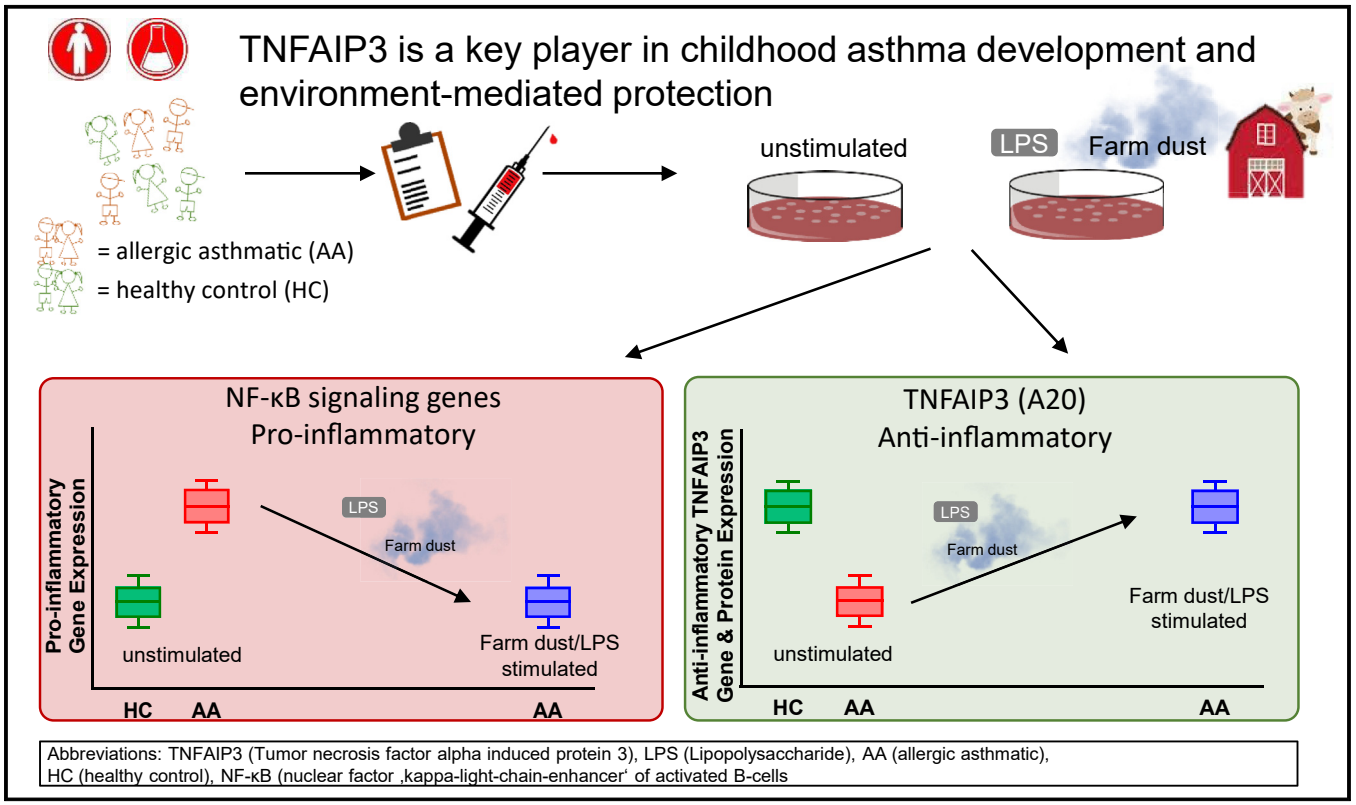
TNF-α–induced protein 3 is a key player in childhood asthma development and environment-mediated protection

Johanna Krusche, MSc,[a,b]* Monika Twardziok, PhD,[a]* Katharina Rehbach,[a]* Andreas Böck, PhD,[a] Miranda S. Tsang, PhD,[c] Paul C. Schröder, PhD,[a] Jörg Kumbrink, PhD,[d,e] Thomas Kirchner, MD,[d,e] Yuhan Xing, MSc,[f] Josef Riedler, MD,[g] Jean-Charles Dalphin, PhD,[h] Juha Pekkanen, MD,[i,j] Roger Lauener, MD,[k,l,m,n] Marjut Roponen, PhD,[o] Jing Li, MD,[p] Chun K. Wong, PhD,[q] Gary W. K. Wong, MD,[f] and Bianca Schaub, MD,[a,b] the PASTURE study group‡

Munich, Marburg, Regensburg, and Ulm, Germany; Hong Kong and Guangzhou, China; Salzburg, Austria; Besançon, France; Kuopio and Helsinki, Finland; Davos, St Gallen, Basel, and Zurich, Switzerland; La Jolla, Calif; and Utrecht, The Netherlands

GRAPHICAL ABSTRACT



From [a]Pediatric Allergology, Department of Pediatrics, Dr von Hauner Children's Hospital, University Hospital, [b]Member of the German Center for Lung Research–DZL, and [d]the Institute of Pathology, Medical Faculty, LMU Munich; [c]the Institute of Chinese Medicine, Chinese University of Hong Kong; [e]German Cancer Consortium (DKTK), Partner Site Munich; [f]the Department of Paediatrics, Faculty of Medicine, Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, N.T., Hong Kong; [g]Children's Hospital Schwarzach, Schwarzach, Austria, Teaching Hospital of Paracelsus Medical Private University Salzburg; [h]University Hospital of Besançon, UMR CNRS 6249 Chrono-Environment, University of Franche-Comté, Besançon; [i]the Department of Health Security, National Institute for Health and Welfare (THL), Kuopio; [j]the Department of Public Health, University of Helsinki; [k]Children's Hospital of Eastern Switzerland, St Gallen; [l]University of Zurich, Zurich; [m]School of Medicine, University of St Gallen, St Gallen; [n]Christine Kühne-Center for Allergy Care and Education, CK-CARE, Davos; [o]Department of Environmental and Biological Sciences, University of Eastern Finland, Kuopio; [p]Guangzhou Institute of Respiratory Health, First Affiliated Hospital of Guangzhou Medical University, Department of Allergy and Clinical Immunology, Guangzhou; [q]Department of Chemical Pathology, Chinese University of Hong Kong, Shatin, NT, Hong Kong; [r]Comprehensive Biomaterial Bank Marburg CBBM, Fachbereich Medizin der Philipps Universität Marburg, Zentrum für Tumor und Immunbiologie ZTI, Marburg; [s]KUNO Childrens University Hospital Regensburg, Department of Pediatric Pneumology and Allergy Campus St Hedwig, Regensburg; [t]the Institute of Epidemiology and Medical Biometry, Ulm University; [u]the Department of Pediatrics, Kuopio University Hospital; [v]the Swiss Tropical and Public Health Institute, and the University of Basel; [w]Children's Hospital, University of Zurich, Zurich; [x]the Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, Davos; [y]the Department of Pediatrics, University Hospital, University of Besanon; and [z]the Institute for Risk Assessment Sciences (IRAS), Division of Environmental Epidemiology, Utrecht University.

*These authors contributed equally to this work as first authors.

‡The PASTURE study group included Markus Ege, MD,[a,b] Martin Depner, PhD,[a] Sabina Illi, PhD,[a] Georg J. Loss, PhD,[p] Harald Renz, MD,[q] Petra I. Pfefferle, PhD,[b,r] Michael Kabesch, MD,[s] Jon Genuneit, MD,[t] Anne M. Karvonen, PhD,[h] Anne Hyvärinen, PhD,[h] Pirkka V. Kirjavainen, PhD,[h] Sami Remes, MD,[u] Charlotte Braun-Fahrländer, MD,[v] Caroline Roduit, PhD,[j,w] Remo Frei, PhD,[h,x] Vincent Kaulek, PhD,[h] Marie-Laure Dalphin, MD,[y] Amandine Divaret-Chauveau, MD,[y] and Gert Doekes, PhD.[z]

Background: Childhood asthma prevalence is significantly greater in urban areas compared with rural/farm environments. Murine studies have shown that TNF-α–induced protein 3 (TNFAIP3; A20), an anti-inflammatory regulator of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, mediates environmentally induced asthma protection.
Objective: We aimed to determine the role of TNFAIP3 for asthma development in childhood and the immunomodulatory effects of environmental factors.
Methods: In a representative selection of 250 of 2168 children from 2 prospective birth cohorts and 2 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-κB signaling–related gene and protein expression was assessed in PBMCs and multiplex gene expression assays (NanoString Technologies) in isolated dendritic cells of schoolchildren and in cord blood mononuclear cells from newborns.
Results: Anti-inflammatory TNFAIP3 gene and protein expression was consistently decreased, whereas proinflammatory Toll-like receptor 4 expression was increased in urban asthmatic patients ($P < .05$), reflecting their increased inflammatory status. *Ex vivo* farm dust or LPS stimulation restored *TNFAIP3* expression to healthy levels in asthmatic patients and shifted NF-κB signaling–associated gene expression toward an anti-inflammatory state ($P < .001$). Farm/rural children had lower expression, indicating tolerance induction by continuous environmental exposure. Newborns with asthma at school age had reduced *TNFAIP3* expression at birth, suggesting TNFAIP3 as a possible biomarker predicting subsequent asthma.
Conclusion: Our data indicate TNFAIP3 as a key regulator during childhood asthma development and its environmentally mediated protection. Because environmental dust exposure conferred the anti-inflammatory effects, it might represent a promising future agent for asthma prevention and treatment. (J Allergy Clin Immunol 2019;■■■:■■■-■■■.)

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

Abbreviations used

AA:	Allergic asthma
CBMC:	Cord blood mononuclear cell
CLARA/CLAUS:	Clinical Asthma Research Association
CT:	Cycle threshold
CTLA4:	Cytotoxic T-lymphocyte associated protein 4
DC:	Dendritic cell
HC:	Healthy control subject
IRAK1:	IL-1 receptor–associated kinase 1
LAL:	Limulus Amoebocyte Lysate
MALT1:	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MANOVA:	Multivariate ANOVA
MYD88:	Myeloid differentiation primary response gene–88
NF-κB:	Nuclear factor kappa-light-chain-enhancer of activated B cells
OTU:	Operational taxonomic unit
PASTURE/EFRAIM:	Protection against allergy: Study in rural environments/Mechanisms of Early Protective Exposures on Allergy Development
PAULINA/PAULCHEN:	Pediatric Alliance for Unselected Longitudinal Investigation of Neonates for Allergies
PDCD1:	Programmed cell death 1
PVDF:	Polyvinylidene fluoride
ROX:	Rhodamine X
TAX1BP1:	Tax1-binding protein 1
TLR4:	Toll-like receptor 4
TNFAIP3:	TNF-α–induced protein 3
TNIP2:	TNFAIP3-interacting protein 2
TRAF6:	TNF receptor–associated factor 6

Asthma is one of the most prevalent chronic diseases in childhood, in which the majority of affected children can be classified as patients with allergic asthma (AA) producing IgE antibodies against environmental antigens.[1] The multifactorial cause 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 increasing, 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, such as LPSs, are protected.[3,4]

The recognition of LPS (endotoxin) by Toll-like receptor 4 (TLR4), which is expressed on innate immune cells, activates inflammatory responses, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling.[5] Because uncontrolled inflammation would cause serious tissue damage, these processes are tightly regulated.[6] Specifically, endotoxin tolerance, an adaptive protective mechanism, controls excessive inflammation through mitigated response to repetitive LPS

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Corresponding author: Bianca Schaub, MD, University Children's Hospital, Dr. von Haunersches Kinderspital, Department of Allergy/Immunology, Lindwurmstraße 4, 80337 Munich, Germany. E-mail: Bianca.Schaub@med.uni-muenchen.de. Gary W. K. Wong, MD, Department of Paediatrics, Faculty of Medicine, Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, N.T., Hong Kong. E-mail: wingkinwong@cuhk.edu.hk.
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stimulation and suppression of proinflammatory signaling and upregulation of anti-inflammatory processes.[5]

TNF-α–induced protein 3 (TNFAIP3; A20), a negative regulator of the inflammatory NF-κB pathway, is important for immune regulation in asthma and its environmentally mediated protection.[7] Amish children living on traditional farms with lower asthma prevalence compared with Hutterite children from highly industrialized farms expressed significantly greater 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 environmentally mediated protection. Specifically, we investigated the following 4 questions: Is *TNFAIP3* and NF-κB signaling gene expression deregulated in PBMCs and dendritic cells (DCs) of steroid-naive asthmatic patients during asthma manifestation? Is it possible to modulate *TNFAIP3* expression and related genes by exposure to "asthma-protective" farm dust extracts *ex vivo*? Do children with constant prior *in vivo* farm dust exposure exhibit modulated *TNFAIP3* gene expression? Can *TNFAIP3* expression at birth serve as a predictive marker for subsequent asthma development?

