

Original article

Maternal allergic disease does not affect the phenotype of T and B cells or the immune response to allergens in neonates

Background: It is hypothesized that the *in utero* environment in allergic mothers can affect the neonatal immune responses. The aim of this study was to analyse the effect of maternal allergic disease on cord blood mononuclear cell (CBMC) phenotype and proliferative responses upon allergen stimulation.

Methods: Peripheral blood mononuclear cells (PBMC) from 12 allergic and 14 nonallergic mothers and CBMC from their children were analysed. In the mothers, we determined cell proliferation, production of IL-4 and expression of FOXP3 in response to allergen stimulation. In the children, we evaluated cell proliferation and FOXP3 expression following allergen stimulation. Furthermore, expression of different homing markers on T cells and regulatory T cells and maturity of the T cells and B cell subsets were evaluated directly *ex vivo*.

Results: The timothy- and birch-allergic mothers responded with increased proliferation and/or IL-4 production towards timothy and birch extract, respectively, when compared to nonallergic mothers. This could not be explained by impairment of FOXP3⁺ regulatory T cells in the allergic mothers. CBMC proliferation and FOXP3 expression in response to allergens were not affected by the allergic status of the mother. Also, phenotype of T cells, FOXP3⁺ regulatory T cells and B cells was not affected by the allergic status of the mother.

Conclusion: Our results suggest that maternal allergic disease has no effect on the neonatal response to allergens or the phenotype of neonatal lymphocytes. The factors studied here could, however, still affect later development of allergy.

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Allergy often starts early in life, and maternal atopy has been reported to confer a higher risk for development of asthma and eczema in children than paternal atopy (1–3). It has been proposed that *in utero* events may affect later allergy development in the child (4). The immunological mechanisms underlying this remain poorly understood. There is evidence for an ability of neonatal T and B cells to respond to a variety of environmental allergens (5, 6). According to some investigators, these responses indicate that neonatal immune responses, thought to be naïve, may in fact be influenced by antenatal exposures (7), while others have challenged the specificity of these responses (8). Two potential routes for antenatal exposure are transplacentally and via the amniotic fluid. An *ex vivo* placental perfusion model has demonstrated that allergens can be transferred across the placenta (9), and allergens have also been detected in the amniotic fluid and cord blood (10).

Impairment of regulatory T cells is believed to be one contributing factor in allergic disease. Regulatory T cells have been studied in cord blood in relation to allergy development and compared in children with high and low risk to develop allergy. Two recent studies reported impairment in regulatory T cells in cord blood of children of atopic mothers following stimulation with bacterial components or polyclonal stimulation (11, 12). Another study demonstrated higher levels of *FOXP3* mRNA expression in blood mononuclear cells at 6 months in infants who developed atopic dermatitis than those who did not develop atopic dermatitis (13).

Allergic reactions in young children take place largely in the skin and the gut. Allergen-reactive T cells play a significant role in the allergic inflammation, and homing receptors are vital in deciding the migration pattern of T cells. Homing of lymphocytes to skin, gut and lymphoid organs has been well studied, however, not in relation to development of allergy. Cutaneous lymphocyte antigen (CLA) is involved in T cell migration to skin, integrin $\alpha 4\beta 7$ to gut and CCR7 and CD62L to secondary lymphoid organs (reviewed in (14)).

B cells are important effector cells in the allergic response, and they have recently been proposed to have a

Abbreviations: β -LG, beta-lactoglobulin; CBMC, cord blood mononuclear cells; CLA, cutaneous lymphocyte antigen; ELISPOT, enzyme-linked immunospot assay; HDM, house dust mite; LPS, lipopolysaccharide; MNC, mononuclear cells; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin.

role in regulating allergic airway disease in mice (15). In humans, B cells have not yet been studied in relation to development of allergy. Antigen-specific cord blood B cell responses to maternal antigen exposures during pregnancy have, however, been described (5). CD27 is a marker for memory B cells, and memory IgM⁺IgD⁺ B cells are considered class switched (16). CD5 is a marker of B1 B cells in mice (17). In humans, CD5 does not define a particular subset, however the number of CD5⁺ B cells decreases with age (17).

To our knowledge, no one has previously studied B cells or different subsets of regulatory T cells in cord blood in relation to maternal allergy. We hypothesize that the intrauterine immunological environment differs between allergic and nonallergic mothers, that this will influence the development of the immune system of the child, and that this will be reflected in the phenotype of the cord blood T and B cells and their responses towards allergens. We here specifically analysed (i) T cell proliferation and expression of FOXP3 in response to allergen stimulation, (ii) expression of homing markers on regulatory T cells and (iii) phenotype and maturity of T and B

cells in cord blood from children of allergic and nonallergic mothers.

