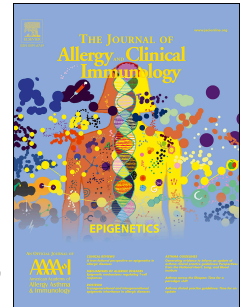


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TNFAIP3 is a key player in childhood asthma development and environment-mediated protection



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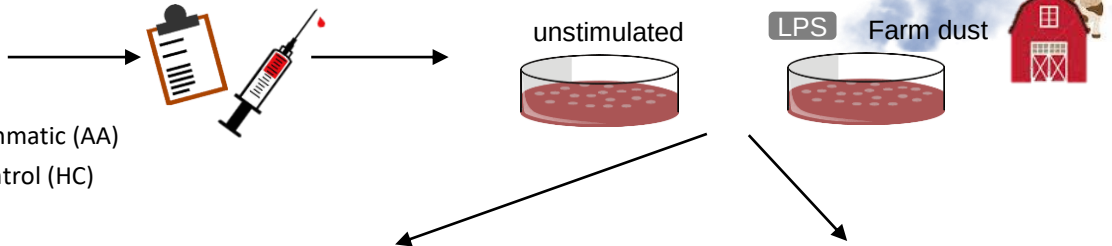


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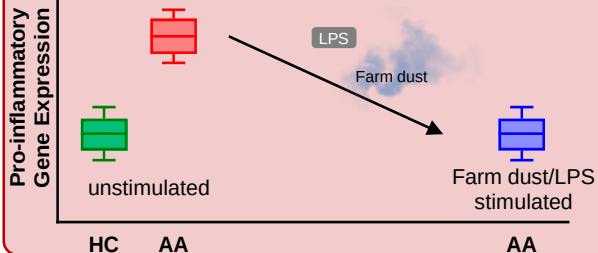


= allergic asthmatic (AA)

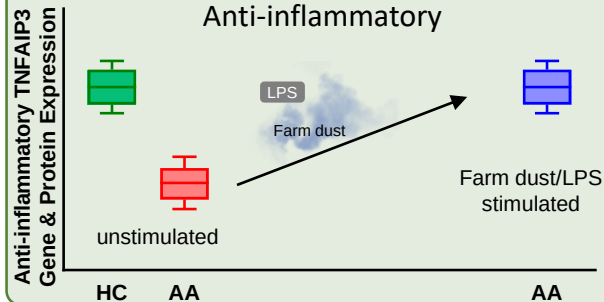
= healthy control (HC)



NF- κ B signaling genes Pro-inflammatory



TNFAIP3 (A20) Anti-inflammatory



Abbreviations: TNFAIP3 (Tumor necrosis factor alpha induced protein 3), LPS (Lipopolysaccharide), AA (allergic asthmatic), HC (healthy control), NF- κ B (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells)

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Abstract

Background

Childhood asthma prevalence is significantly higher in urban areas compared to rural/farm environments. Murine studies have shown that TNFAIP3 (TNF- α -induced protein 3, A20), an anti-inflammatory regulator of NF- κ B signaling, mediates environmental-induced asthma protection.

Objective

We aimed to determine the role of TNFAIP3 for asthma development in childhood and immune modulatory effects of environmental factors.

Methods

In a representative selection of 250 out of 2,168 children from two prospective birth cohort and two cross-sectional studies, we analyzed blood cells of healthy and asthmatic children from urban and rural/farm environments from Europe and China. PBMCs were stimulated *ex vivo* with dust from “asthma-protective” farms or LPS. NF- κ B signaling-related gene and protein expression was assessed in PBMCs and multiplex gene expression assays (NanoString) in isolated dendritic cells of school-children and in CBMCs from newborns.

Results

Anti-inflammatory TNFAIP3 gene and protein expression was consistently decreased while pro-inflammatory *TLR4* expression was increased in urban asthmatics ($p < 0.05$), reflecting their elevated inflammatory status. *Ex vivo* farm dust or LPS-stimulation restored *TNFAIP3* expression to healthy levels in asthmatics and shifted NF- κ B signaling associated gene expression towards an anti-inflammatory state ($p < 0.001$). Farm/rural children showed lower expression indicating tolerance induction by continuous environmental exposure. Newborns with asthma at school-age showed reduced *TNFAIP3* expression at birth, suggesting TNFAIP3 as a possible biomarker predicting subsequent asthma.

Conclusion

Our data indicate TNFAIP3 as key regulator during childhood asthma development and its environment-mediated protection. Since environmental dust exposure conferred anti-inflammatory effects, it may represent a promising future agent for asthma prevention and treatment.

Disclosure of potential conflict of interest

This work was supported by EFRAIM EU FP7- [grant numbers KBBE-2007-1, DFG SCHA 997/3-1 (BS, PS), DFG-SCHA 997/7-1 (BS, MT, JK), SFB TR22, DFG-SCHA 997/8-1 (BS)]; Comprehensive Pneumology Center (BS); FöFoLe (KR); EKFS (BS, AB); Academy of Finland (MR); CK-CARE / Kühne Foundation, Davos, Switzerland (RL, CR); PHRC IR University Hospital of Besancon, France (JCD) and supported by International (Regional) Cooperation and Exchange Program (Cooperation Research-NSFC-AF-DFG) by National Natural Science Foundation of China [grant number 81261130023) (JL). The rest of the authors declare that they have no relevant conflicts of interest.

Key Messages

- Urban asthmatic children express less *TNFAIP3*, a negative regulator of the NF- κ B pathway.
- Farm dust/LPS stimulation downregulates pro-inflammatory genes while anti-inflammatory genes are upregulated.
- Healthy newborns with subsequent asthma expressed less *TNFAIP3* at birth, suggesting *TNFAIP3* as a potential biomarker.

Capsule Summary

Asthmatic children and newborns with subsequent asthma express less *TNFAIP3*, suggesting this negative NF- κ B regulator as a potential biomarker. *Ex vivo* farm dust/LPS stimulation has anti-inflammatory capacities and shifts *TNFAIP3* expression to healthy levels.

Key words

A20, asthma, childhood, immune development, environment, farming, inflammatory, *TNFAIP3*, LPS, protection

121 **Abbreviations**

122	AA	allergic asthmatics
123	ATS	American Thoracic Society
124	A.U.	arbitrary units
125	BSA	bovine serum albumin
126	C	Conghua
127	CBMCs	cord blood mononuclear cells
128	CD	cluster of differentiation
129	cDNA	complementary deoxyribonucleic acid
130	CLARA/CLAUS	CLinical Asthma Research Association
131	CUHK	Chinese University of Hong Kong
132	CREC	Clinical Research Ethics Committee
133	DCs	dendritic cells
134	ECL	enhanced chemiluminescence
135	E.coli	Escherichia coli
136	ERS	European Respiratory Society
137	F	farm
138	FCS	fetal calve serum
139	Fi	Finish
140	Fig.	Figure
141	G	German
142	GINA	Global Initiative for Asthma
143	h	hours
144	HC	healthy controls
145	HK	Hong Kong
146	HRP	Horseradish peroxidase
147	IgE	immunglobuline E

148	IgG	immunoglobuline G
149	IL-18	interleukin-18
150	IQR	inter quartile range
151	LAL	Limulus amoebocyte lysate
152	LPS	lipopolysaccharide
153	MACS	Magnetic Cell Separation
154	MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
155	MANOVA	multivariate analysis of variance
156	MYD88	Myeloid differentiation primary response 88
157	NF	non-farm
158	NF- κ B	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
159	nt	nucleotides
160	PBMCs	peripheral blood mononuclear cells
161	PVDF	Polyvinylidene fluoride
162	RIPA	Radioimmunoprecipitation assay
163	ROX	Rhodamin X
164	RPMI	Roswell Park Memorial Institute
165	RT-PCR	real time polymerase chain reaction
166	SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
167	TAX1BP1	Tax1-binding protein 1
168	TBST	Tris-buffered saline with Tween20
169	TLR4	Toll-like receptor 4
170	TNFAIP3	Tumor necrosis factor alpha induced protein 3
171	TNIP2	TNFAIP3 Interacting Protein 2
172	TRAF6	Tumor necrosis factor receptor associated factor 6
173	y	years

174

175

Introduction

Asthma is one of the most prevalent chronic diseases in childhood, where the majority of affected children can be classified as allergic asthmatics producing immunoglobulin E (IgE) antibodies against environmental antigens.¹ The multifactorial etiology of asthma includes genetic factors and environmental exposures illustrated by remarkable differences in prevalence between children from urban and rural areas.²

Asthma prevalence has been rising particularly in westernized countries with high hygiene standards, whereas children from rural areas in Europe and China and especially farming environments with constant exposure to microbial components like lipopolysaccharides are protected.^{3,4}

The recognition of LPS (endotoxin) by TLR4, expressed on innate immune cells activates inflammatory responses like NF- κ B signaling.⁵ Since uncontrolled inflammation would cause serious tissue damage, these processes are tightly regulated.⁶ Specifically, endotoxin tolerance, an adaptive protective mechanism, controls excessive inflammation by mitigated response to repetitive LPS-stimulation and suppression of pro-inflammatory signaling and upregulation of anti-inflammatory processes.⁵

TNFAIP3 (A20), a negative regulator of the inflammatory NF- κ B pathway, is important for immune regulation in asthma and its environment-mediated protection.⁷ Amish children living on traditional farms with lower asthma prevalence compared to Hutterite children from highly industrialized farms expressed significantly higher levels of *TNFAIP3*.⁸ Furthermore, intranasal instillation of Amish dust extracts significantly inhibited airway hyperreactivity in a murine model.⁸ An allergy-protective effect of farm dust and endotoxin mediated by *TNFAIP3* was also shown in lung epithelial cells.⁷

We aimed to disentangle the role of TNFAIP3 during childhood asthma development and environment-mediated protection. Specifically, we investigated the following four questions: Is *TNFAIP3* and NF- κ B signaling gene expression deregulated in PBMCs and dendritic cells of steroid-naïve asthmatics during

200 asthma manifestation? Is it possible to modulate *TNFAIP3* expression and related genes by exposure to
201 “asthma-protective” farm dust extracts *ex vivo*? Do children with constant prior *in vivo* farm dust exposure
202 exhibit modulated *TNFAIP3* gene expression? And can *TNFAIP3* expression at birth serve as a predictive
203 marker for subsequent asthma development?

