Increased regulatory T-cell numbers are associated with farm milk exposure and lower atopic sensitization and asthma in childhood

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Background: European cross-sectional studies have suggested that prenatal and postnatal farm exposure decreases the risk of allergic diseases in childhood. Underlying immunologic mechanisms are still not understood but might be modulated by immune-regulatory cells early in life, such as regulatory T (Treg) cells.

Objective: We sought to assess whether Treg cells from 4.5-year-old children from the Protection against Allergy: Study in Rural Environments birth cohort study are critical in the atopy and asthma-protective effect of farm exposure and which specific exposures might be relevant.

Methods: From 1133 children, 298 children were included in this study (149 farm and 149 reference children). Detailed questionnaires until 4 years of age assessed farming exposures over time. Treg cells were characterized as upper 20% $CD4^+CD25^+$ forkhead box protein 3 $(FOXP3)^+$ (intracellular) in PBMCs before and after stimulation (with phorbol 12-myristate 13-acetate/ionomycin or LPS), and *FOXP3* demethylation was assessed. Atopic sensitization was defined by specific IgE measurements; asthma was defined by a doctor's diagnosis.

Results: Treg cells were significantly increased in farm-exposed children after phorbol 12-myristate 13-acetate/ionomycin and LPS stimulation. Exposure to farm milk was defined as a relevant independent farm-related exposure supported by higher *FOXP3* demethylation. Treg cell (upper 20% $CD4^+CD25^+$, $FOXP3^+$ T cells) numbers were significantly negatively associated with doctor-diagnosed asthma (LPS stimulated: adjusted odds ratio, 0.26; 95% CI, 0.08-0.88) and perennial IgE (unstimulated: adjusted odds ratio, 0.21; 95% CI, 0.08-0.59). Protection against asthma by farm milk exposure was partially mediated by Treg cells.

Conclusions: Farm milk exposure was associated with increased Treg cell numbers on stimulation in 4.5-year-old children and might induce a regulatory phenotype early in life, potentially contributing to a protective effect for the development of childhood allergic diseases. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

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An early-life farming lifestyle confers protection against asthma, hay fever, and allergic sensitization in childhood.[1,2] Importantly, this allergy-protective farm effect has been reproduced by several international cross-sectional studies.[3] The Allergy and Endotoxin Study (ALEX) and the Prevention of Allergy—Risk Factors for Sensitization In Children Related to Farming and Anthroposophic Lifestyle (PARSIFAL) studies demonstrated that farm exposure protected against allergic diseases, atopic sensitization, and asthma.[2,4] In addition to microbial exposure,[1,5] farm milk consumption[2,6,7] was identified as a relevant specific exposure for the farm effect.

Early-life exposure to farming is relevant for allergy protection, suggesting effects during immune maturation, potentially through specific regulatory mechanisms.[3] Different birth cohort and cross-sectional studies showed that (1) innate immune receptors were involved in the farm effect, such as Toll-like receptor 2 (*TLR2)/CD14* expression, levels of which were increased in farm-raised children at school age[8]; (2) *TLR2/TLR4/CD14* expression in school-aged children was increased by prenatal rather than current exposure to a stable[9]; and (3) prenatal animal contact and gene expression of innate receptors at birth were both inversely associated with atopic dermatitis.[10]

In this context the birth cohort PAULCHEN demonstrated increased and functionally more efficient regulatory T (Treg) cells already in cord blood of neonates exposed to a farm environment *in utero*.[11] This T-cell population is involved in homeostasis of immune regulation and T-cell polarization, potentially affecting early-life T-cell maturation. However, it is unknown whether these effects are still relevant in later childhood and which effects Treg cells might have during subsequent immune maturation, particularly when the first symptoms of atopic diseases start to develop.

In this study we aimed to assess the underlying immunologic mechanisms of exposure in rural environments on immune maturation contributing to this natural model of allergy protection in the first 4.5 years of life. On the basis of our previous findings[11] and the knowledge that T cells and particularly Treg cells are promising candidates for immune modulation toward an allergy-protective profile, we examined activated T cells and Treg cells in PBMCs after different stimulations (phorbol 12-myristate 13-acetate/ionomycin [PI]/LPS–stimulated PBMCs compared with unstimulated PBMCs) and forkhead box protein 3 *(FOXP3)* demethylation at age 4.5 years in a subgroup of farm-exposed versus non–farm-exposed children from the Protection Against Allergy: Study in Rural Environments (PASTURE)/Mechanisms of Early Protective Exposures on Allergy Development (EFRAIM) study.[12] We further assessed which specific farm exposures might be relevant for Treg cell regulation. Finally, we investigated whether Treg cells actually mediate the allergy-protective effect of farm exposure, leading to a lower prevalence of doctor-diagnosed asthma.

METHODS

For more information on methods, see the Methods section in this article's Online Repository at www.jacionline.org.

Abbreviations used

ALEX:	Allergy and Endotoxin Study
EFRAIM:	Mechanisms of Early Protective Exposures on Allergy Development
FITC:	Fluorescein isothiocyanate
FOXP3:	Forkhead box protein 3
OR:	Odds 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
PE:	Phycoerythrin
PI:	Phorbol 12-myristate 13-acetate/ionomycin
SIC:	Specific IgE class
TLR:	Toll-like receptor
Treg:	Regulatory T

Study characteristics

The PASTURE/EFRAIM study includes a prospective birth cohort from rural areas of 5 European countries: Austria, Finland, France, Germany, and Switzerland.[13] In brief, women from rural areas were contacted during pregnancy. Women from family-run livestock farms were assigned to the farm group, whereas women from the same rural areas but not living on a farm were recruited for the reference group. One thousand one hundred thirty-three pregnant women were recruited for the complete PASTURE/EFRAIM study. Of these, a 325-member subgroup of 4.5-year-old German and French children included in the follow-up was selected for assessment of T cells and Treg cells.

Questionnaires

Parents completed questionnaires during pregnancy and when the children were 2, 12, 18, 24, 36, and 48 months old. General health with focus on respiratory and atopic diseases and detailed farm exposures were documented. Questionnaires were based on items from the Asthma Multicenter Infants Cohort Study,[14] ALEX,[2] the PARSIFAL study,[4] and the American Thoracic Society questionnaire.[15] The study was approved by local research ethics committees from each country, and written informed consent was obtained from all parents.

Farm exposures

We primarily focused on the 4-year questionnaire for the respective exposures because the T-cell markers were measured in 4.5-year-old children. Specific farm exposures were used to investigate farm effects (for details, see the Methods section in this article's Online Repository). Farm milk consumption was defined as drinking farm milk within the last 12 months. Stable exposure was defined as spending time in a stable. A single contact with a stable was sufficient to classify the subject as a child with stable contact.

IgE and asthma definitions

Specific IgE levels in serum against 6 food and 13 inhalant allergen (see the Methods section in this article's Online Repository) were assessed at the age of 4.5 years by using the Allergy Screen test panel for atopy (Mediwiss Analytic, Moers, Germany) in a central laboratory. Positive sensitization was defined by using a cutoff for specific IgE of 3.5 IU/mL or greater (specific IgE class [SIC] 3). Asthma was defined as a parent-reported physician's diagnosis of asthma or a recurrent diagnosis of spastic, obstructive, or asthmatic bronchitis at age 4 years (ie, a doctor's diagnosis of asthma). Current asthma at age 4 years was defined by a doctor's diagnosis of asthma and current wheeze at age 4 years.

Isolation and stimulation of PBMCs

Blood samples were collected from the peripheral vein in sodium heparin tubes. PBMCs were isolated within 24 hours by means of density gradient

centrifugation with Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ). Cells were washed and resuspended in RPMI 1640 plus GlutaMAX (Gibco, Carlsbad, Calif) with 10% human serum (Sigma-Aldrich, Steinheim, Germany) at a final concentration of 5×10^6 cells/mL. Cells were incubated for 24 hours unstimulated or stimulated with phorbol 12-myristate 13-acetate (5 ng/mL) and ionomycin (1 μg/mL) or LPS (0.1 μg/mL, Sigma-Aldrich). The stimuli were chosen based on previous findings in the farm environment.[1,16,17] Although PI is a potent polyclonal stimulus strongly activating T-cell proliferation, LPS, an endotoxin from the outer membrane of gram-negative bacteria, is a TLR4 ligand present in high amounts in the farming environment and was demonstrated to be relevant for the atopy-protective farm effect.[1]

Flow cytometry of activated T cells and Treg cells

Intracellular staining, including that for CD4, CD25, and FOXP3, was performed in Germany and France. Treg cells were characterized as upper 20% $CD4^+CD25^+$, $FOXP3^+$ cells (intracellular; for details, see the Methods section in this article's Online Repository; for gate settings, see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). In addition, $CD4^+CD25^+$ cells (activated T cells) were examined (intracellular). In Germany surface CD4, CD25, and CD127 staining was performed (see Fig E1, *B*), and a subjective gate defined by the investigator and named $CD4^+CD25^{high}$ was used ($CD4^+25^{high}CD127^{low/-}$ cells) for additional assessment of Treg cells. Also, $CD4^+CD25^+$ cells (activated T cells) were examined (surface). To guarantee best comparability between centers, optimization experiments in centers, common meetings, and common standard operating procedures were applied. Additionally, testing for comparability of staining and activity of stimuli was performed regularly throughout the study (data not shown).

