

Circulating Dendritic Cells, Farm Exposure and Asthma at Early Age

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Abstract

Farm environment has been shown to protect from childhood asthma. Underlying immunological mechanisms are not clear yet, including the role of dendritic cells (DCs). The aim was to explore whether asthma and farm exposures are associated with the proportions and functional properties of DCs from 4.5-year-old children in a subgroup of the Finnish PASTURE birth cohort study. Myeloid DCs (mDCs), plasmacytoid DCs (pDCs) and CD86 expression on mDCs *ex vivo* ($n = 100$) identified from peripheral blood mononuclear cells (PBMCs) were analysed using flow cytometry. MDCs and production of interleukin (IL)-6 and tumour necrosis factor alpha (TNF- α) by mDCs were analysed after 5 h *in vitro* stimulation with lipopolysaccharide (LPS) ($n = 88$). Prenatal and current farm exposures (farming, stables, hay barn and farm milk) were assessed from questionnaires. Asthma at age 6 years was defined as a doctor's diagnosis and symptoms; atopic sensitization was defined by antigen-specific IgE measurements. Asthma was positively associated with CD86 expression on mDCs *ex vivo* [adjusted odds ratio (aOR) 4.83, 95% confidence interval (CI) 1.51–15.4] and inversely with IL-6 production in mDCs after *in vitro* stimulation with LPS (aOR 0.19, 95% CI 0.04–0.82). *In vitro* stimulation with LPS resulted in lower percentage of mDCs in the farm PBMC cultures as compared to non-farm PBMC cultures. Our results suggest an association between childhood asthma and functional properties of DCs. Farm exposure may have immunomodulatory effects by decreasing mDC proportions.

Introduction

Farm exposure in early childhood or even prenatally protects from childhood asthma and allergies, suggesting effects on maturing immune system. The strong protective factors that have been identified by now are exposure to cowshed and unprocessed cow's milk in early childhood [1].

Dendritic cells (DCs) are the professional antigen-presenting cells, which act as a linkage between innate and adaptive immune system. DCs take up and process antigens, present peptides derived from antigens to T cells, stimulate T cells via expression of distinct costimulatory molecules and quickly migrate to lymphoid tissues and secrete cytokines to initiate immune responses. DCs play a central role in the induction of both T cell immunity and tolerance to inhaled antigens [2] and in the regulation of airway inflammation [3].

There are several DC subtypes, and they differ in function, localization and phenotype. Myeloid DCs

(mDCs) and plasmacytoid DCs (pDCs) are the two main subtypes of peripheral blood DCs [4]. MDCs have been shown to be associated with allergic airway inflammation [5], whereas pDCs have been mainly known for their role in viral infections [6] as well as in the development of tolerance [7,8]. However, the functional plasticity of pDCs and mDCs is challenging the concept of DC subpopulations with distinct functions [9].

Protection against allergic diseases by the traditional farm environment may be associated with early life immunoprogamming of the innate and adaptive immune cells, and these effects have been seen in a typical cytokine pattern [10], relative proportions of regulatory immune cells [11] and upregulated levels of Toll-like receptors (TLRs) between farm-exposed and non-exposed children [12,13]. In a recent study, the lower proportion of circulating mDC subtype 2 at age 6 years was observed in farm children when compared with non-farm children, but the authors were not able to demonstrate whether this

subtype of DCs mediated the allergy-protective effect of farming or whether farm exposures had effect on other DC subtypes [14]. As DCs operate closely with regulatory T cells (Tregs) [15] and they express distinct TLRs [16], it can be hypothesized that DCs might play a special role in the protective effect of farm exposure on childhood asthma.

In vitro generation of human monocyte-derived DCs has been shown to be suppressed by stable dust extract, indicating the immune modulating properties of the stable dust [17]. Another study showed that cowshed dust extract reduced the allergy-inducing capacity of bone marrow DCs of mice [18]. Allergies and asthma have been shown to be associated with the numbers of DCs in adults [19–22] and in children [23–25], but the effect of farm exposure on circulating DCs in epidemiological settings is mainly unexplored.

