

Maternal allergy modulates cord blood hematopoietic progenitor Toll-like receptor expression and function

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Background: Little is known regarding the prenatal determinants of innate immune responses in relation to infant allergic risk. Environmental exposures, including microbial stimuli, might predispose susceptible subjects to atopy and asthma in early infancy or even *in utero*.

Objective: Because Toll-like receptors (TLRs) recognize microbial products and because cord blood (CB) progenitor alterations have been observed in neonates at risk for atopy, we investigated the expression and function of TLRs on CB hematopoietic progenitors in relation to atopic risk, as defined by maternal allergic sensitization.

Methods: Thirty-two (15 with low and 17 with high atopic risk) infant CB samples were assessed for phenotypic and functional alterations in CD34⁺ cells by means of flow cytometry and methylcellulose culture, respectively. CD34⁺ hematopoietic progenitors were stained for TLR-2, TLR-4, TLR-9, GM-CSF receptor α , IL-5 receptor α , and IL-3 receptor α or cultured in methylcellulose assays for hematopoietic cytokine-stimulated eosinophil-basophil (Eo/B) colony-forming units (CFUs) with or without LPS.

Results: High-atopic-risk infants had significantly lower CB CD34⁺ cell TLR-2, TLR-4, and TLR-9 expression ($P = .009$). High-risk infant progenitors gave rise to significantly more Eo/B CFUs ($P = .002$) with hematopoietic cytokine (IL-3, IL-5, or GM-CSF) stimulation *ex vivo*. Although LPS costimulation induced Eo/B CFUs from both low- and high-risk infant CB CD34⁺ cells, this response was significantly ($P = .020$) muted in the high-risk CB progenitors.

Conclusions: Neonatal CB CD34⁺ hematopoietic progenitor cell TLR expression and functional responsiveness are altered in CB

from atopic at-risk infants. Maternal allergic sensitization might modulate hematopoietic progenitor TLR expression and function *in utero*; specifically, Eo/B “lineage priming” at birth might be circumvented through engagement of TLR pathways in early life. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: Atopy, Toll-like receptors, cytokine receptors, cord blood, CD34, eosinophil-basophil

The developmental immune mechanisms in the fetal and neonatal period that influence infant atopic risk are not clearly understood. Immunologic responses to environmental stimuli in early life might influence patterns of immune development.^{1,2} Because studies have provided evidence that antenatal differences in immune function are associated with subsequent allergic disease³ and that childhood allergies manifest in the first years of life,^{4,5} it is possible that the earliest manifestation of allergy results from intrauterine regulation of prenatal responses to environmental stimuli; however, little is known about the prenatal determinants of the innate immune response related to atopic development.

It has been proposed that gestational environmental exposures in the appropriate genetic context might influence the risk of pediatric allergy⁶ and specifically that hematopoietic and/or immune cord blood (CB) profiles might be useful in predicting atopy in early life. For example, perinatal cytokines⁷ and cytokine receptors⁸ have been associated with increased risk of postnatal allergic disease, such as atopic dermatitis. Likewise, we have previously shown that hematopoietic cytokine receptor expression on CB myeloid (CD34⁺/45⁺) progenitor cells is associated with atopic risk, as determined by parental history, maternal allergic sensitization,⁹ or both and first-year atopic and respiratory outcomes.^{10,11} Because maternal allergy is associated with alterations in Toll-like receptor (TLR) expression,¹² CB progenitor TLR expression might also be a potential biomarker identifying children at risk for allergy.

Rural farming environments with high microbial loads have been repeatedly associated with lower childhood allergy,¹³ presumably through immunomodulatory effects on the neonatal innate immune system.^{2,14} Furthermore, there is evidence that microbial stimulation modulates hematopoiesis.¹⁵⁻¹⁸ In fact, we have found that CB progenitors respond to TLR ligation through the induction of eosinophil-basophil (Eo/B) lineage primed cells, activation of CB CD34⁺/45⁺/IL-3 and IL-5 receptor α^{hi} /GM-CSF receptor α^{hi} cells that are highly responsive to hematopoietic cytokine stimulation *ex vivo* (Reece et al, unpublished data). Neonates at risk for allergy also have increased Eo/B “lineage priming”¹⁰; however, whether allergic risk modulates TLR-mediated

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Abbreviations used

CB:	Cord blood
CFU:	Colony-forming unit
Eo/B:	Eosinophil-basophil
GM-CSFR:	GM-CSF receptor
IL-3R:	IL-3 receptor
IL-5R:	IL-5 receptor
MFI:	Median fluorescence intensity
PE:	Phycoerythrin
PerCP:	Peridinin chlorophyll protein
SPT:	Skin prick test
TLR:	Toll-like receptor

effects on these progenitors and their lineage commitment has not yet been studied.