METHODS

Study design

We analyzed blood samples from 2 cross-sectional studies (Clinical Asthma Research Association [CLARA/CLAUS] and TRILATERAL) and 2 prospective birth cohort studies (Protection against allergy: Study in rural environments/Mechanisms of Early Protective Exposures on Allergy Development [PASTURE/EFRAIM] and Pediatric Alliance for Unselected Longitudinal Investigation of Neonates for Allergies [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 1 Δ cycle threshold (CT) differences of TNFAIP3 expression based on an assumed SD of 0.8 ΔCT within groups with an α significance level of .05 and a β value of 0.9 power. Greater numbers of subjects were selected based on RNA availability. Informed written consent was obtained from the parents.

Study population and characteristics

For details on the study population and characteristics, see the Methods section and Fig E1 in this article's Online Repository at www.jacionline.org. The CLARA/CLAUS cohort includes 4- to 15-year-old healthy control subjects (HCs) and children with mild-to-moderate asthma (n = 273 and 334, respectively) recruited at LMU Children's Hospital since January 2009.[9,10] Asthmatic patients received diagnoses according to Global Initiative for Asthma guidelines.[11] Eligibility criteria for asthmatic patients were classical asthma symptoms, at least 3 episodes of wheeze and/or a doctor's diagnosis and/or a history of asthma medication, and lung function indicating significant reversible airflow obstruction according to American Thoracic Society/European Respiratory Society guidelines.[12] Patients with AA were defined by a specific IgE level of 0.35 IU/mL or greater and clinical symptoms. HCs were matched by age (10.2 ± 2.8 years). The analyzed subgroup of 50 children (26 HCs and 24 patients with AA) is representative for the whole cohort regarding age, sex, breast-feeding time, maternal atopy and asthma, smoking, and number of siblings. Approval was obtained from the local ethics board (no. 379-08; LMU Munich, Munich, Germany).

The international birth cohort study PASTURE/EFRAIM includes 1133 children from rural areas in Germany, Switzerland, Austria, Finland, and France from the third trimester of pregnancy until age 10.5 years.[13,14] Asthma was defined as a physician's diagnosis of recurrent spastic, obstructive, or asthmatic bronchitis, asthma, or both reported by parents at the age of 6 years.[15] The selected 6-year-old children (n = 63, 32 farmers [7 asthmatic patients and 25 HCs] and 31 nonfarmers [10 asthmatic patients and 21 HCs]) are representative of the entire cohort, with no significant differences regarding sex, farming, number of siblings, and breast-feeding. Selected asthmatic patients 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; and France, 07/448).

In the Chinese part of the TRILATERAL study, 3,435 and 14,152 children born locally in Hong Kong (7.0 ± 0.7 years) or Conghua (7.2 ± 1.5 years) in Mainland China were recruited between 2013 and 2014. The prevalence of physician-diagnosed asthma was significantly greater in Hong Kong compared with Conghua (5.3% vs 2.6%, $P < .001$). Asthmatic patients were defined as having a history of wheeze ever, current wheeze within 12 months, and physician/hospital-diagnosed asthma. HCs were those without a history of wheeze, physician-diagnosed asthma, allergic rhinitis, atopic dermatitis, and eczema ever. Seventy-nine children from Hong Kong (32 asthmatic patients and 47 HCs) and 66 children from Conghua (19 asthmatic patients and 47 HCs) were recruited for the case-control study (Chinese University of Hong Kong Clinical Research Ethics Committee approval 2013.220). The analyzed subgroup of 112 children (Hong Kong, 54 [32 HCs and 22 patients with AA]; Conghua, 58 [39 HCs and 19 patients with AA]) is representative of 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 to 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 a doctor's diagnosis of asthma until the age of 6 years and/or 10 years by questionnaires. The 25 selected nonfarm children (16 HCs and 9 asthmatic patients) are representative of 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 (ethical approval of "Bayerische Landesärztekammer" [04092]).

Laboratory methods

For more information on laboratory methods, see Table E1 in this article's Online Repository at www.jacionline.org.

Dust collection and extraction. Environmental dust was collected by using standardized methods in which electrostatic dust collectors were placed in cowsheds for 4 weeks in Germany and Finland.[19] Dust was extracted, as described previously.[20]

Cell isolation and stimulation. Cord blood mononuclear cells (CBMCs) and PBMCs were isolated by means of density gradient centrifugation within 24 hours after blood withdrawal, and 5×10^6 cells/mL were cultivated at 37°C in a 5% CO_2 atmosphere. PBMCs from CLARA/CLAUS children were cultured for 24 hours in X-VIVO (Lonza, Walkersville, Md) unstimulated or stimulated with LPS (0.1 μg/mL, *Escherichia coli*–O111:B4) or 40 μg/mL farm dust extract (Germany and Finland). Whole blood cultures from PASTURE/EFRAIM children were incubated for 24 hours in RPMI/10% FCS, either unstimulated or LPS stimulated (0.1 μg/mL, *E coli*–O111:B4). PBMCs from TRILATERAL children were cultured in RPMI/10% FCS, either unstimulated or stimulated, with 1 μg/mL LPS. CBMCs from newborns were cultured for 72 hours unstimulated in RPMI/10% FCS. Differences in LPS concentrations derive from preliminary studies in which ideal conditions were tested to be optimal in each setting.

RNA isolation, cDNA synthesis, and quantitative RT-PCR. CBMC and leukocyte RNA was extracted by using TRIzol or QIAzol and chloroform, whereas RNA from PBMCs and DCs was extracted with the RNeasy Mini Kit. RNA concentration and quality

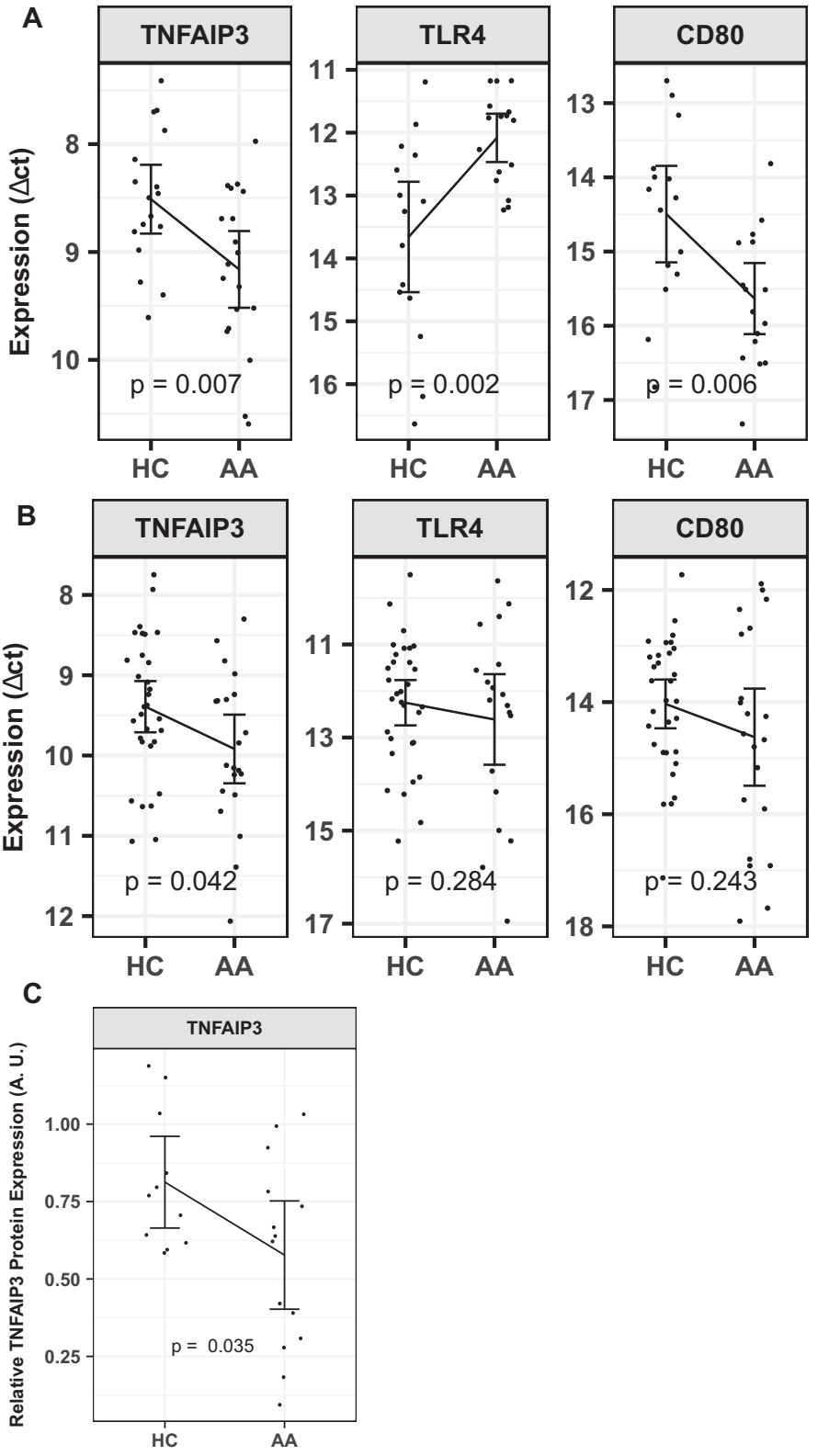


FIG 1. Urban asthmatic children express less *TNFAIP3*. Scatter plots for *TNFAIP3*, *TLR4*, and *CD80* gene (Fig 1, *A* and *B*) and TNFAIP3 protein (Fig 1, *C*) expression are shown. Data are stratified for phenotypes, HCs, and children with AA. Raw data values are represented by *horizontally jittered points*. *Error bars* show 95% CIs around sample means, which were connected by *horizontal lines*. Differences are indicated by the *P* value of 2-sample *t* tests for phenotype comparisons. **A** and **B,** Gene expression (ΔCT) was analyzed by using quantitative PCR after 24 hours of cultivation of unstimulated PBMCs of 4- to 15-year-old children

were tested by using NanoDrop (Thermo Fisher, Waltham, Mass) and/or a Bioanalyzer (Agilent Technologies, Santa Clara, Calif). cDNA was synthetized by using the QuantiTect Reverse Transcription Kit (Qiagen, Venlo, The Netherlands). Gene expression was quantified by using RT-PCR in 96-well plates under standard conditions, including rhodamine X (ROX) as a passive reference, melting curve analysis, and automatic threshold setting with SYBR Green set up in 10 μL containing 6 ng of cDNA and 320 nmol/L primers. 18S levels were used for normalization. Primer sequences are listed in Table E2 in this article's Online Repository at www.jacionline.org.

