

Cord blood cytokines are modulated by maternal farming activities and consumption of farm dairy products during pregnancy: The PASTURE Study

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Background: Traditional farming represents a unique model situation to investigate the relationship of early-life farm-related exposure and allergy protection.

Objectives: To investigate associations between maternal farm exposures and cytokine production in cord blood (CB) mononuclear cells in a prospective multinational birth cohort of 299 farm and 326 nonfarm children and their families.

Methods: Supernatants from phorbol 12-myristate 13-acetate/ionomycin-stimulated CB mononuclear cells were assessed for the production of IFN- γ , TNF- α , IL-5, IL-10, and IL-12.

Results: Significantly higher levels of IFN- γ and TNF- α in farm compared with nonfarm children were found, whereas IL-5, IL-10, and IL-12 levels did not differ between study groups.

Maternal contact with different farm animal species and barns and consumption of farm-produced butter during pregnancy enhanced the production of proinflammatory CB cytokines, whereas maternal consumption of farm-produced yogurt resulted in significant lower levels of IFN- γ and TNF- α in umbilical blood.

Conclusion: Maternal exposure to farming activities and farm dairy products during pregnancy modulated cytokine production patterns of offspring at birth. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Hygiene hypothesis, allergy protection farm, effect, cord blood cytokine pattern

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Allergic diseases have become a major public health problem in westernized societies. Changes in lifestyle conditions and loss or appearance of environmental factors may contribute to the increased prevalence of these diseases since the middle of the last century. Over the last decade, several allergy-protective conditions have been identified, some of which are closely linked to microbial exposures. Traditional farming represents a unique and prototypic environmental situation in which such allergy-protective events can be studied extensively.¹ The so-called “farm effect” that provides protection against respiratory allergies and allergic sensitization has been reproduced worldwide by a number of epidemiologic studies.² Although these studies provide overwhelming evidence for the protective nature of these environments, the characterization of the individual components as well as the exact timing of the underlying immunologic events are far from being understood, because most of these studies have surveyed children at school age in a cross-sectional study design. Nevertheless, the Prevention of Allergy—Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle (PARSIFAL) Study gave hints that protection against allergic diseases coming from farm-related exposures may already start *in utero*.³

These recently reported data led us to hypothesize that protection against allergies by the traditional farm environment is initiated by a prenatal immunoprogamming of cells of the innate and adaptive

Abbreviations used

CB: Cord blood
 EPS: Extracellular polysaccharide
 GMR: Geometric mean ratio
 PARSIFAL: Prevention of Allergy—Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle
 PASTURE: Protection Against Allergy Study in Rural Environments
 PMA: Phorbol 12-myristate 13-acetate

immune system. In neonates born to farm families, these effects should become apparent in a typical cytokine pattern differing from the pattern seen in children born to nonfarm families. According to the hygiene hypothesis, the underlying immunomodulating mechanisms may result in a counterbalance toward a T_H1 -driven or anti-inflammatory cytokine milieu in umbilical blood. Microbes from farming environments, their components and products, and food products manufactured on the farm represent important candidates to play a role in early immunoprogamming. Mucosal exposures, either via the respiratory or the gastrointestinal route are particularly relevant in this context.^{4,5}

To identify relevant environmental factors and to gain a detailed insight into the kinetics of immunomaturization, the international Protection Against Allergy—Study in Rural Environments (PASTURE) birth cohort study has been initiated. The PASTURE project was designed to assess prospectively the role of farm exposures in the development of childhood asthma and allergies in rural environments across Europe.⁶

This analysis aims to investigate the associations of farm-related exposures and consumption of dairy products during pregnancy with early responses of the innate and adaptive immune system of neonates.

METHODS**Study design and population**

The PASTURE study is a birth cohort study conducted in rural areas of 5 European countries: upper Bavaria (Germany), eastern Switzerland, the Salzburg area (Austria), central Finland (Kuopio), and Besançon Franche-Comté (France). Healthy farm and nonfarm pregnant women were identified in the third trimester of pregnancy between August 2002 and March 2005. Farming was characterized by traditional farming conditions such as feeding animals, placing litter, removing dung, milking, cleaning animal sheds, and so forth. In total, 1133 families were included in the study, with an approximate farming/nonfarming ratio of 1:1 (Austria, 220; Finland, 214; France, 203; Germany, 254; Switzerland, 242). Among others, the study modules included a questionnaire, the withdrawal of blood, and the sampling of dust from the participants' homes. Exclusion criteria were as follows: the woman was younger than 18 years, the pregnancy was a multiple pregnancy, the sibling of a child was already included in the study, the mother intended delivery at home or has already delivered at home, the family intended to move away from the area where the study was performed, and the family had no telephone connection. The study was approved by local research ethics committees in each country, and informed written consent was obtained from 1 parent of each child.

Questionnaire

Questionnaires were based on items from the International Study of Asthma and Allergy in Childhood (ISAAC), the Allergy and Endotoxin study (ALEX), and the PARSIFAL study. Parental respiratory health was assessed by using questions derived from the American Thoracic Society questionnaire.⁷ Information on parental atopic diseases and potential confounders such

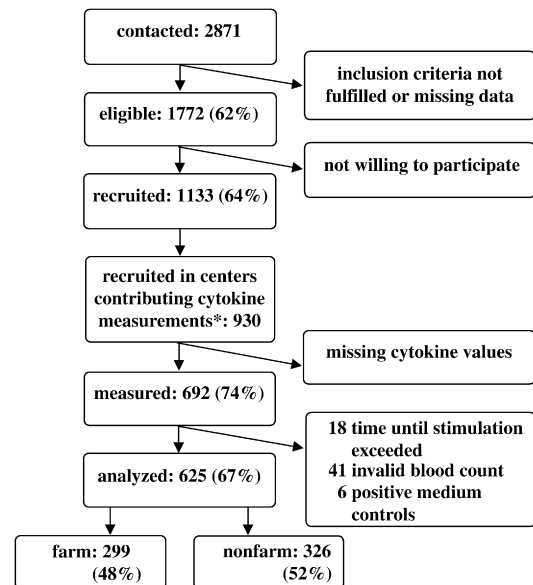


FIG 1. Recruitment of study population. *The study centers in Austria, Finland, Germany and Switzerland contributed to the cytokine measurements.

