

ORIGINAL ARTICLE

EPIDEMIOLOGY AND GENETICS

A switch in regulatory T cells through farm exposure during immune maturation in childhood

P. C. Schröder^{1,a}, S. Illi^{1,a}, V. I. Casaca^{1,a}, A. Lluís², A. Böck¹, C. Roduit^{3,4}, M. Depner¹, R. Frei^{4,5}, J. Genuneit⁶, P. I. Pfefferle^{7,8}, M. Roponen⁹, J. Weber¹, C. Braun-Fahrlander^{10,11}, J. Riedler^{12,13}, J.C. Dalphin¹⁴, J. Pekkanen^{15,16}, R. Lauener^{4,17}, E. von Mutius^{1,18}, B. Schaub^{1,18} & the PASTURE study group^b

¹LMU Munich, University Children's Hospital, Munich, Germany; ²National Jewish Health, Denver, CO, USA; ³Children's Hospital, University of Zürich, Zürich; ⁴Christine Kühne – Center for Allergy Research and Education (CK-CARE); ⁵Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, Davos, Switzerland; ⁶Institute of Epidemiology and Medical Biometry, Ulm University, Ulm; ⁷Comprehensive Biomaterial Bank Marburg CBBM, Fachbereich Medizin der Philipps Universität Marburg, Zentrum für Tumor und Immunbiologie ZTI, Marburg; ⁸Member of the German Centre for Lung Research (DZL), Munich, Germany; ⁹Department of Environmental Science, Inhalation Toxicology Laboratory, University of Eastern Finland, Kuopio, Finland; ¹⁰Swiss Tropical and Public Health Institute; ¹¹University of Basel, Basel, Switzerland; ¹²Children's Hospital Schwarzbach, Schwarzbach; ¹³Teaching Hospital of Paracelsus Medical Private University Salzburg, Salzburg, Austria; ¹⁴Department of Respiratory Disease, University Hospital, University of Besançon, Besançon, France; ¹⁵Department of Health Protection, National Institute for Health and Welfare, Kuopio; ¹⁶Department of Public Health, University of Helsinki, Helsinki, Finland; ¹⁷Children's Hospital of Eastern Switzerland, St. Gallen, Switzerland; ¹⁸Comprehensive Pneumology Centre Munich (CPC-M), German Centre for Lung Research (DZL), Munich, Germany

To cite this article: Schröder PC, Illi S, Casaca VI, Lluís A, Böck A, Roduit C, Depner M, Frei R, Genuneit J, Pfefferle PI, Roponen M, Weber J, Braun-Fahrlander C, Riedler J, Dalphin J-C, Pekkanen J, Lauener R, von Mutius E, Schaub B the PASTURE study group. A switch in regulatory T cells through farm exposure during immune maturation in childhood. *Allergy* 2016; DOI: 10.1111/all.13069.

Keywords

animal-stable; asthma; farming; milk; regulatory T cells (Tregs).

Correspondence

Prof. Bianca Schaub, MD, LMU Munich, University Children's Hospital, Lindwurmstrasse 4, 80337 Munich, Germany.
Tel.: +49 89 4400 5 7856
Fax: +49 89 4400 5 4764
E-mail: Bianca.Schaub@med.uni-muenchen.de

^aThese authors contributed equally to this work.

^bSee Appendix for investigators of the PASTURE study group.

Accepted for publication 8 October 2016

DOI:10.1111/all.13069

Abstract

Background: Farm exposure protects against development of allergies early in life. At 4.5 years, protection against asthma by farm-milk exposure was partially mediated by regulatory T cells (Tregs). The aim of this study was to investigate the critical time window of the 'asthma-protective' farm effect via Tregs during childhood immune maturation.

Methods: Tregs were assessed longitudinally at 4.5 and 6 years in 111 children (56 farm and 55 reference children) from the PASTURE/EFRAIM birth cohort (flow cytometry). Peripheral blood mononuclear cells were cultured unstimulated (U), with phorbol 12-myristate 13-acetate/ionomycin (PI) or lipopolysaccharide (LPS), and stained for Tregs (CD4⁺CD25^{high}FOXP3^{upper20%}). mRNA expression of Treg/Th1/Th2/Th17-associated cell markers was measured *ex vivo*. Suppressive capacity of Tregs on effector cells and cytokines was assessed. Detailed questionnaires assessing farm exposures and clinical phenotypes from birth until age 6 years were answered by the parents.

Results: Treg percentage before and after stimulation and *FOXP3* mRNA expression *ex vivo* decreased from age 4.5 to 6 years ($P(U,LPS) < 0.001$; $P(PI) = 0.051$; $P(FOXP3) < 0.001$). High vs low farm-milk and animal-stable exposure was associated with decreased LPS-stimulated Treg percentage at age 6 years ($P(LPS) = 0.045$). Elevated LPS-stimulated-Treg percentage at age 6 was associated with increased risk of asthma (aOR = 11.29, CI: 0.96–132.28, $P = 0.053$). Tregs

Abbreviations

A, asthmatics; aOR, adjusted odds ratio; BIC, Bayesian information criteria; CI, confidence interval; FOXP3, forkhead box P3; GMR, geometric mean ratio; LCA, latent class analysis; LPS, lipopolysaccharide; NA, nonasthmatics; N, number of subjects; PASTURE/EFRAIM, Protection Against Allergy: Study in Rural Environments/Mechanisms of Early Protective Exposures on Allergy Development; PBMC, peripheral blood mononuclear cells; PI, PMA/ionomycin; PMA, phorbol 12-myristate 13-acetate; SIC, specific IgE class; Tregs, regulatory T cells; U, unstimulated; Yrs, years.

Edited by: De Yun Wang

from asthmatics vs nonasthmatics suppressed IFN- γ ($P = 0.015$) and IL-9 ($P = 0.023$) less efficiently. mRNA expression of Th1/Th2/Th17-associated cell markers decreased between 4.5 and 6 years ($P < 0.001$).

Conclusions: Tregs at the age of 6 years were decreased with farm exposure and increased within asthmatics, opposite to age 4.5 years. This immunological switch defines a critical 'time window' for Treg-mediated asthma protection via environmental exposure before age 6 years.

Accumulating evidence of the asthma/allergy-protective farm effect has been replicated in numerous studies (1). Children living in microbe-rich environments, for example farms, have a lower risk of developing asthma and allergies (2, 3). Regarding the time window, prenatal and early-life exposure exhibited the strongest 'disease-protective' effect (4, 5). In this context, farm-milk consumption was identified as one critical specific exposure accounting in part for the farm effect (4, 6, 7). On the search for relevant underlying mechanisms conferring allergy and asthma protection via rural farm environments, cross-sectional studies implicated a role for innate receptors such as *Toll-like receptor 2* and *CD14* (5, 8). Both were increased in farmer's children (8), and *TLR2/TLR4/CD14* were increased following prenatal animal-stable exposure (5). Importantly adaptive cellular mechanisms, namely regulatory T cells (Tregs), which are crucial for maintenance of immune homeostasis by controlling exuberant immune responses (9), were higher and more suppressive in newborns of farming compared to nonfarming mothers (10). Consistently, a protective effect of farm-milk exposure on asthma, partly mediated by Tregs, was shown in 4.5-year-old farm children of the PASTURE/EFRAIM (Protection Against Allergy: Study in Rural Environments/Mechanisms of Early Protective Exposures on Allergy Development) birth cohort (11). From a longitudinal perspective, farm-associated Treg modulation observed early in life was still present with 4.5 yrs (10, 11).

Our recent study pointed towards a counterregulatory effect of increased and functionally effective Tregs in allergic asthmatics (12). Conversely, other studies on Tregs in asthma showed inconsistent results with up- and downregulated Tregs, however, partly for small sample sizes (13–16).

The objectives of this study were to investigate (i) whether the observed protective effect of an early farm environment on asthma mediated by Tregs at 4.5 yrs is tracking during maturation until 6 yrs and (ii) whether a critical time window for this environmental protection exists that potentially ends before school age. Thus, we assessed Tregs on protein and mRNA level and partly Treg function for the specific nature of farm exposure and the outcome asthma.