Western blotting. PBMCs were lysed in 100 μL of RIPA buffer containing protease inhibitors. Protein concentrations were determined by using the Bradford assay. Cell lysates (50 μg per lane) were separated by using SDS-PAGE. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes, blocked in 5% milk–Tris-buffered saline with Tween 20 and incubated in 5% BSA–Tris-buffered saline with Tween 20 and TNFAIP3 antibody. Blots were incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG and visualized by using enhanced chemiluminescence solution on a ChemiDoc System (Bio-Rad Laboratories, Hercules, Calif). β-Actin was used as a loading control. Protein expression was quantified by using ImageJ software (National Institutes of Health, Bethesda, Md).

NanoString technology. DCs were separated from unstimulated and German dust-stimulated PBMCs of CLARA/CLAUS children by using autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) with the DC isolation kit. In this proof-of-principle experiment, cells were stimulated exclusively with German dust because of the limited material availability. Extracted RNA was concentrated by using the RNA Clean & Concentrator-5 Kit. Concentration, quality, and fragmentation (>300 nucleotides) was assessed with the Bioanalyzer RNA 6000 Nano Kit (Zymo Research, Irvine, Calif). One hundred nanograms of RNA was hybridized (for 18 hours at 65°C), and the PanCancer Immune Profiling Panel was used for NanoString nCounter expression analysis (NanoString Technologies, Seattle, Wash).[21] Data were analyzed by utilizing nSolver Analysis Software v3.0 (NanoString Technologies). Quality control was performed with default settings within the software, positive controls, housekeeper genes, 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 using 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 ANOVA framework (multivariate ANOVA [MANOVA], see the Methods section in this article's Online Repository), which is more powerful than using multiple single ANOVAs for each gene separately. The significance of the global tests was derived by using the F-test, as implemented in the R package program multcomp.[22] Single missing values in gene expression profiles were imputed by using chained equations.[23] After identification of significant global differences for each cohort (see Table E3 in this article's Online Repository at www.jacionline.org), we assessed specific differences by using 2-sample *t* tests. Means were reported with 95% CIs. Outliers were defined as data points having a distance of more than 3 interquartile ranges to the lower/upper data quartile and excluded for parametric tests. An unadjusted *P* value of less than .05 was considered significant. Statistical analysis and visualization were performed with R software (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-κB signaling genes, we assessed gene expression in PBMCs of steroid-naive asthmatic and healthy school-aged children from urban Germany.[9,10] The NF-κB pathway genes *TNFAIP3*, *TLR4*, myeloid differentiation primary response gene–88 *(MYD88)*, mucosa-associated lymphoid tissue lymphoma translocation protein 1 *(MALT1)*, TNF receptor–associated factor 6 *(TRAF6)*, TNFAIP3-interacting protein 2 *(TNIP2)*, and Tax1-binding protein 1 *(TAX1BP1)* and the T cell–associated genes *CD274*, programmed cell death 1 (PDCD1), *CD80*, cytotoxic T-lymphocyte associated protein 4 (CTLA4), *CD86*, and *CD28* were examined.

To compare findings from this European cohort with those from 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* expression compared with HCs ($P \leq .007$; Fig 1, *A*). No phenotypic differences in expression of the other investigated genes were observed under unstimulated conditions (see Fig E2 in this article's Online Repository at www.jacionline.org). Consistently less *TNFAIP3* expression was replicated in asthmatic patients from Hong Kong ($P = .042$; Fig 1, *B*), indicating impaired negative regulation of the NF-κB pathway in asthmatic children across different urban areas. Lower TNFAIP3 expression in asthmatic children was confirmed at protein level ($P = .035$ [Fig 1, *C*]; a representative Western blot is shown Fig E3 in this article's Online Repository at www.jacionline.org).

Ex vivo dust stimulation upregulates TNFAIP3 expression

We analyzed whether 24 hours of stimulation with farm dust extracts or LPS could shift reduced TNFAIP3 levels in asthmatic patients to levels comparable with those at a healthy basal state (levels of healthy children under unstimulated conditions), mimicking asthma-protective farm exposure *ex vivo*. We extracted dust from German and Finnish farms and stimulated PBMCs of children from urban Germany with these dust extracts and LPS, one major component of farm dust, to analyze their immunoregulatory capacities. Limulus Amoebocyte Lysate (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 the Results section in this article's Online Repository at www.jacionline.org). PBMCs of Chinese children have only been stimulated with LPS for logistic reasons.

from Munich, Germany (CLARA/CLAUS cohort, n = 36 [17 HCs and 19 patients with AA]; Fig 1, *A*) and 7-year-old children from Hong Kong, China (TRILATERAL cohort, n = 53 [32 HCs and 21 patients with AA]; Fig 1, *B*). **C,** Relative TNFAIP3 protein level (82 kDa) calculated by using ImageJ software (in arbitrary units) analyzed by means of Western blotting after 24 hours of cultivation of unstimulated PBMCs of 4- to 15-year-old children (CLARA/CLAUS cohort, n = 25 [11 HCs and 14 patients with AA]) normalized to β-actin.

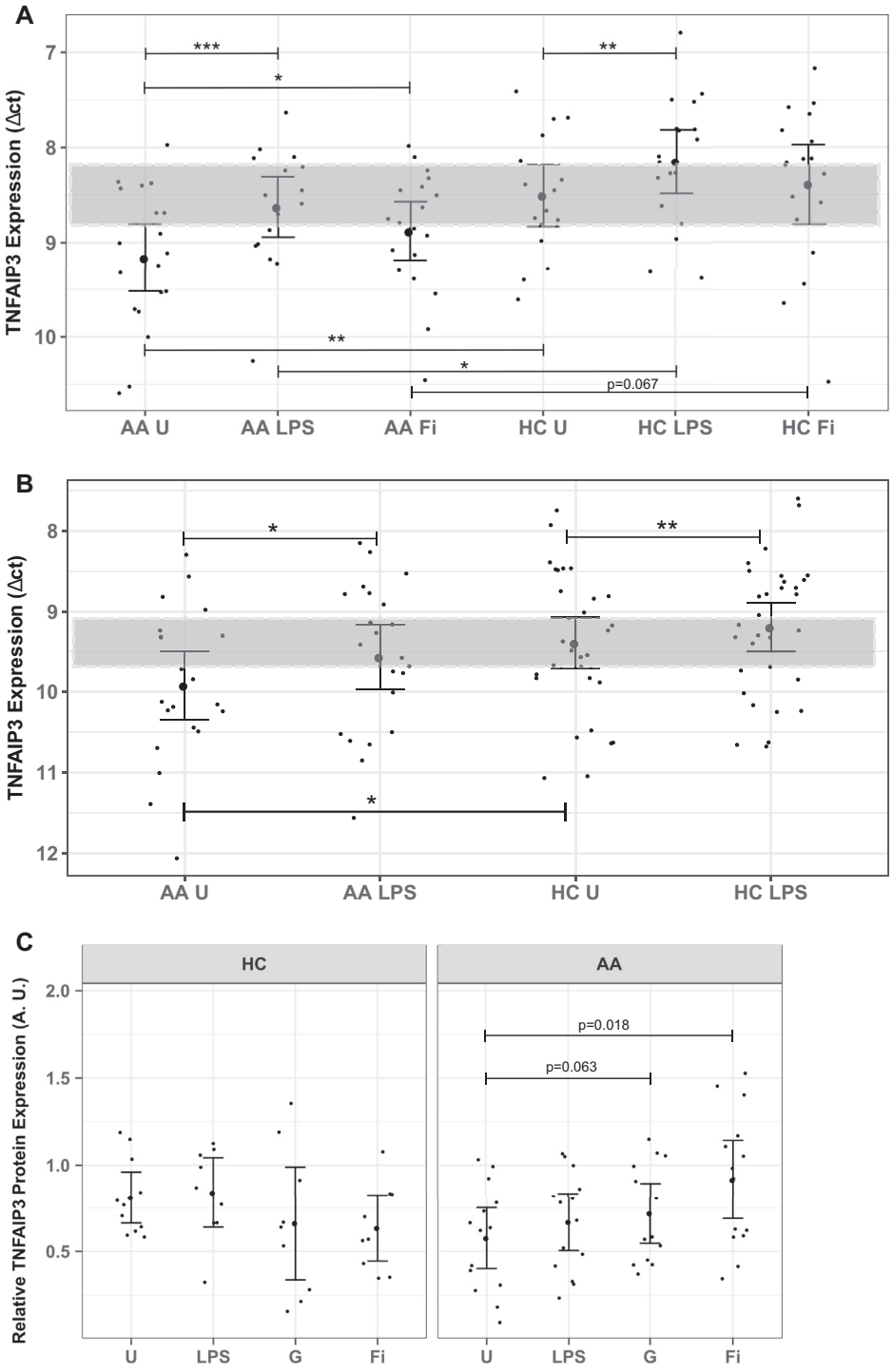


FIG 2. LPS and farm dust stimulation increase TNFAIP3 gene and protein expression in asthmatic children. Effect of LPS and German *(G)* and Finnish *(Fi)* farm dust stimulation compared with unstimulated *(U)* conditions are shown as scatter plots. Data are stratified for phenotypes. Raw data values are represented by *horizontally jittered points*. *Error bars* show 95% CIs around the sample means, which were connected by *horizontal lines*. Differences are indicated by the *P* values of 2-sample *t* tests for phenotype comparisons. **A** and **B,** Gene expression of TNFAIP3 (ΔCT) was analyzed by using quantitative PCR after 24 hours of cultivation of unstimulated or LPS- or Finnish farm dust–stimulated PBMCs of 4- to 15-year-old children from Munich, Germany (CLARA/CLAUS cohort, n = 37 [18 HCs and 19 patients with AA]; Fig 2, *A*) and 7-year-old children from Hong Kong, China (TRILATERAL cohort, n = 53 [31 HCs and 22 patients with AA]; Fig 2, *B*). **C,** TNFAIP3 protein level (82 kDa) calculated by using ImageJ software (in arbitrary units) analyzed by using Western blotting after 24 hours cultivation of unstimulated and LPS- or dust-stimulated PBMCs of 4- to 15-year-old German children (CLARA/CLAUS cohort, n = 25 [11 HCs and 14 patients with AA]) normalized to β-actin. $*P \leq .05$, $**P \leq .01$, and $***P \leq .001$.

On LPS stimulation, asthmatic patients from urban Germany and China reached *TNFAIP3* expression comparable with that of healthy children under unstimulated conditions (Fig 2, *A* and *B*). German dust stimulation also resulted in increased *TNFAIP3* expression, although not significantly (data not shown). Finnish farm dust stimulation also yielded a significant increase in *TNFAIP3* expression to healthy levels. On protein levels, TNFAIP3 upregulation was even stronger on Finnish farm dust stimulation compared with LPS (Fig 2, *C*). Interestingly, for healthy children, no significant upregulation of TNFAIP3 was observed at the protein level.

Taken together, urban asthmatic children showed decreased TNFAIP3 transcriptional and translational levels compared with healthy children, but TNFAIP3 expression could be upregulated on *ex vivo* stimulation with farm dust (Finnish) and LPS.

Ex vivo dust stimulation downregulates proinflammatory genes while upregulating anti-inflammatory genes

Aiming at identifying the immunologic mechanisms underlying the protective farm effect in a more detailed approach, we investigated gene expression of related genes within the NF-κB signaling pathway and costimulatory molecules.

Stimulation with dust extracts from Finnish and German farms or LPS significantly increased expression of anti-inflammatory *CD274*, *CTLA4*, and *CD80* (red) and decreased expression of proinflammatory *CD86*, *CD28*, *TLR4*, and *MYD88* (blue) in healthy and asthmatic children (Fig 3, *A*). This suggests further immunomodulatory characteristics of environmental dust. It is noteworthy that asthmatic patients from urban Germany showed significantly stronger downregulation of *TLR4*, *CD86*, *TRAF6*, and *MALT1* (German) compared with HCs (Fig 3, *A*; $P < .04$, indicated by black rectangle around the respective gene). Increased anti-inflammatory gene expression of *CD274* and *CD80* together with decreased proinflammatory *TLR4* expression on LPS stimulation could be confirmed for children from urban China (Fig 3, *B*). Of note, in preliminary tests we also included urban dust samples as a negative control, resulting in consistent or even decreased TNFAIP3 expression (see Fig E4 in this article's Online Repository at www.jacionline.org).