Materials and methods

Study population

Fifty-eight pregnant women with full-term pregnancies were recruited between January 2004 and January 2006 at the delivery unit of Karolinska University Hospital Solna, Stockholm, Sweden. Women filled out a questionnaire regarding allergic disease, and maternal venous blood was drawn at admission to the delivery unit. Umbilical cord blood was aspirated into heparinized tubes directly following delivery. The women who reported allergic symptoms and were found to have a positive Phadiatop (see later) were included as allergic and those without symptoms and a negative Phadiatop as nonallergic (Table 1). Because of these inclusion criteria and a lack of enough cells in several cases, 12 allergic (21%) and 14 nonallergic (24%) women and their children were analysed in the study. None of the included women had fever during delivery or other disorders than allergy, except two who were on a low dose of Levaxin (mother no. 1 and 13). The study was approved by the Ethics Committee of the Karolinska University Hospital. All mothers gave their informed consent to the study.

Table 1. Characterization of the mothers

Phadiatop* positive mothers no	Age years	Parity	Mode of delivery C/V	Child F/M	Self-reported allergy	Total IgE kU/l†	Timothy kU _A /l‡	Birch kU _A /l‡	Cat kU _A /l‡	HDM kU _A /l‡
1	31	1	C	M	Grass	87	11	3.8	<0.35	<0.35
2	38	1	V	M	Pollen	180	0.7	90	<0.35	<0.35
3	40	3	C	F	Pollen	25	0.6	12	<0.35	<0.35
4	29	2	C	M	Pollen, pets	139	0.4	17	9.3	<0.35
5	39	2	C	M	Pollen, pets, HDM	590	5.6	77	1.1	18
6	34	1	C	F	Grass	13	1.1	<0.35	<0.35	<0.35
7	27	3	C	F	Grass	53	15	<0.35	<0.35	<0.35
8	36	2	C	M	Pollen	80	13	<0.35	<0.35	<0.35
9	36	3	V	F	Birch, mugwort	87	<0.35	36	<0.35	<0.35
10	26	2	V	M	Pollen, grass, birch	33	<0.35	7.7	<0.35	<0.35
11	24	2	V	F	Dust, HDM	8	<0.35	<0.35	<0.35	1.0
12	38	2	C	M	Mould	140	<0.35	<0.35	<0.35	0.5
Median (range)	35 (24-40)	2 (1-3)	8C/4V	5F/7M		83.5 (8.4-590)				
Phadiatop* negative mothers no										
13	26	2	C	F	None	29				
14	35	2	V	M	None	<2				
15	27	2	V	F	None	32				
16	33	1	V	F	None	3.9				
17	28	1	V	M	None	5				
18	35	2	V	M	None	66				
19	30	2	C	F	None	150				
20	48	1	C	F	None	25				
21	42	3	C	M	None	40				
22	26	1	C	M	None	8.6				
23	23	1	C	F	None	67				
24	35	2	C	F	None	26				
25	33	2	C	F	None	15				
26	20	1	C	M	None	14				
Median (range)	31.5 (20-48)	2 (1-3)	9C/5V	8F/6M		25.5 (2-150)				

HDM, house dust mite; C/V, Caesarean (elective)/vaginal delivery; F/M, female/male.

*Phadiatop (Phadia AB) detects allergen-specific IgE against 11 common inhalant allergens. Phadiatop values ≥ 0.35 kU_A/l are considered positive.

†Total IgE was measured with the ImmunoCAP system (Phadia AB). Values above 122 kU/l are considered elevated.

‡Allergen-specific IgE was measured with the ImmunoCAP system (Phadia AB) with a cut-off at 0.35 kU_A/l. Values above cut-off are indicated in bold.

IgE analyses

Maternal plasma was analysed for total IgE and Phadiatop (ImmunoCap system, Phadia AB, Uppsala, Sweden). Phadiatop screens for IgE antibodies against the 11 most common inhalant allergens in Sweden. When the Phadiatop test was positive (≥ 0.35 kU_A/l), allergen-specific IgE against timothy, birch, cat and house dust mite (HDM) were analysed separately. Cut-off for allergen specific IgE was 0.35 kU_A/l

Cell preparation

Peripheral blood mononuclear cells (PBMC) from the women and cord blood mononuclear cells (CBMC) from the umbilical cord were isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) gradient centrifugation within 24 h, except in one case when the time between sampling and isolation of cells was 48 h (mother no. 26). Isolated cells were washed and frozen gradually as previously described (18). Samples were stored at -150°C until analysis.