as sociodemographic conditions, smoking, and family size was collected in separate maternal and paternal questionnaires. Maternal information on pregnancy and delivery as well as on dietary habits during pregnancy was surveyed, emphasizing whether the food had a commercial source or was produced directly on a farm. The consumption of farm milk was assessed in milliliters and categorized into no consumption, below the median of nonzero values (≤ 200 mL), and above that median. Maternal farm-related activities during pregnancy like working on a farm, contact with animal sheds and barns, and different farm animal species were assessed. Farming women were asked in more detail about the exposure to the number of animals, working conditions, and activities (eg, littering, cleaning or moving animals, milking, and so forth), and respiratory symptoms during work. Analyzing those characteristics, values for nonfarmers were imputed with "no." Exposure time in animal sheds and barns was assessed continuously in days per week and hours per week (farmers) or hours per month (nonfarmers) and subsequently dichotomized (yes/no). Contact with farm animal species (cow, pig, poultry, sheep/goat) was assumed if the mother reported contact at least several times per month during pregnancy. The number of different species was modeled continuously (score ranging from 0 to 4).

Assessment of cytokine production

At delivery, cord blood (CB) was collected by midwives and transported at room temperature to the local study center's laboratory. The French group did not contribute to the cytokine data at birth. Cytokine stimulation was standardized in the different study centers by extensive quality control assays and laboratory training (see this article's Method section, Quality control procedures, in the Online Repository at www.jacionline.org). After sample selection (Fig 1), of 684 CB samples (freshly stimulated and analyzed within 27 hours for cytokine production), 625 CB samples remained: Austria, 105; Switzerland, 178; Germany, 147; and Finland, 195.

Briefly, heparinized blood was diluted 1:8 with RPMI cell culture medium supplemented with 1% antibiotics/antimycotics (both Gibco, Karlsruhe, Germany) and 10% FCS (PAA, Laboratories GmbH, Cölbe, Germany). Diluted blood cells (1 mL) were incubated for 24 and 48 hours in 5% CO_2 at 37°C with various stimuli: a combination of phorbol 12-myristate 13-acetate (PMA; 5 ng/mL) and ionomycin (1 μ g/mL; both Sigma, Deisenhofen, Germany), LPS (0.1 μ g/mL; kindly provided by Profs Holst and Brade, Borstel, Germany), and staphylococcal enterotoxin B (0.1 μ g/mL; Sigma). After centrifugation at 800g for 10 minutes, supernatants were frozen at $-80^\circ C$. Concentrations

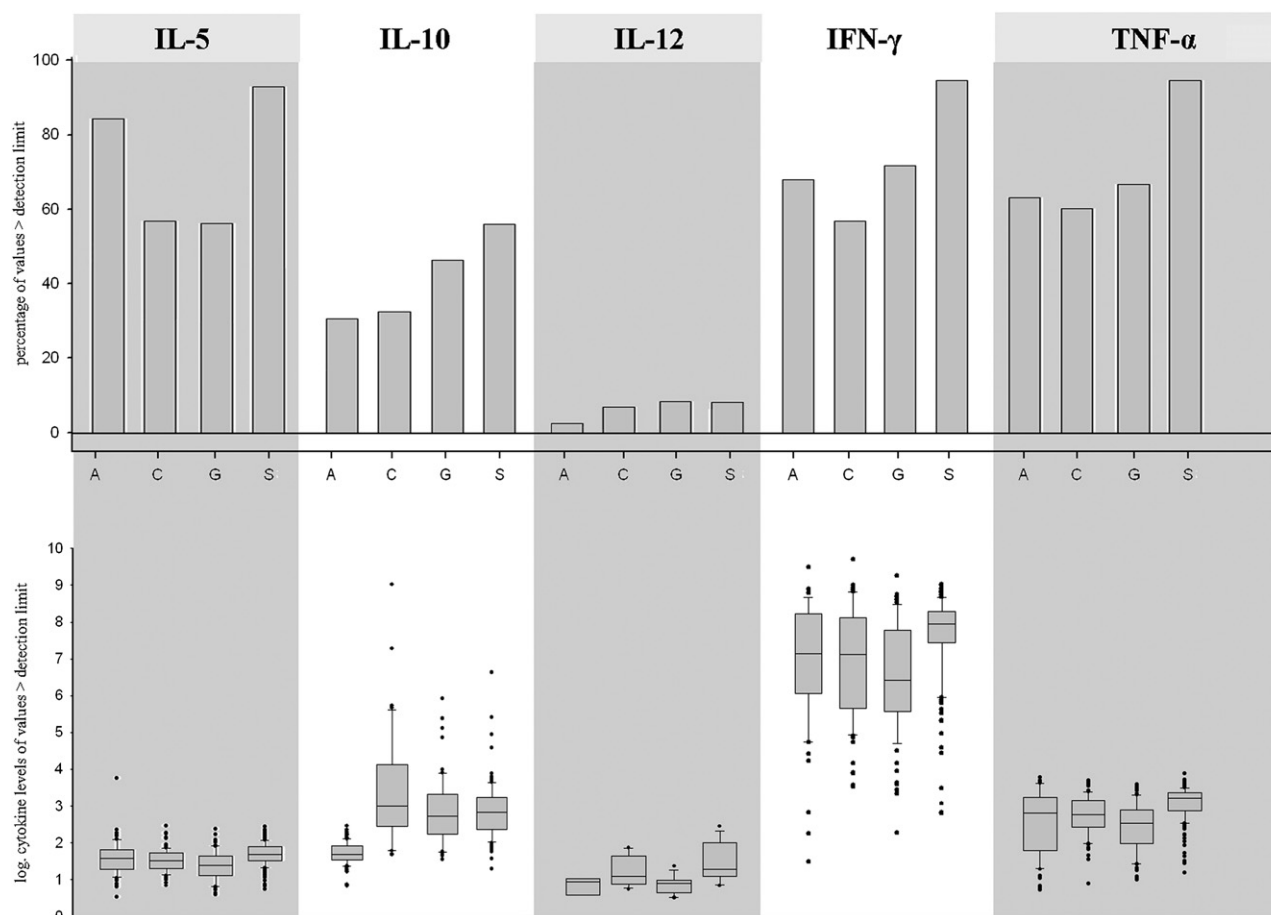


FIG 2. Prevalence and distribution of cytokines above detection limit. A, Austria; C, Switzerland; G, Germany; S, Finland.

