From the: Dr. von Hauner Children´s Hospital, Ludwig-Maximilians-University Munich

Dissertation

to acquire the Doctor of Philosophy (Ph.D.) from the

Medical Faculty of the

Ludwig-Maximilians-University of Munich

Regulation of inflammatory NF-κB and MAPK signaling and the role of innate dendritic cells and adaptive T cells in environment-mediated protection against childhood asthma

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Year:

2022

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List of abbreviations

AA	Allergic asthmatics
A.U.	Arbitrary units
ANOVA	Analysis of variance
APC	Antigen-presenting cell
aOR	Adjusted odds ratio
ATF2	Activating transcription factor 2
ATS	American Thoracic Society
BAFF	B cell activation factor
BAL	Bronchoalveolar Lavage
Breg	Regulatory B cells
BDCA	Blood dendritic cell antigens
BSA	Bovine serum albumin
c	Cells
C	Conghua
CBMC	Cord blood mononuclear cells
CCL	Chemokine ligand
CCR	Chemokine receptor
CI	Confidence interval
cDNA	Complementary DNA
ChIP	Chromatin Immunoprecipitation
CO_2	Carbon dioxide
CD	Cluster of differentiation
cDC	Conventional DC
CFSE	Carboxyfluorescein succinimidyl ester
CLARA/CLAUS	Clinical Asthma Research Association
CT	Cycle Threshold
CyTOF	Cytometry by time of flight
DC	Dendritic cells
DFG	German Research Foundation
DN	Double-negative cells
DUSP1	Dual-specificity phosphatase 1
DZL	German Center for Lung Research

G	German farm dust
GABRIELA	Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community Advanced Study
GATA3	GATA binding protein 3
GINA	Global Initiative for Asthma
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GZMB	Granzyme B
DNA	Deoxyribonucleic acid
EKFS	Else Kröner-Fresenius-Stiftung
ERK	Extracellular signal-regulated kinase
ERS	European Respiratory Society
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal calf serum
FCS-file	Flow cytometry standard file
FEV1	Forced expiratory volume in one second
F	Farm
Fi	Finnish farm dust
FITC	Fluorescein Isothiocyanate
FVC	Forced vital capacity
FOXP3	Forkhead box P3
FlowSOM	Flow Self-Organising Map
h	Hours
H4	Histone 4
HC	Healthy controls
HLA-DR	Human leucocyte antigen D-related
HK	Hong Kong
HRP	Horse radish peroxidase
IκB	Inhibitor of kappa-light-chain-enhancer of activated B cells
IKK	IκB kinase
IFNγ	Interferon γ
IgE	Immunglobulin E
IgG	Immunglobulin G
IL	Interleukin

ILT4	Immunoglobulin-like transcript 4
IRAK	IL-1 receptor-associated kinase
IRF	Interferon regulatory factor
IP	Immunoprecipitation
IU	International units
E.coli	Escherichia coli
HDM	House dust mite
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
LAL	Limulus Amoebocyte Lysate
LpA	Lipid A
LMU	Ludwig-Maximilian University
log	Logarithm
LPS	Lipopolysaccharide
LPS-RS	LPS from Rhodobacter sphaeroides
n	Sample size
MANOVA	Multivariate ANOVA
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAPK	Mitogen-activated protein kinase
MARTHA	Milk Against Respiratory Tract Infections and Asthma
MEM	Minimum Essential Medium
mDC	Myeloid DC
MKP-1	MAPK phosphatase 1
MyD88	Myeloid differentiation primary response gene-88
MHC	Major histocompatibility complex
mRNA	Messenger RNA
moDC	Monocyte-derived DC
NEMO	NF-κB essential modulator
NF	Non-farm
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFKBIA	NF-κB inhibitor alpha
NIK	NF-κB-inducing kinase
NK-cell	Natural killer cells

OR	Odds ratio
OTU	Operational taxonomic unit
PAMP	Pathogen-associated molecular pattern
PARSIFAL	Prevention of Allergy-Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle
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
PBS	Phosphate-buffered saline
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid DC
PVDF	Polyvinylidene fluoride
PE	Phycoerythrin
qPCR	Quantitative polymerase chain reaction
rpm	Revolutions per minute
PRR	Pathogen recognition receptor
rcf	Relative centrifugal force
RIP-1	Receptor-interacting protein kinase 1
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute
rRNA	Ribosomal RNA
RORγ	RAR-related orphan receptor γ
ROX	Rhodamine X
RT	Room temperature
RT-qPCR	Real-time qPCR
RSV	Respiratory Syncytial Virus
RV	Rhinovirus
SCFA	Short chain fatty acid
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphisms
STAT6	Signal transducer and activator of transcription 6

TAB1/2	Transforming growth factor-beta-activated kinase 1/2
TAK1	Transforming growth factor-activated kinase 1
TAX1BP1	Tax1-binding protein 1
TBS	Tris-buffered saline
Tcm	Central memory T cells
Teff	Effector T cells
Tem	Effector memory T cells
TF	Transcription factor
Tfh	Follicular T helper cells
Th	T helper cells
THBD	Thrombomodulin
TNIP	TNFAIP3-interacting protein
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFAIP3	TNF-alpha induced protein 3
TRAF6	TNF receptor-associated factor 6
Treg	Regulatory T cells
TXNIP	Thioredoxin-interacting protein
U	Unstimulated
UGMLC	Universities of Giessen and Marburg Lung Center
UMAP	Uniform Manifold Approximation and Projection
y	Years

List of publications

Original articles:

Mitogen-activated protein kinase signaling in childhood asthma development and environment-mediated protection

Theodorou Johanna; Nowak Elisabeth; Böck Andreas; Salvermoser Michael; Zeber Kathrin, Kulig Paulina; Tsang Miranda S.; Wong Chun-Kwok; Wong Gary W.K.; Roponen Marjut, Kumbrink Jörg; Alhamdan Fahd; Michel Florian; Garn Holger; Tosevski Vinko; Schaub Bianca

Pediatric Allergy and Immunology (IF: 6.377), 33(1):e13657. 2022, doi: 10.1111/pai.13657. Epub 2021 Sep 14

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

Krusche, Johanna*; Twardziok, Monika*; Rehbach, Katharina*; Böck, Andreas; Tsang, Miranda, Schröder, Paul; Kumbrink, Jörg; Kirchner, Thomas; Xing, Yuhan; Riedler, Josef; Dalphin, Jean-Charles; Pekkanen, Juha; Lauener, Roger; Roponen, Marjut; Li, Jing; Wong, Chun K.; Wong Gary; Schaub, Bianca and the PASTURE study group

*shared first authorship

Journal of Allergy and Clinical Immunology (IF: 14.110), 144(6), 1684-1696.e12, 2019, doi:10.1016/j.jaci.2019.07.029

Asthmatic farm children show increased CD3+ CD8low T cells compared to non-asthmatic farm children

Twardziok, Monika; Schröder, Paul C.; **Krusche, Johanna**; Casaca, Vera I.; Illi, Sabina; Böck, Andreas; Loss, Georg J.; Kabesch, Michael; Toncheva, Antoaneta A.; Roduit, Caroline; Depner, Martin; Genuneit, Jon; Renz, Harald; Roponen, Marjut; Weber, Juliane; Braun-Fahrländer, Charlotte; Riedler, Josef; Lauener, Roger; Vuitton, Dominique Angèle; Dalphin, Jean-Charles; Pekkanen, Juha; Mutius, Erika von; Schaub, Bianca; Hyvärinen, Anne; Karvonen, Anne M.; Kirjavainen, Pirkka V.; Remes, Sami; Kaulek, Vincent; Dalphin, Marie-Laure; Ege, Markus; Pfefferle, Petra I.; Doekes, Gert

Clinical Immunology (IF: 3.990) 183, 285-292, 2017, doi:10.1016/j.clim.2017.09.009

A phylogeny of Cephaloziaceae (Jungermanniopsida) based on nuclear and chloroplast DNA markers

Feldberg, Kathrin; Váňa, Jiří; **Krusche, Johanna**; Kretschmann, Juliane; Patzak, Simon D. F.; Pérez-Escobar, Oscar A.; Rudolf, Nicole R.; Seefelder, Nathan; Schäfer-Verwimp, Alfons; Long, David G.; Schneider, Harald; Heinrichs, Jochen

Organisms Diversity & Evolution (IF: 2.313) 16 (4), 1–16, 2016, doi:1007/s13127-016-0284-4

Letter:

Mass cytometry-based identification of a unique T cell signature in childhood allergic asthma
Raifer Hartmann*, Schulz Axel R.*, **Theodorou Johanna***, Romero-Olmedo Addi J., Böck Andreas, Bertrams Wilhelm, Schmeck Bernd T., Chung Ho Ryun, Lohoff Michael, Chang Hyun-Dong, Schaub Bianca, Mei Henrik E., Huber Magdalena
***shared first authorship**

Allergy (IF: 13.146), 77(1): 313-316. 2022, doi: 10.1111/all.15110. Epub 2021 Oct 4. 2021

Reviews:

EAACI Position Paper: Comparing the skin microbiome in human and veterinary individuals and its influence on allergic or atopic diseases – Chapter: The skin microbiome: Are pets a good choice to prevent allergy development in human individuals (mini-farm effect)? And vice versa?

Theodorou, Johanna; Schaub, Bianca

Allergy (IF: 13.146), submitted, 2021

Book chapter: Immunology and defence mechanisms

Theodorou, Johanna; Schaub, Bianca

ERS Handbook of Paediatric Respiratory Medicine 2nd edition, The European Respiratory Society, 2021, doi:10.1183/9781849841313.002519

Special Edition on asthma: Role of early immune regulation in asthma development

Krusche, Johanna; Basse, Sarah; Schaub, Bianca

Seminars in Immunology (IF: 9.35), 42(1):29-42, 2020, doi:10.1007/s00281-019-00774-z

Frühe Umweltexposition im Leben – Schutz oder Risiko für allergische Erkrankungen

Krusche, Johanna; Schaub, Bianca

Allergologie 41 (8), 348-358, 2018, doi:10.5414/ALX02021

Unpublished manuscripts:

Farm-dust mediated protection of childhood asthma: Identification of unique cellular and molecular regulatory mechanisms

Theodorou Johanna; Salvermoser Michael; Böck Andreas; Claudia Beerweiler; Zeber Kathrin; Kulig Paulina; Kumbrink Jörg; Tosevski Vinko; Schaub Bianca

Manuscript in preparation, 2022

1. Contribution to the publications

1.1 Contribution to paper I

In the publication “TNFAIP3 is a key player in childhood asthma development and environment-mediated protection”, the author of this dissertation, Johanna Theodorou née Krusche, Dr. rer. nat. Monika Twardziok and Dr. med. Katharina Rehbach contributed equally to the work resulting in a shared first authorship. Dr. Rehbach described selected parts of this paper in the framework of her medical dissertation.[1] Johanna Theodorou contributed substantially to the publication of this study by participating in the design of the study, performing experiments, analyzing and interpreting the data, and writing the manuscript. In detail, Johanna Theodorou prepared the RNA samples from isolated dendritic cells including concentration and quality measurements as well as condensation and hybridization of the samples for subsequent NanoString measurements. In addition, Johanna Theodorou performed qPCR measurements of the CLARA/CLAUS cohort. Moreover, the major part of data interpretation, the design of the conceptional structure of the final version of the manuscript, and the majority of manuscript writing were done by Johanna Theodorou. Monika Twardziok performed qPCR measurements of the PAULINA/PAULCHEN cohort, interpreted the data, and wrote the first version of the manuscript. Katharina Rehbach performed experiments, specifically qPCR and Western Blot measurements of the CLARA/CLAUS and PASTURE/EFRAIM cohorts, recruited samples, and interpreted the data.

1.2 Contribution to paper II

The first author of the manuscript entitled “Mitogen-activated protein kinase signaling in childhood asthma development and environment-mediated protection“, Johanna Theodorou, contributed substantially to this study by participating in the design of the study, performing the experiments partly together with the collaborating co-authors of the manuscript, interpretation of the data, conceptionally structuring and writing of the manuscript. In detail, Johanna Theodorou performed qPCR measurements of the CLARA/CLAUS and TRILATERAL cohort together with Elisabeth Nowak and Miranda S. Tsang. Elisabeth Nowak included some parts on gene expression measured in PBMCs in her medical dissertation.[2] In addition, qPCR measurements of the PAULINA/PAULCHEN cohort, LPS-blocking experiments, and Western Blots were done by Johanna Theodorou. Moreover, Johanna Theodorou recruited children together with the study doctor and the medical students and prepared the samples for NanoString, CyTOF, and histone acetylation measurements that were performed at the respective facilities. Data analysis was performed by Johanna Theodorou in cooperation with the biostatisticians of the group. Compilation, data interpretation, conceptual design, and writing of the manuscript were done by Johanna Theodorou.

1.3 Contribution to the unpublished manuscript (Appendix)

Johanna Theodorou contributed significantly to this publication by participation in the design of the study, performing the experiments, analysis, and interpretation of the data, and compiling them together to the manuscript. Specifically, Johanna Theodorou recruited the children together with the study doctor and the medical students and prepared the samples for NanoString and CyTOF measurements. Moreover, all experiments on monocyte-derived dendritic cells, and Treg suppression assays were done by Johanna Theodorou. Additional validation experiments are

currently performed by Claudia Beerweiler. Data analysis and interpretation of the cell culture experiments were performed by Johanna Theodorou and for the NanoString and CyTOF experiments in close cooperation with the biostatisticians (Dr. rer nat. Andreas Böck, Michael Salvermoser). Moreover, Johanna Theodorou interpreted the data and structurally designed and wrote the manuscript.

2. Introductory summary

2.1 Childhood asthma

Asthma is a complex disease in which patients suffer from chronic inflammation of the airways accompanied by airway obstruction with limited expiratory airflow.[3] The resulting symptoms include wheeze, dyspnea, chest tightness, and/or cough that vary in their occurrence and intensity.[3] Symptoms and acute exacerbations are often triggered by external factors including viral infections, exercise, exposure to allergens, and chemical-irritative substances.[3]

The prevalence of asthma is with over 300 million affected people the most common respiratory disease worldwide.[4] The disease often origins in early life. With the high and constantly rising worldwide prevalence, asthma represents the most common chronic disease in childhood.[3] Childhood asthma is often classified in non-allergic and allergic phenotypes. The latter is accompanied by the production of immunoglobulin E (IgE) to rather harmless inhaled environmental substances.[5]

The development of asthma is dependent on both genetic and environmental factors as well as their complex interactions. Family history is one of the strongest risk factors influencing the susceptibility of this and other allergic diseases by polygenic heredity.[6] The effect of genetic variants can also be modulated by environmental factors through complex gene-environment interactions.[7] These gene-environment interactions might present a possible explanation why some children that harbor risk SNP variants develop asthmatic symptoms while others stay asymptomatic.[8] In addition, epigenetic modifications including DNA methylation and histone acetylation might present one important pillar in the development of childhood asthma.[9] Although genetics and epigenetics are strong influencing factors in the etiology of childhood asthma, rapid increases in the prevalence of asthma and other allergic diseases as well as significant regional differences even in populations with similar genetic ancestry, underscore the importance of environmental factors in the development of allergic diseases. While some environmental influences like air pollution, caesarian section, exposure to tobacco smoke, mold, maternal stress in pregnancy, and use of antibiotics have detrimental effects on asthma development, other influences can even result in the protection against the disease.[10] Based on the extent and timing of the exposure some factors might even be categorized in both groups.[8]

2.2 Epidemiological evidence of environment-mediated asthma protection

In contrast to the listed rather harmful environmental factors for childhood asthma development, others including balanced diet during pregnancy, early contact with other children and especially exposure to farming environments was associated with strong asthma and allergy protection.[8] In 1989, David Strachan introduced the term “hygiene hypothesis” by describing an inverse relation-

ship of birth order and symptoms of hay fever.[11] He suggested that an increasing number of siblings being associated with less hygienic conditions followed by more frequent infections might reduce the development of allergies.[11] The high increase in allergy prevalence during the last decades is suggested to be associated with the loss of symbiotic relationships with parasites and bacteria.[12] Over the last 150 years, enormous hygienic interventions, as well as the development of antibiotics and vaccinations significantly reduced the prevalence of infectious diseases, while allergic diseases increased in parallel.[13,14] In fact, infections to certain pathogens might induce protection against allergic diseases.[15] E.g. Italian cadets that were seropositive for orally acquired pathogens like *Toxoplasma gondii*, Hepatovirus A, and *Helicobacter pylori* had a highly decreased likelihood to suffer from an allergic disease.[16] Also, helminth infections were associated with a reduced risk for allergic diseases.[17,18] In addition, changes in lifestyle with a more industrialized and westernized way of living were associated with reduced environmental biodiversity and changes in the composition of the microbiome[13], resulting in an insufficient maturation of the child´s immune system due to loss of instructive signals.[19] In this context, the terms of the "old friends hypothesis" and "biodiversity hypothesis" were proposed, with the shared idea of increased prevalence of allergic diseases due to loss of symbiotic relationships with bacteria.[20,21] However, not all pathogens and parasites are protective. Infections with RSV and RV are rather associated with an increased risk of airway diseases and some helminth infections are even associated with bronchial hyperreactivity and asthma severity.[22–24]

The strongest protection in asthma and allergy development is conveyed by exposure to farming environments in early childhood as shown by numerous epidemiological studies around the world.[25–29] Data from two European pediatric studies have shown a significantly lower asthma prevalence of children growing up on farms compared to those without farming exposure in early life with an adjusted odds ratio (aOR) and corresponding 95% confidence interval (CI) of 0.49 (0.35-0.69; PARSIFAL study) and 0.76 (0.65-0.89; GABRIELA study), respectively.[26] The reduction of the risk for atopy with early life farming exposure was even stronger (aOR 0.24 (0.18-0.34; PARSIFAL) and 0.51 (0.46-0.57; GABRIELA).[26] Another prominent example of the so-called "farm effect" is the great difference in asthma prevalence between children of the Amish people and the Hutterites. These two farming populations from the US share similar characteristics that might influence asthma risk regarding genetic background, the number of siblings, nutrition and breastfeeding, vaccination rates, and exposure to air pollution, tobacco smoke, and pets.[30] However, while the Amish people live on traditional farms, farms of the Hutterites are highly industrialized.[30] The difference in childhood asthma prevalence is with 5.2% (Amish) and 21.3% (Hutterite) strikingly high.[30–32] Another study investigating the effect of farm exposure on the risk of allergy in children from Saskatchewan (Canada) revealed a significant atopy risk reduction for livestock farming with an aOR of 0.38 (0.17-0.88).[33] In addition, also rural areas in China were associated with a lower prevalence of childhood allergy and asthma. By comparing the prevalence of children from urban and rural areas in and around Beijing in China there was a significantly reduced risk for asthma (6.3% vs. 1.1%) and a 3.22 fold lower risk for atopy when living in rural areas.[34,35] Regarding the protective components within these farming environments, several exposures

might convey beneficial effects including exposure to farm animals, stable or barn exposure, and the consumption of unprocessed raw farm milk.[25,36–40]

Indeed, consumption of farm milk was shown to be even protective for allergic diseases independently of other farm-related exposures.[25,38,39,41,42] A recent meta-analysis revealed a reduced risk for asthma (OR=0.58 (0.49-0.69)) and atopic sensitization (OR=0.76 (0.62-0.95)) upon early-life consumption of raw cow's milk[41]. Due to potential contamination of unprocessed raw cow´s milk with harmful pathogens like *Listeria*, *Salmonella*, or *Escherichia coli*, its consumption is not recommended in many countries.[41,43] Therefore, minimally processed milk that provides safety with low destruction of relevant heat-sensitive proteins[44] might be a promising agent in asthma prevention that is currently tested in the clinical intervention study MARTHA ("Milk Against Respiratory Tract Infections and Asthma").[41]

Moreover, the exposure to stables of farm animals is inversely associated with asthma and allergy risk.[25,45,46] The protective effect of *in utero* stable exposure was shown in the cross-sectional PARSIFAL study.[45,47] Children whose mothers were exposed to stables during pregnancy had significantly less atopic sensitization (aOR=0.58; 0.39-0.86).[45] In addition to prenatally conferred protection, farm exposure in the first year of life is especially relevant for a potent induction of protective mechanisms.[25] Stable exposure in the first year of life was associated with reduced risk for asthma (aOR=0.51 (0.14-1.86), hey fever (aOR=0.25 (0.05-1.13)), and atopic sensitization (aOR=0.56 (0.25-1.27)) even when no farm milk was consumed.[25] Specifically, early-life contact to cows and staying in their stables was associated with reduced asthma (aOR=0.74 (0.62-0.89); aOR=0.79 (0.65-0.95)), hay fever (aOR=0.52 (0.41-0.66); aOR=0.66 (0.52-0.85)) and atopic sensitization (aOR=0.75 (0.65-0.88); aOR=0.78 (0.67-0.92)).[46] The protective effect of components of farm dust extracts collected from stables on the development of allergic airway inflammation was also demonstrated in a murine model with reduced airway hyperresponsiveness and eosinophilia upon intranasal application of the polysaccharide arabinogalactans isolated from farm dust extracts.[48] In addition, also dust extracts from farmers' homes were shown to contribute to asthma protective mechanisms. While intranasal application of house dust extracts from the Hutterite, which were associated with modernized farming lifestyles and higher childhood asthma prevalence[30], led to induction of eosinophilia and airway hyperresponsiveness, Amish house dust extracts inhibited these asthma features in a mouse asthma model.[30] In addition, it has shown that increased similarity in the composition of the bacterial microbiome of indoor dust from non-farming families to those from farm-homes is associated with increased asthma protection.[49]

In the search of the specific protective components contained in farming environments, endotoxin emerged as an important component, and its beneficial immune-modulating role was demonstrated in several studies.[29,50,51] Endotoxin, also lipopolysaccharide (LPS), is a cell wall component of gram-negative bacteria and occurs frequently in farming environments. Although LPS reflects, in general, a pro-inflammatory stimulus triggering inflammatory signaling cascades by binding to the toll-like receptor 4 (TLR4) on the cell surface, repetitive and constant LPS exposure induces an "adaptive tolerance mechanism" called endotoxin tolerance.[50] By mitigated response

to LPS and the suppression of inflammation, the immune system controls harmful effects of constant inflammatory conditions including tissue destruction.[50] Braun-Fahrländer et al. demonstrated a strong inverse association of endotoxin load in the children's mattresses and the development of asthma (aOR=0.48 (0.28-0.81)) and atopic sensitization (aOR=0.76 (0.58-0.98)).[29] This effect was even observable in children from non-farming households (asthma: aOR=0.52 (0.25-1.07); allergic sensitization: aOR=0.73 (0.51-1.04)).[29] The low prevalence of childhood asthma within the Amish population was also at least partially contributed to endotoxin exposure, as they show 6.8 times higher levels of endotoxin in their house dust samples compared to those of the Hutterites.[30] However, not only LPS but also components of gram-positive bacteria like Peptidoglycan as well as mycoplasma, and hypomethylated CpG motifs within bacterial DNA were associated with reduced risk for allergy and/or asthma.[26,52–54]

2.3 Immunological mechanisms underlying the farm effect

2.3.1 The role of innate immune responses and inflammation in environment-mediated asthma protection

The immune system recognizes microbes by binding so-called pathogen-associated microbial patterns (PAMPs) via pathogen recognition receptors (PRRs) that are expressed on the surface of cells of the innate immune system. The most studied PRRs are the class of toll-like receptors (TLRs). The impact of TLR-signaling in the development and protection against allergic diseases has been demonstrated in several studies.[55] TLR4 polymorphisms have been associated with increased asthma and allergy severity.[56] In addition, increased expression of TLR4, -5, and -6 of children consuming farm milk in the first year of life underline the role of TLR signaling as demonstrated in the multicenter European birth cohort study PASTURE/EFRAIM.[57,58]

Upon TLR-ligand binding, antigen-presenting cells (APC) express different kinds of co-stimulatory molecules, cytokines, and induce specific signaling cascades including the inflammatory NF-κB and the connected MAPK signaling pathway. Under unstimulated conditions, NF-κB is held in check by binding of IκBα and IκBβ resulting in cytosolic continuance of the transcription factor (TF).[59] Upon stimulation of TLR4, the adaptor protein MyD88 triggers the activation of the kinase IRAK1/4 which subsequently activates the E3 ubiquitin ligase TRAF6, which ubiquitinylates and thereby activates the downstream target TAK1. Consequently, the IKK complex gets activated via phosphorylation by TAK1 resulting in ubiquitin-dependent proteasomal degradation of the inhibitory regulator IκBα.[60] The released TF NF-κB can enter the nucleus and bind specific promoter regions of pro-inflammatory genes, and thereby trigger inflammatory gene expression.[61] The NF-κB pathway is regulated at multiple levels by complex phosphorylation and ubiquitination processes and anti-inflammatory regulators like IκBα or TNFAIP3.[62] The gene product of TNFAIP3, known as A20, is an ubiquitin-editing enzyme that restricts pro-inflammatory signaling by removing ubiquitin chains of important targets within the NF-κB signaling pathway including TRAF6.[63] Two binding partners, TNIP1/2 and TAX1BP1 are involved in exerting the anti-inflammatory effect

of A20 by recruiting the E3 ubiquitin ligase to specific substrates.[63] TNFAIP3 itself is regulated at multiple levels including cleavage by the para-caspase MALT1.[64]

Exposure to farming environments might also influence the regulation of inflammatory pathways. In fact, significant differences in the expression of innate genes of the NF-κB signaling pathway were described between the Hutterite and the Amish people.[30] Moreover, the asthma-protective effect of farm dust exposure was shown to be at least partially regulated via innate signaling pathways since the protective effect of intranasal application of the Amish home dust as shown by attenuated airway hyperresponsiveness and eosinophilia was diminished when mice were deficient for MyD88 and TRIF.[30] In another mouse model, the protective effect of LPS was demonstrated to be dependent on TNFAIP3, since TNFAIP3-deficient mice were not able to induce LPS-mediated suppression of asthma features as shown in wildtype mice.[65] Association of polymorphisms of TNFAIP3 with asthma risk in children of the GABRIELA study further emphasized the role of this negative regulator of NF-κB signaling in environment-mediated asthma protection.[65] Furthermore, TNFAIP3 was significantly less expressed in the Hutterite compared to the "asthma-protected" Amish children.[30]

In addition, ligation of LPS to TLR4 induces the highly connected MAPK signaling pathway.[66] Upon stimulation, a cascade of serine/threonine kinases is set in motion accumulating in the phosphorylation of the central MAP kinases MAPK1/2 (ERK1/2), MAPK8/9 (JNK/JNK2), and MAPK11/14 (p38-2/p38). To inhibit excessive pro-inflammatory signaling, the MAPK pathway has to be tightly regulated at various levels. The pro-inflammatory regulators can either be directly targeted by post-translational modifications or by anti-inflammatory regulators like MAPK phosphatases.[67,68] The dual-specificity phosphatase 1 (DUSP1, also MKP-1) is the most studied MAPK phosphatase that inhibits ERK1/2, JNK, and p38 through dephosphorylation of the threonine and tyrosine residues within their MAPK activation motif.[69] Since phosphorylation is required for MAPK activation, phosphatases sufficiently deactivate their substrates.