FOXP3 demethylation

DNA methylation of *FOXP3* was assessed by means of pyrosequencing in a subsample (n = 43) of the German arm of the study. Blood samples were taken at age 4.5 years, and genomic DNA was extracted from whole blood by using the FlexiGene DNA Kit (Qiagen, Hilden, Germany). Details of the sodium bisulfite conversion (EpiTect Bisulfite kit, Qiagen) and the pyrosequencing experiments were previously published (see the Methods section in this article's Online Repository).[18] Eight CpGs in the TSDR region[19] were analyzed by using the following PCR primers: forward, TGGGTTAAGTTTGTTG TAGGATAGG; reverse, TCCCTTTCTAACTAAATTTCTCAAAAAC.

Statistical analysis

T-cell subpopulations (in percentages) related to total lymphocyte numbers were used as Treg cell markers and shown as log-transformed percentages for association analyses because of slight deviations of normal distribution. Association between Treg cell markers and farm-related variables were analyzed by using linear regression, and associations between asthma or atopic sensitization and Treg cells markers were analyzed by using logistic regression. Nonparametric tests were also applied for confirmation and testing of differences in demethylation status. Statistical significance was defined by a P value of less than .05. We adjusted for multiple comparisons with a procedure taking into account the correlated structure of our variables introduced by Nyholt[20] and calculated independent significance thresholds, which involve this adjustment for intracellular or surface staining. The Pearson correlation coefficient was used.

Path analysis was used to evaluate the mediation of effects between exposure and outcome by Treg cell markers. The path analysis technique is an extension of regression analysis used in a context in which we suppose mediated effects.[21] For this, a total effect is decomposed into direct and indirect effects, and all effects can be tested for significance (see the Methods section in this article's Online Repository).

RESULTS

Data on Treg cells were available from 157 German and 141 French children among the 325 included children. Twenty-seven children were excluded because of a low amount of cells or technical problems (Fig 1). Table I shows the prevalence of typical farm exposures and outcomes stratified by country. According to the study design, half of these subjects were farm children. Moreover, half of the study group drank farm milk, mostly drinking it exclusively, with a predominance of consumption of unboiled milk in Germany. Despite an overlap between farming and farm exposures, some farm children did not consume farm milk, whereas some of the reference children drank farm milk (see Fig E2 in this article's Online Repository at www.jacionline.org). Of all children, 6% had an asthma diagnosis at age 4 years; 13.1% were atopic for inhalant IgE at age 4.5 years (8.4% for seasonal and 7.1% for perennial allergens), whereas 4.4% were sensitized to food allergens (increased specific IgE levels by using SIC3, Table I).

Treg cell numbers were quantitatively increased in farm compared with reference children

Fig 2 shows the distribution of intracellular staining of $CD4^+$ cells, $CD4^+CD25^+$ (activated) T cells, and Treg cells characterized as upper 20% $CD4^+CD25^+$, $FOXP3^+$ Treg cells, including their medians (as a percentage). Fig E3 in this article's Online Repository at www.jacionline.org shows the distribution of $CD4^+$, $CD4^+CD25^+$, and $CD4^+CD25^{high}CD127^{low/-}$ cells (surface staining). Both upper 20% $CD4^+CD25^+$, $FOXP3^+$ (Fig 2) and surface $CD4^+CD25^{high}CD127^{low/-}$ (see Fig E3) cells in unstimulated conditions were expressed at low concentrations (median, 0.2%; Fig 2 and see Fig E3), and numbers of the former were slightly increased on LPS (median, 0.3%) and PI (median, 0.4%) stimulation (both Fig 2). After PI stimulation, activated T-cell numbers were increased.

Treg cell numbers (percentage relative to lymphocytes) were consistently increased in farm compared with nonfarm children (Table II). This effect was significant after LPS and PI stimulation, most pronounced on LPS stimulation ($P = .001$), and still significant after adjustment for multiple testing. Moreover, activated $CD4^+CD25^+$ T cells were significantly increased on PI and LPS stimulation in farm children. In the German population Treg cell numbers were increased in farm children in unstimulated conditions when the marker $CD4^+CD25^{high}CD127^{low/-}$ was assessed ($P = .025$, see Table E1 in this article's Online Repository at www.jacionline.org).

Farm milk consumption was associated with increased Treg cell numbers

To disentangle which specific farm exposures were relevant, we examined associations of 2 typical farm exposures (a stable and farm milk) at age 4 years with Treg cell markers. After adjustment for farming, only current farm milk consumption was associated with increased activated T-cell numbers (U [unstimulated], PI, and LPS) and Treg cell numbers (PI and LPS, $P \leq .01$, Table II), with the latter still significant after adjustment for multiple testing. Additionally, increased Treg cell numbers (surface $CD4^+25^{high}CD127^{low/-}$) were observed in children with farm milk exposure on PI and LPS stimulation (see Table E1). These associations were unchanged after inclusion of potential confounders (sex and siblings). In contrast, the increase in Treg cell numbers in farm children was diminished after adjustment for farm milk consumption (data not shown).

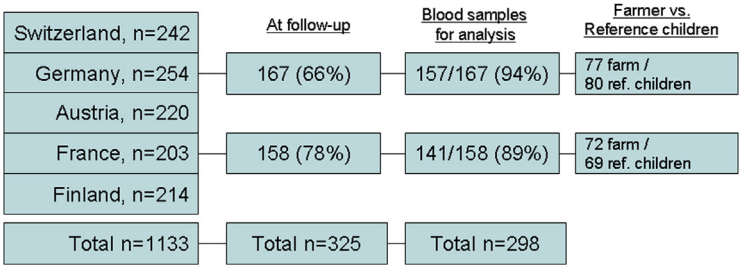

FIG 1. Study design for assessment of Treg cells.

TABLE I. Prevalences of outcomes/exposures at 4 years of age stratified by center

	All			Germany			France		
	No, no.	Yes, no.	Yes (%)	No, no.	Yes, no.	Yes (%)	No, no.	Yes, no.	Yes (%)
Exposures									
Farmer	149	149	50.0	80	77	49.0	69	72	51.1
Farm milk consumption	150	148	49.7	79	78	49.7	71	70	49.6
Only shop milk		135	47.7		73	48.3		62	47.0
Shop or farm milk		48	17.0		27	17.9		21	15.9
Exclusive farm milk		100	35.3		51	33.8		49	37.1
Only shop milk		135	50.4		73	51.0		62	49.6
Farm milk always boiled		51	19.0		17	11.9		34	27.2
Farm milk unboiled		82	30.6		53	37.1		29	23.2
Stay in stable	127	171	57.4	65	92	58.6	62	79	56.0
Regular contact with hay	167	131	44.0	81	76	48.4	86	55	39.0
Outcome age 4 y									
Atopy (SIC3*)									
Inhalant IgE	259	39	13.1	133	24	15.3	126	15	10.6
Seasonal IgE	273	25	8.4	139	18	11.5	134	7	5.0
Perennial IgE	277	21	7.1	144	13	8.3	133	8	5.7
Food IgE	285	13	4.4	147	10	6.4	138	3	2.1
Atopy (SIC2*)									
Inhalant IgE	214	84	28.2	101	56	35.7	113	28	19.9
Seasonal IgE	247	51	17.1	125	32	20.4	122	19	13.5
Perennial IgE	247	51	17.1	121	36	22.9	126	15	10.6
Food IgE	239	59	19.8	124	33	21.0	115	26	18.4
Atopy (SIC1*)									
Inhalant IgE	186	112	37.6	86	71	45.2	100	41	29.1
Seasonal IgE	231	67	22.5	121	36	22.9	110	31	22.0
Perennial IgE	228	70	23.5	108	49	31.2	120	21	14.9
Food IgE	204	94	31.5	111	46	29.3	93	48	34.0
Doctor-diagnosed asthma	280	18	6.0	147	10	6.4	133	8	5.7
Current asthma	282	16	5.4	149	8	5.1	133	8	5.7

*SIC3, 3.5 IU/mL or greater; SIC2, 0.7 IU/mL or greater; and SIC1, 0.35 IU/mL or greater.