Methods

Study characteristics

The PASTURE/EFRAIM Study is an international birth cohort including 1133 children from rural areas of five European countries (Germany, Switzerland, Austria, Finland, France) (17) with follow-up from the third trimester of

pregnancy until age 6 yrs, comprising children who live and do not live on farms. These children and their families were invited to participate in follow-ups that included questionnaires and/or blood sampling. Questionnaires were administered at the end of pregnancy and when the children were 2 months and 1, 1.5, 2, 3, 4, 5 and 6 years old (Fig. S1). Furthermore, blood samples were taken at the age of 4.5 and 6 yrs. Specific IgE was measured in all blood samples, and mRNA expression was measured in all blood samples with sufficient material left. In this study, children with measurements of mRNA expression at both time points are included in the 'mRNA-population'. In all German children with blood sampling at age 4.5 or 6 yrs, Treg expression was assessed. Of note, children with measurements of Treg expression at both time points are included in the 'Treg-population'.

The study was approved by the local research ethics committee from each country, and written informed consent was obtained from all parents.

Questionnaires and clinical definitions

Extensive questionnaires (4, 17, 18) were completed by the parents at the respective follow-ups. The questionnaires assessed information on general health, with a focus on doctor's diagnosis of atopic and respiratory diseases and on farm exposures.

A child was considered a 'farm child' when parents answered 'yes' to the question 'Does your child live on a farm?' and whose family ran the farm. Specific farm exposures were assessed longitudinally and included, among others, consumption of farm-milk and regular stays in an animal-stable, as well as farm characteristics comprising types of animal breeding and cultivation (described in (11)). Other factors such as socioeconomic status, family history of atopy and maternal smoking were noted. Children not living on a farm could be exposed also to farm animals if they live next to or near to a farm.

Asthma was assessed at age 4 and 6 yrs when the clinical phenotype of asthma is rather stable (19). Current asthma at a given age was defined as a doctor's diagnosis of asthma ever plus current wheeze (in the last 12 months), both assessed at the given age. To define a lifetime diagnosis of asthma, we combined current asthma at either age 4 or 6 yrs.

Specific IgE

Sensitization to allergens was measured as previously described (11) (details in Supporting Information).

mRNA expression of T-cell markers

mRNA expression was assessed in blood samples collected at 4.5 and 6 yrs of age and expressed as $\Delta\Delta C_t$ values (details in Supporting Information).

Cell isolation and stimulation

Peripheral blood mononuclear cells (PBMC) for flow cytometry were isolated within 24 h, kept unstimulated (U) and stimulated for 24 h with PMA (5 ng/ml) and ionomycin (1 μ g/ml) (PI), or LPS (0.1 μ g/ml; details in Supporting Information). Based on cell availability, sample number varied between stimulation conditions for the Treg-population (Fig. S1).

Treg quantity and functional assessment

Quantitative Treg (defined as percentage of $CD4^+CD25^{high}FOXP3^{upper20\%}$ in lymphocytes) and functional Treg assessment was performed as previously described in (11) and (12), respectively (details in Supporting Information). Supernatants from functional Tregs assays were collected for assessment of IFN- γ , IL-13, IL-5, IL-9, IL-10, IL-2 and IL-1 β and by LUMINEX technology using the Human Cytokine-Multiplex-Assay-Kit following the manufacturer's instructions (Bio-Rad, Munich, Germany; details in Supporting Information).

Statistical analysis

Differences between population characteristics were tested with chi-square test. mRNA expression and Tregs (%) were depicted as median values. Due to non-normality, differences in mRNA expression and Tregs (%) between exposure groups or phenotypes were assessed by nonparametric tests, that is the Wilcoxon test, and differences between ages 4.5 and 6 yrs by paired Wilcoxon signed-rank test. The effect of log-transformed Tregs (%) on asthma was assessed by farm-adjusted logistic regression. Cytokine levels in Treg suppression assays were log-transformed, and comparison before vs after adding Tregs was conducted separately for each exposure and phenotype using a paired *t*-test. Comparison between exposures or phenotypes was conducted using a two-sample *t*-test. In case of non-normally distributed cytokine data, a Wilcoxon signed-rank test or the Wilcoxon rank-sum test was used (details in Supporting Information). For plotting purposes, data shown in forest plots were retransformed (antilog) to original units and presented as geometric mean ratios (GMR) and confidence intervals.

To define a lifetime pattern for farm-milk and animal-stable exposure, we performed latent class analyses (LCAs) with a four-class solution – the solution with the highest Bayesian information criteria (BIC) – based on all available exposure data on farm-milk and animal-stable exposure (i.e. pregnancy, 1, 1.5, 2, 3, 4, 5, 6 yrs). All participants of the PASTURE/EFRAIM study with at least seven of eight available data points per exposure (farm-milk, animal-stable) were

included. Individuals were assigned to the class for which they had the highest posterior probability of belonging. Three separate LCAs were performed including farm-milk exposure over time, animal-stable exposure over time and both exposures together, as farm-milk and animal-stable are highly correlated. The resulting four latent classes from the latter LCA were labelled 'high farm-milk and animal-stable exposure', 'high farm-milk and low animal-stable exposure', 'low farm-milk and high animal-stable exposure' and 'low farm-milk and animal-stable exposure'. Figure S2 shows the prevalence of farm-milk and animal-stable exposure over time within these four latent classes.

Because the study was based on previous knowledge and the variables were highly correlated, corrections for multiple testing were not performed (20). A *P*-value 0.05 or lower was considered significant. Statistical analysis was performed using SAS 9.3 software (SAS Institute, Inc., Cary, NC, USA) and R.

Results

Population characteristics

Of the 1133 enrolled children in the PASTURE/EFRAIM cohort, 920 children participated in both the 4- and 6-year questionnaires (Fig. S1). Furthermore, 628 participated in blood sampling at 4.5 and 6 yrs. Specific IgE was measured in all of these available blood samples. The sample size for mRNA measurements ($N = 549$, 'mRNA-population') was reduced due to insufficient amounts of blood available. We report Tregs ('Treg-population') only in the German arm of the study, comprising about one-fifth of the total study population. Due to cell availability, only $N = 111$ were in the final 'Treg-population' (56 farmer's children, 55 reference non-farming children), and sample size differed between stimulation conditions.

Characteristics of the PASTURE/EFRAIM birth cohort and relevant subpopulations are given in Table 1. As observed in other cohorts (21), repeated participation in blood sampling was significantly higher for children with parental atopy. Also, farm children were more willing to participate in repeated blood sampling. However, the Treg-population did not differ significantly from other subpopulations.

Tregs decreased during immune maturation from age 4.5 to 6 yrs

We assessed immune maturation of Tregs between 4.5 and 6 yrs in all children with available Treg data at both time points. Overall, Tregs (%) significantly decreased from age 4.5 to 6 yrs (Fig. 1A–C). In unstimulated and LPS-stimulated cells, Tregs decreased significantly from a median of 0.20% (4.5 yrs) to 0.13% (6 yrs, $P < 0.001$) and 0.27% to 0.13% ($P < 0.001$), respectively. In PMA/ionomycin-stimulated cells, Tregs decreased from a median of 0.59% to 0.41% ($P = 0.051$). Coherent with Treg flow cytometry data from unstimulated cells, mRNA expression of Treg-associated marker *FOXP3* measured in cells *ex vivo* (unstimulated)