Both pathways were highly associated with asthma and allergy development.[70,71] In mouse models, the importance of NF-κB in the development of allergic airway diseases was shown as mice deficient in one of the NF-κB subunits were protected from eosinophilic airway inflammation.[72,73] In line, in the airway tissue and peripheral blood cells of asthmatics, increased NF-κB translocation into the nucleus and binding to DNA promoters have been demonstrated.[74,75] Moreover, a higher level of phosphorylated and degraded IκBα and the IKK complex was observed in the airways of asthmatics.[74,75] Inhibition of NF-κB signaling by glucocorticoids that are effectively used in asthma therapy further suggests the role of the pro-inflammatory TF in the immunopathological mechanisms of the disease.[76] Also the pro-inflammatory MAPK signaling pathway was shown to play a role in the development of asthma. Increased levels of phosphorylated ERK1/2 (pERK1/2) and p38 (pp38) were observed when lung tissue and epithelial cells of the bronchi of asthmatic adults were investigated.[77,78] Induced pp38 expression levels upon allergen-challenge further indicating the role of this pathway in the pathophysiology of asthma.[79] Since glucocorticoids applied in asthma therapy exert their anti-inflammatory effect by elevated acetylation of histone H4 in the

glucocorticoid response elements region of the DUSP1 promoter, this negative regulator is also in the focus of asthma research.[80,81]

One major group of APC are dendritic cells (DC), which are also associated with asthma development.[82,83] Based on their developmental origin and their function, DC can be classified into plasmacytoid (pDC) and myeloid (mDC, also called conventional cDC) DC, while the latter can again be subdivided into mDC1 and mDC2.[84] In the context of allergy, pDC are associated with tolerance induction and Treg control, while mDC are linked to the development of Th2-driven allergic response.[83] Not only lung DC but also circulating DC are associated with allergic diseases including asthma.[85–87] Increased mDC2 numbers within peripheral blood of allergic asthmatics and their induction upon allergen challenge indicate their role in Th2-promoting allergic inflammation.[87,88] Moreover, modulated levels of DC-associated co-stimulatory molecules like ILT4 and CD86 molecules were demonstrated in asthmatic patients and asthmatics with acute exacerbation, respectively.[89,90] Besides their role in asthma development, DC have also been associated with immunological mechanisms upon farm exposure. For instance, children growing up on farms had significantly lower numbers of mDC2 compared to non-farm children.[89] Within the TRILATERAL study, a multicenter cohort including children from European farms (in Germany and Finland as an extended subgroup of the PASTURE cohort) and rural China, an increase of cells expressing the co-stimulatory molecule CD80 upon farm dust stimulation of PBMCs as reported in the Finnish population.[91] *In vitro* experiments in mice have shown that stimulation with farm dust extracts is followed by a reduced capacity of DC to induce allergic immune responses[92] and suppressed differentiation of human monocyte-derived DC (moDC).[93] Stimulation of DC with CpG oligodeoxynucleotides that mimics the microbial CpG DNA leads to DC-mediated activation of Tregs.[94] Moreover, endotoxin stimulation before HDM sensitization has been shown to inhibit Th2-response and IgE production by suppressing the migration of DC to the lymph node.[95]

2.3.2 The involvement of adaptive T cell responses in asthma protective mechanisms conferred by farming exposure

Upon activation, APC communicate with adaptive T cells by the interaction of the antigen-presenting MHC complex with the T cell receptor as well as via co-stimulatory molecules and specific cytokines. The interaction of co-stimulatory molecules expressed on APC and T cells can either result in stimulation or inhibition of the subsequent T cell response, dependent on the receptors and ligands. While ligation of the T cell surface protein CD28 results in effective activation of T cells, CTLA-4 (CD152) mediated T cell inhibition.[96,97] Also the interaction of CD274 (PD-L1) on APC with CD279 (PD-1) on the T cell surface mediates inhibitory signals.[98] CD80 (B7-1) and CD86 (B7-2), expressed on APC can even function in both directions depending on their T cell ligand.[99] Upon specific stimulation, naïve T-helper cells differentiate into certain subtypes including Th1, Th2, Tregs, Th9, Th17, Th22, or Tfh cells that exert different functions. While Type 1 T-helper cells (Th1) secrete high levels of Interferon gamma (IFNγ) and IL-2 and are characterized

by expression of the transcription factors (TF) T-bet and Hlx, Th2 cells, that are associated with allergic immune response, produce mainly IL-4, IL-5, and IL-13 and express the TF GATA3 and STAT6.[100–103] Moreover, also Th17 cells are associated with the pathophysiology of asthma, especially with severe forms and neutrophilic phenotypes.[104] Regulatory T cells (Treg) characterized by high CD25 expression and intracellular expression of the TF FOXP3 are highly important in maintaining an immunological balance and avoidance of harmful excessive inflammation due to their capacity to suppress immunity to specific pathogens and maintain tolerance to self-antigens.[105,106] While some studies report decreased Treg numbers in asthmatic children[107,108], higher Treg numbers were demonstrated for children with manifest allergic asthma in the cross-sectional CLARA/CLAUS cohort.[109] Increased Treg numbers are potentially induced through counterregulatory processes necessary to control the excessive inflammation during the allergic response.[109] Besides the differences in Treg numbers, also differences in their functionality were shown to be associated with asthma. Already at birth, newborns of allergic mothers had not only lower Treg numbers but this cell type had also lower suppressive capacity compared to offspring of non-atopic mothers as shown in the longitudinal PAULINA/PAULCHEN cohort including healthy newborns from Munich city and the surrounding area and rural Germany.[110] Moreover, a decreased functionality of Treg from children with allergic asthma was demonstrated, as assessed by their suppression of Teff proliferation.[111] Also, the effect of exposure to microbial substances on the immune system is not limited on innate cells, but is also associated with modulated adaptive T cell responses. While the pathophysiology of asthma is iter alia associated with a Th2-shifted Th1/Th2 dysbalance, endotoxin exposure was associated with reduced Th2-driven immune response in both mouse and human models.[65] Children with higher endotoxin levels in their homes had higher levels of IFNγ-positive T-helper cells upon mitogen stimulation.[112] The Th1-associated cytokines IFNγ and TNFα levels were consistently shown to be increased in the supernatants of mitogen-stimulated cord blood cells of farm children.[113] The Th1-promoting features of LPS were also shown in human DC upon stimulation with gram-negative *Acinetobacter lwoffii*.[114] This bacterial species, isolated from cowsheds has been shown to prevent the development of murine allergic inflammation.[114] Also Treg that interfere in the homeostasis between Th1 and Th2 immune responses have been associated with the farm effect in several studies. Newborns that were exposed to a farming environment *in utero* had elevated Treg numbers in their cord blood.[115] However, a specific time window of Treg-mediated asthma-protection via farm environments is suggested.[116] While at year 4.5, farm milk and stable exposure were positively associated with Treg numbers upon LPS stimulation and reduced risk of childhood asthma with an aOR of 0.26 (0.08-0.88), the opposite was demonstrated at age 6 years (aOR=11.29 (0.96-132.28)).[116,117] Farm-associated features like farm milk consumption, farm exposure during pregnancy, and elevated duration of breastfeeding have been linked with increased Treg levels.[115,117,118] Microbial components have been shown to promote the generation and the suppressive capacity of Tregs. Not only LPS but also CpG DNA has been demonstrated to induce the development of Tregs.[94,119] Moreover, the SCFA butyrate, a main bacterial metabolite that is associated with asthma protection[120] has been shown to promote the generation of Tregs.[121]

2.4 Objectives

Due to the high burden of childhood asthma and the side effects of currently used treatment options, preventive strategies and safe treatment alternatives are urgently needed. While the protective effect of rural and farming environments has been demonstrated epidemiologically, its immunological mechanisms and potential application for clinical asthma prevention and treatment are central and current focus of intense research.

This thesis aimed to **investigate underlying immunological mechanisms focusing on inflammatory regulation and the role of innate and adaptive immune responses of farm dust-mediated asthma protection.**

Specifically, the regulation of pro- and anti-inflammatory processes induced by microbial exposure and their relevance for asthma development was examined focusing on the inflammatory NF-κB and MAPK signaling pathways with TNFAIP3 and DUSP1 as their central anti-inflammatory regulators. Moreover, the effect of *ex vivo* farm dust stimulation on immunological regulation was investigated in a broader context by examining the contribution of innate and adaptive immune responses in environment-mediated asthma protection. Focus was placed on innate dendritic cells (DC) and adaptive T cell subpopulations on molecular, cellular, and functional levels using a broad spectrum of methods. For this purpose, in-depth characterized children of the following two cross-sectional asthma cohorts and two longitudinal birth cohort studies were included:

CLARA/CLAUS is a German cross-sectional asthma cohort including 4-17-year-old children with mild-to-moderate asthma and healthy controls.[109,122–124] In the European, multicenter prospective birth cohort study PASTURE/EFRAIM, pregnant women and their children from rural areas were recruited and follow-ups were conducted.[25,58,123] The international cross-sectional TRILATERAL study comprised children from three countries: Germany and Finland - as an extension of the PASTURE study – and China. In this thesis, the Chinese part of the TRILATERAL cohort was included, that comprised 7-year-old healthy and asthmatic children from urban Hong Kong and rural Conghua in China.[123,124] The PAULINA/PAULCHEN cohort is a German longitudinal birth cohort study including healthy newborns from Munich and surrounding areas and rural Germany.[110,123–126]

In this thesis, the following approaches were pursued:

a) Differences in gene and protein expression of NF-κB and MAPK signaling genes and co-stimulatory molecules were measured by qPCR and Western Blotting in peripheral blood mononuclear cells (PBMCs) of healthy (HC) and allergic asthmatic (AA) school-age children in two distinct populations (CLARA/CLAUS**,** TRILATERAL) at asthma manifestation and b) at birth, by analyzing gene expression in cord blood of healthy newborns with consideration of their subsequent asthma development (PAULINA/PAULCHEN). c) The functional stimulatory effect of farm dust on inflammatory regulation was investigated by *ex vivo* stimulation of PBMCs with dust extracts from asthma-protected environmental areas around the world. d) The effects of *in vivo* farming exposure on inflammatory gene expression were examined in 6-year-old children with and without *in*

vivo farming exposure of the international PASTURE/EFRAIM cohort. e) Cell-type-specific detailed regulation in AA versus HC following dust stimulation was examined in isolated DC and Treg and on single-cell level using state-of-the-art technologies including the multiplex gene expression technology NanoString and mass cytometry (Cytometry by time of flight, CyTOF). f) Additional detailed functional analyses included farm dust stimulation of monocyte-derived DC (moDC), kinetic experiments, LPS-blocking, and Treg suppression assays.

Using this experimental setup, **the following research aims were pursued:**

1.) To identify a differential **inflammatory balance** regarding NF-κB and MAPK signaling at baseline
    a. in peripheral blood of school-age children with **manifest AA** compared to HC in two cross-sectional asthma cohorts (CLARA/CLAUS, TRILATERAL)
    b. **at birth,** comparing gene expression in cord blood of healthy newborns with subsequent asthma development in a longitudinal birth cohort (PAULINA/PAULCHEN).
2.) To examine, if **farm dust exposure** *in vivo* and *ex vivo* can modulate or even counteract a dysbalanced inflammatory regulation of AA by comparing immune responses of children from three different pediatric cohorts (CLARA/CLAUS, TRILATERAL, PASTURE/EFRAIM).
3.) To disentangle the detailed role of **innate dendritic cells** for environment-mediated childhood asthma protection following *ex vivo* farm dust stimulation on gene/protein, cellular and functional level.
4.) To investigate the effects of *ex vivo* farm dust stimulation on **adaptive immune responses** for childhood asthma development, including inflammatory and asthma-associated gene/protein expression, cellular composition, and activation of T cell subpopulations.

2.5 Summary

In my thesis, baseline regulation was investigated in peripheral blood of children with allergic asthma (AA) and healthy controls (HC) at birth in cord blood and at asthma manifestation including four pediatric cohorts (1.). Moreover, the effects of *in vivo* and/or *ex vivo* farm dust stimulation on immunological mechanisms including inflammatory regulation (2.), innate immune responses of dendritic cells (DC) (3.), and subsequent T cell responses (4.) were investigated.

An elevated inflammatory baseline status regarding NF-κB and MAPK signaling was demonstrated for AA at manifestation and even before asthma development at birth. Children with *in vivo* farm exposure demonstrated a hyporesponsive immune state with low expression of both pro- and anti-inflammatory genes. Anti-inflammatory regulation upon *ex vivo* stimulation with dust extracts from asthma-protective farming environments and LPS was consistently shown in peripheral blood cells of HC and AA on gene and confirmed on protein levels. Moreover, detailed functional analyses revealed the farm dust-mediated induction of immunological tolerance mechanisms demonstrated by the generation of a more suppressive phenotype of innate DC with decreased antigen presentation, reduced T cell activation, and downregulation of allergy-associated Th2- and Th17-mediated immune responses. The main findings are summarized in a graphical abstract (Figure 1).

1.) Asthmatic children demonstrated increased inflammation at disease manifestation and even already at birth

School-age children with manifest allergic asthma demonstrated higher baseline inflammation as shown by higher pro-inflammatory TLR4 expression and lower levels of TNFAIP3 - the anti-inflammatory negative regulator of the NF-κB pathway. This was identified in the German cross-sectional CLARA/CLAUS cohort and replicated in the Chinese arm of the TRILATERAL cohort. In line, DUSP1, negatively regulating of the highly connected pro-inflammatory MAPK signaling pathway, was significantly less expressed in manifest allergic asthmatic children. TNFAIP3 was even significantly lower expressed at birth in healthy newborns that developed asthma until 10 years of age compared to those who stayed healthy.

2.) Dysbalanced inflammatory regulation of children with allergic asthma could be counteracted by *ex vivo* farm dust stimulation

When peripheral blood cells of AA and HC were stimulated *ex vivo* with dust extract collected in several farming environments around the world and LPS, **anti-inflammatory regulation** was revealed as assessed by expression of genes of the pro-inflammatory NF-κB and MAPK signaling pathways. While stimulation of PBMCs with farm dust or LPS **downregulated pro-inflammatory targets** of the pathways including *TLR4* and phosphorylated MAPKs in both HC and AA, the **anti-inflammatory regulators *TNFAIP3* and *DUSP1* were significantly upregulated**. These anti-inflammatory stimulatory effects of farm dust were not only attributable to its major component LPS - the cell-wall component of gram-negative bacteria - but went beyond the effects of endo-

toxin shown by LPS blocking experiments. Children growing up on farms with *in vivo* farm exposure demonstrated a hyporesponsive immune status with reduced gene expression of all investigated genes but induced anti-inflammatory regulation upon acute LPS stimulation.

3.) Farm dust stimulation affected innate immune regulation by induction of a suppressive DC phenotype and reduced antigen presentation

On molecular expression level, anti-inflammatory regulation of farm dust stimulation was especially demonstrated in isolated DC and monocyte-derived DC. In addition, the T cell activating capacity of DC was suppressed as shown by the upregulation of co-stimulatory molecules that are associated with inhibitory immune responses like CD274 and downregulation of activating molecules like CD86. On cellular level, the numbers of antigen-presenting DC and monocytes were significantly reduced following farm dust stimulation in both HC and AA analyzed by mass cytometry via manual gating and verified by semi-supervised clustering. Characteristics of antigen presentation were reduced as identified by downregulation of the MHC class II surface receptor HLA-DR, the specific reduction of mDC, a subtype of DC associated with antigen presentation, and a shift towards rather tolerogenic pDC.

4.) Farm dust stimulation modulates adaptive T cell responses with reduced T cell activation and downregulation of Th2/Th17-associated immune response

Anti-inflammatory regulation of MAPK signaling following farm dust stimulation was not only disentangled in innate immune cells but also in several T cell subpopulations. Reduced T cell activation was also confirmed by single-cell mass cytometry by upregulation of inhibitory co-stimulatory molecules and downregulation of activating receptors like CD28 on the surface of T cells. Moreover, the transcription factors GATA3 and RORγ were both downregulated upon farm dust stimulation in T cells of AA pointing to the downregulation of allergy-associated Th2 and Th17 immunity. On cellular level, numbers of effector memory T cell populations were induced following farm dust stimulation while central memory phenotypes were reduced. Moreover, stimulation with farm dust extracts resulted in reduced proliferation of effector T cells independent of Tregs demonstrated by in-depth functional experiments.

Conclusion:

Children with manifest allergic asthma demonstrated elevated baseline inflammation with reduced expression of the anti-inflammatory key players of NF-κB and MAPK signaling - TNFAIP3 and DUSP1. *Ex vivo* stimulation with farm dust extracts could counteract this inflammatory dysbalance by induction of anti-inflammatory regulation. Moreover, farm dust stimulation resulted in the induction of a suppressive, tolerogenic DC phenotype characterized by anti-inflammatory and T cell inhibitory signaling and reduced antigen presentation. Also, the adaptive arm of the immune system was affected by farm dust stimulation, demonstrated by decreased T cell activation and allergy-associated Th2- and Th17-associated immune responses. These anti-allergic properties may suggest the use of farm dust components as an auspicious approach for preventive strategies and potentially for long-term treatment options against childhood asthma.

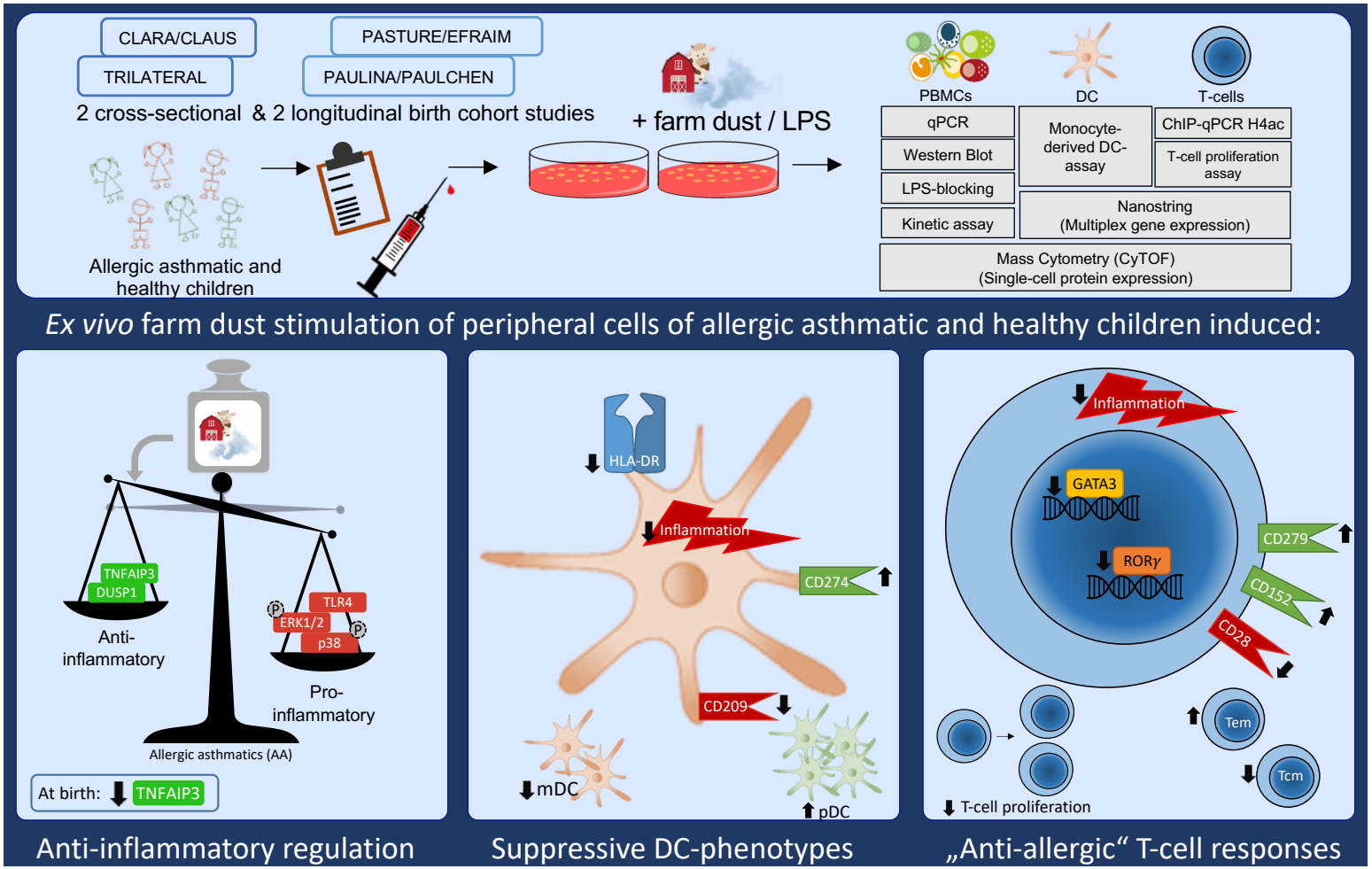

Figure 1: Graphical abstract summarizing the effects of *ex vivo* farm dust stimulation of peripheral blood cells of allergic asthmatic and healthy children on inflammatory regulation, DC phenotypes and T cell responses

3. Paper I: TNFAIP3 is a key player in childhood asthma development and environment-mediated protection

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

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Journal of Allergy and Clinical Immunology (IF: 14.110), 144(6), 1684-1696.e12, 2019, doi:10.1016/j.jaci.2019.07.0

Environmental and occupational disease

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

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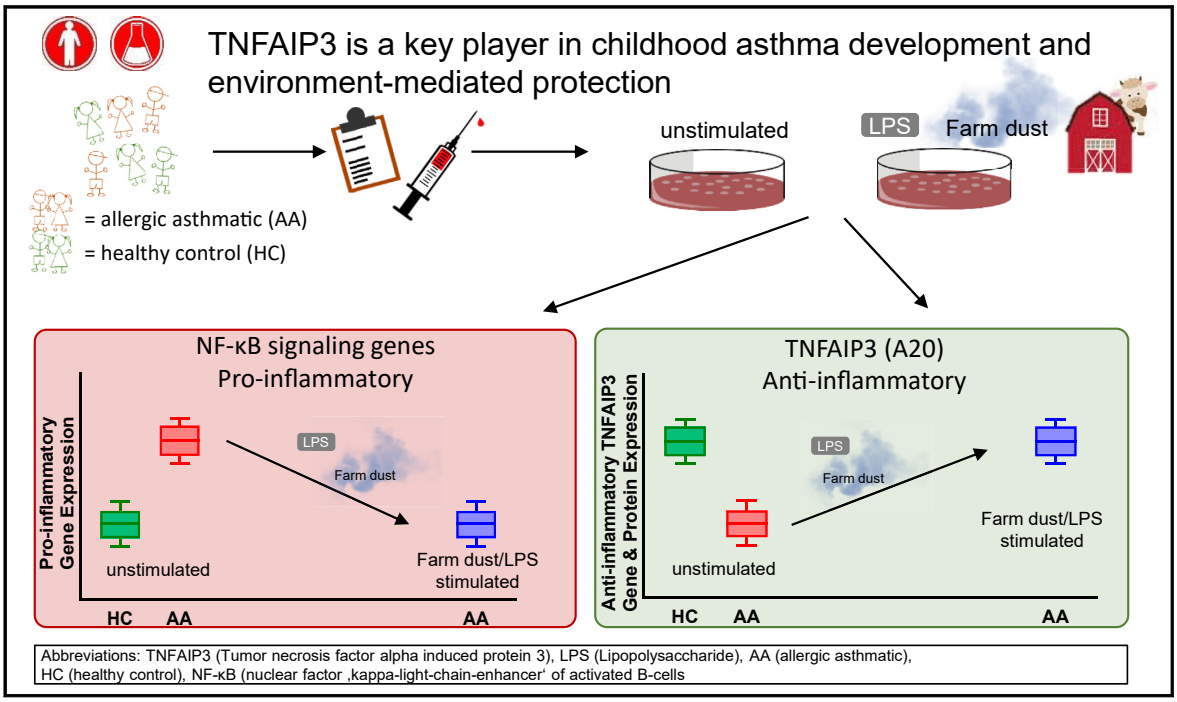
GRAPHICAL ABSTRACT



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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;144:1684-96.)