Methods

Study design

We analyzed blood samples from two cross-sectional studies (CLARA/CLAUS, TRILATERAL) and two prospective birth cohort studies (PASTURE/EFRAIM, PAULINA/PAULCHEN). A case-control design was chosen (see below for each study population). Power calculation revealed a required group size of 14 children per phenotype for one Δ ct differences of TNFAIP3 expression based on an assumed standard deviation of 0.8 Δ ct within group with $\alpha=0.05$ significance and $\beta=0.9$ power. Higher subject numbers were selected, based on RNA availability. Informed written consent was obtained from the parents.

Study population and characteristics (Details see Supplement and Fig. E1)

The CLARA/CLAUS cohort includes healthy (HC) and mild-to-moderate asthmatic 4-15-year-old children (N=273/N=334) recruited at LMU children's hospital since 01/2009.^{9,10} Asthmatics were diagnosed according to GINA-guidelines.¹¹ Eligibility criteria for asthmatics were classical asthma symptoms, at least 3 episodes of wheeze and/or doctor's diagnosis and/or history of asthma medication and lung function indicating significant reversible airflow obstruction according to ATS/ERS-guidelines.¹² Allergic asthmatics (AA) were defined by specific IgE level ≥ 0.35 IU/mL and clinical symptoms. HC were matched by age (10.2 ± 2.8 y). The analyzed subgroup of 50 children (26 HC/24 AA) is representative for the whole cohort regarding age, sex, breastfeeding time, maternal atopy and asthma, smoking and number of siblings. Approval was obtained from the local ethics board, LMU Munich, Germany (Nr. 379-08).

The international birth cohort study PASTURE/EFRAIM includes 1,133 children from rural areas in Germany, Switzerland, Austria, Finland and France from the third trimester of pregnancy until 10.5y.^{13,14} Asthma was defined as physician's diagnosis of recurrent spastic, obstructive, or asthmatic bronchitis and/or asthma reported by the parents at the age of 6.¹⁵ The selected 6-year-old children (N=63, 32 farmers (7 Asthmatics/25 HC), 31 non-farmers (10 Asthmatics/21 HC) are representative for the entire cohort with no significant differences regarding sex, farming, number of siblings, breast feeding. Selected

asthmatics showed more familiar atopy. The study was approved by the local research ethics committee from each country (Ethics numbers: Germany: 02046; Austria: 401; Switzerland EKSG: 021056; Finland: 10/2008; France: 07/448).

In the Chinese part of the TRILATERAL study, N=3435 and N=14152 children born locally in Hong Kong ($7.0 \pm 0.7y$) or Conghua ($7.2 \pm 1.5y$) in Mainland China were recruited between 2013-2014. Prevalence of physician diagnosed asthma was significantly higher in Hong Kong compared to Conghua (5.3% vs. 2.6%, $p<0.001$). Asthmatics were defined as having a history of wheeze ever, current wheeze within 12 months and physician/hospital diagnosed asthma. HC were those without a history of wheeze, physician diagnosed asthma, allergic rhinitis, atopic dermatitis and eczema ever. 79 children from Hong Kong (32 asthmatics/47 HC) and 66 children from Conghua (19 asthmatic/47 HC) were recruited for the case-control study (Approval number: CUHK CREC number 2013.220). The analyzed subgroup of 112 children (HK: 54 (32 HC/22 AA); C: 58 (39 HC/19 AA)) is representative for the whole cohort.

In the combined birth cohort study PAULINA/PAULCHEN, cord blood samples from newborns from the Munich area (N=190) and rural Germany (N=93) were collected from 2004-2008.¹⁶⁻¹⁸ Eligibility criteria were an uncomplicated pregnancy and healthy neonates. Exclusion criteria were preterm delivery, perinatal infections and maternal use of antibiotics during the last trimester of pregnancy and chronic maternal diseases. Subsequent asthma in children was defined as doctor's diagnosis of asthma until the age of 6y and/or 10y by questionnaires. The 25 selected non-farm children (16 HC/9 Asthmatics) are representative for the entire cohort showing no significant difference in sex, birth weight, birth length, and gestational age, maternal age, asthma and smoking or paternal atopy. Approval was obtained from the human ethics committee of the Bavarian Ethical Board, LMU Munich, Germany (Ethical approval of „Bayerische Landesärztekammer“: 04092).

Laboratory methods (details Suppl. Table E1)**Dust collection and extraction**

Environmental dust was collected by standardized methods, where electrostatic dust collectors were placed in cowsheds for 4 weeks in Germany (G) and Finland (Fi).¹⁹ Dust was extracted as described before.²⁰

Cell isolation and stimulation

CBMCs and PBMCs were isolated by density gradient centrifugation within 24 h after blood withdrawal and 5×10^6 cells/ml were cultivated at 37 °C, 5% CO₂. PBMCs from CLARA/CLAUS children were cultured for 24 h in X-Vivo unstimulated or stimulated with LPS (0.1 µg/ml, E.coli-O111:B4) or 40 µg/ml farm dust extract (G, Fi). Whole blood cultures from PASTURE/EFRAIM children were incubated for 24 h in RPMI/10% FCS unstimulated or LPS-stimulated (0.1 µg/ml, E.coli-O111:B4). PBMCs from TRILATERAL children were cultured in RPMI/10% FCS unstimulated or stimulated with 1 µg/ml LPS. CBMCs from newborns were cultured for 72 h unstimulated in RPMI/10% FCS. Differences in LPS-concentrations derive from preliminary studies where ideal conditions were tested to be optimal in each setting.

RNA isolation, cDNA synthesis and quantitative RT-PCR

CBMC and leukocyte RNA was extracted using TRIzol or QIAzol, and chloroform, while RNA from PBMCs and DCs was extracted using RNeasy Mini Kit. RNA concentration and quality was tested via Nanodrop and/or Bioanalyzer. cDNA was synthesized using the QuantiTect Reverse Transcription Kit. Gene expression was quantified by RT-PCR in 96-well plates under standard conditions including ROX as passive reference, melt curve analysis and automatic threshold setting using SYBR Green, set up in 10 µl containing 6 ng cDNA and 320 nM primers. 18S levels were used for normalization. The primer sequences are listed in the supplement (Suppl. Table E2).

Western blotting

PBMCs were lysed in 100 μ l RIPA Buffer containing protease inhibitors. Protein concentration was determined by Bradford assay. Cell lysates (50 μ g/lane) were separated by SDS-PAGE. Proteins were transferred to PVDF membranes, blocked in 5% milk-TBST and incubated in 5% BSA-TBST and TNFAIP3 antibody. Blots were incubated with HRP-conjugated goat anti-rabbit IgG and visualized by ECL solution on a ChemiDocTM System. β -Actin was used as loading control. Protein expression was quantified using ImageJ Software.

Nanostring

Dendritic cells were separated from unstimulated and German dust-stimulated PBMCs of CLARA/CLAUS children by AutoMACS using the dendritic cell isolation kit. In this proof-of-principle experiment, cells were stimulated exclusively with German dust, due to limited material availability. Extracted RNA was concentrated using the RNA Clean & ConcentratorTM-5 Kit. Concentration, quality and fragmentation (>300nt) was assessed with the Bioanalyzer RNA 6000 Nano Kit. 100 ng RNA were hybridized (18 h, 65 °C) and the PanCancer Immune Profiling Panel was used for NanoString nCounter expression analysis.²¹ Data were analyzed utilizing nSolver Analysis Software v3.0. Quality control was performed using default settings within the software, positive controls, housekeeper, and total (excluding controls) counts and binding densities in each sample. A standard curve based on positive controls was used for standardization. Expression was normalized to 33 housekeeping genes.

Statistical analysis

Differences between population characteristics were tested with Wilcoxon tests for continuous variables and Fisher tests for contingency tables. Gene expression was analyzed by global tests for the null hypothesis of no effect by stimulation, farm exposure and phenotype. The parallel assessment of several genes within each subject was taken into account by using the multivariate extension of the analysis-of-variance framework (MANOVA, Supplement), which is more powerful than multiple single ANOVA for each gene separately. The significance of the global tests was derived via the F-test as implemented in R

package multcomp.²² Single missing values in the gene expression profiles were imputed by chained equations.²³ Following identification of significant global differences for each cohort (Suppl. Table E3), we assessed specific differences by two-sample t-tests. The mean was reported with 95% confidence intervals. Outliers were defined as data points having a distance of more than three IQR to the lower/upper data quartile and excluded for parametric tests. An unadjusted p-value less than 0.05 was considered as significant. Statistical analysis and visualization was performed using R version 3.3.1 and the ggplot package.^{24,25}

Results

Asthmatic urban children express less TNFAIP3

To investigate the regulation of *TNFAIP3* and related NF- κ B signaling genes, we assessed gene expression in PBMCs of steroid-naïve asthmatic and healthy school-age children from urban Germany.^{9,10} NF- κ B pathway genes *TNFAIP3*, *TLR4*, *MYD88*, *MALT1*, *TRAF6*, *TNIP2*, *TAX1BP1* and T-cell-associated genes *CD274*, *CD279*, *CD80*, *CD152*, *CD86* and *CD28* were examined. To compare findings from this European cohort with the international TRILATERAL cohort we assessed gene expression of *TNFAIP3*, *TLR4*, *MYD88*, *MALT1*, *TRAF6*, *TNIP2*, *TAX1BP1*, *CD274*, *CD80* and *CD86* in urban Chinese children. Under unstimulated conditions, German asthmatic children displayed significantly decreased *TNFAIP3* and *CD80* expression and increased *TLR4* levels compared to healthy controls (Fig.1A, $p \leq 0.007$). No phenotypical differences in expression of the other investigated genes were observed under unstimulated conditions (Suppl. Fig. E3). Lower *TNFAIP3* levels of asthmatic children were confirmed on protein levels (Fig.1C, $p = 0.035$, representative Western Blot in Suppl. Fig. E2). Consistently, less *TNFAIP3* expression was replicated in asthmatics from Hong Kong (Fig.1B, $p = 0.042$), indicating an impaired negative regulation of the NF- κ B pathway in asthmatic children across different urban areas.