In support of this, *FOXP3* demethylation at the TSDR region, which is specific for natural Treg cells,[19] was consistently higher in whole blood of children with farm milk consumption but not farm exposure in a subgroup of 43 children (median for all CpGs, $P = .08$, see Fig E4 in this article's Online Repository at www.jacionline.org). Correlation analysis revealed a significant positive correlation of *FOXP3* demethylation (in detectable CpGs) with Treg cell expression (upper 20% $CD4^+CD25^+$, $FOXP3^+$ cells) for PI and LPS stimulation (data not shown). Of note, PI-stimulated *FOXP3* demethylation was 99% in isolated $CD4^+CD25^{high}FOXP3^+$ cells versus 1% in isolated $CD4^+CD25^{low}$ cells assessed in a child with a sufficient amount of cells, thus representing Treg cells.

Assessing the subgroups of farm and reference children, Treg cell numbers after PI and LPS stimulation were consistently positively associated with farm milk consumption and most prominent in reference children without stable exposure (see Table E2 in this article's Online Repository at www.jacionline.org).

We could not identify different effects of specific patterns of farm milk consumption (farm milk vs mixed milk and boiling status of milk) on Treg cell numbers (see Table E3 in this article's Online Repository at www.jacionline.org). We observed an association between Treg cell numbers and farm milk exposure during pregnancy and childhood (see Table E4 in this article's Online Repository at www.jacionline.org). Because of low numbers with farm milk consumption during pregnancy only, it was not possible to disentangle an effect depending on timing. For other farm-related variables, including regular contact with hay, type of farming (pigs, poultry, or others), and number of animal species, no

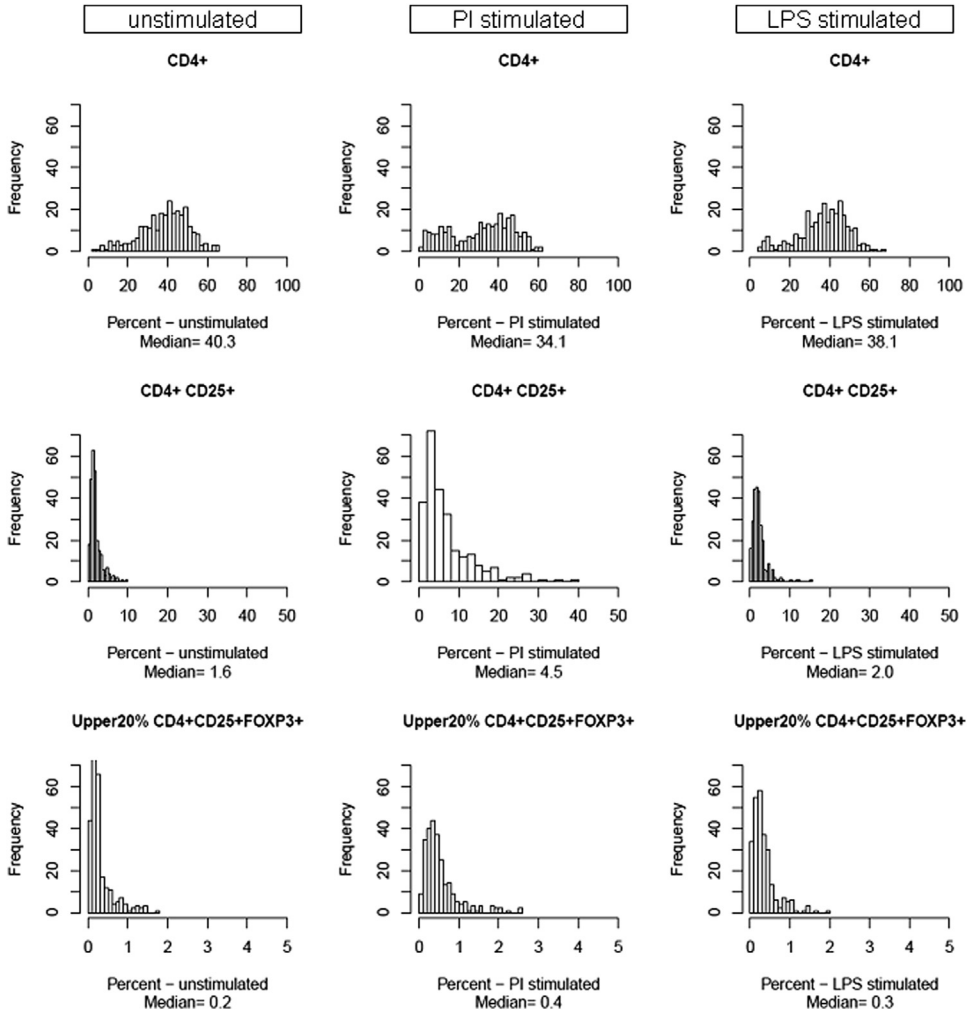

FIG 2. Distribution of $CD4^+$ T cells, activated T cells, and Treg cells relative to lymphocytes (as a percentage). Intracellular expression of $CD4^+$, $CD4^+CD25^+$, and upper 20% $CD4^+CD25^+$, $FOXP3^+$ cells in unstimulated, PI-stimulated, and LPS-stimulated cells (n = 298, Germany and France).

effect on Treg cell numbers remained significant on adjustment for farm milk consumption.

Effect of farm milk on atopic sensitization and asthma

Farm milk consumption at 4 years was negatively associated with sensitization against seasonal IgE allergens in the whole cohort (SIC3; odds ratio [OR], 0.56; 95% CI, 0.34-0.94; P =.029; n = 711); however, the effect was less pronounced in the subsample of children with available Treg cell data (called the Treg cell subsample; OR, 0.78; 95% CI, 0.34-1.78; n = 298; P = .552). Farm milk consumption showed a protective trend against perennial atopy in both the whole cohort (SIC3; OR, 0.67; 95% CI, 0.37-1.22; P = .192; n = 711) and the Treg cell subsample (OR, 0.60; 95% CI, 0.24-1.50; P = .275). A significant protective effect of farm milk consumption on doctor-diagnosed asthma at age 4 years was present in both the whole cohort (OR, 0.45; 95% CI, 0.23-0.88; P = .019; n = 988) and in the Treg cell subsample (OR, 0.27; 95% CI, 0.09-0.84; P = .024).

Treg cell numbers were associated with lower perennial IgE levels and asthma at age 4 years

Treg cell numbers (upper 20% $CD4^+CD25^+$, $FOXP3^+$) at baseline (unstimulated) and after PI and LPS stimulation were mostly negatively associated with sensitization to inhalant or food allergens (Table III). Unstimulated Treg cell numbers were significantly associated with lower inhalant IgE levels (not shown), with a stronger association with perennial than seasonal allergens (perennial IgE, P = .003, Table III). In addition, activated T cells also showed a protective effect against perennial sensitization (P = .040). Although less pronounced, the effects for perennial sensitization were also observed in lower SICs (data not shown). Furthermore, LPS-stimulated Treg cell numbers were inversely associated with a doctor's diagnosis of asthma (P = .03, Table III). A similar pattern was observed for current asthma (data not shown).