Table 1 Population characteristics

	Total population; N = 1133		Questionnaire population (questionnaire at 4 and 6 yrs); N = 920		Blood sample pop- ulation (blood sam- ple at 4.5 and 6 yrs); N = 628		mRNA-population (mRNA at 4.5 and 6 yrs); N = 549		Treg-popu- lation (Treg at 4.5 and 6 yrs); N = 111	
	n/N	%	n/N	%	n/N	%	n/N	%	n/N	%
Parental atopy	591/1099	53.8	495/915	54.1	353/625	56.5*,**	316/546	57.9*,**,*	56/110	50.9
Maternal atopy	371/1132	32.8	305/920	33.1	223/628	35.5*,**	200/549	36.4*,**	37/111	33.3
Paternal atopy	342/1082	31.6	290/910	31.9	211/620	34.0*,**	190/541	35.1*,**	36/109	33.0
≥2 older sibs	379/1133	33.4	310/920	33.7	216/628	34.4	189/549	34.4	31/111	27.9
Females	530/1090	48.6	445/918	48.5	296/626	47.3	262/547	47.9	53/111	47.7
Maternal smoking in pregnancy	155/1126	13.8	112/914	12.2*	76/623	12.2	68/544	12.5	10/111	9.0
Living on a farm	530/1133	46.8	441/920	47.9	310/628	49.4*	280/549	51.0*,**,*	56/111	50.4
Farm-milk/animal-stable exposure (LCA)										
High farm-milk, high animal-stable	358/938	38.2	340/897	37.9	242/611	39.6	210/532	39.5	46/110	41.8
High farm-milk, low animal-stable	87/938	9.3	83/897	9.2	57/611	9.3	48/532	9.0	6/110	5.4
Low farm-milk, high animal-stable	121/938	12.9	118/897	13.1	73/611	11.9	70/532	13.2	16/110	14.5
Low farm-milk, low animal-stable	372/938	39.7	356/897	39.7	239/611	39.1	204/532	38.3	42/110	38.2
Asthma†	–	–	83/920	9.0	63/623	10.1	57/544	10.5	15/111	13.5
Seasonal sensitization (SIC 3)‡	–	–	–	–	96/628	15.3	86/548	15.7	20/111	18.0
Any sensitization (SIC 3)‡	–	–	–	–	150/628	23.9	131/548	23.9	25/111	22.5

* $P < 0.05$ vs total population; ** $P < 0.05$ vs questionnaire population; *** $P < 0.05$ vs blood sample population.

†At age 4 and/or 6 yrs where questionnaires were available.

‡At age 4.5 and/or 6 yrs where questionnaires and blood samples were available.

LCA, latent class analysis; yrs, years.

decreased between age 4.5 and 6 yrs in the Treg-population (median of 1.22 (4.5 yrs) to 0.62 (6 yrs), $P < 0.001$; Fig. 1D) and mRNA-population (median of 1.39 (4.5 yrs) to 0.80 (6 yrs), $P < 0.001$; Fig. 1E).

Immunological switch: only high, but not low, farm-milk intake and animal-stable exposure was associated with decreased LPS-stimulated Tregs at age 6 yrs

To define a lifetime pattern for farm-milk and animal-stable exposure, we performed latent class analyses (LCAs) with a four-class solution. Most children were classified as low ($n = 372$; 39.7%) or high ($n = 358$; 38.2%) farm-milk/animal-stable-exposed in the total PASTURE/EFRAIM population, which did not differ significantly from the mRNA- and Treg-population (Table 1).

We compared Treg percentages over time for both extreme exposure classes from the four-class LCA solution, low vs high farm-milk/animal-stable (Fig. 2), and both exposures independently, low vs high farm-milk (Fig. S3) and animal-stable exposure (Fig. S4). At 4.5 yrs, children with high farm-milk/animal-stable exposure showed higher Tregs (%) (in PMA/ionomycin- and LPS-stimulated conditions) compared to low farm-milk/animal-stable-exposed (median 0.67% vs 0.51%, $P = 0.064$ for PMA/ionomycin; median 0.38% vs 0.16%, $P = 0.005$ for LPS; Fig. 2B, C). At 6 yrs, the opposite was found and LPS-stimulated Tregs (%) were higher in low compared to high farm-milk/animal-stable-exposed children (median 0.18% vs 0.14%, $P = 0.045$;

Fig. 2C). When analysed independently, similar effects were found for animal-stable exposure (Fig. S4), but not farm-milk (Fig. S3).

When Tregs (%) were considered longitudinally from 4.5 to 6 yrs, a significant decrease was found in high farm-milk/animal-stable-exposed (median 0.67% vs 0.32%, $P = 0.003$ for PI; median 0.38% vs 0.14%, $P < 0.001$ for LPS), but not in PMA/ionomycin- and LPS-stimulated low farm-milk/animal-stable-exposed children (Fig. 2B, C). Consistent with unstimulated Treg flow cytometry data, *ex vivo* mRNA expression of Treg-associated marker *FOXP3* decreased from 4.5 to 6 yrs but was independent of farm-milk/animal-stable exposure in the Treg- and mRNA-population (Fig. 2D, E). A similar pattern was detected for farm-milk and animal-stable exposure (Figs S3 and S4) on protein (flow cytometry) and mRNA expression.

Asthmatic compared to nonasthmatic children exhibited higher Treg percentage with 6 yrs

Previously, we showed an inverse association of LPS-stimulated Tregs at age 4.5 yrs with asthma (aOR (farm-adjusted) = 0.26, CI: 0.08–0.88, $P = 0.030$) (11). Strikingly, LPS-stimulated Tregs at age 6 yrs showed a strong positive association with asthma (aOR (farm-adjusted) = 11.29, CI: 0.96–132.28, $P = 0.053$). LPS-stimulated Tregs (%) at age 6 yrs were higher in asthmatic children (A) compared to nonasthmatics (NA) (median 0.20% vs 0.11%, $P = 0.011$; Fig. 3C).

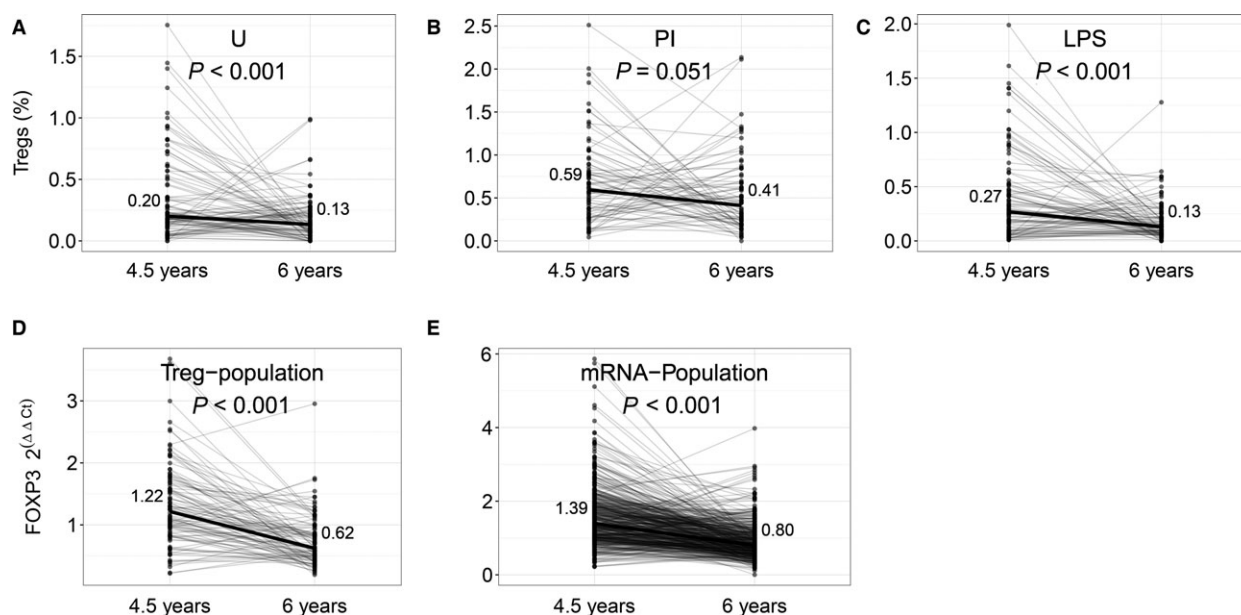


Figure 1 Decrease in Tregs (%) and *FOXP3* mRNA expression between 4.5- and 6-year-old children. U, unstimulated; PI, PMA/ionomycin; LPS, lipopolysaccharide; Panels A–C (flow cytometry): percentage of U-, PI- and LPS-stimulated Tregs where cells were available at 4.5- and 6-year-old PASTURE/EFRAIM children.

Panels D and E (mRNA expression): Treg-associated marker *FOXP3* measured in the Treg-population (D) and mRNA-population (E). $n(A/B/C) = 95/80/100$; $n(D/E) = 102/544$. Flow cytometry and mRNA expression data for both ages are presented in median values.