We aimed to investigate associations between phenotype and functional properties of DCs, asthma and farm exposures in the international PASTURE/EFRAIM birth cohort study. Peripheral blood mononuclear cells (PBMCs) collected at age 4.5 years in a subgroup of farm-exposed and non-farm-exposed Finnish children were analysed for *ex vivo* DC phenotype: the percentages of mDCs and pDCs and the expression of costimulatory molecule CD86 on mDCs. To investigate the functional capacity of mDCs, PBMCs were stimulated with lipopolysaccharide (LPS) and the intracellular production of the cytokines interleukin (IL)-6 and tumour necrosis factor alpha (TNF- α) in mDCs was analysed.

Materials and methods

Study characteristics. The study population consisted of Finnish children ($n = 100$, 50 from farming families, Fig. 1) who belong to the Finnish PASTURE (also called LUKAS1, $n = 214$). The PASTURE/EFRAIM is a prospective birth cohort study in five European countries (Austria,

Finland, France, Germany and Switzerland), and the aim is to investigate protective and risk factors in early life influencing the development of atopic diseases. Briefly, pregnant women were contacted in the third trimester of pregnancy. Women who lived or worked on farms with livestock were assigned to the farm group. The reference group was composed of women from the same rural areas, but not living or working on a farm. The design of the PASTURE has been described in detail elsewhere [26]. Written informed consent was obtained from all parents. The experimental plan was approved by the Research Ethics Committee in Kuopio, Finland (number 33/2002).

IgE and asthma definitions. In this subgroup of the Finnish PASTURE, clinical phenotypes were assessed at age 6 years when they are more stable. Immunoglobulin E (IgE) for 6 food allergens (hen's egg, cow's milk, peanut, hazelnut, carrot and wheat) and 13 common inhalant allergens (*Dermatophagoides pteronyssius*, *D. farinae*, cat, horse, dog, *Alternaria alternata*, mugwort, plantain, alder, birch pollen, hazel pollen, rye pollen and a grass pollen mix) were assessed from peripheral blood samples by the Allergy Screen test panel for atopy (Mediawiss Analytic, Moers, Germany) in a central laboratory. Positive sensitization was defined by the cut-off concentration 0.7 IU/ml. Asthma was defined as a doctor's diagnosis of asthma at least once per lifetime and/or repeated diagnosis of obstructive bronchitis reported by the parents at age 6 years and/or unremitting wheeze 18 months – 6 years and/or LCA-asthma (latent class analysis) [27].

Exposure definitions. We investigated farm exposures (yes/no) during pregnancy (prenatal exposure) and during the past 12 months (current exposure at age 4 years) from questionnaires. We investigated specific farm exposures (yes versus no) during the past 12 months as the DCs and their functional properties were assessed at age 4.5 years. All the studied children were living either on a dairy and/

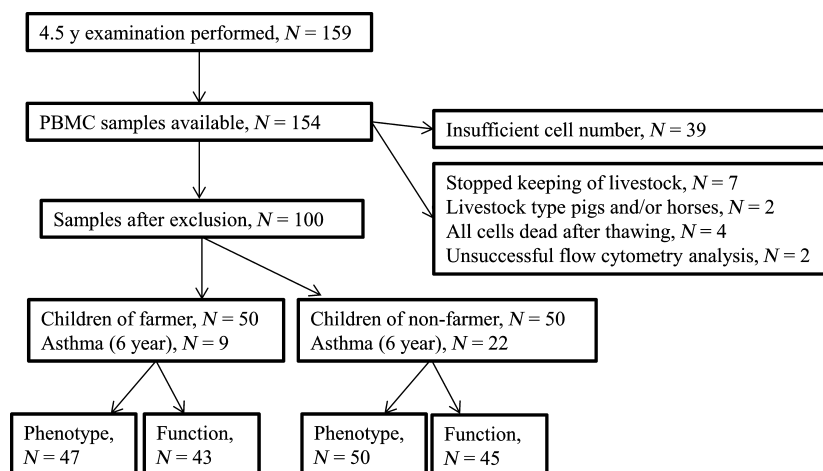


Figure 1 Selection and number of samples. 154 peripheral blood mononuclear cell (PBMC) samples were available but due to given reasons number of analysed samples was 100 (children of farmer $n = 50$).