To investigate the effect of simultaneously increased anti-inflammatory and decreased proinflammatory expression on dust and LPS stimulation, we calculated an "inflammation ratio" by relating proinflammatory to anti-inflammatory gene expression. The average ΔCT value of all genes assembling anti-inflammatory properties (*TNFAIP3*, *CD274*, *CD80*, *TNIP2*, *TAX1BP1*, *CTLA4*, and *PDCD1*) was divided by the average ΔCT value of all proinflammatory genes (*TLR4*, *MYD88*, *MALT1*, *TRAF6*, *CD86*, and *CD28*). Under unstimulated conditions, asthmatic patients had a significantly greater inflammation ratio, indicating a stronger proinflammatory basal state of asthmatic patients compared with healthy children ($P = .005$; Fig 3, *C*, and see the Results section in this article's Online Repository). On stimulation, this inflammation ratio was highly significantly reduced, indicating the anti-inflammatory capacity of 24 hours of stimulation with farm dust and LPS for both healthy and asthmatic children (Fig 3, *C*).

IL18 gene expression was analyzed to investigate downstream NF-κB signaling. IL-18 is induced through the TLR4-MYD88 axis and is associated with asthma pathogenesis by inducing type 2 cytokines, allergic inflammation, and eosinophilic influx into the airways.[26,27] Under unstimulated conditions, asthmatic children had significantly greater *IL18* expression in line with their greater inflammatory status at baseline ($P = .002$; Fig 3, *D*). However, when stimulating PBMCs with German and Finnish farm dust, proinflammatory *IL18* expression was significantly downregulated in both healthy and asthmatic children (Fig 3, *D*), whereas asthmatic children downregulated *IL18* expression even stronger on LPS ($P = .001$) and German farm dust ($P = .045$) stimulation (*P* values not shown in Figure). These data emphasize an anti-inflammatory capacity of farm dust and LPS stimulation, even downstream of NF-κB, at the cytokine level.

TNFAIP3 expression in DCs

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

Under unstimulated conditions, asthmatic patients had less *TNFAIP3* expression in DCs, although this was not significant ($P = .27$), whereas *TLR4* expression was significantly increased ($P = .01$, see Fig E5 in this article's Online Repository at www.jacionline.org).

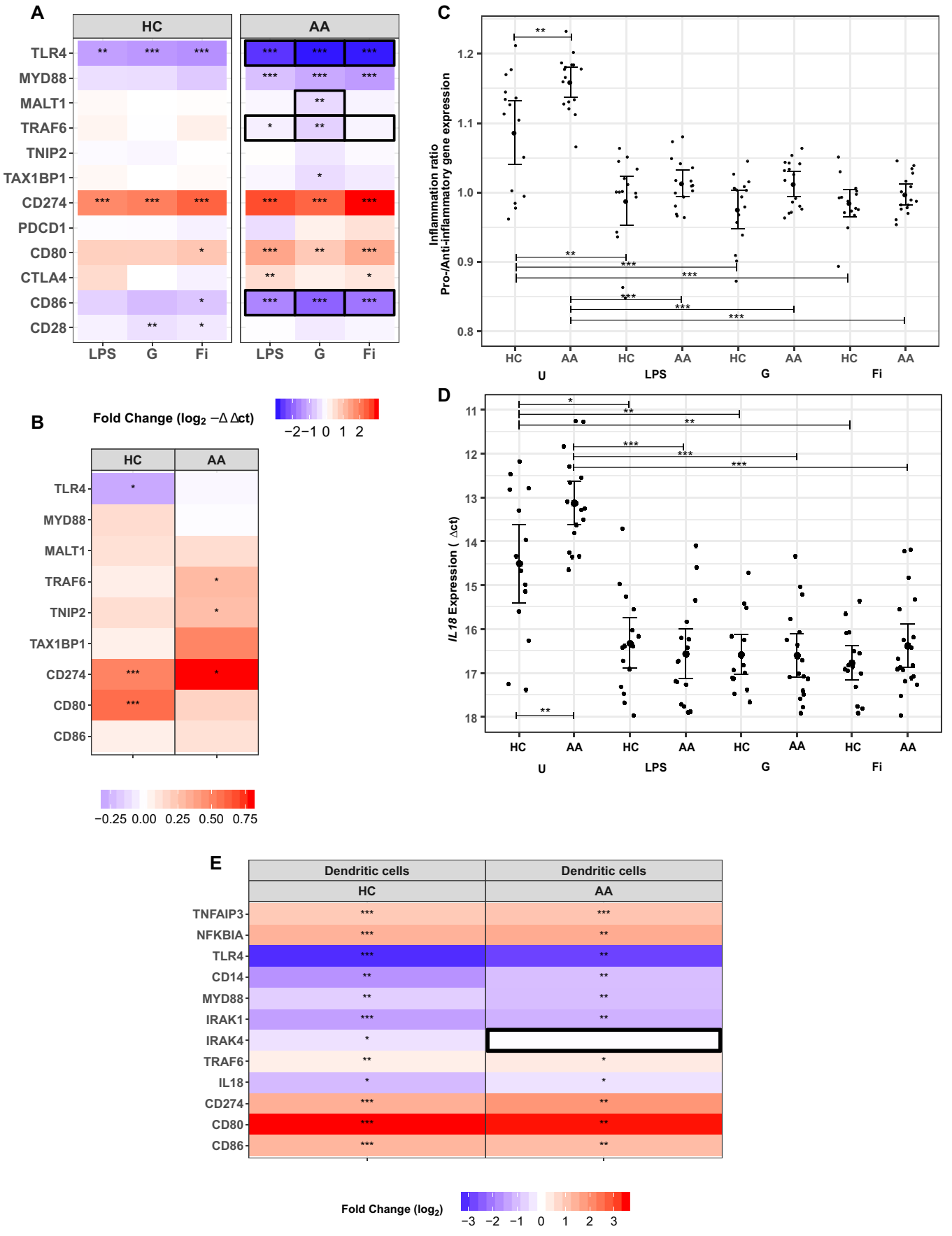
However, on farm dust stimulation, expression of proinflammatory signaling genes, such as *TLR4*; its interacting partner *CD14*; and *MYD88*, IL-1 receptor–associated kinase 1 *(IRAK1)*, *IRAK4*, and *IL18*, was downregulated in DCs, whereas expression of anti-inflammatory signaling genes, such as *TNFAIP3*, the NF-κB inhibiting protein *NFKBIA (IKBα)*, *CD80*, and *CD274*, was significantly upregulated (Fig 3, *E*) in both healthy and asthmatic children.

These findings confirm the results obtained for PBMCs and underline the regulatory role of DCs in the context of environmentally mediated asthma protection.

Living in rural areas downregulates NF-κB 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 nonfarm children (both healthy subjects and asthmatic patients) in unstimulated or 24-hour 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 nonfarm children independent of phenotype, farm children expressed significantly less *TNFAIP3* and *CD274* under unstimulated conditions ($P \leq .035$; Fig 4, *A*).

To assess whether this effect is unique for European farming environments, we investigated gene expression in PBMCs of children from urban (Hong Kong) and rural (Conghua) China, another asthma-protective environment.[30] Consistently, there was no significant difference between asthmatic and healthy children from rural China under unstimulated conditions. Comparing urban with rural children, rural children expressed significantly lower levels of all investigated genes ($P \leq .002$; Fig 4, *B*),


A
HC
AA
TLR4
MYD88
MALT1
TRAF6
TNIP2
TAX1BP1
CD274
PDCD1
CD80
CTLA4
CD86
CD28
LPS
G
Fi
B
Fold Change (log2 −Δ Δct)
−2 −1 0 1 2
HC
AA
TLR4
MYD88
MALT1
TRAF6
TNIP2
TAX1BP1
CD274
CD80
CD86
−0.25 0.00 0.25 0.50 0.75
C
Inflammation ratio
Pro-/Anti-inflammatory gene expression
1.2
1.1
1.0
0.9
0.8
HC
AA
U
LPS
G
Fi
D
IL18 Expression (Δct)
11
12
13
14
15
16
17
18
E
Dendritic cells
HC
AA
TNFAIP3
NFKBIA
TLR4
CD14
MYD88
IRAK1
IRAK4
TRAF6
IL18
CD274
CD80
CD86
Fold Change (log2)
−3 −2 −1 0 1 2 3

suggesting a lower basal inflammatory response pattern for rural children and 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* and downregulated *TLR4* expression (Fig 5) 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-κB pathway, including *TNFAIP3*, compared with nonfarm/nonrural children. Importantly, the capacity to increase anti-inflammatory gene expression (*TNFAIP3*, *CD80*, and *CD274*) and decrease proinflammatory *TLR4* expression was preserved after 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 expression of NF-κB pathway genes and T cell–associated genes in CBMCs of healthy newborns from the German birth cohort PAULINA/PAULCHEN.[16-18] Subsequent asthma diagnosis at school age until 10 years was assessed by a physician.

In fact, newborns with subsequent asthma had significantly lower *TNFAIP3* expression and dysregulated NF-κB signaling gene expression already at birth ($P = .037$, Fig 6). Furthermore, expression of the T cell–associated genes *CD274* and *CD80* was decreased in newborns with subsequent asthma compared with that in healthy children, although not significantly ($P = .055$ and $P = .084$, respectively; see Fig E6 in this article's Online Repository at www.jacionline.org). This suggests an important role of *TNFAIP3* for regulation of inflammation and its involvement in early determination of asthma development.

DISCUSSION

In this study comprising data of 4 pediatric cohorts (total of 2168 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 proinflammatory NF-κB pathway at the transcriptional and translational levels. Notably, lower *TNFAIP3* expression levels were already present at birth in children with asthma by 10 years, 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 proinflammatory gene expression. These anti-inflammatory capacities might indicate a potential therapeutic role for farm dust exposure, even in patients with manifest asthma. Another piece of support for this concept is shown by an anti-inflammatory shift in the most potent antigen-presenting cells, namely DCs. Farm children with long-term *in vivo* farm exposure showed overall less gene expression of all investigated genes compared with urban children. Nevertheless, the anti-inflammatory capacity of acute e*x vivo* LPS stimulation was also present in farm children.