The protective farm milk effect on asthma is partially mediated by Treg cells

Using path analysis, we showed that the protective effect of farm milk exposure on asthma (total effect, P = .018)

TABLE II. Association of activated T cells and Treg cells (intracellular staining) with farming and typical farm-related exposures in children living on a farm and reference children of the same area at 4.5 years of age

Stimuli	Intracellular marker	Farm vs reference children		Staying in stables		Farm milk consumption		No. of children†
		GMR (95% CI)	*P* value*	GMR (95% CI)	*P* value*	GMR (95% CI)	P value*	
		Adjusted for center		Adjusted for center		Adjusted for center		
U	$CD4^+CD25^+$	1.15 (0.97-1.36)	.117	1.10 (0.92-1.31)	.295	**1.28 (1.08-1.51)**	**.005‡**	263
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$	1.15 (0.91-1.44)	.237	1.01 (0.80-1.27)	.938	1.23 (0.98-1.54)	.071	262
PI	$CD4^+CD25^+$	**1.30 (1.11-1.54)**	**.002‡**	**1.27 (1.08-1.50)**	**.005‡**	**1.37 (1.17-1.61)**	**<.001‡**	258
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$	**1.23 (1.04-1.46)**	**.015**	**1.25 (1.05-1.48)**	**.011‡**	**1.36 (1.15-1.60)**	**<.001‡**	258
LPS	$CD4^+CD25^+$	**1.32 (1.10-1.57)**	**.003‡**	**1.27 (1.06-1.52)**	**.010‡**	**1.41 (1.18-1.69)**	**<.001‡**	261
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$	**1.44 (1.15-1.79)**	**.001‡**	**1.35 (1.08-1.68)**	**.008‡**	**1.57 (1.27-1.95)**	**<.001‡**	260
				Additional adjustment for farming		Additional adjustment for farming		
U	$CD4^+CD25^+$			0.97 (0.75-1.27)	.836	**1.31 (1.05-1.65)**	**.020**	263
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$			0.80 (0.57-1.14)	.216	1.24 (0.91-1.67)	.172	262
PI	$CD4^+CD25^+$			1.09 (0.85-1.42)	.493	**1.29 (1.03-1.60)**	**.027**	258
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$			1.16 (0.89-1.50)	.279	**1.36 (1.08-1.70)**	**.008‡**	258
LPS	$CD4^+CD25^+$			1.07 (0.81-1.40)	.639	**1.33 (1.06-1.69)**	**.016**	261
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$			1.06 (0.76-1.48)	.726	**1.46 (1.10-1.94)**	**.010‡**	260

GMR, Geometric mean ratio; *U*, unstimulated.
**P* value of regression analysis in the log-transformed data: significant results ($P < .05$) are shown in boldface.
†Numbers can vary because of availability of samples and parameters.
‡Significant after adjustment for multiple testing.

TABLE III. Association of asthma and atopic sensitization (SIC3) with activated T-cell and Treg cell numbers in children living on a farm and reference children of the same area at 4.5 years of age

Stimuli	Intracellular marker	Asthma*		Atopic sensitization					
				Food IgE*		Seasonal IgE*		Perennial IgE*	
		OR (95% CI)	*P* value†	OR (95% CI)	*P* value†	OR (95% CI)	*P* value†	OR (95% CI)	*P* value†
U	$CD4^+CD25^+$	0.22 (0.03-1.34)	.099	0.94 (0.15-5.96)	.944	1.04 (0.27-4.04)	.956	**0.19 (0.04-0.93)**	**.040**
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$	0.53 (0.16-1.80)	.309	0.88 (0.21-3.76)	.862	0.45 (0.17-1.19)	.108	**0.21 (0.08-0.59)**	**.003‡**
PI	$CD4^+CD25^+$	1.07 (0.18-6.41)	.944	0.16 (0.02-1.09)	.062	0.92 (0.21-3.99)	.912	0.57 (0.13-2.56)	.464
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$	1.22 (0.21-7.23)	.827	0.16 (0.02-1.05)	.056	0.33 (0.08-1.33)	.118	0.36 (0.08-1.52)	.164
LPS	$CD4^+CD25^+$	**0.22 (0.05-1.02)**	**.054**	1.57 (0.26-9.48)	.622	0.99 (0.27-3.55)	.984	0.60 (0.15-2.34)	.462
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$	**0.26 (0.08-0.88)**	**.030**	0.70 (0.18-2.66)	.597	0.41 (0.15-1.12)	.082	0.49 (0.17-1.43)	.191

U, Unstimulated.
*All analyses were adjusted for farm and center.
†*P* value of logistic regression analysis in the log-transformed data: significant results ($P < .05$) are shown in boldface.
‡Significant after adjustment for multiple testing.

was partially mediated by Treg cells (upper 20% $CD4^+CD25^+$, $FOXP3^+$) because we detected a borderline effect ($P = .079$) for the path of exposure (farm milk consumption) through Treg cells on asthma (indirect effect; Fig 3, *A*). For activated T cells ($CD4^+CD25^+$), similar effects were seen; however, they were not significant (Fig 3, *B*). Of note, Treg cells are contained in the population of $CD4^+CD25^+$ cells.

DISCUSSION

In this birth cohort study we showed that Treg cell numbers were significantly increased in 4.5-year-old farm-exposed compared with nonexposed children on innate LPS and mitogenic PI stimulation. These effects were primarily detected for farm milk exposure, with no clear difference between the various treatments of the milk (ie, boiling). Exposure during pregnancy and childhood showed the strongest effect for an increase in Treg cell numbers. Regarding the effect of farming on atopic diseases at 4 years, unstimulated Treg cells were negatively associated with sensitization to perennial IgE, and LPS-stimulated Treg cells were negatively associated with doctor-diagnosed asthma.

Several cross-sectional studies have demonstrated the protective effect of farming against development of atopic diseases in childhood.[2,4,22] However, the underlying mechanism is still not clear. Several points might be relevant. One main factor is the identification of the specific type or types of exposure mediating

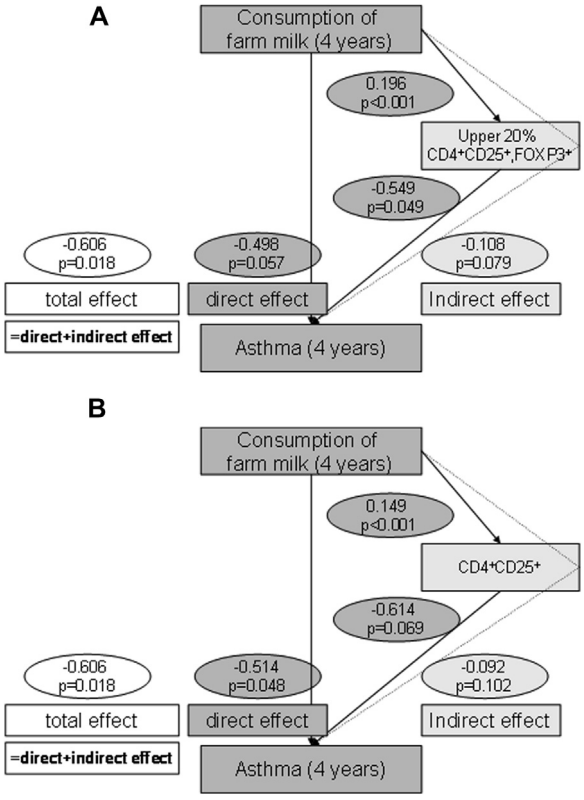

FIG 3. Mediation of the farm milk effect on asthma (doctor's diagnosis at age 4 years, n = 18) by LPS-stimulated Treg cells and activated T cells. The total effect of farm milk on asthma at age 4 years is divided into a direct and an indirect path (estimates and *P* values of each effect are indicated separately). Upper 20% $CD4^+CD25^+$, $FOXP3^+$ **(A)** versus $CD4^+CD25^+$ **(B)** cells are shown as a possible indirect link between farm milk exposure and asthma, with a more pronounced effect through upper 20% $CD4^+CD25^+$, $FOXP3^+$ cells ($\beta = -0.108$, $P = .079$).

similar or potentially different regulatory mechanisms. Further factors include the timing and duration of exposure and finally the effect on clinical atopic phenotypes, such as atopic sensitization and asthma.