or cattle farm when the child was 4 years old. Children whose family had stopped keeping of livestock ($n = 7$) or the main type of livestock had been changed to pigs or/and horses ($n = 2$) before the age of 4 years were excluded from this study (Fig. 1). Farming was defined as a family living on a farm with livestock when the child was born. Exposures to stables (cowshed) and hay barn were defined as exposure of at least a quarter of an hour per week. Consumption of farm milk was defined as mean consumption of at least 10 ml cow milk per day. Farm milk included all other cow milk except shop milk.

Isolation and cryopreservation of PBMCs. PBMCs at age 4.5 years were isolated from EDTA blood (Vacutainer, BD, Plymouth, UK) by Ficoll–Paque technique (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and cryopreserved. Freshly isolated PBMCs were resuspended with 10% FBS Gold (PAA Laboratories GmbH, Pasching, Austria) in RPMI 1640 (Invitrogen, Grand Island, NY, USA) and mixed 1:1 with 15% DMSO (Sigma Aldrich Company, St. Louis, MO, USA) in FBS Gold. Cryotubes were stored in -80°C overnight for gradual temperature reduction. The next day, the tubes were transferred to a liquid nitrogen cryopreservation tank.

Immunophenotyping and functional experiments. Cells were thawed and resuspended with 10% human AB serum (Innovative Research, Novi, MI, USA) in RPMI 1640 (Invitrogen). Viability and the number of PBMCs were determined by Trypan Blue exclusion after thawing. The mean live PBMC count after thawing was 6.2×10^6 (Standard deviation, SD 2.26×10^6). The mean percentage of dead cells was 7.9% (SD 4.4%). One portion of thawed PBMCs was stained for DC phenotyping, and another was used for functional experiments. In functional experiments, the PBMCs were stimulated with TLR ligand, LPS, lipopolysaccharides from *Escherichia coli* 0111:B4 (0.5 $\mu\text{g}/\text{ml}$, Sigma Aldrich Company). PBMCs were cultivated with or without LPS in 10% human AB serum in RPMI 1640 at 37°C and 5% CO_2 for 5 h. In functional experiments, monensin (GolgiStop; BD, San Diego, CA, USA) was added to cultures to block intracellular protein transport processes. After 5 h, the cells were harvested, stained for expression of surface molecules, permeabilized (BD Cytotfix/Cytoperm Plus Kit, GolgiStop; BD) and then stained for intracellular cytokines. Samples were analysed immediately after staining by flow cytometry.

Flow cytometry. PBMCs were analysed by CyAn ADP flow cytometer (Beckman Coulter Inc., Fullerton, CA, USA) equipped with two lasers (solid state 488 nm and 633) and capabilities to analyse 7 colours simultaneously (530/40, 575/25, 630/20, 680/30, 750LP, 665/20, 750LP). The analyses and manual compensations were carried out using the Summit v4.3 software application.

Erythrocytes and debris were excluded, as were $\text{CD}19^{+}$ B cells, which also express $\text{CD}1\text{c}$, and $\text{CD}14^{+}$ monocytes,

which also express $\text{CD}11\text{c}$ (Fig. S1). Dead cells were excluded on the basis of propidium iodide (PI) staining (0.05 $\mu\text{g}/\text{ml}$, Sigma Aldrich Company) in phenotype protocol, and doublets were excluded on the basis of their FS Area/FS Lin characteristics. Frequencies of mDCs ($\text{CD}14^{-}\text{CD}19^{-}\text{CD}11\text{c}^{+}\text{CD}1\text{c}^{+}$) and pDCs ($\text{CD}14^{-}\text{CD}19^{-}\text{CD}123^{+}\text{CD}303^{+}$) were expressed as percentages of live PBMCs. The expression of $\text{CD}86$ on mDCs *ex vivo* (median fluorescence intensity, MFI) and the production of IL-6 and $\text{TNF-}\alpha$ in mDCs after stimulation with LPS (a fold change of MFI over the unstimulated sample) were studied. For details, see online supporting information S1.

