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Asthmatic farm children show increased $CD3^+CD8^{low}$ T-cells compared to non-asthmatic farm children☆

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ABSTRACT

Several studies report an important role of $CD8^+$ cytotoxic T-cells in atopy. Farm children show protection against atopy development, partly explained by $CD4^+$ T-cell subtypes. Additional effects of $CD8^+$ T-cells are unknown being investigated in this study within the PASTURE/EFRAIM birth cohort in PBMCs from farming and non-farming 6-year-old ($N = 76$) German children. $CD3^+CD8^+CD25^+$ T-cells were analyzed by flow cytometry. Genotyping of 17q21 locus-SNPs associated with childhood asthma was performed. No differences in $CD8^+$ T-cell subsets were seen between farmers and non-farmers regardless of asthma. Among farm children, asthmatics displayed increased $CD3^+CD8^{low}(CD25^+)$ T-cells compared to non-asthmatics. Asthmatic farm children exhibited a lower PI-induced stimulatory capacity of $CD3^+CD8^{low}(CD25^+)$ cells and a lower IFN-γ secretion than non-asthmatic farm children. Among farm children with *GSDMB* and *ORMDL3* risk alleles, asthmatics displayed higher $CD3^+CD8^{low}$ cells than non-asthmatics. Our data indicates a specific role of $CD8^{low}$ T-cells in asthmatic farm children.

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Abbreviations: MAF, Minor allele frequency; NK cell, Natural Killer cell; PBMCs, Peripheral blood mononuclear cells; PMA, Phorbol 12-myristate 13-acetate; PI, Phorbol 12-myristate 13-acetate/ionomycin; U, unstimulated; yrs, years.

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1. Introduction

Farm exposure during pregnancy and early childhood has been associated with asthma and allergy protection in children [1–3]. Underlying immune mechanisms of protection involve a wide range of different cell types, non-immune, innate and adaptive immune cells; however the contribution of distinct cell types is yet not fully understood. Consumption of not pasteurized farm-milk influences innate dendritic cells, specifically myeloid dendritic cells [4]. Adaptive $CD4^+$ T-helper cells play a crucial and established role in development/maintenance of allergic diseases and asthma [5–7]. Specifically, an asthma-protective effect by farm-milk consumption is in part mediated by regulatory T-cells in childhood [8]. The contribution of $CD8^+$ T-cells in allergic diseases and asthma is not fully understood. While some studies describe a protective role by suppressing $CD4^+$ T-helper 2 cells and inducing IFN-γ secretion [9–11], other studies suggested that $CD8^+$ T-cells contribute to the development of allergies and inflammation after cockroach sensitization [12] and increased IL-4 production by peripheral blood $CD8^+$ T-cells in asthmatics [13]. A potential protective mechanism has been proposed by chronic exposure to microbial burden which may induce maturation of $CD8^+$ T-cells [14]. Thus, we aimed to examine, whether farm-exposure modulates $CD8^+$ T-cells and their subtypes in 6-year-old children from the international PASTURE/EFRAIM birth cohort study.

2. Methods

2.1. Study characteristics

The PASTURE/EFRAIM Study is an international birth cohort study including 1133 children from rural areas in Germany, Switzerland, Austria, Finland and France [15] with follow-up from the third trimester of pregnancy until 10.5 yrs, comprising children who live and do not live on farms. These children were repeatedly invited to participate in follow-ups that included questionnaires and/or blood sampling. Questionnaires were administered at end of pregnancy or birth and when the children were 2 months, 1, 1.5, 2, 3, 4, 5, 6 and 10.5 yrs of age [8]. In German children with available blood for flow cytometry at age 6 yrs, CD8 expression was analyzed (CD8-group, $N = 76$, 41 farmer with 4 asthmatics, 35 non-farmer with 6 asthmatics, Table 1). The study was approved by the local research ethics committee from each country (Germany ethics number 02046, original number and amendment 2014; Austria ethics number E Nr. 401; Switzerland EKSG 021056; Finland 10/2008; France 07/448), and written informed consent was obtained from all parents.

2.2. Questionnaires and clinical definitions

Extensive questionnaires based on previous studies [15,16] 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 asthma and 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 [8]. Other factors such as socioeconomic status, family history of atopy, and maternal smoking were noted. Asthma was assessed at age 6 yrs when the clinical phenotype of asthma is rather stable [17]. Asthma was defined as physician's diagnosis of asthma at least once per lifetime and/or recurrent diagnoses of spastic, obstructive, or asthmatic bronchitis as reported by the parents at age 6 yrs.

2.3. Cell isolation and stimulation

PBMCs were isolated within 24 h after blood withdrawal by density gradient centrifugation with Ficoll-Paque™ PLUS (GE Healthcare, Piscataway, USA). Cells were washed and resuspended in RPMI 1640 + GlutaMax (Gibco, Carlsbad, USA) with 10% human serum (Sigma-Aldrich, Steinheim, Germany) at a final concentration of 5×10^6 cells/ml. PBMCs were then stimulated with PMA (5 ng/ml)/Ionomycin (1 μg/ml) (PI) or were cultured without stimulation (U) at 37 °C with 5% CO_2. For intracellular staining, 1 μl of 1000× Brefeldin A (eBioscience, San Diego, USA) was added to 5×10^6 cells after 23 h. After a total incubation time of 24 h, cells were washed and prepared for quantitative cell assessment by flow-cytometric analysis. Based on cell availability, sample number varied between stimulation conditions. $N = 76$ children were cultured without stimulation and $N = 73$ were cultured with PI stimulation (3 non-asthmatic non-farm children were not stimulated with PI).

Table 1
Population characteristics.