Ex vivo dust stimulation upregulates TNFAIP3 expression

We analyzed whether 24 h stimulation with farm dust extracts or LPS could shift reduced *TNFAIP3* levels in asthmatics to levels comparable to a healthy basal state (*TNFAIP3* levels of healthy children under unstimulated conditions), mimicking asthma-protective farm exposure *ex vivo*. We extracted dust from German (G) and Finnish (Fi) farms and stimulated PBMCs of children from urban Germany with these dust extracts and LPS, one major component of farm dust, to analyze their immune regulatory capacities. LAL-testing of the dust samples revealed high endotoxin concentrations with a bacterial composition of mainly Proteobacteria (90.1%), but also gram-positive Firmicutes (8.6%) and Actinobacteria (1%) (see Supplement). PBMCs of Chinese children have only been stimulated with LPS, due to logistic reasons.

Upon LPS-stimulation, asthmatics from urban Germany and China reached *TNFAIP3* levels comparable to those of healthy children under unstimulated conditions (Fig.2A, 2B). German dust stimulation also resulted in increased *TNFAIP3* expression, yet not significant (data not shown). Finnish farm dust (Fi) stimulation also yielded a significant increase of *TNFAIP3* to healthy levels. On protein levels, *TNFAIP3* upregulation was even stronger upon Finnish farm dust stimulation compared to LPS (Fig.2C). Interestingly, for healthy children no significant upregulation of *TNFAIP3* was observed on protein levels. Taken together, urban asthmatic children showed decreased *TNFAIP3* transcriptional and translational level compared to healthy children but *TNFAIP3* expression could be upregulated upon *ex vivo* stimulation with farm dust (Fi) and LPS.

***Ex vivo* dust stimulation downregulates pro-inflammatory genes while upregulating anti-inflammatory genes**

Aiming at identifying the immunological mechanisms underlying the protective farm effect in a more detailed approach, we investigated gene expression of related genes within the NF- κ B signaling pathway and co-stimulatory molecules.

Stimulation with dust extracts from Finnish (Fi) and German (G) farms or LPS significantly increased expression of anti-inflammatory *CD274*, *CD152* and *CD80* (red), while decreased expression of pro-inflammatory *CD86*, *CD28*, *TLR4* and *MYD88* (blue), in healthy and asthmatic children (Fig.3A, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). This suggests further immune modulatory characteristics of environmental dust. Noteworthy, asthmatics from urban Germany showed significantly stronger downregulation of *TLR4*, *CD86*, *TRAF6*, and *MALT1* (G) as compared to HC (Fig.3A, $p < 0.04$, indicated by black rectangle around the respective gene). Increased anti-inflammatory gene expression of *CD274* and *CD80* together with decreased pro-inflammatory *TLR4* expression upon LPS-stimulation could be confirmed for all children from urban China (Fig.3B). Of note, in preliminary tests we also included urban dust samples as a negative control resulting in consistent or even decreased *TNFAIP3* expression (Suppl. Fig. E4).

To investigate the effect of simultaneously increased anti-inflammatory and decreased pro-inflammatory expression upon dust and LPS stimulation, we calculated an “inflammation ratio” by relating pro- to anti-inflammatory gene expression. The average Δ CT-value of all genes assembling anti-inflammatory properties (TNFAIP3, CD274, CD80, TNIP2, TAX1BP1, CD152, CD279) was divided by the average Δ CT-value of all pro-inflammatory genes (*TLR4*, *MYD88*, *MALT1*, *TRAF6*, *CD86*, *CD28*). Under unstimulated conditions (U), asthmatics had a significantly higher inflammation ratio, indicating a stronger pro-inflammatory basal state of asthmatics compared to healthy children (Fig.3C, $p=0.005$, see Supplement-results). Upon stimulation, this inflammation ratio was highly significantly reduced, indicating the anti-inflammatory capacity of 24 h stimulation with farm dust and LPS for both healthy and asthmatic children (Fig.3C, $**p<0.01$, $***p<0.001$).

IL-18 gene expression was analyzed to investigate downstream NF- κ B signaling. *IL-18* is induced via the TLR4-MYD88 axis and is associated with asthma pathogenesis by inducing Type2-cytokines, allergic inflammation and eosinophilic influx into the airways.^{26,27} Upon unstimulated conditions, asthmatic children showed significantly higher levels of *IL-18* in line with their higher inflammatory status at baseline (Fig.3D, $p=0.002$). However, when stimulating PBMCs with German and Finnish farm dust, pro-inflammatory *IL-18* was significantly downregulated in both healthy and asthmatic children (Fig. 3D, $*p\leq 0.05$, $**p\leq 0.01$, $***p\leq 0.001$), while asthmatic children downregulated *IL-18* expression even stronger upon LPS ($p=0.001$) and German farm dust ($p=0.045$) stimulation. These data emphasizes an anti-inflammatory capacity of farm dust and LPS stimulation even downstream of NF- κ B on cytokine level.

***TNFAIP3* expression in dendritic cells**

To investigate whether the findings are related to the most potent antigen presenting cells, we examined dendritic cells (DCs), as we have previously shown the important role of DCs for environment-mediated asthma protection.^{28,29} We isolated dendritic cells from unstimulated and farm dust stimulated (G) PBMCs of healthy and asthmatic children from urban Germany and assessed multiplex gene expression in a proof-of-principle experiment.

Under unstimulated conditions, asthmatics displayed less *TNFAIP3* expression in dendritic cells, yet not significant ($p=0.26$) while *TLR4* expression was significantly increased (Suppl. Fig. E5, $p=0.01$).

However, upon farm dust stimulation, pro-inflammatory signaling genes like *TLR4*, its interacting partner *CD14* and *MYD88*, *IRAK1*, *IRAK4* and *IL-18* were downregulated in dendritic cells, while anti-inflammatory signaling genes like *TNFAIP3*, the NF- κ B inhibiting protein *NFKBIA* (*IKK α*), *CD80* and *CD274* were significantly upregulated (Fig.3E, *** $p<0.001$, ** $p<0.01$, * $p<0.05$) in both healthy and asthmatic children.

These findings confirm the results obtained for PBMCs and underline the regulatory role of dendritic cells in the context of environment-mediated asthma protection.

Living in rural areas downregulates NF- κ B signaling gene expression

Next, we aimed to determine whether natural *in vivo* farm exposure modulates *TNFAIP3* expression and its associated pathway genes. Therefore, we examined gene expression in a subset of farm and non-farm children (both healthy and asthmatics) in unstimulated or 24 h LPS-stimulated whole blood cultures (N=61) in our European PASTURE/EFRAIM cohort. No difference in *TNFAIP3* expression was present between healthy and asthmatic farm children. However, comparing farm and non-farm children independent from phenotype, farm children expressed significantly less *TNFAIP3* and *CD274* under unstimulated conditions (Fig.4A, $p\leq 0.035$). To assess if this effect is unique for European farming environments, we investigated gene expression in PBMCs of children from urban (Hong Kong) and rural China (Conghua), another asthma-protective environment.³⁰ Consistently, there was no significant difference between asthmatic and healthy children from rural China under unstimulated conditions. Comparing urban to rural children, rural children expressed significantly lower levels of all investigated genes (Fig.4B, $p\leq 0.003$), suggesting a lower basal inflammatory response pattern for rural children, replicating the effects observed in farm children from Europe. *Ex vivo* LPS-stimulation for children from both European farms and rural China resulted in a strong upregulation of anti-inflammatory *TNFAIP3*,

CD80 and *CD274* while downregulated *TLR4* expression (Fig.5A/B, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$) in line with the results observed in the urban cohorts.

In summary, children from European farms and rural China showed decreased gene expression of the NF- κ B pathway including *TNFAIP3* compared to non-farm/non-rural children. Importantly, the capacity to increase anti-inflammatory genes (*TNFAIP3*, *CD80* and *CD274*) and decrease pro-inflammatory *TLR4* was preserved following LPS-stimulation *ex vivo*.

Newborns with subsequent asthma express less *TNFAIP3*

Finally, to assess whether dysregulation of *TNFAIP3* is already present at birth, we analyzed the expression of NF- κ B pathway genes and T cell-associated genes in CBMCs of healthy newborns from the German birth cohort PAULINA/PAULCHEN.¹⁶⁻¹⁸⁸ Subsequent asthma diagnosis at school-age until 10y was assessed by a physician.

In fact, newborns with subsequent asthma displayed significantly lower *TNFAIP3* expression dysregulated NF- κ B signaling gene expression already at birth (Fig.6, $p = 0.037$). Furthermore, the T-cell associated genes *CD274* and *CD80* were decreased in newborns with subsequent asthma compared to the healthy children, although not significant (Suppl. Fig. E6, $p = 0.055$ respectively $p = 0.084$). This suggests an important role of *TNFAIP3* for the regulation of inflammation and its involvement in the early determination of asthma development.

