

Low neonatal Toll-like receptor 4-mediated interleukin-10 production is associated with subsequent atopic dermatitis

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Summary

Background Atopic dermatitis (AD) and respiratory syncytial virus lower respiratory tract infection (RSV LRTI) are common diseases during early life. Impaired Th1-cell polarizing Toll-like receptor (TLR) responses play an important role in the pathogenesis of both diseases. Neonatal TLR-mediated production of Th1-type cytokines is decreased at birth, but rapidly increases during the first month of life.

Objective To determine whether decreased TLR-mediated production of Th1-polarizing cytokines, at the age of 1 month is associated with subsequent AD or RSV LRTI.

Methods A prospective healthy birth cohort study was performed. Whole blood concentrations of innate immune cells and TLR-mediated cytokine responses were measured at the age of 1 month in 291 neonates. AD was determined by a physician questionnaire at the age of 1 year and RSV LRTI was defined as parent-reported respiratory symptoms and presence of RSV RNA in a nose–throat specimen.

Results Of participating neonates, 45 (15%) developed AD and 41 (14%) developed RSV LRTI. Risks of AD and RSV LRTI were not associated (χ^2 , $P = 1.00$). AD was associated with decreased concentrations of basophils (7.6 vs. $14.0 \times 10^6/\text{mL}$, $P = 0.002$) and plasmacytoid dendritic cells (17.0 vs. $20.5 \times 10^6/\text{mL}$, $P = 0.04$), increased concentrations of NK-cells (79.7 vs. $45.1 \times 10^6/\text{mL}$, $P = 0.03$), and twofold lower TLR4-mediated IL-10 production ($P = 0.001$). In contrast, RSV LRTI was associated neither with neonatal concentrations of innate immune cells, nor with TLR-mediated TNF- α , IL-12p70, IL-10 or IFN- α production.

Conclusions and Clinical Relevance Atopic dermatitis, but not RSV LRTI, is associated with distinct pre-symptomatic differences in the innate immune system. We hypothesize that decreased neonatal IL-10-mediated immune regulation during early life might play a causal role in the initiation of AD.

Keywords allergic disease, atopy, bronchiolitis, endotoxin, hygiene hypothesis, newborn, eczema, Toll-like receptor

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Introduction

Toll-like receptors (TLR) are key pathogen recognition receptors of the innate immune system that initiate the early antimicrobial response and orchestrate the ensuing adaptive response [1]. We and others have shown that neonatal TLR responses are characterized by decreased production of Th1-polarizing cytokines (IL-12p70, TNF- α , IFN- α) and increased production of

the regulatory cytokine IL-10 compared with adult responses [2–7]. Polarized neonatal cytokine responses are thought to prevent potentially harmful allo-immune responses towards maternal antigens *in utero*, and to facilitate microbial colonization after birth [3]. However, impaired generation of Th1-polarizing responses leaves the neonate highly susceptible to infection [8]. In addition, bias towards Th2-type immune responses at the neonatal and infant age has been associated

with increased risk of allergic disease in later life [9–11].

Atopic dermatitis (AD) is a chronic inflammatory skin disorder, with a lifetime prevalence of 20% in westernized countries [12]. AD occurs in the first 6 months of life in 45% of children and before the age of one in 60% [12]. Hyper-reactive Th2-type immune responses play a central role in the pathogenesis of AD [13]. Acute eczematous skin lesions are infiltrated with Th2-cells expressing high levels of IL-4, IL-5 and IL-13 [14]. In addition, patients with AD have elevated IL-13-expressing T cells in the peripheral blood [14], increased eosinophils and high serum IgE [15]. This Th2-bias might be dictated by the innate immune system, as both genetic and functional defects in TLRs and their downstream signalling molecules are associated with AD [16–18].

Respiratory syncytial virus (RSV) is the most common cause of childhood acute lower respiratory tract infection world-wide, affecting 90% of children before the age of 2 years [19, 20]. Respiratory syncytial virus disease severity varies from mild upper respiratory tract infection to lower respiratory tract infection (LRTI) and respiratory insufficiency necessitating hospitalization. Specific patterns of TLR-mediated cytokine responses might impact disease severity [21–23]. Whereas most respiratory viruses induce robust Th1-type immune responses, immune responses in infants with acute RSV infection are characterized by low levels of Th1-type cytokines IFN- γ and IL-12p70 [21, 22]. In addition, infants with severe RSV bronchiolitis have an increased IL-10/IL-12 mRNA ratio in nasal lavage fluid and elevated IL-4/IFN- γ mRNA ratios in nasal lavage fluid and in stimulated PBMC [23] compared with infants with upper respiratory tract infection. Decreased serum concentrations of IFN- γ were observed in RSV-infected infants, but not in infants infected with parainfluenza-virus or adenovirus [22], suggesting that insufficient production of pro-inflammatory or Th1-type cytokines is a specific feature of severe RSV infection [24, 25].