Therefore we investigated whether maternal allergic sensitization (conferring high atopic risk to their infants by definition) affects CB progenitor TLR-2, TLR-4, and TLR-9 expression and function. We hypothesized that progenitor cell TLR expression and function are altered in atopic high-risk infants. To test this, we quantified the level of TLR expression and the role of TLR ligation in CB hematopoietic progenitor differentiation in a population of women participating in an Australian birth cohort study.

METHODS

Study design and population

Pregnant female subjects booked for antenatal appointments in local metropolitan hospitals (including St John of God Hospital, King Edward Memorial Hospital, and Osborne Park Hospital) between August 2002 and October 2004 were screened by a researcher using a telephone or interview questionnaire. Healthy pregnant volunteers ($n = 32$) were recruited into the study, including similar numbers of nonatopic ($n = 15$) and atopic ($n = 17$) participants. Subjects were excluded from analysis if they were delivered preterm (<36 weeks' gestation) or if there were any clinically important neonatal morbidity or disease. Maternal allergy status was assessed by means of skin prick tests (SPTs) to common allergens (house dust mite, grasses, moulds, cat, dog, feathers, and cockroach). These extracts were purchased from Hollister-Stear Laboratories (Spokane, Wash). A wheal size of 3 mm or more larger than that elicited by the negative control was considered positive. Mothers were defined as allergic if they had a history of disease and 1 or more positive SPT responses. All volunteers recruited into the study received verbal and written information about the study and signed written consent forms. For laboratory assessments, research personnel were unaware of maternal allergy status. Infant CB samples were considered high risk if the mothers had at least 1 positive SPT response and low risk if the mothers had a negative SPT response. Allergic symptoms were reported by the mothers as doctor diagnosed, but this was not confirmed by the investigators. As such, these symptoms were not considered in the inclusion criteria. The study was approved by the Princess Margaret Hospital Ethics Committee, Perth, Australia, and by the Hamilton Health Sciences/McMaster Faculty of Health Sciences Research Ethics Board in Hamilton, Ontario, Canada.

CB collection and sample cryopreservation

CB was collected at delivery into an equivolume of RPMI (Gibco, Burlington, Ontario, Canada) plus heparin (Sigma Aldrich Ltd, Oakville, Ontario, Canada). Mononuclear cells were isolated with Lymphoprep (Nycomed Pharma AS, Oslo, Norway) gradient centrifugation at 500g for 30 minutes and washed twice in RPMI and then once in RPMI containing 2% FCS (Gibco). Cells were enumerated with a hemocytometer (Neubauer

Chamber; Hausser Scientific, Blue Bell, Pa) and diluted to 40×10^6 cells/mL RPMI/2% FCS and placed on ice.

Cryopreservation and shipment of CB samples

Cells were diluted to 40×10^6 /mL RPMI/2% FCS (MultiSer; Biosciences Pty, Ltd, Baulkham Hills, Australia) and placed on ice. An equal amount of cooled 15% dimethyl sulfoxide (BDH, Victoria, Australia) in FCS was added dropwise. Samples were frozen in 1-mL aliquots, each containing 20×10^6 cells, and were cooled to -80°C at a rate of $1^\circ\text{C}/\text{min}$ in a freezing container (Nalgene cryocontainer) containing isopropyl alcohol (BDH) and transferred to a liquid nitrogen tank for storage and shipment. We and others have shown reliably and reproducibly that cryopreservation and thawing in this way do not distort cellular immune responses, including functional and phenotypic characteristics of $\text{CD}34^+$ progenitor cells,¹⁰ or alter the pattern of TLR cellular function.¹⁹ Mononuclear cells stored in liquid nitrogen (2×10^7 CB mononuclear cells per patient) were transported from Australia to Hamilton by courier.

Cells were thawed by means of dropwise application of RPMI complete, resuspended in McCoy's 3+ media (Gibco), and incubated (37°C in 5% CO_2) for 2 hours in a plastic flask to remove adherent mononuclear cells. Nonadherent mononuclear cells were then resuspended in 1 mL of Iscove's 2+ culture medium (for methylcellulose cultures) or 1 mL of FACS buffer (for flow cytometry), and a cell count was performed. Average viability was greater than 98%, and average recovery was 81.5% of the total number of cells frozen.

TLR ligand used for methylcellulose cultures

LPS from *Escherichia coli* 0111:B4 was purchased from Sigma (Mississauga, Ontario, Canada). Optimal culture conditions for LPS were determined, including optimal cell numbers and ligand doses. Preliminary experiments with the maximal number of Eo/B colony-forming units (CFUs) as an end point measure revealed that 10 $\mu\text{g}/\text{mL}$ LPS was an optimal concentration for all assays performed.