IgA analysis in cord blood

Total IgA levels in cord blood plasma were analysed using an ELISA according to the manufacturer's instruction (Bethyl Laboratories, Montgomery, TX, USA). All samples were measured in duplicates, and a standard, ranging between 7.8 and 500 ng/ml, was included. The level of IgA detected in cord blood plasma ranged from 2.0 to 25 $\mu\text{g/ml}$ (median 5.9 $\mu\text{g/ml}$, $n = 26$).

In vitro stimulation of cells and proliferation assay

On day 0 of the analysis, cells stored at -150°C were thawed in a 37°C water bath and washed in 37°C culture medium. The viability of the cells was around 80%. Mononuclear cells (MNC) were cultured in a humidified atmosphere with 6% CO_2 at 37°C in round-bottom 96-well plates (Falcon, $2.5 \times 10^5/200$ $\mu\text{l/well}$ in duplicates or triplicates). Cells were cultured at 37°C alone or with optimal stimulatory concentrations of extracts of timothy (10 $\mu\text{g/ml}$), birch pollen (1 $\mu\text{g/ml}$), cat dander (1 $\mu\text{g/ml}$), house dust mite (HDM, 10 $\mu\text{g/ml}$) or β -lactoglobulin (β -LG 30 $\mu\text{g/ml}$), all from ALK-Abelló, Hørsholm, Denmark, except for the β -LG which was from Sigma-Aldrich (Steinheim, Germany). Lipopolysaccharide (LPS) content was measured in all allergen extracts by the chromogenic Limulus amoebocyte lysate assay (Charles River Laboratories, L'arbrèsle, France). Because of high contamination, LPS was removed from the β -LG preparation using detoxigel (Pierce, Rockford, IL, USA). After the detoxigel treatment, LPS content in

the β -LG extract was 0.007 ng/ml for the end concentration used in the cell cultures. For the other extracts, the LPS end concentrations were as follows: timothy = 0.008 ng/ml, birch = 0.15 ng/ml, cat = 0.013 ng/ml and HDM = 0.013 ng/ml. Cells were also stimulated with phytohaemagglutinin (PHA, 1 $\mu\text{g/ml}$ for PBMC, 10 $\mu\text{g/ml}$ for CBMC; Remel Inc, Lenexa, KS, USA) as a positive control and LPS 0.15 ng/ml from *Escherichia coli* (Sigma-Aldrich) as a control for the highest LPS contamination among the extracts. Cell proliferation was measured following pulse labelling using 1 μCi [methyl- ^3H]thymidine (Amersham, Buckinghamshire, UK) for 16–18 h before harvest on day 5. Results are expressed as the ratio between cpm of stimulated and unstimulated cells (stimulation index).

ELISPOT

Production of cytokines by PBMCs was measured by enzyme-linked immunospot assay (ELISPOT). PBMCs were precultured for 20 h in round-bottom 96-well plates (Falcon) with stimulation as described earlier except for the PHA stimulation where 1×10^5 cells/well was used. After 20 h, cells were transferred to ELISPOT plates (Mabtech, Stockholm, Sweden) precoated with mouse monoclonal anti-human IL-4 antibodies (Mabtech). Cells were cultured in the ELISPOT plate for another 20 h. Plates were developed according to the manufacturers' instructions. Spots were counted using an image analysis system (Autoimmun Diagnostika GmbH, Straßberg, Germany). The number of spots in the unstimulated wells (range 0–3 spots) was subtracted from the number of spots in stimulated wells. Results are presented as number of spots per 2.5×10^5 cells.

Flow cytometric analysis

At day 0 and after 5 days of culturing with or without stimulation as described earlier, cells (triplicate of $2.5 \times 10^5/200$ $\mu\text{l/well}$, pooled before staining) were phenotyped by flow cytometry. Optimized amounts of fluorochrome-conjugated MoAbs for surface markers were added to the different staining panels (Table 2): anti-CCR7 PE-Cy7, anti- β 7 PE, anti-CD4 PE-Cy7, anti-CD4 AmCyan, anti-CD25 PE, anti-CD4 PerCP, anti-IgD PE, anti-IgM APC, anti-CD5 PE-Cy7, anti-CD27 FITC, anti-CD31 PE and anti-CD3 Pacific Blue (BD Pharmingen, San Jose, CA, USA), anti-CLA FITC, anti-CD19 APC-Cy7 and anti-CD62L APC-Cy7 (BioLegend, San Diego, CA, USA), anti-CD14 PE-Cy5 (Abcam, Cambridge, UK) and CD45RA APC (Miltenyi, Bergisch Gladbach, Germany). For staining of FOXP3 with an APC-conjugated anti-FOXP3 antibody (clone PCH101; eBioscience, San Diego, CA, USA), the cells were