Farm milk exposure was associated with increased Treg cell numbers

Different mechanisms, including upregulation of innate immune receptors, have been reported as relevant for the farming effect, particularly in the context of prenatal exposure.[8,9] Additionally, Treg cells, as an important immunoregulatory T-cell population, were increased in number and functionally more efficient in cord blood of neonates of farm families,[11] supporting the hypothesis that the atopy-protective farming effect starts already *in utero*. Here we demonstrated in a large cohort that the effect observed early in life on Treg cells was still present during early childhood, namely that Treg cell numbers were increased on stimulation in farm and particularly farm milk–exposed children at age 4 years. In addition to Treg cells, activated T cells seem to be critical as well. Although in human studies several markers were suggested to best reflect Treg cell numbers, such as CD25 expression in high amounts, low CD127 surface expression,[23] or intracellular FOXP3 expression,[24] $CD4^+CD25^{high}FOXP3^+$ (in our study objectively defined as upper 20% $CD4^+CD25^+$, $FOXP3^+$) is currently the most specific marker for functional Treg cells, which were used as a Treg cell marker in this study.[25] Although CD127 was identified as a Treg cell surface marker inversely correlating with FOXP3 expression,[23,26] low CD127 expression might not be an intrinsic characteristic of Treg cells, and differential CD127 expression on Treg cells might depend on their localization and activation status.[27,28] These latter findings might explain differences in intracellular and surface staining. In support of our findings for Treg cells characterized by intracellular upper 20% $CD4^+CD25^+$, $FOXP3^+$ T cells, PI-stimulated *FOXP3* was completely demethylated in isolated $CD4^+CD25^{high}FOXP3^+$ T cells. *FOXP3* demethylation without stimulation in whole blood, a specific Treg cell marker,[19,29] was also greater in a subgroup of farm milk–exposed children and positively correlated with upper 20% $CD4^+CD25^+$, $FOXP3^+$ cell numbers on stimulation, supporting an effect of Treg cells for farm milk exposure. In parallel to higher *FOXP3* demethylation in whole blood of farm milk–exposed children, Treg cell numbers were increased in unstimulated cells, although not significantly. Of note, this discrepancy might be explained by using the 2 different methods to investigate Treg cells at the epigenetic or protein expression levels. However, Treg cells and activated T cells both seem to be relevant, and in-depth functional assessment in future studies is critical.

Regarding specific farm exposures, a critical role for farm milk consumption on Treg cell expression on PI and LPS stimulation was shown. In several cross-sectional studies (eg, ALEX and PARSIFAL), farm milk consumption in the first year or ever in life was associated with reduced risk for childhood atopic diseases.[2,6,7] Other previously reported atopy-protective farm-related exposures, such as exposure to stables[2,9,30] or increasing numbers of farm animal species,[9,11,17] were not related to increased Treg cell expression at age 4.5 years in this subgroup of the PASTURE study. Yet because of high correlations between protective factors, it is difficult to disentangle specific components of the farm effect. However, analyzing all children with farm milk consumption in different subgroups, the effect on Treg cell numbers was even more pronounced in reference children, particularly without stable contact, indicating a true effect of farm milk consumption. In contrast, in reference children with stable exposure but without farm milk consumption, no increase in Treg cell numbers was seen. One possible explanation is that atopy protection through farm milk is mediated by Treg cells, whereas other farm exposures, such as stable exposure, might mediate an allergy-protective effect through additional or different mechanisms not yet identified. Strong protection in reference children might also be an indicator of no previous continuous exposure in contrast to a potential "saturation effect" in farm children. In support of this, a lower prevalence of atopy in children with consumption of unpasteurized milk early in life was reported.[31] In a stratified analysis this association was observed among urban but not rural children.[31]

Farm milk and specifically raw milk consumption has been discussed as a key protective exposure contributing to the farm effect.[3,32] Several studies from different countries described a negative association of unboiled farm milk consumption with asthma, hay fever,[6] eczema,[33,34] and atopy.[6,31,34,35] Raw milk can contain more bacteria, including pathogenic but also protective strains, than boiled milk.[36] Milk treatments, such as heating and homogenization, can affect heat-sensitive components or the physical structure of milk fat and abrogate the farm milk effect on allergy protection.[3,32] However, our study did not reveal major

differences in Treg cell numbers, depending on the boiling status of farm milk. Thus Treg cells could potentially be modulated by heat-resistant farm milk components and act synergistically with other mechanisms involving other farm milk components to contribute to the farm effect.

Timing of exposure and specific stimulation

Regarding timing and length of exposure, farm milk exposure during pregnancy alone and both pregnancy and childhood was associated with a consistent pattern of increased Treg cell numbers. Because of few children with farm milk exposure during pregnancy only and a high correlation between the time points, we cannot disentangle the effect of pregnancy exposure only.

The strongest effect of farm exposures on Treg cell numbers was observed on stimulation with LPS (endotoxin), a cell-wall component of gram-negative bacteria present in high amounts in the farming environment.[1] For protection against atopy, LPS stimulation might be an important stimulus from the farm environment, suggesting previous contact, and might promote a strong Treg cell response in later childhood. Interestingly, farm milk–exposed children from the reference group without contact with stables showed a strong response to LPS compared with that seen in milk-exposed farm children, which might additionally point to an additive effect for nonfarm children, although this and the specifics of stable contact need to be confirmed and the underlying mechanisms need to be elucidated in detail. Yet an effect in children not raised on a farm is truly important and would be transferable to a larger percentage of the population.

Association of Treg cell numbers with asthma and atopic sensitization

Our findings point to an atopy- and asthma-protective role for farm-related exposure, as modulated by Treg cells, which has been previously suggested as an intriguing concept but not proved to date.[3] At the age of 4.5 years, the prevalence of atopic sensitization in childhood is known to be less than at school age, and thus the number of children with specific IgE against common allergens of greater than 3.5 IU/mL (SIC3) was still low in our study. Yet a negative association with Treg cell numbers was detected also in children with lower specific IgE levels, strongly suggesting a true effect. Of note, a doctor's diagnosis of asthma at age 4 years has the risk of including children with transient wheeze. However, showing a similar effect for children with current asthma at age 4 years, excluding the potential transient wheezer, confirms our findings for doctor-diagnosed asthma. Nevertheless, our data obtained at preschool age need to be confirmed in later childhood, and potential atopy-protective mechanisms of farm exposures on asthma will be determined at an age when lung function can be measured reliably.

Conclusions

In summary, this study indicates that the protective farm effect on asthma and atopy can be modulated by Treg cells. We have previously shown that Treg cell numbers were increased in cord blood of farm children.[11] In this study we demonstrated that Treg cell numbers and activated T-cell numbers were increased at 4.5 years of age on stimulation in the context of farm and specifically farm milk exposure. However, the specific components of the farm milk that alter Treg cell immune responses on activation still need to be identified. Potential candidates might be part of the fat fraction, such as fatty acids, bacteria, or vitamins. Furthermore, Treg cells were inversely associated with early diagnoses of atopy and asthma, supporting the hypothesis of a disease-related mechanism, even if the association between farm milk consumption and atopy was less pronounced in this subsample than in other farm studies.

The strength of this study is the detailed investigation of Treg cells in a large number of children in relation to different farm exposures in childhood, including a follow-up to assess immune maturation of Treg cells (including *FOXP3* demethylation) and T cells and their role on the development of atopic diseases later in childhood. Because immune maturation is still ongoing and the prevalence of atopic sensitization will be higher and clinically relevant in later childhood, a follow-up of these children is critical to assess whether farming exposures persistently shape the immune responses mediated by Treg cells toward an atopy-protective status in later childhood.

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Key messages

- **Farm exposure and specifically farm milk intake was associated with increased Treg cell numbers at 4.5 years of age.**
- **Treg cell numbers were negatively associated with atopic sensitization and asthma.**
- **The protective farm milk effect on asthma was partially mediated by Treg cells.**

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METHODS

Farm exposures

Further details of specific farm exposure were as follows. First, cow's milk consumption was either described as shop milk consumption when it was purchased at a shop or as farm milk consumption when consumed directly from a farm. Exposure was grouped into the following categories: (1) exclusive shop milk exposure, (2) mixed milk exposure (exposure to both shop and farm milk), and (3) exclusive farm milk exposure. Farm milk exposure was further divided into subgroups of "only boiled farm milk" and "any unboiled farm milk," describing either consumption of exclusive boiled farm milk or of both unboiled and boiled farm milk. "Farm milk in childhood" refers to farm milk consumption in the second, third, or fourth years of life, whereas a consistent pattern of no farm milk consumption built the control group. A 4-category variable showing the pattern of farm milk consumption in childhood, pregnancy, or both was also created.

Second, stable contact includes contact for farm and nonfarm children, for the latter indicating whether they stayed on another farm regularly and how much time they spent in the stable.

Third, regular hay contact was defined as at least weekly contact with hay, such as by playing in the hay.

Fourth, the type of farm was assessed by the type of stock breeding (cows, pigs, poultry, or other animals).

Fifth, the number of animal species was estimated based on the presence of cows, pigs, horses, or poultry on a farm and contemporaneously a child's contact with the stable.

Some other relevant exposures were defined as potential confounders: number of older siblings, dichotomized into children with at least 2 siblings or children with 0 to 1 sibling; smoking in pregnancy defined by any maternal smoking in 1 of the trimesters; duration of breast-feeding assessed by months children were breast-fed in the first 18 months of life; and parental atopy status defined as the presence of asthma, hay fever, and/or atopic dermatitis in at least 1 of the parents.