Decreased *TNFAIP3* expression in PBMCs of asthmatic schoolchildren from 2 distinct urban areas (Germany and China) together with increased *TLR4* expression indicates an impaired negative regulation of NF-κB signaling with simultaneous excessive activation of this pathway. Increased expression of the LPS-recognizing *TLR4* receptor was also identified in DCs of asthmatic patients. These alterations might 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 immunologic 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. Because 24 hours of 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 expression of TNFAIP3, the central negative regulator of NF-κB signaling, in healthy and asthmatic children. Notably, stimulation even restored the lower levels of asthmatic patients by shifting its expression to a "healthy level." Anti-inflammatory properties through LPS and farm dust were

FIG 3. LPS and farm dust stimulation downregulate proinflammatory (*TLR4*, *MYD88*, *CD86*, *TRAF6*, and *CD28*) and upregulate anti-inflammatory (*CD274*, *CD80*, and *CTLA4*) gene expression. Upregulation and downregulation on 24 hours of stimulation with German *(G)* or Finnish *(Fi)* farm dust or LPS are color coded in heat maps (Fig 3, *A*, *B*, and *E*) with a red/blue gradient. Saturation reflects strength in terms of the fold change ($\log_2$ scale). Significant regulation compared with that in unstimulated cells is marked with asterisks (*$P < .05$, **$P < .01$, and ***$P < .001$) based on 2-sample *t* test analysis. Different regulation between phenotypes (patients with AA vs HCs) is marked by a *black rectangle* around the respective gene. **A** and **B,** Fold change of gene expression (ΔCT) in PBMCs of 4- to 15-year-old children from Munich, Germany (n = 34 [16 HCs and 18 patients with AA]; Fig 3, *A*) and 7-year-old children from Hong Kong, China (n = 49 [29 HCs and 20 patients with AA]; Fig 3, *B*). **C,** Scatter plots of inflammation score defined as ratio of proinflammatory (*TLR4*, *MYD88*, *MALT1*, *TRAF6*, *CD86*, and *CD28*) against anti-inflammatory (*TNFAIP3*, *CD274*, *CD80*, *TNIP2*, *TAX1BP1*, *CTLA4*, and *PDCD1*) gene expression in unstimulated *(U)* and LPS-stimulated or farm dust–stimulated (German and Finnish) PBMCs of 4- to 15-year-old children from Munich, Germany (n = 31 [15 HCs and 16 patients with AA]). **D,** Scatter plots of *IL18* gene expression (ΔCT) analyzed by using quantitative PCR after 24 hours of cultivation of unstimulated and LPS- or German and Finnish farm dust–stimulated PBMCs of 4- to 15-year-old children from Munich, Germany (n = 36 [17 HCs and 19 patients with AA]) from the CLARA/CLAUS cohort. Data are shown stratified for phenotype. *Error bars* show 95% CIs around the mean. *P* values from *post hoc t* test analysis are indicated for comparisons of HCs to patients with AA. *$P \leq .05$, **$P \leq .01$, and ***$P \leq .001$; Fig 3, *D*. **E,** Fold change of relative expression in isolated DCs of unstimulated and German dust stimulated PBMCs of 4- to 15-year-old children from Munich, Germany (n = 12 [7 HCs and 5 patients with AA]) measured with NanoString multiplex expression analysis.

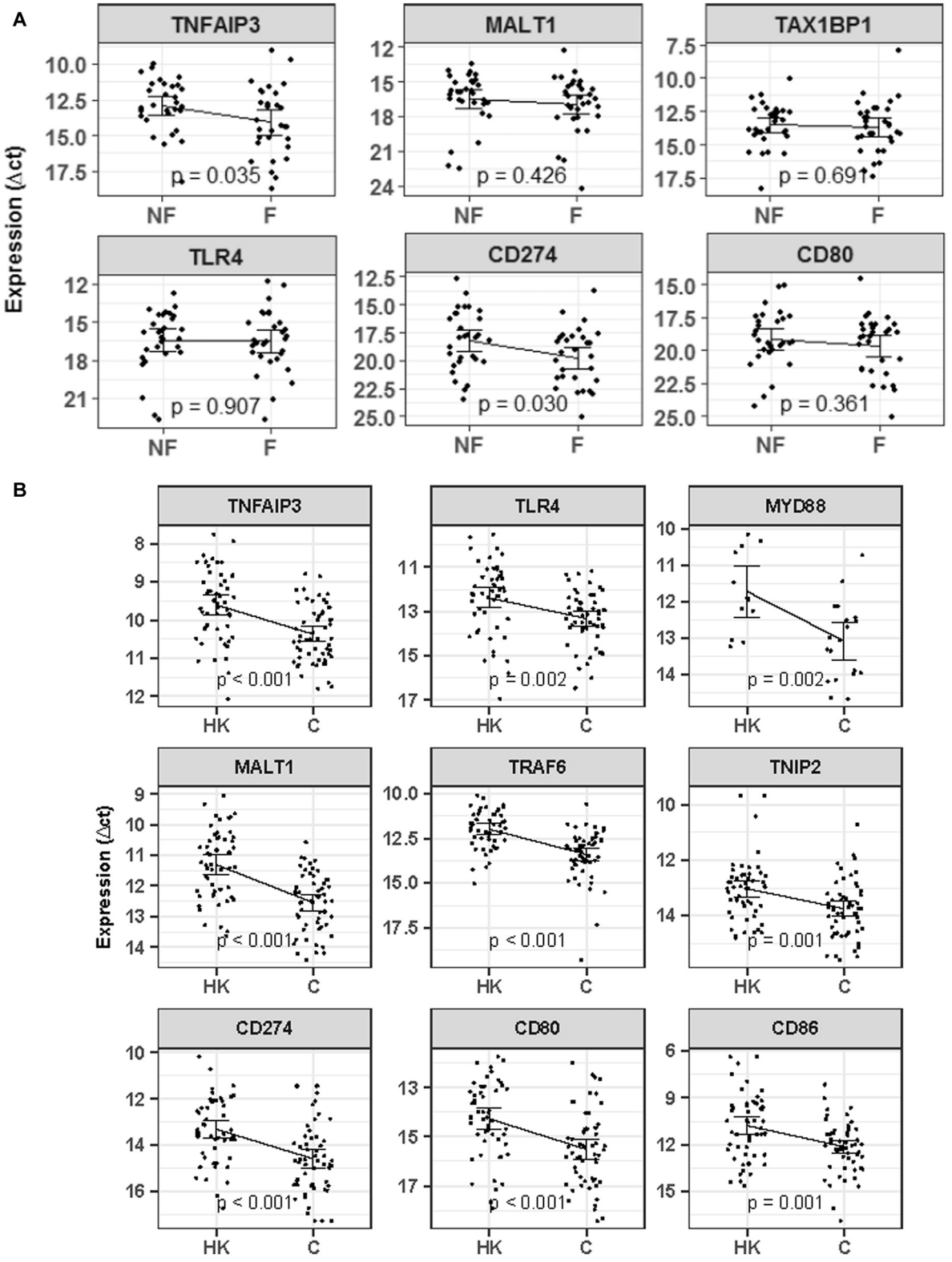


FIG 4. Farm/rural children show lower NF-κB signaling gene expression. Scatter plots of gene expression (ΔCT) were analyzed by using quantitative PCR after 24 hours of cultivation of unstimulated leukocytes of 6-year-old farm (*F*; n = 32) and nonfarm (*NF*; n = 31) healthy and asthmatic children from the PASTURE/EFRAIM cohort **(A)** and 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 **(B)**. Data are shown stratified by environment. *Error bars* show 95% CIs around the mean. *P* values from *post hoc t* test analysis are indicated for comparisons of nonfarm versus farm and Conghua vs Hong Kong, respectively.

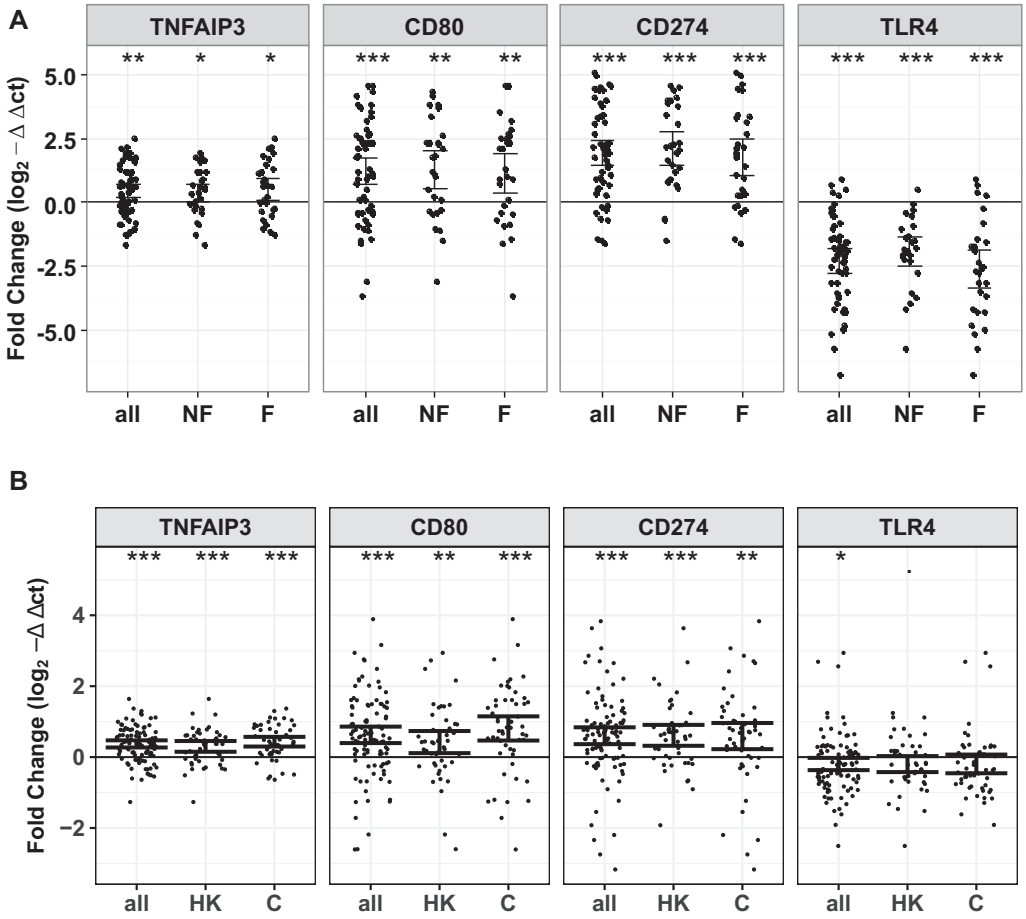


FIG 5. LPS stimulation in farm/rural children results in upregulation of *TNFAIP3, CD80,* and *CD274* and downregulation of *TLR4* compared with unstimulated blood cells. Fold change in gene expression was analyzed by using quantitative PCR after 24 hours of cultivation of unstimulated and LPS-stimulated leukocytes of 6-year-old farm (*F;* n = 29) and nonfarm (*NF;* n = 29) healthy and asthmatic children from the PASTURE/EFRAIM cohort **(A)** and 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 **(B)**. Data are shown stratified by environment. *Error bars* show 95% CIs around the mean. *P* values from *post hoc* 2-sample *t* test analysis are indicated for comparisons of LPS-stimulated versus unstimulated conditions (fold change). $*P < .05$, $**P < .01$, and $***P \leq .001$.

also mediated by upregulating *CD274*, a suppressive T-cell ligand, and *CD80*. Although CD80 interacts with a greater affinity with inhibitory molecules (CTLA4 and CD274), CD86 executes T-cell activation through interaction with CD28.[31,32] In fact, stimulation resulted in significant downregulation of pro-inflammatory *CD28* in healthy children, whereas suppressive *CTLA4* was upregulated in asthmatic patients.

Moreover, stimulation resulted in downregulation of proinflammatory regulators, such as *TLR4*, the initial activator of the NF-κB pathway. This downregulation of the LPS receptor indicates that environmentally 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*, which activates the NF-κB inducing IKK complex, were reduced on stimulation in asthmatic patients. The stimulatory effects of LPS could also be replicated in our urban Chinese cohort, although to a lower extent. Nevertheless, the key finding of upregulation of anti-inflammatory *TNFAIP3*, *CD274*, and *CD80* and downregulation of proinflammatory *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 farm environments is beneficial for all children and potentially for allergy prevention and is even able to decrease the increased inflammatory baseline status of patients with AA.