of all cytokines were measured by using the Opteia ELISA kits (BD, Heidelberg, Germany) and 384-well ELISA plates (Nunc, Langensfeld, Germany) according to the manufacturer's instructions except a reduced reaction volume (50 μ L) and a higher concentrated first standard of 1000 pg/mL, respectively. Each sample was run at least undiluted and in a 1:20 dilution in duplicate. In addition, higher concentrated samples were run in a 1:40 dilution.

To assess T-cell effector status as well as cytokine production of antigen presenting cells the signature cytokines IFN- γ , TNF- α , IL-5, IL-10 and IL-12 were measured quantitatively in the central laboratory. Sensitivities were as follows: 10.0 pg/mL for IFN- γ , 7.7 pg/mL for TNF- α , 8.0 pg/mL for IL-5, 7.2 pg/mL for IL-12, and 11.4 pg/mL for IL-10.

The interval between blood collection and stimulation did not differ between CB samples from farming and nonfarming newborns. Intra-assay variance ranged from 1.2% to 5.2% (coefficient of variation) for the 3 different dilutions (samples per concentration, $n = 10$). Stimulation assays in whole-blood samples require strong stimuli for obtaining sufficient concentrations of cytokines in supernatants. This condition was best met with PMA/ionomycin stimulation for 48 hours. Kinetics were monitored in an interval from 12 to 72 hours to determine the time point maximum of cytokine production for each of the 5 cytokines. Except for TNF- α (maximum plateau production, 12-48 hours), all cytokines levels peaked at 48 hours, and TNF- α values taken for 24 hours and 48 hours correlated strongly ($r = 0.79$). Among the 4 stimuli, PMA/ionomycin yielded in the highest number of detectable cytokine levels. Therefore, PMA/ionomycin stimulation from 48 hours was chosen for further analysis.

Measurements in dust samples

When children were 2 months old, dust samples were collected in 1092 families by fieldworkers from maternal mattresses and the living room

floors—that is, wall-to-wall carpets (only in Switzerland and Germany) or smooth floors with or without additional rugs according to Schram et al.⁸ Endotoxin was measured with the Limulus Amebocyte Lysate test by using the same batch of reagents and standards for all analyses according to the manufacturer's instructions (Bio Whittaker, Walkersville, Md). Extracellular polysaccharides (EPSs) were analyzed centrally with a sandwich immunoassay as described by Douwes et al.⁹ Concentrations were expressed as units per square meter of sampling surface. In combination with cytokine data, 600 samples from maternal mattresses, 68 samples from wall-to-wall carpets, 544 smooth floor samples, and 338 rug samples remained for analyses.

Statistical analysis

Prevalences of exposures and confounders were compared among farmers and nonfarmers by using the χ^2 or Mann-Whitney U test. Levels of cytokines (produced after PMA/ionomycin stimulation) were standardized on the number of leukocytes and log-transformed to reach satisfactory approximation to normal distribution for detected values. IL-12 was only included in descriptive statistical analyses because of the high number of values below the detection limit. Cytokine levels were categorized (below detection limit, below the center-specific median of detected levels, and above that median; for center-specific medians, see Fig 2). Furthermore, cytokine levels were modeled continuously in multivariable Tobit regression models to account for the left censoring caused by values below the detection limit (Procedure QLIM in SAS Institutes, Cary, NC).

To test for additive or synergistic effects of endotoxin and EPS exposure levels, measured values were normalized by transforming into z scores. These scores were summed for each of the biomarker sources (mattress, floor, rug, carpet) and finally included in the models as new exposure variables.

TABLE I. Differences between newborns from farm and nonfarm families in cytokine categories of CB samples

	Nonfarm families		Farm families		Trend test†
	No. of		No. of		
	samples	Percentage	samples	Percentage	P
IFN-γ					
Below detection limit	87	28.7	67	24.4	
≤ Median‡	123	40.6	90	32.7	
> Median‡	93	30.7	118	42.9	.012**
TNF-α					
Below detection limit	88	31.0	59	22.6	
≤ Median‡	99	34.9	102	39.1	
> Median‡	97	34.1	100	38.3	.064*
IL-5					
Below detection limit	90	28.5	75	26.0	
≤ Median‡	112	35.4	110	38.1	
> Median‡	114	36.1	104	36.0	.70
IL-10					
Below detection limit	163	54.9	157	59.0	
≤ Median‡	68	22.9	54	20.7	
> Median‡	66	22.2	55	20.3	.38
IL-12					
Below detection limit	283	93.4	253	92.3	
≤ Median‡	11	3.6	10	3.7	
> Median‡	9	3.0	11	4.0	.53

*Significant differences between farm and nonfarm newborns: * $P < .1$; ** $P < .05$.

† P value of Cochran-Armitage trend test.

‡Center-specific medians were used for categorization.

Geometric mean ratios (GMRs) with 95% CIs were calculated by exponentiation of the regression coefficients and their 95% CIs. Exploratory tests were considered to be significant at a P value of .05 and borderline significant at .1.

All models were adjusted for center and farming (yes/no) to account for center effects and the sampling design. After testing various covariates individually, a combination of variables was included in the models if joint confounding—that is, a change in GMR of more than 10%—occurred. The block was made up of sex, maternal smoking, parental education, number of siblings, maternal age, maternal asthma, and hay fever. All available exposure variables regarding farming, and microbial or dairy products were screened for potential effects on cytokine levels. At least borderline significant results were then included in a backward selection model with a selection level of $P = .10$.