Statistical analysis. Distributions of DC parameters were mostly normally distributed. $\text{TNF-}\alpha$ expression in mDCs after stimulation with LPS was log-transformed in order to reach satisfactory approximation to normal distribution. Associations of exposures with asthma and asthma with DCs were analysed using logistic regression and expressed as odds ratios (OR) and 95% confidence interval (CI). Analyses were adjusted for farming, number of older siblings, gender, prenatal exposure to cat(s) and/or dog(s) and paternal allergic disease, which were selected based on change in estimated effects of DCs by more than 10% (Table S2). Associations between DCs and asthma were not linear, and therefore, DC parameters were categorized into two groups using 66th percentile cut-off point. Associations between exposures and DC parameters were analysed using *t*-test and general linear model. Possible confounders were tested but not reported as they did not influence the main results. Associations of DCs and asthma with exposures to stables, hay barn and farm milk were not adjusted for farming due to low number of exposed non-farming children. Statistical significance was defined by $P < 0.05$. SPSS Statistics 19.0 (IBM Corporation, New York, NY, USA) was used for the statistical analysis.

Results

Association of farm exposures with asthma and atopic sensitization

Farming, prenatal exposure to stables and current exposure to stables and hay barn were inversely associated with asthma (crude odds ratios, cORs, between 0.20 and 0.37, data not shown), and after adjustment for potential confounders (number of older siblings, gender, prenatal exposure to cat(s) and/or dog(s) and paternal allergic disease), farming and current exposure to stables were inversely associated with asthma ($P = 0.028$ and 0.033 , Table 1). When associations in all Finnish children in the cohort ($n = 214$ at age 6 years) were studied, the cORs were similar to our Finnish subsample but less pronounced (data not shown).

Table 1 Adjusted odds ratios for the associations of prenatal and current (at age 4 years) farm exposures with asthma and atopic sensitization at age 6 years.

	Asthma ^a			Atopic sensitization ^b		
	N (n) ^c	aOR (95% CI)	P ^d	N (n) ^c	aOR (95% CI)	P ^d
Prenatal exposure						
Farmer	49 (9)	0.32 (0.11–0.88)	0.028	45 (28)	0.87 (0.33–2.28)	0.780
Stay in stables	48 (9)	0.42 (0.15–1.17)	0.097	44 (29)	1.30 (0.50–3.42)	0.591
Stay in hay barn	27 (5)	0.53 (0.15–1.81)	0.309	23 (15)	1.28 (0.43–3.76)	0.660
Farm milk consumption	34 (8)	0.56 (0.20–1.58)	0.270	32 (20)	0.87 (0.33–2.29)	0.773
Current exposure						
Stay in stables	46 (8)	0.33 (0.12–0.91)	0.033	42 (27)	1.04 (0.40–2.71)	0.939
Stay in hay barn	19 (2)	0.28 (0.05–1.41)	0.122	17 (11)	0.91 (0.28–3.02)	0.881
Farm milk consumption	18 (5)	0.92 (0.26–3.20)	0.889	18 (9)	0.55 (0.18–1.70)	0.300

Adjusted odds ratio (aOR) with 95% confidence interval (CI); reference group, non-exposed.

^aAsthma was defined as a doctor's diagnosis of asthma at least once per lifetime and/or repeated diagnosis of obstructive bronchitis reported by the parents at age 6 years and/or unremitting wheeze 18 months – 6 years and/or LCA-asthma (latent class analysis) [27].