These anti-inflammatory properties of *ex vivo* farm dust stimulation demonstrated in PBMCs were attributable to those in isolated DCs, supporting our novel concept of a protective mechanism. As major antigen-presenting cells, DCs are in primary contact with 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 environmentally mediated protection.[28,29] Although epidemiologic studies have consistently shown the *in vivo* asthma-protective effect of farm exposure, our immunologic findings might suggest possible therapeutic effects of farm dust,

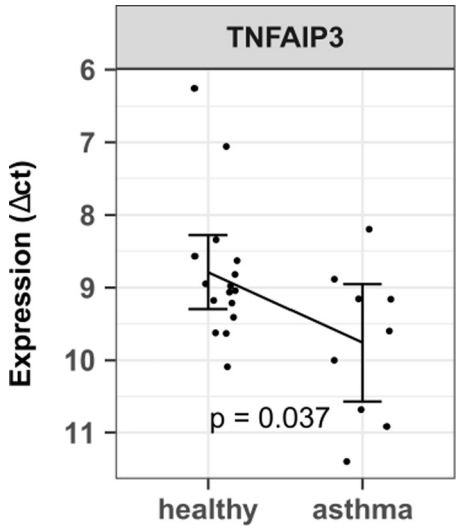


FIG 6. Newborns with subsequent asthma express less *TNFAIP3*. Scatter plots of gene expression (ΔCT) analyzed by using quantitative PCR after 72 hours of cultivation of unstimulated CBMCs from the PAULINA/PAULCHEN cohort are shown. Data are stratified for phenotype (n = 25 [16 HCs and 9 asthmatic patients]). *Error bars* show 95% CIs around the mean. *P* values from *post hoc t* test analysis are indicated for comparisons of HCs and asthmatic patients.

even at the time of early symptomatic disease. However, aiming at use of farm dust as a preventive or therapeutic approach, it is critical to overcome the challenges of dose, composition, administrative route, and safety of farm dust extracts.

In comparison with 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 through unresponsiveness, together with direct downregulation of basal inflammation, to keep harmful inflammation under control. Also, reduced protein expression of both T_H1 and T_H2 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 proinflammatory 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 nonfarming areas. This could explain the greater differences between rural and urban China compared with the European cohort.

Despite the protective environmental exposure, a small number of farm children still have asthma. Thus additional factors, such as genetic predisposition, might be responsible for their asthma development. This might explain why we could not detect any significant differences in *TNFAIP3* expression and the investigated NF-κB 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 data). Family history of atopy, age, and sex was relevant for asthma prediction for both farmers and nonfarmers. However, although asthma prediction was most specific when including genetics for farm children, for nonfarm children, environmental influences were decisive.

On stimulation, both farm/rural and urban children had a similar upregulation of *TNFAIP3*, *CD274*, and *CD80* and downregulation *TLR4*, suggesting there is no difference in the 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 an important factor explaining the rural asthma-protective effect. Farm dust stimulation restores TNFAIP3 expression to healthy levels and increased anti-inflammatory and decreased proinflammatory gene expression. Furthermore, healthy newborns who had asthma at school age expressed lower levels of *TNFAIP3* already at birth. Thus this key regulator can potentially serve as a marker predicting childhood asthma development.

We thank the families for participation in the studies. We acknowledge Isolde Schleich and Tatjana Netz for excellent technical assistance and all study nurses for recruitment and taking blood samples.

Key messages

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

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METHODS

The reagents used for this study are listed in Table E1. RT-PCR was performed in 96-well plates under standard conditions (2 minutes at 95°C, 20 seconds at 95°C, and 30 seconds at 62.5°C, 40 times), including ROX as a passive reference, melting curve analysis, and automatic threshold setting by using the SYBR Green set up in 10 μL containing 6 ng of cDNA and 320 nmol/L primers. The sequences are listed in Table E2. A representative Western blot is shown in Fig E3.

Bacterial 16S rRNA gene analysis

The V3–V5 region of the bacterial 16S rRNA gene was amplified with the forward primer V3-5 forward (CCTATCCCCTGTGTGCCTTGGCAGTCT CAGCCTACGGGAGGCAGCAG) and the reverse primer Bac04xx (CCATCTCATCCCTGCGTGTCTCCGACTCAG-barcode- CCGTCAATTC MTTTRAGT) from Eurofins (Eurofins Genomics, Ebersberg, Germany). The 12-bp barcode for this sample was as follows: GTCGCTGTCTTC (primer Bac0421). Triplicate 25-μL PCRs were set up. For amplification, the Fast Start High Fidelity PCR System (Roche, Mannheim, Germany), adding 2.0 μL (5 μmol/L) of each primer and 1 μL of BSA (10 mg/mL; Sigma-Aldrich, St Louis, Mo), was used. For every sample, a PCR-negative control was included. One microliter of a 1:5 dilution of the original provided DNA extract was used as a PCR template at the end. PCRs were run at 95°C for 120 seconds, followed by 28 cycles of 94°C for 20 seconds, 50°C for 30 seconds, and 72°C for 5 minutes.

Furthermore, amplicons were purified with Agencourt AMPure XP beads (Beckmann Coulter, Fullerton, Calif). Amplicon purity and concentration were evaluated on an agarose gel. Unidirectional sequencing of the 16S rRNA gene fragments was performed by using the 454-GS FLX Titanium protocol (Roche). Amplicons were sequenced at the Wellcome Trust Sanger Institute (Cambridge, United Kingdom). Raw data were processed by using the 454 data processing pipeline (version 2.9) for shotgun reads. Denoising and removal of chimeras were achieved by using AmpliconNoise. Sequences were clustered in operational taxonomic units (OTUs) with 97% sequence similarity by using UCLUST with the *de novo* picking method in QIIME version 1. For taxonomic assignment, OTUs were aligned against the SILVA database (version 111 NR). Concentrations of the purified PCR product from the extract was 22.6 ng/μL, which was determined by using PicoGreen.

Determination of LPS concentrations in farm dust samples

The LAL test QCL-1000 (Lonza) was used according to the instructions, including β-G-Blocker and spike to increase the test's endotoxin specificity, to determine the LPS concentration within our farm dust extracts. 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 using standardized methods in which electrostatic dust collectors were placed in cowsheds for 4 weeks in Germany and Finland, whereas urban dust was collected in the sleeping room in an apartment located in Munich.[E1] Dust was extracted, as described previously.[E2] PBMCs of 6 healthy adults were isolated by means of density gradient centrifugation, and 100 μL of cell suspension (5×10^6 cells/mL) was cultivated in RRMI/10% FCS/1% penicillin-streptomycin at 37°C in a 5% CO_2 atmosphere unstimulated or stimulated with 40 μg/mL German, Finnish, and urban dust. $CD4^+CD25^-$ and $CD4^-CD14^+CD19^+$ cells were isolated by using the DC Isolation Kit II by Miltenyi Biotech, and RNA was extracted by using the RNeasy Mini Kit. cDNA was synthesized with the QuantiTect Reverse Transcription Kit. Gene expression of *TNFAIP3* and *18S* was quantified by using RT-PCR in 96-well plates under standard conditions, including ROX as a passive reference, melting curve analysis, and automatic threshold setting with SYBR Green set up in 10 μL containing 6 ng of cDNA and 320 nmol/L primers. *18S* levels were used for normalization. Primer sequences are listed in Table E2.

Study populations and characteristics

A flow diagram visualizing all study subjects within the 4 analyzed pediatric cohorts is shown in Fig 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. Children with mild-to-moderate asthma from the CLAUS cohort, which is an ongoing replicative cohort of CLARA, were selected for this study based on significantly decreased lung function and high total IgE levels. HCs had more paternal asthma and greater IgE levels compared with HCs of the whole cohort. We had to exclude 1 HC from our *TNFAIP3* gene expression analysis because of technical problems. Informed written consent was obtained from the parents for participation and blood collection.

PASTURE/EFRAIM. The population of recruited children was composed of half farm and half nonfarm children who were repeatedly invited to participate in follow-ups that included questionnaires, blood sampling, or both 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 and 1, 1.5, 2, 3, 4, 5, 6, and 10.5 years of age.[E3,E4] 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. Because of 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 of the whole cohort with regard to center distribution. Furthermore, our selected asthmatic children had more family atopy.

TRILATERAL. Questionnaires based on the International Study of Asthma and Allergies in Childhood were collected from parents. The participation rate was 82% and 86%, respectively. Ethnically, Chinese children born locally in Hong Kong (n = 3,118) or Conghua (n = 10,642) were selected for analysis. The mean age of children who participated in the survey was 7.0 ± 0.7 and 7.2 ± 1.5 years, with male sex of 52.6% and 53.2%, respectively, from Hong Kong and Conghua. Prevalence rates of the following diseases were significantly greater in Hong Kong compared to Conghua children: physician-diagnosed asthma: HK: 5.3% vs C: 2.6% (***), current wheeze (within 12 months): HK: 7.8% vs C: 1.7% (***), rhinoconjunctivitis: HK: 22.3% vs C: 2.9% (***), and flexural dermatitis: HK: 8.5% vs C: 4.3% (***) (all ***$P < .001$). 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 asthmatic patients and 47 HCs [mean age, 6.8 ± 0.6 years] and 32 cases [mean age, 6.8 ± 0.7 years]) and 66 children from Conghua (19 asthmatic patients and 47 HCs [mean age, 7.5 ± 0.7 years] 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 a doctor's diagnosis of asthma and/or allergic rhinitis and/or atopic dermatitis. Total IgE levels along with specific IgE (RAST) levels were measured. The farming group in the PAULCHEN cohort was defined as families having lived, worked, or both on a farm during pregnancy, whereas the nonfarming group was defined as having lived in a nonrural and nonfarming environment during pregnancy. Informed consent was obtained from the mothers for their participation in the studies.

Statistical analysis

Global tests were performed for the null hypothesis that average gene expression is not affected by stimulation, farm exposure, and phenotype (Table E3). The parallel assessment of several genes within each subject is taken into account by using MANOVA, which ensures capture of the potentially high correlation of the data and therefore is more powerful than multiple single ANOVAs for each gene separately. For the described first step, single missing values in gene expression profiles were imputed by using chained equations.

The null hypothesis of 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, 13 genes were measured unstimulated and on stimulation with LPS and German and Finnish farm dust in a single phenotype comparison (patients with AA vs HCs), leading to $13 \times 4 \times 1 = 52$ hypotheses.

For the TRILATERAL cohort, 8 genes were measured unstimulated and on stimulation with LPS in a single phenotype comparison (asthmatic patients vs HCs), leading to $8 \times 2 \times 1 = 16$ hypotheses).

For the PASTURE/EFRAIM cohort, 6 genes were measured unstimulated and on stimulation with LPS in a single phenotype comparison (asthmatic patients vs HCs), leading to $6 \times 2 \times 1 = 12$ hypotheses.

For the PAULINA/PAULCHEN cohort, 10 genes were measured unstimulated in a 2-phenotype comparison (healthy and asthmatic), leading to 10 hypotheses.

RESULTS

Farm dust composition

16S rRNA sequencing was performed, revealing that 8564 sequences were available for the farm dust sample, to unravel the bacterial taxonomic composition. 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. Fifty-four of the OTUs are doubletons or singletons. The majority of the sequences belonged to the phyla of gram-negative Proteobacteria (90.1%), whereas 8.6% and 1% belonged to the gram-positive Firmicutes or Actinobacteria, respectively. When analyzing LPS concentrations within farm dust samples by using LAL testing, a very high endotoxin concentration was measured for the German and Finnish farm dust extracts, respectively, exceeding the highest standard of the test by far.

Statistical assessment of global effects

For the CLARA/CLAUS cohort, MANOVA revealed significant global differences for phenotype effect, stimulus effect, and the effect of phenotype on changes caused by stimulation through fold change. Therefore subsequent *post hoc t* test analyses were performed to identify specific effects by comparing the 2 phenotypes (Fig 1, *A*), stimulus effects (stimulated vs unstimulated; Fig 3, *A*), and effects of phenotype on changes caused by stimulation (Fig 3, *A*, boxes marked with bold black borders).

For the two farming/rural cohorts, PASTURE/EFRAIM and TRILATERAL, MANOVA revealed significant global differences for exposure (Fig 4, *A* and *B*) and stimulus effects (Figs 3, *B*, and 5, *A* and *B*), and for TRILATERAL, MANOVA revealed a significant effect of exposure on changes caused by 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 using MANOVA for phenotype effects. Subsequent *post hoc t* test analyses were performed to identify specific effects by comparing 2 phenotypes each (Fig 6).