Discussion

In this study comprising data of four pediatric cohorts (total of 2,168 children) from urban and rural/farm areas in Germany and China, we demonstrated that asthmatic children from both Germany and China expressed less *TNFAIP3*, a central negative regulator of the pro-inflammatory NF- κ B pathway on transcriptional and translational levels. Notably, lower *TNFAIP3* expression levels were already present at birth in children who developed asthma by 10y, suggesting *TNFAIP3* as a potential biomarker for asthma prediction. Moreover, *ex vivo* stimulation of PBMCs with farm dust extracts and LPS upregulated expression of *TNFAIP3* and other anti-inflammatory regulators, while decreasing pro-inflammatory genes. These anti-inflammatory capacities may indicate a potential therapeutic role for farm dust exposure even in manifest asthmatics. Another support for this concept is shown by an anti-inflammatory shift in the most potent antigen-presenting cells, namely dendritic cells. Farm children with long-term *in vivo* farm exposure showed overall less gene expression of all investigated genes compared to urban children. Nevertheless, the anti-inflammatory capacity of acute *ex vivo* LPS-stimulation was also present in farm children.

Decreased *TNFAIP3* expression in PBMCs of asthmatic school-children from two distinct urban areas (Germany and China) together with increased *TLR4* expression indicates an impaired negative regulation of NF- κ B signaling with simultaneous excessive activation of this pathway. Increased expression of the LPS-recognizing *TLR4*-receptor was also identified in dendritic cells of asthmatics. These alterations may contribute to airway inflammation resulting in clinical symptoms of asthma.

Farm and rural environments protect children from asthma and allergic diseases.³ To identify the immunological mechanisms underlying this “farm effect”, we performed stimulation experiments with farm dust and its main component LPS. Of note, 16S rRNA sequencing revealed a high bacterial richness of the farm dust extracts comprising mainly gram-negative Proteobacteria but also gram-positive bacteria without any LPS in their cell walls. Since 24 h LPS-stimulation results in a tolerogenic state, we used this model to mimic *in vivo* farm exposure. Indeed, *ex vivo* stimulation of PBMCs with LPS or farm dust

activated anti-inflammatory processes by increasing TNFAIP3, the central negative regulator of NF- κ B signaling, in healthy and asthmatic children. Notably, stimulation even restored the lower levels of asthmatics by shifting its expression to a “healthy level”. Anti-inflammatory properties via LPS and farm dust were also mediated by upregulating *CD274*, a suppressive T-cell ligand and *CD80*. Whereas *CD80* interacts with a greater affinity with inhibitory molecules (*CD152*, *CD274*), *CD86* executes T-cell activation through the interaction with *CD28*.^{31,32} In fact, stimulation resulted in significant downregulation of pro-inflammatory *CD28* in healthy children while suppressive *CD152* was upregulated in asthmatic subjects.

Moreover, stimulation resulted in downregulation of pro-inflammatory regulators like *TLR4*, the initial activator of the NF- κ B pathway. This downregulation of the LPS receptor indicates that environment-mediated asthma protection by chronic/prolonged LPS exposure is driven by hyporesponsiveness subsequently leading to anti-inflammatory immune responses. In addition, its adaptor *MYD88* and *MALT1*, the negative regulator of TNFAIP3 and *TRAF6* that activates the NF- κ B inducing IKK-complex, were reduced upon stimulation in asthmatics. The stimulatory effects of LPS stimulation could also be replicated in our urban Chinese cohort, although in a lower extent. Nevertheless, the key finding, i.e. upregulation of anti-inflammatory *TNFAIP3*, *CD274* and *CD80* while downregulation of pro-inflammatory *TLR4* has been consistently shown in both cohorts.

The beneficial effects of farm dust and LPS stimulation were demonstrated for both healthy and asthmatic children. Therefore, we believe that exposure to farming environments is beneficial for all children and potentially for allergy prevention and is even able to decrease an elevated inflammatory baseline status of allergic asthmatics.

These anti-inflammatory properties of *ex vivo* farm dust stimulation demonstrated in PBMCs were attributable to isolated dendritic cells (DCs), supporting our novel concept of a protective mechanism. As major antigen-presenting cells, DCs are in primary contact to antigens contained in farm dust extracts with the crucial ability to activate T-cells. Moreover, several other farming studies have shown the central role

of DCs, suggesting that specifically signaling through DCs is decisive in environment-mediated protection.^{28,29} While epidemiological studies consistently showed the *in vivo* asthma-protective effect of farm exposure, our immunological findings may suggest possible therapeutic effects of farm dust even at the time of early symptomatic disease. However, aiming at the use of farm dust as preventive or therapeutic approaches it is critical to overcome the challenges of dose, composition, administrative route and safety of farm dust extracts.

In comparison to our *ex vivo* model, farm children are consistently exposed to a range of antigens and high endotoxin levels triggering inflammatory responses. The continuous activation of immune responses induces tolerance mechanisms via unresponsiveness together with direct downregulation of basal inflammation to keep harmful inflammation under control. Also reduced protein expression of both Th1 and Th2 cytokines as a result of constant LPS exposure has been associated with subsequent asthma protection in farm children.^{4,7,33} This could explain our findings of lower pro- and anti-inflammatory gene expression for children from rural China and European farms. In contrast to the Chinese cohort, in which children from rural areas were compared with children from a big city (Hong Kong), the reference group to the farm children in the European PASTURE/EFRAIM cohort comprised children from non-farming areas. This could explain the greater differences between rural and urban China compared to the European cohort.

Despite the protective environmental exposure, a small number of farm children still develop asthma. Thus, additional factors such as genetic predisposition may be responsible for their asthma development. This may explain why we could not detect any significant differences in expression of *TNFAIP3* and the investigated NF- κ B signaling genes, when comparing asthmatic and healthy children from farm/rural areas. This hypothesis is supported by a recent study on prediction of childhood asthma risk (Krautenbacher et al., unpublished). Family history of atopy, age and sex were relevant for asthma prediction for both, farmer and non-farmer. However, whereas asthma prediction was most specific including genetics for farm children, for non-farm children environmental influences were decisive.

Upon stimulation, both farm/rural and urban children showed a similar upregulation of *TNFAIP3*, *CD274* and *CD80* and downregulation *TLR4*, suggesting there is no difference in acute response to inflammatory triggers, although the basal immune status - as illustrated under unstimulated conditions - is generally lower in farm/rural children. Of note, for feasibility a representative subgroup was investigated for functional experiments. Based on sufficient power, confirmation in more than 1 cohort and significant effects despite subgroups, we are confident that these results are translational to the whole cohorts.

In summary, our data confirm *TNFAIP3* as a key regulator of childhood asthma development and important factor explaining the rural asthma-protective effect. Farm dust stimulation restores *TNFAIP3* expression to healthy levels and increased anti-inflammatory while decreased pro-inflammatory gene expression. Furthermore, healthy newborns, who developed asthma at school-age expressed lower levels of *TNFAIP3* already at birth. This key regulator can thus potentially serve as a marker predicting childhood asthma development.

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Contributors

JK, MT and KR interpreted the data and wrote the manuscript. AB analyzed the data. JK, PCS, KR, MST, MT, YX, JKu performed experiments. BS designed the study and contributed to analysis, interpretation and final version of the manuscript. TK contributed to the study design. JR, JCD, JP, RL and MR are PIs and/or contributed to the PASTURE/EFRAIM birth cohort study. JL, CKW, GWKW are PIs of the TRILATERAL project and contributed with design, recruitment of samples, analysis and revisions of the manuscript. The PASTURE study group delivered patient samples and KR recruited patients.

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Figure Legends**Figure 1: Urban asthmatic children express less *TNFAIP3*.**

Scatter plots for *TNFAIP3*, *TLR4* and *CD80* gene (A and B) and *TNFAIP3* protein (C) expression. Data is shown stratified for phenotypes, healthy (HC) and allergic asthmatic (AA) children. Raw data values are represented by horizontally jittered points. Error bars show 95% confidence intervals around the sample means, which were connected by horizontal lines. Differences are indicated by the p-value of two-sample t-tests for phenotype comparisons. Gene expression (Δ ct) was analyzed by qPCR after 24 h cultivation of unstimulated PBMCs of A) 4-15-year-old children from Munich, Germany (CLARA/CLAUS cohort, N=36 (17 HC/19 AA)) and B) 7-year-old children from Hong Kong, China (TRILATERAL cohort N=53 (32 HC/21 AA)). C) Relative *TNFAIP3* protein level (82 kDa) calculated by Image J software (arbitrary units, A.U.) analyzed by Western blot after 24 h cultivation of unstimulated PBMCs of 4-15-year-old children (CLARA/CLAUS cohort, N=25 (11 HC/14 AA)) normalized to β -Actin.

Figure 2: LPS and farm dust stimulation increase *TNFAIP3* gene and protein expression in asthmatic children.

Effect of LPS and German (G) and Finnish (Fi) farm dust stimulation compared to unstimulated (U) conditions shown as scatter plots. Data is shown stratified for phenotypes. Raw data values are represented by horizontally jittered points. Error bars show 95% confidence intervals around the sample means, which were connected by horizontal lines. Differences are indicated by the p-value of two-sample t-tests for phenotype comparisons. Gene expression of *TNFAIP3* (Δ ct) was analyzed by qPCR after 24 h cultivation of unstimulated or LPS- or Fi-farm dust stimulated PBMCs of A) 4-15-year-old children from Munich, Germany (CLARA/CLAUS cohort, N=37 (18 HC/19 AA)) and B) 7-year-old children from Hong Kong, China (TRILATERAL cohort N=53 (31 HC/22 AA)). C) *TNFAIP3* protein level (82 kDa) calculated by Image J software (arbitrary units, A.U.) analyzed by Western blot after 24 h cultivation of unstimulated (U) and LPS or dust-stimulated PBMCs of 4-15 year old German children (CLARA/CLAUS cohort, N=25 (11 HC/14 AA)) normalized to β -Actin.

Figure 3: LPS and farm dust stimulation downregulates pro-inflammatory (*TLR4*, *MYD88*, *CD86*, *TRAF6*, *CD28*) and upregulates anti-inflammatory (*CD274*, *CD80*, *CD152*) gene expression.