		Total population $N = 1133$		German population $N = 254$		CD8 group $N = 76$	
		n/N	%	n/N	%	n/N	%
Available information at 6 yrs:							
Questionnaire		934/1133	82.4	212/254	83.5	76/76	100.0
SNPs		895/1133	79.0	174/254	68.5	56/76	73.7
IFNγ PI-stimulated		638/1133	56.3	148/254	58.3	75/76	98.7
Characteristics:							
Parental atopy		591/1099	53.8	142/244	58.2	41/75	54.7
Maternal atopy		371/1132	32.8	93/254	36.6	28/76	36.8
Paternal atopy		342/1082	31.6	82/239	34.3	27/75	36.0
≥2 older sibs		379/1133	33.5	68/254	26.8†	23/76	30.3
Females		530/1090	48.6	117/244	48.0	36/76	47.4
Maternal smoking in pregnancy		155/1126	13.8	37/254	14.6	9/76	11.8
Living on a farm		530/1133	46.8	112/254	44.1	41/76	54.0‡
Asthma ever at 6 yrs		78/928	8.4	18/212	8.5	10/76	13.2
rs2290400	C C	247/899	27.4	51/191	26.7	19/76	25.0
	T C	448/899	49.8	103/191	53.9	43/76	56.6
	T T	204/899	22.7	37/191	19.4	14/76	18.4
rs7216389	C C	253/901	28.1	51/193	26.4	18/76	23.7
	C T	446/901	49.5	105/193	54.4	44/76	57.9
	T T	202/901	22.4	37/193	19.2	14/76	18.4
rs8076131	A A	235/904	26.0	45/189	23.8	20/74	27.0
	G A	448/904	49.6	107/189	56.6	42/74	56.8
	G G	221/904	24.4	37/189	19.6	12/74	16.2

p-values for characteristics: †$p < 0.05$ vs total population, ‡$p < 0.05$ vs German population. N = whole population with available information, n = number of children with distinct characteristics.

2.4. CD8 T-cells, B-cells, NK and intracellular IFN-γ assessment

Quantitative $CD8^+$ T-cell assessment (defined as percentage of $CD3^+CD8^+$ T-cells or $CD3^+CD8^+CD25^+$ for activated $CD8^+$ T-cells, in lymphocytes and % of cells with low $CD8^+$ expression to assess $CD3^+CD8^{low}$ cells and $CD3CD8^{low}CD25^+$) was performed as follows: 1×10^6 cells were incubated with 2 μl CD3-PE (#AO7747, Beckman Coulter), 2 μl CD8-FITC (#AO7756, Beckman Coulter) and 2 μl CD25-PC5 antibodies (#IM 2646, Beckman Coulter) for samples obtained at 6 yrs. For intracellular IFN-γ staining (in samples at age 10.5 yrs) cells were permeabilized (Human Regulatory T Cell Staining Kit, eBioscience, San Diego, US) and stained with 5 μl IFN-γ-APC (#17-7319-82, eBioscience). For B-cells (defined as $CD3^-CD19^+$) and NK-cells (defined as $CD3^-CD16/CD56^+$) 2 μl CD19-FITC (#130-091-328, Miltenyi Biotec) and 5 μl CD3-FITC/CD16 + CD56-PE (#A07735, Beckman Coulter) were used. For the corresponding isotype controls 0.5 μl IgG1-PE (130-098-106, Miltenyi Biotec), 0.5 μl IgG1-FITC (130-098-105, Miltenyi Biotec), 0.25 μl IgG2a-PC5 (#A09148, Beckman Coulter) and 2.5 μl IgG1-APC (#17-4015-80, eBioscience) were used. Cells were measured with BD FACSCanto™ II (BD Biosciences, Heidelberg, Germany). Data was analyzed with BD FACSDiva™ version 6.1.3 software (BD Biosciences, Heidelberg, Germany) and FCS Express version 4 Flow Research Edition.

2.5. Cytokine assessment

Whole blood supernatants from 6-year-old children were collected after 24 h stimulation with PI (5 ng/ml PMA, 1 μg/ml Ionomycin). IFN-γ was measured in the supernatants by multiplexed cytometric bead array according to the manufacturer's instructions (BD) in Marburg, Germany. The detection limit was 0.01 pg/ml and the cytokine level below the detection limit was set to zero. IFN-γ concentration was standardized by the number of leukocytes (calculated using Sysmex KX-21N blood cell analyzer; Sysmex Corporation, Kobe, Japan).

2.6. Genotyping

Three SNPs at the 17q21 locus (*GSDMB* rs2290400 and rs7216389, *ORMDL3* rs8076131) related to asthma development were genotyped in PASTURE (n/N = 895/1133). Genotyped German children within the CD8-group (n/N = 76/76) were considered for association of SNPs with CD8 T-cells. Genotyping was performed either by RFLP assay (n/N = 20/76) or by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (n/N = 56/76; Sequenom Inc., San Diego, Calif). Detailed information including specific amplification and extension primers for all genotyping procedures is available on request or in [18].