Longitudinally, unstimulated and LPS-stimulated Tregs (%) of nonasthmatics decreased from 4.5 to 6 yrs (median 0.19% vs 0.13, $P = 0.002$, U; median 0.29% vs 0.11%, $P < 0.001$ LPS; Fig. 3A, C), while no changes were observed within asthmatics. Consistent data within 4.5 or 6 yrs and longitudinally between 4.5 and 6 yrs were found for mRNA expression of Treg-associated marker *FOXP3* in the Treg- and mRNA-population (Fig. 3D, E), while the longitudinal decrease for asthmatics in flow cytometry data was not significant (0.21% vs 0.16%, $P = 0.146$, Fig. 3A).

No differences were observed for stimulated or unstimulated Tregs (%) in sensitized vs nonsensitized children (any inhalant sensitization SIC 3) at 4.5 and 6 yrs (not shown). Longitudinally, from 4.5 to 6 yrs, only nonsensitized children presented decreased Tregs (U: median 0.22% vs 0.12%, $P < 0.001$; PMA/ionomycin: median 0.61% vs 0.34%, $P = 0.022$; LPS: median 0.31% vs 0.13%, $P < 0.001$), but not sensitized children (SIC 3, U: median 0.15% vs 0.21%, $P = 0.666$; PMA/ionomycin: median 0.58% vs 0.51%, $P = 0.832$; LPS: median 0.17% vs 0.15%, $P = 0.419$; see also Table S5).

Drinking farm-milk, animal-stable exposure and asthma status influenced cytokine modulation through Tregs in 6-year-old children

Tregs from children with high vs low farm-milk/animal-stable exposure did not differ in their suppressive capacity, but both

could efficiently suppress effector cell division ($n(\text{high/low}) = 6/10$; $P = 0.03/0.001$, not shown). However, Tregs from children with high or low exposure to farm-milk/animal-stable were functionally different in their capacity to suppress cytokine secretion. While Tregs from children with high and low exposure to farm-milk/animal-stable significantly suppressed IFN- γ and IL-13 (Fig. 4A; GMR before/after Treg addition, high: $P(\text{IFN-}\gamma) = 0.035$, $P(\text{IL-13}) = 0.034$; low: $P(\text{IFN-}\gamma) = 0.049$, $P(\text{IL-13}) = 0.004$), IL-5, IL-9 and IL-10 were solely suppressed by Tregs from high-exposed (Fig. 4A; $P(\text{IL-5}) = 0.046$, $P(\text{IL-9}) = 0.026$, $P(\text{IL-10}) = 0.04$) and IL-2 by Tregs from low-exposed children (Fig. 4A; $P = 0.029$). IL-1 β was not significantly changed in both groups. No differences for any cytokine were found when high- vs low-exposed children were compared. Similar effects on cytokines were observed for farm-milk and animal-stable exposure (not shown).

Tregs from children with asthma and without asthma did not differ in their suppressive capacity (not shown). Additionally, Tregs of nonasthmatics suppressed cytokine secretion of IFN- γ , IL-13, IL-9, IL-10 and IL-2 (Fig. 4B; $n(\text{asthma/nonasthma}) = 4/13$; GMR: before/after Treg addition, $P(\text{IFN-}\gamma) = 0.003$, $P(\text{IL-13}) < 0.001$, $P(\text{IL-9}) = 0.008$, $P(\text{IL-10}) = 0.033$, $P(\text{IL-2}) = 0.030$), and Tregs from asthmatics significantly suppressed only IFN- γ (Fig. 4B; $P(\text{IFN-}\gamma) = 0.018$). For IFN- γ and IL-9, Treg-mediated suppression was significantly different between nonasthmatics and asthmatics (Fig. 4B; $P(\text{IFN-}\gamma) = 0.015$, $P(\text{IL-9}) = 0.023$).

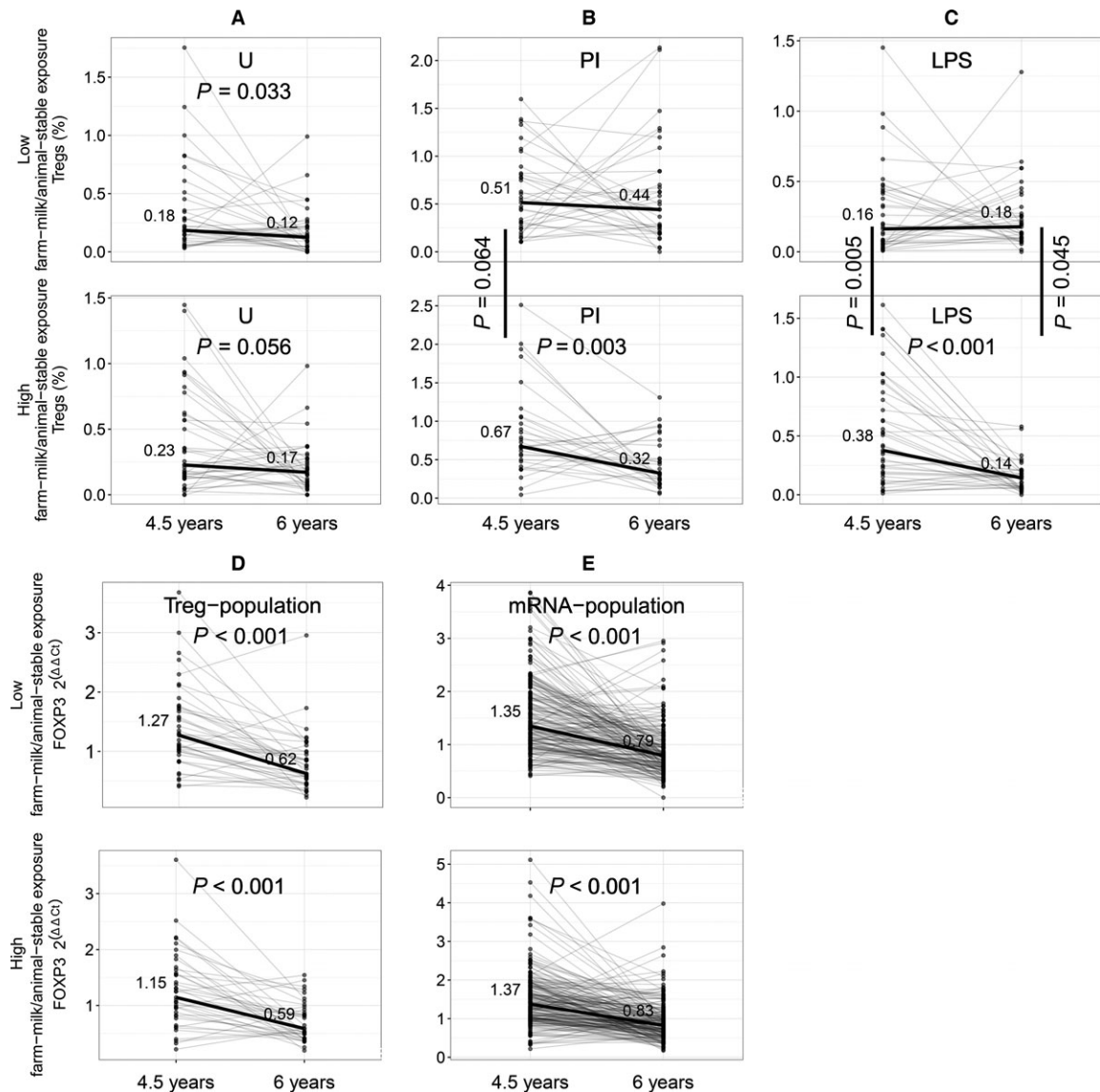


Figure 2 At age 6 yrs, LPS-stimulated Tregs (%) were lower when children had high compared to low farm-milk/animal-stable exposure. Tregs (%) and *FOXP3* mRNA expression of 4.5- and 6-year-old PASTURE/EFRAIM children stratified for farm-milk/animal-stable exposure (low vs high) are shown. U, unstimulated; PI, PMA/ionomycin; LPS, lipopolysaccharide. Panels A–C (flow cytometry): percentage of U-, PI- and LPS-stimulated Tregs where cells were available at 4.5- and 6-year-old children. Panels D and E (mRNA

expression): Treg-associated marker *FOXP3* measured in the Treg-population (D) and mRNA-population (E). *n*(A/B/C low farm-milk/animal-stable exposure) = 36/35/37; *n*(A/B/C high farm-milk/animal-stable exposure) = 39/31/41; *n*(D/E low farm-milk/animal-stable exposure) = 41/201; *n*(D/E high farm-milk/animal-stable exposure) = 39/209. Flow cytometry and mRNA expression data for both ages are presented in median values.