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

This work was supported by EFRAIM EU FP7- (grants KBBE-2007-1, DFG SCHA 997/3-1 [to B.S. and P.C.S.], DFG-SCHA 997/7-1 [to B.S., M.T., and J.K.], SFB TR22, and DFG-SCHA 997/8-1 [to B.S.]); the Comprehensive Pneumology Center (to B.S.); FöFoLe (to K.R.); EKFS (to B.S. and A.B.); the Academy of Finland (to M.R.); CK-CARE/Kühne Foundation, Davos, Switzerland (to R.L. and C.R.); PHRC IR University Hospital of Besancon, France (to J.-C.D.); and the International (Regional) Cooperation and Exchange Program (Cooperation Research-NSFC-AF-DFG) by the National Natural Science Foundation of China (grant 81261130023 [to J.L.]).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication February 15, 2019; revised July 16, 2019; accepted for publication July 17, 2019.

Available online August 2, 2019.

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0091-6749/$36.00

https://doi.org/10.1016/j.jaci.2019.07.029

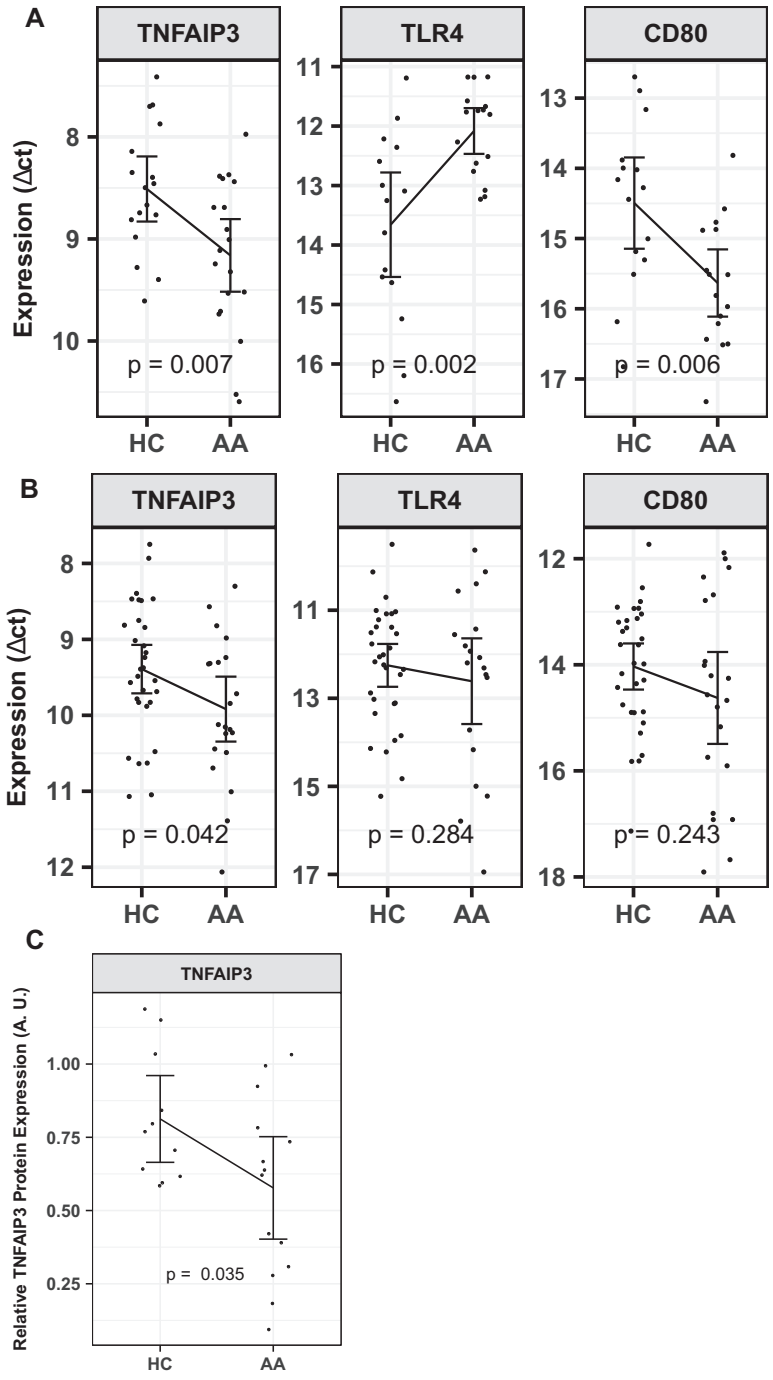

FIG 1. Urban asthmatic children express less *TNFAIP3*. Scatter plots for *TNFAIP3, TLR4,* and *CD80* gene (Fig 1, *A* and *B*) and TNFAIP3 protein (Fig 1, *C*) expression are shown. Data are stratified for phenotypes, HCs, and children with AA. Raw data values are represented by *horizontally jittered 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

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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

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.

(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 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.

On LPS stimulation, asthmatic patients from urban Germany and China reached *TNFAIP3* expression comparable with that of

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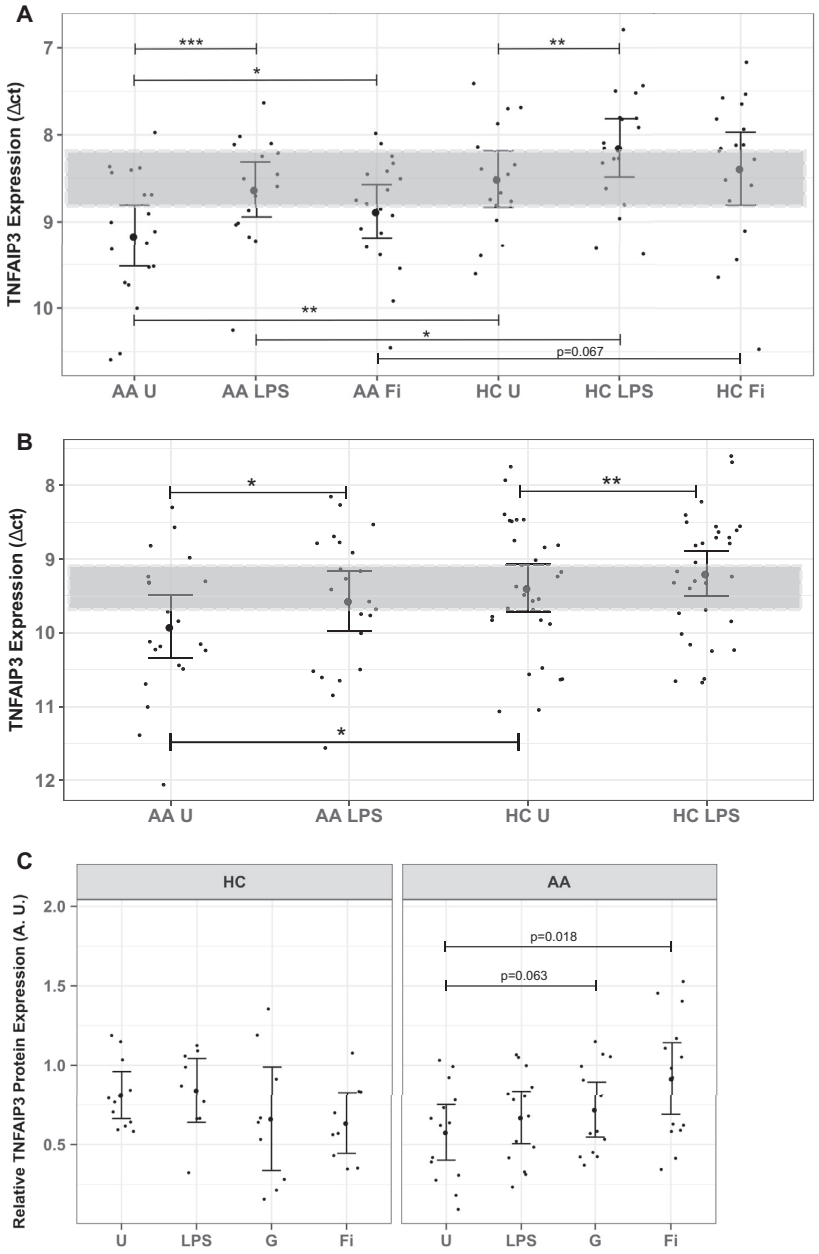
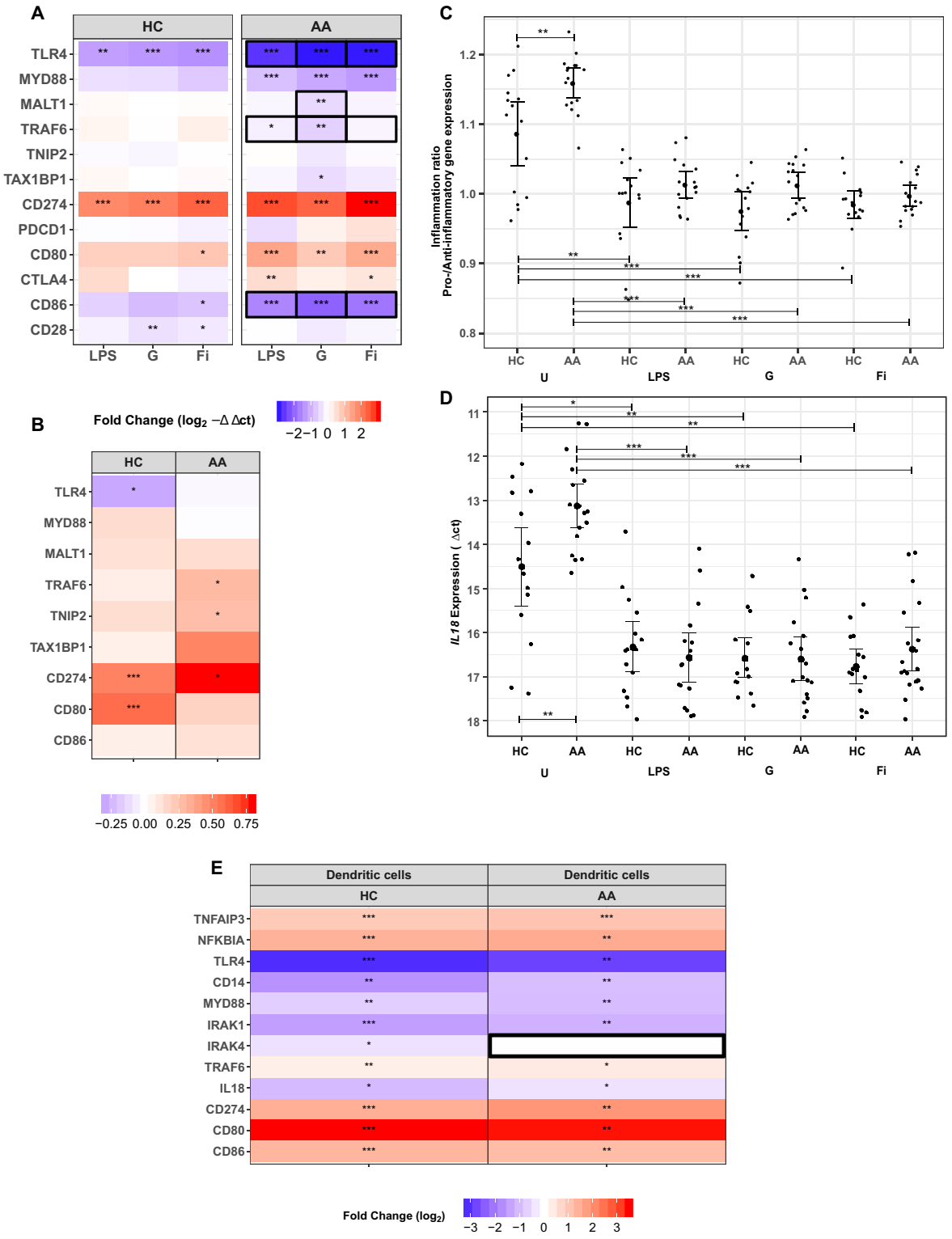


FIG 2. LPS and farm dust stimulation increase TNFAIP3 gene and protein expression in asthmatic children. Effect of LPS and German *(G)* and Finnish *(Fi)* farm dust stimulation compared with unstimulated *(U)* conditions are shown as scatter plots. Data are stratified for phenotypes. Raw data values 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 $***P \leq .001$.


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
−0.25 0.00 0.25 0.50 0.75
C
Inflammation ratio
Pro-/Anti-inflammatory gene expression
U
LPS
G
Fi
D
IL18 Expression (Δct)
E
Dendritic cells
TNFAIP3
NFKBIA
TLR4
CD14
MYD88
IRAK1
IRAK4
TRAF6
IL18
CD274
CD80
CD86
Fold Change (log2)
−3 −2 −1 0 1 2 3

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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

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.

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*), 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 *ex 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 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

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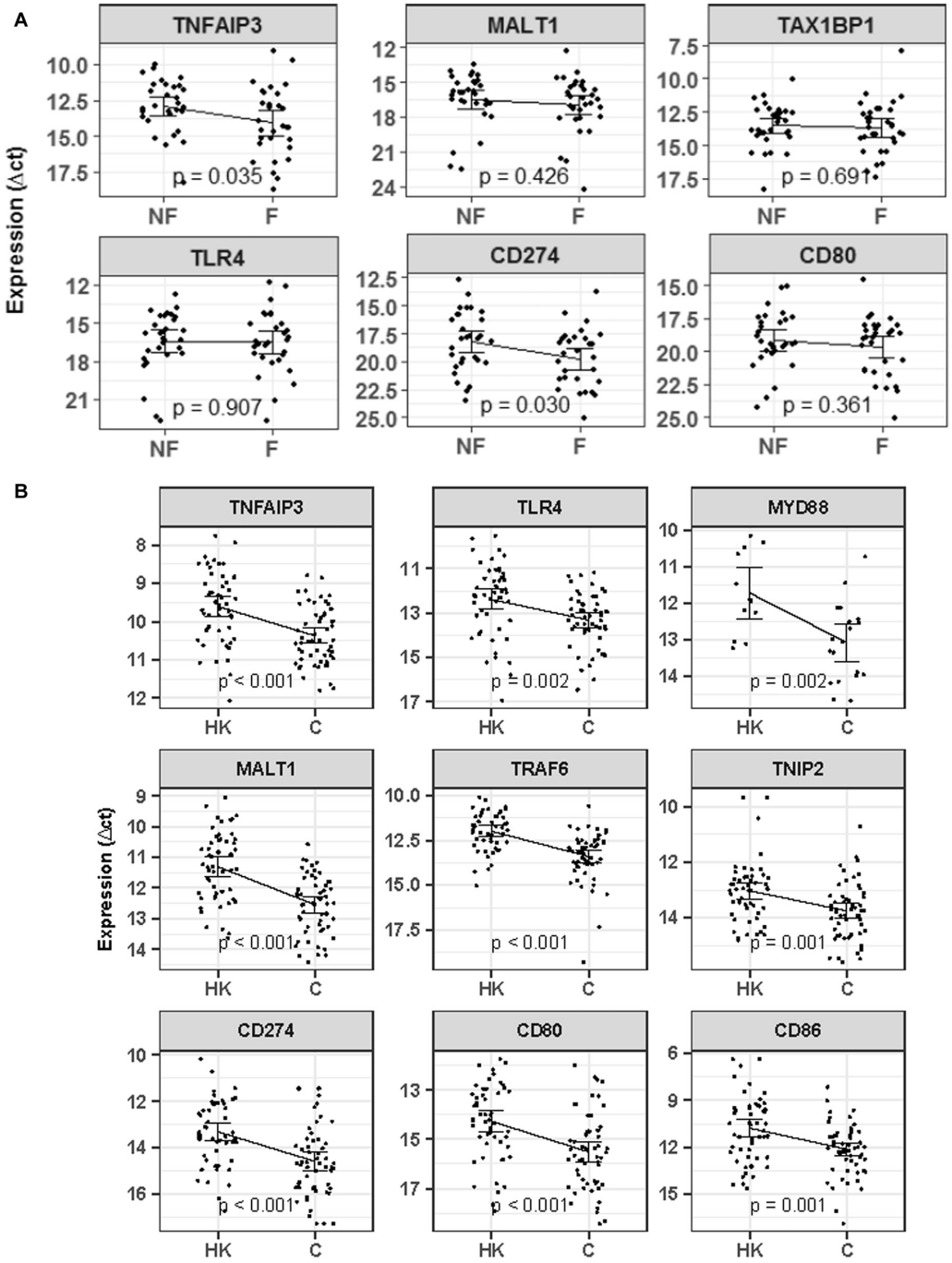

FIG 4. Farm/rural children show lower NF-κB signaling gene expression. Scatter plots of gene expression (ΔCT) were analyzed by using quantitative PCR after 24 hours of cultivation of unstimulated leukocytes of 6-year-old farm (*F*; n = 32) and nonfarm (*NF*; n = 31) healthy and asthmatic children from 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.

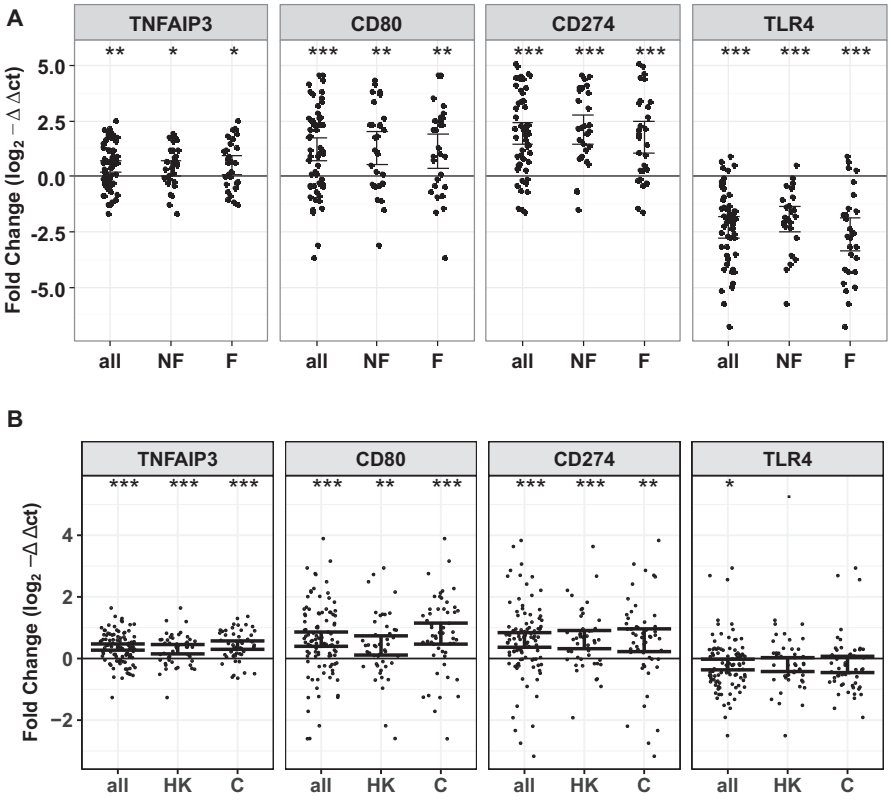

FIG 5. LPS stimulation in farm/rural children results in upregulation of *TNFAIP3, CD80,* and *CD274* and downregulation of *TLR4* compared with unstimulated blood cells. Fold change in gene expression was analyzed by using quantitative PCR after 24 hours of cultivation of unstimulated and LPS-stimulated 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 $***P \leq .001$.

proinflammatory *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, 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.

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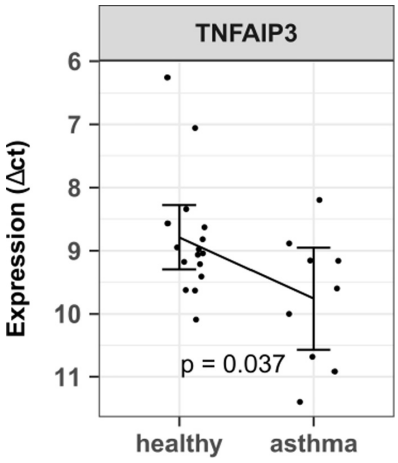

FIG 6. Newborns with subsequent asthma express less *TNFAIP3*. Scatter plots of gene expression (ΔCT) analyzed by using quantitative PCR after 72 hours of cultivation of unstimulated CBMCs from the PAULINA/PAULCHEN cohort are shown. Data are stratified for phenotype (n = 25 [16 HCs and 9 asthmatic patients]). *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.

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 $\pm$ 0.76) compared with children with asthma alone (ΔCT, 8.74 $\pm$ 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.

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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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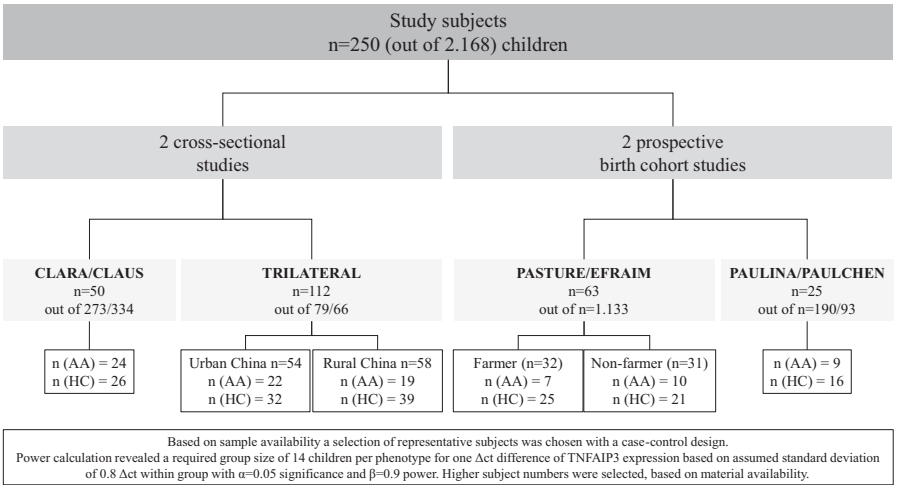

FIG E1. Flow diagram of study subjects. Flow diagram of all subjects included in this study comprising 4 pediatric cohorts, 2 cross-sectional studies (CLARA/CLAUS and TRILATERAL), and 2 birth cohort studies (PASTURE/EFRAIM and PAULINA/PAULCHEN), with a total of 250 of 2168 children.

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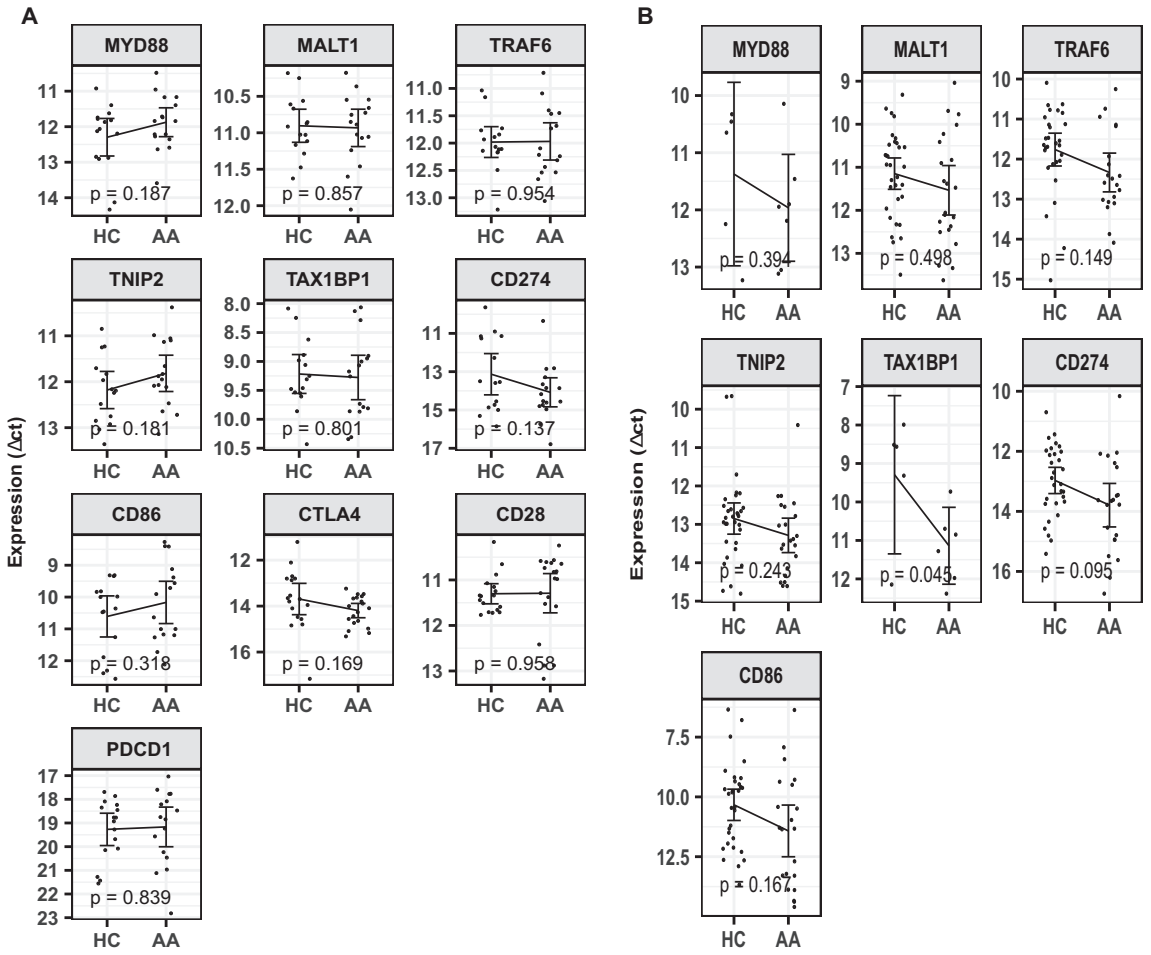

FIG E2. Gene expression of *MYD88, MALT1, TRAF6, TNIP2, TAX1BP1*, CD274, *CD86, CD28, CTLA4*, and *PDCD1* between healthy and asthmatic children under unstimulated conditions. Scatter plots for gene expression of *MYD88, MALT1, TRAF6, TNIP2, TAX1BP1, CD274*, and *CD86* (Fig E2, *A* and *B*) and *CD28, 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**).