We have previously identified the first month of life as a critical period for the development of the TLR system. During this period, TLR-mediated cytokine responses rapidly mature from a Th2-biased profile to wards increased Th1-type responses characteristic of later life [26]. Post-natal maturation of TLR responses is probably guided by exposure to innate immune stimuli, which might thus modulate the risk of subsequent disease [27]. In the current study, we hypothesized that neonates with delayed maturation of TLR responses during the first month of life, resulting in persistent impairments in Th1-immunity, may have increased susceptibility to RSV LRTI and AD in infancy and childhood. We therefore studied the impact of different TLR responses at the age of 1 month on the subsequent risk

of AD and RSV LRTI in the first year of life in a healthy birth cohort.

Methods

Study design and population

A prospective observational birth cohort study was performed in two urban hospitals in Utrecht, the Netherlands, from March 2006 to February 2010. Study design and inclusion criteria have been published previously [28]. Eligible subjects were healthy neonates born after uncomplicated pregnancy of ≥ 37 weeks through either vaginal delivery or elective caesarean section. Data on clinical characteristics were collected from hospital charts and standardized parental questionnaires [29]. At the age of 1 month, neonatal venous blood was obtained in sterile sodium heparin tubes. Samples were processed within 5 h (range 0.5–5) of blood collection. None of the participants had any signs or symptoms of infectious disease (e.g. runny nose, fever) or AD in the 2 weeks prior to sampling. The study was approved by the ethical review boards of the University Medical Center Utrecht and the Diaconessen Hospital Utrecht. All parents provided written informed consent for study participation.

Innate immune cellularity

Whole blood concentrations of innate immune cells were determined as previously described [5]. Concentrations of neutrophils, eosinophils and basophils were obtained from differential leucocyte count. Concentrations of monocytes, natural killer (NK)-cells, myeloid dendritic cell (mDC) and plasmacytoid dendritic cells (pDC) were determined by flow cytometry. Monocytes were identified as HLA-DR⁺/CD14⁺, NK-cells as CD3⁺/CD16⁺/CD56⁺, mDC as HLA-DR⁺/lineage[−]/CD11c⁺ and pDC as HLA-DR⁺/lineage[−]/CD123⁺ (Table S1) [5]. To obtain absolute concentrations, the percentage of cells in the lymphocyte/monocyte gate (as determined by forward-sideward scatter properties) was multiplied by the total numbers of lymphocytes and monocytes from the differential leucocyte count.

Toll-like receptor stimulation

In vitro TLR stimulation was performed using optimal concentrations of TLR agonists and incubation times for cytokine measurements, as titrated in pilot experiments and described previously (Table S2) [5]. To study cytokine responses to multiple TLR agonists in limited blood volume, whole blood was diluted 1 : 14 in serum-free RPMI prior to *in vitro* TLR stimulation. After a 4-h (TNF- α) or 24-h (IFN- α , IL-12p70, IL-10) incubation at

37°C and 5% CO₂, samples were centrifuged at 1000 g for 5 min. Supernatants were collected and stored at -80°C, until further analysis.

Cytokine protein detection

Cytokine readouts were chosen based upon their key Th1- (IL-12p70, IFN- α) or Th2-polarizing capacity (IL-10). The TLR2-mediated TNF- α was included because previous reports related impairments in TLR2-mediated TNF- α to increased risk of neonatal infection [3, 30]. Cytokine concentrations in culture supernatants were determined by ELISA according to the manufacturer's instructions: TNF- α , IL-10 (both from Sanquin/CLB, Amsterdam, the Netherlands), IL-12p70 (Diacor Research, Besançon, France) and IFN- α (Bender Medsystems, Burlingame, CA, USA, detects all natural isoforms of human IFN- α , except for IFN- α 1). The lower limits of detection were 1.0 pg/mL (TNF- α and IL-10) and 2.0 pg/mL (IL-12p70 and IFN- α). For samples in which the cytokine concentration was below the detection limit, the concentration was arbitrarily defined as half of the detection limit.