^bAtopic sensitization was defined as a positive result to any of the studied allergens, that is, antigen-specific IgE concentration of 0.7 IU/ml or greater at age 6 years.

^cNumbers can vary because of availability of parameters. N; total number of children exposed, n; number of exposed and asthmatic children.

^dAdjusted (number of older siblings, gender, prenatal exposure to cat(s) and/or dog(s) and paternal allergic disease) p values of logistic regression: significant results ($P < 0.05$) are shown in boldface.

Association of DCs with atopic sensitization and asthma

As farming was inversely associated with asthma in this small study population, we next studied the associations of DCs with atopic sensitization and asthma. The increased expression of CD86 on mDCs *ex vivo* was associated with asthma at age 6 years ($P = 0.008$, Table 2) after adjustment for potential confounders (farming, number of older siblings, gender, prenatal exposure to cat(s) and/or dog(s) and paternal allergic disease) and as a trend with atopic sensitization against inhalant allergens (aOR 2.87, 95% CI 0.91–9.06, $P = 0.073$). MDCs produced less IL-6 in the PBMC cultures of asthmatic children as compared to the PBMC cultures of non-asthmatic children after *in vitro* stimulation with LPS ($P = 0.026$).

Association of DCs with farm exposures

In vitro stimulation with LPS resulted in lower percentage of mDCs in the farm PBMC cultures as compared to the non-farm PBMC cultures ($P = 0.015$, Table 3). Similar but borderline significant association was observed between farming and percentage of mDCs *ex vivo* ($P = 0.069$).

To further investigate the effect of specific farm-related exposures on DCs, we studied associations between DC parameters and prenatal and current farm exposures. In general, all farm-related exposures were associated with decreased percentages of DCs, but only few statistically significant associations were found (Table 3). Percentages of pDCs *ex vivo* and mDCs after *in vitro* stimulation with LPS were lower in the PBMCs of children with prenatal

exposure to farm milk as compared to the non-exposed ($P = 0.042$ and 0.043). *In vitro* stimulation with LPS resulted in lower mDC percentage and higher production of TNF- α by mDCs in PBMC cultures of children with current exposure to hay barn ($P = 0.035$ and 0.043).

Discussion

The protective effect of early life farm exposure on the development of atopic diseases has been demonstrated in several cross-sectional studies [1,28–32]. However, the underlying immunological mechanisms still need to be identified. The effects of farm exposure and asthma on DC proportions and on their functional properties at an early stage of childhood immune maturation, namely age 4.5 years, have not been published previously in an epidemiological setting. In the present study, the most interesting associations were seen between asthma and the functional properties of DCs. We also found some evidence on the associations of farm exposures with the decreased proportions of DCs.

The roles of DCs in bridging innate and adaptive immunity and in the induction of immunity and tolerance suggest that they may have a role also in the asthma-protective effect of farming. Overexpression of CD86, indicating increased T cell stimulatory capacity, has been shown to be associated with asthma and allergies [33], and this was also demonstrated in our study. CD86 expression on mDCs may be modulated by Tregs, which have been shown to be lower in asthmatic children than in healthy children [11] and to inhibit the upregulation of CD80 and

	Asthma ^b				Atopic sensitization ^c		
	<i>n</i> ^d	aOR (95% CI)	<i>P</i> ^e		<i>n</i> ^d	aOR (95% CI)	<i>P</i> ^e
<i>Ex vivo</i>							
mDC ^f	11	0.96 (0.33–2.77)	0.935	18	1.02 (0.39–2.68)	0.964	
pDC ^f	11	0.98 (0.33–2.85)	0.963	16	0.64 (0.25–1.67)	0.365	
CD86 on mDC ^f	14	4.83 (1.51–15.4)	0.008	17	1.59 (0.57–4.48)	0.377	
<i>In vitro</i> stimulation with LPS							
mDC ^f	11	1.63 (0.53–5.01)	0.392	16	1.16 (0.41–3.27)	0.782	
IL-6 in mDC ^f	3	0.19 (0.04–0.82)	0.026	13	1.30 (0.45–3.71)	0.627	
TNF- α in mDC ^f	6	0.87 (0.25–3.00)	0.822	15	1.11 (0.38–3.20)	0.851	

Adjusted odds ratio (aOR) with 95% confidence interval (CI).