Expression of Th2-, Th1- and Th17-associated markers decreases during immune maturation independent of exposure and asthma phenotype

To assess the influence of farm-milk/animal-stable exposure and asthma phenotype on the behaviour of Th2-, Th1- and Th17-associated markers during immune maturation, mRNA expression of *GATA3*, *STAT6* (A, B, C isoforms (22)), *TBX21*

and *RORC* was assessed in the mRNA-population *ex vivo*. Overall, Th2 (*GATA3*, *STAT6A*, *STAT6B*, *STAT6C*)-, Th1 (*TBX21*)- and Th17 (*RORC*)-associated markers decreased from 4.5 to 6 yrs ($P < 0.001$; Fig. 5). This pattern remained unchanged when stratified for farm-milk/animal-stable exposure or asthma (Figs S5 and S6). However, the Th1-associated marker *TBX21* with 6 yrs was increased in nonasthmatics vs asthmatics ($P = 0.027$; Fig. S6).

Discussion

Protection against childhood asthma in a farming environment, partly mediated via Tregs, was demonstrated previously by our group (11). In this study, we now identified an immunological switch during immune maturation occurring between age 4.5 and 6 yrs: first, at age 6 yrs children with high compared to low farm exposure exhibited lower Tregs, while the opposite occurred at 4.5 yrs. Second, a previously published inverse association of Tregs with asthma at 4.5 yrs (11) switches into a strong positive association at 6 yrs. Tregs at 6 yrs were higher in asthmatics compared to nonasthmatics, contrary to what was occurring at 4.5 yrs (11). Thus, asthma protection through farm exposure partly mediated by Tregs present at 4.5 yrs (11) vanished at 6 yrs defining a critical time window in immune maturation between 4.5 and 6 yrs. Third, we identified a trajectory of significantly decreasing Tregs in parallel with decreasing *FOXP3* *ex vivo* mRNA expression during immune maturation between 4.5 and 6 yrs in the PASTURE/EFRAIM birth cohort. This was most prominent for children with high compared to low farm exposure.

Several cross-sectional studies have demonstrated the protective effect of farming against development of atopic diseases in childhood (4, 18, 23). The ALEX and PARSIFAL studies reported a reduced risk for childhood atopic diseases when farm-milk was consumed in the first year or ever in life (4, 6, 7). Recently, our group showed an asthma-protective effect via farm-milk exposure partly mediated by Tregs at age 4.5 yrs (11). The present study now enhances the 'asthma-protective' role of Tregs before age 6 yrs via farm exposure identifying an immunological switch of Tregs (%) between 4.5 and 6 yrs. Different to age 4.5 yrs where Tregs were increased in high vs low farm-exposed children, at age 6 yrs Tregs were lower in high farm- vs low farm-exposed children. The fact that farm-milk- and animal-stable-exposed children expressed similar high levels of LPS-stimulated Tregs at age 4.5 yrs compared to low-exposed children points towards a Treg induction, potentially to ensure healthy immune balance. This is no longer the case with 6 yrs defining a so-called window of opportunity, a critical time period for immune regulation. Different to age 4.5 yrs (11), at the age of 6 yrs Tregs from children with asthma were increased compared to healthy children, which is strongly supported by our own study on school-age asthma with increased Tregs in asthmatics compared to healthy children (12). These findings potentially indicate a counterregulatory mechanism in which Tregs from asthmatic compared to nonasthmatic children get increased during maturation to counterbalance exuberant allergic responses.

Our findings in the large epidemiological PASTURE/EFRAIM cohort of Tregs decreasing between 4.5 and 6 yrs are in accordance with previous reports showing a decline in Tregs during the first 36 months of life (24). While in cord blood compared to adults higher proportions of Tregs were found (25, 26), studies are inconsistent reporting either a minor change on circulating Tregs throughout ageing (27) or increases in thymic Tregs with age in atopic and

nonatopic children (age 1 week to 14 yrs) who underwent cardiopulmonary bypass (28). Our previous data of increased Tregs in asthmatic children in another cohort are supporting the fact that the switch here between age 4.5 and 6 yrs is still present at the median age of 9.5 yrs in the CLARA study (12). Tregs of farm-exposed children or of children without asthma decreased over time; thus, only 'protected children' were affected by a decrease in Tregs upon stimulation over time. Similarly, Tregs (%) did not change in sensitized children between 4.5 and 6 yrs, whereas Tregs from nonsensitized children decreased from 4.5 to 6 yrs. Several explanations are possible: with efficient induction of Tregs and healthy immune regulation, a further increase may not be required in healthy children. In case of allergic inflammation as in asthma and inefficient immune balance, it may actually be critical to induce Tregs for potentially controlling ongoing allergic inflammation. However, increased numbers may not be sufficient; thus, Treg-mediated cytokine suppression may be impaired. From a longitudinal perspective, these results may indicate no requirement for increased Tregs in the absence of asthma or allergic sensitization during maturation. During an early period in life, the immune system may be more susceptible to modulation through farm exposure, while during further maturation a 'tolerance phenomenon', induced by continuous stimulation, may occur.

Importantly, the decrease in Tregs (unstimulated), assessed by flow cytometry, was supported by decreased *ex vivo* mRNA expression of Treg-associated marker *FOXP3* in the larger mRNA-population, underpinning the robustness of our results. Additionally, LPS stimulation seems crucial to detect differences with 6 yrs in Tregs between high- and low-exposed children to farm-milk/animal-stable and animal-stable only and between nonasthmatics and asthmatics. Th1-, Th2- and Th17-associated cell markers *TBX21*, *GATA3*, *STAT6A*, *STAT6B*, *STAT6C* and *RORC* decreased over time following the same pattern as the Treg-associated marker *FOXP3* and Tregs. Increase in Th1-associated marker *TBX21* in nonasthmatics may potentially indicate a shift towards Th1. Further experiments are required for proving changes associated with cell-specific regulation. Overall and apart from *STAT6C* in the German population, a centre effect for mRNA expression of Treg-, Th1-, Th2- or Th17-associated cell markers via stratified analyses was not found (Table S1).

In this study, Treg suppressive activity *per se* was not different between high- and low-exposed farm-milk/animal-stable children, nor between children with and without asthma. Although functional examination was only feasible in a limited number of children in this study, our recent cross-sectional asthma study supports similar functional Treg suppression in asthmatics vs nonasthmatics (12). Yet, Treg functional capacity to suppress cytokines was significantly suppressed in children with high but not low exposure to farm-milk/animal-stable and in nonasthmatics but not in asthmatics for IL-9, a cytokine being preferentially secreted by Th9 cells. Suppression of IFN- γ (Th1) by Tregs in asthmatics was significantly lower than in nonasthmatics.