Clinical outcomes

The primary outcome of RSV LRTI was defined as (i) parent-reported LRTI symptoms, and (ii) simultaneous presence of RSV RNA in a nose-throat specimen. Throughout the first year of life, parents completed prospective logs to record presence and severity of respiratory symptoms. LRTI was defined by two independent researchers, using strict criteria: moderate or severe cough or wheeze of any severity lasting for at least 2 days. At every respiratory episode, parents obtained a nose-throat swab [31]. Samples were sent to the researchers in viral transport medium and upon

receipt, frozen at 80°C. The presence of RSV RNA was determined by real-time polymerase chain reaction (PCR) as described previously [32]. Secondary outcomes were 'physician-attended RSV infection' and 'atopic dermatitis'. Physician-attended RSV infection was defined as an episode of respiratory tract symptoms for which the general practitioner or paediatrician was visited, and simultaneous presence of RSV RNA in a nose-throat swab. At the age of 1 year, general physicians of all participants were contacted to answer a standardized, four-item questionnaire that was based on the international classification of primary care codes (ICPCC). AD was assessed by the question 'Was this participant diagnosed with one or multiple episodes of "atopic eczema" (ICPCC S87) in his/her first year of life?' For sensitivity analysis, parent-reported AD was derived from validated parental questionnaire at the age of 1 year [29], and defined as a positive answer to the question 'Did your child experience any eczema or pruritic skin rash in the first year of life?'

Statistical analysis

Whole blood concentrations of innate immune cells and TLR-mediated cytokine responses were logarithmically transformed to obtain normal distribution. Means of logarithmic transformed variables were compared between groups using two-sided Student's *t*-test. Differences between dichotomic variables were determined using the χ^2 -test. Logistic regression analysis was used to determine the effect of innate immune determinants on outcome variables, while adjusting for possible confounding factors. Based on results from univariate analysis, 'birth weight', 'birth season' and 'day care' were considered potential confounders for the association between innate immune variables and RSV LRTI. Similarly, 'birth season', 'day care' and 'parental atopy'

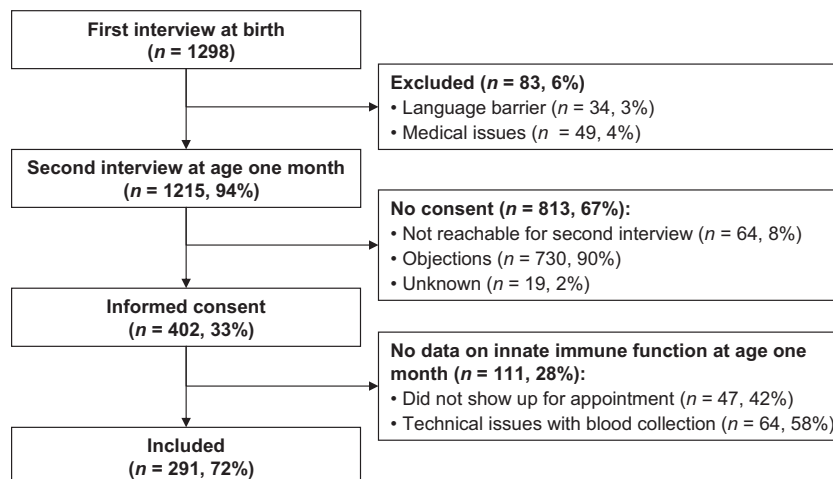


Fig. 1. Flowchart of study population.

were considered potential confounders for the association between innate immune variables and AD.

Results

During the study period, 1298 neonates were eligible for study participation (Fig. 1). Of these, 402 gave informed consent. Due to missed appointments ($n = 47$) or technical reasons (e.g. failure in blood collection, blood clotting, $n = 64$), innate immune parameters at the age of 1 month were not available for 111 (28%) of participants, resulting in a final cohort of 291 neonates. Baseline characteristics did not differ between participating neonates and non-participants (Table S3). At the age of 1 year, 45 participants had AD [absolute risk (AR) 15%, Table 1]. Baseline characteristics were similar between neonates with and without AD. In addition, 41 participants had experienced an episode of RSV LRTI (AR 14%, Table 1), and 22 participants had physician-attended RSV infection (AR 7%). Neonates who developed RSV LRTI had a higher birth weight and gestational age compared with those who did not, as observed previously [28]. There was no relationship between risks of AD and RSV LRTI (χ^2 , $P = 1.00$).

Atopic dermatitis

Distinct neonatal concentrations of innate immune cells are associated with subsequent AD. Whole blood concentrations of innate immune cells at the age of 1 month were compared between neonates with and those without subsequent AD (Fig. 2). Neonates with subsequent AD had decreased concentrations of basophils (7.6 vs. $14.0 \times 10^6/L$, $P = 0.002$) and pDCs (17.0 vs. $20.5 \times 10^6/L$, $P = 0.04$), and increased concentrations of NK-cells (79.7 vs. $45.1 \times 10^6/L$, $P = 0.03$) compared with neonates without subsequent AD. All associations remained significant after adjustment for potential confounders (Table 2) and an additional borderline significant association was found between whole blood concentrations of eosinophils and subsequent risk of AD ($P = 0.05$).