Methylcellulose colony assay

Methylcellulose colony assays were performed as previously described.¹¹ Briefly, CB nonadherent mononuclear cells were cultured in duplicates with 2×10^5 cells/35 mm $\times$ 10 mm culture disks (Falcon Plastics, Oxnard, Calif) in 0.9% methylcellulose (Sigma) with Iscove's 2+ (modified Dulbecco's medium [Gibco] and 20% FCS supplemented with 1% penicillin-streptomycin and 5×10^{-5} mol/L 2-mercaptoethanol) in the presence of recombinant human cytokines at optimal doses, IL-3 (1 ng/mL), IL-5 (1 ng/mL), or GM-CSF (10 ng/mL) (BD Biosciences, Mississauga, Ontario, Canada), or equal amounts of Iscove's 2+ culture medium as a control. Cultures were incubated for 14 days (37°C in 5% CO_2) in the presence of IL-3, IL-5, or GM-CSF. For TLR stimulation, 10 $\mu\text{g}/\text{mL}$ LPS was added to the hematopoietic cytokine-stimulated cultures. Eo/B CFUs responsive to each cytokine were enumerated from duplicate cultures by using inverted light microscopy (colonies were defined as >40 cells). The researcher counting colonies was blinded to any information about the subjects.

Antibodies

Receptors of the study include phycoerythrin (PE)-anti-human TLR-2 (CD282), PE-anti-human TLR-4 (CD284), PE-anti-human TLR-9 (CD289), PE-anti-human IL-3 receptor (IL-3R) α (CD123), PE-anti-human IL-5 receptor (IL-5R) α (CDw125), and PE-anti-human GM-CSF receptor (GM-CSFR) α (CD116). TLR antibodies were purchased from eBioscience (San Diego, Calif), and hematopoietic cytokine receptor antibodies were purchased from BD PharMingen (San Jose, Calif). Isotype control for TLR-2 and TLR-4 was PE-mouse immunoglobulin G2a (IgG2a κ). Isotype control for TLR-9 was PE-rat IgG2a κ . Isotype control for hematopoietic cytokine receptors was PE-mouse IgG1a κ . Peridinin chlorophyll protein (PerCP)-conjugated CD34 antibody was used to label progenitor cells. Leukocyte labeling and compensation were carried out using fluorescein isothiocyanate-conjugated CD45, allophycocyanin-conjugated CD45, PerCP-conjugated CD45 and PE-conjugated CD45 antibodies.

Cell staining

Four-color immunofluorescent staining was performed by using an adapted procedure.⁹ The CD34-enriched cells were resuspended in 1 mL of FACS buffer (PBS containing 0.1% sodium azide) and placed in aliquots in individual tubes at a concentration of 1×10^5 cells per tube. Cells were resuspended in murine block (PBS containing 0.2% sodium azide, 2.5% murine serum, and 2.5% human serum) and incubated in the refrigerator for 30 minutes. Optimal amounts of PE-labeled anti-IL-3R α , IL-5R α , GM-CSFR α , TLR-2, TLR-4, or their respective isotype controls were added to the tubes, and they were incubated for 30 minutes in the refrigerator. Cells were then washed with FACS buffer and fixed in 250 μ L of 1% paraformaldehyde. Compensation tubes were likewise prepared, staining cells individually with anti-CD45–fluorescein isothiocyanate, anti-CD45–PE, anti-CD45–allophycocyanin, and anti-CD45–PerCP. For intracellular analysis of TLR-9, cells were resuspended in a fixing solution (Caltag Laboratories, Carlsbad, Calif) for 20 minutes at room temperature, washed with FACS buffer, and resuspended in permeabilization buffer (Caltag Laboratories). Antibodies for PE–anti-human TLR-9 and rat isotype control were added to the permeabilization solution and incubated for 30 minutes at room temperature. Cells were then washed and resuspended in 250 μ L of 1% paraformaldehyde. All samples were covered with aluminum foil and refrigerated until ready for acquisition.

Acquisition and analysis

Acquisition was performed with an LSR II flow cytometer (BD Bioscience). Five thousand progenitors were collected for analysis. To enumerate CD34⁺ cells by means of flow cytometry, we used an established multiparameter gating strategy⁹ and a 4-color staining protocol to assess the expression of TLR and hematopoietic cytokine receptors. Flow Jo software (Tree Star, Inc, Ashland, Ore) was used for the analysis of data. CD34⁺ cells were identified as having high CD34 expression, low-intermediate CD45 expression, and low forward and side scatter, which is consistent with lymphoblastoid cells. For gating analysis, quadrant markers were set so that 2% of cells were stained with isotype control (negative staining). Cytokine receptor expression was calculated as the percentage of CD34⁺ cells staining positively for the receptor. This was obtained by subtracting the staining achieved by the designated isotype control from the staining of the CD34⁺ cells with the receptor of interest. Absolute numbers of CD34 cells were not determined because of the use of an enrichment protocol. Specific median fluorescence intensity (MFI), which represents the number of receptors per cell, was calculated by subtracting the MFI of the isotype from the MFI of the receptor of interest.