2.7. Statistical analysis

Differences between population characteristics were tested with Chi^2 test or Fisher's exact test in case of small numbers. Due to non-normal distribution, differences in CD8 T-cells (%) and cytokine IFN-γ of PI-stimulated whole blood supernatants between exposure groups or phenotypes were assessed by Wilcoxon rank sum test (Mann Whitney U). Differences in intracellular IFN-γ levels between CD8 T-cells (and subtypes), B-cells and NK cells were assessed by linear regression; pairwise comparisons between cell types were performed by t-tests and adjusted for multiple testing using the single step method of Hothorn et al., [19]. Association of asthma phenotype and farm status with presence of SNPs was tested with Fisher's exact test on the respective contingency tables. A p-value 0.05 or lower was considered as significant. We did not adjust for multiple testing as it may inflate type II errors and our analysis was based on an a priori hypothesis. Boxplots show first and third quartiles (box) and median (line). Whiskers are extended to the most extreme data point that is no $>1.5 \times$ IQR (inter quartile range) from the edge of the box. Data beyond the end of the whiskers are plotted as points. Statistical analysis was performed using SAS 9.3 software (SAS Institute, Inc., Cary, NC) and R software/environment (http://www.R-project.org/).

3. Theory

A naïve $CD8^+$ T-cell can differentiate into different subsets of an effector cell depending on cytokine milieu and antigen exposure [20,21]. One of these options is becoming either a "normal" CD8 expressing cell or, alternatively, a $CD8^{low}$ T-cell. $CD8^{low}$ T-cells have lower CD8 surface protein and mRNA expression and are poorly cytolytic. They express low perforin and granzyme B and C levels, but express IFN-γ and IL-4 [20]. An association or contribution of $CD8^+$ T-cells and their subtypes in asthma and allergy development and modulation through farm exposure is lacking in the current literature. However, since $CD8^+$ memory T-cells prevent allergic sensitization during chronic microbial burden [14] and $CD8^{low}$ T-cells showed an activated effector phenotype during chronic antigen contact [20], an asthma-protective role of $CD8^+$ T-cells induced by farm exposure is conceivable.

Asthma has significant genetic contributions. Single-nucleotide polymorphisms (SNPs) in *GSDMB* (Gasdermin B) and *ORMDL3* (ORMDL sphingolipid biosynthesis regulator 3) at the 17q21 asthma locus are strongly associated with childhood asthma [18,22–24]. ORMDL3 protein is a negative regulator of sphingolipid synthesis [25], while the function of GSDMB protein is not fully understood, but an involvement in secretory pathways is suggested [26]. Although both genes are not directly linked to immune cell function, they might influence immune cells by affecting immune-related signaling pathways like Ca^{2+}-signaling. Yet to date, no association with immune cells, e.g. $CD8^+$ T-cells, was shown.

Due to the tremendous increase of allergic diseases worldwide [27], creating a substantial social and financial burden, it is important to better understand the underlying immunological mechanisms of protection. Dendritic cells and regulatory T-cells can only partly explain the asthma-protective farm effect [8,28]. Thus, we aimed to investigate the role of $CD8^+$ T-cells in asthmatic and non-asthmatic farm children.

4. Results

4.1. Study population

Of the 1133 enrolled children in the PASTURE/EFRAIM cohort, 934 children participated in the 6 yrs questionnaires (Table 1). We report CD8 T-cells only within the German arm of the study (N = 254), comprising about one fifth of the total study population. Due to limited cell availability, data on CD8 T-cells was only available in N = 76 at age 6 yrs (41 farmer with 4 asthmatics, 35 non-farmer with 6 asthmatics). Since cell numbers were limited, not all samples were cultured under all stimulation conditions. No significant differences were found between the whole population, the German subpopulation and CD8-group of the PASTURE/EFRAIM cohort in regards to maternal, paternal or parental atopy, sex, smoking during pregnancy and asthma at age 6 yrs (Table 1). However, the German subpopulation displayed a significant difference in regard to the number of siblings (Total 33.5%, Germany 26.8%), while the CD8-group contained more farm children (Total 46.8%, CD8-group 53.3%). The SNP frequencies were slightly varying between the CD8-group and the total PASTURE/EFRAIM population, but not significantly different. Clinical symptoms of asthmatic farm and non-farm children were comparable regarding wheeze, with no significant differences in their family-history atopy and specific IgE levels against common allergens.

4.2. Asthmatic farm children exhibit more $CD3^+CD8^{low}CD25^+$ T-cells compared to non-asthmatic farm children

When we only stratified for farm exposure, there were no significant differences between farm and non-farm children (unstimulated and PI-stimulated). When stratified for farm-exposure and phenotype, an increase in total $CD3^+CD8^+$ T-cells (%) from asthmatic compared to non-asthmatic children was found only within farm-exposed children after PI stimulation (asthmatics [median (Q1; Q3)] = [27.4% (24.82; 29.20)] versus non-asthmatics = [21.7% (18.44; 25.01)], $p = 0.041$, PI-stimulated, Fig. 1A). Investigations on correlation of $CD3^+CD8^+$ T-cells with peripheral and central obstruction (FEF25 and 75) before provocation respectively FEV1 following exercise testing revealed a highly negative correlation ($r = -0.997$, $p = 0.0045$ for FEF25 and 75; $r = -0.999$, $p = 0.033$ for FEV1). When $CD3^+CD8^{normal}$ cells were assessed (defined as $CD3^+CD8^+$ T-cells minus the $CD3^+$ cells with low CD8 expression), they were increased in asthmatic farm children upon PI-stimulation (asthmatics = [21.2% (18.07; 24.95)] versus non-asthmatic children = [14.1% (11.08; 16.52)], $p = 0.009$, PI-stimulated, Fig. 1B). By contrast, $CD3^+CD8^{low}$ T-cells (%, defined as $CD3^+$ cells with low CD8 expression) were increased without stimulation among farm-exposed asthmatic children (asthmatics = [5.5% (4.29; 6.00)] vs non-asthmatic children = [3.2% (2.74; 4.32)], $p = 0.046$, unstimulated, Fig. 2A). When cells were additionally gated for the T-cell activation marker CD25 ($CD3^+CD8^{low}CD25^+$), the results were even more evident displaying an increase of these cells in asthmatic farm children (asthmatics = [0.86% (0.48; 1.35)] versus non-asthmatics