The basic farm effect model and each further model were tested for heterogeneity between study centers. Heterogeneity was assumed if the Cochran Q-test resulted in a P value $< .05$ and suspected for P values $< .15$. In addition, all models with assumed or suspected center heterogeneity were stratified for center.

To exclude collinearity of the farmer variable with other farm-related variables in the respective multivariable models, variance inflation factors and Eigenvalues were checked.

All statistical computations were performed by SAS 9.2.

RESULTS

A total of 1133 pregnant mothers were enrolled, and 47% of them were farm women. The analyzed population was made up of 625 newborns with valid cytokine measurements (Fig 1). Samples were analyzed for 2 relevant parameters of neonatal cytokine expression: detectability and cytokine production level. As illustrated by the upper panel of Fig 2, the proportion of values

above the detection limit differed among the 5 CB cytokines from 73.4% for TNF- α to 7.8% for IL-12 and varied across centers by 35 percentage points at most. For all cytokines, the medians of the detected levels differed significantly between the centers (Fig 2, lower panel).

In qualitative analysis, TNF- α was more often detectable in CB from farm newborns than in nonfarm children (odds ratio, 1.52; 95% CI, 1.01-2.28). However, this farm effect was heterogeneous between countries (see this article's Table E1 in the Online Repository at www.jacionline.org).

The quantitative analysis revealed that higher levels of IFN- γ were produced among farm newborns than nonfarm children, whereas for levels of TNF- α production, a nonsignificant trend ($P = .064$) was observed (Table I).

There were differences between farm and nonfarm families (Table II), and some of those characteristics showed an association with the CB cytokine production. For TNF- α production, a sex effect was observed, with higher levels for boys (data not shown). Higher CB cytokine levels of IL-10 and IFN- γ production were found for newborns with older siblings. Production of IL-5 in CB showed a highly significant inverse association with maternal smoking during pregnancy and a positive association with a maternal history of hay fever. However, adjusting for these demographic variables did not explain the observed farm effect on IFN- γ and TNF- α .

To elucidate further this farm effect, several farm-related activities of the mother were tested in bivariate models controlling for center and farming: contact of the pregnant mother to number of farm animal species, working on a farm, and stay in a barn showed positive associations with CB IFN- γ and TNF- α production (see this article's Table E2 in the Online Repository at www.jacionline.org). In addition, stay in animal sheds was associated with TNF- α . After inclusion of these farm variables in the models, the described differences between farm and nonfarm newborns disappeared in favor of the respective exposures. The original heterogeneity between countries regarding the farm effect was not seen for the farm-related activities. After backward selection in multivariate models, the same pattern of associations for farm-related activities emerged (Fig 3). However, the effect size for cytokine production diminished slightly, and working on a farm and contact with animal sheds no longer reached statistical significance.

Consistent and strong associations were observed between the farm-related activities (working on a farm, staying in a barn, and contact with farm animal species) and the levels of endotoxin and EPSs in indoor dust samples (data not shown). However, associations between the levels of microbial compounds and cytokine levels were much weaker and less consistent (Table III). Higher IFN- γ production was observed with higher endotoxin load in wall-to-wall carpet samples. A higher TNF- α production was related to EPS in indoor floor and rug samples, to endotoxin in rug samples, and to the maximum endotoxin load of the home. All significant associations changed marginally after additional adjustment for farm-related activities (data not shown).

Combining endotoxin with EPS to one exposure variable revealed solely a significant association for TNF- α versus exposure on rugs: GMR, 1.13 (95% CI 1.02, 1.26); after adjustment for farm-related activities: GMR, 1.11 (95% CI 1.00, 1.24).

Another source of the observed farm effect was found for the consumption of farm-produced dairy products (Table E2). Consumption of farm milk was associated with higher levels of

TABLE II. Sociodemographic and farming characteristics of study participants

	Nonfarm families (N = 326, 52.2%)	Farm families (N = 299, 47.8%)	P value
Sex of newborn child (boy vs girl)	170 (53.0)	146 (49.3)	.37
Gestational age of newborn (wk), median (min-max)	40 (35-43)	40 (36-43)	.96*
Birth weight of newborn (g), median (min-max)	3445 (1990-4850)	3550 (1900-5100)	.0002*
Maternal age (y), median (min-max)	31.0 (19-44)	31.2 (21-48)	.074*
Maternal BMI (kg/m ²), median (min-max)	22.5 (16-37)	23.6 (18-44)	<.0001*
Maternal smoking during pregnancy (yes/no)	60 (18.4)	18 (6.0)	<.0001
Lower parental education level (yes/no)	146 (44.7)	175 (58.5)	.0006
First child of mother (yes/no)	153 (46.9)	70 (23.4)	<.0001
Maternal history of atopic diseases (yes/no)	162 (49.6)	124 (41.4)	.039
Paternal history of atopic diseases (yes/no)	155 (47.5)	101 (33.7)	.0002
Farm milk consumption during pregnancy			
As much as 200 mL per day	32 (9.9)	109 (36.6)	
More than 200 mL per day	12 (3.7)	114 (38.3)	<.0001
if yes, unboiled milk consumption (yes/no)	33 (80.5)	184 (83.6)	.62
if yes, unskimmed milk consumption (yes/no)	34 (82.9)	183 (82.8)	.98
Consumption of dairy products during pregnancy at least once a week			
Butter made of farm milk	5 (1.5)	40 (13.4)	<.0001
Yogurt made of farm milk	8 (2.5)	37 (12.4)	<.0001
Cheese made of farm milk	18 (5.5)	55 (18.5)	<.0001
Shop butter	210 (64.4)	205 (68.6)	.27
Shop yogurt	279 (85.6)	253 (84.6)	.73
Active working on a farm during pregnancy (yes/no)	28 (13.3)	281 (87.5)	<.0001
Average time spent in animal shed during pregnancy (h/wk), median (min-max)	0 (0-19.4)	11.3 (0-60.7)	<.0001*
Average time spent in barn during pregnancy (h/wk), median (min-max)	0 (0-19.4)	1.2 (0-42)	<.0001*
Contact to any farm animal species during pregnancy (at least several times per month)			
Contact with cow	64 (19.6)	266 (88.9)	<.0001
Contact with pig	14 (4.2)	63 (21.0)	<.0001
Contact with poultry	42 (12.8)	95 (31.7)	<.0001
Contact with sheep/goat	26 (7.9)	63 (21.0)	<.0001

Statistics given are N and % unless otherwise noted.