Statistical analysis

The sample size for this study ($n = 32$) was based on feasibility consideration because the overall goal of the study was exploratory for hypothesis generation. The demographic and prognostic characteristics of the patients were analyzed by using descriptive statistics reported as means (SDs) or medians (minimums-maximums) for continuous variables and counts for categorical variables. We used the *t* test to compare the 2 groups (low and high atopic risk defined by maternal sensitization) on the basis of CD34⁺ cell phenotypic biomarkers or functional outcomes. We further applied the Mann-Whitney *U* test to assess the sensitivity or robustness of the results, and the results remained the same. The criterion for statistical significance was set *a priori* at an α value of .050. We did not adjust the α value for multiple testing because the analyses were primarily exploratory (ie, for hypothesis generation).²⁰ The results are reported as estimates of the difference (95% CI) and associated *P* values. All *P* values are reported to 3 decimal places, with *P* values of less than .001 reported as a *P* value of less than .001. All analyses were performed with PASW Statistics (formerly named SPSS) version 18 (SPSS, Inc, Chicago, Ill).

RESULTS

Characteristics of maternal and neonatal populations

The study participants included 15 nonatopic mothers and 17 atopic mothers (Table I). There was no statistical difference in

TABLE I. Characteristics of the study population

	Low-risk infants (n = 15)	High-risk infants (n = 17)	<i>P</i> value
Maternal characteristics			
Maternal age (y)	32.3 (5.2)	33.5 (3.9)	.433
Parity	1.4 (0.8)	1.0 (0.0-4.0)*	.248
Method of delivery			
Vaginal	12	12	
Caesarean	3	5	.437
Allergic phenotype			
Asthma	0	5 (29.4%)	<.001
Allergic rhinitis	0	16 (94.1%)	<.001
Any positive SPT response	0	17 (100%)	<.001
Positive HDM SPT response	0	14 (82.3%)	<.001
Positive grass pollen SPT response	0	12 (70.6%)	<.001
Positive animal dander SPT response	0	3 (17.6%)	<.001
Neonatal characteristics			
Gestational age (d)	278.1 (6.3)	276.5 (9.2)	.590
Birth weight (kg)	3.45 (0.4)	3.50 (0.4)	.708
Birth length (cm)	49.6 (1.8)	49.5 (1.6)	.819
Total IgE (kU/L)	0.17 (0.00)	0.17 (0.18-2.48)*	.183
Female sex	11	10	.405
Male sex	4	7	

Data are shown as means (SDs) or *medians (minimum-maximum). Data illustrating maternal atopic variables are represented as the number of subjects (percentage of group).

HDM, House dust mite.

maternal age or parity between the mothers in the low-atopic-risk and high-atopic-risk groups. In terms of neonatal characteristics, there were no statistically significant differences in the methods of delivery, gestational age, birth length or weight, head circumference, or total IgE level between the 2 groups (Table I).

Presence of maternal allergy is associated with reduced CB progenitor CD34⁺ cell TLR expression

We first sought to determine whether the expression profiles of 3 TLRs that have been associated with allergy (TLR-2, TLR-4, and TLR-9) were altered on human CB progenitor cells in relation to maternal allergic sensitization. As shown in Table II and Fig 1, in high-risk infants born to allergic mothers, CB CD34⁺ cells had significantly reduced TLR-2 (mean difference, 8.18; 95% CI, 2.15-14.22; *P* = .012), TLR-4 (mean difference, 13.38; 95% CI, 2.83-23.94; *P* = .017), and TLR-9 (mean difference, 56.89; 95% CI, 16.77-97.02; *P* = .009) expression when compared with that seen in low-risk (ie, no maternal allergy) infant CB. Hematopoietic cytokine receptor percentage expression on CB CD34⁺ cells was also significantly decreased in the high-atopic-risk group for IL-3R α (mean difference, 6.24; 95% CI, 1.85-10.64; *P* = .009) and IL-5R α (mean difference, 23.15; 95% CI, 13.32-32.97; *P* < .001; Table II and Fig 2) expression. This high-risk group also had higher specific MFIs for the respective cytokine receptors (data not shown; ie, the selective presence of subpopulations of CD34⁺/CD45⁺/IL-5R α ^{hi} cells and similarly for cells expressing IL-3R α and GM-CSFR α).