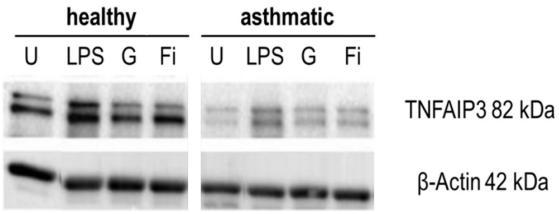

FIG E3. Representative Western blot. One representative image of TNFAIP3 Western blot analysis with β-actin as a loading control (*left*, healthy children; *right*, asthmatic children right) *U*, unstimulated; *G* and *Fi*, and after stimulation with LPS or farm dust (German and Finnish, respectively).

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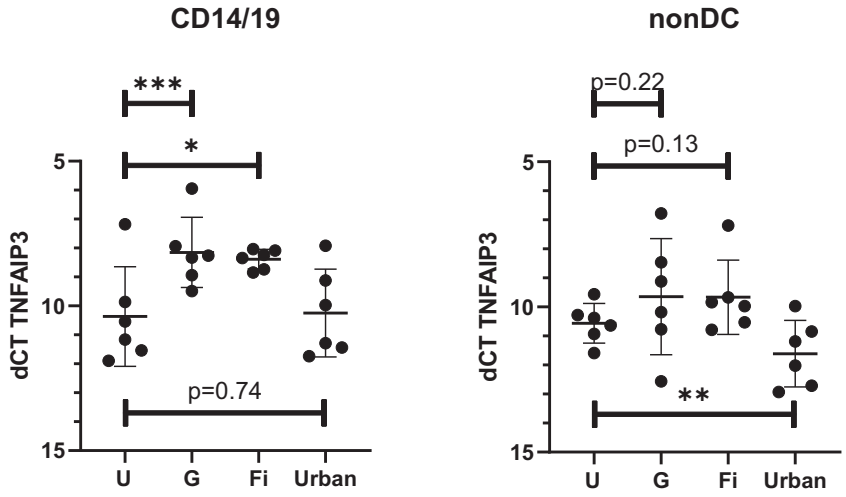

FIG E4. Farm dust stimulation upregulates. *TNFAIP3* gene expression with urban dust stimulation results in consistent or even downregulated TNFAIP3 levels. Scatter plots of gene expression (ΔCT) of *TNFAIP3* in isolated CD14/CD19 cells and non-DCs analyzed by using quantitative PCR after 24 hours of 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.

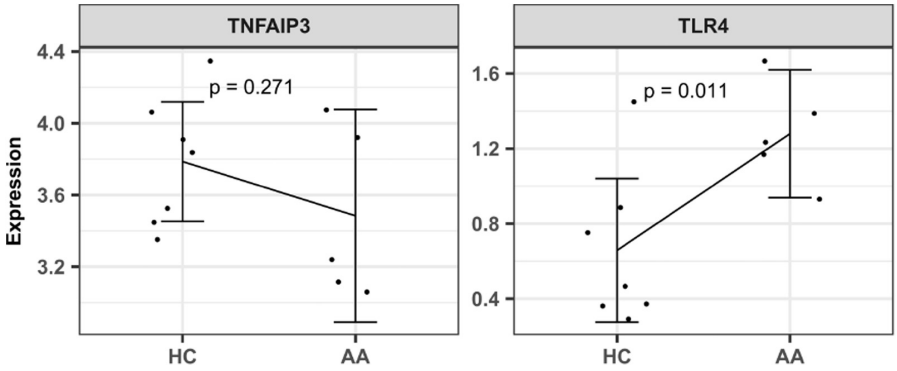

FIG E5. DCs of asthmatic patients express greater levels of TLR4 under unstimulated conditions. Scatter plots of gene expression of *TNFAIP3* and *TLR4* in isolated DCs analyzed by using NanoString technology. DCs were isolated by using magnetic cell separation after 24 hours of cultivation of unstimulated 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.

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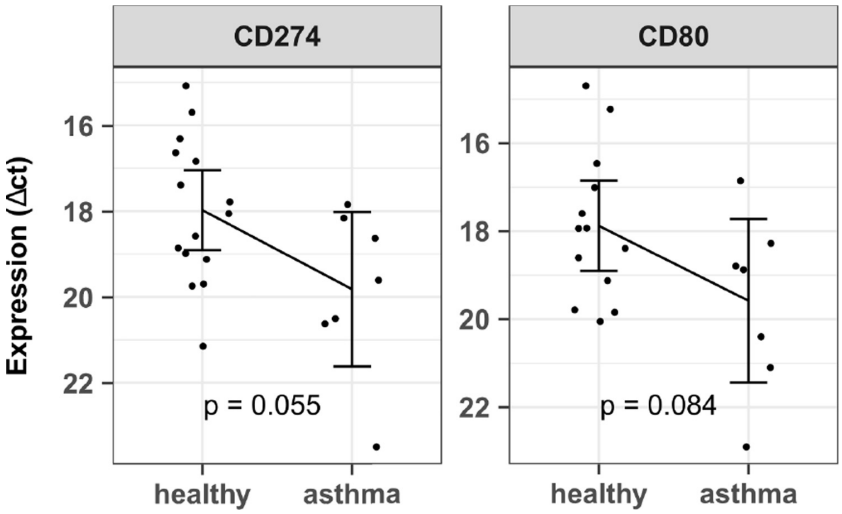

FIG E6. Newborns with subsequent asthma express less CD274 and CD80. Scatter plots of gene expression (ΔCT) of *CD274* and *CD80* analyzed by using quantitative PCR after 72 hours of cultivation of unstimulated CBMCs from the PAULINA/PAULCHEN cohort. Data are stratified for phenotype (n = 20-22 [13-15 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

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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.

4. Paper II: Mitogen-activated protein kinase signaling in childhood asthma development and environment-mediated protection

Mitogen-activated protein kinase signaling in childhood asthma development and environment-mediated protection

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Pediatric Allergy and Immunology (IF: 6.377), 33(1):e13657. 2022, doi: 10.1111/pai.13657. Epub 2021 Sep 14.

Received: 18 May 2021 | Revised: 14 August 2021 | Accepted: 19 August 2021

DOI: 10.1111/pai.13657

ORIGINAL ARTICLE

WILEY

Mitogen-activated protein kinase signaling in childhood asthma development and environment-mediated protection

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Funding information
E. Nowak reports grants from Förderprogramm für Forschung und Lehre Ludwig-Maximilians-Universität München, during the conduct of the study. Dr. Roponen reports grants from The Academy of Finland, during the conduct of the study; Dr. Schaub reports grants from DFG (DFG-SCHA 997/3-1, DFG-SCHA 997/7-1, DFG-SCHA 997/8-1), BMBF 01GL1742A, grants from SFB TR22, grants from Comprehensive Pneumology Center, grants from EKFS, during the conduct of the study; grants from DFG, personal fees from Consultancy, outside the submitted work.

Editor: Ömer KALAYCI

Abstract

Background: While childhood asthma prevalence is rising in Westernized countries, farm children are protected. The mitogen-activated protein kinase (MAPK) pathway with its negative regulator dual-specificity phosphatase-1 (DUSP1) is presumably associated with asthma development.

Objectives: We aimed to investigate the role of MAPK signaling in childhood asthma and its environment-mediated protection, including a representative selection of 232 out of 1062 children from two cross-sectional cohorts and one birth cohort study.

Methods: Peripheral blood mononuclear cells (PBMC) from asthmatic and healthy children were cultured upon stimulation with farm-dust extracts or lipopolysaccharide. In subgroups, gene expression was analyzed by qPCR (PBMCs, cord blood) and NanoString technology (dendritic cells). Protein expression of phosphorylated MAPKs was measured by mass cytometry. Histone acetylation was investigated by chromatin immunoprecipitation.

Results: Asthmatic children expressed significantly less *DUSP1* ($p = .006$) with reduced acetylation at histone H4 ($p = .012$) compared with healthy controls. Farm-dust stimulation upregulated *DUSP1* expression reaching healthy levels and downregulated inflammatory MAPKs on gene and protein levels (PBMCs; $p \leq .01$). Single-cell protein

Linked article: This article is commented on by Ömer Kalayci & Philippe A. Eigenmann, in this issue. To view this editorial comment visit https://onlinelibrary.wiley.com/doi/10.1111/pai.13715.

analysis revealed downregulated pMAPKs upon farm-dust stimulation in B cells, NK cells, monocytes, and T-cell subpopulations.

Conclusion: Lower *DUSP1* baseline levels in asthmatic children and anti-inflammatory regulation of MAPK in several immune cell types by farm-dust stimulation indicate a regulatory function for *DUSP1* for future therapy contributing to anti-inflammatory characteristics of farming environments.

KEYWORDS
allergy, asthma, childhood, farming, immunology, MAPK, protection

1 | INTRODUCTION

Asthma is the most common chronic childhood disease.[1] Early farming exposure is protective for asthma and other allergic diseases.[2–4] Constant exposure to microorganisms and their products like lipopolysaccharide (LPS) conveys beneficial effects by inducing tolerance mechanisms.[5] *Ex vivo* stimulation of peripheral blood mononuclear cells (PBMCs) of children with farm-dust extracts and LPS exerts anti-inflammatory capacities by upregulating anti-inflammatory TNFAIP3, while downregulating pro-inflammatory genes of the NF-κB pathway.[6] In close connection, the pro-inflammatory MAPK pathway is associated with adult asthma development.[7] Kinase cascades accumulating in the phosphorylation of central MAPKs activate transcription factors that trigger inflammation. To control harmful inflammation, feedback mechanisms control these processes via post-translational modification or induction of inhibitors like MAPK phosphatases.[8,9] Especially, the archetype dual-specificity phosphatase DUSP1 (MKP-1) is important in the complex regulation by deactivating MAPKs through dephosphorylation.[10] Glucocorticoids used in asthma therapy act via DUSP1 through enhanced H4 acetylation in the glucocorticoid response element region in the DUSP1 promoter.[11,12] However, the role of MAPK signaling in childhood asthma development and its preventive mechanisms is still unclear.

Our study aimed to identify differences in the expression of key regulators of MAPK signaling in peripheral blood cells of asthmatic and healthy children to further disentangle the immunological mechanisms underlying asthma development in childhood.

Besides the involved pathways and cell populations, multiple levels of interactions including transcriptional, translational, and post-translational regulation are involved in asthma protective mechanisms.

Therefore, we specifically investigated anti-inflammatory MAPK regulation in individual cell populations and multiple regulation levels by investigating MAPK signaling genes on mRNA, protein, and post-transcriptional, epigenetic levels including data of three international pediatric cohorts—two cross-sectional and one birth cohort study.

Key message

Our study, comprising data of three pediatric cohorts, contributes to the understanding of an inflammatory balance in childhood asthma development. We demonstrate decreased expression of DUSP1, the anti-inflammatory regulator of MAPK signaling in peripheral blood in children with manifest asthma, suggesting its potential role as a biomarker for childhood asthma development. Ex vivo farm-dust stimulation conveyed anti-inflammatory characteristics in several immune cell subsets of asthmatic and healthy children by upregulating anti-inflammatory DUSP1 and downregulating pro-inflammatory MAPK on gene and protein level. Thus, farm-dust application might be a promising therapeutic approach in future.

Synonyms

DUSP1: MKP-1; HVH1; CL100
ERK1: MAPK3; p44mapk; p44erk1
ERK2: MAPK1; ERK; p41mapk; MAPK2
ERK5: MAPK7; BMK1
JNK1: MAPK8 JNK; SAPK1
JNK2: MAPK9; p54a; SAPK
p38: MAPK14; PRKM14; Mxi2; PRKM15
TAK1: MAP3K7
NIK: MAP3K14; HSNIK; FTDCR1B; HS

2 | METHODS

2.1 | Study design

Blood samples of 232 children from two international cross-sectional studies and a birth cohort study were analyzed based on material availability. For the cross-sectional studies, a case-control study design of allergic asthmatic (AA) versus healthy children (HC) was chosen. HC had no history of wheeze, asthma, allergic rhinitis, allergic dermatitis, and/or eczema, and no chronic diseases. Based on an assumed standard deviation of 0.8 Δct per group, 14 children per group were required to detect one Δct difference of gene expression with $\alpha = .05$ significance and $\beta = 0.9$ power. The selected subpopulations (shown in Table S1A-C) were checked to be representative compared with the whole cohorts (Appendix S1). Due to restricted amount of children´s blood, a limited number of experiments could be performed per sample, resulting in different subpopulations per approach. Written informed consent for participation was obtained from the parents for all studies.

2.2 | Study population and characteristics

CLARA/CLAUS

For this cross-sectional study, N = 273/361 4- to 15-year-old children were recruited at the LMU children´s hospital since January 2009.[6,13,14] AA were defined with classical asthma symptoms and at least 3 wheezing episodes, a physician´s diagnosis of asthma (GINA guidelines), asthma medication use, lung function indicating significant reversible airflow obstruction (ATS/ERS guidelines), and specific IgE levels ≥0.35 IU/ml.[13] A subgroup of *N* = 87 (35AA/52HC) was selected for this study, based on material availability.

PAULINA/PAULCHEN

Our birth cohort study recruited *N* = 283 (190 PAULINA/93 PAULCHEN) pregnant women at the LMU hospital from 2004 to 2008.[15,16] Subsequent asthma was defined as a doctor´s diagnosis of asthma and/or doctor`s diagnosis of spastic, obstructive, or asthmatic bronchitis and relevant asthma medication until the age of 10 years as reported by the parents. Samples of 46 newborns (23 Asthma/23 HC) were included in this study based on RNA availability.

TRILATERAL

In the Chinese arm of the cross-sectional TRILATERAL study, healthy and asthmatic children from urban Hong Kong (*N* = 3435) age 7.0 ± 0.7 years and rural Conghua (*N* = 14,152) in China age 7.2 ± 1.5 years were recruited from 2013 to 2014 at The Chinese University of Hong Kong.[6] Of the 145 children included in the case-control study, a representative subgroup of 99 children [45 from Hong Kong (17 AA/28 HC); 54 from Conghua (15 AA/39 HC)] were analyzed in this study. AA had a history of wheeze ever, current wheeze (last 12 months), physician/hospital-diagnosed asthma, and positive skin prick test to at least one allergen.

2.3 | Laboratory methods

Gene expression was assessed in cord blood mononuclear cell (CBMCs, PAULINA/PAULCHEN) or PBMCs (CLARA/CLAUS, TRILATERAL) by qPCR and isolated dendritic cells (DCs) (CLARA/CLAUS, NanoString). Histone acetylation was measured by ChIP-qPCR in *N* = 20 children of the CLARA/CLAUS cohort. Moreover, protein expression was assessed by mass cytometry (CyTOF) in *N* = 20 children (CLARA/CLAUS; Figure S1, and details below).

2.4 | Dust collection and extraction

Environmental dust was extracted by standardized cold extraction from separate electrostatic dust collectors placed in cowsheds in Germany (G) or Finland (Fi) for 4 weeks.[6,17,18] LAL testing and 16S rRNA sequencing revealed high levels of LPS and a bacterial composition of mainly gram-negative Proteobacteria and gram-positive Firmicutes and Actinobacteria as previously published.[6]

2.5 | Cell isolation and stimulation

PBMCs (CLARA/CLAUS) were isolated within 24 h after blood withdrawal and cultivated (5 x 10^6/ml) in X-Vivo for 24 h (37°C, 5% CO_2) under unstimulated conditions (U) or stimulated with 0.1 μg/mL LPS (E.coli-O111:B4) or 40 μg/ml G or Fi farm-dust extract. PBMCs (TRILATERAL) were stimulated with 1.0 μg/ml LPS (E.coli-O111:B4), since preliminary studies revealed different optimal concentrations for each setting. Isolated CBMCs (PAULINA/PAULCHEN) were cultivated unstimulated in RPMI+10% human serum or stimulated with 0.1 μg/mL lipid A (LpA) for 72 h (37°C, 5%CO_2).

2.6 | RNA isolation, cDNA synthesis, and quantitative RT-PCR

RNA extraction, cDNA synthesis, and quantitative real-time PCR were performed as previously described.[6] Gene expression was normalized to 18S expression. Primer sequences are listed in Table S2.

2.7 | NanoString

DCs were separated by AutoMACS (DC isolation kit) after 24-h cultivation of PBMCs of *N* = 12 (5AA/7HC) children (CLARA/CLAUS) with or without German farm-dust. Hybridized RNA (100 ng, 18h, 65°C) was analyzed using the NanoString nCounter expression analysis (PanCancer Immune Profiling Panel). Data analysis was performed (nSolver Analysis Software v3.0)[19] using default settings. Positive controls, expression of housekeeping genes, total counts (excluding controls), and binding densities were assessed for quality testing in each sample. Standardization and normalization were based on the expression of positive controls and housekeeping genes within the panel.

2.8 | Mass cytometry (CyTOF)

Thawed unstimulated and German-dust stimulated (24 h) PBMCs (2 x 10^6) from *N* = 20 (10 AA/10 HC; CLARA/CLAUS) were stained with cisplatin ^{195}Pt viability stain and barcoded. Pooled samples were stained with an antibody surface stain cocktail (Table S3, details see supplement), fixed, and permeabilized followed by intracellular antibody staining. Cells were fixed with 1.6% formalin fixative and stained with $^{191/193}$Iridium. Samples (1 x 10^6 cells/ml) were resuspended in 0.1 x EQ four element calibration beads and acquired on a CyTOF-2.1 mass cytometer (Fluidigm) controlled by Helios software version 6.5.358 and

with a standard acquisition flow rate of 0.03 ml/min. FCS files were normalized using standalone MATLAB application Normalizer 0.3[20], visualized and manually gated with FlowJo (Figure S9, Table S4), and analyzed using the R packages CytoML and flowWorkspace.[21–23]

2.9 | ChIP-qPCR

$CD4^+$ cells were separated ($CD4^+$ T-cell Isolation Kit, AutoMACS) from 24-h cultivated PBMCs of $N = 20$ (10 AA/10 HC; CLARA/CLAUS), as histone acetylation within this cell population was already associated with asthma development.[24,25] Cross-linked cells were analyzed with the True MicroChIP kit. Chromatin lysate was sonicated and incubated overnight with H4ac polyclonal rabbit antibody and IgG as isotype control followed by incubation with protein A-coated magnetic beads. The immunoprecipitated chromatin was eluted from beads and de-crosslinked. Purified DNA (Microchip Diapure columns kit) was analyzed by qPCR (details supplement).

2.10 | Statistical analysis

Gene expression (qPCR) was assessed for stimulation effects and differences between phenotypes within a linear regression framework, technical, and population-related conditions were included as covariates leading to adjusted estimates of differences. qPCR data from CBMC were adjusted for sex. PBMC data were adjusted for age and sex and laboratory location (Germany, Hong Kong) for the TRILATERAL cohort. Association between genes was reported as Pearson correlations. Differences in acetylation measures were assessed by linear regressions adjusting for age, sex, and processing time. NanoString data were unadjusted due to the small sample size with two-sample *t* tests for phenotype differences and one-sample tests on fold change values for stimulation effects. Due to high correlation or limited sample size, data were not corrected for multiple testing.

3 | RESULTS

3.1 | Asthmatic children express less anti-inflammatory DUSP1 at mRNA and H4 acetylation levels

Gene expression analysis in PBMCs of allergic asthmatic (AA) and healthy (HC) school-age children of our CLARA/CLAUS cohort[9,21,22] (Figure S1) revealed that AA had significantly lower levels of anti-inflammatory *DUSP1* under unstimulated conditions ($p = .006$), that correlated inversely with specific IgE classes to house dust mite allergens (Figure S3A/B). *DUSP1*-specific H4 acetylation, that was associated with anti-inflammatory effects of asthma-therapeutic glucocorticosteroids,[11,12] was significantly lower in AA ($p = .012$) consistent with our findings on mRNA level.[26] By investigating a possible connection of anti-inflammatory regulation between MAPK (Figure S2) and NF-κB signaling, we revealed a strong correlation of *DUSP1* and *TNFAIP3* (Figure 1C, $r = 0.759$, $p < .0001$).

3.2 | Asthmatic children express higher MAPK levels

The pro-inflammatory counterparts of DUSP1, namely ERK1/2 and p38, were measured by mass cytometry on phosphorylated protein level to subdivide the individual subpopulations of peripheral blood. While no phenotypical differences were identified under unstimulated conditions (Figure S4), significantly higher pp38 levels were demonstrated in naïve T cells of AA (Figure 2A, $p = 0.03$).

Healthy newborns that developed asthma until the age of 10 expressed significantly more pro-inflammatory *ERK2* ($p = .049$) and trendwise higher *p38* levels in CBMCs at birth upon LpA stimulation (Figure 2B, $p = 0.085$).

3.3 | LPS and farm-dust stimulation upregulate *DUSP1* expression while MAPK expression was downregulated in PBMCs

When PBMCs of our CLARA/CLAUS cohort were cultivated for 24 h with and without farm-dust extracts or LPS, a significant upregulation of anti-inflammatory *DUSP1* was shown for AA upon all investigated stimuli, reaching levels comparable to unstimulated baseline levels of HC (Figure 3A). HC upregulated *DUSP1* expression only upon LPS stimulation. Significant *DUSP1* upregulation upon LPS stimulation was also shown for AA ($p = .014$) and HC ($p = .047$) in our Chinese Trilateral cohort (Figure 3B). Kinetic analysis of farm-dust stimulation revealed that prolonged stimulation (24 h) resulted in maximal anti-inflammatory regulation with upregulated anti-inflammatory *DUSP1* and *TNFAIP3*, downregulation of pro-inflammatory *TLR4* and *IL-18*, and an increased Th1/Th2 ratio (Figure S5). LPS blocking experiments demonstrated stimulatory effects of farm-dust stimulation beyond the effect of LPS (Figure S6). A whole protein concentration of 33.75 (G) and 101.43 (Fi) μg/ml was determined via Bradford assay and the presence of bovine betalactoglobulin was demonstrated in our farm-dust samples via Western blotting in line with data already described in the literature (details Appendix S1).[27]

Besides upregulation of anti-inflammatory *DUSP1*, farm-dust stimulation significantly downregulated phosphorylated protein expression of pro-inflammatory ERK1/2 and p38 in PBMCs of AA (Figure 3C, $p \leq .03$), while no evidence for differences in total non-phosphorylated ERK1/2 and p38 protein expression was shown (Figure S7). Gene expression levels of *ERK2*, *ERK5*, *JNK2*, *p38*, and *TAK1* were significantly downregulated in HC, but not in AA upon stimulation (Figure 3D).

In addition to anti-inflammatory signaling induction upon *ex vivo* farm-dust stimulation, *in vivo* exposure to rural environments resulted in significant lower pro-inflammatory MAPK14 and trendwise lower MAPK1 expression when gene expression was compared

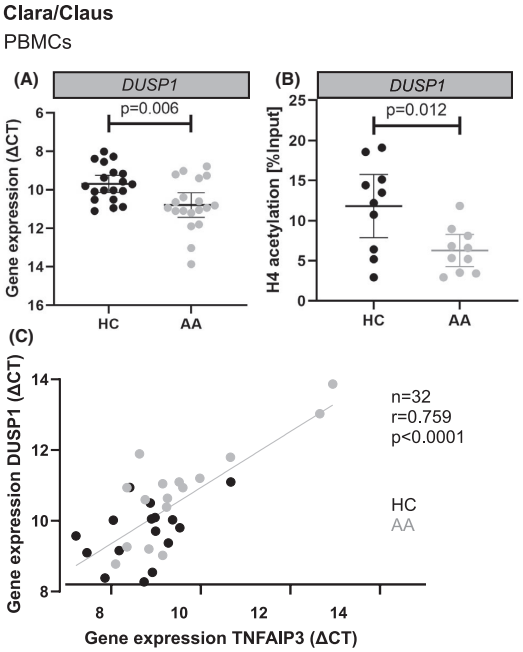

FIGURE 1 Asthmatic children express less DUSP1. Scatter plot for (A) *DUSP1* gene expression measured by qPCR and (B) *DUSP1*-specific histone H4 acetylation levels measured by ChIP-qPCR stratified for phenotypes, healthy (HC), and allergic asthmatic (AA) children. (C) Correlation of *TNFAIP3* and *DUSP1* gene expression (qPCR). 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 adjusted for sex and age. Histone acetylation measurements were additionally adjusted for processing time. Unstimulated PBMCs of 4- to 15-year-old children of the CLARA/CLAUS cohort were analyzed after 24-h cultivation on (A and C) gene expression level (Δct) by qPCR in $N = 38$ (19 HC/19 AA) and (B) histone H4 acetylation levels calculated as % recovery = $100 \cdot 2^{[(Ct(input) - 3{,}32) - Ct(IP)]}$ by chromatin immunoprecipitation followed by qPCR for *DUSP1* expression ($N = 20$ (10 HC/10 AA))

between children from rural Conghua and urban Hong Kong in the TRILATERAL cohort (Figure S8).