BMI, Body mass index; min-max, minimum-maximum.

*Wilcoxon-tests; otherwise, χ^2 tests.

IFN- γ in models controlled for country and farming. Consumption of unskimmed compared with skimmed farm milk resulted in significantly higher levels of IFN- γ and TNF- α . The consumption of unboiled compared with boiled farm milk failed to reach statistical significance.

Highly significant associations (mutually adjusted) with CB cytokines were detected for the consumption of farm yogurt and farm butter (each $n = 45$), but in opposite directions (Fig 3). Furthermore, an additive effect was observed for the combined consumption of butter and unskimmed milk on IFN- γ and TNF- α (adjusted GMRs, 5.72 [95% CI 2.83, 11.56] and 2.75 [95% CI 1.42, 5.35]), respectively.

The GMRs indicated that positive associations for farm butter were stronger for TNF- α and IFN- γ , whereas inverse associations for farm yogurt were stronger for IL-5 (farm butter vs IFN- γ /IL-5: GMR, 3.17 [95% CI 2.08, 4.82]; farm butter vs TNF- α /IL-5: GMR, 1.58 [95% CI 1.07, 2.33]; farm yogurt vs IL-5/IFN- γ : GMR, 0.79 [95% CI 0.65, 0.96], farm yogurt vs IL-5/TNF- α : GMR, 0.82 [95% CI 0.65, 1.02]). The overlap of mothers consuming both farm butter and farm yogurt during pregnancy was 40%, indicating that the aforementioned effects on cytokines arose from different subgroups.

We also explored a potential farm effect regarding IL-5 and IL-10; however, the production of these CB cytokines did not differ between farm and nonfarm newborns (data not shown).

DISCUSSION

The cross-sectional PARSIFAL study suggested that maternal farm exposures such as contact with farm animals during pregnancy may have a protective effect on the development of allergies in the offspring later in life.³ However, these associations are vulnerable to misclassification because of the retrospective study design. In turn, the PASTURE study was designed as a prospective birth cohort enrolling mothers prenatally and assessing their exposure before the birth of the index child. The associations between elevated IFN- γ and TNF- α production in CB and maternal farm exposures during pregnancy support the previous PARSIFAL findings and extend the observed associations toward potentially underlying mechanisms.

The PASTURE birth cohort is a multicenter collaboration across 5 countries. Extended quality control assays verified that the study protocol by which frozen cell culture supernatants were transported to and analyzed in a central laboratory provided reproducible and comparable cytokine measures in samples from the different study centers.¹⁰ Measurement of CB cytokine production after *in vitro* stimulation ensured that contamination by maternal cytokines did not affect the detected associations.¹¹ Although stimulation assays performed from whole blood samples do not allow an allocation of the measured cytokines to specific cell populations, this method provides a valid overview of cytokine pattern at birth. The polyclonal stimulus PMA/ionomycin

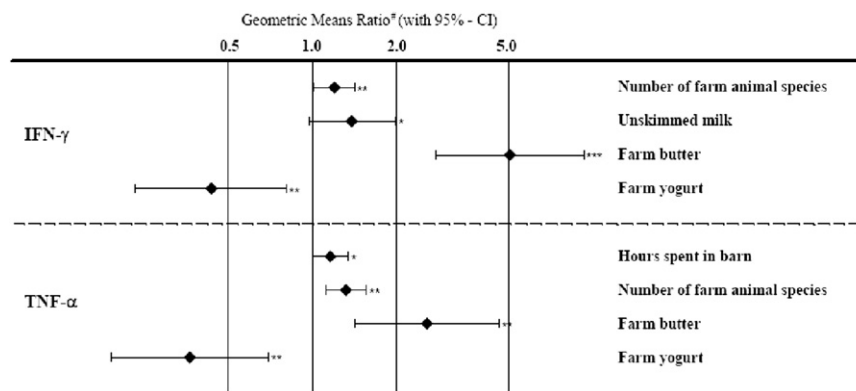


FIG 3. Effect of farming exposure and consumption of dairy products on cytokine levels. Number of farm species—per animal species. Unskimmed milk—yes vs no. Farm butter—consumption of butter made from farm milk (at least once a week). Farm yogurt—consumption of yogurt made from farm milk (at least once a week). Hours spent in barn—per 5 hours.

#Geometric means ratios (GMRs) were calculated by EXP(β 1) in Tobit regression models adjusting for center and farming status. The relevant farm exposure variables were then identified by backward selection in the mutually adjusted Tobit regression models and are shown with 95% CIs.

* $P \leq .1$, ** $P \leq .05$, *** $P \leq .001$.

seems to be the most appropriate and reliable agent to monitor the production of a wide spectrum of cytokines representing the balance between T_H1 and T_H2 immune responses.^{10,12}

Proinflammatory CB cytokines may be influenced by the mode of delivery. Vaginal delivery was found to be associated with higher levels of proinflammatory CB cytokines compared with delivery by cesarean section.^{11,13-15} In the PASTURE population, however, the mode of delivery had no significant effect on cytokine levels, thereby excluding confounding by mode of birth (data not shown).

Children born to mothers living on a farm showed significantly higher levels of CB IFN- γ after stimulation with PMA/ionomycin than children born to mothers from the same rural areas but not living on a farm. This farm effect could be attributed to maternal contact with different farm animal species and the consumption of unskimmed farm milk and farm-produced butter.