Specific IgE assessment

Specific IgE levels were assessed at the age of 4.5 years in serum by using the Allergy Screen test panel for atopy (Mediwiss Analytic, Moers, Germany) in a central laboratory. This method has previously been validated against the *in vitro* IgE CAP system (Pharmacia, Freiburg, Germany) and the skin prick test.[E1] The 6 food allergens included hen's egg, cow's milk, peanut, hazelnut, carrot, and wheat flour. The 13 inhalant allergens comprised *Dermatophagoides pteronyssius*; *Dermatophagoides farinae*; cat; horse; dog; *Alternaria* species; mugwort, plantain, alder, birch, hazel, and rye pollen; and grass pollen mix. Positive sensitization against perennial, seasonal, or food allergens was defined by using the cutoff for specific IgE of 3.5 IU/mL or greater (SIC3). In addition, different cutoffs were tested (ie, ≥0.35 IU/mL [SIC1] and ≥0.7 IU/mL [SIC2]).

Isolation and stimulation of PBMCs

Blood samples were collected from the peripheral vein in sodium heparin tubes. The laboratory investigators were blind to clinical information.

Flow cytometry of Treg cells

Treg cell numbers were assessed by means of flow cytometry with 2 different staining methods: surface and intracellular staining. Treg cells were characterized as upper 20% $CD4^+CD25^+$, $FOXP3^+$ cells (intracellular). Natural Treg cells have been mainly defined by the expression of their transcription factor, *FOXP3*.[E2] However, some other T cells might also express *FOXP3* without being functionally suppressive. It has been demonstrated that in human subjects the suppressive natural Treg cell population expresses high amounts of CD25,[E3] and therefore the current best marker available to yield the functional natural Treg cell population is $CD4^+CD25^{high}FOXP3^+$.

For surface staining, 2.5×10^5 cells were stained with 2 μL of CD4–fluorescein isothiocyanate (FITC)/1 μL of CD25-PC5 (both from Beckman Coulter, Fullerton, Calif) and 2 μL of CD127–phycoerythrin (PE; eBioscience, San Diego, Calif) antibodies. The corresponding isotype controls IgG_1-FITC, IgG_1-PE (both from Dako Cytomation, Glostrup, Denmark), and IgG_{2a}-PC5 (Beckman Coulter) were used. For intracellular FOXP3 staining, the Human Regulatory T Cell Staining Kit (eBioscience) or Human FoxP3 Buffer Set (BD PharMingen, San Jose, Calif) were used. Cells (10^6) were stained with 8 μL of CD4-FITC, 4 μL of CD25-PC5 (both from Beckman Coulter), and 10 μL of FOXP3-PE (clone PCH101, eBioscience) antibodies in Germany or 3 μL of CD4-FITC, 3 μL of CD25-allophycocyanin, and 3 μL of FOXP3-PE (all from BD PharMingen; FOXP3 clone 259D/C7) in France. Prior optimization experiments showed comparable results. The corresponding FOXP3 isotype antibodies IgG_{2a}-PE (eBioscience) and IgG_1-PE (BD PharMingen) were used as controls, respectively.

By using 3- or 4-color flow cytometry, acquisition was performed with CellQuest or CellQuest Pro Software (BD Biosciences) for FACScan and FACSCalibur and with FACSDiva software for FACSCanto II (BD Biosciences). Analyses were performed with FCS express version 3 (De Novo Software, Los Angeles, Calif) and FACSDiva software (BD Bioscience). The objective gate defined as upper 20% $CD4^+CD25^+$, $FOXP3^+$ was used for intracellular staining. For surface staining, a subjective gate, defined as $CD4^+CD25^{high}CD127^{neg/low}$, was used. The subjective gate is based on the shape of the $CD4^+CD25^+$ cell gate. Only the group with the highest CD25 expression is included.

FOXP3 demethylation

Experiments were performed with the PSQ 96MD Pyrosequencing system by using the PyroGold SQA Reagent Kit (Qiagen) and Q-CpG software (version 1.0.9; Biotage, Uppsala, Sweden).

Statistical analysis

Percentages of T-cell subpopulations related to lymphocytes were used as Treg cell markers. Because the distribution of some Treg cell markers (in percentages) partly deviated from normal distribution, they were $\log_{10}$ transformed, and very few 0 values in percentages were set to a minimal value of 0.005 before log transformation.

Data analyses for Treg cells using surface staining were only assessed in Germany, whereas intracellular expression data were analyzed for the pooled sample of both countries. Association between Treg cell markers and farm-related variables were analyzed by means of linear regression analyses of the log-transformed variables. Geometric mean ratios and 95% CIs were reported. Nonparametric Wilcoxon or Kruskal-Wallis tests were applied to confirm the results and also used to test for differences in demethylation status.

Associations between asthma or atopic sensitization and Treg cell markers were analyzed by means of logistic regression. ORs and 95% CIs were presented on the $\log_{10}$ scale.

All analyses were adjusted for center and farming. Additional confounders, such as sex, number of siblings, smoking in pregnancy, breast-feeding, and parental atopy, were included in some models but not reported because they did not influence the main results.

Statistical significance was defined by a P value of less than .05. Corrections for multiple testing were performed with a procedure introduced by Nyholt.[E4] This procedure takes correlation of the variables into account, which is applicable for our correlated markers. The Pearson correlation coefficient was used to calculate the correlation in this context.

Path analysis was used to evaluate the mediation of protective effects between exposure and outcome by Treg cell markers, if regression of outcome on Treg cells and regression of Treg cells on farm exposures were significant. The path analysis technique is an extension of regression analysis used in a context in which we suppose mediated effects. For this, a total effect is decomposed into direct and indirect effects, and all effects can be tested for significance in the same model. Thereby the indirect effects are calculated as the product of the direct effects on the path, such as farm milk to Treg cells and Treg cells to asthma. Adding all indirect and all direct effects provides the total effect.

Path analysis was done with Mplus 5.21 software (Muthen & Muthen, Los Angeles, Calif), whereas other analyses were performed with SAS 9.2 software (SAS Institute, Cary, NC).

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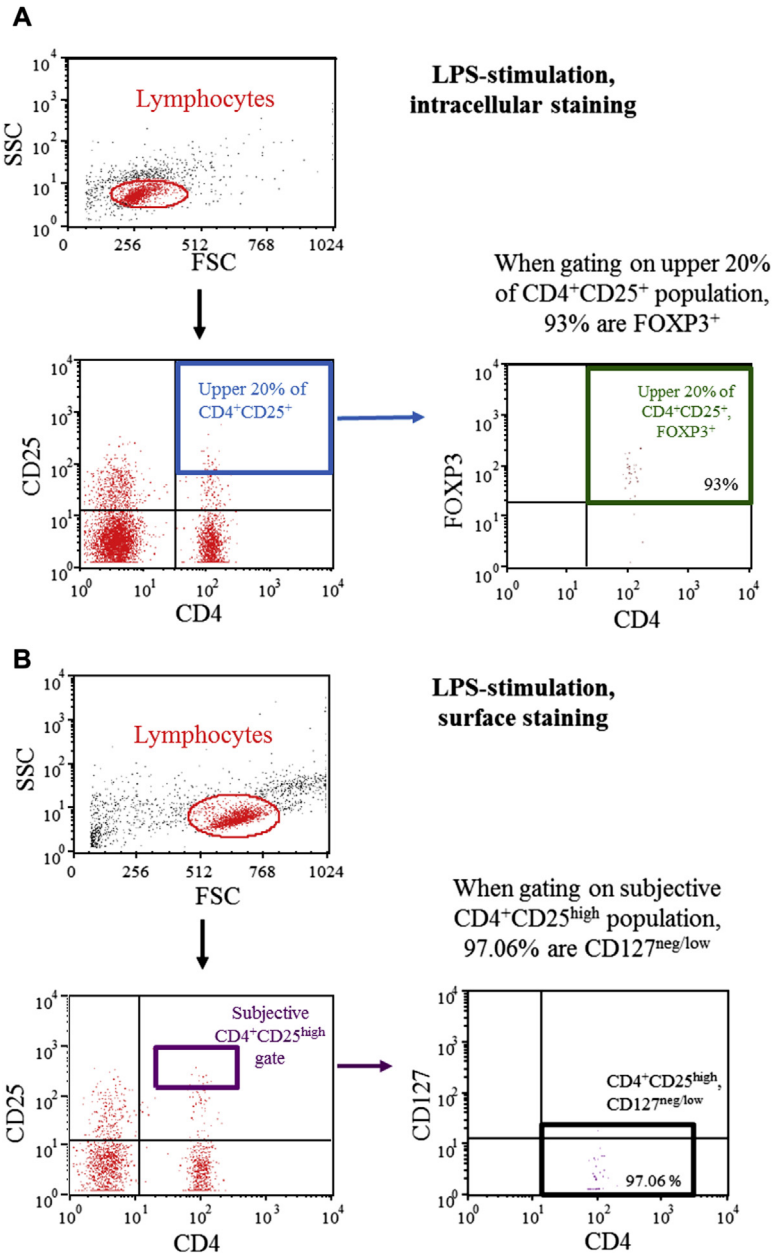

FIG E1. Gating strategies for flow cytometry. **A,** Assessing Treg cell numbers by means of intracellular staining (upper 20% $CD4^+CD25^+$, $FOXP3^+$). **B,** Assessing Treg cell numbers by means of surface staining ($CD4^+CD25^{high}CD127^{-/low}$ cells).