3.4 | Farm-dust stimulation downregulates pro-inflammatory MAPK expression in DCs, B cells, monocytes, natural killer cells, and several T-cell subpopulations

By investigating whether farm-dust stimulation has general, anti-inflammatory capabilities on several immune cell types, pERK1/2 and pp38 expression was assessed in B cells, monocytes, and NK cells as distinguished by the expression of lineage markers and subsequent manual gating (Figure S9). While downregulation of pERK1/2 was

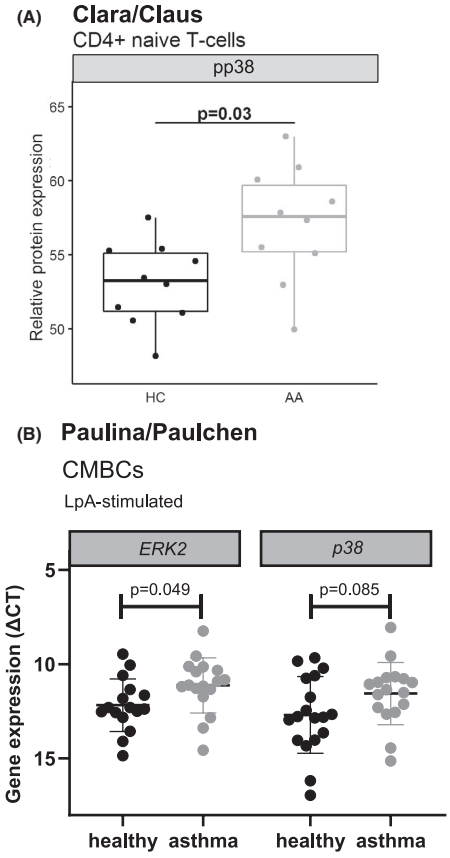

FIGURE 2 Asthmatic children express higher pro-inflammatory pp38 levels in naïve T cells at manifestation and ERK2 mRNA at birth. (A) Protein expression of phosphorylated p38 levels in gated naïve T helper cells measured by mass cytometry (CyTOF) in 24-h cultivated unstimulated PBMCs of $N = 20$ (10 HC/10 AA) 4- to 15-year-old children of the CLARA/CLAUS cohort. 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 (HC versus AA). (B) Scatter plots of gene expression *ERK2* and *p38* were analyzed by qPCR. CBMCs of healthy newborns (PAULINA/PAULCHEN cohort) were cultured for 72 h upon LpA stimulation (0.1 μg/mL). Data are shown as Δct value normalized to 18S gene expression, stratified for phenotype, and adjusted for sex ($N = 33$–35 (16–18/17 (HC/Asthma)). Raw data values are represented by horizontally jittered points. Error bars show 95% confidence intervals around the mean and adjusted *p*-values derived *t* test analysis comparing of children that stayed healthy to those developing asthma until the age 10

further demonstrated in monocytes of both phenotypes and B cells of AA, pp38 was decreased in B cells and NK cells of HC and AA (Figure 4A). Significant downregulation of pERK1/2 in T-cell subpopulations including CD8+ T cells, CD4+ T cells, Th2, and Tregs was

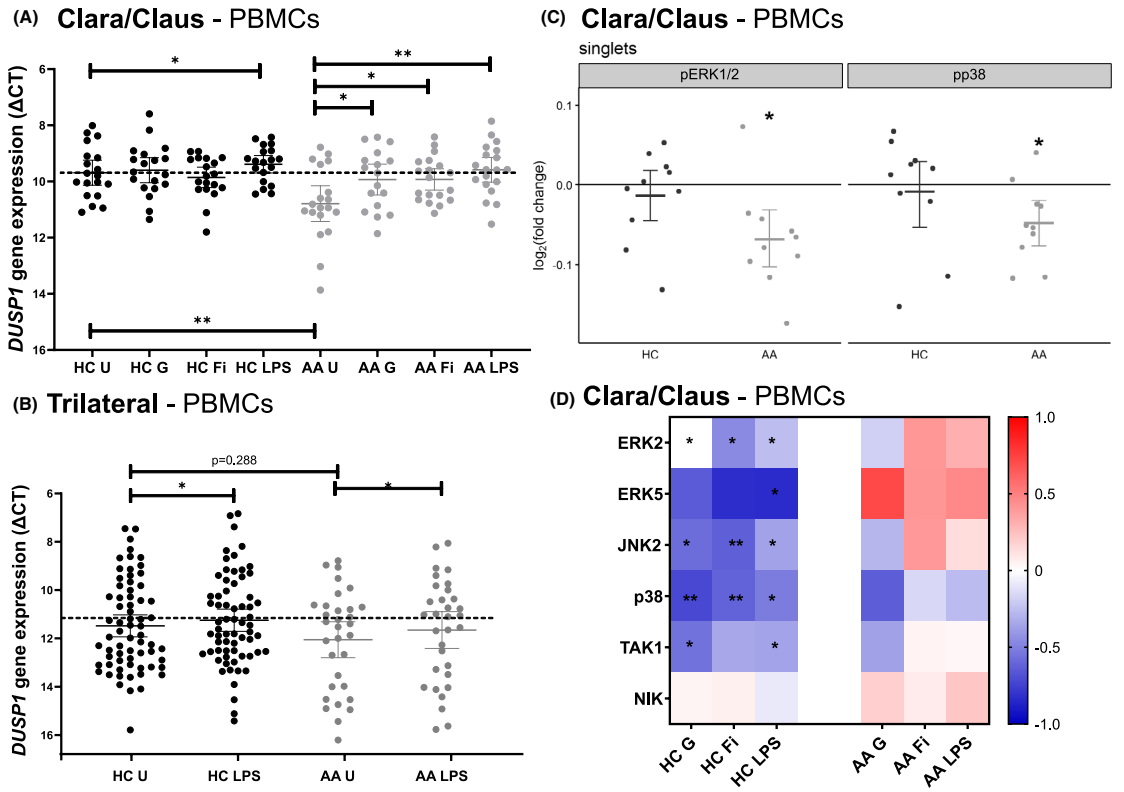

FIGURE 3 Stimulation of PBMCs with LPS and farm-dust upregulates anti-inflammatory *DUSP1* while downregulating pro-inflammatory MAPK expression. Scatter plot of *DUSP1* gene expression (Δct) measured by qPCR in PBMCs of (A) $N = 38$ (19 HC/19 AA) children of the CLARA/CLAUS cohort upon unstimulated (U) and farm-dust (G, Fi) or LPS-stimulated conditions and (B) $N = 99$ (67HC/32AA) children of the TRILATERAL cohort upon unstimulated (U) and LPS-stimulated conditions. (C) Fold change (German farm-dust stimulated vs. unstimulated conditions, $\log_2$) of phosphorylated protein expression measured by mass cytometry in PBMCs of $N = 20$ (10 HC/10 AA) children from the CLARA/CLAUS cohort. Only significant regulation differences compared with the unstimulated cells are indicated with asterisks (* $p < .05$, ** $p < .01$, *** $p < .001$) based on two-sample *t* test analysis. 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. (D) Fold change (stimulated vs. unstimulated conditions) of gene expression (Δct, qPCR) in PBMCs of 4- to 15-year-old children of the CLARA/CLAUS cohort ($N = 23$-35 (11-19 HC/ 12-16 AA)). Gene expression regulation upon 24-h stimulation with German (G) or Finnish (Fi) farm-dust or LPS color-coded in heat map. Blue color indicates lower gene expression (downregulation), while red indicates higher gene expression (upregulation) upon stimulation compared to unstimulated conditions (fold change, $\log_2$ scale). Strength of up- and downregulation is reflected by color saturation. Asterisks (* $p < .05$, ** $p < .01$) mark significant stimulatory regulation based on two-sample *t* test analysis

exclusively shown for AA (Figure 4B). Since DCs were already associated with environment-mediated asthma protection of childhood asthma in previous studies, multiplex gene expression analysis was performed in isolated DC by NanoString technology.[28,29] Farm-dust stimulation resulted in upregulation of anti-inflammatory *DUSP1*, while *p38* and *ERK2* were downregulated on gene expression level in DCs (Figure 4C).

Taken together, pro-inflammatory ERK1/2 and p38 expression were downregulated on gene and protein levels in almost all investigated cell populations, namely PBMCs, DCs, B cells, or NK cells, monocytes, CD8+ T cells, CD4+ T cells, Th2 cells, and Tregs (Figure 5A), suggesting an anti-inflammatory role of farm-dust exposure together with the impact on both innate and adaptive parts of the immune system (Figure 5B).

4 | DISCUSSION

Immunological mechanisms underlying childhood asthma protection via farm exposures include suppression of pro-inflammatory pathways to attenuate inflammatory tissue destruction as we previously demonstrated for NF-κB-signaling.[6] We now investigated

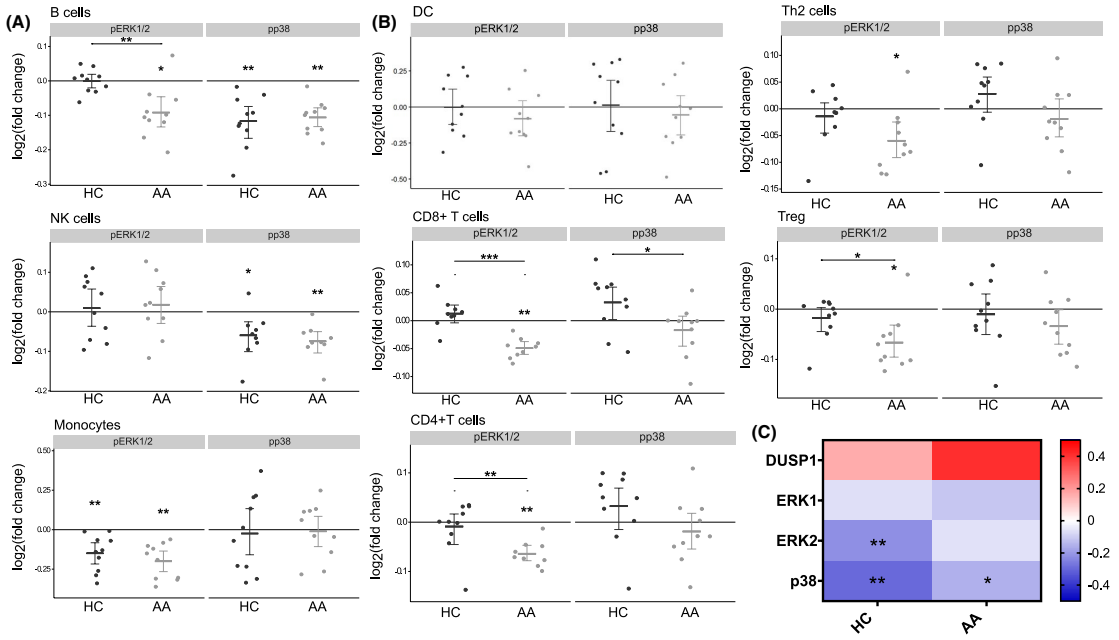

FIGURE 4 Farm-dust stimulation downregulates pro-inflammatory pMAPK expression in several blood cell populations. Fold change (German farm-dust stimulated vs. unstimulated conditions) of relative phosphorylated protein expression in gated (A) B cells, monocytes, and natural killer cells (NK) and (B) DC and T-cell populations including $CD8^+$ T cells, $CD4^+$ T cells, Th2 T cells, and Tregs measured by mass cytometry in $N = 20$ (10 HC/10 AA) children from the CLARA/CLAUS cohort. Data are shown stratified by phenotype. Error bars show 95% confidence intervals around the mean. Differences are indicated by the p-value of two-sample *t* tests for phenotype comparisons. Only significant regulation differences compared to the unstimulated cells are indicated with asterisks (* $p < .05$, ** $p < .01$, *** $p < .001$) based on two-sample t test analysis. Significant differences in regulation between the AA and HC are marked with an arrow between the phenotypes (* $p < .05$, ** $p < .01$, *** $p < .001$). (C) Downregulation (blue) of gene expression of *ERK1*, *ERK2*, and *p38* and upregulation (red) of *DUSP1* in isolated dendritic cells (DC) of unstimulated and German-dust stimulated PBMCs of 4- to 15-year-old children from the CLARA/CLAUS cohort ($N = 12$ [7 HC/5 AA]) measured with NanoString multiplex expression analysis (*ERK1*, *ERK2*, *and p38*) and qPCR (*DUSP1*, $N = 11$ [7HC/4AA]). Color saturation reflects the strength in terms of the fold change ($\log_2$ scale)

the pro-inflammatory MAPK signaling pathway, which is in close connection with NF-κB-signaling in three pediatric cohorts focusing on involved immune cell subpopulations. We could demonstrate decreased baseline *DUSP1* expression and histone H4 acetylation levels in asthmatic children and anti-inflammatory regulation upon farm-dust stimulation on transcriptional and phosphorylation levels in all investigated cell types.

DUSP1 is highly important for efficient suppression of inflammatory processes by inhibiting the key players within the MAPK signaling pathway, namely ERK1/2, JNK1, and p38.[8] Thus, lower H4 acetylation and linked mRNA expression of *DUSP1* in asthmatic patients may enhance inflammation due to insufficient MAPK suppression. Increased MAPK levels in cord blood further indicate the contribution of MAPK signaling to inflammatory properties of asthmatic children already at birth. In children with manifest asthma, higher levels of pp38 in naïve T cells displayed increased baseline inflammation.

Baseline MAPK differences under unstimulated conditions might be rather visible at the central site of inflammation compared with peripheral blood, as previously demonstrated in adult lung biopsies and unstimulated bronchial epithelial cells.[30,31] Here, we could show that children of our CLARA/CLAUS cohort expressed significantly less *DUSP1* systemically at baseline. With the advantage of expression assessment in easily accessible blood, DUSP1 could function as potential biomarker for asthma development together with TNFAIP3.

Consistent with our previous findings regarding NF-κB signaling[6], we now demonstrated that farm-dust stimulation had further anti-inflammatory capacity beyond TNFAIP3 signaling by regulating the connected MAPK pathway. Upregulation of anti-inflammatory *DUSP1* reaching healthy baseline levels and downregulation of phosphorylated pro-inflammatory MAPK proteins reflect sufficient inhibitory dephosphorylation by DUSP1. Differential regulation levels were observed between the phenotypes. While significant MAPK downregulation was demonstrated for HC on mRNA levels, regulation was implemented on phosphorylation levels in AA. Since HC already started with high *DUSP1* levels, we suggest that further increase is not required, resulting in weak reduction of MAPK phosphorylation. In contrast, the significant increase of *DUSP1* in AA leads to enhanced dephosphorylation with decreased

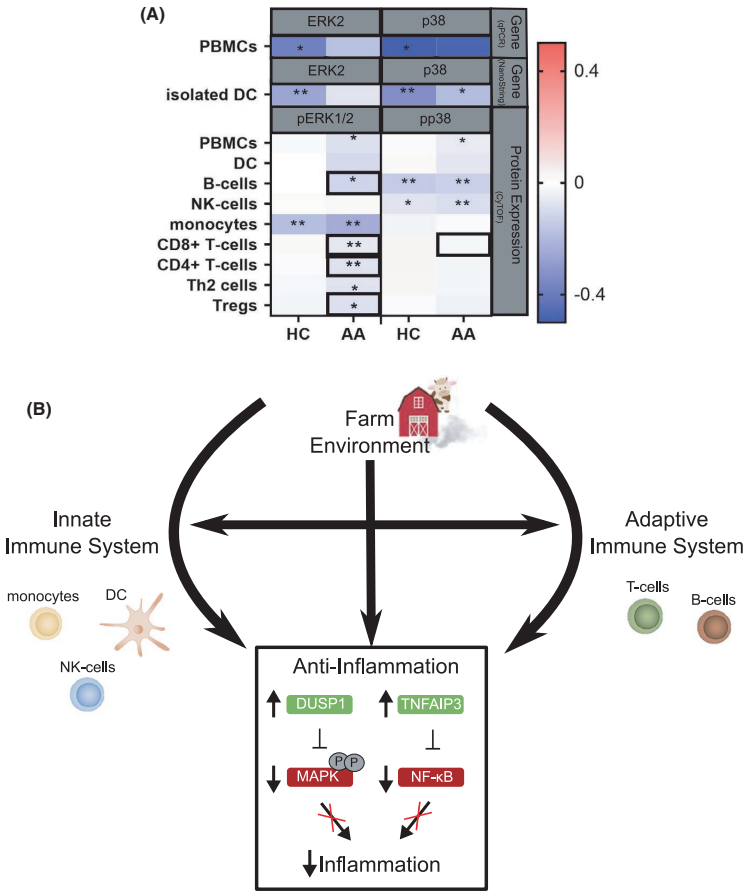

FIGURE 5 Summary of anti-inflammatory capacity of farm-dust stimulation on MAPK expression in several subpopulations and expression levels. (A) Downregulation of gene or phosphorylated protein expression of ERK1/2 and p38 in German-dust stimulated PBMCs (compared to unstimulated conditions), isolated DCs (gene) and gated DCs (protein), B cells, NK cells, monocytes, and T-cell subpopulations (CyTOF) of 4- to 15-year-old children from the CLARA/CLAUS cohort measured with qPCR (mRNA in PBMC, $N = 38$), NanoString (mRNA in isolated DCs, $N = 12$), and mass cytometry (phosphorylated protein levels, $N = 20$). Color saturation reflects the strength in terms of the fold change ($\log_2$ scale). (B) Schematic concept of immunological mechanisms underlying the protective effects of farming environments

MAPK levels. In turn, pMAPK levels restrict the *de novo* gene synthesis via negative feedback loops, resulting in weak MAPK mRNA upregulation.

The discordance of gene and phosphorylation data might also arise from different kinetics and temporal profiles of AA and HC. Due to logistic reasons, we measured all expression levels at the same time point. Further studies, investigating the phenotypical differences in temporal and feedback dynamics in the tightly regulated MAPK signaling pathway, are relevant. We are aware that the MAPK pathway consists of a cascade of numerous kinases. We have focused on DUSP1, ERK1/2, and p38 as they regulate inflammatory downstream processes and were previously associated with asthma development.[32] Since the efficiency of MAPK inhibitors is relatively limited in therapeutic asthma research, DUSP1 induction is suggested to be auspicious[8]; thus, farm-dust application might be a promising agent.

As in our previous study, the differences in DUSP1 expression were stronger in our clinical CLARA/CLAUS cohort, where children were recruited during early manifestation in our outpatient asthma clinic.[6] In comparison, Chinese children were recruited in an epidemiological approach, using questionnaire-based phenotyping rather than clinically assessed asthma classification. Also, genetic and ethical background might influence the baseline DUSP1 expression. Of note, different concentrations of LPS were used in the cohorts as these concentrations were proven to be optimal in the corresponding setting in preliminary experiments. However, since stimulation of the isolated cells with farm-dust extracts or LPS resulted in consistent upregulation of DUSP1 in both cohorts and epidemiological studies have demonstrated the farm effect in multiple areas worldwide, we are confident of anti-inflammatory characteristics of farming environments around the world. Additional effects of farm-dust extracts, for example, via bovine betalactoglobulin or other proteins are possible and require functional proof.

By analyzing MAPK expression in individual immune cell subpopulations and applying several different methods to cover various levels of expression, we were able to provide further proof of a universal anti-inflammatory capacity of *ex vivo* farm-dust stimulation in children with manifest asthma, suggesting an important role of inflammatory regulation in this concept. Of note, due to the high correlation of the data and the explorative nature of the study, data were not adjusted for multiple testing. In conclusion, this study further contributes to our understanding of the immunological mechanisms

of environment-mediated asthma protection in children. This might involve ubiquitously induction of anti-inflammatory key regulators like DUSP1 and inhibiting central inflammatory pathways including NF-κB and MAPK signaling in several immune cell populations as one immune regulatory mechanism. Thereby, this study confirms our previous concept of efficient anti-inflammatory properties mediated by environmental available stimuli even in manifest asthmatic children[6] indicating that it is also relevant for the potential of modifying established disease and is not limited to asthma prevention. Finally, if this concept can be confirmed in future studies and translated into safe and efficient application, this may be a valuable and simple option for anti-inflammatory activation in established asthmatic patients.

ACKNOWLEDGMENTS

We would like to thank all participating families. Special thank also to Isolde Schleich and Tatjana Netz for their technical support and the study nurses for blood sampling.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Johanna Viktoria Theodorou contributed to data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); writing—original draft (equal). **Elisabeth Nowak** contributed to data curation (equal); formal analysis (equal); investigation (equal); methodology (equal). **Andreas Böck** contributed to formal analysis (equal); investigation (equal); methodology (equal). **Michael Salvermoser** contributed to formal analysis (equal); investigation (equal); methodology (equal). **Claudia Carina Beerweiler:** contributed to data curation (supporting); methodology (supporting). **Kathrin Zeber** contributed to data curation (equal); methodology (equal). **Paulina Kulig** contributed to investigation (equal); methodology (equal). **Miranda S Tsang** contributed to data curation (equal); investigation (equal); methodology (equal). **Chun K Wong** contributed to conceptualization (equal); supervision (equal). **Gary WK Wong (N/A)** contributed to conceptualization (equal); supervision (equal). **Marjut Roponen** contributed to conceptualization (equal). **Joerg Kumbrink** contributed to methodology (equal). **Fahd Alhamdan** contributed to methodology (equal). **Florian Michel** contributed to methodology (equal). **Holger Garn** contributed to conceptualization (equal). **Vinko Tosevski** contributed to conceptualization (equal); methodology (equal). **Bianca Schaub** contributed to conceptualization (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); project administration (equal); resources (equal); supervision (equal); writing—review and editing (equal).

ETHICAL APPROVAL AND TRIAL REGISTRATION

Ethical approval for the CLARA/CLAUS study was obtained by the local ethics board, LMU Munich, Germany Nr. 379-08 (DRKS00004635). Ethics for the PAULINA/PAULCHEN study was obtained by the human ethics committee of the Bavarian Ethical Board, LMU Munich, Germany #04092 (DRKS00015204). Ethics approval for the Chinese arm of the TRILATERAL study was obtained by The Chinese University of Hong Kong Clinical Research Ethics Committee #2013.220.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/pai.13657.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Theodorou J, Nowak E, Böck A, et al. Mitogen-activated protein kinase signaling in childhood asthma development and environment-mediated protection. *Pediatr Allergy Immunol*. 2022;33:e13657. https://doi.org/10.1111/pai.13657

Supplementary information

Mitogen-activated protein kinase signaling in childhood asthma development and environment-mediated protection

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Methods

Study population and characteristics

This study comprises data of three pediatric cohorts, two cross-sectional cohorts (CLARA/CLAUS, TRILATERAL), and one birth cohort study (PAULINA/PAULCHEN) comparing data of N=232 out of 1062 total children (Figure S1).

CLARA/CLAUS

Table S1A shows the population characteristics of each subpopulation used in this study. The healthy controls were recruited in schools or the Dr. von Hauner Children´s Hospital before outpatient daily procedures (e.g. fracture, hernia operation). The subgroup of 38 children (19 AA/19HC) analyzed by qPCR did not differ significantly from the whole CLARA/CLAUS cohort in terms of sex, age, smoking in pregnancy, parental asthma, pets, levels of FEV1/FVC, and IgE. The subgroup of 20 children (10 AA/10 HC) investigated by mass cytometry (CyTOF) had more male subjects in both groups, however, distributed equally between the two phenotypes within the subgroup. The 5 asthmatic children investigated by NanoString had higher FEV1/FVC levels compared to the asthmatics of the whole CLARA/CLAUS cohort, thus representing mild asthmatics. The subgroup of 20 children (10 AA/10 HC) included in the acetylation measurements was significantly younger, however not different between phenotypes in the analyzed subgroup. Acetylation levels were not significantly associated with age. The parents answered questionnaires on general health and socioeconomic status.

PAULINA/PAULCHEN

There were no significant differences in the subpopulation included in this study compared to the whole PAULINA/PAULCHEN cohort regarding sex, number of siblings, maternal age, smoking in pregnancy, and family history. Table S1B shows the characteristics of the subpopulation.

TRILATERAL

No significant deviations could be observed between the analyzed subpopulation and the whole TRILATERAL cohort in terms of region, number of siblings, and sex. Population characteristics are shown in Table S1C.

Laboratory methods

Gene expression data were analyzed by qPCR in unstimulated and LpA-stimulated (72h) cord blood cells of N=46 healthy newborns (23HC/23Asthma). Gene expression was also measured by qPCR in unstimulated (U), German (G) and Finnish (Fi) farm dust-stimulated and LPS-stimulated (24h) PBMCs of HC and AA children of the German CLARA/CLAUS cohort (N=38 (19HC/19AA). qPCR measurements in unstimulated and LPS-stimulated (24h) PBMCs were replicated in the Chinese TRILATERAL cohort including HC and AA children from urban (Hong Kong, N=45 (28HC/17AA)) and rural (Conghua, N=54 (39HC/15AA)) China. Histone H4 acetylation levels of DUSP1 were measured by ChIP-qPCR in unstimulated isolated $CD4^+$ T-cells of the CLARA/CLAUS cohort (N=20 (10HC/10AA)). Gene expression was measured in isolated unstimulated and German (G) farm dust stimulated (24h) dendritic cells in N=12 (7HC/5AA) for *ERK1, ERK2,* and *p38* by NanoString and *DUSP1* by qPCR analysis. Phosphorylated protein expression levels were analyzed by mass cytometry (CyTOF) in unstimulated and German (G) farm dust stimulated (24h) PBMCs and their manually gated subpopulations in N=20 (10HC/10AA) children of the CLARA/CLAUS cohort. The cohorts, performed analysis and experimental conditions are summarized in a Flow Chart (Figure S1). In Figure S2 the complex MAPK signaling pathway and part of its interaction with the NF-kB pathway are summarized with anti-inflammatory DUSP1 and TNFAIP3 labeled in green and pro-inflammatory targets like TLR4 and MAPKs labeled in red.