Expression of *MYD88, MALT1, TRAF6, TNIP2, TAX1BP1, CD274, CD86, CD28, CTLA4,* and *PDCD1* between healthy and asthmatic children under unstimulated conditions

When comparing gene expression of HCs to children with AA 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*, *CTLA4*, *CD86*, *PDCD1*, and *CD86* (see Fig E2, *A*). When comparing these findings with those of 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* (see Fig E2, *B*), yet asthmatic Chinese children expressed significantly less anti-inflammatory *TAX1BP1*.

Comorbid allergic diseases

To assess the relevance of other atopic comorbid allergic diseases, such as atopic dermatitis, food allergy, and allergic rhinoconjunctivitis, in addition to asthma, we performed a stratified analysis. Of note, gene expression levels of *TNFAIP3* were significantly ($P = .035$) decreased in children of the CLARA/CLAUS cohort with asthma and additional allergic rhinoconjunctivitis (ΔCT, 9.41 ± 0.76) compared with children with asthma alone (ΔCT, 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 with children with AA 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 does not indicate 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 non-DC cells were isolated by using magnetic cell separation. RNA was extracted, cDNA was synthetized, and quantitative PCR for *TNFAIP3* was performed.

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

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

Significant differences between asthmatic and healthy children regarding the "inflammation ratio" relating all investigated proinflammatory genes to anti-inflammatory expression was also observable when the ratio was built by the 2 central genes of the pathway: *TLR4* and *TNFAIP3*. Because 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 CD80 expression, which yielded consistent results, as described in the article.

TNFAIP3 and TLR4 expression in isolated DCs

By investigating gene expression of NF-κB–related genes in isolated DCs by using the multiplex NanoString method, asthmatic children showed lower expression levels of *TNFAIP3* under unstimulated conditions compared with healthy children ($P = .27$, Fig E5). In comparison with the significant difference of TNFAIP3 expression in PBMCs of asthmatic versus healthy children, expression levels obtained in isolated DCs did not reach significance. This might be caused by the low number of children included in this proof-of-principle experiment or because of a relevant additional cell population. Yet the consistent results in both DCs and PBMCs with a significantly increased expression of *TLR4* in asthmatic patients compared with healthy children indicate the importance of DCs, even under unstimulated conditions ($P = .01$, Fig E5).

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 had asthma until the age of 10 years compared with those who stayed healthy, although not significantly ($P \leq .084$, Fig E6).

Sex-specific effects

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

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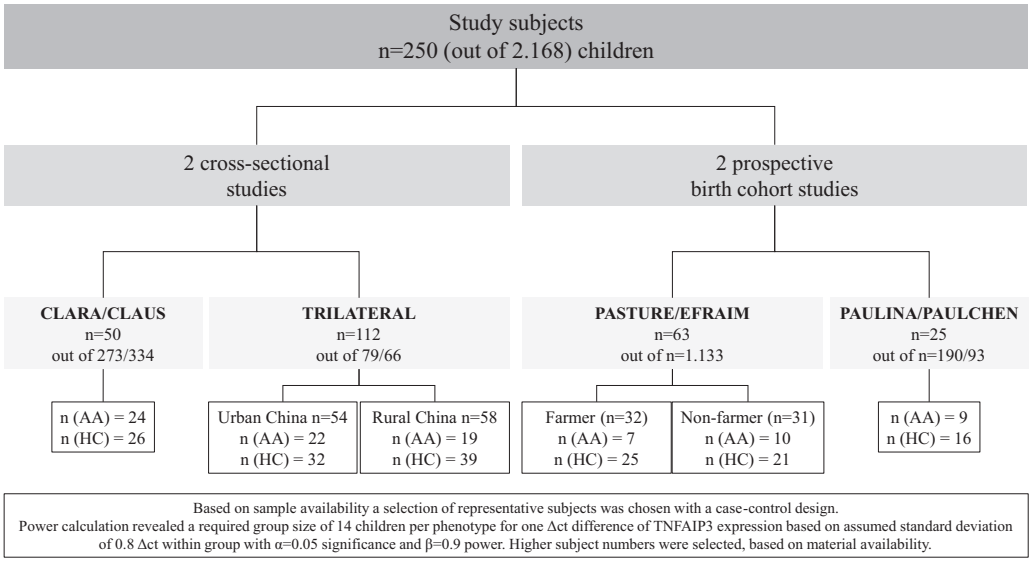

FIG E1. Flow diagram of study subjects. Flow diagram of all subjects included in this study comprising 4 pediatric cohorts, 2 cross-sectional studies (CLARA/CLAUS and TRILATERAL), and 2 birth cohort studies (PASTURE/EFRAIM and PAULINA/PAULCHEN), with a total of 250 of 2168 children.

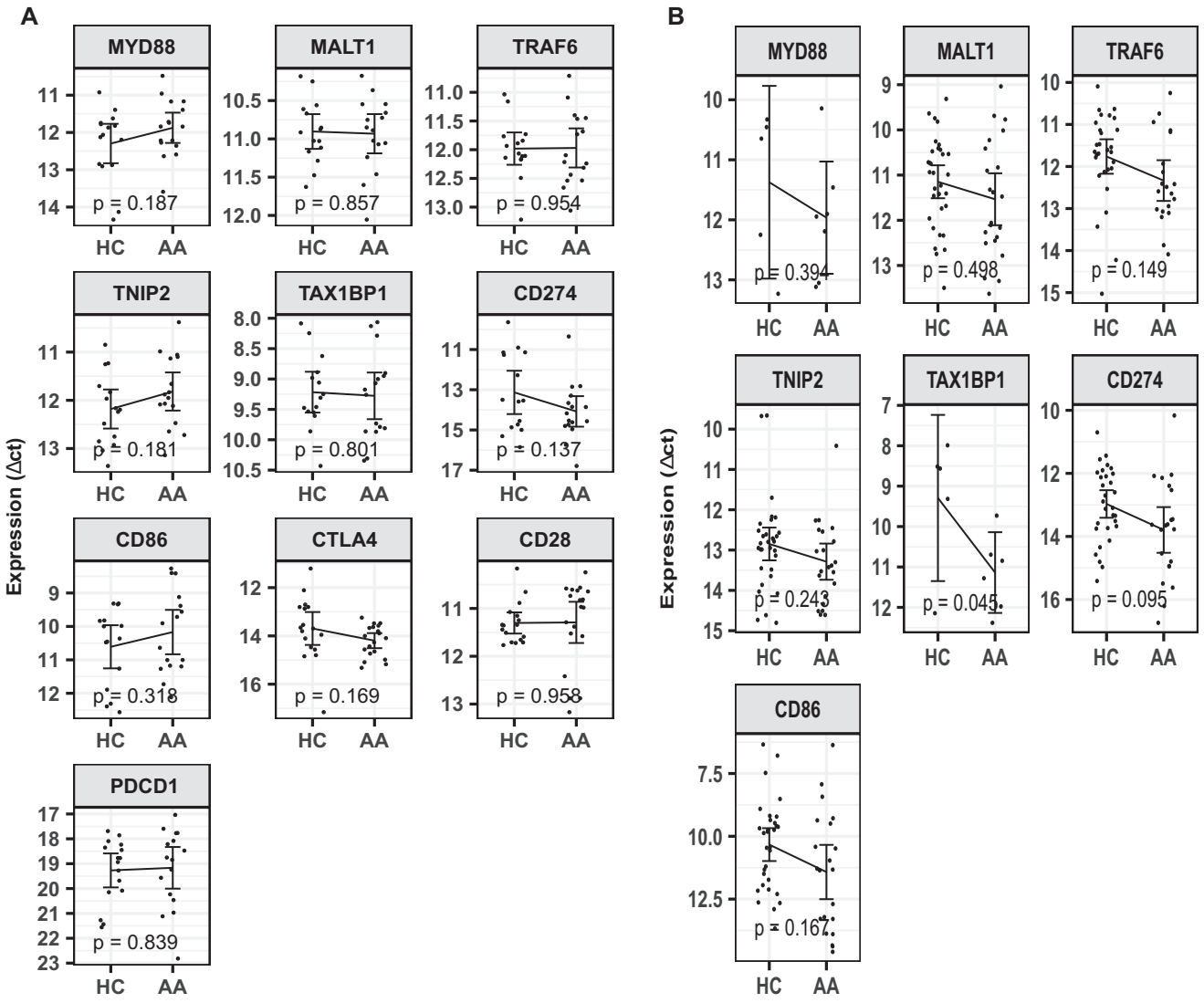

FIG E2. Gene expression of *MYD88*, *MALT1*, *TRAF6*, *TNIP2*, *TAX1BP1*, CD274, *CD86*, *CD28*, *CTLA4*, and *PDCD1* between healthy and asthmatic children under unstimulated conditions. Scatter plots for gene expression of *MYD88*, *MALT1*, *TRAF6*, *TNIP2*, *TAX1BP1*, *CD274*, and *CD86* (Fig E2, *A* and *B*) and *CD28*, *CTLA4*, and *PDCD1* (Fig E2, *A*). Data are stratified for phenotypes, HCs, and asthmatic children. Raw data values are represented by *horizontal jittered points. Error bars* show 95% CIs around sample means, which are connected by *horizontal lines*. Differences are indicated by *P* values of 2-sample *t* tests for phenotype comparisons. Gene expression (ΔCT) was analyzed by using quantitative PCR after 24 hours of cultivation of unstimulated PBMCs of 4- to 15-year-old children from Munich, Germany (CLARA/CLAUS cohort, n = 36 [17 HCs and 19 patients with AA]; **A**) and 7-year-old children from Hong Kong, China (TRILATERAL cohort, n = 53 [32 HCs and 21 asthmatic patients]; **B**).

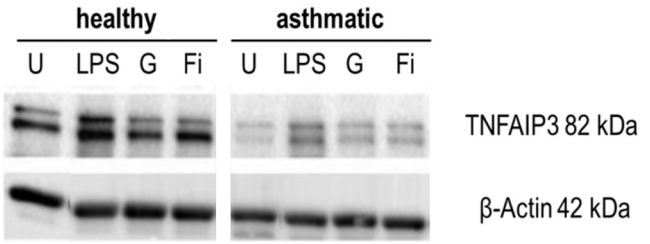

FIG E3. Representative Western blot. One representative image of TNFAIP3 Western blot analysis with β-actin as a loading control (*left,* healthy children; *right*, asthmatic children right) *U*, unstimulated; *G* and *Fi*, and after stimulation with LPS or farm dust (German and Finnish, respectively).