Up-/ and down regulation upon 24 h stimulation with German (G) or Finnish (Fi) farm dust or LPS is color-coded in heat maps (A, B and E) with a red-/blue gradient. Saturation reflects the strength in terms of the fold change ($\log_2$ scale). Significant regulation compared to the unstimulated cells is marked with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) based on two-sample t-test analysis. Different regulation between phenotypes (AA vs HC) is marked by a black rectangle around the respective gene. Fold change

of gene expression (Δ ct) in PBMCs of A) 4-15-year-old children from Munich, Germany (N=34 (16 HC/18 AA)) and B) 7-year-old children from Hong Kong, China (N=49 (29 HC/20 AA). C) Scatter plots of inflammation score defined as ratio of pro-inflammatory (*TLR4*, *MYD88*, *MALT1*, *TRAF6*, *CD86*, *CD28*) against anti-inflammatory expression (*TNFAIP3*, *CD274*, *CD80*, *TNIP2*, *TAX1BP1*, *CD152*, *CD279*) in unstimulated (U) and LPS or farm dust (G, Fi) stimulated PBMCs of 4-15-year-old children from Munich, Germany (N=31 (15 HC/16 AA)). D.) Scatter plots of *IL-18* gene expression (Δ ct) analyzed by qPCR after 24 h cultivation of unstimulated and LPS or German (G) and Finnish (Fi) farm dust stimulated PBMCs of 4-15-year-old children from Munich, Germany (N=36 (17 HC/19 AA)) the from CLARA/CLAUS cohort. Data is shown stratified for phenotype. Error bars show 95% confidence intervals around the mean. P-values from post-hoc t-test analysis are indicated for comparisons of healthy controls to allergic asthmatics. E) Fold change of relative expression in isolated dendritic cells of unstimulated and G stimulated PBMCs of 4-15-year-old children from Munich, Germany (N=12 (7 HC/5 AA)) measured with NanoString multiplex expression analysis.

Figure 4: Farm/rural children show lower NF- κ B signaling gene expression.

Scatter plots of gene expression (Δ ct) was analyzed by qPCR after 24 h cultivation of unstimulated A) leucocytes of 6-year-old farm (F) (N=32) and non-farm (NF) (N=31) healthy and asthmatic children from the PASTURE/EFRAIM cohort and B) PBMCs of 7-year-old healthy and asthmatic children from rural Conghua (C) (N=58) and Hong Kong (HK) (N=54) from the TRILATERAL cohort. Data is shown stratified by environment. Error bars show 95% confidence intervals around the mean. P-values from post-hoc t-test analysis are indicated for comparisons of NF vs F and C vs HK, respectively.

Figure 5: LPS stimulation in farm/rural children results in upregulation of *TNFAIP3*, *CD80* and *CD274* and downregulation of *TLR4* compared to unstimulated blood cells.

Fold change in gene expression analyzed by qPCR after 24 h cultivation of unstimulated and LPS stimulated A) leucocytes of 6-year-old farm (F) (N=29) and non-farm (NF) (N=29) healthy and asthmatic children from the PASTURE/EFRAIM cohort and B) PBMCs of 7-year-old healthy and asthmatic children from rural Conghua (C) (N=55) and Hong Kong (HK) (N=49) from the TRILATERAL cohort. Data is shown stratified by environment. Error bars show 95% confidence intervals around the mean. P-values from post-hoc two-sample t-test analysis are indicated for comparisons of LPS vs U (fold change; * $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$).

659 **Figure 6: Newborns with subsequent asthma express less *TNFAIP3*.**

660 Scatter plots of gene expression (Δct) analyzed by qPCR after 72 h cultivation of unstimulated CBMCs
661 from PAULINA/PAULCHEN cohort. Data is shown stratified for phenotype (N=25 (16 HC/9
662 Asthmatics)). Error bars show 95% confidence intervals around the mean. P-values from post-hoc t-test
663 analysis are indicated for comparisons of healthy controls to asthmatics.

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Figure 1

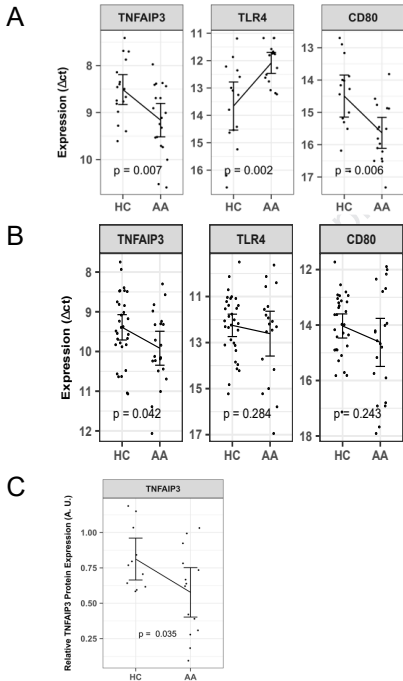
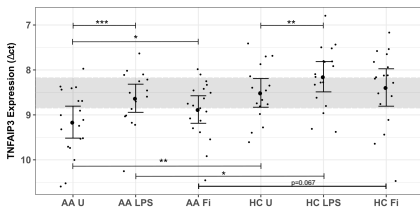
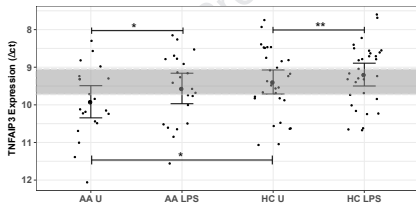


Figure 2

A



B



C

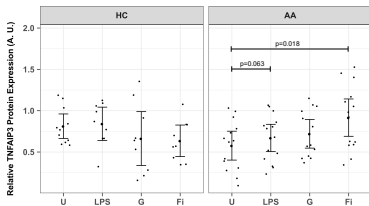


Figure 3

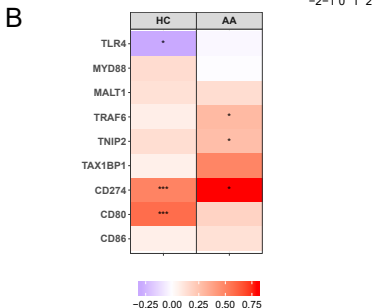
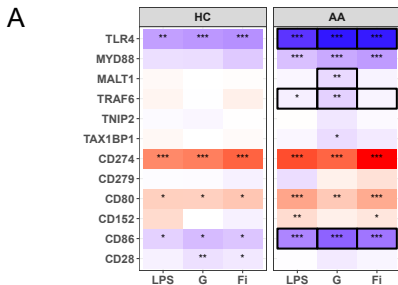
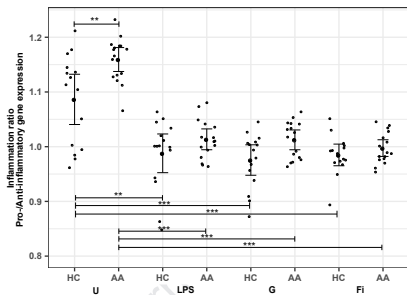


Figure 3

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D

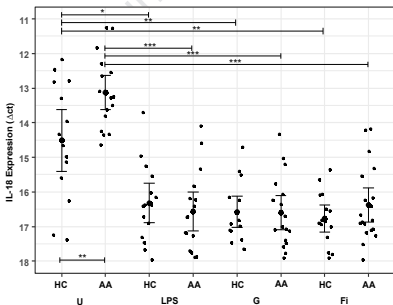


Figure 3

E

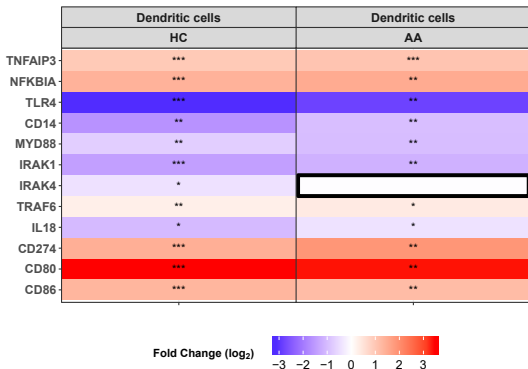
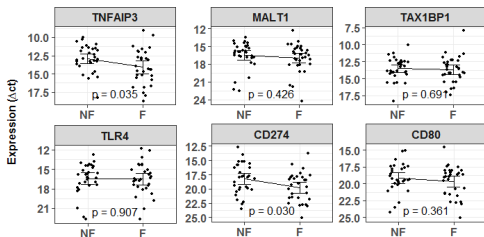


Figure 4

A



B

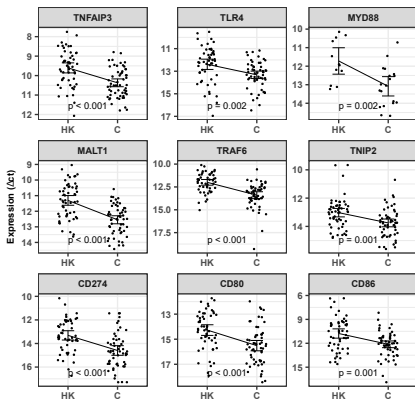
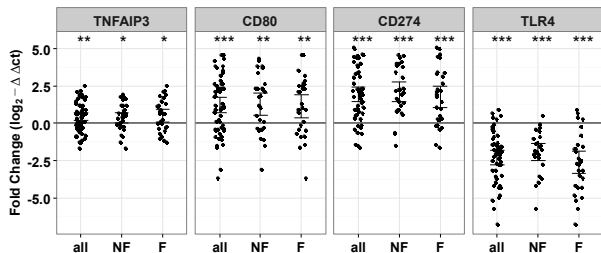


Figure 5

A



B

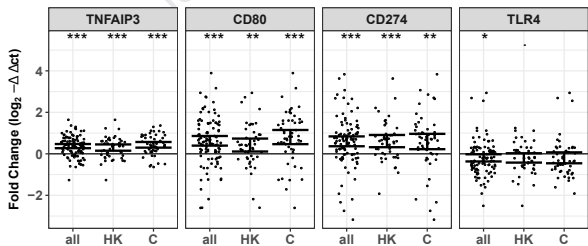
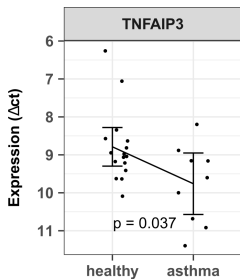


Figure 6



Supplementary Table E1: List of used reagents and according companies.