Quantitative RT-PCR

Standard conditions used for qPCR were as follows: 2 min, 95°C; 20 sec, 95°C and 30 sec, 62.5°C, 40x including ROX as a passive reference, melt curve analysis, and automatic threshold setting. Each well of the 96-well plate contained 6 ng cDNA, 320 nM primers and SYBR Green in a total of 10 µL. The used primer sequences as listed in Table S2.

Kinetic experiments

PBMCs ($5x10^6$ cells/ml) of 3 healthy children were cultivated under unstimulated condition or stimulated with 0.1 µg/mL LPS (E.coli-O111:B4) or 40 µg/mL German farm-dust (G). RNA was harvested after 0.5h, 1h, 2.5h, 6h, 12h and 24h with varying sample size (1h, 12h, 24h: N=3; 2.5h: N=2, 0.5h, 6h: N=1). Fold change values (stimulated vs. unstimulated) were graphically summarized for each stimulation time-point.

CyTOF sample preparation, acquisition and manual gating

Metal-tagged antibodies (Table S3) were purchased from Fluidigm or conjugated using X8 MaxPar Antibody Labeling kit (Fluidigm), titrated, validated, and stained with Cisplatin ^{195}Pt-viability stain (Fluidigm). All samples were barcoded-using Cell-ID 20-Plex Pd Barcoding-kit (Fluidigm) according to manufacture specifications. Cells were fixed and permeabilized using Foxp3/Transcription factor kit (eBioscience). $^{191/193}$Iridium, four element calibration beads were purchased from Fluidigm. For acquisition, the following signal processing settings were used: default thresholding scheme, lower convolution threshold of 800 intensity units [IU], minimum event duration of 8 pushes, maximum event duration of 100 pushes, with noise reduction active. Normalized FCS files were loaded into FlowJo software (10.6.1). Cells were distinguished from beads and debris by removing ^{140}Ce (bead-marker) and/or ^{198}Pt (dead-marker) positive events. The viability was comparable between unstimulated and farm dust stimulated samples. Live singlet cells were gated by removing events with high event length and extreme DNA content ($^{191/193}$Ir). The gating strategy is shown in Figure S9. The beads-negative, live, and singlets gates were adjusted to each sample individually. All other gates were fixed and applied for all samples. Manual gating of living singlets cells was based on the expression of the corresponding markers (Table S4).

Chromatin Immunoprecipitation qPCR (ChIP-qPCR)

Cells were cross-linked with 1% formaldehyde (Sigma-Aldrich, no. 252549) to a final concentration and incubated for 8 minutes at room temperature. The cross-linking reaction was quenched with 125 mM Glycine (Roth, no. 3908.2) to a final concentration and incubated for 5 minutes at room temperature. Subsequently, the cross-linked cells were pelleted and rinsed with HBSS (Gibco, no. 14065049) containing

protease inhibitor mix (Diagenode, no. C12010012). The cross-linked cells were pelleted again and kept on ice from now onwards. The Chromatin Immunoprecipitation (ChIP) analysis was performed using the True MicroChIP kit (Diagenode, no. C01010130). According to the manufacturer's protocol for every 100,000 cells, the pelleted cross-linked cells were re-suspended in Lysis Buffer tL1 containing protease inhibitor mix and incubated for 5 minutes on ice, and then the lysate was diluted 1:4 with HBSS containing Protease inhibitor mix. Afterwards, the chromatin lysate was sheared using the Bioruptor® Pico sonication device combined with the Bioruptor® Water cooler (Diagenode) for 13 cycles using a 30" [ON] 30" [OFF] settings. Shearing was conducted in 1.5 ml Bioruptor® Pico Microtubes with Caps (Diagenode, no. C30010016). The sheared chromatin was diluted 1:1 with ChIP Buffer tC1 containing protease inhibitor mix and incubated overnight on a rotator at 40 rpm at 4C° for immunoselection using the following amounts of antibodies:1 µg of H4ac polyclonal rabbit antibody (Active Motif, no. 39925) and 1 µg of IgG rabbit antibody (Diagenode, no. C15410206) as an isotype control. The antibody ligated chromatin was further incubated with Protein-A coated magnetic beads on a rotator at 40 rpm for 2 hours at 4 C° and subsequently washed once with each of the following washing buffers: Wash Buffer tW1, tW2, tW3, and tW4, respectively. Afterwards, the immunoprecipitated chromatin was eluted from the beads by incubating with Elution Buffer tE1 on a rotator at 40 rpm for 30 minutes at room temperature. The de-crosslinking reaction was performed by adding Elution Buffer tE2 to the eluted chromatin and incubated overnight at 65 C° in a water bath. DNA was purified using Microchip Diapure columns kit (Diagenode, no. C03040001) According to the manufacturer's protocol: 5 volumes of ChIP DNA binding buffer was added to each volume of DNA solution and washed twice with DNA wash buffer. DNA was eluted with DNA elution buffer stored at - 20 C°. The DNA sequences of the desired primers were downloaded from UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly and the primers were designed using the online tool Primer-BLAST with the amplicon size between 60 bp to 150 bp and an optimal annealing temperature of 60 C°. The primers were tested using different annealing temperatures and using positive (genomic DNA) and blank (H2O) controls. qPCR analysis was done by using SYBR Green master (total volume of 25 µl/reaction) (Qiagen, no. 1054605) with the following reaction PCR program: 7 minutes denaturation step

at 95°C, followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72 °. The used primer sequences were specific for the promoter region of DUSP1: ACGTGCCACCTGCCTTG (forward primer); CCCTTTCGGTTCAGGTCGG (reverse primer). The results were analyzed using the following equation: $\%\, recovery = 100 \cdot 2^{[(Ct(input)-3{,}32)-Ct(IP)]}$. No significant differences were demonstrated between healthy and asthmatic children for the negative control IgG (not shown).

Detection of bovine beta-lactoglobulin (BLG) in farm dust samples

Supernatants (centrifugation: 4°C, 800 rcf, 10 min) from dissolved farm dust samples were separated using SDS-Page (15%). Concentrations of 5, 25 and 50 mg/mL farm dust extract were used. As a positive control, 5 µg/mL BLG (Sigma-Aldrich, #L0130-1G, courtesy of Dr. Pali-Schöll, Vienna, Austria) was used, while 5 µg/mL OVA (Sigma-Aldrich, # A5503) and 50 mg/mL dust extract from an urban apartment served as negative controls. Proteins were transferred to PVDF membranes and blocked in 5% BSA-TBST. Beta-lactoglobulin specific immunoblots were performed using a rabbit polyclonal anti-BLG antibody (Abcam plc #ab229780) and a goat anti-rabbit IgG H&L (HRP) (Abcam plc #ab205718; courtesy of Dr. Pali-Schöll, Vienna, Austria) according to the manufacturer's instructions. Visualization was performed with chemiluminescence solution (Amersham™ ECL™ Prime Western Blotting Detection Reagent) on a ChemiDoc™ System (Bio-Rad Laboratories, Hercules, California).

LPS-blocking

Isolated PBMCs of N=6 (3HC/3AA) children were cultivated for 24h under unstimulated conditions or stimulated with German or Finnish farm dust or LPS. LPS-blocking was achieved by co-stimulation of the unstimulated or stimulated cells with 0.3 µg/ml LPS-RS. Upon cell harvesting, RNA was extracted and gene expression of *DUSP1*, *TNFAIP3* and *TLR4* was measured in synthesized cDNA. The concentration of 0.3 µg/mL of the blocking reagent LPS-RS was chosen based on prior testing of doses between 10 to 10.000 ng/mL.

Western Blot analysis

Unstimulated (U), German (G) and Finnish (Fi) farm dust and LPS stimulated PBMCs ($6x10^5$) of N=8 (5HC/3AA) children of the CLARA/CLAUS cohort were lysed with 100µL RIPA-buffer containing protease and phosphatase inhibitors. Protein concentration was measured by Bradford assay. Each sample was loaded on four different gels with 20µg protein per lane and separated by SDS-Page and blotted on a polyvinylidene fluoride membrane. Blocking was performed for 1h at room temperature with 5%milk-TBST (Tris-buffered saline with 1% Tween 20). Each membrane was incubated overnight at 4°C with one of the following primary antibodies: ERK1/2, pERK1/2, p38 or pp38 respectively (Cell signaling: ERK1/2: #4695T, pERK1/2: #4370T, p38: #8690T and pp38: #4511T) diluted 1:1000 or 1:2000 (ERK1/2), respectively with 5% BSA-TBST. Upon washing with TBST, incubation with the HRP-linked secondary antibody (#7074S Cell signaling, anti-rabbit IgG, 1:1000 in 5% milk-TBST) was performed for 30 minutes at room temperature and again washed three times with TBST. The signal was visualized by enhanced chemiluminescence solution on a ChemiDoc Imaging System. After three washing steps, the membranes were incubated overnight at 4°C with the primary antibody Vinculin (Sigma-Aldrich: #V9131, diluted 1:6000 in 5% BSA-TBST) that was used as a loading control, washed and incubated with the HRP-linked secondary antibody (Invitrogen, #32430, anti-mouse, 1:1000 diluted in 5% milk-TBST). After washing and incubation with the enhanced chemiluminescence solution, the signal was detected on the ChemiDoc. Quantification of the corresponding bands was performed with the Image Lab software. The total band volume intensity of each lane was related to the intensity of the corresponding Vinculin band to normalize the expression to the amount of loaded protein.

Results

Correlation of *DUSP1* with clinical IgE classes to house dust mite allergens

To assess, if DUSP1 expression is linked with clinical characteristics, we performed a correlation analysis of *DUSP1* gene expression levels (dCT) to IgE classes to the house dust mite allergens Dermatophagoides pteronyssinus and Dermatophagoides farinae, measured with the MediWiss Allergy Screen in the serum of

the children. *DUSP1* mRNA levels correlated inversely (r=-0.3641 and r=-0.3522) to allergen IgE classes of D. pteronyssinus (p=0.0246) and D. farinae (p=0.0301) (Figure S3A/B).

No phenotypical differences of pERK1/2 and pp38 in PBMCs under unstimulated conditions

When we compared the differences in phosphorylated protein expression of ERK1/2 and p38 of asthmatic and healthy school-aged children in isolated PBMCs using mass cytometry, no phenotypical differences could be observed under unstimulated condition (Figure S4).

Immune-regulatory capacity of farm-dust stimulation for 24 hours indicated an "anti-allergic" Th1-shifted cytokine pattern

Since DUSP1 was described as an early response gene, we investigated the expression kinetics within 24 hours of farm-dust/LPS stimulation. *DUSP1* expression was upregulated upon stimulation at early time points (0.5h) with a declining extent over time reaching baseline levels at 1h upon stimulation, as described earlier [Chi et al. 2006 Dynamic regulation of pro- and anti-inflammatory cytokines by MAPK phosphatase 1 (MKP-1) in innate immune responses]. After 2h of stimulation, *DUSP1* expression was even strongly downregulated. However, with prolonged stimulation, *DUSP1* expression was continuously upregulated over time, reaching its maximum at 24h (Figure S5A). Similar kinetic patterns were observable for *TNFAIP3* (Figure S5B). Also, pro-inflammatory *TLR4* and *IL18* were continuously downregulated with increasing stimulation duration, with maximum downregulation after 24 hours (Figure S5C-D).

In order to investigate the stimulatory effects on expression and kinetics of downstream mediators, gene expression of *IL4, IL10, IFNγ, IL1α, IL1β* was measured. All investigated cytokines, except for *IL18* (Figure S5D) were upregulated upon farm-dust and LPS stimulation (Figure S5E). When focusing on *IL4* and *IFNγ*, as representatives of Th2 and Th1 cytokines, respectively, an alternating pattern regarding the kinetics of stimulatory effects was observable. While Th2-associated *IL4* was strongly induced early after stimulation with declining intensity, Th1-associated *IFNγ* was gradually upregulated. At early time points (0.5h-2h) Th2-associated cytokine expression was dominant, yet prolonged stimulation caused a Th1-polarization

with rising and dominating IFNγ levels with a maximal Th1/Th2 ratio at 24h (Figure S5F). Since we aimed to investigate tolerance mechanisms associated with the farm effect rather than acute responses to microbial substances and demonstrated optimal anti-inflammatory immune-modulatory effects of farm-dust following 24h stimulation, we chose this duration for our stimulation experiments.

Farm dust samples contain bovine beta-lactoglobulin (BLG)

As bovine BLG was already associated with the beneficial effects of farming environments and demonstrated to be present in farm dust studies by Pali-Schöll et al.(Mayerhofer & Pali-Schöll The farm effect revisited: from β-lactoglobulin with zinc in cowshed dust to its application, Allergo J Int 2021), we checked if our used farm dust samples (G, Fi) contain bovine BLG by Western Blotting. In fact, within our farm dust extracts, bovine BLG could be detected while no BLG was present in our negative controls (OVA and urban apartment dust).

The anti-inflammatory effect of farm dust stimulation goes beyond the effect of LPS

Since LPS and the farm dust extracts had similar immunomodulatory capacities, we aimed to investigate if farm dust stimulation can exert its anti-inflammatory properties even when the impact of LPS was blocked. Therefore, we co-stimulated PBMCs of 3 healthy and 3 asthmatic children with German and Finnish farm dust extracts or LPS as a control together with 0.3 µg/mL LPS-RS that blocks the effect of LPS-signaling by competing with the TLR4 ligand without stimulating its downstream signaling cascade due to its underacetylated lipid A structure. In fact, when stimulating cells only with LPS-RS, no significant change in gene expression was observed, proofing the capacity of this reagent to compete with LPS without triggering the signaling cascade (Figure S6). Stimulation with dust extracts from German (G) and Finnish (Fi) farms resulted in the known expression patterns of upregulated anti-inflammatory *TNFAIP3* and downregulation of pro-inflammatory *TLR4* as we have already published before (Krusche et al. TNFAIP3 is a key player in childhood asthma development and environment-mediated protection, JACI 2019). In line, anti-inflammatory *DUSP1* was upregulated. As expected, when co-stimulating the cells with LPS-RS the stimulatory effect of LPS was highly reduced or even completely abolished. In contrast, the anti-

inflammatory effects of farm dust stimulation were reduced but still visible, indicating that although farm dust extracts confer a major part of their anti-inflammatory effects via LPS, they still harboring other components involved in anti-inflammatory signaling.**Total ERK1/2 and p38 levels remained unchanged upon farm dust and LPS stimulation, while phosphorylated ERK1/2 was downregulated in asthmatic children**

In order to investigate whether the effect of dust stimulation actually leads to reduced phosphorylation and not to a reduction in total protein, we investigated the expression of total MAPK and phosphorylated MAPK by western blotting in healthy and asthmatic children (N=8 (5HC/3AA). The reduced expression of phosphorylated ERK1/2 upon LPS and farm dust stimulation in asthmatic children was confirmed by this method (Figure S7A). Total ERK1/2 and total p38 expression was not changed or showed even slight upregulation upon stimulation in the same samples, suggesting that stimulation is indeed affecting phosphorylation rather than total MAPK expression (Figure S7A). Four representatives blots for one healthy and one asthmatic child for each target protein (ERK1/2, pERK1/2, p38, and pp38) and their corresponding Vinculin expression are shown in (Figure S7B), confirmed by a total of N=8 (5HC/3AA). Due to the limited amount of blood and the requirement of specific nuclear protein extraction, analysis of DUSP1 was not feasible on mRNA and protein level in parallel in this setting of a large cross-sectional cohort study, however, data on DUSP1 acetylation were investigated.

***In vivo* exposure to rural environments downregulates pro-inflammatory *MAPK14* expression**

To investigate the influence of *in vivo* exposure to rural environments on the expression of MAPK signaling genes, *DUSP1*, *MAPK1* and *MAPK14* expression was compared between children from rural Conghua and from urban Hong Kong. While *DUSP1* was not significantly different between the two groups, children from Conghua expressed significantly less pro-inflammatory *MAPK14* (p=0.003) and trend-wise lower *MAPK1* (p=0.085) levels compared to children with exposure to urban Hong Kong (Figure S8). This suggests that exposure to farming and rural areas do not only induce anti-inflammatory signaling by *ex vivo*

254 farm dust stimulation, but also downregulates pro-inflammatory signaling in children with *in vivo* exposure
255 to the "asthma-protective" environments.

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Table S1: Population characteristics study cohorts

A) CLARA/CLAUS cohort

	Study population			qPCR (MAPK genes)			Acetylation			Mass cytometry			NanoString		
Parameter	**HC**	**AA**	**P value**	**HC**	**AA**	***P* value**	**HC**	**AA**	***P* value**	**HC**	**AA**	***P* value**	**HC**	**AA**	***P* value**
N	52	35		19	19		10	10		10	10		7	5	
Female sex, no. (%)	23 (44.2)	16 (45.7)	0.891[1]	11 (57.9)	9 (47.4)	0.516[1]	5 (50.0)	6 (60.0)	0.653[1]	1 (10.0)	1 (10.0)	1.000[1]	6 (85.7)	1 (20.0)	**0.023**[1]
Age (y)	9.0 (3.2)	8.6 (2.9)	0.559[2]	9.5 (3.5)	9.1 (3.2)	0.737[2]	6.9 (1.4)	6.6 (2.2)	0.751[2]	9.3 (2.9)	7.6 (2.1)	0.173[2]	9.3 (2.4)	9.0 (2.5)	0.820[2]
Smoking during pregnancy, no. (%)	4 (7.7)	1 (2.9)	0.342[1]	0 (0.0)	0 (0.0)	1.000[1]	2 (20.0)	0 (0.0)	0.136[1]	1 (10.0)	0 (0.0)	0.305[1]	1 (20.0)	1 (20.0)	1.000[1]

Breastfeeding duration (month)	9.1 (7.2)	10.2 (7.7)	0.576[2]	8.3 (7.3)	9.8 (8.1)	0.567[2]	8.4 (9.1)	10.6 (6.8)	0.562[2]	9.3 (6.9)	7.0 (2.6)	0.379[2]	8.4 (5.3)	24 (-)	**0.041**[2]
Pets no. (%)	14 (26.9)	7 (20.0)	0.459[1]	7 (36.8)	3 (15.8)	0.141[1]	2 (20.0)	1 (10.0)	0.531[1]	2 (20.0)	2 (20.0)	1.000[1]	0 (0.0)	1 (20.0)	0.217[1]
Maternal diagnosis of asthma (ever), no. (%)	4 (7.8)	12 (34.3)	**0.002**[1]	1 (5.3)	4 (21.1)	0.150[1]	1 (10.0)	5 (50.0)	0.051[1]	0 (0.0)	1 (10.0)	0.330[1]	2 (28.6)	2 (40.0)	0.679[1]
Maternal diagnosis of hay fever	6 (11.8)	12 (35.3)	**0.009**[1]	2 (10.5)	6 (33.3)	0.092[1]	1 (10.0)	5 (50.0)	**0.**051[1]	0 (0.0)	4 (40.0)	**0.033**[1]	2 (28.6)	2 (40.0)	0.679[1]

(ever), no. (%)														
Maternal diagnosis of atopic eczema (ever), no. (%)	3 (5.9)	7 (20.0)	**0.045**[1]	0 (0.0)	2 (10.5)	0.146[1]	0 (0.0)	4 (40.0)	**0.025**[1]	0 (0.0)	1 (10.0)	0.330[1]	1 (14.3)	0 (0.0)	0.377[1]
Paternal diagnosis of asthma (ever), no. (%)	2 (4.3)	4 (11.8)	0.213[1]	1 (5.6)	1 (5.3)	0.969[1]	0 (0.0)	1 (10.0)	0.305[1]	0 (0.0)	1 (10.0)	0.357[1]	0 (0.0)	0 (0.0)	1.000[1]
Paternal diagnosis of hay fever	6 (12.5)	12 (35.3)	**0.014**[1]	4 (22.2)	5 (26.3)	0.772[1]	1 (10.0)	5 (50.0)	0.051[1]	0 (0.0)	4 (40.0)	**0.043**[1]	1 (16.7)	1 (20.0)	0.887[1]

(ever), no. (%)															
Paternal diagnosis of atopic eczema (ever), no. (%)	2 (4.2)	4 (12.1)	0.179[1]	1 (5.6)	2 (11.1)	0.546[1]	1 (10.0)	2 (20.0)	0.531[1]	0 (0.0)	1 (10.0)	0.357[1]	0 (0.0)	1 (20.0)	0.251[1]

Total number of subjects might differ slightly because of available data. Data are indicated as mean (SD). p-value derives from [1]Pearson´s Chi-squared test or [2]Linear Model ANOVA.

267 **B) PAULINA/PAULCHEN cohort**

Parameter	HC	Asthma	*P* value
N	23	23	
Female sex, no. (%)	12 (52.2)	11 (47.8)	0.768[1]
Smoking during pregnancy, no. (%)	1 (4.3)	2 (8.7)	0.550[1]
Maternal diagnosis of asthma (ever), no. (%)	1 (4.3)	4 (17.4)	0.155[1]
Maternal diagnosis of hay fever (ever), no. (%)	4 (17.4)	5 (21.7)	0.710[1]
Maternal diagnosis of atopic eczema (ever), no. (%)	4 (17.4)	2 (8.7)	0.381[1]
Paternal diagnosis of asthma (ever), no. (%)	1 (4.3)	2 (8.7)	0.550[1]
Paternal diagnosis of hay fever (ever), no. (%)	7 (30.4)	7 (30.4)	1.000[1]
Paternal diagnosis of atopic eczema (ever), no. (%)	2 (8.7)	2 (8.7)	1.000[1]

268

269 Total number of subjects might differ slightly because of available data. Data are indicated as mean (SD). p-value derives from [1]Pearson´s Chi-squared

270 test.

C) TRILATERAL cohort

Parameter	HC	AA	*P* value
N	67	32	
Rural region (Conghua), no (%)	39 (58.2)	15 (46.9)	0.289[1]
Female sex, no. (%)	36 (53.7)	10 (31.2)	**0.036**[1]
Age (y)	7.5 (0.8)	7.6 (1.1)	0.596[2]
Skin-prick test positive[3], no (%)	29 (43.3)	32 (100.0)	<0.001[1]

Total number of subjects might differ slightly because of available data. Data are indicated as mean (SD). p-value derives from [1]Pearson´s Chi-squared test or [2]Linear Model ANOVA. [3]For skin-prick testing, a wheal size ≥ 3 mm was defined as a positive reaction to at least one of the eight aeroallergens: *Dermatophagoides pteronyssinus* (Der p), *Dermatophagoides farinae* (Der f), *Blomia tropicalis*, cat and dog dander, cockroach, ragweed, and mugwort pollen

Table S2: Sequences of forward primers used for qPCR analysis

Gene	Primer sequence (forward primer, 5´- 3´)
18S	AGTCCCTGCCCTTTGTACACA
DUSP1	CTCAAAGGAGGATACGAAGCG
ERK2	GATCTCAAGATCTGTGACTTTGG
ERK5	ACCAGTCTTTCGACATGGG
IFNγ	TGGGTTCTCTTGGCTGTTACTG
IL1α	GCCCAAGATGAAGACCAACCAG
IL1β	TGGCAATGAGGATGACTTGTTC
IL4	TCTTTGCTGCCTCCAAGAACAC
IL10	CTACGGCGCTGTCATCGAT
IL18	AACAAACTATTTGTCGCAGGAAT
JNK2	CATAGAGATTTGAAGCCTAGCA
NIK	CTTGGTTGGGGAGATCGGC
p38	ACCAGACAGTTGATATTTGGTCAG
TAK1	TGTAGAGCTTCGGCAGTTATCC
TNFAIP3	GCCCAGGAATGCTACAGATACCC
TLR4	CTCAACCAAGAACCTGGACCTG

Table S3: List of antibodies used for mass cytometry.

Target	Clone	Mass number	Metal	Source	Dilution
CD11b	ICRF44	209	Bi	Fluidigm	1:200
CD28	CD28.2	160	Gd	Fluidigm	1:400
CD69	FN50	144	Nd	Fluidigm	1:200
CD44	BJ18	166	Er	Fluidigm	1:1600
CCR7=CD197	G043H7	159	Tb	Fluidigm	1:200
CD14	M5E2	175	Lu	Fluidigm	1:1600
CD19	HIB19	142	Nd	Fluidigm	1:3200
CD192 (CCR2)	K036C2	153	Eu	Fluidigm	1:3200
CD25 (IL-2R)	2A3	149	Sm	Fluidigm	1:800
CD3	UCHT1	170	Er	Fluidigm	1:1600
CD4	RPA-T4	145	Nd	Fluidigm	1:1600
CD45RA	HI100	169	Tm	Fluidigm	1:400
CD45RO	UCHL1	165	Ho	Fluidigm	1:100
CD56	NCAM16.2	176	Yb	Fluidigm	1:3200
CD8	RPA-T8	146	Nd	Fluidigm	1:3200
HLA-DR	L243	174	Yb	Fluidigm	1:1600
phospho-p44/42 MAPK (Erk1/2)[T202/Y204]	D13.14.4E	167	Er	Fluidigm	1:400
phospho-p38(T180/Y182)	D3F9	156	Gd	Fluidigm	1:200
Helios	22F6	143	Nd	Biolegend	1:3200
FOXP3	PCH101	162	Dy	Fluidigm	1:200

Table S4: Lineage marker expression criteria for manual gating of cell populations

Cell population	Parent Population	Expression of lineage markers
PBMCs	All events	$Bead^-$, $dead^-$, $long\_eventlength^-$, extreme_DNA
Cytotoxic T-cells	PBMCs	$CD3^+$, $CD19^-$, $CD4^-$, $CD8^+$
T-helper cells	PBMCs	$CD3^+$, $CD19^-$, $CD8^-$, $CD4^+$
Regulatory T-cells (Tregs)	T-helper cells	$CD25^+$, $FOXP3^+$
T-helper type 2 cells (Th2)	T-helper cells	$CD11b^-$, $CD14^-$, $GATA3^+$, $Helios^+$
Naive T-helper cells	T-helper cells	$CCR7^+$, $CD45RO^-$
B-cells	PBMCs	$CD3^-$, $CD19^+$, $CD69^+$
Natural Killer (NK)-cells	PBMCs	$CD3^-$, $CD19^-$, $CD14^-$, $HLA\text{-}DR^-$, $CD69^+$, $CD56^+$
Monocytes	PBMCs	$CD3^-$, $CD19^-$, $CD14^+$, $HLA\text{-}DR^+$, $CD11b^+$
Dendritic cells (DC)	PBMCs	$CD3^-$, $CD19^-$, $CD14^-$, $HLA\text{-}DR^+$, $CD56^-$

Figure Legends

Figure S1: Flow Chart of study participants

Flow diagram summarizing all experiments and study participants of the three pediatric cohorts, two cross-sectional studies (CLARA/CLAUS, TRILATERAL), and a birth cohort study (PAULINA/PAULCHEN) with a total of 232 out of 1062 children.