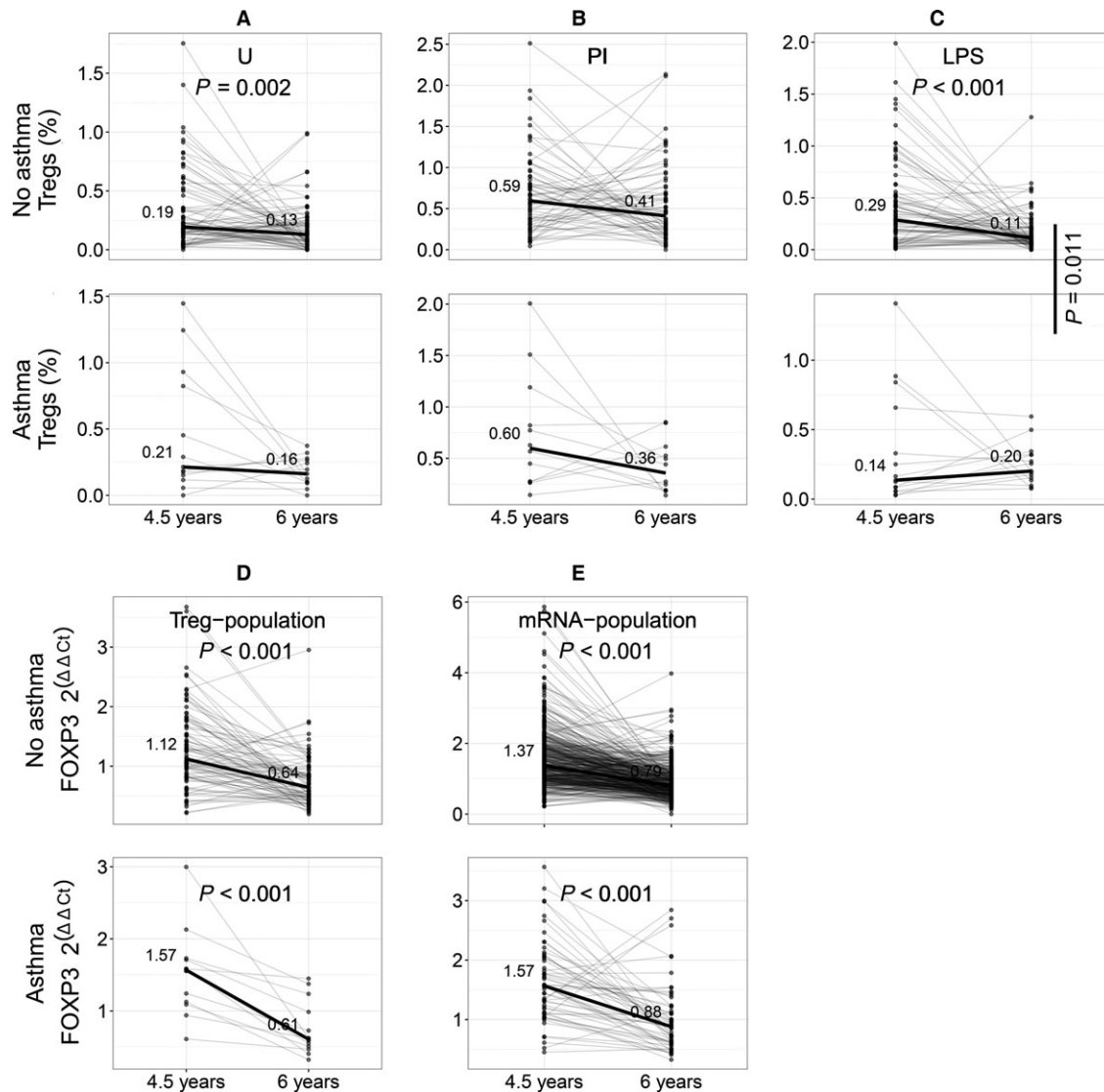


Figure 3 Tregs (%) at age 6 yrs were increased in asthmatic but not in nonasthmatic children. Tregs (%) and *FOXP3* mRNA expression of 4.5- and 6-year-old PASTURE/EFRAIM children stratified for asthma (A) vs no asthma (NA). U, unstimulated; PI, PMA/ionomycin; LPS, lipopolysaccharide. Panels A–C (flow cytometry): percentage of U-, PI- and LPS-stimulated Tregs where cells were available at 4.5- and 6-year-old children. Panels D and E (mRNA

expression): Treg-associated marker *FOXP3* measured in the Treg-population (D) and mRNA-population (E). $n(A/B/C)$ of no asthma = 82/68/85; $n(A/B/C)$ of asthma = 13/12/15; $n(D/E)$ of no asthma = 88/483; $n(D/E)$ of asthma = 14/56. Flow cytometry and mRNA expression data for both ages are presented in median values.

Therefore, IFN- γ levels in asthmatics vs nonasthmatics were higher, being in accordance with increased IFN- γ in BAL cells in asthmatic subjects (29) and increased levels of IFN- γ -producing CD8⁺ T cells in asthmatics (30).

A few important aspects of the study require consideration. Tregs were defined as CD4⁺CD25^{high}FOXP3⁺ upper 20%, which presented a *FOXP3* demethylation status of 99.0% on the Treg-specific demethylated region and is most specific for humans as reported (9, 11). Tregs were assessed in PBMC as in many epidemiological studies in human childhood, thus

not directly investigating local tissues. Due to the limited number of asthma cases or low cell availability, more detailed analysis, for example further subgroup analysis of phenotypes, identification of the origin of the cytokine-secreting subpopulations of effector T cells in Treg suppression assays or comparison for Treg function between age 4.5 and 6 yrs, was not feasible.

The main strength of this study from PASTURE/EFRAIM is the comprehensive assessment of farm-related exposures and disease development until 6 yrs in a large

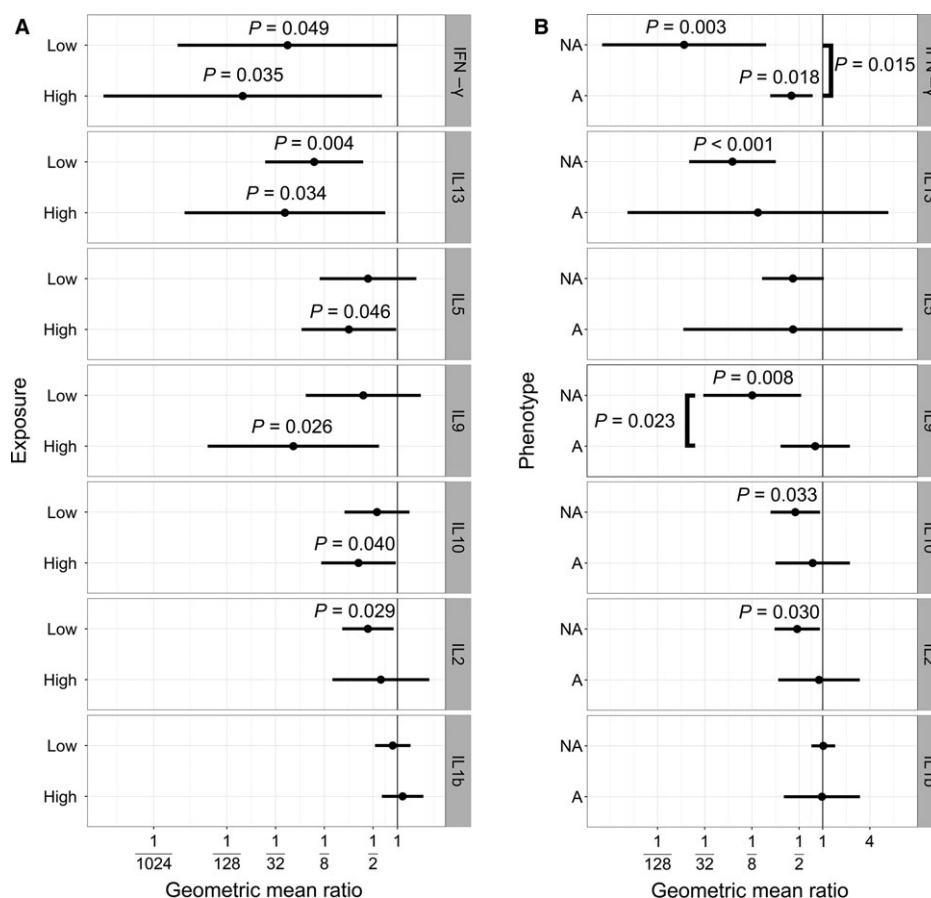


Figure 4 Cytokine modulation through Tregs is influenced by farm-milk/animal-stable exposure and asthma at the age of 6 yrs. Forest plot for cytokine levels of Treg suppression assays in (A) low/high farm-milk/animal-stable-exposed children and (B) children with asthma (A) and without asthma (NA). $n(\text{high/low/A/NA}) = 6/9/4/13$; Geometric mean ratio (following Treg addition/no Treg addition). The dots and horizontal line show the geometric mean ratio (GMR)

group of well-characterized children in relation to quantitative and qualitative assessment of Tregs in children at two time points, namely 4.5 and 6 yrs using the current most specific Treg marker $\text{CD4}^+ \text{CD25}^{\text{high}} \text{FOXP3}^{+ \text{upper}20\%}$, being examined identically over time in the same laboratory.