A number of previous studies had reported that decreased levels of IFN- γ at birth predicted the onset of allergies later in life, and likewise, maternal atopy has been shown to decrease IFN- γ levels at birth.¹⁶⁻¹⁸ Otherwise, detectable levels of IFN- γ at birth were observed to be associated with a reduced risk for atopy and asthma later in life.¹⁹ Therefore, the increased CB IFN- γ levels seen in the farm environment are suggestive of mechanisms contributing to allergy protection later in life. The further follow-up of the PASTURE cohort will clear up this assumption.

Besides IFN- γ production, TNF- α levels were also increased in CB from children born to farming mothers. TNF- α is mainly produced by cells of the innate immune system as a response to the recognition of microbial components. The strong and numerous associations between elevated TNF- α production in CB and farming environmental exposures during pregnancy might indicate that this cytokine plays an important and genuine role at the frontline of interaction between the fetus and maternal exposures. It has been shown that both placental and fetal tissues and cells are capable of producing TNF- α during all trimesters of pregnancy.²⁰ This Janus-faced cytokine was identified as playing a pivotal role as a mediator of apoptosis in fetal maldevelopment caused by detrimental stimuli as well as a protector of the normal

embryogenesis in cell populations residing at the fetomaternal interface. A strong stimulation of TNF- α -producing cells in the placenta caused by bacterial infection of the fetoplacental compartment results in preterm labor and miscarriage. Low production of TNF- α induces regulatory processes in growth and differentiation of the progeny.²¹⁻²⁴ Between these poles of TNF- α production, microbial stimuli from noninfectious or low-dose maternal exposures might prime CB TNF- α production without leading to inflammation in the fetoplacental compartments.

Allergy-protective effects of early endotoxin exposure have been reported from epidemiologic studies and confirmed in experimental settings by using mouse models of experimental asthma.²⁵⁻²⁷ Furthermore, there is increasing evidence that fungal agents may also provide protection against allergic outcomes²⁸ and modulate the capacity of human PBMCs to produce cytokines.²⁹ Here we found only weak associations between maternal domestic endotoxin and EPS exposure and elevated production of proinflammatory CB cytokines. These findings might indicate that direct contact with farm animals and barn exposure seems to be more effective than maternal exposures to house dust, because domestic endotoxin and EPS exposure may not reflect the whole and complex spectrum of the microbial environment on a traditional farm.

Previous farm studies have indicated that the allergy-protective farm effect may be mediated in part by the consumption of farm-produced dairy products.³⁰⁻³² The data presented herein confirm these observations. Cow's milk produced on the farms does not undergo pasteurization and homogenization, but skimming and/or boiling is common. In our hands, cytokine production in CB was mostly affected by consumption of unskimmed milk, suggesting that the fat content may contribute to immune activation. This notion is also supported by the fact that the consumption of farm-produced butter by the pregnant mother increased levels of CB IFN- γ and TNF- α after stimulation with PMA/ionomycin. These findings implicate a critical role for farm milk processing. In contrast with homogenized shop milk, unskimmed farm milk and butter contain the natural amount of milk fat and polyunsaturated fatty acids, and maternal consumption of these farm

TABLE III. Effect of microbial exposure in dust samples (per ln[IU/m²]) on cytokines

Dust extractions	IFN- γ			TNF- α			IL-5			IL-10		
	GMR† (95% CI)	<i>P</i>		GMR† (95% CI)	<i>P</i>		GMR† (95% CI)	<i>P</i>		GMR† (95% CI)	<i>P</i>	
In samples of maternal mattress												
Endotoxin (per ln[IU/m ²])	1.03 (0.91-1.16)	.65		1.08 (0.96-1.21)	.21		1.02 (0.96-1.09)	.47		0.97 (0.88-1.08)	.62	
EPS (per ln[IU/m ²])	1.06 (0.95-1.18)	.28		1.01 (0.90-1.12)	.91		1.01 (0.96-1.07)	.74		0.99 (0.90-1.08)	.76	
In wall-to-wall carpet samples (only in Switzerland, Germany)												
Endotoxin (per ln[IU/m ²])	1.61 (1.06-2.44)	.024		1.36 (0.88-2.09)	.16		1.05 (0.84-1.30)	.69		0.89 (0.61-1.31)	.55	
EPS (per ln[IU/m ²])	1.23 (0.69-2.2)	.49		0.88 (0.48-1.59)	.66		1.01 (0.74-1.39)	.93		1.61 (0.90-2.9)	.11	
In smooth floor samples												
Endotoxin (per ln[IU/m ²])	1.01 (0.90-1.12)	.92		1.01 ** (0.92-1.12)	.78		0.98 (0.93-1.03)	.45		0.99 (0.90-1.08)	.77	
EPS (per ln[IU/m ²])	1.03 (0.93-1.14)	.57		1.10 (1.00-1.21)	.040		0.98 (0.93-1.03)	.48		0.97 (0.89-1.07)	.54	
In rug samples												
Endotoxin (per ln[IU/m ²])	1.07 (0.94-1.22)	.32		1.14 (1.01-1.28)	.027		1.01 (0.95-1.08)	.68		1.05 (0.93-1.18)	.45	
EPS (per ln[IU/m ²])	1.09 (0.93-1.29)	.28		<i>1.15 * (0.99-1.34)</i>	<i>.067</i>		1.02 * (0.94-1.10)	.66		1.02 (0.87-1.19)	.83	
Maximum load in all samples‡												
Endotoxin (per ln[IU/m ²])	1.07 (0.95-1.19)	.27		<i>1.10 (0.99-1.22)</i>	<i>.072</i>		1.03 (0.97-1.08)	.37		1.02 (0.93-1.13)	.65	
EPS (per ln[IU/m ²])	1.07 (0.95-1.21)	.26		1.08 (0.96-1.22)	.20		0.98 (0.92-1.05)	.60		0.95 (0.86-1.06)	.36	

Significant results are shown as follows: Boldface, $P < .05$, Italic, $P < .1$. p_Q = P -value of Cochran's Q-test.*Suspicion of heterogeneity with $P_Q < .15$.**Assumption of heterogeneity with $P_Q < .05$; center-specific results given in Table E1.†GMR calculated by EXP(B₁) in Tobit regression models. CI adjusted at least for center and farming status and in addition—when necessary—for sex, maternal smoking during pregnancy, number of older siblings, maternal age at birth, maternal history of asthma, and hay fever.