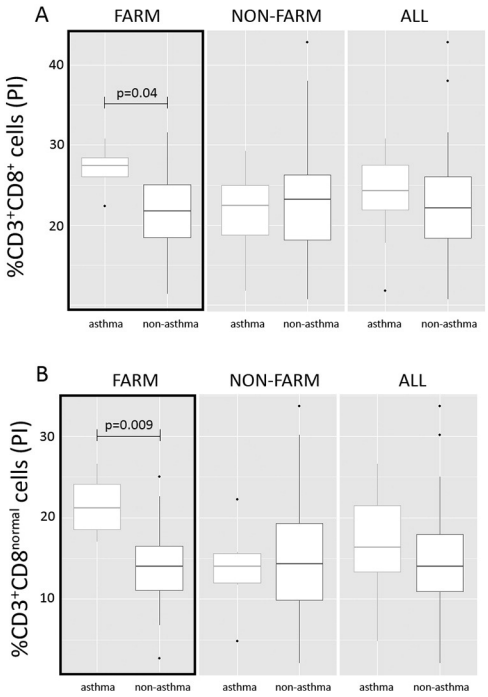

Fig. 1. Asthmatic farm children have more $CD3^+CD8^+$ and $CD3^+CD8^{normal}$ T-cells upon PI stimulation. Boxplots show % of PI-stimulated $CD3^+CD8^+$ (**A**) and $CD3^+CD8^{normal}$ T-cells (**B**) in 6-year-old children. Data is shown overall and stratified for exposed (F) and non-exposed (NF) children. Asthmatic (A) children are shown in red, non-asthmatic (NA) children are shown in blue. Bars above boxplots display significant Wilcoxon-test comparing asthma against no asthma. N(overall) = 76. N(A-F;NA-F;A-NF;NA-NF) = 4;37;6;29.

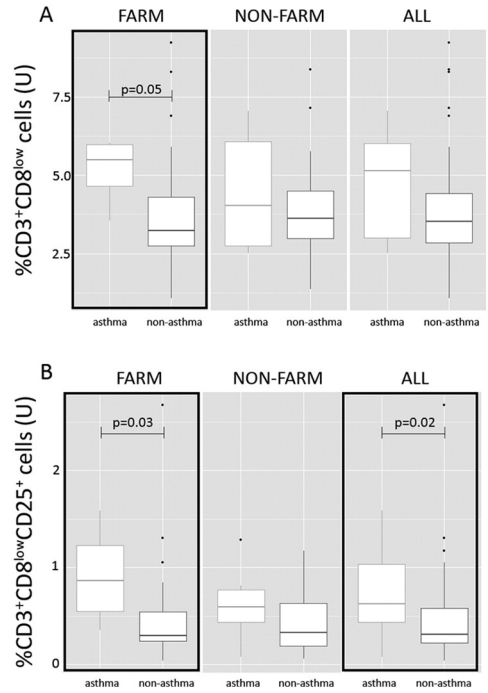

Fig. 2. Asthmatic farm children exhibit more $CD3^+CD8^{low}$ T-cells. Boxplots show % of unstimulated $CD3^+CD8^{low}$ (**A**) and $CD3^+CD8^{low}CD25^+$ T-cells (**B**) in 6-year-old children. Data is shown overall and stratified for exposed (F) and non-exposed (NF) children. Asthmatic (A) children are shown in red, non-asthmatic (NA) children are shown in blue. Bars above boxplots display significant Wilcoxon-test comparing asthma against no asthma. N(overall) = 76. N(A-F;NA-F;A-NF;NA-NF) = 4;37;6;29.

= [0.30% (0.24; 0.54)], $p = 0.03$, unstimulated, Fig. 2B). However, without farming stratification similar significant results were obtained (Fig. 2B). Upon PI stimulation, healthy farm children showed not significantly higher levels of $CD8^{low}$ (median 6.8% vs 4.4% $p = 0.237$) and $CD8^{low}CD25^+$ (median 5.3% vs 3.7% $p = 0.122$) T-cells compared to asthmatic farm children. We further evaluated the stimulatory capacity of $CD8^{low}$ T-cells calculating PI/U ratio. Among farmers, asthmatic children exhibited a significantly lower increase in $CD3^+CD8^{low}$ T-cell percentage (PI/U) than non-asthmatics (asthmatics = [1.19 (0.80; 1.56)]; non-asthmatics = [2.11 (1.64; 2.62)], $p = 0.009$, PI/U, Fig. 3A) and $CD3^+CD8^{low}CD25^+$ (asthmatics = [4.81 (2.59; 9.39)]; non-asthmatics = [16.4 (9.03; 28.08)], $p = 0.01$, PI/U, Fig. 3B). Without farming stratification similar significant results were obtained (Fig. 3). By comparing farm and non-farm asthmatics, the $CD8^{low}$ cell profile of asthmatic farm children revealed slightly higher numbers as compared to asthmatic non-farm children, however not significantly. The gating strategy for $CD8^{low}$ T-cells is shown in Figs. S1, S2 and S5.