^aDC data were dichotomized using 66th percentile cut-off points. Low (<66%) category was set as the reference group.

^bAsthma was defined as a doctor's diagnosis of asthma at least once per lifetime and/or repeated diagnosis of obstructive bronchitis reported by the parents at age 6 years and/or unremitting wheeze 18 months – 6 years and/or LCA-asthma (latent class analysis) [27].

^cAtopic sensitization was defined as a positive result to any of the studied allergens, that is, antigen-specific IgE concentration of 0.7 IU/ml or greater at the age 6 years.

^dNumbers can vary because of availability of samples and parameters. *n*; number of asthmatic children in upper tertile.

^eAdjusted (farming, number of older siblings, gender, prenatal exposure to cat(s) and/or dog(s) and paternal allergic disease) *p* value of logistic regression: significant results (*P* < 0.05) are shown in boldface.

^fMyeloid DCs (mDCs) and plasmacytoid DCs (pDCs) are expressed as percentages of live peripheral blood mononuclear cells, *ex vivo* expression of CD86 on mDCs as median fluorescence intensity (MFI) and IL-6 and TNF- α in mDCs as a fold change of MFI in lipopolysaccharide (LPS)-stimulated sample over the MFI in unstimulated sample.

Table 2 Adjusted odds ratios for the associations of asthma and atopic sensitization at age 6 years with dendritic cells (DCs), comparing high levels (upper tertile)a with low levels of DCs at age 4.5 years.

CD86 on immature DCs possibly resulting in tolerance [34].

We also measured the production of IL-6 and TNF- α in mDCs after stimulation with LPS as general markers of mDC activation. Lebre *et al.* [35] showed decreased expression of stimulated blood T helper (Th) 1-associated cytokine IL-12, TNF- α and IFN- α in mDCs and pDCs from patients with atopic dermatitis. Also, Chen *et al.* [36] observed increased expression of CD86 and decreased production of IL-12 and the regulatory cytokine IL-10 in monocyte-derived DCs of asthmatic subjects. In another study, the number of TNF- α producing mDCs was not shown to be associated with asthmatic children, but the number of IL-12 producing mDCs was lower in asthmatic children [37]. Increased levels of IL-6 have been found in serum, bronchoalveolar lavage fluid and induced sputum of asthmatic patients [38]. In contrast to these findings, we demonstrated an inverse association between asthma and the production of IL-6 in mDCs after stimulation with LPS. To support our results, DC-derived IL-6 production has been shown to restrict Th2-cell differentiation in an experimental model [39]. Moreover, Bratke *et al.* [21] showed that TLR7-mediated IL-6 production in pDCs was inversely associated with asthma. To conclude the associations of asthma and DCs, increased expression of CD86 and decreased production of IL-6 by mDCs of the asthmatic children may have promoted the induction and/or maintenance of Th2-skewed responses. The causal

effect of DCs on asthma could not be confirmed in our cross-sectional study.

Previous studies have shown an inverse association between the numbers of blood mDCs and allergic diseases in children [24] and adults [22], although also, as in our study, results with no associations [19,23] and controversial results have been reported [20]. Blood pDC numbers have been reported to be decreased in asthmatic children [23,25] and increased in asthmatic adults [19,20], but we could not show the association of pDC proportions with asthma. Furthermore, we observed no associations between farm exposures and atopic sensitization or between any DC marker and atopic sensitization in this study population.