Decreased TLR4-mediated IL-10 production is associated with increased risk of subsequent atopic dermatitis. We next investigated whether TLR-mediated cytokine responses at the age of 1 month were associated with prospective susceptibility to AD (Fig. 3). Neonates who developed physician-diagnosed AD had 1.8-fold lower TLR4-mediated IL-10 responses at the age of 1 month

Table 1. Characteristics of study population

	RSV LRTI			Atopic dermatitis		
	Yes ($n = 41$)	No ($n = 250$)	P -value	Yes ($n = 45$)	No ($n = 246$)	P -value
Gestational age, weeks (SEM)	40.3 (0.2)	39.9 (0.1)	0.01	40.1 (0.1)	39.9 (0.1)	0.27
Birth weight, g (SEM)	3834 (73)	3574 (29)	0.001	3653 (71)	3603 (29)	0.52
Gender, n (%)			0.61			0.87
Male	18 (44)	123 (49)		21 (47)	120 (49)	
Female	23 (56)	127 (51)		24 (53)	126 (51)	
Delivery mode, n (%)			0.09			1.00
Vaginal	39 (95)	212 (85)		39 (87)	212 (86)	
Elective caesarean section	2 (5)	38 (15)		6 (13)	34 (14)	
Birth season, n (%)			0.06			0.12
Winter	8 (20)	62 (25)		5 (12)	65 (26)	
Spring	9 (22)	67 (27)		16 (37)	60 (24)	
Summer	17 (41)	55 (22)		11 (26)	61 (25)	
Fall	7 (17)	66 (26)		13 (30)	60 (24)	
Siblings, n (%)	25 (61)	161 (66)	0.73	29 (64)	157 (64)	1.00
Pets, n (%)	12 (29)	80 (33)	0.72	16 (36)	65 (33)	0.73
Feeding, n (%)						
Ever breastfed	24 (85)	211 (87)	0.81	37 (84)	209 (85)	0.63
Exclusive breastfeeding >1 mo	24 (59)	130 (54)	0.61	20 (46)	134 (54)	0.25
Age at start formula, mo (SEM)	4.3 (0.8)	4.2 (0.3)	0.93	4.8 (0.9)	4.1 (0.3)	0.38
Day care attendance, n (%)	30 (79)	128 (64)	0.09	31 (78)	127 (51)	0.07
Maternal atopy*, n (%)	12 (29)	93 (38)	0.30	15 (34)	90 (37)	0.74
Parental atopy*, n (%)	23 (56)	137 (56)	1.00	24 (55)	136 (55)	0.87
Maternal atopic dermatitis, n (%)	10 (24)	46 (19)	0.40	9 (20)	47 (19)	0.84
Parental atopic dermatitis, n (%)	68 (28)	14 (34)	0.46	17 (39)	65 (26)	0.15
Parental smoking, n (%)	4 (10)	69 (28)	0.10	8 (18)	65 (26)	0.26

*Parental atopy was defined as parent-reported physician-diagnosed asthma, atopic dermatitis or hayfever. SEM, standard error of the mean.