TABLE II. Differences between low- and high-atopic-risk infant CD34⁺ cell phenotypes

Phenotype	Groups, mean (SD)		Mean difference (95% CI)	P value
	LR (n = 6)	HR (n = 9)		
TLR-2	8.36 (8.54)	0.17 (0.34)	8.18 (2.15 to 14.22)	.012
TLR-4	14.4 (14.8)	1.02 (1.80)	13.38 (2.83 to 23.94)	.017
TLR-9	86.57 (6.04)	29.67 (44.66)	56.89 (16.77 to 97.02)	.009
GM-CSFR α	18.38 (5.61)	11.29 (6.70)	7.09 (−0.09 to 14.27)	.053
IL-3R α	7.89 (6.00)	1.64 (1.30)	6.24 (1.85 to 10.64)	.009
IL-5R α	32.46 (8.86)	9.31 (8.48)	23.15 (13.32 to 32.97)	<.001

Data were analyzed by using *t* tests. Significant (*P* < .050) results are shown in boldface.

HR, High atopic risk; LR, low atopic risk.

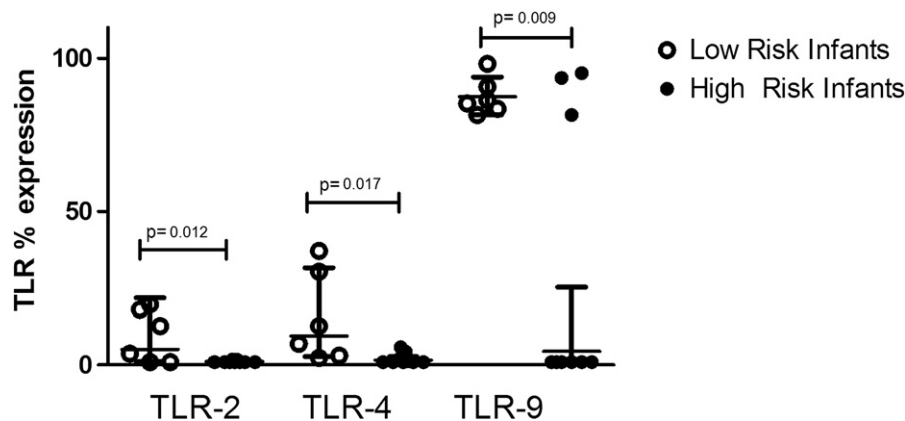


FIG 1. Differential CB CD34⁺ progenitor cell expression of TLR-2, TLR-4, and TLR-9 between high- and low-risk infants. CB mononuclear cells from high- and low-risk infants were stained with antibodies to surface TLR-2 and TLR-4 and intracellular TLR-9. CD34⁺ progenitor cells were gated and analyzed for differences in TLR expression. Data are presented as geometric means (95% CIs), and significant findings are indicated on the graph.

Differences between low- and high-risk infants in Eo/B differentiation capacity of TLR and hematopoietic cytokine–costimulated CB CD34⁺ cells

Because we (Reece et al, unpublished data) and others^{15–18} had previously demonstrated that TLR ligation of hematopoietic progenitor cells modulates their differentiation fates, we sought to determine what effect maternal allergic status had on Eo/B lineage commitment before and after TLR stimulation. First, we assessed Eo/B lineage commitment of CB CD34⁺ progenitor cells in low- and high-risk infants to hematopoietic cytokine stimulation alone. Stimulation of CB CD34⁺ cells from high-risk infants with the hematopoietic cytokines resulted in significantly higher IL-3–responsive (mean difference, −22.09; 95% CI, −34.87 to −9.31; *P* = .002) and GM-CSF–responsive (mean difference, −18.53; 95% CI, −30.35 to −6.70; *P* = .004) Eo/B CFUs compared with Eo/B CFUs from hematopoietic cytokine–stimulated low-atopic-risk CB CD34⁺ cells (Table III and Figs 3–5).

Because maternal allergy is associated with antenatal alterations in TLR expression⁵ and function^{3,21} on monocytes and CB progenitors (Fig 1), we next investigated whether TLR-mediated CD34⁺ cell Eo/B differentiation was also influenced by maternal allergic status. Interestingly, when high-risk infant CB CD34⁺ cells were assessed for their functional responsiveness to LPS and hematopoietic cytokine costimulation, these progenitors responded with significantly decreased Eo/B CFU production to LPS and IL-5 (mean difference, 4.88; 95% CI, 0.96–8.81; *P* =

.020) and GM-CSF (mean difference, 12.92; 95% CI, 1.42–24.41; *P* = .032) when compared with low-risk infant progenitors (Table III and Figs 3–5).