Table 2. Staining panels for flow cytometry

Fluorochrome	Treg panel Antigen, clone	Treg homing Antigen, clone	RTE panel Antigen, clone	B cell panel Antigen, clone
FITC	CD14, M ϕ P9	CLA, HECA-452		CD27, M-T271
PE	CD25, M-A251	β 7, FIB504	CD31, L133.1	IgD, IA6-2
PerCP	CD4, SK3			
PE-Cy5		CD14, 61D3	CD14, 61D3	
PE-Cy7		CCR7, 3D12	CD4, SK3	CD5, L17F12
APC	FOXP3, PCH101	FOXP3, PCH101	CD45RA, T6D11	IgM, G20-127
APC-Cy7		CD62L, DREG-56		CD19, HIB19
Pacific Blue	CD3, UCHT1	CD3, UCHT1	CD3, UCHT1	
AmCyan		CD4, SK3		

RTE, Recent thymic emigrants; CLA, Cutaneous lymphocyte antigen.

fixed and permeabilized using a fixation/permeabilization kit according to the manufacturer's protocol (eBioscience). A separate experiment using BioLegend FOXP3 antibody, clone 206D on two CBMC and two PBMC samples showed a similar staining pattern. Data were acquired directly after staining, or cells were fixed in paraformaldehyde and acquired the next day on a FACSaria (BD Biosciences, San Jose, CA, USA). Data were analysed using DIVA software version 5.0.2 (BD Biosciences, San Jose, CA, USA). Isotype controls or unstained cells were used to set gates for positive and negative populations. Bi-exponential transformation was used to analyse the data. A lymphocyte gate was set using forward and side scatter, followed by a singlet gate excluding doublets. Between 1×10^5 and 4×10^5 events were collected depending on staining panel. Populations smaller than 100 events were not included in the statistical analysis.

Statistical analysis

Statistical analysis was performed using GRAPHPAD PRISM software version 4.03 (Graphpad Software Inc, San Diego, CA, USA). D'Agostino and Pearson omnibus normality test was used to test the data for normal distribution. In the majority of cases, the data were not normally distributed, and between-group comparisons were performed using the Mann–Whitney *U*-test for two groups and Kruskal–Wallis with Dunn's multiple comparisons test for three groups. Spearman rank test was used for testing correlations. Some of the Foxp3-expression data were normally distributed, and in these cases, Student's *t*-test was used for between-group comparisons. $P < 0.05$ was considered significant.

Results

Allergic mothers respond to their respective allergens, but this can not be explained by differences in FOXP3 expression in T cells

Most of the allergic mothers had timothy and/or birch-specific plasma IgE (Table 1). Only two of the allergic mothers had cat-specific IgE, and three had HDM-specific IgE. The IL-4 production as measured using ELISPOT following stimulation of PBMC with timothy extract was significantly higher in allergic mothers with specific IgE towards timothy compared to nonallergic mothers (Fig. 1A). Similarly, the IL-4 production following stimulation with birch extract was significantly higher in allergic mothers with specific IgE towards birch when compared to nonallergic mothers (Fig. 1B). When analysing PBMC proliferation following allergen stimulation, allergic mothers with specific IgE towards timothy had a significantly higher proliferation in response to stimulation with timothy extract compared to the nonallergic mothers (Fig. 1C). There was, however, no significant difference when analysing the mothers with birch-specific IgE (Fig. 1D). Of all extracts, the birch extract had the highest LPS contamination (0.15 ng/ml). Control stimulations with LPS alone at 0.15 ng/ml for all individuals revealed no significant difference in IL-4 production or proliferation between birch IgE-positive mothers and nonallergic mothers (data not shown).

The percentage of FOXP3⁺ cells of the CD3⁺CD4⁺ cells following stimulation with allergen was determined

by flow cytometry. There were no differences between allergic and nonallergic mothers (Fig. 2A). Also, when specifically analysing birch-allergic mothers following stimulation with birch extract or timothy-allergic mothers following timothy extract stimulation, there were no differences in their percentages of FOXP3⁺ cells compared to nonallergic mothers (data not shown). Upon PHA stimulation, virtually all cells stained positive for FOXP3, and therefore, we have not included these data in the analysis. Analysis of CD4⁺CD25^{high} cells indicated similar results (data not shown).

Proliferation or FOXP3 expression in CBMC is not affected by maternal allergic disease

There were no significant differences in proliferation between the CBMCs of children with birch- or timothy-allergic mothers and nonallergic mothers following stimulation with birch or timothy extract, respectively (Fig. 1C,D). This was also true when analysing the other allergen stimulations and comparing the CBMCs from all 12 children of allergic mothers with all 14 children of nonallergic mothers (data not shown). When correlating the stimulation index of CBMCs with stimulation index of PBMCs, no significant correlation was detected (data not shown). When comparing the expression of FOXP3 in cord blood from children of allergic and nonallergic mothers, there was no significant difference either following stimulation or in unstimulated cells (Fig. 2B).