Upregulation of innate immune receptors, namely TLRs [12] as well as Tregs [11], has been shown in children of farmers, suggesting that these mechanisms are relevant for the farm effect. In our study, upregulated responses were observed in association with current regular contact with hay, which was associated with TNF- α production in mDCs after stimulation with LPS. In a recent study, phorbol 12-myristate 13-acetate/ionomycin-stimulated cord blood levels of Th1 cytokine IFN- γ and TNF- α were shown to be increased in children of farmers and TNF- α levels were also associated with prenatal exposure to hay barn [10]. In an experimental setting, farm-originated bacteria affected maturation and cytokine profiles of human monocyte-derived DCs by increasing expression of CD40, CD80, CD86, TNF- α and IL-6 [40]. In another study,

Table 3 Effect of prenatal and current farm-related exposures on dendritic cells (DCs) at age 4.5 years.

	<i>Ex vivo</i>							<i>In vitro</i> stimulation with LPS						
	mDC ^b			pDC ^b		CD86 on mDC ^b		mDC ^b			IL-6 in mDC ^b		TNF- α in mDC ^{b,c}	
	<i>n</i> ^a	Mean (SD)	<i>P</i> ^d	Mean (SD)	<i>P</i> ^d	Mean (SD)	<i>P</i> ^d	<i>n</i> ^a	Mean (SD)	<i>P</i> ^d	Mean (SD)	<i>P</i> ^d	GMR (95%CI)	<i>P</i> ^d
<i>Prenatal exposure</i>														
Farmer														
No	48	0.33 (0.15)	0.069	0.36 (0.18)	0.312	9.90 (2.46)	0.677	45	0.43 (0.20)	0.015	1.18 (0.30)	0.904	0.95 (0.76–1.18)	0.633
Yes	47	0.28 (0.11)		0.33 (0.17)		9.72 (1.60)		43	0.34 (0.11)		1.18 (0.32)		1	
Stay in stables														
No	44	0.32 (0.15)	0.378	0.36 (0.17)	0.405	10.1 (2.45)	0.334	40	0.42 (0.20)	0.136	1.16 (0.30)	0.903	0.89 (0.71–1.12)	0.314
Yes	46	0.30 (0.12)		0.33 (0.17)		9.69 (1.65)		42	0.36 (0.12)		1.17 (0.32)		1	
Stay in hay barn														
No	64	0.33 (0.14)	0.086	0.35 (0.18)	0.304	10.0 (2.30)	0.331	59	0.41 (0.18)	0.122	1.17 (0.28)	0.915	0.82 (0.64–1.06)	0.130
Yes	26	0.27 (0.11)		0.31 (0.13)		9.56 (1.37)		23	0.34 (0.13)		1.17 (0.37)		1	
Farm milk consumption														
No	61	0.32 (0.14)	0.121	0.37 (0.19)	0.042	9.83 (2.19)	0.976	57	0.41 (0.19)	0.043	1.22 (0.30)	0.108	1.05 (0.84–1.31)	0.653
Yes	32	0.28 (0.11)		0.29 (0.13)		9.81 (1.91)		29	0.34 (0.11)		1.10 (0.31)		1	
<i>Current exposure</i>														
Stay in stables														
No	51	0.32 (0.15)	0.256	0.36 (0.17)	0.319	9.92 (2.36)	0.595	48	0.41 (0.20)	0.072	1.19 (0.30)	0.703	0.92 (0.74–1.14)	0.435
Yes	44	0.29 (0.11)		0.33 (0.17)		9.69 (1.69)		40	0.35 (0.12)		1.17 (0.32)		1	
Stay in hay barn														
No	76	0.32 (0.14)	0.086	0.36 (0.17)	0.220	9.81 (2.08)	0.978	69	0.40 (0.18)	0.035	1.18 (0.31)	0.918	0.76 (0.59–0.99)	0.043
Yes	19	0.26 (0.09)		0.30 (0.17)		9.82 (2.07)		19	0.34 (0.08)		1.19 (0.30)		1	
Farm milk consumption														
No	77	0.31 (0.14)	0.436	0.36 (0.18)	0.095	9.82 (2.21)	0.947	73	0.39 (0.17)	0.355	1.19 (0.32)	0.493	1.12 (0.83–1.49)	0.459
Yes	18	0.29 (0.10)		0.28 (0.14)		9.78 (1.35)		15	0.35 (0.13)		1.13 (0.23)		1	

Mean with standard deviation (SD).

