data shown are mean CD14⁺ monocyte percentage. Bars represent ± 1 SEM. * $P < 0.05$ (unpaired *t*-test). SEM, standard error of the mean.

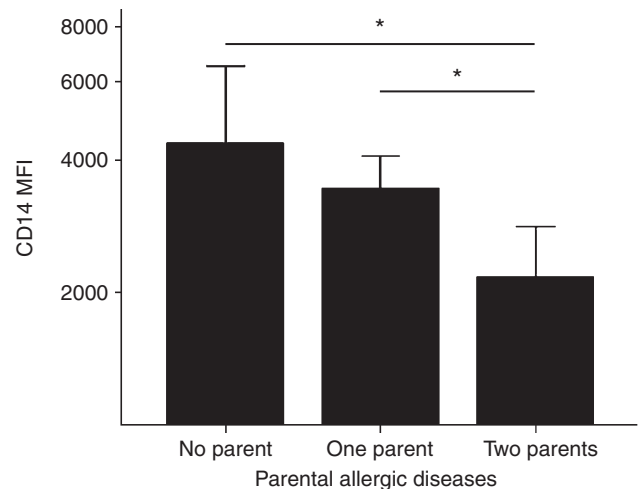


Fig. 2. CD14 expression in infants at different risk of allergic disease. Mean fluorescence intensity of CD14 expression on viable mononuclear cells in uncultured cord blood mononuclear cells from infants at low risk (no parent affected by allergic disease; $n = 13$), intermediate risk (one parent affected; $n = 53$) or high risk (two parents affected; $n = 25$) of developing allergic disease – data shown are median CD14 mean fluorescence intensity. Bars represent 95% CI. * $P < 0.05$ (Mann–Whitney *U*-test). CI, confidence interval.

was also no evidence of difference between infants at intermediate or high risk of allergic disease in resting CBMC TLR2 (mean $\log_{10}$ expression 0.96 one parent with allergic disease; 1.05 both parents; $P = 0.4$) or TLR4 (mean $\log_{10}$ expression 0.70 one parent with allergic disease;

0.75 both parents; $P=0.7$) mRNA expression (uncultured CBMC). Likewise there was no difference in expression of these genes in CBMC cultured with different stimuli, nor when analyses were performed according to the presence of maternal or paternal allergic disease alone (data not shown). TLR gene expression data were not available for low-risk infants (no parent with allergic disease) due to limited cell numbers in these CBMC samples.

Dendritic cell percentage and phenotype in relation to risk of allergic disease

In freshly thawed CBMC and CBMC cultured with a range of stimuli *in vitro*, we evaluated the percentage and phenotype of cord blood DC according to risk of developing allergic disease (parental history of allergic disease). We found a significant association between risk of allergic disease and low DC number in all stimulated cultures with the exception of HKL (Table 2). We found increases in pDC and mDC percentages in infants at high risk of allergic disease as compared with those at low or intermediate risk under some culture conditions, but these data were not consistent, cell numbers included in analyses were small and there was no evidence of difference between groups in CD123 MFI or CD11c MFI for the total DC population nor in iDC percentage (data not shown). There was little evidence of inter-group difference in total DC percentage, pDC or mDC%, or MFI of CD123 and CD11c for fresh uncultured CBMC or CBMC cultured in medium alone. Likewise there was no significant difference in the ratio of pDC:mDC in uncultured CBMC, nor in CBMC cultured with LTA, LPS, OVA, HKL, α -CD3 or medium alone. DC

Table 2. Dendritic cell percentage in cord blood according to number of parents affected by allergic disease

Stimulus	No parent	One parent	Two parents	<i>P</i> -value
<i>N</i>	14	53	25	
Uncultured	1.8 (1.3–2.9)	1.7 (1.0–2.1)	1.6 (0.8–2.2)	0.2
Medium only	2.1 (1.2–2.8)	1.7 (1.1–2.9)	1.3 (0.9–2.4)	0.3
LTA	1.9 (1.2–3.0)	1.9 (1.2–2.5)	1.1 (0.6–1.9)	0.02
LPS	1.9 (1.3–2.4)	1.7 (0.9–2.5)	1.0 (0.5–1.5)	0.01
OVA	2.3 (1.2–3.1)	2.0 (1.2–2.8)	1.2 (0.8–2.4)	0.04
HKL	1.8 (1.2–3.7)	2.1 (1.2–3.0)	1.5 (0.9–2.0)	0.2
Anti-CD3	2.0 (1.0–2.7)	1.4 (0.9–2.5)	0.9 (0.5–1.6)	0.03