Phenotype of cord blood T and B cells is not affected by maternal allergic disease

Unstimulated FOXP3⁺ T cells in cord blood were analysed for their expression of the homing markers $\beta 7$, CLA, CCR7 and CD62L. The most prominent regulatory T cell subset was CD62L⁺CCR7⁺ (Fig. 3A). In addition, $\beta 7$ ⁺ regulatory T cells were more common than $\beta 7$ [−], while CLA⁺ regulatory T cells were virtually absent. However, no significant differences were detected between children of allergic and nonallergic mothers (Fig. 3A). CD3⁺ T cells were also phenotyped for expression of the same homing markers. There were no significant differences between cord blood from children of allergic and nonallergic mothers (Fig. 3B). A similar pattern of homing marker distribution was apparent in CD3⁺ T cells and FOXP3⁺ T cells. The maturity of the cord blood T cells was evaluated by analysis of CD45RA expression and the presence of CD31⁺ recent thymic emigrants (Fig. 4A). Strikingly, the majority of the CD4⁺ cells were CD31[−], while the CD4[−] cells on the other hand were preferably CD31⁺. However, there were no differences in the percentages of the different subpopulations between children of allergic and nonallergic mothers. The vast majority of the B cells in the cord blood were naïve CD27[−]IgM⁺IgD⁺ (Fig. 4B). Also, a

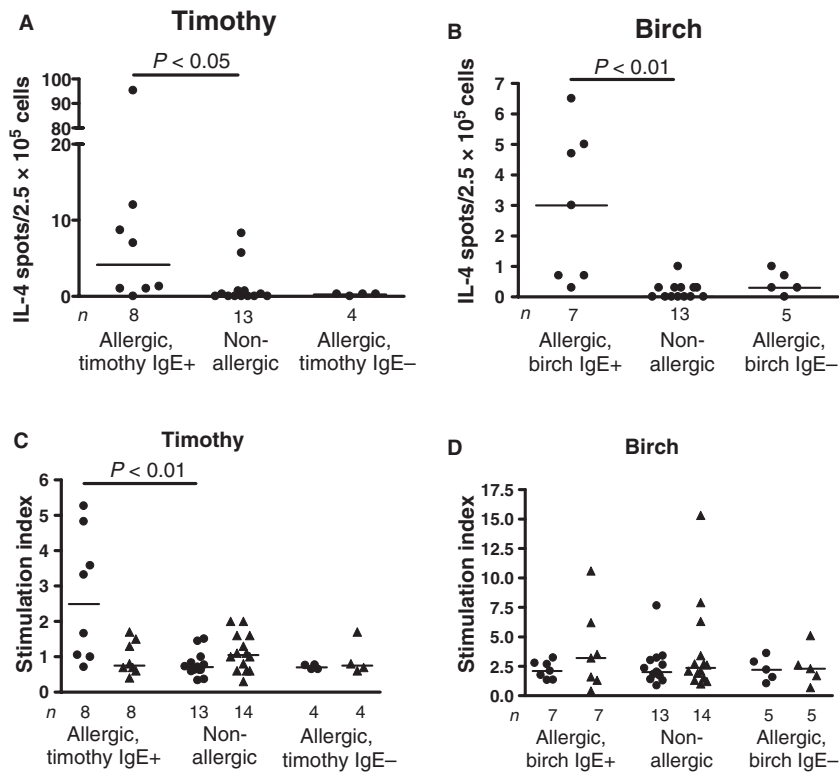


Figure 1. (A–D) IL-4 production in maternal peripheral blood mononuclear cells (PBMC) cultures and PBMC and cord blood mononuclear cells (CBMC) cell proliferation following allergen stimulation. Cells were stimulated with timothy (A, C) or birch extract (B, D). IL-4 production in PBMC (A, B) was measured with enzyme-linked immunospot assay (Mabtech AB). Mothers (Table 1) were divided in three groups: allergic with allergen specific IgE, nonallergic and allergic without allergen specific IgE to the respective allergen used for stimulation. Kruskal–Wallis ANOVA indicated a significant difference among the three groups for both timothy ($P = 0.016$) and birch ($P = 0.003$) stimulation and Dunn's post-test indicated that the significant difference was between allergic mothers with specific IgE compared to nonallergic mothers (A timothy $P < 0.05$, B birch, $P < 0.01$). For the cell proliferation as measured by thymidine incorporation (C, D), the three groups were compared by Kruskal–Wallis ANOVA for PBMC (●) and CBMC (▲), separately. For timothy stimulation, there was a significant difference in proliferation among the three groups in PBMC ($P = 0.006$). Dunn's post-test indicated that the significant difference was between allergic mothers with specific IgE and nonallergic mothers ($P < 0.01$). There were no significant differences in proliferation among CBMC groups. Data are expressed as the ratio between cpm of stimulated and unstimulated cells (stimulation index).