Figure S2: MAPK signaling pathway

Pro-inflammatory (labeled in red) and anti-inflammatory (labeled in green) key players of MAPK signaling and interacting NF-κB pathway.

Figure S3: Inverse correlation of *DUSP1* gene expression and HDM-allergen IgE classes

Correlation of *DUSP1* gene expression (Δct) measured by qPCR in unstimulated PBMCs after 24h of cultivation and IgE classes to the HDM-allergens A) D. pteronyssinus and B) D. farinae measured in serum by MediWiss AllergyScreen in N=38 (19 HC/19 AA). Correlation is given by Pearson r. The corresponding p-value derived from two-tailed t-test.

Figure S4: PBMCs of healthy and asthmatic school-age children do not differ in their expression of pERK1/2 and pp38 under unstimulated conditions.

Scatter plots of phosphorylated protein expression of A) ERK1/2 and B) p38 in unstimulated PBMCs of school-age children (CLARA/CLAUS cohort, N=20 (10 HC/10 AA)) analyzed by mass cytometry (CyTOF). 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 means. Phenotypical comparison and corresponding p-values were calculated by t-test analysis.

Figure S5: Time dependent stimulatory effects of farm-dust and LPS with optimal immunomodulation upon 24 hours

Up-/ Downregulation of gene expression as fold change (log_2) of farm-dust (dark grey) and LPS (light grey) stimulated to unstimulated conditions at various time points (0.5, 1, 2.5, 6, 12, 24 hours) of A) *DUSP1* B) *TNFAIP3* C) *TLR4* D) *IL18* E) *IL-4, IFNγ, IL1α, IL1β* and *IL10*. The charts show the sample means with the corresponding standard deviation (SD). F) *IFNγ* and *IL4* upon German farm-dust and LPS stimulation. Gene expression was measured by qPCR in PBMCs of three healthy children (1h, 12h, 24h: N=3; 2.5h: N=2, 0.5h, 6h: N=1).

Figure S6: Anti-inflammatory effects of farm dust stimulation are beyond the effects of LPS

Fold change of gene expression measured by qPCR in PBMCs of N=7 (4HC/3AA) children of the CLARA/CLAUS cohort without stimulation (U) and upon German(G)/Finnish(Fi) farm dust or LPS stimulation with (+) or without (-) co-stimulation of LPS-blocking LPS-RS for 24h. **Figure S7: Farm dust or LPS stimulation downregulates pERK1/2 expression while total ERK1/2 and p38 expression is not decreased**

A) Scatter plot of the protein expression of ERK1/2, pERK1/2, p38, and pp38 measured in unstimulated (U), German (G), and Finnish (Fi) farm dust or LPS stimulated PBMCs (24h) in N=8 (5 HC/ 3 AA) by Western Blot analysis. Protein expression is normalized to the loading control Vinculin and shown as a fold change (log2) compared to the unstimulated condition. Significant up- or downregulation of protein expression compared to unstimulated conditions is marked by asterisks (*$p<0.05$). P-values derived from a paired t-test. B) Four representative images of Western blot analysis for total and phosphorylated protein expression of ERK1/2 and p38 and their corresponding Vinculin expression in unstimulated (U) and German (G) and Finnish (Fi) farm dust and LPS stimulated PBMCs (24h) of one healthy control (left) and one asthmatic child (right). Each lane contained 20µg total protein.

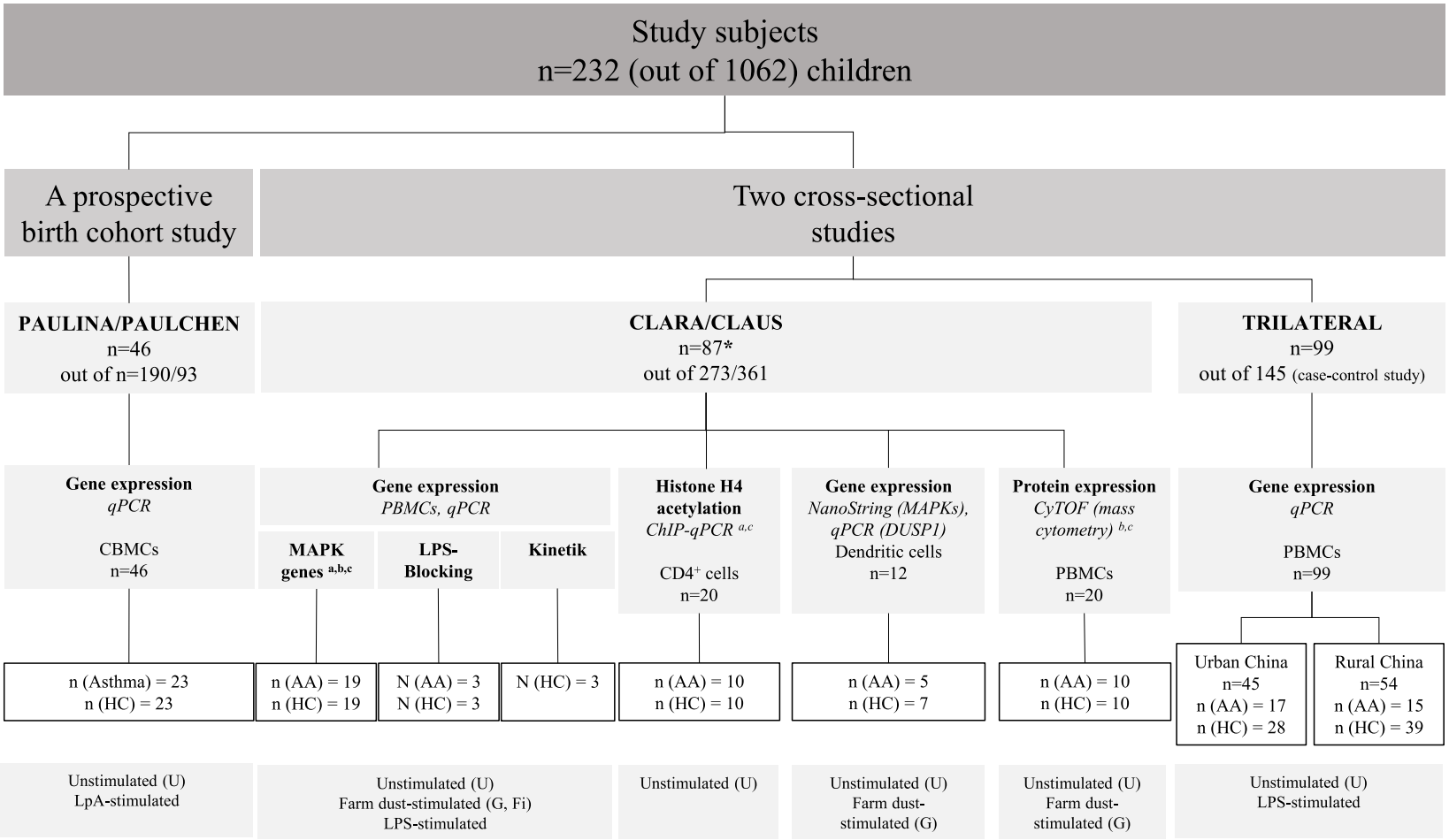
Figure S8: *In vivo* exposure to rural environments downregulates pro-inflammatory *MAPK14* expression

Scatter plot for A) *DUSP1*, B) *MAPK1* and C) *MAPK14* gene expression stratified for region (urban Hong Kong and rural Conghua, China) measured by qPCR in unstimulated PBMCs of 7-year-old children of the TRILATERAL cohort analyzed after 24h cultivation in N=99 (45 Hong Kong/54 Conghua). 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 adjusted for sex, age and lab location.

Figure S9: Gating strategy for analysis of mass cytometry data

PBMCs were incubated with different metal-labeled antibodies and measured by mass cytometry (CyTOF). Generated FCS files were normalized and manually gated by the FlowJo software based on the expression of the corresponding lineage markers. Population frequencies are related to the parent population.

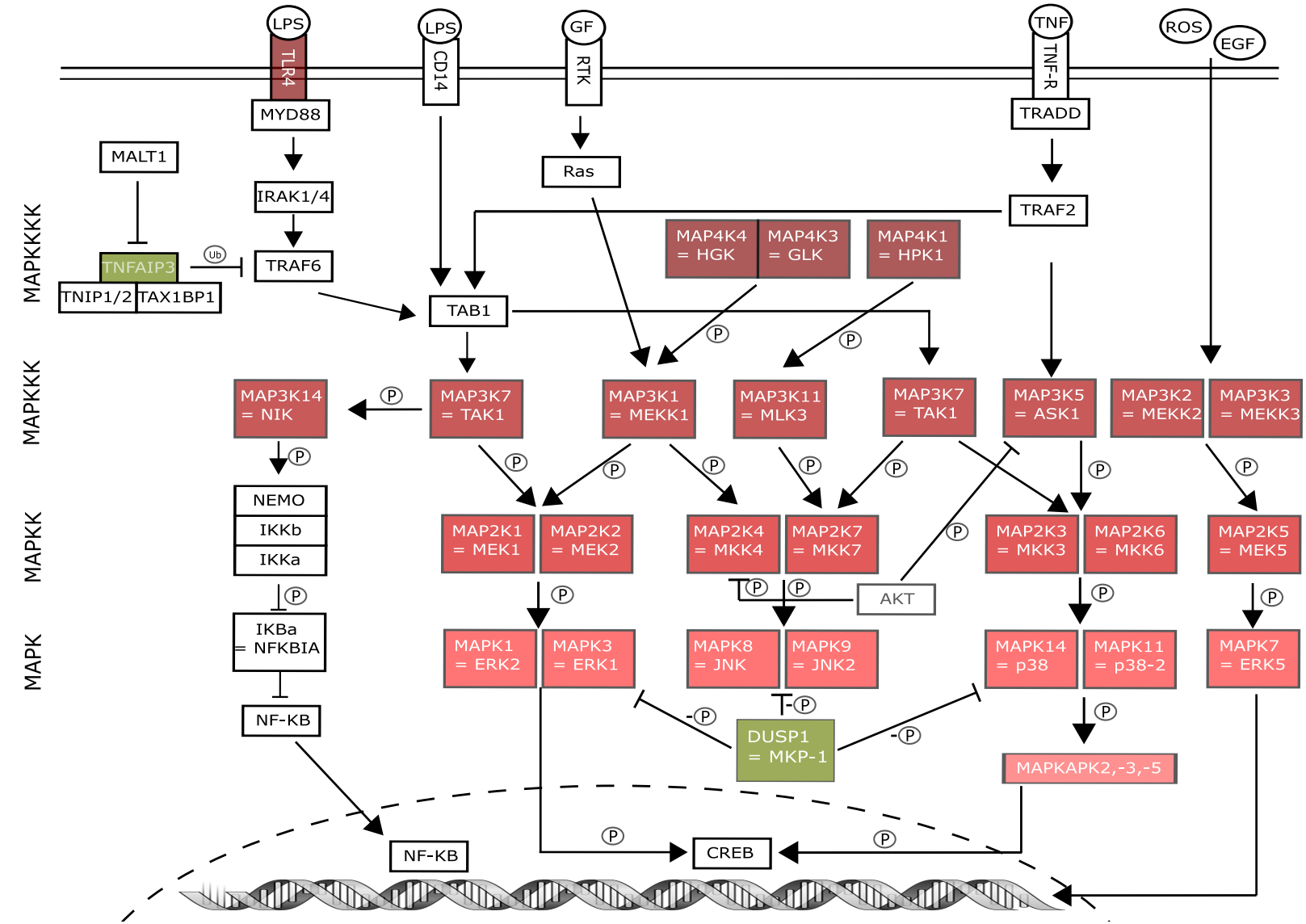
Supplementary Figure S1



*the total number of children included in the CLARA/CLAUS cohort for this study differs from the sum of the case numbers of the sub-analyses because some children could be included for more than one analysis when enough material was available. Some sub-analysis were possible in up to 3 identical children.

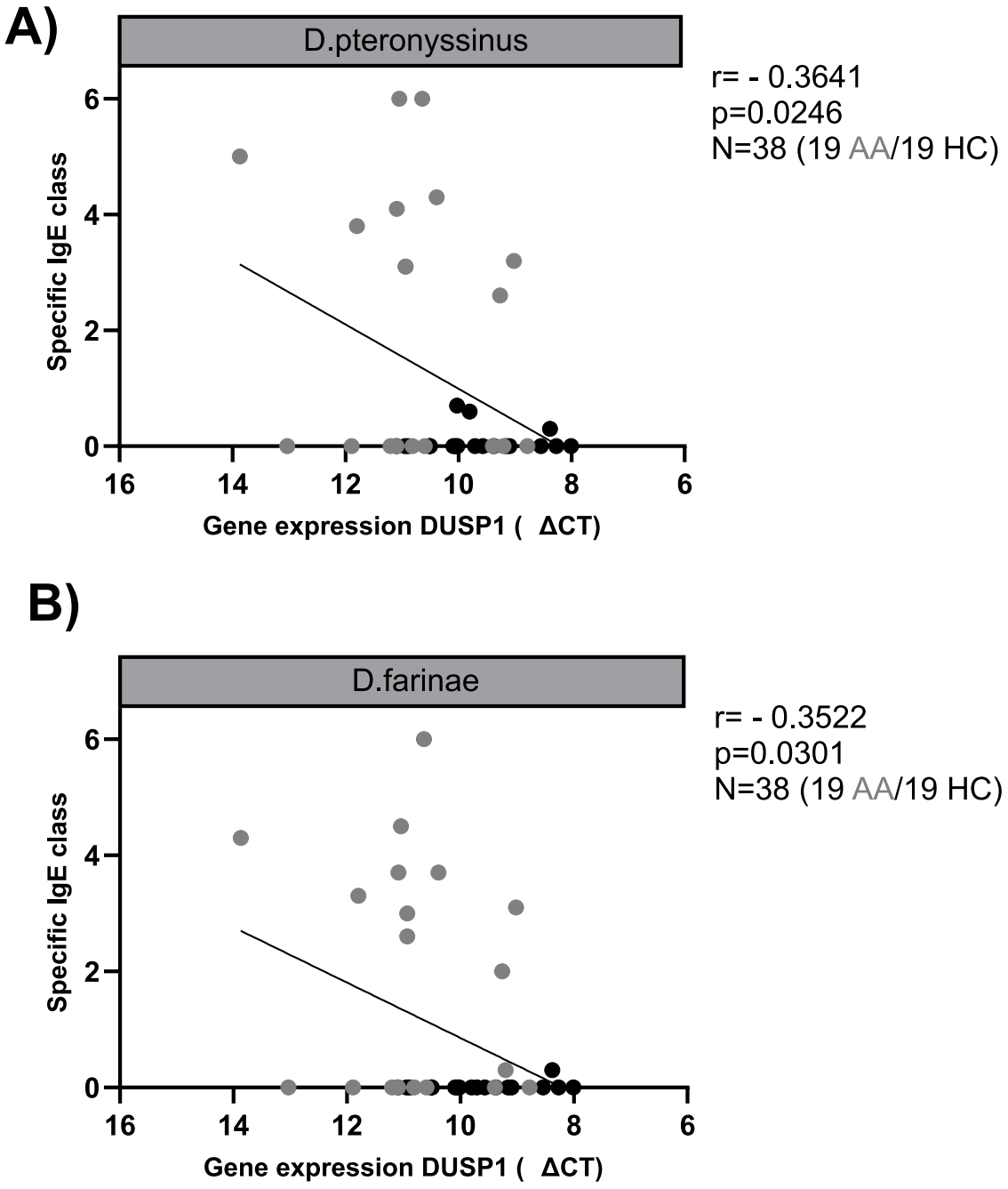
[a,b,c] The overlap of samples used for different approaches was as following: a) N=1 AA sample was used for qPCR (MAPK genes) and histone H4 acetylation measurements, b) N=4 AA samples were used for qPCR (MAPK genes) and CyTOF analysis and c) N=2 AA samples were used for qPCR (MAPK genes), histone H4 acetylation and CyTOF measurements.