‡In addition, adjusted for sample type.

products was associated with significantly increased IFN- γ and TNF- α in CB. Frequent dietary uptake of products containing high amounts of milk fat were shown to modulate cytokine expression and might reduce the risk of allergies and asthma in children.^{33,34} In our cohort, the traditional dairy processes to produce butter and yogurt are restricted to the Alpine region. Milk and butter from these regions are characterized by a wide spectrum and a specific pattern of polyunsaturated fatty acids, especially conjugated linoleic acids,³⁵ which are capable of modulating cytokines such as TNF- α ³⁶ and may attenuate the development of allergic airway reactions.^{37,38}

In turn, fermented farm milk products consumed by the pregnant mother may have an immunosuppressive effect on CB cytokine production. Natural unpasteurized yogurt is rich in a wide spectrum of bioactive lactic acid bacteria. Probiotic strains derived from fermented milk products were shown to possess allergy-protective and immunomodulating properties in human and animal studies.³⁹⁻⁴¹ Dependent on the mixture of probiotic strains, fermented dairy products may obtain suppressive properties on proallergic immune responses or effects to bias the immune profile in the T_H1 direction.⁴²

Interestingly, only a weak and nonsignificant association between unboiled milk and cytokine production was found, suggesting a smaller role for microbial components in the immune activation of the neonate. This notion is supported by previous findings from the PASTURE study showing that milk samples collected from farm families contained significantly lower amounts of endotoxin than samples drawn from shop-bought milk.⁴³ However, we could not exclude combined effects of leaving the milk unskimmed and unboiled because of small sample sizes of the respective exposure categories.

No effect of maternal farm exposures on IL-10 after stimulation with PMA/ionomycin was seen. This finding is consistent with data from a study investigating the role of regulatory T cells in CB

of newborns of farming and nonfarming mothers. In these highly characterized samples, no difference between CB IL-10 production in farmer and nonfarmer children could be detected after stimulation with PMA/ionomycin.⁴⁴ Further analysis of forkhead box protein 3 mRNA expression as a specific marker of regulatory T-cell activity might give a more detailed insight into the role of regulatory T cells in this context.

Statistical analyses in epidemiologic studies testing a number of environmental exposures on multiple outcome variables can generate hypotheses but cannot prove with any certainty effects of environmental exposures on the early development of the immune system. These findings should therefore be replicated in further studies.

Maternal farming lifestyle in close contact with livestock and self-produced dairy products seems to induce mechanisms in the progeny that result in a T_H1-skewed cytokine pattern at birth. This new finding might represent the initial step for allergy protection later in life. The follow-up of the PASTURE cohort will allow the verification of this notion once children attain school age, when clinical allergic outcomes will have become apparent.

This work is dedicated to Prof Stephan K. M. Weiland, who died on March 19, 2007.

Clinical implications: The effect of maternal environmental exposure on CB immune responses suggests a prenatal onset of immune maturation.

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METHODS

Quality control procedures

In the context of epidemiologic studies, immunologic investigations using human samples collected in the field entail various challenges concerning quality control. In our study, samples had to be transported from the sampling site to the respective study center's laboratory ("country laboratory"). For samples collected in Germany, samples were transported to Munich, in Austria to Salzburg, in Finland to Kuopio, in France to Besançon, and in Switzerland to Zurich. In these country laboratories, blood serum was obtained by centrifugation and frozen in aliquots, whereas blood tubes were dedicated for later RNA and DNA and environmental exposure measurements. These tubes were stored in the different country laboratories at -20°C and then shipped to specialized laboratories for subsequent analyses (unpublished data, PASTURE Study Group, November 2003). The stimulation experiments described in the current article were performed on arrival of the blood samples in the different country laboratories. Supernatants of the cell cultures were then harvested, frozen at -20°C , and shipped on dry ice to Marburg (central laboratory study center), where cytokine concentrations were measured. To assure consistent handling of the samples and adherence to the standard operating procedures (SOPs) in the different laboratories and countries during the prolonged period in which the children participating in this study were born, a strict quality control program was established. It consisted of the following elements:

SOPs. For all experimental procedures, SOPs were worked out in face-to-face meetings of all heads of the laboratories involved before blood sampling was initiated.

Standardization of materials used. All the laboratories used the same plastic wares from the same manufacturers, although the wares were ordered in their individual countries. The stimuli used for the cell culture experiments were bought by the Marburg laboratory, which prepared stock solutions shipped to the different countries. To ensure that the activity of the stimuli was unchanged during the period the study was carried out, at the end of the study, an aliquot of the stimuli was sent by each country to a central laboratory, and the activity of the stimuli sent in by the different laboratories was tested in a stimulation assay.

Centralized training. To ensure that the procedures described in the SOPs were consistently applied in all laboratories, 2 central training meetings for the laboratory workers in the different countries contributing to the initial workup of the samples were realized. During these meetings, laboratory workers carried out the experimental procedures according to the SOPs.

Quality control experiments to ensure consistency between the different laboratories. All laboratories participated at least 2 times during the study period in an interlaboratory comparison in which aliquots of blood samples from healthy volunteers were stimulated according to the SOPs in parallel in the different country laboratories, and the supernatants were subsequently analyzed centrally in 1 laboratory.