4.3. IFN-γ secretion is decreased in asthmatic compared to non-asthmatic farm-exposed children

IFN-γ secretion has been shown to be increased in $CD8^{low}$ T-cells in comparison to $CD8^{normal}$ T-cells after stimulation with soluble anti-CD2, anti-CD3 and anti-CD28 monoclonal antibody [20]. Therefore, we measured IFN-γ in whole blood supernatants of PI-stimulated cells from 6-year-old children in the CD8-group and in available supernatants of blood samples of the German subpopulation ($N = 148$). Notably, there was no selection bias between the whole PASTURE/EFRAIM population, the German subpopulation and the CD8-group (Table 1). Among farm-exposed children, asthmatics showed significantly lower IFN-γ levels (pg/ml) than non-asthmatics in the CD8-group and the German subpopulation (CD8-group, $N = 76$, asthmatics [median (Q1; Q3)] = [10,834.75 pg/ml (699.42; 29,672.44)]; non-asthmatics = [32,948.36 pg/ml (21,437.24; 47,226.37)], $p = 0.051$, not shown; German subpopulation, $N = 148$, asthmatics = [957.39 pg/ml (403.90; 20,342.96)] versus non-asthmatics = [30,317.27 pg/ml (8161.55; 49,623.54)], $p = 0.03$, Fig. 4A).

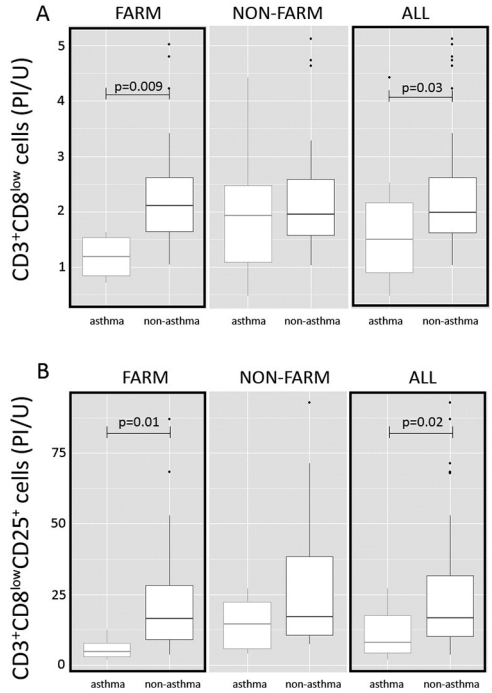

Fig. 3. $CD3^+CD8^{low}$ T-cells in asthmatic farm children display a lower PI/U ratio. Boxplots show the ratio of PI-stimulated/unstimulated $CD3^+CD8^{low}$ (**A**) and $CD3^+CD8^{low}CD25^+$ T-cells (**B**) in 6-year-old children. Data is shown overall and stratified for exposed (F) and non-exposed (NF) children. Asthmatic (A) children are shown in red, non-asthmatic (NA) children are shown in blue. Bars above boxplots display significant Wilcoxon-test comparing asthma against no asthma. N(overall) = 76. N(A-F;NA-F;A-NF;NA-NF) = 4;37;6;29.

The levels of the type-2 cytokine IL-4 was increased in asthmatic vs. non-asthmatic non-farming children after PI stimulation in our CD8-group (median AA: 57.05 vs. median HC: 39.65, $p = 0.050$) as well as the German subpopulation with increased IL-4 (median AA: 57.05 vs. median HC: 33.61, $p = 0.045$) as well as IL-13 (median AA: 144.18 vs. median HC: 91.62, $p = 0.037$). We next wanted to unravel whether decreased IFN-γ levels in asthmatics were attributed to a higher number of low IFN-γ-producing $CD8^{low}$ T-cells. Since recruitment at age 6 yrs preceded this analysis, intracellular IFN-γ production in $CD3^+CD8^{normal}$, $CD3^+CD8^{low}$ and in other IFN-γ-producing cells (B-cells and Natural Killer (NK) cells) upon PI stimulation was measured in farm children from the CD8-group at the age of 10.5 yrs ($N = 13$, farmer only). Importantly, comparable $CD8^{low}$ T-cell results were observed in 10.5 year-old children although the case numbers were very small (data not shown). We found significant differences between the different cell types producing IFN-γ. While among $CD3^+CD8^-$, $CD3^+CD8^+$ and NK cells were more IFN-γ producing cells, among $CD3^+CD8^{low}$ and $CD3^+CD8^{low}CD25^+$ T-cells and B-cells only few cells displayed IFN-γ production (Fig. 4B). Since the 13 farm children included only one asthmatic child, this study was not powered to detect differences for IFN-γ production between 10.5-year-old asthmatic and non-asthmatic farm children in any cell type. The gating strategy for IFNγ-producing $CD8^+$, $CD8^{normal}$, $CD8^{low}$, NK- and B-cells is shown in Figs. S3–S5.

4.4. Asthmatic farm children with asthma risk alleles have more $CD3^+$ $CD8^{low}$ T-cells than non-asthmatic children with risk alleles

To analyze the role of genetic asthma predisposition on $CD3^+CD8^{low}$ cells, genotyping of SNPs on 17q21 locus associated with childhood asthma (*GSDMB* rs2290400 and rs7216389, *ORMDL3* rs8076131) was performed in children of the CD8-group ($N = 76$, Table 1). The minor allele frequency (MAF) in the total population and in the CD8-group was about 45–47% (Table 2). Linkage disequilibrium was high between the SNPs ($r^2 = 0.97$ for *GSDMB* rs2290400 and rs7216389, $r^2 = 0.70$ for *GSDMB* rs2290400 and *ORMDL3* rs8076131 and $r^2 = 0.72$ for *GSDMB* rs7216389 and *ORMDL3* rs8076131) in our subgroup. We analyzed the amount of $CD3^+CD8^{low}$ T-cells in asthmatic and non-asthmatic children overall and stratified for farming and genotype. Asthmatic farm children with asthma risk alleles (*GSDMB* rs2290400 and rs7216389, *ORMDL3* rs8076131) displayed more $CD3^+CD8^{low}$ T-cells than non-asthmatic farm children with risk alleles (rs2290400 medians 5.49/3.21, $N = 4/26$, $p = 0.02$; rs7216389 medians 5.49/3.21, $N = 4/26$, $p = 0.02$; rs8076131 medians 5.49/3.25, $N = 4/30$, $p = 0.04$) suggesting an asthma- and farm-dependent mechanism (Fig. 5). When analyzing differences of $CD8^{low}$ T-cells between asthmatic children with risk alleles of farm vs non-farm, we did not detect significant differences for the risk alleles GSDMB_rs2290400 (CT_TT), ORMDL_rs8076131 (AG_GG) and GSDMB_rs7216389 (CT_TT) with *p*-values of $p = 0.51$, $p = 0.10$ and $p = 0.51$. Of note, the number of analyzed children is limited with a maximum of $n = 10$ children in total. Table 3 gives detailed information about the number of individuals in each group.