Reagent	Company
Ficoll-Paque™ PLUS	GE Healthcare, Piscataway, USA
RPMI	Gibco, Carlsbad, USA
X-Vivo	Lonza Walkersville, Inc., Walkersville, USA
FCS	Sigma-Aldrich, St. Louis, Missouri, USA (PASTURE/EFRAIM) Gibco, Carlsbad, USA (TRILATERAL)
LPS (E.coli-O111:B4)	Sigma-Aldrich, St. Louis, Missouri, USA
TRIzol	Invitrogen, Karlsruhe, Germany
QIAzol	Qiagen, Venlo, the Netherlands
chloroform	Sigma-Aldrich, St. Louis, Missouri, USA
RNeasy Mini Kit	Qiagen, Venlo, the Netherlands
QuantiTect Reverse Transcription Kit	Qiagen, Venlo, the Netherlands
BIO-RAD CFX96 System	Bio-Rad, Hercules, California, USA
SsoAdvanced Universal SYBR Green Supermix	Bio-Rad, Hercules, California, USA
Vector NTI software Version 10 Advance 11.5	Invitrogen, Carlsbad, USA
cComplete Protease Inhibitor Cocktail	Roche, Basel, Switzerland
Bradford assay	Carl Roth, Karlsruhe, Germany
TNFAIP3 antibody 4625S	Cell Signalling Technology, Cambridge, UK
HRP-conjugated goat anti-rabbit IgG, 7074S	Cell Signalling Technology, Cambridge, UK
ECL solution	ThermoFisher, Waltham, Massachusetts, USA
ChemiDoc™ MP System	Bio-Rad, Hercules, California, USA
Image Lab software	Bio-Rad, Hercules, California, USA
β-Actin (sc-47778 HRP	Santa Cruz Biotechnologies, Santa Cruz, USA
ImageJ Software	National Institutes of Health, Bethesda, USA
AutoMACS	Miltenyi Biotec, Bergisch Gladbach, Germany
human blood dendritic cell isolation Kit II	Miltenyi Biotec, Bergisch Gladbach, Germany

RNA Clean & Concentrator™-5 Kit	ZymoResearch, Irvine, California, USA
Bioanalyzer RNA 6000 Nano Kit	Agilent Technologies, Santa Clara, California, USA
NanoString nCounter expression analysis	NanoString Technologies, Seattle, USA
Limulus Amebocyte Lysate (LAL) test QCL-1000	Lonza Walkersville, Inc., Walkersville, USA

Supplementary Table E2: Sequences of used primers for RT-PCR.

Gene	Primer sequence (forward primer, 5' - 3')
18S	AGTCCCTGCCCTTTGTACACA
CD274	ACCACCACCAATTCCAAGAGAG
CD80	CTGGCTGGTCTTTCTCACTTCTGTTC
CD86	GCGGCTTTTATCTTCACCTTTC
MALT1	CTCAGCCCCCAGGAATAAAG
MYD88	TGCCTTCATCTGCTATTGCCCC
TAX1BP1	GCAGCAGAGGCAGATTTTGACATAG
TLR4	CTCAACCAAGAACCTGGACCTG
TNFAIP3	GCCCAGGAATGCTACAGATACCC
TNIP2	CAAAGGAATGTGGGGGAGAGAAGTC
TRAF6	TGATGTAGAGTTTGACCCACCCCTG
CD28	CCATGTGAAAGGGAAACACCTT
CD152	TGGCCCTGCACTCTCCTGT
CD279	GCTCAGGGTGACAGAGAGAAG
IL-18	AACAAACTATTTGTCGCAGGAAT

Supplementary Table E3: Results of the global MANOVA for effects of phenotype, exposure, and stimulus. P-values derived from F-test.

	CLARA/CLAUS	TRILATERAL	PASTURE/EFRAIM	PAULINA/ PAULCHEN
Effect of Phenotype	40 hypotheses $p = 1.806 \cdot 10^{-10}$ Subsequent t-test analysis comprised in Figure 1A	Stratified for farm exposure: 32 hypotheses $p = 1.919 \cdot 10^{-2}$ Subsequent t-test analysis comprised in Figure 1B adjusted for farm region: $p = 2.398 \cdot 10^{-1}$ n.s., no further t-test analysis without stratification or adjustment: 16 hypotheses $p = 1.649 \cdot 10^{-1}$ n.s., no further t-test analysis	Stratified for farm exposure: 24 hypotheses $p = 5.081 \cdot 10^{-4}$ adjusted for farm exposure: $p = 5.645 \cdot 10^{-1}$ n.s., no further t-test analysis without stratification or adjustment: 12 hypotheses $p = 5.739 \cdot 10^{-1}$ n.s., no further t-test analysis	10 hypotheses $p = 3.076 \cdot 10^{-6}$ Subsequent t-test analysis comprised in Figure 6
Effect of Exposure	-	16 hypotheses $p = 3.309 \cdot 10^{-7}$ Subsequent t-test analysis comprised in Figure 4B	12 hypotheses $p = 1.827 \cdot 10^{-2}$ Subsequent t-test analysis comprised in Figure 4A	-
Effect of Stimulus (via fold change)	30 hypotheses $p = 1.568 \cdot 10^{-18}$ Subsequent t-test analysis comprised in Figure 3A	8 hypotheses $p = 9.832 \cdot 10^{-21}$ Subsequent t-test analysis comprised in Figure 3B and 5B	6 hypotheses $p = 1.063 \cdot 10^{-16}$ Subsequent t-test analysis comprised in Figure 5A	-
Effect of phenotype on changes due to stimulation (via fold change)	30 hypotheses $p = 2.723 \cdot 10^{-12}$ Subsequent t-test analysis comprised in Figure 3A	8 hypotheses $p = 5.247 \cdot 10^{-2}$ n.s., no further t-test analysis	6 hypotheses $p = 7.323 \cdot 10^{-1}$ n.s., no further t-test analysis	-
Effect of exposure on changes due to stimulation (via fold change)		8 hypotheses $p = 1.852 \cdot 10^{-3}$ Post-hoc t-test analysis revealed no significance	6 hypotheses $p = 4.044 \cdot 10^{-1}$ n.s., no further t-test analysis	

Supplemental files

TNFAIP3 is a key player in childhood asthma development and environment-mediated protection

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Methods

Reagents used for this study are listed in Supplementary Table E1.

RT-PCR was performed in 96-well plates under standard conditions (2 min, 95°C; 20 sec, 95°C and 30 sec, 62.5°C, 40x) including ROX as passive reference, melt curve analysis and automatic threshold setting using SYBR Green, set up in 10 µl containing 6 ng cDNA and 320 nM primers. The sequences are listed in Supplementary Table E2.

A representative Western blot is shown in Supplementary Figure E2.

Bacterial 16S rRNA gene analysis

The V3–V5 region of the bacterial 16S rRNA gene was amplified with the forward primer V3-5Forward (CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCCTACGGGAGGCAGCAG) and the reverse primer Bac04xx (CCATCTCATCCCTGCGTGTCTCCGACTCAG-barcode- CCGTCAATTCMTTTRAGT) from Eurofins (Eurofins Genomics, 85560 Ebersberg, Germany). The 12bp barcode for this sample was: GTCGCTGTCTTC (primer Bac0421). Triplicate 25µl Polymerase Chain Reactions (PCRs) were set up. For the amplification Fast Start High Fidelity PCR System (Roche, Germany) adding 2.0µl [5µM] of each primer and 1µl of bovine serum albumin [10mg/ml] (Sigma-Aldrich) was used. For every sample a PCR negative control was included. 1µl of a 1:5 dilution of the original provided DNA extract was used as PCR template at the end. PCRs were run at 95°C for 120 seconds, followed by 28 cycles of 94°C for 20s, 50°C for 30s, 72°C for 5 min.

Furthermore, the amplicons were purified with Agencourt AMPure XP beads (Beckmann Coulter, USA). Amplicon purity and concentration was evaluated on an agarose gel. Unidirectional sequencing of the 16S rRNA gene fragments was performed using 454-GS FLX Titanium (Roche) protocol. Amplicons were sequenced at the Wellcome Trust Sanger Institute (Cambridge, UK). Raw data were processed by the 454 data processing pipeline v2.9 for shotgun reads. Denoising and removal of chimera was achieved by

Ampliconnoise. Following sequences were clustered in Operational Taxonomic Units (OTUs) with 97% sequence similarity by UCLUST using the de-novo picking method in QIIME version 1. For taxonomy assignment OTUs were aligned against SILVA database version 111 NR. The concentration of the purified PCR product from the extract was 22.6 ng/μl, determined using PicoGreen.

Determination of LPS concentration in farm dust samples

To determine the LPS concentration within our farm dust extracts, the Limulus Amebocyte Lysate (LAL) test QCL-1000 by Lonza was used according to the instructions including β-G-Blocker and spike to increase the test's endotoxin specificity. For the LAL-test German and Finnish farm dust extracts were diluted 1:100 with PBS.

Urban versus farm dust stimulation

Environmental dust was collected by standardized methods, where electrostatic dust collectors were placed in cowsheds for 4 weeks in Germany (G) and Finland (Fi) whereas urban dust was collected in the sleeping room in an apartment located in Munich.¹⁹ Dust was extracted as described before.²⁰ PBMCs of six healthy adults were isolated by density gradient centrifugation and 100 μL cell suspension (á 5x10⁶ cells/ml) were cultivated in RRMI/10%FCS/1%Penicillin-Streptomycin at 37 °C, 5% CO₂ unstimulated or stimulated with 40 μg/mL G, Fi and urban dust. CD4+CD25- as well as CD4-CD14+CD19+ cells have been isolated by the DC isolation Kit II by Miltenyi Biotech and RNA was extracted using RNeasy Mini Kit. cDNA was synthesized using the QuantiTect Reverse Transcription Kit. Gene expression of TNFAIP3 and 18S was quantified by RT-PCR in 96-well plates under standard conditions including ROX as passive reference, melt curve analysis and automatic threshold setting using SYBR Green, set up in 10 μl containing 6 ng cDNA and 320 nM primers. 18S levels were used for normalization. The primer sequences are listed in the supplement (Supplementary Table E2).