TABLE E1. Center-specific associations for models with assumed ($P_Q < .05$) or suspect ($P_Q < .15$) heterogeneity

Cytokine	Exposure	Center	Center-specific GMR† with 95% CI	Cochran P_Q	I^2 (%)
IFN- γ	Farming status	A	0.89 (0.37-2.17)	.12	49
		C	2.62 (1.14-6.00)		
		G	1.10 (0.57-2.13)		
		S	0.90 (0.67-1.20)		
TNF- α	Farming status	A	1.62 (0.67-3.92)	.049	62
		C	2.57 (1.18-5.60)		
		G	1.29 (0.68-2.44)		
		S	0.89 (0.68-1.16)		
IL-10	Stay in barn	A	1.85 (1.01-3.40)	.10	53
		C	0.75 (0.51-1.10)		
		G	0.94 (0.68-1.29)		
		S	1.12 (0.64-1.96)		
TNF- α	Monitoring farm animals*	A	3.86 (0.96-15.56)	.12	49
		C	2.38 (0.74-7.67)		
		G	0.70 (0.25-1.9)		
		S	0.96 (0.59-1.55)		
IL-5	Consumption of butter made of farm milk	A	0.67 (0.33-1.36)	.13	51
		C	1.74 (0.96-3.15)		
		G	1.20 (0.49-2.95)		
IFN- γ	Consumption of shop butter	A	1.47 (0.35-6.29)	.077	56
		C	0.36 (0.15-0.86)		
		G	0.67 (0.28-1.59)		
		S	1.12 (0.82-1.53)		
TNF- α	Endotoxin in smooth floor sample	A	1.33 (1.00-1.77)	.030	67
		C	0.67 (0.45-0.99)		
		G	1.08 (0.86-1.36)		
		S	0.96 (0.87-1.05)		
IL-5	EPS in rug sample	A	1.05 (0.84-1.31)	.063	59
		C	1.59 (1.08-2.36)		
		G	0.85 (0.66-1.09)		
		S	0.99 (0.91-1.07)		
TNF- α	EPS in rug sample	A	1.57 (0.79-3.10)	.075	57
		C	3.23 (1.10-9.49)		
		G	1.14 (0.78-1.67)		
		S	0.96 (0.84-1.11)		

p_Q = P -value of Cochran's Q-test.

A, Austria; C, Switzerland; G, Germany, I^2 , variance component between study centers; S, Finland.

*Values for nonfarm women were imputed with "no."

†GMR calculated by $\text{EXP}(\beta_1)$ in Tobit regression models, adjusted for farming status.

TABLE E2. Effect of farm-related activities and consumption of dairy products on cytokines

	IFN- γ		TNF- α		IL-5		IL-10	
	GMR* (95% CI)	P	GMR* (95% CI)	P	GMR* (95% CI)	P	GMR* (95% CI)	P
Farm-related activities								
Active working on a farm (yes/no)	1.59 (0.99-2.54)	.055	1.74 (1.14-2.65)	.010	1.14 (0.90-1.44)	.30	0.73 (0.49-1.07)	.11
Stay spent in animal shed (yes/no)	1.28 (0.83-1.98)	.26	1.41 (0.94-2.12)	.096	1.20 (0.97-1.48)	.096	0.99 (0.69-1.43)	.96
Hours spent in barn (per 5 h)	1.18 (1.00-1.41)	.056	1.19 (1.03-1.38)	.022	1.03 (0.95-1.12)	.52	0.91§ (0.76-1.07)	.25
Contact with farm animal species (score, 0-4)	1.28 (1.08-1.52)	.0052	1.29 (1.10-1.51)	.0021	0.97 (0.89-1.07)	.58	0.95 (0.81-1.1)	.48
Cleaning animals† (yes/no)	1.09 (0.7-1.7)	.71	1.05 (0.69-1.59)	.82	1.12 (0.9-1.41)	.30	0.64 (0.44-0.95)	.026
Monitoring animals† (yes/no)	1.40 (0.88-2.23)	.16	1.19§ (0.77-1.85)	.44	1.23 (0.97-1.56)	.083	1.09 (0.72-1.64)	.68
Littering† (yes/no)	1.20 (0.7-2.04)	.50	1.01 (0.62-1.64)	.97	1.36 (1.05-1.78)	.022	0.77 (0.48-1.23)	.28
Consumption of farm-produced dairy products								
Farm milk (none, $\leq$ 200 mL, >200 mL)	1.33 (1.04-1.71)	.024	1.11 (0.88-1.40)	.37	1.08 (0.95-1.23)	.22	0.97 (0.78-1.19)	.75
Unskimmed farm milk (vs skimmed milk)‡	2.13 (1.06-4.26)	.034	2.49 (1.31-4.72)	.0053	1.06 (0.76-1.47)	.75	1.34 (0.72-2.49)	.36
Unboiled farm milk (vs boiled milk)‡	1.77 (0.88-3.53)	.11	1.68 (0.89-3.18)	.11	1.34 (0.96-1.88)	.086	1.08 (0.58-2.01)	.82
Butter made of farm milk (at least once a week)	4.74 (2.63-8.56)	<.0001	2.43 (1.39-4.23)	.0017	1.28§ (0.94-1.74)	.12	0.90 (0.52-1.56)	.71
Yogurt made of farm milk (at least once a week)	0.72 (0.39-1.35)	.31	0.62 (0.34-1.13)	.12	0.55 (0.39-0.76)	.0003	0.53 (0.30-0.96)	.037
Cheese made of farm milk (at least once a week)	1.18 (0.70-1.98)	.53	1.34 (0.83-2.16)	.23	0.72 (0.55-0.93)	.013	1.08 (0.69-1.70)	.72
Consumption of dairy products bought in a shop								
Shop butter (at least once a week)	0.70§ (0.49-1.00)	.047	0.84 (0.61-1.18)	.32	0.93 (0.78-1.11)	.44	1.18 (0.87-1.59)	.28
Shop yogurt (at least once a week)	0.99 (0.65-1.53)	.98	1.02 (0.69-1.52)	.91	1.05 (0.85-1.30)	.65	1.53 (1.04-2.23)	.030

Significant results are shown as follows: Boldface, $P < .05$, Italic, $P < .1$.*GMR calculated by EXP(β_1) in Tobit regression models adjusted at least for center and farming status and in addition—when necessary—for sex, maternal smoking during pregnancy, number of older siblings, maternal age at birth, maternal history of asthma, and hay fever.

†Asked only of farm women. Nonfarm women were imputed with “no.”

‡In $n = 267$ with milk consumption.§Suspicion of heterogeneity with $P_Q < .15$; center-specific results given in Table E1.

||No consumption of butter made of farm milk in Finland.