DC percentage in uncultured and cultured CBMC from infants at low risk (no parent affected by allergic disease), intermediate risk (one parent affected) or high risk (two parents affected) of developing allergic disease. Values shown are median (IQR) percentage lineage^{neg}HLA-DR^{pos} cells in the total viable mononuclear cell population. *P*-values are derived from the Kruskal–Wallis *H*-statistic for a difference between the three groups under each culture condition.

CBMC, cord blood mononuclear cells; DC, dendritic cell; IQR, inter-quartile range; HLA, human leucocyte antigen; LPS, lipopolysaccharide; OVA, ovalbumin; LTA, lipoteichoic acid; HKL, heat-killed *Lactobacillus rhamnosus* GG.

data were also analysed according to the presence of maternal or paternal allergic disease separately. There was no evidence of association with total DC percentage, DC subtypes or pDC:mDC ratio according to maternal history of allergic disease. In contrast, infants with a paternal history of allergic disease had reduced total DC numbers under all culture conditions but not in uncultured CBMC (data not shown).

CD25^{hi}FoxP3^{hi} CD4 T cell percentage in relation to risk of allergic disease

We evaluated the number of CD25^{hi}FoxP3^{hi} CD4⁺ T cells in CBMC samples from infants in the three risk strata. In freshly thawed CBMC there was weak evidence of increased percentage of CD25^{hi}FoxP3^{hi} regulatory T cells in infants at increased risk of allergic disease (median 3.4% no parent affected by allergic disease; 3.95% one parent; 4.8% both parents; $P=0.09$; Kruskal–Wallis *H*-statistic; Fig. 3). There was no significant difference between groups in CD25 MFI or FoxP3 MFI on the total CD4⁺ T cell population in uncultured CBMC. The increased percentage of Tregs in infants at higher risk of allergic disease was still seen when data were analysed according to maternal history of allergic disease (median 3.5% maternal allergic disease absent; 4.5% present; $P=0.09$; Mann–Whitney *U*-test) but not when analysed according to paternal history of allergic disease (median 4.3% paternal allergic disease absent; 4.4% present; $P=0.9$;

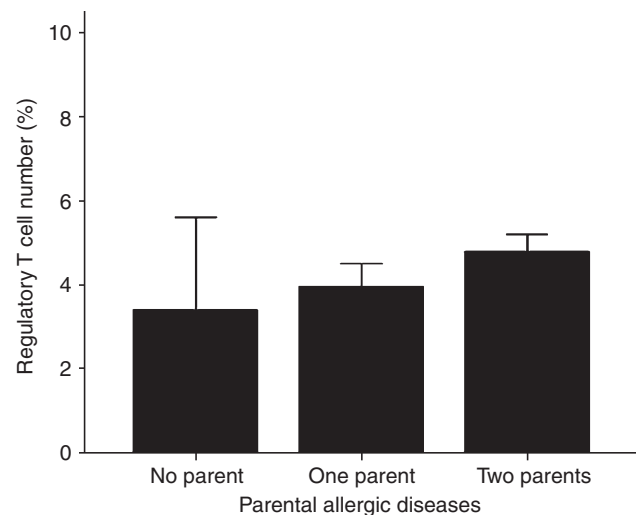


Fig. 3. CD25^{hi}FoxP3^{hi} Tregs in infants at different risk of allergic disease. CD25^{hi}FoxP3^{hi} regulatory T cell percentage in uncultured cord blood mononuclear cells from infants at low risk (no parent affected by allergic disease; $n=13$), intermediate risk (one parent affected; $n=54$) or high risk (two parents affected; $n=25$) of developing allergic disease. Data shown are median Treg percentage in total CD4⁺ T cells. Bars represent 95% CI. There is a non-significant trend to increased Treg percentage in infants at increased risk of allergic disease ($P=0.09$; Kruskal–Wallis *H*-statistic). CI, confidence interval.