few $CD27^+IgM^+IgD^+$ B cells, recently demonstrated to be recirculating marginal zone B cells (19), were observed. A minor fraction of class-switched $CD27^+IgM^-IgD^-$ B cells could be detected in five individuals. CD5 expression was not different between children of allergic mothers (median 38.6%, range 20.5–65.5%) and children of nonallergic mothers (median 36.4%, range 15.8–61%). There were no differences in any of the analysed subsets of B cells between children of allergic and nonallergic mothers.

Discussion

This is the first study to investigate B cell subsets and phenotype of regulatory T cells in cord blood in relation to maternal allergy. We demonstrate here that maternal allergic disease does not have an effect on the neonatal

response to allergens or the phenotype of neonatal T and B cells.

In contrast to our data on regulatory T cells, it has been demonstrated previously that maternal atopy can influence cord blood regulatory T cells. Schaub et al. (12) recently reported impairment of regulatory T cells in cord blood of children of atopic mothers. However, they analysed $CD4^+CD25^{high}$ cells upon innate stimulation with Lipid A/peptidoglycan. Also, the impairment in suppressive function was seen following mitogen stimulation (12). Taylor et al. on the other hand demonstrated that *FOXP3* mRNA expression in blood MNC at 6 months of age was higher in infants who developed atopic dermatitis (13). One important reason for the conflicting data on the role of regulatory T cells in allergy development is the lack of a specific marker. *FOXP3* is considered the most specific marker for regulatory T cells in humans, but still, *FOXP3* can be transiently induced in

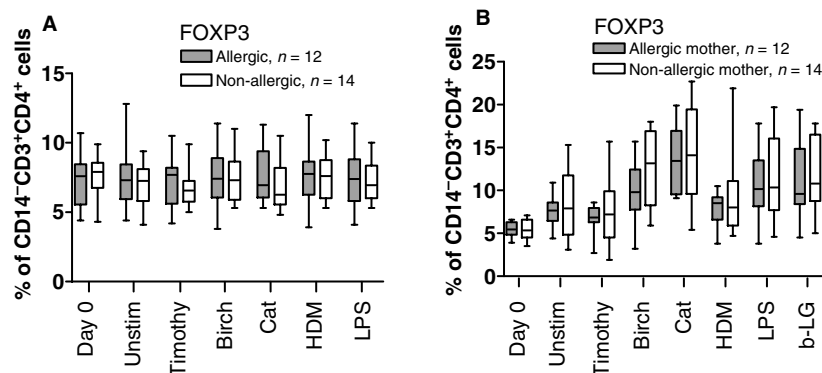


Figure 2. (A–B) FOXP3 expression in peripheral blood mononuclear cells (PBMC) and cord blood mononuclear cells (CBMC) following allergen stimulation. Cells were cultured in medium alone or in the presence of allergen extracts or lipopolysaccharide for 5 days. Expression of FOXP3 was evaluated on day 0 and day 5 by flow cytometry with intracellular staining, combined with surface staining for CD14, CD3 and CD4. Data are expressed as percentage of CD14[−]CD3⁺CD4⁺ T cells. PBMC from allergic and nonallergic mothers (A). CBMC from children of allergic and non-allergic mothers (B). Boxes indicate data between the 25th and 75th percentiles, the central line being the median. Lines extend out to max and min values. Student's *t*-test was used to compare PBMC from allergic and nonallergic mothers for all the stimulations and for all CBMC stimulations except HDM, where Mann–Whitney *U*-test was used. No significant differences were identified.

nonregulatory T cells and does not always predict suppressive function (20). Therefore, we have also analysed CD4⁺CD25^{high} cells.