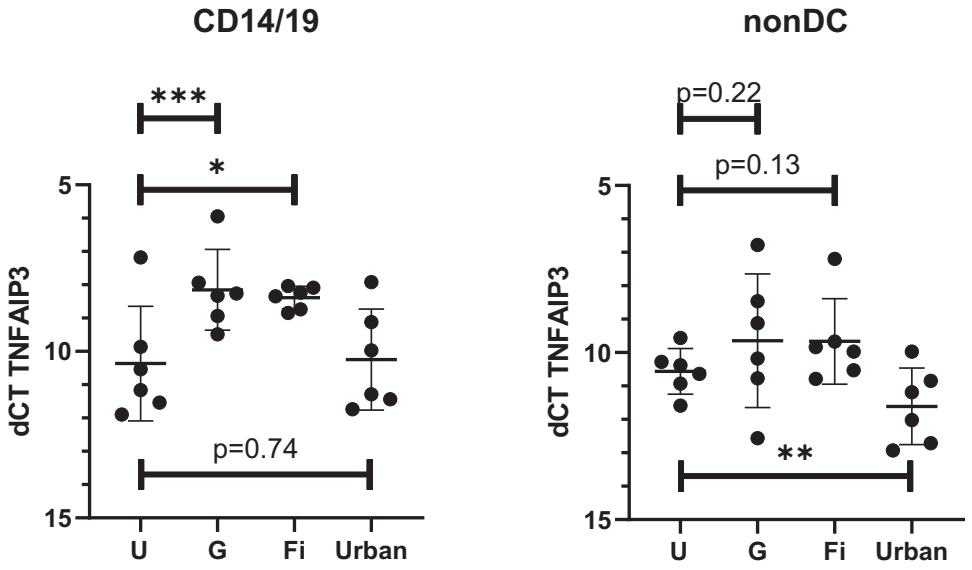

FIG E4. Farm dust stimulation upregulates. *TNFAIP3* gene expression with urban dust stimulation results in consistent or even downregulated TNFAIP3 levels. Scatter plots of gene expression (ΔCT) of *TNFAIP3* in isolated CD14/CD19 cells and non-DCs analyzed by using quantitative PCR after 24 hours of cultivation of unstimulated and LPS- or German *(G)* and Finnish *(Fi)* farm dust–stimulated PBMCs of 6 healthy adults. *Error bars* show 95% CIs around the mean. Significant regulation compared with unstimulated cells is marked with asterisks (*$P < .05$, **$P < .01$, and ***$P < .001$) based on 2-sample *t* test analysis.

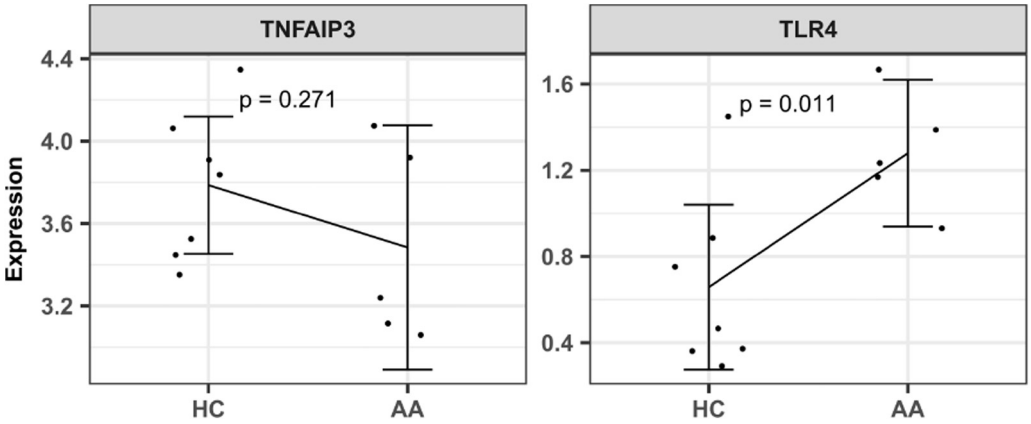

FIG E5. DCs of asthmatic patients express greater levels of TLR4 under unstimulated conditions. Scatter plots of gene expression of *TNFAIP3* and *TLR4* in isolated DCs analyzed by using NanoString technology. DCs were isolated by using magnetic cell separation after 24 hours of cultivation of unstimulated PBMCs of 4- to 15-year-old children from Munich, Germany (n = 12 [7 HCs and 5 patients with AA]). Data are shown stratified for phenotype. *Error bars* show 95% CIs around the mean. *P* values from *post hoc t* test analysis are indicated for comparison of HCs and asthmatic patients.

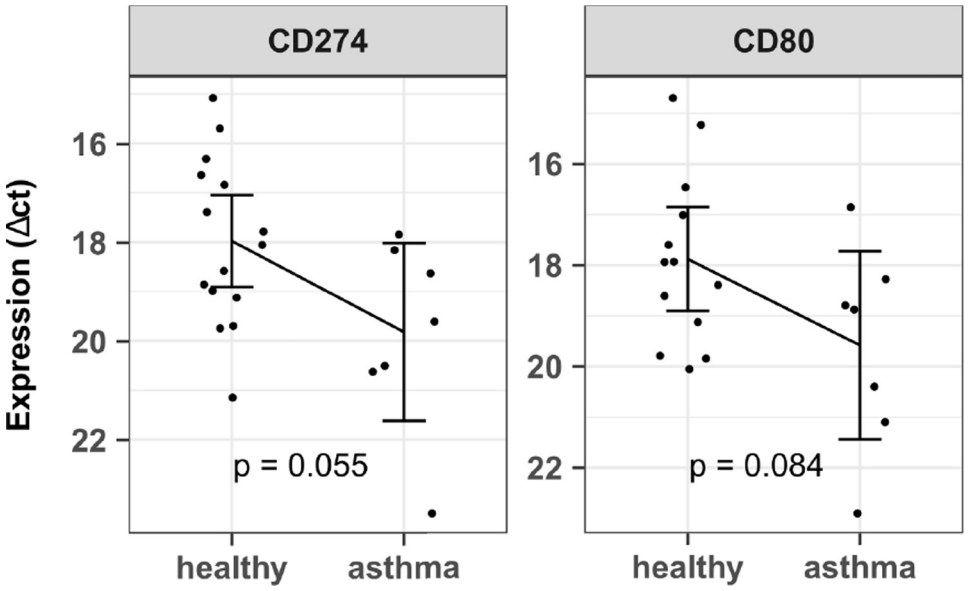

FIG E6. Newborns with subsequent asthma express less CD274 and CD80. Scatter plots of gene expression (ΔCT) of *CD274* and *CD80* analyzed by using quantitative PCR after 72 hours of cultivation of unstimulated CBMCs from the PAULINA/PAULCHEN cohort. Data are stratified for phenotype (n = 20-22 [13-15 HCs and 9 asthmatic patients). *Error bars* show 95% CIs around means. *P* values from *post hoc t* test analysis are indicated for comparisons of HCs to asthmatic patients.

TABLE E1. List of used reagents according companies

Reagent	Company
Ficoll-Paque PLUS	GE Healthcare, Piscataway, NJ
RPMI	Gibco, Carlsbad, Calif
X-VIVO	Lonza Walkersville, Walkersville, Md
FCS	Sigma-Aldrich, St Louis, Mo (PASTURE/EFRAIM) Gibco, Carlsbad, Calif (TRILATERAL)
LPS (*E coli*–O111:B4)	Sigma-Aldrich
TRIzol	Invitrogen, Karlsruhe, Germany
QIAzol	Qiagen, Venlo, The Netherlands
Chloroform	Sigma-Aldrich
RNeasy Mini Kit	Qiagen
QuantiTect Reverse Transcription Kit	Qiagen
BIO-RAD CFX96 System	Bio-Rad Laboratories, Hercules, Calif
SsoAdvanced Universal SYBR Green Supermix	Bio-Rad Laboratories
Vector NTI software, version 10, advance 11.5	Invitrogen, Carlsbad, Calif
cOmplete Protease Inhibitor Cocktail	Roche, Basel, Switzerland
Bradford assay	Carl Roth, Karlsruhe, Germany
TNFAIP3 antibody 4625S	Cell Signaling Technology, Cambridge, United Kingdom
Horseradish peroxidase–conjugated goat anti-rabbit IgG, 7074S	Cell Signaling Technology
Enhanced chemiluminescence solution	Thermo Fisher, Waltham, Mass
ChemiDoc MP System	Bio-Rad Laboratories
Image Lab software	Bio-Rad Laboratories
β-Actin (sc-47778 horseradish peroxidase	Santa Cruz Biotechnologies, Santa Cruz, Calif
ImageJ software	National Institutes of Health, Bethesda, Md
autoMACS	Miltenyi Biotec, Bergisch Gladbach, Germany
Human Blood Dendritic Cell Isolation Kit II	Miltenyi Biotec
RNA Clean & Concentrator-5 Kit	Zymo Research, Irvine, Calif
Bioanalyzer RNA 6000 Nano Kit	Agilent Technologies, Santa Clara, Calif
NanoString nCounter expression analysis	NanoString Technologies, Seattle, Wash
LAL test QCL-1000	Lonza, Walkersville, Md

TABLE E2. Sequences of primers used for RT-PCR

Gene	Primer sequence (forward primer, 5′-3′)
18S	AGTCCCTGCCCTTTGTACACA
CD274	ACCACCACCAATTCCAAGAGAG
CD80	CTGGCTGGTCTTTCTCACTTCTGTTC
CD86	GCGGCTTTTATCTTCACCTTTC
MALT1	CTCAGCCCCCCAGGAATAAAG
MYD88	TGCCTTCATCTGCTATTGCCCC
TAX1BP1	GCAGCAGAGGCAGATTTTGACATAG
TLR4	CTCAACCAAGAACCTGGACCTG
TNFAIP3	GCCCAGGAATGCTACAGATACCC
TNIP2	CAAAGGAATGTGGGGGAGAGAAGTC
TRAF6	TGATGTAGAGTTTGACCCACCCCTG
CD28	CCATGTGAAAGGGAAACACCTT
CTLA4	TGGCCCTGCACTCTCCTGT
PDCD1	GCTCAGGGTGACAGAGAGAAG
IL18	AACAAACTATTTGTCGCAGGAAT

TABLE E3. Results of global MANOVA for effects of phenotype, exposure, and stimulus

	CLARA/CLAUS	TRILATERAL	PASTURE/EFRAIM	PAULINA/PAULCHEN
Effect of phenotype	52 hypotheses, $P = 1.214 \cdot 10^{-10}$ Subsequent *t* test analysis comprised in Fig 1, *A*	Stratified for farm exposure: 32 hypotheses, $P = 1.766 \cdot 10^{-2}$, subsequent *t* test analysis in Fig 1, *B* Adjusted for region, 16 hypotheses, $P = 2.180 \cdot 10^{-1}$ n.s., no further *t* test analysis Without stratification or adjustment: 16 hypotheses, $P = 1.713 \cdot 10^{-1}$, n.s., no further *t* test analysis	Stratified for farm exposure: 24 hypotheses, $P = 1.543 \cdot 10^{-4}$ Adjusted for farm exposure, 12 hypotheses, $P = 4.901 \cdot 10^{-1}$, n.s., no further *t* test analysis Without stratification or adjustment: 12 hypotheses, $P = 4.852 \cdot 10^{-1}$, n.s., no further *t* test analysis	10 hypotheses, $P = 1.996 \cdot 10^{-5}$, subsequent *t* test analysis in Fig 6
Effect of exposure	—	16 hypotheses, $P = 1.675 \cdot 10^{-9}$, subsequent *t* test analysis in Fig 4, *B*	12 hypotheses, $P = 4.694 \cdot 10^{-2}$, subsequent *t* test analysis in Fig 4, *A*	—
Effect of stimulus (via fold change)	39 hypotheses, $P = 1.114 \cdot 10^{-23}$, subsequent *t* test analysis in Fig 3, *A*	8 hypotheses, $P = 2.832 \cdot 10^{-17}$, subsequent *t* test analysis in Figs 3, *B*, and 5, *B*	6 hypotheses, $P = 2.943 \cdot 10^{-18}$, subsequent *t* test analysis in Fig 5, *A*	—
Effect of phenotype on changes due to stimulation (via fold change)	39 hypotheses, $P = 1.084 \cdot 10^{-22}$, subsequent *t* test analysis comprised in Fig 3, *A*	8 hypotheses, $P = 3.875 \cdot 10^{-1}$, n.s., no further *t* test analysis	6 hypotheses, $P = 6.323 \cdot 10^{-1}$, n.s., no further *t* test analysis	—
Effect of exposure on changes due to stimulation (via fold change)		8 hypotheses, $P = 1.389 \cdot 10^{-4}$, *post hoc t* test analysis revealed no significance	6 hypotheses, $P = 3.877 \cdot 10^{-1}$, n.s., no further *t* test analysis	

P values were derived from the F test.
n.s., Not significant.