Table 3. CD25^{hi}FoxP3^{hi} CD4⁺ T cell percentage in cord blood according to number of parents affected by allergic disease

Culture condition	No parent	One parent	Two parents	P-value
N	14	53	25	
Medium only	3.7 (3.5–4.9)	4.3 (3.2–5.7)	4.7 (4.0–6.0)	0.11
LTA	2.3 (1.6–2.9)	3.0 (2.1–4.3)	3.0 (2.2–4.1)	0.06
LPS	2.7 (2.0–3.9)	3.5 (2.6–4.9)	3.4 (3.0–4.8)	0.14
OVA	5.0 (4.0–6.0)	4.5 (3.7–6.1)	6.0 (4.7–6.5)	0.15
HKL	6.3 (5.6–9.5)	7.6 (5.6–10.4)	8.3 (6.0–12.4)	0.39
Anti-CD3	28.6 (22.2–37.2)	29.2 (18.9–44.0)	27.8 (19.5–45.5)	0.91

CD25^{hi}FoxP3^{hi} CD4⁺ T cell percentage in cultured CBMC from infants at low risk (no parent affected by allergic disease), intermediate risk (one parent affected) or high risk (two parents affected) of developing allergic disease. Values shown are median (IQR) percentage CD25^{hi}FoxP3^{hi} cells in the total CD4⁺ T cell population. P-values are derived from the Kruskal–Wallis H-statistic for a difference between the three groups under each culture condition.

CBMC, cord blood mononuclear cells; DC, dendritic cell; IQR, interquartile range; LPS, lipopolysaccharide; OVA, ovalbumin; LTA, lipoteichoic acid; HKL, heat-killed *Lactobacillus rhamnosus* GG.

Mann–Whitney U-test). In cultured CBMC there was no significant difference between groups in percentage of CD25^{hi}FoxP3^{hi} CD4⁺ T cells with the different stimuli studied (Table 3). There was however weak evidence of increased percentage of CD25^{hi}FoxP3^{hi} CD4⁺ T cells after culture with LTA ($P=0.06$), attributable to a difference between those at low risk and those at either intermediate or high risk of allergic disease. There was no significant difference between groups in FoxP3 MFI on CD4⁺ T cells after culture with LTA nor under any other culture condition. We also found no difference between groups in CD25 MFI on CD4⁺ T cells after culture with any of the stimuli used (data not shown).

Discussion

We have shown for the first time that infants at high inherited risk of developing allergic disease based on family history have a deficiency in CD14⁺ monocyte percentage and reduced DC percentage when compared with infants at lower risk. Our data also suggest that such infants may have increased CD25^{hi} FoxP3^{hi} regulatory T cells present at birth. The alterations of monocytes and Tregs appear to be most strongly associated with maternal allergic disease. However, the majority of family history data was obtained from mothers, so information about paternal allergic disease may be less reliable. Together these results confirm that infants at high risk of allergic disease have altered immune function at the time of birth, and that such differences are reflected in a range of immune parameters including markers of innate immune responsiveness. We found high-risk infants to have reduced membrane bound CD14 ex-

pression. If this is important in the pathogenesis of allergic disease, then early life interventions to prevent such disease might aim to induce increased innate immune responses in the high-risk infant.

A wide range of immune abnormalities has been reported in human umbilical cord blood associated with either family history of allergic disease or its subsequent development (reviewed in [40]). We found low CD14 expression on mononuclear cells to be associated with family history of allergic disease, and this is consistent with an emerging body of evidence surrounding the importance of CD14 in allergic disease. Other investigators have found reduced CD14mRNA transcription in CBMC in association with parental allergic disease but did not report any evaluation of membrane bound CD14 [21]. Our data are consistent with these findings. Two other groups have specifically reported the relationship between CD14⁺ monocytes and family history of allergic disease – the first of these examined just 23 subjects, and found no relationship between family history of allergic airways disease and cord blood CD14⁺ monocyte numbers [41]. The second group studied 45 mother/infant pairs and reported no relationship between leucocyte CD14 MFI or CD14⁺ monocyte numbers in fresh whole blood and family history of allergic disease [42]. This group did not report the statistics for this outcome, had a smaller sample size than our study and used different methodology. There may be important differences between measuring CD14⁺ monocytes as a percentage of total leucocytes in fresh whole blood, and measuring them as a percentage of mononuclear cells in freeze thawed blood. Examination of soluble and membrane bound CD14 expression in relation to allergic disease remains an important area for further investigation, particularly in view of the associations between CD14 gene polymorphisms and environmental determinants of allergic disease reported by several groups [31, 43–45]. We failed to support the previously reported association of low HLA-DR MFI on CD14⁺ monocytes with risk of subsequent allergic disease, found in a study of 36 infants [15].