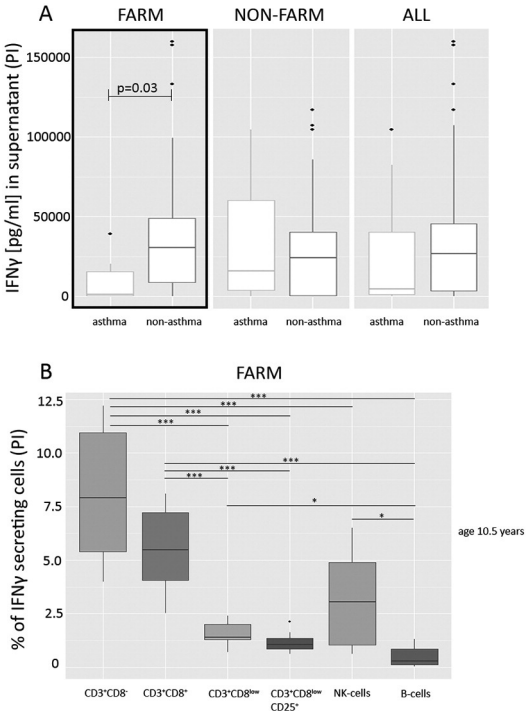

Fig. 4. Asthmatic farm children secrete less IFN-γ (PI). (**A**) Boxplots show IFN-γ levels (pg/ml) measured in the supernatants of whole blood PI-stimulated cells in the German subpopulation of 6-year-old children. Data is shown overall and stratified for exposed (F) and non-exposed (NF) children. Asthmatic (A) children are shown in red, non-asthmatic (NA) children are shown in blue. Bars above single boxplots display result from Wilcoxon-test comparing asthma against no asthma. *N*(overall) = 148. *N*(A-F,NA-F,A-NF,NA-NF) = 6;67;10;65. (**B**) Boxplots show % of PI-stimulated cell subsets ($CD3^+CD8^-$, $CD3^+CD8^+$, $CD3^+CD8^{low}$, $CD3^+CD8^{low}CD25$, NK and B-cells) of 10.5-year-old farm-exposed children producing IFN-γ. Bars above single boxplots display result from pairwise comparisons between cell types by *t*-tests adjusted for multiple testing with $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. $N = 13$ with 12 NA and 1A.

5. Discussion

In a subgroup of the large international PASTURE/EFRAIM birth cohort, we demonstrate that asthmatic 6-year-old farm children have lower IFN-γ levels (in PI-stimulated whole blood supernatants) and more $CD3^+CD8^{low}$ and $CD3^+CD8^{low}CD25^+$ T-cells than non-asthmatic farm children. These cells have a lower capacity to proliferate when stimulated with a potent T-cell stimulus (PI). No differences were seen between farmers and non-farmers. Differences in $CD3^+CD8^+$ T-cell subsets between asthmatic and non-asthmatic farm children suggest a distinct mechanism based upon the farm environment. Moreover, an inverse association of $CD8^+$ T-cells and hyper-reactivity, described in a murine cockroach model by Schaller et al. [12] is indirectly suggested in this study as correlations of $CD8^+$ T-cells are inversely associated with reduced lung function parameters (pulmonary obstruction).

5.1. Role of $CD8^{low}$ T-cells in asthma

The role of $CD3^+CD8^{low}$ T-cells is not fully understood, and no relation to asthma and farming was reported yet. An increase of $CD3^+$ $CD8^{low}$ T-cells was also observed in patients with progressive form of multiple sclerosis [29] and in patients with chronic or persistent antigen exposure like HIV [30] or chronic HBV infections [31], after transplantations [32] and in mice with Trypanosoma cruzi infection [33]. There, $CD3^+CD8^{low}$ T-cells were reported to be type-2 polarized and they exhibited a poor cytolytic, rather suppressive (IL-10 and TGF-ß mediated) phenotype. In naïve $CD3^+CD8^{low}$ (and $CD3^+CD8^{normal}$) T-cells from healthy human donors or mice a type-2 polarization was only achieved after stimulation with anti-CD2, anti-CD3, and anti-CD28 monoclonal antibody or phytohemagglutinin [20] or IL-4 [21,34] indicating the

Table 2
Description of 17q21 locus polymorphisms.

SNP ID	rs2290400	rs7216389	rs8076131
Gene	GSDMB	GSDMB	ORMDL3
Alleles	T/C	T/C	G/A
Minor allele	T	T	G
Risk allele	T	T	A
Position in chromosome[a]	17:39909987	17:39913696	17:39924659
Location in gene	Intron	Intron	Intron
Minor allele frequency PASTURE	0.476	0.472	0.492
Minor allele frequency CD8- group	0.467	0.474	0.446

[a] dbSNP GRCh38.p2.