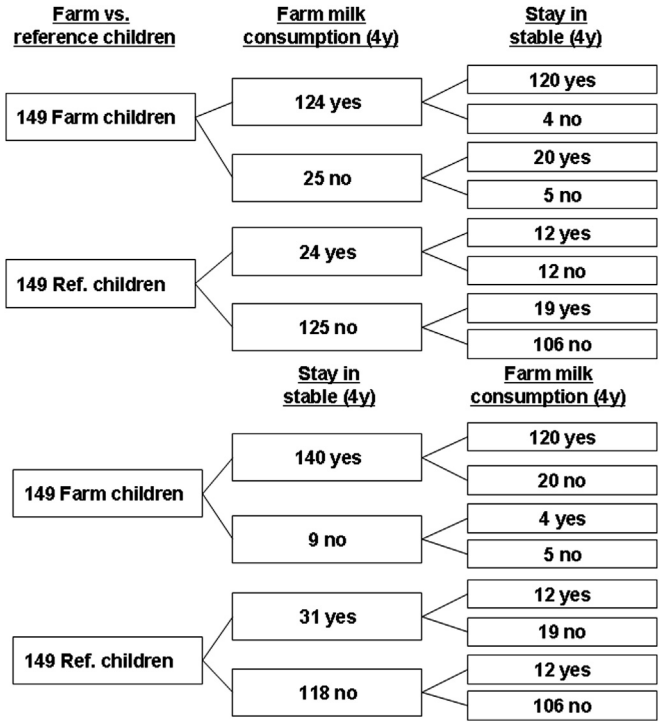

FIG E2. Distribution of children age 4 years stratified by farm and farm milk consumption and stable contact **(A)** and stay in a stable and farm milk consumption **(B)**. There are children who consume farm milk and do not have stable contact (n = 12 in reference children) and children with stable contact who do not drink farm milk (n = 19 in reference children).

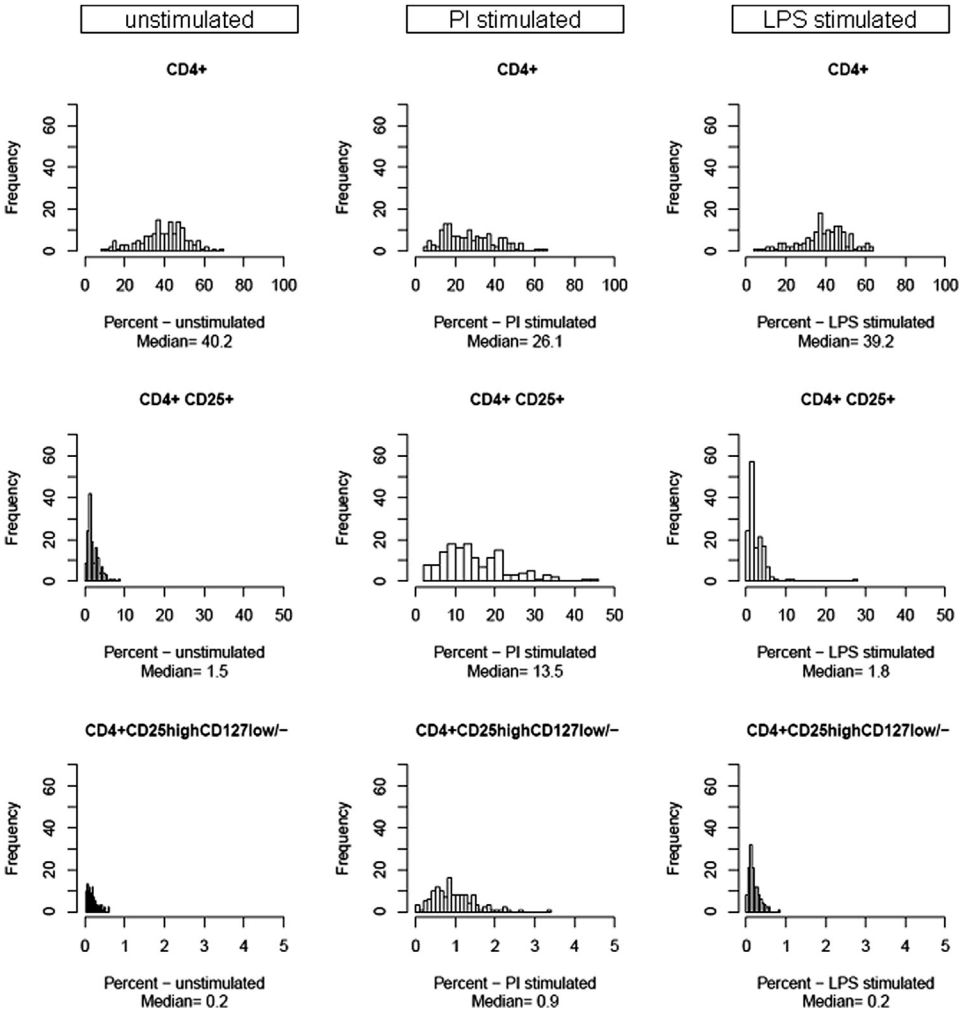

FIG E3. Distribution of $CD4^+$, activated T cells, and Treg cells relative to lymphocytes (as a percentage): surface expression of $CD4^+$, $CD4^+CD25^+$, and $CD4^+CD25^{high}CD127^{low/-}$ cells (n = 157, Germany).

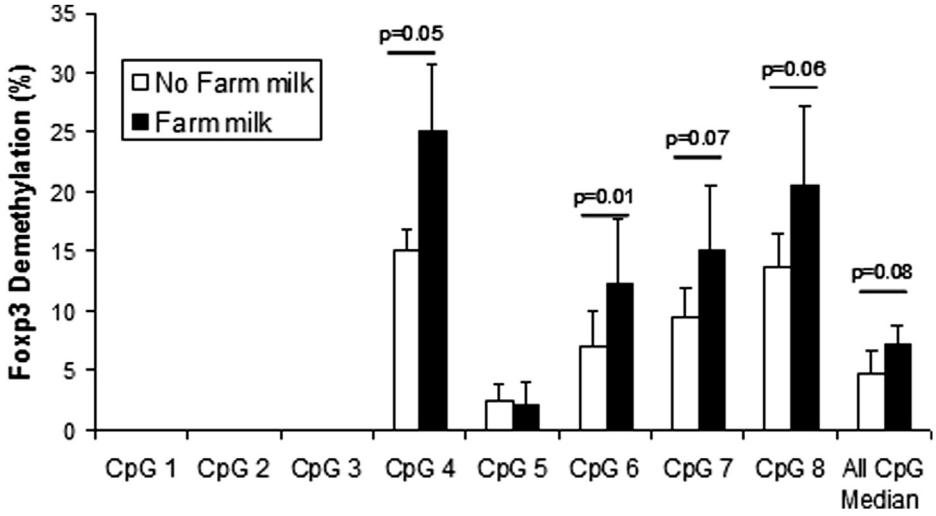

FIG E4. *FOXP3* demethylation (CpGs 1-8 and median of all CpGs) in the TSDR region proximal to the promoter. Demethylation was detectable in CpGs 4 to 8 and was generally higher in children with farm milk exposure compared with those with no farm milk exposure (n = 43 German children, median ± interquartile range).