Study populations and characteristics

A flow diagram visualizing all study subjects within the four analyzed pediatric cohorts is shown in Supplementary Figure E1.

CLARA/CLAUS

Parents completed a detailed questionnaire assessing health data on allergy, asthma, and socioeconomic factors. The study population contains healthy children and patients with mild-to-moderate asthma. Mild-to-moderate asthmatic children from the CLAUS cohort, which is an ongoing replicative cohort of CLARA, were selected for this study based on the significantly decreased lung function and high total IgE levels. HC had more paternal asthma and higher IgE-level compared to HC of the whole cohort. We had to exclude one healthy control from our TNFAIP3 gene expression analysis due to technical problems. Informed written consent was obtained from the parents for participation and blood collection.

PASTURE/EFRAIM

Recruited children, comprising of half farm and non-farm children, who were repeatedly invited to participate in follow-ups, that included questionnaires and/or blood sampling based on previous studies. Questionnaires were administered at the end of pregnancy or birth between 2002 and 2004 and when the children were 2 months, 1, 1.5, 2, 3, 4, 5, 6 and 10.5 years of age.^{13,14} The questionnaires assessed information on general health, socioeconomic status, family history of atopy, and maternal smoking, with a focus on doctor's diagnosis of asthma and farm exposure. Due to the stratified sampling within center, aiming for a comparable sample size (German N=24, Finnish N=18, and French N=21), our selection is not representative for the whole cohort with regards to center distribution. Furthermore, our selected asthmatic children showed more familiar atopy.

TRILATERAL

Questionnaires based on ISAAC (The International Study of Asthma and Allergies in Childhood) were collected from the parents. The participation rate was 82% and 86%, respectively. Ethnically, Chinese

children born locally in Hong Kong (N=3118) or Conghua (N=10642) were selected for analysis. The mean age of children that participated in the survey was 7.0 ± 0.7 and 7.2 ± 1.5 years with male gender 52.6% and 53.2%, respectively from Hong Kong and Conghua. Prevalence of physician diagnosed asthma, current wheeze (within 12 months), rhino-conjunctivitis and flexural dermatitis was significantly higher in Hong Kong compared to Conghua (*(5.3% vs. 2.6%), *(7.8% vs. 1.7%), *(22.3% vs. 2.9%) and *(8.5% vs. 4.3%), in which the prevalence rates in Hong Kong were significantly higher than those in Conghua children (* $p < 0.001$). After completion of the questionnaire, Chinese children born in Hong Kong or Conghua were invited for a case-control study. A total of 79 children from Hong Kong (32 Asthmatics/47 HC, mean age 6.8 ± 0.6 and 32 cases, mean age 6.8 ± 0.7) and 66 children from Conghua (19 Asthmatics/47 HC), mean age 7.5 ± 0.7 and 19 cases, mean age 7.3 ± 0.9) were recruited for the case-control study.

PAULINA/PAULCHEN

Maternal atopy in both cohorts was defined as doctor's diagnosis of asthma and/or allergic rhinitis and/or atopic dermatitis. A total IgE along with specific IgE (RAST) was measured. The farming group in the PAULCHEN cohort was defined as the family having lived and/or worked on a farm during pregnancy, whereas the non-farming group was defined as having lived in a non-rural and non-farming environment during pregnancy. Informed consents were obtained from the mothers for their participation in the studies.

Statistical analysis

Global tests were performed for the null hypothesis that the average gene expression is not affected by stimulation, farm exposure and phenotype (Supplementary Table 3). The parallel assessment of several genes within each subject is taken into account by using the multivariate extension of the analysis-of-variance framework (MANOVA). It ensures to capture the potentially high correlation of the data and therefore is more powerful than multiple single ANOVA for each gene separately. For the described first step, single missing values in the gene expression profiles were imputed by chained equations.

The null hypothesis of the global tests comprises the intersection of multiple single hypotheses reflected by the number of measured genes per condition and group comparisons (phenotypes/exposure). For the CLARA/CLAUS cohort, 10 genes were measured unstimulated and upon stimulation with LPS, German and Finnish farm dust (G, Fi) in a single phenotype comparison (AA vs. HC), leading to $10 \cdot 4 \cdot 1 = 40$ hypotheses.

For the TRILATERAL cohort, 8 genes were measured unstimulated and upon stimulation with LPS in a single phenotype comparison (Asthmatics vs. HC), leading to $8 \cdot 2 \cdot 1 = 16$ hypotheses.

For the PASTURE/EFRAIM cohort, 6 genes were measured unstimulated and upon stimulation with LPS in a single phenotype comparison (Asthmatics vs. HC), leading to $6 \cdot 2 \cdot 1 = 12$ hypotheses.

For the PAULINA/PAULCHEN cohort, 10 genes were measured unstimulated in a two phenotype comparison (healthy, asthma), leading to 10 hypotheses.

Results

Farm dust composition

To unravel the bacterial taxonomic composition, 16S rRNA sequencing was performed revealing that 8564 sequences were available for the farm dust sample. After denoising with AmpliconNoise there were 7019 sequences clustered into 84 OTUs. The 10 most frequent OTUs in the samples accounted for 97.25% of the total sequences. 54 of the OTUs are doubletons or singletons. The majority of the sequences belonged to the phyla of gram-negative proteobacteria (90.1%), while 8.6% and 1% belonged to the gram-positive firmicutes or actinobacterien, respectively. When analyzing the LPS concentration within the farm dust samples by Limulus amoebocyte lysate (LAL) testing, a very high endotoxin concentration has been measured for the German and Finnish farm dust extract, respectively exceeding the highest standard of the test by far.

Statistical assessment of global effects

For the CLARA/CLAUS cohort, the multivariate analysis of variance (MANOVA) revealed significant global differences for phenotype effect, stimulus effect as well as the effect of phenotype on changes due to stimulation via fold change. Therefore, subsequent post-hoc t-test analysis were performed to identify specific effects by comparing the two phenotypes (Figure 1A), stimulus-effects (stimulated vs. unstimulated, Figure 3A) and effect of phenotype on changes due to stimulation (Figure 3A, boxes marked with bold black borders).

For the two farming/rural cohorts PASTURE/EFRAIM and TRILATERAL, the multivariate analysis of variance (MANOVA) revealed significant global differences for exposure (Figure 4A and 4B) and stimulus effects (Figure 3B, Figure 5A and 5B) and for TRILATERAL a significant effect of exposure on changes due to stimulation. However, subsequent t-test analysis revealed no significant result for this comparison. Moreover, significant phenotype effects were only seen in a farm exposure-stratified analysis.

For the birth cohort PAULINA/PAULCHEN, significant global differences were shown by the multivariate analysis of variance (MANOVA) for phenotype effect. Subsequent post-hoc t-test analyses were performed to identify specific effects by comparing two phenotypes each (Figure 6).

Expression of *MYD88*, *MALT1*, *TRAF6*, *TNIP2*, *TAX1BP1*, *CD274*, *CD86*, *CD28*, *CD152* and *CD279* between healthy and asthmatic children under unstimulated conditions

When comparing gene expression of healthy controls to allergic asthmatic children of the Munich CLARA/CLAUS cohort under unstimulated conditions, no significant differences in gene expression could be observed for *MYD88*, *MALT1*, *TRAF6*, *TNIP2*, *TAX1BP1*, *CD274*, *CD86*, *CD28*, *CD152* and *CD279* (Supplementary Figure E3A). When comparing these findings to the replication cohort TRILATERAL, analyzing gene expression of children from urban China (Hong Kong) consistently no differences in gene expression could be observed at baseline (unstimulated) for *MYD88*, *MALT1*, *TRAF6*, *TNIP2*, *CD274* and *CD86* (Supplementary Figure E3B), yet asthmatic Chinese children expressed significantly less anti-inflammatory *TAX1BP1*.

Comorbid allergic diseases

In order to assess the relevance of other atopic comorbid allergic diseases like atopic dermatitis, food allergy and allergic rhinoconjunctivitis in addition to asthma, we performed stratified analysis. Of note, gene expression levels of *TNFAIP3* were significantly ($p=0.035$) decreased in children of the CLARA/CLAUS cohort with asthma and additional allergic rhinoconjunctivitis (dCT: 9.41 (+/-0.76)) compared to children with asthma alone (dCT: 8.74 (+/-0.50)). However, there was no significant difference in expression of any of the investigated genes between asthmatic children with atopic dermatitis or food allergy compared to children suffering from allergic asthma only. Also for the TRILATERAL cohort, no significant differences in gene expression data were observable after stratification for medication for allergic rhinoconjunctivitis or eczema respectively. Thus, we are confident that lower *TNFAIP3* gene expression is not indicating an atopic status per se but is indeed asthma mediated.

Urban dust stimulation results in constant or decreased TNFAIP3 expression

To control for the possibly negative effects of urban dust exposure, we included dust from a bedroom (located in urban Germany) as a negative control in our preliminary tests. PBMCs of healthy adults have been cultivated under unstimulated conditions or stimulated with farm dust extracts from German and Finnish farms or urban dust respectively for 24 hours. Subsequently, CD14/CD19 cells and nonDC cells have been isolated by magnetic cell separation. RNA has been extracted, cDNA synthesized and qPCR for TNFAIP3 has been performed.