^aNumbers can vary because of availability of samples and parameters.

^bMyeloid DCs (mDCs) and plasmacytoid DCs (pDCs) are expressed as percentages of live peripheral blood mononuclear cells, *ex vivo* expression of CD86 on mDCs as median fluorescence intensity (MFI) and IL-6 and TNF- α in mDCs as a fold change of MFI in lipopolysaccharide (LPS)-stimulated sample over the MFI in unstimulated sample.

^cLog-transformed values were used in statistical analysis. Geometric mean ratio (GMR) with 95% confidence interval (CI) was calculated with general linear model.

^dUnadjusted P value of t-test: significant results ($P < 0.05$) are shown in boldface.

mouse bone marrow DCs treated with stable dust extract 2 days before they were pulsed with Ovalbumin and used to sensitize mice via the airways expressed CD86 and produced high amounts of IL-10, IL-12 and TNF- α [18]. Furthermore, they were not able to prime allergic responses in mice, emphasizing the role of innate immunity as a regulator of asthma development.

Tregs have been shown to be increased in children exposed to farm milk and decreased in asthmatic children at age 4.5 years in the PASTURE study [11]. Loss *et al.* [41] showed a positive association between blood mRNA expression of TLR4, 5 and 6 and farm milk consumption at the age of 1 year in the PASTURE study. In our study with Finnish subsample of the PASTURE study, farm milk consumption, especially during pregnancy, was associated with decreased pDC proportions. It is speculated that pDCs have a role in tolerance and in control of airway inflammation [8]. However, as we investigated circulating DCs: decreased numbers of these cells in the peripheral blood of farming children may be related to the intense exposure to the variety of stimulants present in a farm

environment, meaning that DCs might have migrated from blood to other tissues, which are in contact with environment.

Farming, prenatal farm milk consumption and current regular contact with hay were associated with decreased mDC proportions after stimulation with LPS. Also, a trend for decreased mDC proportions *ex vivo* was observed in farm-exposed children. Myeloid DCs may play a role in the development of Th2 responses and airway inflammation [5,8]. Farm exposure and subsequent exposure to LPS may have an effect on the development of tolerance by decreasing the numbers of mDCs, which present antigens to reactive naïve T cells. Farm-originated dust extract has been shown to suppress the differentiation of human monocytes to DCs [17], but we could not confirm in our study whether decreased mDC proportions in farm-exposed children were related to impaired *ex vivo* differentiation. Considering correlation between farm exposures in this small population, it is not possible to conclude whether stable, hay barn or farm milk specifically affected the percentage of circulating DCs.

We used cryopreserved cells in our experiments due to logistical reasons. It is possible that freezing, storage in liquid nitrogen and thawing of cells have affected the phenotype or functional capacity of PBMCs, and therefore, associations between early life exposures, DC markers and asthma may need to be confirmed using fresh samples. However, previous studies have shown that these processes do not distort cellular immune responses [42,43]. Some of the statistically significant associations may be due to chance because of the multiple exposures vs. DC comparisons. However, they all point towards same direction, which may indicate that the associations are not chance findings.

DCs were investigated in a Finnish subpopulation of the PASTURE study, whereas associations between farm milk exposure, increased Tregs and lower risk of asthma have been shown in German and French children of the PASTURE cohort study [11]. Further studies are needed to disentangle the role of DC regulation on Tregs and how it may contribute to the lower risk of asthma in farm-exposed children.

In conclusion, we have first time described phenotype and functional properties of circulating DCs in association with asthma and farm exposure at 4.5-year-old children. Our results suggest an association between childhood asthma and the expression of functional markers by mDCs. Farm exposure may have immunomodulatory effects by decreasing mDC proportions. This study provides important insights for the future studies investigating underlying immunological mechanisms of farm-related asthma-protection.

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