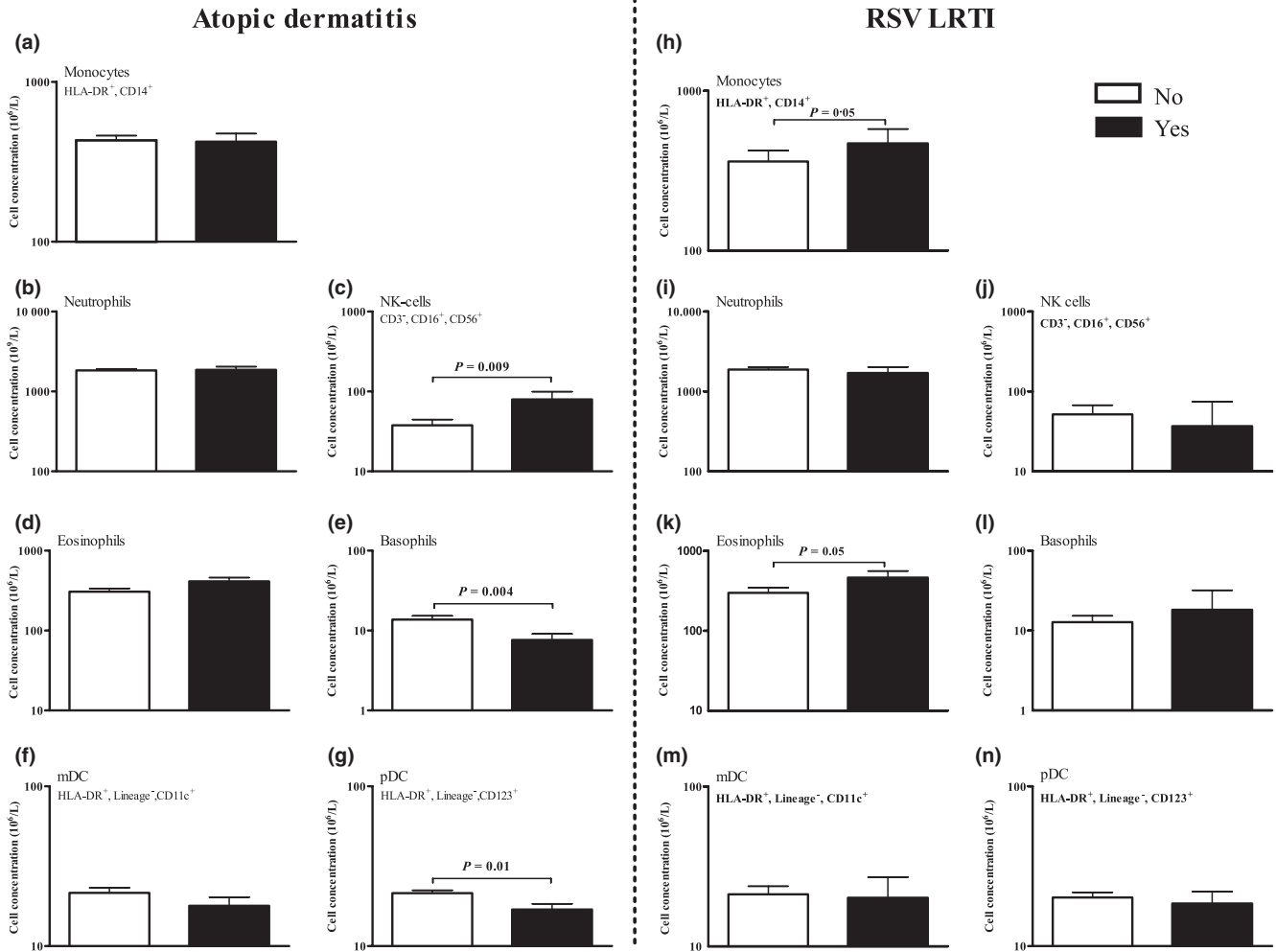


Fig. 2. Atopic dermatitis is associated with distinct concentrations of innate immune cells at the age of 1 month. Whole blood concentrations of innate immune cells at the age of 1 month were measured by flow cytometry and compared between neonates with subsequent atopic dermatitis ($n = 45$) and those without ($n = 246$) (a–g), or between neonates with subsequent RSV LRTI ($n = 41$) and those without ($n = 250$) (h–n). Bars represent geometric means and 95% confidence intervals derived from log transformed data.

Table 2. Independent associations between concentrations of innate immune cells and TLR-mediated cytokine responses at the age of 1 month and the subsequent risk of atopic dermatitis

	OR (95% CI)	P-value
pDC	0.19 (0.40–0.91)	0.04
NK cells	1.83 (1.12–3.02)	0.02
Eosinophils	3.00 (1.00–8.97)	0.05
Basophils	0.28 (0.12–0.71)	0.01
TLR4-mediated IL-10	0.13 (0.04–0.41)	<0.001

Multivariable logistic regression analysis was performed comparing concentrations of innate immune cells and TLR-mediated cytokine responses between neonates with subsequent atopic dermatitis ($n = 45$) and those without ($n = 246$), while adjusting for birth season, parental atopy and day care as potential confounders. Only significant associations are shown.

compared with those who did not (533 pg/mL vs. 954 pg/mL, $P = 0.003$). Logistic regression analysis, adjusting for birth season, parental atopy and day care

as potential confounders, showed similar results [$B = 0.13$ (95% CI: 0.04–0.41), $P < 0.001$] (Table 2). Sensitivity analyses using 'parent-reported atopic dermatitis' (defined as eczema or pruritic skin rash, derived from parental questionnaire at the age of 1 year) rendered similar findings, both in univariate analysis (588 pg/mL vs. 995 pg/mL, $P = 0.001$) and in multivariate analysis [$B = 0.24$ (0.10–0.62), $P = 0.003$]. No significant correlations were found between concentrations of NK-cells, pDC, basophils, eosinophils and TLR4-mediated IL-10 production.