In this pilot, stimulation with dust but not urban extract from German and Finnish farms resulted in significant upregulation of TNFAIP3 gene expression in CD14/CD19 cells. In non-dendritic cells, urban dust stimulation resulted even in significant downregulation of TNFAIP3 expression while farm dust stimulation upregulated TNFAIP3 expression, yet not significant in this cell fraction (Suppl. Fig. E4).

Inflammation ratio by relating *TLR4* to *TNFAIP3* gene expression

The significant differences between asthmatic and healthy children regarding the “inflammation ratio” relating all investigated pro-inflammatory genes to anti-inflammatory expression was also observable when the ratio was built by the two central genes of the pathway *TLR4* and *TNFAIP3*. Since CD80 cannot always act as an anti-inflammatory regulator, depending on its binding partners, we have calculated the inflammation ratio in a further approach without including the CD80 expression, yielded consistent results as described in the manuscript.

TNFAIP3 and TLR4 expression in isolated dendritic cells

By investigating gene expression of NF- κ B related genes in isolated dendritic cells by the multiplex NanoString method, asthmatic children showed lower expression levels of *TNFAIP3* under unstimulated conditions compared to healthy children (Supplementary Figure E5, $p=0.26$). In comparison to the significant difference of TNFAIP3 expression in PBMCs of asthmatic versus healthy children, the expression levels obtained in isolated dendritic cells did not reach significance. This might be caused by

the low number of children included in this proof-of-principle experiment or due to a relevant additional cell population. Yet, the consistent results in both dendritic and peripheral blood mononuclear cells with a significantly increased expression of *TLR4* for asthmatics compared to healthy children indicate the importance of dendritic cells even under unstimulated conditions (Supplementary Figure E5, $p=0.01$).

Newborns with subsequent asthma express less *CD274* and *CD80*

By investigating gene expression in CMBCs from healthy newborn children, decreased expression of *CD274* and *CD80* could be observed for newborns who developed asthma until the age of 10y compared to those who stayed healthy, yet not significant (Supplementary Figure E6, $p\leq 0.084$).

Gender-specific effects

Of note, as childhood asthma rates are known to be gender-dependent, we performed the analysis stratified for gender. However, stratified analysis did not show different findings, although the overall significance is partly lost in the gender subgroups, likely due to the lower sample size.

Supplementary Figure Legends

Supplementary Figure E1: Flow diagram of study subjects

Flow diagram of all subjects included in this study comprising four pediatric cohorts, two cross-sectional studies (CLARA/CLAUS, TRILATERAL) and two birth cohort studies (PASTURE/EFRAIM, PAULINA/PAULCHEN) with a total of 250 out of 2,168 children.

Supplementary Figure E2: Representative Western Blot

One representative image of TNFAIP3 Western blot analysis with β -Actin as loading control (healthy children left, asthmatic children right, unstimulated (U) and after stimulation with LPS or farm dust (G, Fi).

Supplementary Figure E3: Gene expression of *MYD88*, *MALT1*, *TRAF6*, *TNIP2*, *TAX1BP1*, *CD274*, *CD86*, *CD28*, *CD152* and *CD279* between healthy and asthmatic children under unstimulated conditions

Scatter plots for gene expression of *MYD88*, *MALT1*, *TRAF6*, *TNIP2*, *TAX1BP1*, *CD274*, *CD86* (A and B) and *CD28*, *CD152* and *CD279* (A). Data is shown stratified for phenotypes, healthy (HC) and asthmatic children. Raw data values are represented by horizontally jittered points. Error bars show 95% confidence intervals around the sample means, which were connected by horizontal lines. Differences are indicated by the p-value of two-sample t-tests for phenotype comparisons. Gene expression (Δ ct) was analyzed by qPCR after 24 h cultivation of unstimulated PBMCs of A) 4-15-year-old children from Munich, Germany (CLARA/CLAUS cohort, N=36 (17 HC/19 AA)) and B) 7-year-old children from Hong Kong, China (TRILATERAL cohort N=53 (32 HC/21 Asthmatics)).

Supplementary Figure E4: Farm dust stimulation upregulates TNFAIP3 gene expression while urban dust stimulation results in consistent or even downregulated TNFAIP3 levels

Scatter plots of gene expression (Δct) of *TNFAIP3* in isolated CD14/19 cells and non-dendritic cells (nonDC) analyzed by qPCR after 24 h cultivation of unstimulated and LPS or German (G) and Finnish (Fi) farm dust stimulated PBMCs of six healthy adults. Error bars show 95% confidence intervals around the mean. Significant regulation compared to the unstimulated cells is marked with asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) based on two-sample t-test analysis.

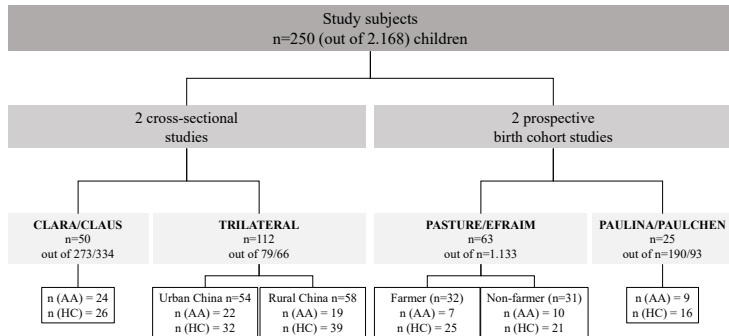
Supplementary Figure E5: Dendritic cells of asthmatics express higher levels of TLR4 under unstimulated conditions.

Scatter plots of gene expression of *TNFAIP3* and *TLR4* in isolated dendritic cells analyzed by NanoString. Dendritic cells were isolated by magnetic cell separation after 24 h cultivation of unstimulated PBMCs of 4-15-year-old children from Munich, Germany (N=12 (7 HC/5 AA)). Data is shown stratified for phenotype. Error bars show 95% confidence intervals around the mean. P-values from post-hoc t-test analysis are indicated for comparisons of healthy controls to asthmatics.

Supplementary Figure E6 Newborns with subsequent asthma express less CD274 and CD80.

Scatter plots of gene expression (Δct) of *CD274* and *CD80* analyzed by qPCR after 72 h cultivation of unstimulated CBMCs from PAULINA/PAULCHEN cohort. Data is shown stratified for phenotype (N=20-22 (13-15/9 (HC/Asthma))). Error bars show 95% confidence intervals around the mean. P-values from post-hoc t-test analysis are indicated for comparisons of healthy controls to asthmatics.

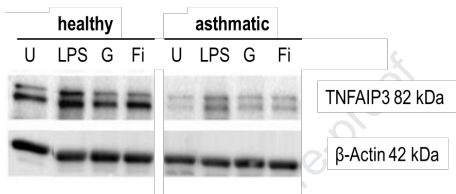
Supplementary Figure E1



Based on sample availability a selection of representative subjects was chosen with a case-control design.

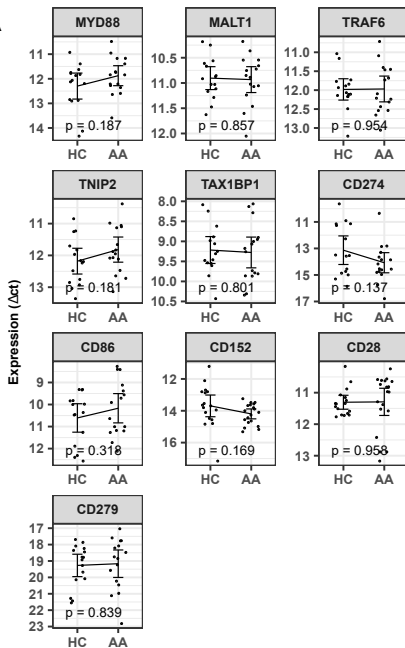
Power calculation revealed a required group size of 14 children per phenotype for one Δ ct difference of TNFAIP3 expression based on assumed standard deviation of 0.8 Δ ct within group with $\alpha=0.05$ significance and $\beta=0.9$ power. Higher subject numbers were selected, based on material availability.

Supplementary Figure E2



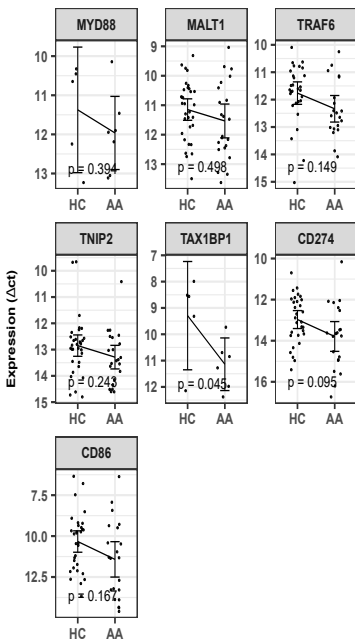
Supplementary Figure E3

A

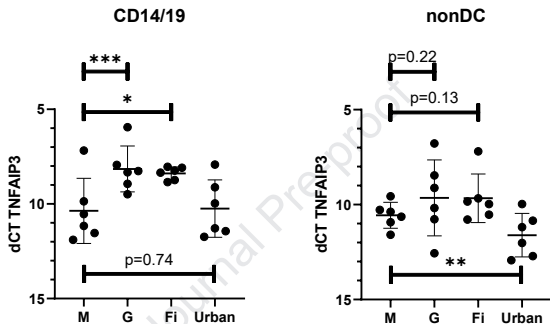


Supplementary Figure E3

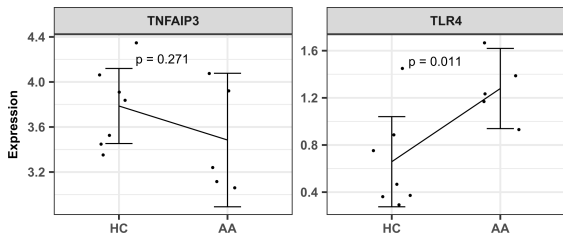
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Supplementary Figure E4



Supplementary Figure E5



Supplementary Figure E6