DISCUSSION

There is an increasing body of evidence supporting antenatal differences in TLR-related immune function and subsequent infant allergic disease. For example, it has recently been shown that infant allergic disease is associated with presymptomatic differences in TLR function at birth.³ Maternal environmental exposures to farm animals⁵ and cigarette smoke¹⁹ have likewise been shown to modulate neonatal TLR expression and function. We have previously shown that neonatal CB progenitor cells display altered phenotype and function with respect to parental atopic history and maternal atopic status^{9,10}; however, it is unknown whether the intrauterine environment of an allergic mother influences neonatal progenitor TLR expression and function. Our current study demonstrates that maternal allergic sensitization is associated with alterations in TLR expression and function on CB progenitor cells. We found that infant CB progenitor cell TLR-2, TLR-4, and TLR-9 expression was significantly reduced in high-risk infants and that *ex vivo* ligation of TLR-4 with LPS significantly decreased the functional responsiveness and thus the Eo/B lineage commitment of CB progenitor cells.

That high-risk infants' (ie, born to allergic mothers) CB progenitor cells have significantly decreased TLR-2, TLR-4, and

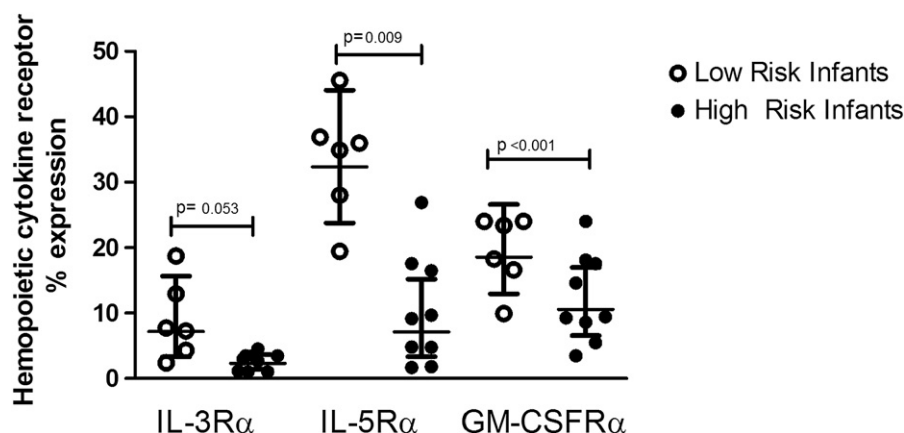


FIG 2. Differential CB CD34⁺ progenitor cell expression of IL-3R α , IL-5R α , and GM-CSFR α between high- and low-risk infants. CB mononuclear cells from high- and low-risk infants were stained with antibodies to surface IL-3R α , IL-5R α , and GM-CSFR α . CD34⁺ progenitor cells were gated and analyzed for differences in hemopoietic cytokine receptor expression. Data are presented as geometric means (95% CIs), and significant findings are indicated on the graph.

TABLE III. Differences between low- and high- atopic-risk infant CD34⁺ cell differentiation function

Eu/B CFU production	Groups, mean (SD)		Mean difference (95% CI)	P value
	LR (n = 9)	HR (n = 8)		
Hematopoietic cytokines				
IL-5 responsive	4.22 (4.03)	5.50 (5.93)	-1.28 (-6.47 to 3.91)	.607
IL-3 responsive	9.72 (6.04)	31.81 (16.87)	-22.09 (-34.87 to -9.31)	.002
GM-CSF responsive	4.72 (4.16)	23.25 (16.11)	-18.53 (-30.35 to -6.70)	.004
	LR (n = 6)	HR (n = 5)		
Addition of LPS				
IL-5 responsive	6.08 (3.71)	1.20 (1.15)	4.88 (0.96 to 8.81)	.020
IL-3 responsive	13.83 (12.61)	4.60 (3.79)	9.23 (-4.10 to 22.57)	.152
GM-CSF responsive	14.92 (11.00)	2.00 (2.67)	12.92 (1.42 to 24.41)	.032

Data were analyzed by using *t* tests. Significant results (*P* < .050) are shown in boldface.

HR, High atopic risk; LR, low atopic risk.

TLR-9 expression suggests that factors affecting CB CD34⁺ cell TLR expression might be operative *in utero*. Maternal allergic sensitization is typified by an atopic milieu consisting of increased ambient levels of the cytokines IL-4, IL-5, and IL-13, as well as higher IgE levels. It has been shown recently that peripheral blood dendritic cell TLR-9 suppression can be induced by IgE activation.²² Whether²³ or not²⁴ maternal IgE can cross the placenta has been debated, but IgE produced in the neonate, through cross reactivity with maternally derived allergens,^{24,25} has been demonstrated and thus could explain the observed decreased TLR-9 expression in our high-risk infants. However, this might be unlikely because there were no differences between circulating total CB IgE levels in the 2 groups (Table I). Likewise, T_H2 cytokines, such as IL-4 and IL-13, both of which have been detected in placental tissue,^{26,27} have been shown to downregulate TLR-4 expression and signaling²⁸ and could explain our finding of decreased CB CD34⁺ cell expression of that TLR. Alternatively, genetic factors might also be involved in the modulated expression of these TLRs on CB progenitors. In fact, it has recently been shown that maternal genotype, in particular the ability to signal through TLR-2, TLR-3, TLR-4, TLR-7, and TLR-9, was of vital importance in the subsequent protection of offspring against asthma.²⁹