Taken together, our data suggest that farm exposure, and in particular the combination of farm-milk and animal-stable exposure shape immune maturation of Tregs early during childhood. The asthma-protective ‘window of opportunity’ occurring in the farming environment is partially modulated via Tregs at least until 4.5 yrs, while with 6 yrs the effect has vanished and switches to the opposite direction. Childhood asthma was associated with increased Tregs with 6 yrs. Future follow-up studies until later childhood are underway to follow additional atopic phenotypes during later school age. Future studies are required to replicate functional Treg capacity in a large number of children to disentangle the role of environmental exposure and asthma development during further childhood immune maturation.

and 95% confidence interval. Solid vertical line denotes GMR of 1, values <1 describe Treg-mediated suppression of the respective cytokine, and values >1 describe increased secretion; x-axis on log scale labels reflect original units. Cytokines were measured in supernatants from Treg suppression assays, without Tregs and with Tregs (Teff : Tregs = 1 : 0.5).

Acknowledgments

We thank all the children and their families for the participation in the study. We are grateful to the fieldworkers. We would also like to thank Isolde Schleich and Tatjana Netz for expert technical assistance with sample processing and cytokine measurements.

Author contributions

P.C. Schröder had responsibility for statistical analysis, interpretation and wrote the final version of the manuscript. S. Illi and A. Böck performed statistical analysis. V.I. Casaca, A. Lluís, C. Roduit, M. Depner, R. Frei, J. Genuneit, P.I. Pfefferle, M. Roponen, J. Weber were involved in data collection. C. Braun-Fahrlander, J. Riedler, J.C. Dalphin, J. Pekkanen, R. Lauener and E. von Mutius contributed to data collection, study management, critical review of the article and are responsible for the design of the PASTURE study. B.

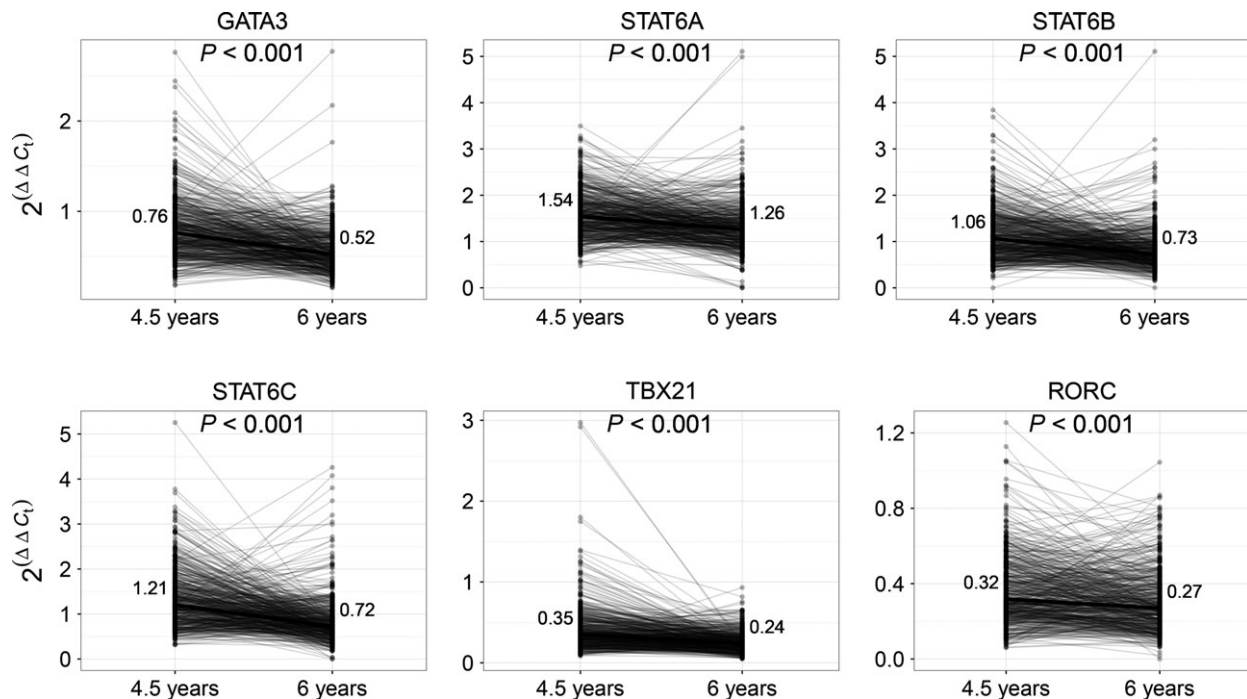


Figure 5 Th2-, Th1- and Th17-associated cell markers decrease between age 4.5 and 6 yrs. mRNA expression of Th2 (GATA3, STAT6A, STAT6B, STAT6C)-, Th1 (TBX21)- and Th17 (RORC)-associated cell markers where unstimulated cells were available at 4.5

and 6 yrs (mRNA-population). $n(\text{GATA3/STAT6A/STAT6B/STAT6C/TBX21/RORC}) = 544/547/548/548/544/548$. Median values are shown for both ages.

Schaub was responsible for the study design of this article, analysis, interpretation and the final version of the manuscript. THE PASTURE study group contributed with data collection and interpretation. All authors approved the final version of the submitted manuscript.

Funding

This work was supported by EFRAIM EU FP7-KBBE-2007-1, DFG SCHA 997/3-1 (BS, PS), Bavarian Research Association (VC), SFB TR22 (BS, AL), Marie Curie Grant (MEST-CT-2005-020524-GALTRAIN, AL) and Comprehensive Pneumology Centre (BS). The funding sources did not influence the study design; the collection, analysis and interpretation of data; the writing of the manuscript; and the decision to submit the manuscript for publication. The corresponding author had full access to all of the data in this study and takes complete responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of interest

Paul C. Schröder, Vera I. Casaca, Sabina Illi, Andreas Böck, Caroline Roduit, Anna Lluís, Remo Frei, Petra Ina Pfefferle, Marjut Roponen, Charlotte Braun-Fahrlander, Josef Riedler and Martin Depner have nothing to disclose. Jon Genuneit reports grants from European Union during the conduct of the study. Juliane Weber reports grants

from European Commission during the conduct of the study. Roger Lauener reports grants from Kühne Foundation and EU outside the submitted work. Jean-Charles Dalphin reports grants from Novartis Pharma, personal fees from Novartis Pharma, Chiesi, Intermune, GSK, AstraZeneca and Boehringer Ingelheim and nonfinancial support from Novartis, GSK, AstraZeneca, Intermune, Chiesi, Boehringer Ingelheim and Stallergenes outside the submitted work. Juha Pekkanen has nothing to disclose. Erika von Mutius reports personal fees from GlaxoSmithKline, Novartis, Astellas Pharma Europe Ltd, ALK-Abelló, *Journal of Allergy and Clinical Immunology* and *New England Journal of Medicine* and grants from Friesland Campina outside the submitted work. Michael Kabesch is a consultant for Sanofi and is on the speakers' bureau of Roxall, Glaxo Wellcome, Novartis and Allergopharma. Bianca Schaub reports funding from Comprehensive Pneumology Centre, and funding through grants from EU and DFG outside the submitted work.

Supporting Information

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

Figure S1. Flow chart of longitudinal data assessment in the PASTURE/EFRAIM cohort relevant for current analyses.

Figure S2. Prevalence of farm-milk and animal-stable exposure over time, within LCA classes.

Figure S3. At age 6 years, Tregs (%) did not change when children were exposed to high or low farm-milk.

Figure S4. At age 6 years, LPS-stimulated Tregs (%) were lower when children were exposed to high compared to low animal-stable.

Figure S5. Th2-, Th1- and Th17-associated cell markers decreased between age 4.5 and 6 years in high and low farm-milk/animal-stable-exposed children.

Figure S6. Th2-, Th1- and Th17-associated cell markers decreased between age 4.5 and 6 years in asthmatic and nonasthmatic children.

Table S1. Stratification by centre of mRNA expression data for Treg-, Th1-, Th2- and Th17-associated cell markers.

Table S2. Median, IQR (Q1/Q3), for Fig. 1 for relevant differences between 4.5 and 6 years of age in the respective stimulation conditions.

Table S3. Median, IQR (Q1/Q3), for Fig. 2 for relevant differences between 4.5 and 6 years of age in the respective stimulation conditions.

Table S4. Median, IQR (Q1/Q3), for Fig. 3 for relevant differences between 4.5 and 6 years of age in the respective stimulation conditions.