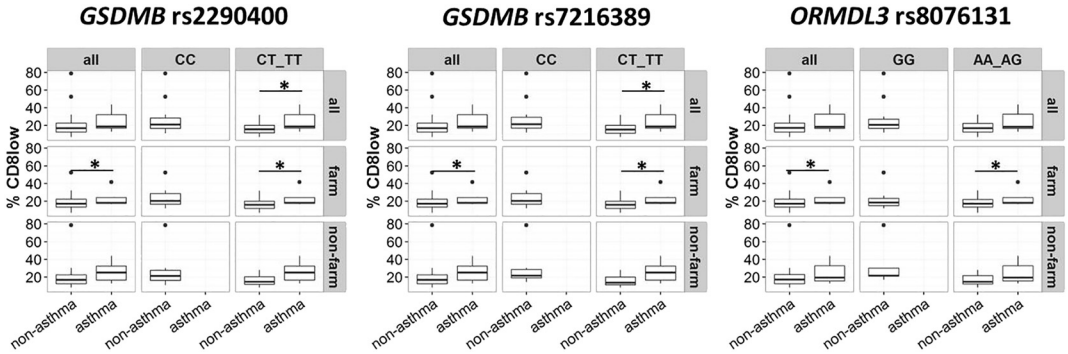

Fig. 5. Asthmatic farm children with asthma risk alleles have more $CD3^+CD8^{low}$ T-cells than healthy children with risk alleles. (**A**) Boxplots show % of unstimulated $CD3^+CD8^{low}$ T-cells in 6-year-old children comparing non-asthmatics (NA) and asthmatics (A) with risk alleles (GSDMB_rs2290400 and rs7216389: risk allele CT_TT; ORMDL3 rs8076131: risk allele AA_AG). Data are shown overall and stratified for risk allele (non-risk = NR, risk = R) and farming exposure (farm = F, non-farm = NF). Bars above single boxplots display result from Wilcoxon-test of NA versus A with $^*p < 0.05$. N(overall) = 76. *N*(NR-F, NR-NF, R-F,R-NF) = 11,8,30,27/11, 7, 30, 28/7, 5, 41, 33.

need of stimulation for $CD3^+CD8^{low}$ T-cells to exhibit type-2 phenotype or even suppressor function. Of note, since we have not cultured isolated $CD8^+$ T-cells but PBMCs in this study, diverse effects of different cell populations on $CD8^{low}$ T-cells influencing each other, are conceivable. Asthma is a chronic inflammatory disease and a farming environment offers constant antigen exposure giving $CD3^+CD8^{low}$ T-cells of farm children the opportunity to possibly polarize towards a suppressive effector cell. Therefore, one could hypothesize that the increase of $CD3^+CD8^{low}$ T-cells in asthmatic farm children might be the result of a protective "asthma-compensating" mechanism. The ratio of PI/U $CD3^+CD8^{low}$ T-cells is higher in non-asthmatic as compared to asthmatic farm children, indicating their potential to upregulate suppressive $CD8^{low}$ T-cell expression upon stimulation. Thereby, the protective farm effect mediated by suppressive CD3 + CD8low T-cells might remain inefficient. On the other hand, the stimulatory capacity (e.g. ratio of cells with and without PI stimulation) does not directly reflect suppressor function. Yet, this group of asthmatic farm children is an important subgroup to study and to learn from, since protection does not work as efficiently as in the large group of protected non-asthmatic farm children.

5.2. Role of IFN-γ secretion by $CD8^{low}$ T-cells in asthma

Since IFN-γ secretion was shown to be markedly different between $CD8^{normal}$ and $CD8^{low}$ T-cells [20], we measured IFN-γ in whole blood supernatants of PI-stimulated cells in 6-year-old children with available CD8 data (CD8-group) and the whole German subpopulation. Among farm-exposed children, asthmatics showed significantly lower IFN-γ levels than non-asthmatics and also non-farm children displayed at least a similar direction, yet not significant, matching a type-2 shifted immune state in asthmatic children. IFN-γ is a Th1/type-1 cytokine promoting enhanced phagocytic activity, while type-2 immune responses are characterized by e.g. IL-4 and IL-13 secretion and high antibody titers including IgE [35] being associated with asthma and allergy. Low IFN-γ expression levels postnatally are related to higher risk for allergies, atopic disease and airway obstruction [36,37]. Intracellular staining of IFN-γ in PI-stimulated PBMCs from 10.5-year-old farm children revealed that $CD3^+CD8^{low}$ and $CD3^+CD8^{low}CD25^+$ T-cells are beside B-lymphocytes the subpopulation with the smallest IFN-γ production compared to $CD3^+CD8^+$, $CD3^+CD8^-$, and NK cells matching suppressor or type-2 polarized cells. However, the number of cases was small ($N = 13$). Trautmann and colleagues, by contrast, reported a higher IFN-γ secretion by $CD3^+CD8^{low}$ cells than by $CD3^+CD8^{normal}$ cells after stimulation with anti-CD2/CD3/CD28 indicating a stimulus-dependent IFN-γ response [20].

Table 3
Detailed information about the number of all genotyped individuals.