TABLE E1. Association of activated T-cell and Treg cell numbers (surface staining) with farming and typical farm-related exposures at 4.5 years of age

		Farm vs reference children		Staying in stables		Farm milk consumption		No. of children
Stimuli	**Surface marker**	**GMR (95% CI)**	***P* value***	**GMR (95% CI)**	***P* value***	**GMR (95% CI)**	***P* value***	**No.†**
		Adjusted for center		**Adjusted for center**		**Adjusted for center**		
U	$CD4^+CD25^+$	0.96 (0.76-1.22)	.763	1.01 (0.79-1.28)	.948	1.01 (0.79-1.27)	.965	151
	$CD4^+25^{high}CD127^{low/-}$	**1.31 (1.04-1.66)**	**.025**	**1.20 (0.94-1.53)**	.139	**1.28 (1.01-1.62)**	**.046**	134
PI	$CD4^+CD25^+$	0.97 (0.80-1.18)	.779	0.99 (0.81-1.20)	.887	1.11 (0.91-1.35)	.298	149
	$CD4^+25^{high}CD127^{low/-}$	1.10 (0.88-1.38)	.411	1.03 (0.82-1.29)	.808	**1.28 (1.03-1.60)**	**.028**	131
LPS	$CD4^+CD25^+$	0.87 (0.68-1.12)	.290	0.92 (0.71-1.18)	.507	0.94 (0.73-1.20)	.608	148
	$CD4^+25^{high}CD127^{low/-}$	1.10 (0.83-1.46)	.492	1.06 (0.79-1.41)	.692	**1.43 (1.09-1.88)**	**.012**	132
				Additional adjustment for farming		**Additional adjustment for farming**		
U	$CD4^+CD25^+$			1.07 (0.76-1.51)	.691	1.05 (0.77-1.44)	.751	151
	$CD4^+25^{high}CD127^{low/-}$			0.98 (0.70-1.37)	.906	1.13 (0.83-1.53)	.441	134
PI	$CD4^+CD25^+$			1.01 (0.76-1.35)	.931	1.23 (0.95-1.59)	.113	149
	$CD4^+25^{high}CD127^{low/-}$			0.92 (0.67-1.28)	.636	**1.35 (1.03-1.79)**	**.034**	131
LPS	$CD4^+CD25^+$			1.03 (0.71-1.49)	.877	1.03 (0.75-1.43)	.838	148
	$CD4^+25^{high}CD127^{low/-}$			0.97 (0.65-1.47)	.900	**1.60 (1.13-2.26)**	**.009‡**	132

GMR, Geometric mean ratio; *U*, unstimulated.

**P* value of regression analysis in the log-transformed data: significant results ($P < .05$) are shown in boldface.

†Numbers can vary because of availability of sample and parameters.

‡Significant after adjustment for multiple testing.

TABLE E2. Association of activated T-cell and Treg cell numbers with farm milk consumption at age 4.5 years stratified by different subgroups

		Farm children (n = 124 farm milk vs 25 nonfarm milk)*		All reference children (n = 24 farm milk vs 125 nonfarm milk)*		Reference children with contact with stables (n =12 farm milk vs 19 nonfarm milk)*		Reference children without contact with stables (n = 12 farm milk vs 106 nonfarm milk)*	
Stimuli	**Intracellular marker**	**GMR (95% CI)**	***P* value†**	**GMR (95% CI)**	***P* value†**	**GMR (95% CI)**	***P* value†**	**GMR (95% CI)**	***P* value†**
U	$CD4^+CD25^+$	1.33 (0.99-1.77)	.060	1.32 (0.93-1.89)	.124	1.62 (0.89-2.94)	.126	1.37 (0.85-2.21)	.199
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$	1.16 (0.76-1.78)	.492	1.33 (0.87-2.06)	.194	1.78 (0.85-3.74)	.143	1.54 (0.87-2.71)	.138
PI	$CD4^+CD25^+$	1.10 (0.82-1.48)	.532	**1.54 (1.11-2.13)**	**.011‡**	1.74 (1.00-3.03)	.065	1.54 (1.00-2.38)	.054
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$	1.14 (0.83-1.55)	.422	**1.66 (1.21-2.29)**	**.002‡**	1.68 (0.90-3.14)	.119	**1.79 (1.19-2.71)**	**.007‡**
LPS	$CD4^+CD25^+$	1.29 (0.93-1.79)	.123	**1.44 (1.04-2.00)**	**.031**	1.26 (0.69-2.30)	.458	**1.82 (1.17-2.82)**	**.009‡**
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$	1.40 (0.98-2.02)	.067	**1.60 (1.04-2.47)**	**.035**	1.21 (0.53-2.78)	.658	**2.32 (1.31-4.12)**	**.005‡**

GMR, Geometric mean ratio; *U*, unstimulated.

*All analyses are adjusted for center.

†*P* value of regression analysis in the log-transformed data: significant results ($P < .05$) are shown in boldface.

‡Significant after adjustment for multiple testing.

TABLE E3. Association of markers for activated T cells or Treg cells with specific milk exposures

		Mixed milk vs shop milk (n = 183, 48 vs 135)*		Farm milk vs shop milk (n = 235, 100 vs 135)*		Boiled farm milk vs shop milk (n = 186, 51 vs 135)*		Unboiled farm milk vs shop milk (n = 217, 82 vs 135)*	
Stimuli	**Intracellular marker**	**GMR (95% CI)**	***P* value†**	**GMR (95% CI)**	***P* value†**	**GMR (95% CI)**	***P* value†**	**GMR (95% CI)**	***P* value†**
U	$CD4^+CD25^+$	1.25 (0.96-1.64)	.104	1.32 (1.00-1.75)	.051	1.30 (0.97-1.74)	.082	1.15 (0.87-1.52)	.312
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$	1.12 (0.78-1.60)	.532	1.31 (0.91-1.91)	.148	1.15 (0.78-1.69)	.489	1.07 (0.74-1.55)	.702
PI	$CD4^+CD25^+$	**1.38 (1.06-1.78)**	**.016**	1.14 (0.87-1.49)	.332	1.12 (0.85-1.47)	.426	1.23 (0.94-1.60)	.130
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$	**1.43 (1.10-1.86)**	**.008‡**	1.31 (0.99-1.72)	.054	1.21 (0.91-1.60)	.185	1.30 (0.99-1.71)	.057
LPS	$CD4^+CD25^+$	1.22 (0.93-1.61)	.150	**1.37 (1.03-1.81)**	**.030**	1.14 (0.85-1.52)	.394	1.25 (0.95-1.64)	.109
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$	1.29 (0.93-1.80)	.131	**1.51 (1.08-2.13)**	**.017**	1.27 (0.89-1.80)	.187	1.32 (0.95-1.83)	.104

GMR, Geometric mean ratio; *U*, unstimulated.

*All analyses are adjusted for center.

†*P* value of regression analysis in the log-transformed data: significant results ($P < .05$) are shown in boldface.

‡Significant after adjustment for multiple testing.

TABLE E4. Association of activated T-cell and Treg cell numbers with pattern of milk consumption

		In pregnancy, yes; in childhood, no (n = 10)		In pregnancy, no; in childhood, yes (n = 54)*		In pregnancy, yes; in childhood, yes (n = 122)*	
Stimuli	**Intracellular marker**	**GMR (95% CI)**	***P* value†**	**GMR (95% CI)**	***P* value†**	**GMR (95% CI)**	***P* value†**
U	$CD4^+CD25^+$	1.03 (0.62-1.71)	.919	1.02 (0.78-1.34)	.863	1.18 (0.91-1.54)	.215
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$	1.12 (0.57-2.19)	.742	0.94 (0.66-1.33)	.717	1.14 (0.81-1.62)	.444
PI	$CD4^+CD25^+$	1.45 (0.87-2.41)	.156	1.23 (0.96-1.59)	.105	1.19 (0.93-1.52)	.178
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$	1.40 (0.83-2.35)	.205	**1.35 (1.04-1.75)**	**.022**	**1.34 (1.04-1.73)**	**.024**
LPS	$CD4^+CD25^+$	1.44 (0.79-2.62)	.233	1.03 (0.78-1.35)	.847	1.19 (0.91-1.55)	.198
	Upper 20% $CD4^+CD25^+$, $FOXP3^+$	1.44 (0.70-2.99)	.322	1.05 (0.76-1.46)	.765	1.38 (1.00-1.90)	.053

GMR, Geometric mean ratio; *U*, unstimulated.

*The reference group were 111 children who themselves and whose mothers did not drink farm milk; analyses are adjusted for center and farming.

†*P* value of regression analysis in the log-transformed data: significant results ($P < .05$) are shown in boldface.