In agreement with previous studies, we detected very few CLA⁺ regulatory T cells in our cord blood samples (21). Also, our low level of CD27⁺IgM⁺IgD⁺ cells are in agreement with previous findings of an age-dependent increase in this population, starting at around 1% in cord blood (19). It has been speculated that these B cells could be derived from naïve cells responding to T-dependent antigens (22). The majority of the B cells found in our cord blood samples were CD27[−]IgM⁺IgD⁺ B cells, which are considered to be pregerminal centre B cells. A previous study has demonstrated that induction of integrin α E β 7 on cord blood T cells upon stimulation with α s1-casein precedes the development of atopic eczema in infants (23). Another study reported that the percentage of proliferating T cells expressing CCR4 or integrin α E (CD103) in cord blood following stimulation with β -LG was significantly reduced in children with a high risk to develop allergy (24). Allergy development has been connected to a delayed maturation of the immune system in several different studies. It has for example been demonstrated in atopic dermatitis that more immature T cells are present in skin of patients than in skin from healthy individuals (25). CD31 is a marker for recent thymic emigrants (26). In our cord blood samples, the majority of the CD4⁺ T cells were naïve CD45RA⁺ cells, but negative for CD31. None of the homing or maturation markers we analysed were differently expressed in the cord blood of children of allergic mothers compared to children of nonallergic mothers. It would have been interesting to also analyse different T and B cell markers following stimulation with allergens; however, this was

not possible in our study because of insufficient cell numbers.

When using IgA analysis for detection of maternal contamination of cord blood, it is common to use a cut-off of 10 μ g/ml as indication of contamination (27), but also 32 μ g/ml is used (28). In our study, four of our cord blood samples had IgA levels higher than 10 μ g/ml, but all were below 32 μ g/ml (11.6, 12.8, 13.6 and 25 μ g/ml, mothers No. 16, 3, 11 and 8, respectively, see Table 1). The data generated from cord blood samples with IgA levels > 10 μ g/ml were not deviating from the other cord blood samples. Thus we consider it unlikely that maternal blood contamination has influenced our data.

We have included samples from both vaginal delivery and elective caesarean section in this study. Labour could potentially affect the different immunological parameters we are studying, because it induces foetal stress and release of pro-inflammatory cytokines and inflammatory mediators (29). However, the proportion of vaginal deliveries was similar in the allergic and nonallergic groups (Table 1) and should therefore have equally affected both groups.

In conclusion, we have demonstrated that despite a maternal *in vitro* PBMC response to allergen stimulation, the maternal allergic status does not affect the phenotype of cord blood T or B cells, presence of regulatory T cells or the proliferation of CBMC in response to allergen stimulation. The factors we have studied here could, however, still play a role in relation to later development of allergy in the children. We are presently collecting maternal, paternal and children's samples in a large prospective study where we will have the possibility to investigate the impact of neonatal immune functions on later allergy development.