Although the prenatal determinants predisposing an infant to allergy are relatively unknown, elucidating the mechanisms

regarding poorly acquired immune deviation in children at risk for allergy might prove helpful.³⁰ Although innate immune responses to TLR ligation depend on a number of variables (eg, age³¹), it is generally accepted that such stimulation results in the secretion of cytokines that favor T_H1 immunity³²; stimulation with TLR-2³³ or TLR-4³⁴ agonists directly activates T_H1-mediated responses. Interestingly, a study by Pfefferle et al¹⁴ demonstrated that maternal farming activities modulate CB cytokine profiles in the same fashion. Specifically, the authors showed that children born to mothers with regular farming contact and presumably more microbial exposure had increased IFN- γ and TNF- α levels at birth; both of these are cytokines associated with T_H1 immunity. Reduced TLR expression on immunocompetent cells, possibly in combination with reduced T_H1 cytokine production at birth,¹⁴ might therefore result in a lower degree of immune deviation and a T_H2-skewed immune system, increasing the risk of allergies in later life. Our observation of reduced TLR expression in high-risk infant CB hematopoietic progenitors not only agrees with other studies on CB monocytes⁵ but might reflect a more general phenotypic alteration across several immune compartments and cells (eg, monocytes), which are crucially involved in immune deviation.

Our finding that costimulation of CB hematopoietic progenitors with LPS and hematopoietic cytokines in high-risk children

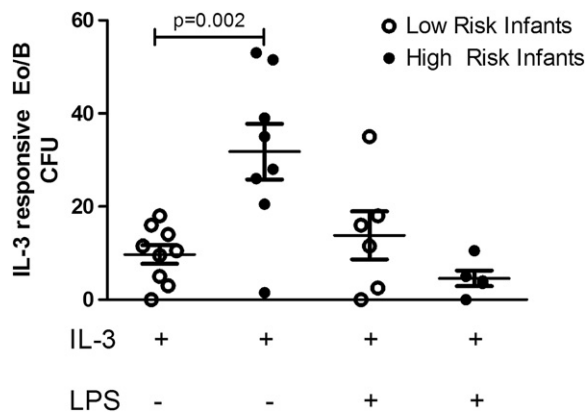


FIG 3. Responses to hematopoietic cytokines (IL-3) with or without LPS costimulation of CB CD34⁺ progenitor cells in high- and low-risk infants. CB mononuclear cells from low- and high-risk infants were enriched for CD34⁺ cells and stimulated with the hematopoietic cytokine IL-3 (1 ng/mL) or IL-3 and LPS (10 μ g/mL) for 14 days (5% CO₂ in 37°C). Data are presented as means $\pm$ SEMs, and significant findings are indicated on the graph.

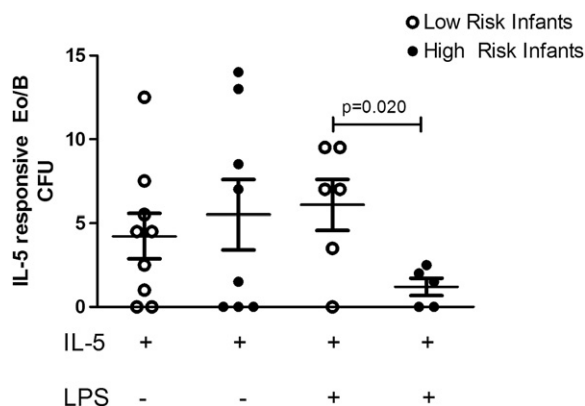


FIG 4. Responses to hematopoietic cytokine (IL-5) with or without LPS costimulation of CB CD34⁺ progenitor cells in high- and low-risk infants. CB mononuclear cells from low- and high-risk infants were enriched for CD34⁺ cells and stimulated with the hematopoietic cytokine IL-5 (1 ng/mL) or IL-5 and LPS (10 μ g/mL) for 14 days (5% CO₂ in 37°C). Data are presented as means $\pm$ SEMs, and significant findings are indicated on the graph.