Table S5. Median; IQR (Q1/Q3), for sensitization for relevant differences between 4.5 and 6 years of age in the respective stimulation conditions.

References

1. von Mutius E, Vercelli D. Farm living: effects on childhood asthma and allergy. *Nat Rev Immunol* 2010;**10**:861–868.
2. Ege MJ, Mayer M, Normand AC, Genuneit J, Cookson WO, Braun-Fahrlander C et al. Exposure to environmental microorganisms and childhood asthma. *N Engl J Med* 2011;**364**:701–709.
3. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 2002;**347**:869–877.
4. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* 2001;**358**:1129–1133.
5. Ege MJ, Bieli C, Frei R, van Strien RT, Riedler J, Ublagger E et al. Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children. *J Allergy Clin Immunol* 2006;**117**:817–823.
6. Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G et al. The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. *J Allergy Clin Immunol* 2011;**128**:766–773.
7. Waser M, Michels KB, Bieli C, Floistrup H, Pershagen G, von Mutius E et al. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. *Clin Exp Allergy* 2007;**37**:661–670.
8. Lauener RP, Birchler T, Adamski J, Braun-Fahrlander C, Bufer A, Herz U et al. Expression of CD14 and Toll-like receptor 2 in farmers' and non-farmers' children. *Lancet* 2002;**360**:465–466.
9. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. *Cell* 2008;**133**:775–787.
10. Schaub B, Liu J, Hoppler S, Schleich I, Huehn J, Olek S et al. Maternal farm exposure modulates neonatal immune mechanisms through regulatory T cells. *J Allergy Clin Immunol* 2009;**123**:774–782.
11. Lluís A, Depner M, Gaugler B, Saas P, Casaca VI, Raedler D et al. Increased regulatory T-cell numbers are associated with farm milk exposure and lower atopic sensitization and asthma in childhood. *J Allergy Clin Immunol* 2014;**133**:551–559.
12. Raedler D, Ballenberger N, Klucker E, Böck A, Otto R, Prazeres Da Costa O et al. Identification of novel immune phenotypes for allergic and nonallergic childhood asthma. *J Allergy Clin Immunol* 2015;**135**:81–91.
13. Hartl D, Koller B, Mehlhorn AT, Reinhardt D, Nicolai T, Schendel DJ et al. Quantitative and functional impairment of pulmonary CD4 + CD25hi regulatory T cells in pediatric asthma. *J Allergy Clin Immunol* 2007;**119**:1258–1266.
14. Ling EM, Smith T, Nguyen XD, Pridgeon C, Dallman M, Arbery J et al. Relation of CD4 + CD25 + regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 2004;**363**:608–615.
15. Tiemessen MM, Van Hoffen E, Knulst AC, Van Der Zee JA, Knol EF, Taams LS. CD4 CD25 regulatory T cells are not functionally impaired in adult patients with IgE-mediated cow's milk allergy. *J Allergy Clin Immunol* 2002;**110**:934–936.
16. Bellinghausen I, Klostermann B, Knop J, Saloga J. Human CD4 + CD25 + T cells derived from the majority of atopic donors are able to suppress TH1 and TH2 cytokine production. *J Allergy Clin Immunol* 2003;**111**:862–868.
17. Von Mutius E, Schmid S. The PASTURE project: EU support for the improvement of knowledge about risk factors and preventive factors for atopy in Europe. *Allergy Eur J Allergy Clin Immunol* 2006;**61**:407–413.
18. Alfvén T, Braun-Fahrlander C, Brunekreef B, von Mutius E, Riedler J, Scheynius A et al. Allergic diseases and atopic sensitization in children related to farming and anthroposophic lifestyle—the PARSIFAL study. *Allergy* 2006;**61**:414–421.
19. Depner M, Fuchs O, Genuneit J, Karvonen AM, Hyvärinen A, Kaulek V et al. Clinical and epidemiologic phenotypes of childhood asthma. *Am J Respir Crit Care Med* 2014;**189**:129–138.
20. Rothman KJ. No adjustments are needed for multiple comparisons. *Epidemiology* 1990;**1**:43–46.
21. Illi S, von Mutius E, Lau S, Niggemann B, Gruber C, Wahn U. Perennial allergen sensitization early in life and chronic asthma in children: a birth cohort study. *Lancet* 2006;**368**:763–770.
22. Schedel M, Frei R, Bieli C, Cameron L, Adamski J, Lauener R et al. An IgE-associated polymorphism in STAT6 alters NF- κ B binding, STAT6 promoter activity, and mRNA expression. *J Allergy Clin Immunol* 2009;**124**:583–589.
23. Von Ehrenstein OS, Von Mutius E, Illi S, Baumann L, Böhm O, Von Kries R. Reduced risk of hay fever and asthma among children of farmers. *Clin Exp Allergy* 2000;**30**:187–193.
24. Sullivan KE, McDonald-McGinn D, Zackai EH. CD4(+) CD25(+) T-cell production in healthy humans and in patients with thymic hypoplasia. *Clin Diagn Lab Immunol* 2002;**9**:1129–1131.
25. Wing K, Ekmark A, Karlsson H, Rudin A, Suri-Payer E. Characterization of human CD25 + CD4 + T cells in thymus, cord and adult blood. *Immunology* 2002;**106**:190–199.
26. Schönland SO, Zimmer JK, Lopez-Benitez CM, Widmann T, Ramin KD, Goronzy JJ et al. Homeostatic control of T-

- cell generation in neonates. *Blood* 2003;**102**:1428–1434.
27. Raynor J, Lages CS, Shehata H, Hildeman DA, Chougnet CA. Homeostasis and function of regulatory T cells in aging. *Curr Opin Immunol* 2012;**24**:482–487.
 28. Tulic MK, Andrews D, Crook ML, Charles A, Tourigny MR, Moqbel R et al. Changes in thymic regulatory T-cell maturation from birth to puberty: differences in atopic children. *J Allergy Clin Immunol* 2012;**129**:199–206.
 29. Krug N, Madden J, Redington AE, Lackie P, Djukanovic R, Schauer U et al. T-cell cytokine profile evaluated at the single cell level in BAL and blood in allergic asthma. *Am J Respir Cell Mol Biol* 1996;**14**: 319–326.
 30. Magnan AO, Mély LG, Camilla CA, Badier MM, Montero-Julian FA, Guillot CM et al. Assessment of the Th1/Th2 paradigm in whole blood in atopy and asthma. Increased IFN-gamma-producing CD8(+) T cells in asthma. *Am J Respir Crit Care Med* 2000;**161**:1790–1796.

Appendix

Investigators of the PASTURE study group: Anne Hyvärinen and Anne M. Karvonen (Department of Health Protection, National Institute for Health and Welfare, Kuopio, Finland), Maija-Riitta Hirvonen (Department of Environmental Science, Inhalation Toxicology Laboratory, University of Eastern Finland, Kuopio, Finland), Pekka Tiittanen (Department of Health Protection, National Institute for Health and Welfare, Kuopio, Finland), Sami Remes (Department of Pediatrics, Kuopio University Hospital, Kuopio, Finland), Vincent Kaulek (Department of Respiratory Disease, University Hospital, University of Besançon, Besançon, France), Marie-Laure Dalphin (Department of Pediatrics, University Hospital, University of Besançon, Besançon, France), Markus J. Ege (LMU Munich, University Children's Hospital;

Comprehensive Pneumology Centre Munich (CPC-M), German Centre for Lung Research (DZL), Munich, Germany), Georg J. Loss (LMU Munich, University Children's Hospital, Munich, Germany; Department of Pediatrics, University of California, San Diego, La Jolla, CA, USA), Harald Renz (Member of the German Centre for Lung Research (DZL), Munich; Department of Clinical Chemistry and Molecular Diagnostics, Philipps University of Marburg, Marburg, Germany), Gert Doekes (Division of Environmental Epidemiology, Institute for Risk Assessment Sciences (IRAS), Utrecht University, Utrecht, The Netherlands), and Michael Kabesch (Department of Pediatric Pneumology and Allergy, KUNO Children's University Hospital Regensburg, Regensburg; Hannover Medical School, Clinic for Pediatric Pneumology and Neonatology, Hannover, Germany).