Respiratory syncytial virus lower respiratory tract infection

Neonatal whole blood concentrations of innate immune cells are not associated with the development of RSV LRTI. The primary outcome of community-based RSV

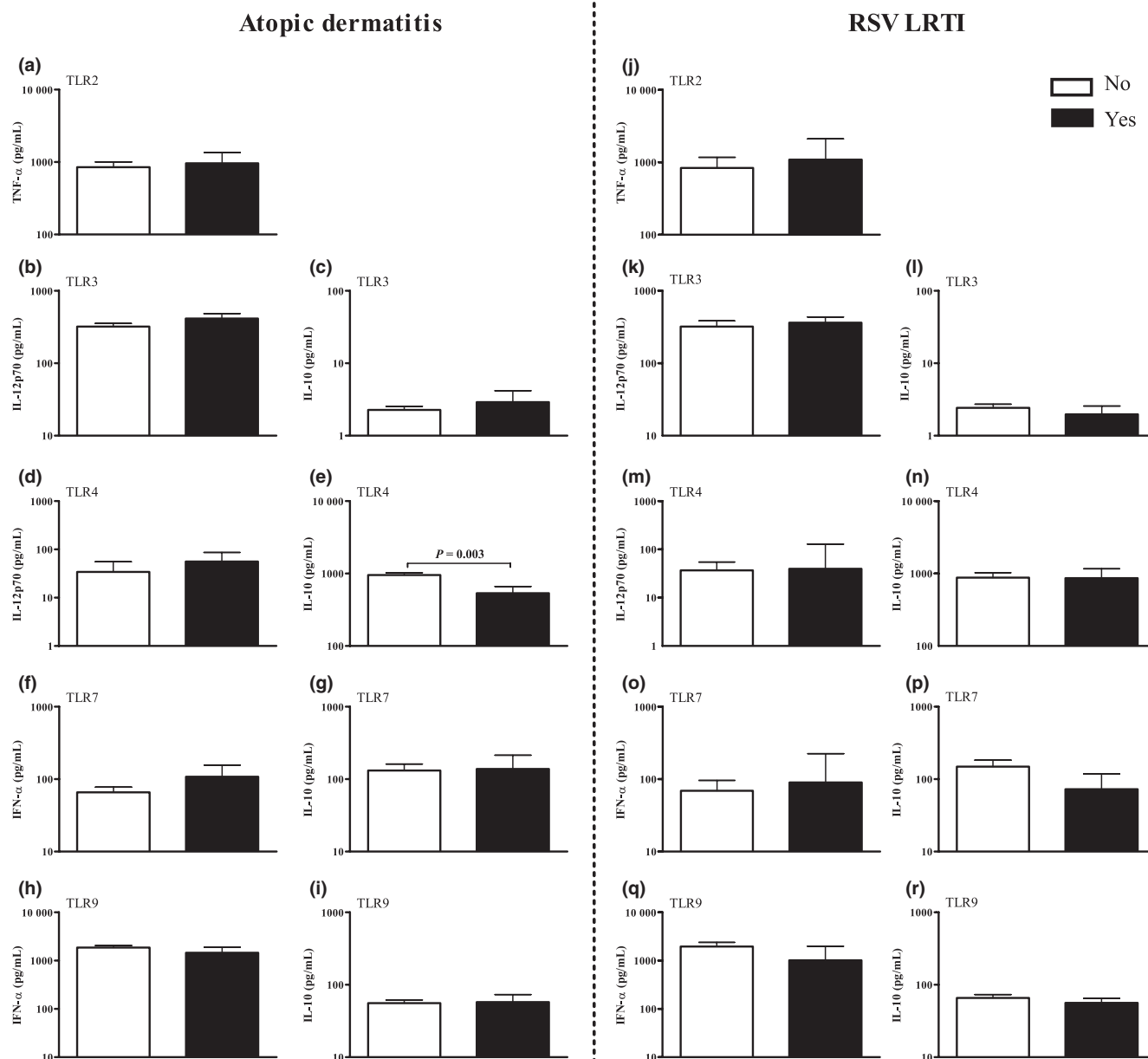


Fig. 3. Decreased TLR4-mediated IL-10 production in neonates with subsequent atopic dermatitis. Whole blood TLR-mediated cytokine production at the age of 1 month was measured by ELISA and compared between neonates with subsequent atopic dermatitis ($n = 45$) and those without ($n = 246$) (a–i), or between neonates with subsequent RSV LRTI ($n = 41$) and those without ($n = 250$) (j–r). Bars represent geometric means and 95% confidence intervals derived from log transformed data.

LRTI in the first year of life was analysed in relation to neonatal whole blood concentrations of innate immune cells (Fig. 2). There were no differences in concentrations of mDCs, pDCs, neutrophils and basophils at the age of 1 month between neonates with and those without subsequent RSV LRTI. Neonates who developed RSV LRTI had increased concentrations of monocytes (468 vs. $363 \times 10^6/L$, $P = 0.05$) and eosinophils (437 vs. $363 \times 10^6/L$, $P = 0.05$) compared with those without RSV LRTI, but significance was lost after adjustment for potential confounders. No differences in

cell concentrations were found in relation to the secondary outcome physician-attended RSV (data not shown). Analyses using cell percentages instead of absolute concentrations (e.g. eosinophils as a percentage of total leucocytes) rendered similar results (data not shown).