No effect of maternal allergy on neonatal lymphocytes

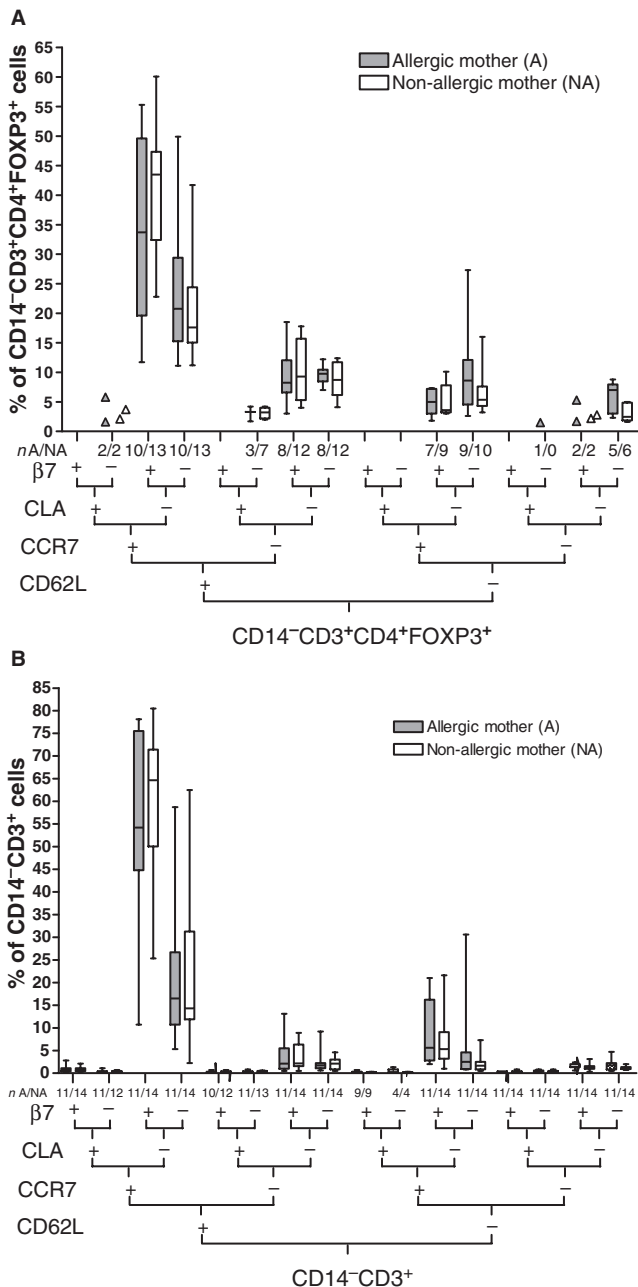


Figure 3. (A–B) Homing markers on regulatory T cells and T cells. Cord blood mononuclear cells (CBMC) from children of allergic and nonallergic mothers were stained for the expression of the homing markers $\beta 7$ integrin, CLA, CCR7 and CD62L on FOXP3⁺ regulatory T cells (A) and T cells (B) and acquired on a FACSaria. Data were analysed using Diva software. All data are from unstimulated cells at Day 0. Boxes indicate data between the 25th and 75th percentiles, the central line being the median. Lines extend out to max and min values. In case of few observations, individual values are depicted as triangles. Student's *t*-test or Mann–Whitney *U*-test was used to evaluate differences between children of allergic and nonallergic mothers. Populations with fewer than 100 events were not included in the analysis. No significant differences were identified.

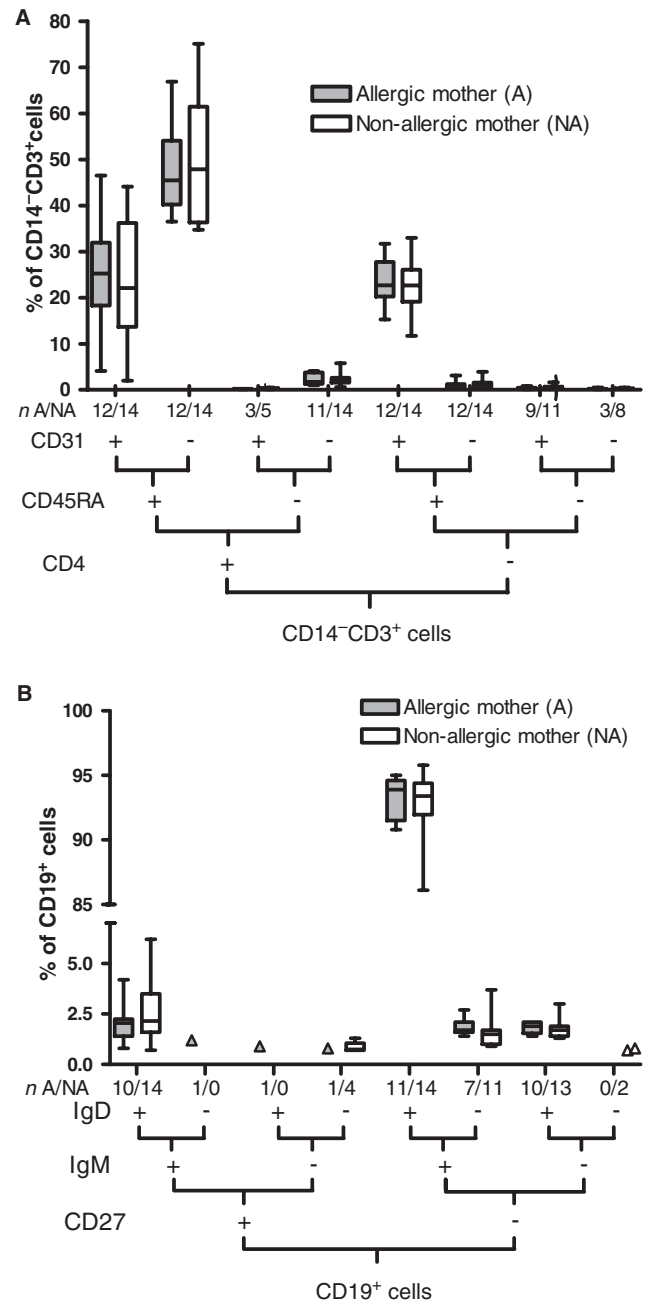


Figure 4. (A–B) Phenotype of T and B cells. Cord blood mononuclear cells (CBMC) from children of allergic and nonallergic mothers were stained for the expression of markers for naïve T cells and recent thymic emigrants CD45RA and CD31, respectively (A), or the B cell markers CD19, CD27, IgM and IgD (B) and acquired on a FACSaria. Data were analysed using Diva software. All data are from unstimulated cells at Day 0. Boxes indicate data between the 25th and 75th percentiles, the central line being the median. Lines extend out to max and min values. In case of few observations, individual values are depicted as triangles. Student's *t*-test or Mann–Whitney *U*-test was used to evaluate differences between CBMC of children of allergic and nonallergic mothers. Populations with fewer than 100 events were not included in the analysis. No significant differences were identified.

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