We were not able to assess gene transcription of TLR2 and TLR4 in all three risk groups in this study due to limited cell numbers – this may have reduced our ability to detect important differences in transcription of these genes in relation to risk of allergic disease. We found no significant difference in CBMC TLR2 and TLR4 transcription between infants with one or two parents affected by allergic disease in freshly thawed CBMC. This adds to the mixed data from two previous studies investigating human cord blood TLR2 and TLR4 expression in relation to parental allergic disease, and does not therefore exclude the possibility that TLR2 and TLR4 protein expression is deficient in infants at high risk of allergic disease [18, 21].

The total DC percentage in cultured CBMC was decreased in those at highest risk of allergic disease, but

there was no consistent difference in expression of the phenotypic DC markers CD123 and CD11c. A previous study of 18-cord blood samples reported no difference in either total DC or DC subtype percentage between infants at high and low risk of allergic disease [46]. The statistical power of our study was much greater, and this might account for the difference in outcome regarding total DC percentage. Our data regarding CD14⁺ monocytes and total DC percentage may be related. Monocytes play a role as DC precursors under specific culture conditions [47]. Although the culture conditions used in our experiments were different, it is possible in principle that the reduced CD14⁺ monocytes before culture reduced the pool of cells available for *in vitro* DC development during culture. Together these data suggest a reduced capacity for effective antigen presentation in those at highest risk of allergic disease, and this might contribute to the development of aberrant immune responses.

There was weak evidence of increased FoxP3^{hi} Treg percentage in the freshly thawed CBMC of infants at high risk of allergic disease ($P = 0.09$). In view of the number of separate statistical comparisons made, these findings need to be interpreted with caution. One other group has evaluated neonatal FoxP3 expression in relation to risk of allergic disease, and found increased FoxP3 mRNA expression in CBMC of high-risk infants (maternal atopy) after culture with a TLR2 ligand, compared with low-risk infants [14]. In a second study the same group found no difference in FoxP3 expression between high- and low-risk infants [48]. These data cast doubt on the significance of the trend to increased Treg percentage found in high-risk infants in our study. Several studies have found increased CD25^{hi}FoxP3^{hi} Treg cells in those with established allergic disease – it is unclear whether this immune abnormality is already present at birth, and this is an area worthy of further study [49].

Several groups have found other immune alterations associated with family history of allergic disease that were not specifically examined in this study [40]. The most consistent abnormality reported by our laboratory and others is reduced mitogen and allergen-induced IFN- γ secretion in CBMC from high-risk infants [17, 19, 20]. A similar defect in IFN- γ secretion has been found later in life in those with allergic diseases such as eczema and asthma and in those with high total IgE levels [50]. Prescott et al found the maturation of IFN- γ responses to be delayed in those with allergic disease, and our laboratory has found that resolution of asthma in adulthood is associated with a normalization of IFN- γ responses [17, 51]. Together these previous findings of deficient IFN- γ responses and our new finding of reduced CD14⁺ monocyte and DC percentages suggest that those at highest risk of allergic disease have a deficiency of pro-inflammatory immune responses that is already present at birth. One might speculate that in high-risk infants deficient innate

immune responses result in aberrant and persistent inflammatory responses to environmental stimuli via alternative mechanisms. Similar paradigms have been proposed for the pathogenesis of some other auto-inflammatory diseases [52].

In summary we have reported for the first time a clear association between CD14 expression at birth and inherited risk of allergic disease development. Associated with this we have found reduced DCs in culture and weak evidence of increased CD25^{hi}FoxP3^{hi} Tregs. In combination with our laboratory's previous finding of defective IFN- α secretion and the work of others, these data suggest that infants at risk of allergic disease have a significant deficiency in PRR expression and APC function which is present from birth. If early life interventions are able to correct these defective immune responses, then they may be able to prevent the development of allergic disease in high-risk infants [31].

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