Neonatal TLR-mediated cytokine responses are not associated with the development of RSV LRTI. No differences in cytokine responses to agonists for TLR2, TLR3, TLR4, TLR7 and TLR9 were found between

neonates who developed RSV LRTI in the first year of life and those who did not (Fig. 3). The secondary outcome, 'physician-attended RSV infection', was associated with higher TLR3-mediated IL-12p70 production at the age of 1 month (676 pg/mL vs. 316 pg/mL, $P = 0.015$). This remained statistically significant after adjustment for potential confounders [$B = 7.48$ (1.67–33.4, $P = 0.021$)].

Discussion

This prospective unselected birth cohort study demonstrates that AD is preceded by marked differences in the innate immune system at the age of 1 month. Neonates who subsequently developed AD had decreased TLR4-mediated IL-10 production, decreased concentrations of basophils and pDC and increased concentrations of NK-cells compared with those who did not. On the contrary, except for a modest association with increased TLR3-mediated IL-12p70 production, RSV LRTI was associated with pre-symptomatic differences neither in innate immune cellularity nor in TLR-mediated cytokine responses at the age of 1 month.

Dysregulated TLR-mediated cytokine responses during early life may play an important role in the pathogenesis of AD [33]. At birth, cord blood mononuclear cells (CBMC) from atopic mothers produce higher TLR2-, TLR3- and TLR4-mediated IL-12 and IFN- γ compared with those from non-atopic mothers [33]. In addition, in high-risk neonates, increased TLR2-, TLR4- and TLR5-mediated IL-6 and TNF- α production by CBMC is associated with increased risk of childhood AD [33]. Thus, cord blood TLR hyperresponsiveness, either due to genetic factors or environmental exposures, may play a causal role in the pathogenesis of AD.

Disrupted post-natal development of the TLR system might also contribute to AD pathogenesis. Lack of early life exposure to innate immune stimuli, including the TLR4 agonist endotoxin, contributes to the subsequent development of asthma, allergic sensitization and AD [27, 34, 35]. In addition, atopic children demonstrate an inverse pattern of innate immune maturation from non-atopic children, decreasing, rather than increasing, TLR-mediated production of TNF- α , IL-12, IL-6 and IL-1 β , from birth to the age of five [36]. Impaired production of Th1-polarizing innate immune signals may contribute to the persistent Th2-skewed allergen-specific immune responses (i.e. increased IL-4, IL-13, decreased IFN- γ) observed in atopic children [37, 38].

In summary, disrupted early life TLR development might induce persistent dysfunction of innate and adaptive immunity, thus predisposing to atopy. Previously, we identified the first month of life as a period of rapid development of the TLR system, providing a

potential window of opportunity for interventions aimed at preventing atopy [5]. The association between TLR4-mediated IL-10 at the age of 1 month and subsequent risk of AD suggests that strategies aimed at increasing IL-10 production during this highly sensitive period might restore immune balance and protect from subsequent AD.

Several explanations may account for the association between low neonatal TLR4-mediated IL-10 production and AD. First, IL-10 is an important regulatory cytokine [39]. In mice, intradermal injections of IL-10 plasmid DNA suppress the development of AD skin lesions [40]. In humans, subcutaneous allergen-specific immunotherapy increases IL-10 expression in AD skin, coinciding with clinical improvement [41]. Thus, deficient IL-10-mediated immune regulation might predispose to AD. Second, decreased neonatal IL-10 production to TLR4 stimulation might reflect a general dysregulation of responses mediated by TLR4. Several allergens associated with AD [42, 43] show structural and functional homology to components of the TLR4 receptor complex [44–46]. Similarities in the structure and biological functions of LPS and allergens might account for the association between neonatal TLR4-mediated IL-10 and AD. However, we found no association between TLR4-mediated IL-12p70 production and AD, suggesting that other mechanisms also play a role. Third, differences in microbial flora between atopic and non-atopic neonates might account for the association between neonatal innate immune responses and AD [3]. Atopic dermatitis patients have distinct microbial flora of their skin [47] and gut [48] compared with non-atopic subjects. Differences in gut colonization precede the development of clinical AD [48], suggesting that dysregulated microbial-induced immune development might play a causal role in the initiation of AD.

Atopic dermatitis was also associated with decreased whole blood concentrations of basophils and (in regression analysis) increased concentrations of eosinophils. A potential cause of this preferential differentiation of eosinophil/basophil precursor cells towards the eosinophil lineage is the key Th2-cytokine IL-5 [49]. Previous studies strengthen this hypothesis, demonstrating that increased IL-5 production during the neonatal and infant period is associated with elevated concentrations of eosinophils and total IgE [50] and increased risk of atopy at the age of 1 year [51].