resulted in decreased Eo/B CFU production is consistent with previous observations that TLR ligands can influence cell differentiation fates.¹⁵⁻¹⁸ Moreover, the finding of reduced CB Eo/B CFU production in high-risk infants after TLR stimulation suggests that TLR engagement on CB CD34⁺ cells has the capacity to suppress Eo/B lineage priming of myeloid progenitors, decreasing the likelihood of development of allergic eosinophilic/basophilic inflammation, as well as disease maintenance or progression. Our results suggest that there might exist a window of opportunity to revert Eo/B lineage priming in children at risk for allergy through engagement of progenitor cell TLR pathways in early life. Although the operative mechanisms underlying these observations remain elusive, reduced TLR expression on CB progenitors has implications for the signaling cascade triggered by TLR ligation,^{35,36} specifically those pathways involved in eosinophilopoiesis.³⁷ Likewise, early-life programming of the developing fetal immune system through epigenetic modulation is also

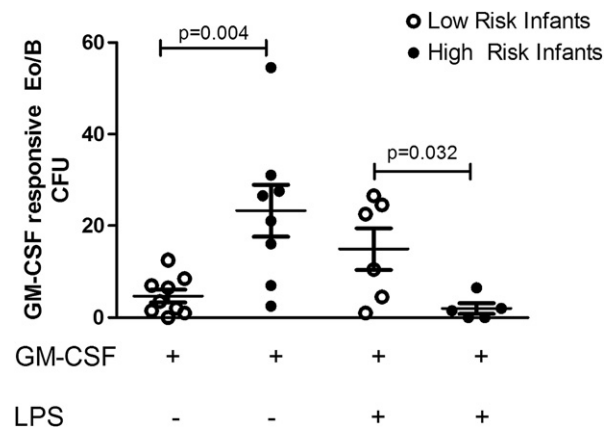


FIG 5. Responses to hematopoietic cytokine (GM-CSF) with or without LPS costimulation of CB CD34⁺ progenitor cells in high- and low-risk infants. CB mononuclear cells from low- and high-risk infants were enriched for CD34⁺ cells and stimulated with the hematopoietic cytokine GM-CSF (10 ng/mL) or GM-CSF and LPS (10 μ g/mL) for 14 days (5% CO₂ in 37°C). Data are presented as means $\pm$ SEMs, and significant findings are indicated on the graph.

possible because bacterial products might alter methylation processes³⁸ or acetylation of histone proteins.³⁹

We thus propose a novel role for TLR-mediated microbial (environmental) regulation of the allergic response: modulation of CB CD34⁺ myeloid progenitor cell Eo/B differentiation function and thus of the capacity to mount an allergic inflammatory (eg, eosinophilic) tissue response independent of T cells. LPS stimulation appears to have a unique suppressor effect on hematopoietic cytokine-mediated Eo/B differentiation *ex vivo*, thereby providing evidence for a novel TLR-mediated mechanism of immune modulation (ie, downregulation of eosinophilopoiesis in early life in children at risk for allergy). Although it has previously been reported that LPS stimulation of the nasal mucosa of atopic children suppresses allergen-induced T_H2 responses³⁴ and that LPS exposure in early life prevents the rate of wheeze in children,^{40,41} this is the first report of such suppressive effects on neonatal hematopoietic progenitor cell differentiation function in infants at risk for atopy.

It is also important to discuss the statistical considerations that were undertaken in this study. Of note, the exploratory nature of this study, which is generally hypothesis generating, justifies the use of a smaller sample size and no adjustment of the type I error for multiple testing (eg, by using the Bonferroni adjustment).²⁰ There are strong statistical arguments against the use of stringent correction factors in such studies.²⁰ The fact that we observed some signals of significant difference between these 2 groups supports and strengthens the biological plausibility of the hypotheses. With this consideration, the findings presented here warrant further investigation and validation in large studies (ie, by using larger sample sizes) in which these correction factors would be more accurately applied to avoid type I (α) errors. It is also noteworthy statistically that despite the small sample size, the sensitivity analyses using nonparametric methods resulted in similar significant findings, which adds to the robustness of the reported findings.

In conclusion, this report demonstrates that high-risk infant CB hematopoietic progenitors have not only significantly reduced TLR-2, TLR-4, and TLR-9 expression but that this expression is negatively correlated with maternal allergic sensitization.

Moreover, costimulation with hematopoietic cytokines and TLR agonists results in reduced Eo/B differentiation *in vitro*, identifying a novel immunomodulatory effect of TLR stimulation on CB progenitors in relation to allergic risk. Our results suggest that not only might CB progenitor TLR expression potentially identify children at risk for allergy but that the maternal atopic milieu could be regulating TLR expression and function on CB progenitor cells *in utero*, a finding that might shed light on how environmental and genetic factors influence the development of allergies, even before birth.

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Clinical implications: Because hematopoietic processes at birth might play an important role in the development of atopy, investigating progenitor cell phenotypic and functional alterations might reveal novel pathways and predictors of early childhood atopic outcomes.

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