GSDMB_rs2290400	**Farming**	**Asthma**	**CC**	**CT_TT**
	farm	asthma	0	4
	farm	non-asthma	11	26
	non-farm	asthma	0	6
	non-farm	non-asthma	8	21
	Sum	Sum	19	57
GSDMB_rs7216389	**Farming**	**Asthma**	**CC**	**CT_TT**
	farm	asthma	0	4
	farm	non-asthma	11	26
	non-farm	asthma	0	6
	non-farm	non-asthma	7	22
	Sum	Sum	18	58
ORMDL3_rs8076131	**Farming**	**Asthma**	**GG**	**AA_AG**
	farm	asthma	0	4
	farm	non-asthma	7	30
	non-farm	asthma	0	5
	non-farm	non-asthma	5	23
	Sum	Sum	12	62

5.3. Impact of genotype for $CD8^{low}$ T-cells and asthma

The chromosome 17q21 locus with the co-expressed genes *ORMDL3* and *GSDMB* is known as major genetic risk locus for childhood asthma [38]. While ORMDL3 protein is a negative regulator of sphingolipid synthesis [25], GSDMB is a member of the gasdermin-domain containing protein family. Its protein function is not fully understood, but an involvement in secretory pathways is suggested [26]. Furthermore, it was recently shown that GSDMB is highly expressed in lung bronchial epithelium and its overexpression in primary human bronchial epithelium increased expression of genes important to airway remodeling (TGF-β1, 5-lipoxygenase) and airway-hyperresponsiveness [39]. Loss et al. have demonstrated that the 17q21 locus is related to episodes of acute airway obstruction in asthma, but underlying mechanisms are still unknown [40]. A possible mechanism for the relation of 17q21 locus and episodes of acute airway obstruction might be mediated by $CD8^{(normal)}$ T-cells, which belong to the innate immune system and eliminate particularly virus-infected cells by cytolytic enzymes. In contrast, $CD3^+CD8^{low}$ T-cells were shown to be poorly cytolytic and rather suppressive [20,21]. In our study, among children with asthma risk alleles (*GSDMB* rs2290400 and rs7216389, *ORMDL3* rs8076131) asthmatics displayed more $CD3^+CD8^{low}$ T-cells than non-asthmatics (overall and among farm children). Thus, disease and genetic risk for asthma may be related to increased $CD3^+CD8^{low}$ T-cells, being inefficient for healthy immune balance despite their general suppressive capacity. Alternatively, increased $CD3^+CD8^{low}$ T-cells point towards a pro-inflammatory state during chronic airway inflammation. Of note, we were not able to disentangle the role of $CD3^+CD8^{low}$ T-cells in asthmatic children without genetic predisposition as these children were not available in this subgroup demonstrating the association of asthma

risk alleles with asthma development, as shown before [18]. Importantly, among the whole PASTURE study population over 87% of asthmatic children (and 100% of asthmatic children in the CD8-group) had the analyzed asthma risk alleles encompassing significantly more risk alleles in asthmatic children than in non-asthmatic children (not shown). In conclusion, in this cohort of protected children the role of chromosome 17 genotype on $CD8^{low}$ T-cells cannot finally be disentangled for asthma development. Either $CD3^+CD8^{low}$ T-cells play a protective (suppressive) role in asthma induced by farm exposure or $CD3^+CD8^{low}$ T-cells are asthma-promoting, pro-inflammatory cells pointing towards a specific asthma-related mechanism in asthmatic farm children despite continuous antigen exposure in a farm environment.

5.4. Strengths and limitations of our study

Notably, our study's limitation are the small case numbers due to the study design in an "asthma-protective farm environment", but still our results point to a clear role for $CD3^+CD8^{low}$ T-cells in asthma development. However, our data requires replication in future studies. We may rather have an effect of underestimation. Furthermore, similar characteristics between our whole population, the German subpopulation and the German CD8-group of the PASTURE/EFRAIM cohort regarding parental atopy, sex, smoking during pregnancy and asthma at age 6 yrs point to an important role of $CD3^+CD8^{low}$ T-cells in this environmental setting. It may be interesting to compare our findings to other, asthma-protective environments, where large epidemiological studies including functional assessment of $CD3^+CD8^{low}$ T-cells, asthma and genetic asthma risk in childhood are feasible.

5.5. Conclusion

In summary, we report an increase of $CD3^+CD8^{low}$ and activated $CD3^+CD8^{low}CD25^+$ T-cells together with a decrease of IFN-γ levels in asthmatic compared to non-asthmatic farm children suggesting a specific role of $CD8^{low}$ T-cells in asthmatic farm children, which might represent either counteracting anti-inflammatory suppressor cells or pro-inflammatory asthma-promoting cells. The amount of $CD8^{low}$ T-cells indicates a farm-dependent mechanism associated with childhood asthma. Further functional analyses and cytokine profiling of $CD3^+CD8^{low}$ T-cells are under way to illuminate the role and function of $CD3^+CD8^{low}$ T-cells in farming children dependent on asthma and asthma risk genotype.

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Author contributions

PS, MT, JK, VC, SI, AB, AT and GL performed experiments and/or analyzed the data. KB, CR, MD, JG, HR, MR and JW provided samples and data for the study. CBF, JR, RL, DAV, JCD, JP, EM, BS designed the study and MT, PS, JK and BS wrote the manuscript.

Conflict of interest

MT, PC, JK, AB, SI, GL, MD, JW, AT, VK, GD, JR, RL, CR, MR, VC, CBF, DV, JCD have nothing to disclose. BS received a grant from German Research Foundation; EM received a European Commission Grant for the EFRAIM project. MK received a Grant from the European Union, German Ministry of Education and Research and German Research Foundation. AMK received a Grant from the Academy of Finland, Juho Vainio Foundation and the Foundation for Pediatric Research. JG received a European Commission Grant for the EFRAIM project. JP received an EU Grant and minor grants from Finnish foundations supporting medical research (Juho Vainio Foundation, Päivikki and Sakari Sohlberg Foundation, Finnish Cultural Foundation).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2017.09.009.

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