Why are neonatal innate immune responses associated with AD, but not RSV LRTI? The lack of associations between neonatal innate immune parameters measured in this study and subsequent RSV LRTI might indicate that the immune dysfunction observed during severe RSV infection [21–23] is the result, rather than the cause, of severe RSV disease. Alternatively, dysregulation of TLR-mediated cytokine responses

might be specific for infants with hospital-admitted (as opposed to community-based) RSV infection. Insight into the reciprocal interactions between RSV and the host immune response is highly needed for treatment of severe RSV bronchiolitis and prevention of post-bronchiolitis wheeze.

This study has several strengths, including (i) we were able to follow a large cohort of healthy neonates born at term after uncomplicated gestation and delivery prospectively; (ii) venous blood was collected at the age of 1 month, preventing any potential artefacts present in cord blood (e.g. hypoglycaemia, low pH, circulating maternal cells), and (iii) all respiratory symptoms suspected to arise from RSV infection were confirmed by PCR. Potential limitations also deserve discussion. First, we did not identify the cell type responsible for TLR-mediated cytokine production. Consequently, the observed association between TLR4-mediated IL-10 and AD might be secondary to differences in innate immune cell concentrations. However, the cell types that were relatively decreased in atopic neonates either lack TLR4 (pDC) [52] or are incapable of responding to LPS *in vitro* (basophils) [53], and adjustment for concentrations of innate immune cells rendered a similar significant association between TLR4-mediated IL-10 and AD (data not shown). Second, the *in vitro* whole blood model is very different from the *in vivo* response in AD skin or RSV-infected airways. Third, our comprehensive characterization of neonatal innate immunity includes multiple

comparisons. To minimize the risk of false discovery, we focused on associations that were highly significant, that remained significant after adjustment for potential confounders and that were present for multiple related outcomes (e.g. physician-diagnosed AD and parent-reported AD). No attempt was made to correct for multiple testing, and future studies will be needed to validate our findings.

In summary, this study demonstrates that atopic dermatitis, but not RSV LRTI, is associated with distinct pre-symptomatic TLR-mediated cytokine responses at the age of 1 month. Future studies aimed at characterizing the basic mechanisms and clinical factors modulating neonatal TLR4-mediated IL-10 production might identify potential targets for prevention of AD.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Markers used to identify innate immune cells by flow cytometry

Table S2. Agonists used for *in vitro* TLR stimulation

Table S3. Baseline characteristics of study population compared to all eligible neonates

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Online Supplementary material

Supplementary table 1: Markers used to identify innate immune cells by flow cytometry

Cell type	Marker	Fluorochrome	Clone
Monocytes	HLA-DR ⁺	PerCP	L243
	CD14 ⁺	FITC	MoP9
NK-cells	CD3 ⁻	APC	SK7
	CD16 ⁺	PE	B73.1
	CD56 ⁺	FITC	NCAM16.2
Plasmacytoid DC	HLA-DR ⁺	PerCP	L243
	CD123 ⁺	PE	9F5
	Lineage ⁻	FITC	
	CD3		SK7
	CD16		3G8
	CD19		SJ25C1
	CD20		L27
	CD14		MoP9
	CD56		NCAM16.2
	HLA-DR ⁺	PerCP	L243
	Lineage ⁻	FITC	
Myeloid DC	CD3		SK7
	CD16		3G8
	CD19		SJ25C1
	CD20		L27
	CD14		MoP9
	CD56		NCAM16.2
	CD11c ⁺	APC	S-HCL-3

Supplementary Table 2: Agonists used for *in vitro* TLR stimulation

TL	Agonist used	Concentration	Manufacturer
R			
2	Pam ₃ Cys SSNA	1 µg/mL	Bachem Biosciences, Weil am Rhein, Germany
3	Polyinosinic:polycytidylic acid (Poly I:C)	200 µg/mL	Invivogen, San Diego, CA
4	Ultrapure lipopolysaccharide derived from <i>E. Coli</i>	100 ng/mL	Invivogen
	Interferon-γ (IFN-γ)	20 ng/mL	Preprotech Inc, Rocky Hill, NJ
7	Loxoribine	1 mM	Invivogen
9	CpG oligodeoxynucleotide (CpG ODN)	30 µg/mL	Invivogen

Supplementary Table 3: Baseline characteristics of study population compared to all eligible neonates

	Included in study population (n=291)	Not included in study population (n=1007)	p-value
Birth weight, g (SE)	3611 (27)	3571 (36)	0.56
Gestational age, wks (SE)	40.0 (0.06)	40.0 (0.03)	0.94
Siblings, n (%)	186 (64)	622 (62)	0.63
Male gender, n (%)	141 (48)	517 (51)	0.39
Birth Season, n (%)			0.93
Winter	70 (24)	248 (25)	
Spring	76 (26)	246 (24)	
Summer	72 (25)	263 (26)	
Fall	73 (25)	250 (25)	