Supplementary Figure S2



Supplementary Figure S3

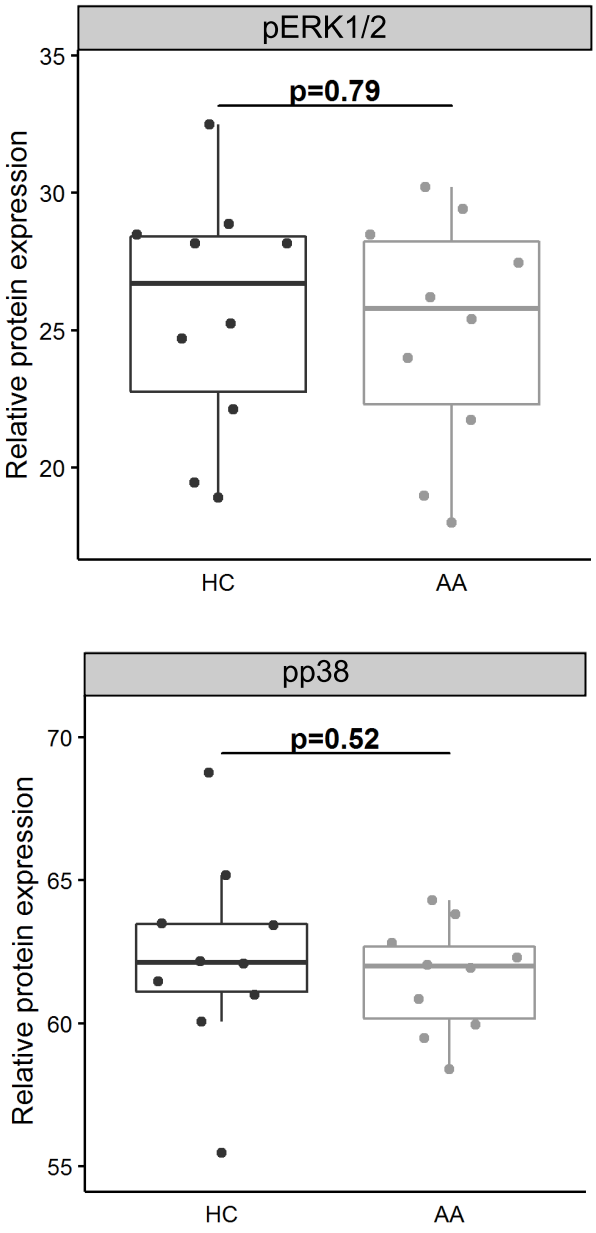
Clara/Claus



Supplementary Figure S4

Clara/Claus

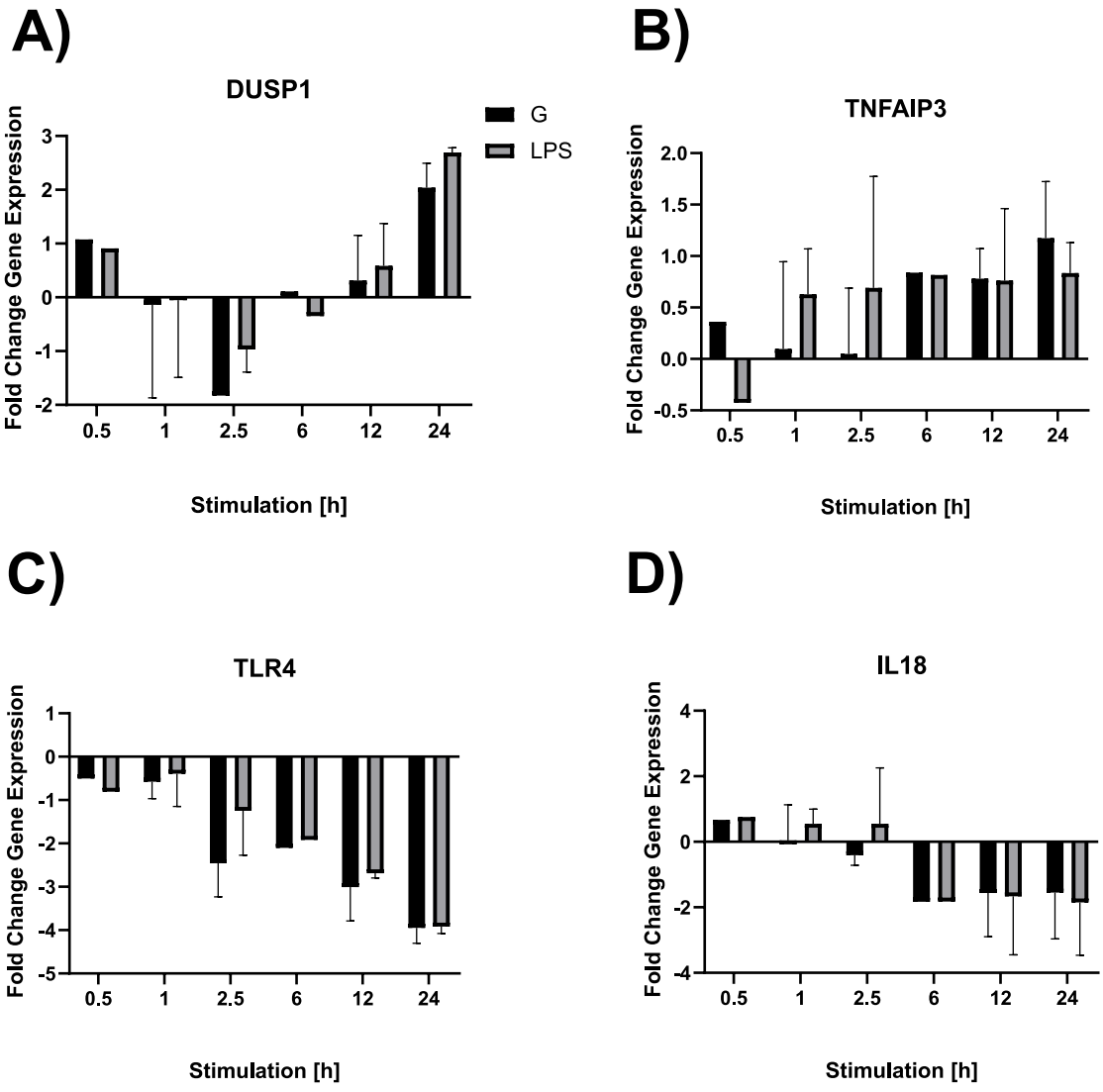
PBMC

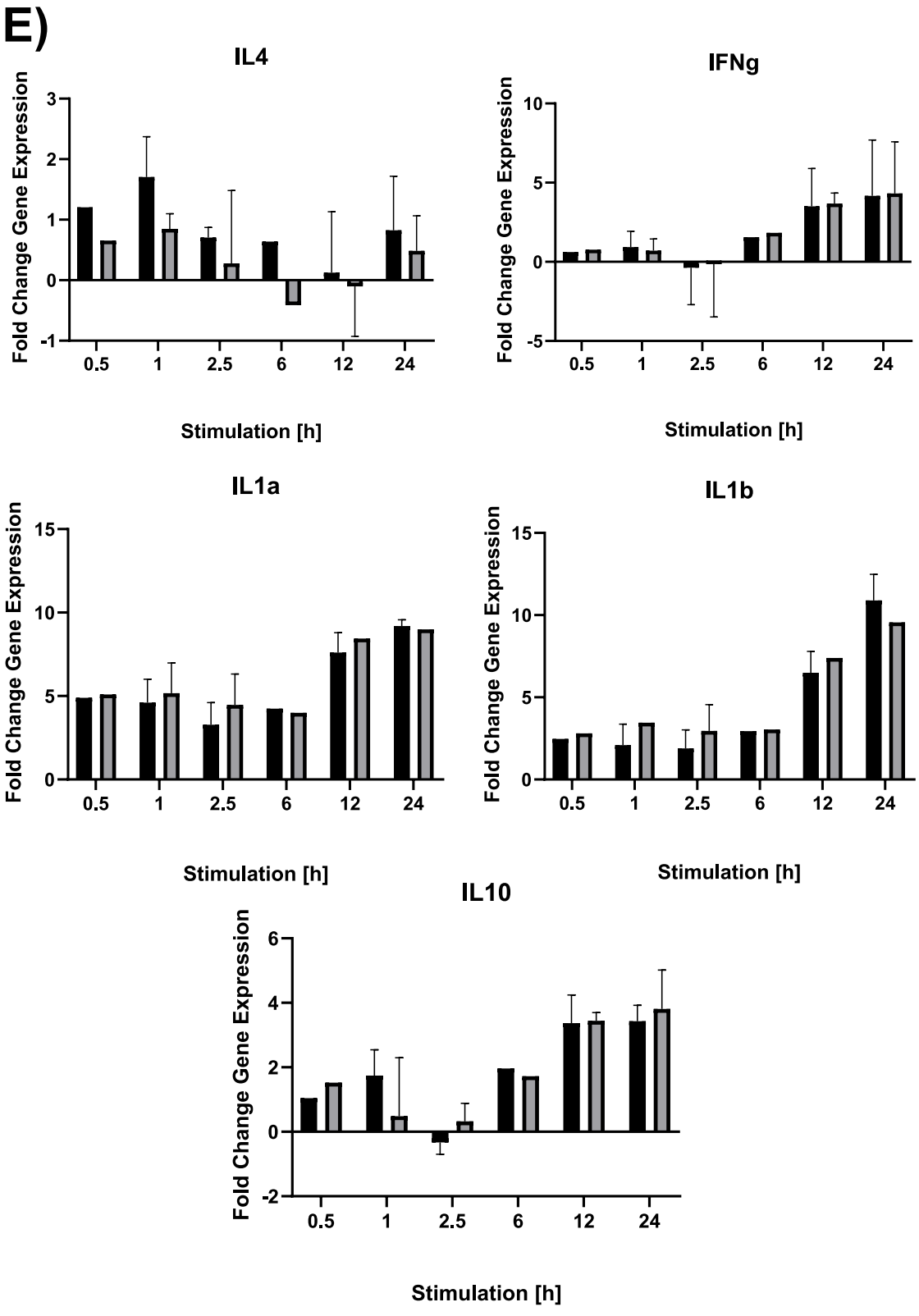


Supplementary Figure S5

Clara/Claus

PBMCs




E)
IL4
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Stimulation [h]
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Stimulation [h]
IL1a
Fold Change Gene Expression
15
10
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Stimulation [h]
IL1b
Fold Change Gene Expression
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Stimulation [h]
IL10
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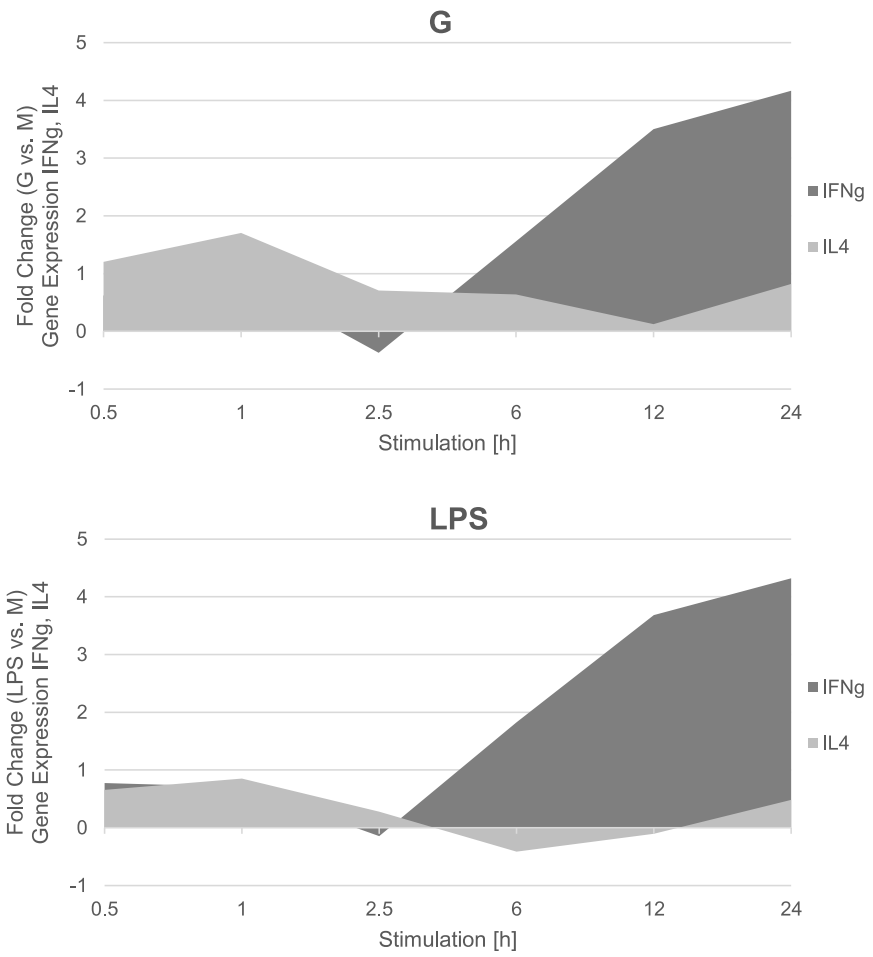
Stimulation [h]

F)


G
Fold Change (G vs. M)
Gene Expression IFNg, IL4
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Stimulation [h]
IFNg
IL4
LPS
Fold Change (LPS vs. M)
Gene Expression IFNg, IL4
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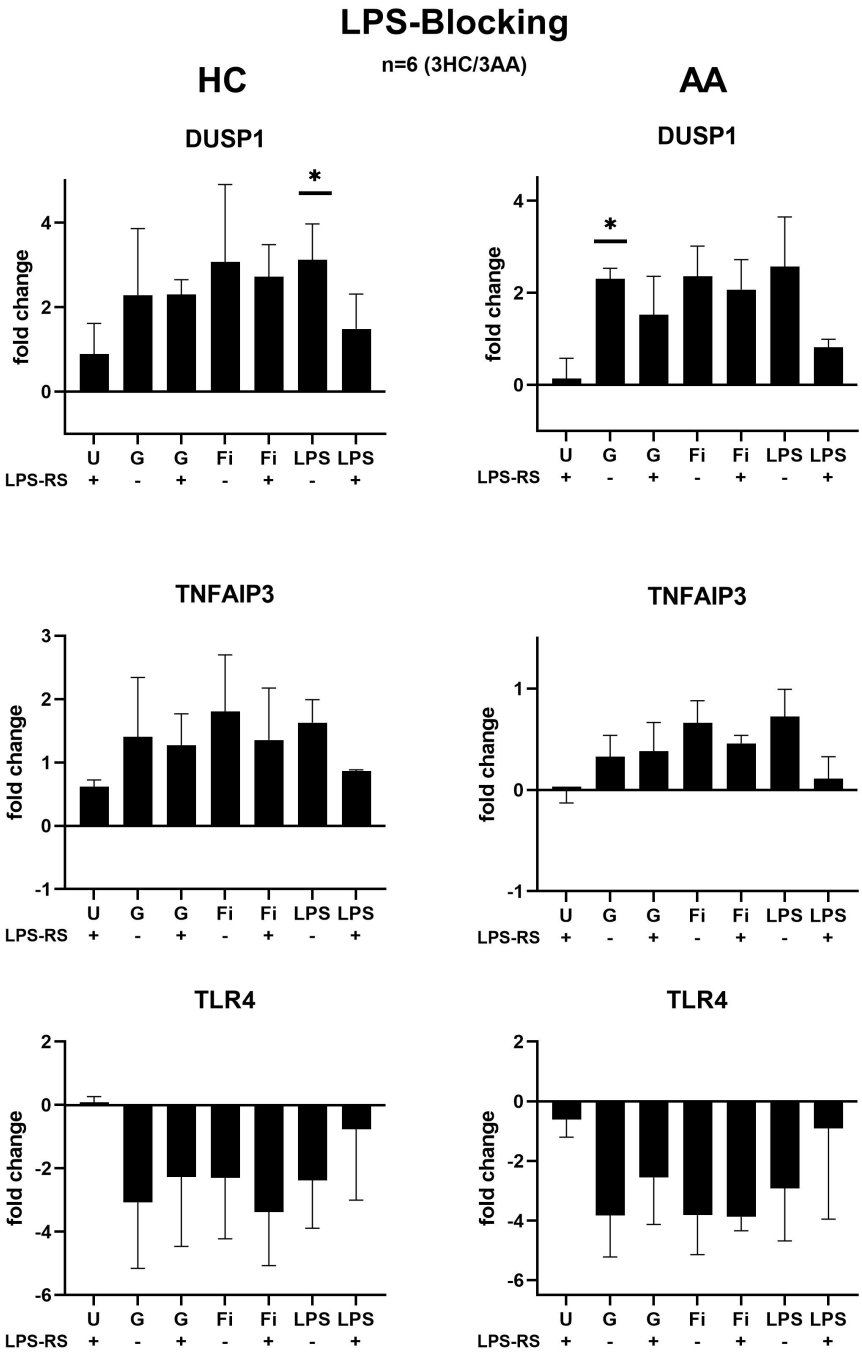
Stimulation [h]
IFNg
IL4

Supplementary Figure S6
Clara/Claus

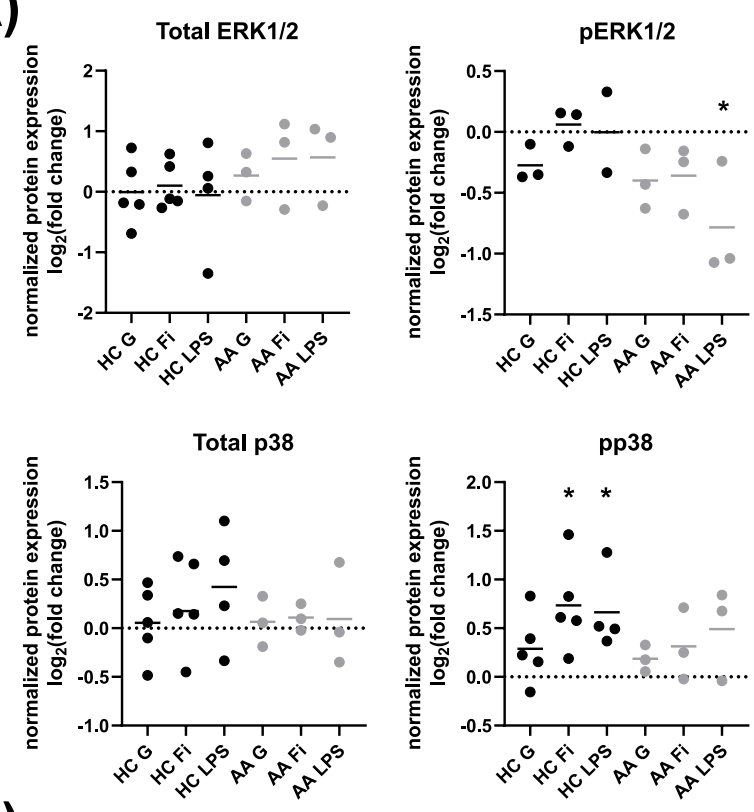
PBMCs



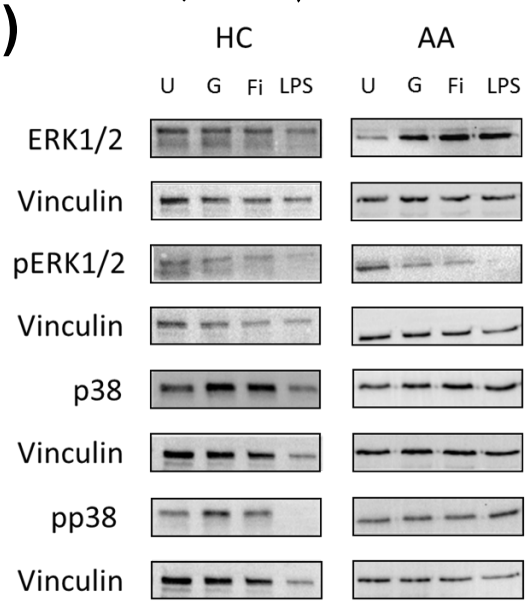
Supplementary Figure S7 Clara/Claus

PBMCs

A)

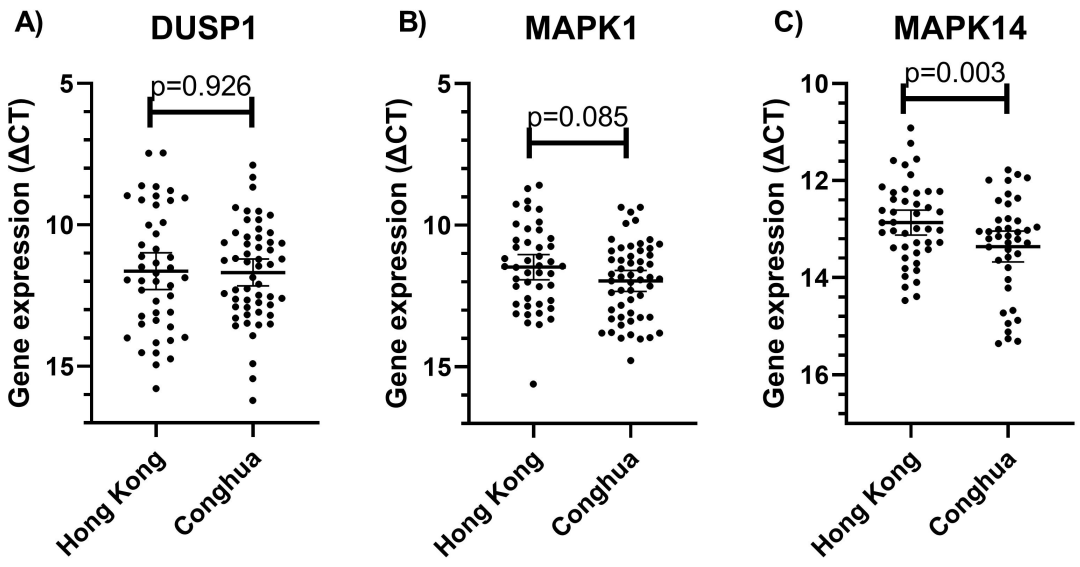


B)

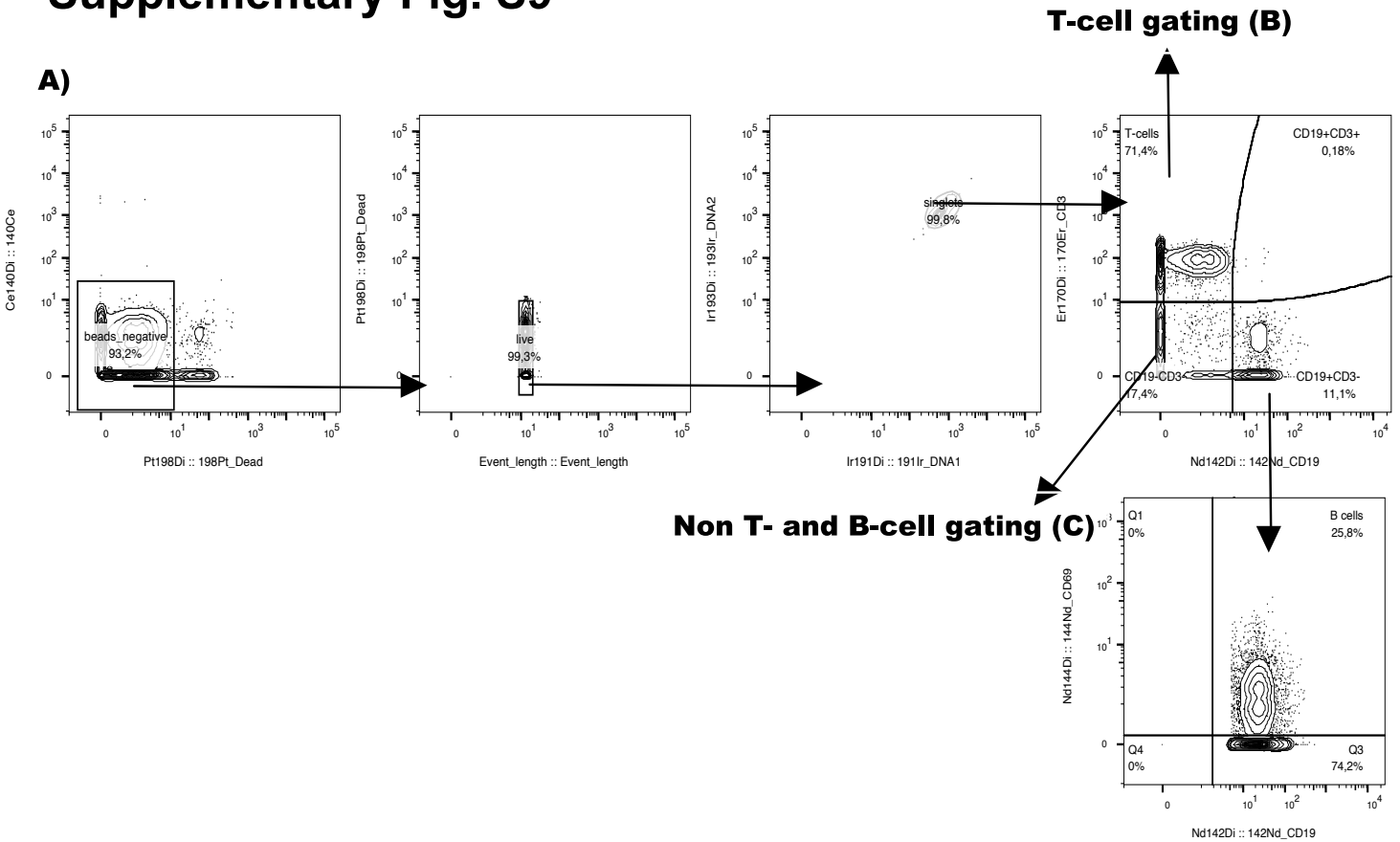


Supplementary Figure S8
TRILATERAL

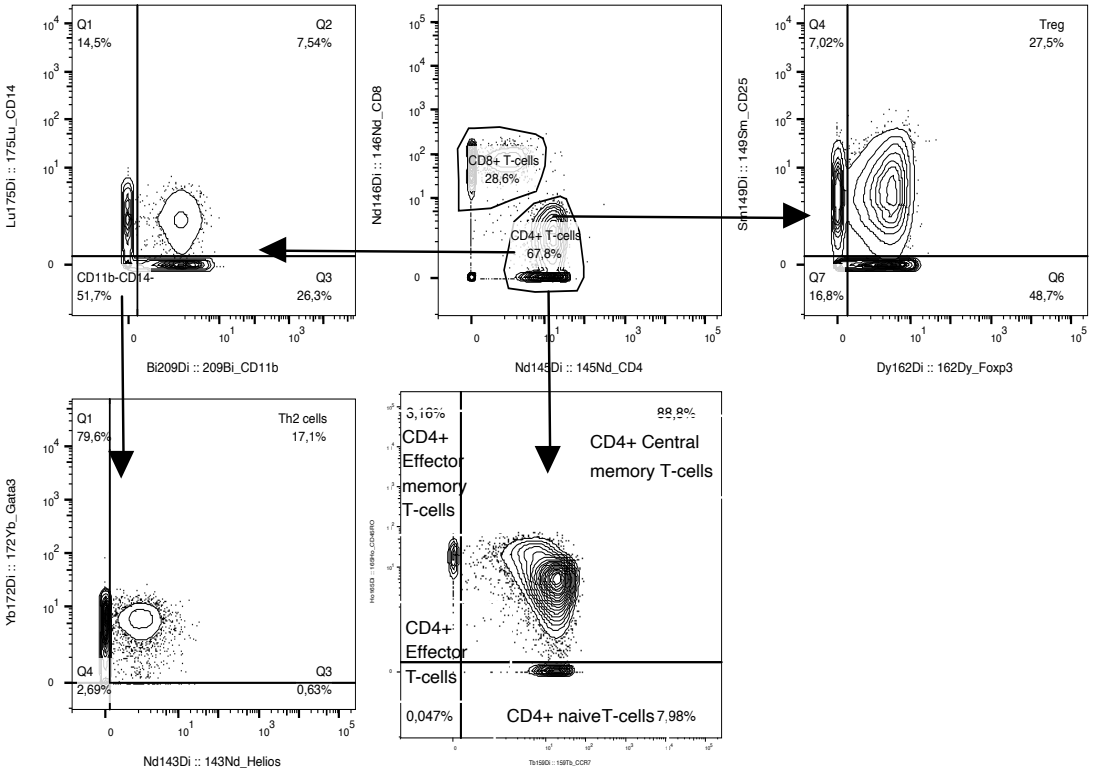
PBMCs



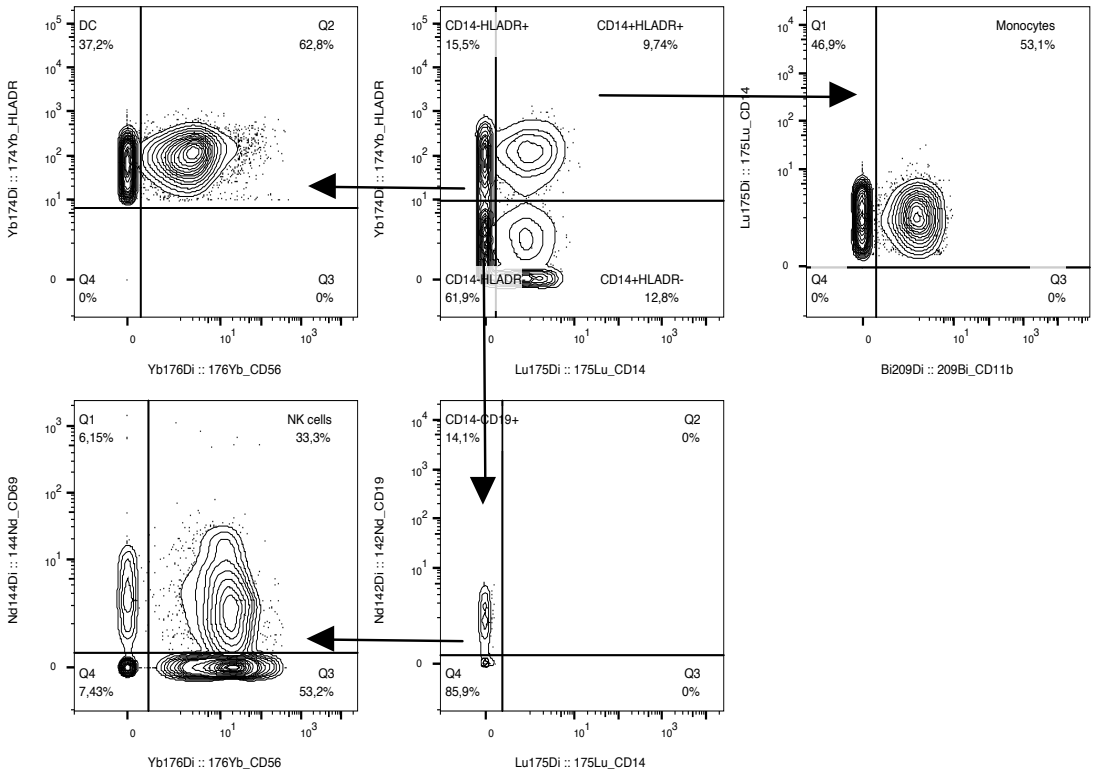
Supplementary Fig. S9



B) T-cell gating



C) Non T- and B-cell gating



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Appendix A: Unpublished manuscript: Farm-dust mediated protection of childhood asthma: Identification of unique cellular and molecular regulatory mechanisms

Farm-dust mediated protection of childhood asthma: Identification of unique cellular and mo-lecular regulatory mechanisms

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Manuscript in preparation, 2022

Farm-dust mediated protection of childhood asthma: Identification of unique cellular and molecular regulatory mechanisms

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Abstract

The protective farm effect on childhood asthma was replicated numerously in epidemiologic studies. This study aimed to identify detailed immunological mechanisms underlying the farm effect by investigating peripheral blood mononuclear cells of school-age allergic asthmatics (AA) and healthy children (HC) that were cultivated *ex vivo* with and without farm-dust extracts on cellular, molecular and functional levels. Single-cell protein expression was analyzed by mass cytometry (CyTOF). Isolated dendritic (DC) and regulatory T cells (Treg) were analyzed on multiplex gene expression level (NanoString), functional *ex vivo* stimulation of monocyte-derived DC (moDC) was performed and T cell proliferation was monitored.

Ex vivo farm-dust stimulation induced immunological tolerance mechanisms by a) anti-inflammatory regulation, b) reduced antigen presentation and c) mitigated T cell response. Decreased APC numbers and their HLA-DR-expression, induction of anti-inflammatory NF-κB signaling, and co-stimulatory molecules in moDC were demonstrated in HC but also manifest AA. Moreover, subsequent T cell activation was inhibited as shown by downregulation of TCR, the activating co-stimulatory molecule CD28, and reduced T cell proliferation. While B cell numbers and central memory T cell were induced, effector memory T cells were downregulated upon stimulation. Exclusively in AA, NK-cells, cytotoxicity-related protein expression, and Th2- and Th17-associated GATA3 and RORγ were downregulated in CD4+ and/or CD8+ T cell subpopulations upon dust stimulation. While dust-stimulation increased Treg-frequency exclusively in HC, oxidative stress-related TXNIP was downregulated in Tregs of both phenotypes.

Our study which investigated the immunological mechanisms of the farm effect on a multidimensional level using state-of-the-art technologies revealed in-depth immunological tolerance induction. Farm-dust stimulation resulted in decreased innate antigen presentation, anti-inflammatory regulation, decreased catalytic NK-function, downregulation of Th2/Th17 immunity, together with inhibition of T-effector-cell activation and proliferation in manifest AA. These anti-allergic tolerogenic properties suggest a potential future therapeutic value of the strong “asthma-protective environment”.

Introduction

Childhood asthma belongs to the most common chronic diseases in childhood. The pathophysiology involves complex mechanisms and several immune cells, including innate dendritic cells (DC), but also Th2-cells and regulatory T cells (Treg). Contrary to the high prevalence in industrialized regions, children from rural and especially farming areas suffer significantly less from allergic diseases including asthma.[1–4] Microbial substances common in farming environments like lipopolysaccharide (LPS), the cell wall component of gram-negative bacteria are involved in this protection.[3,5] DC and other antigen-presenting cells (APC) play an important role by representing the first contact with microbial substances, interacting with T cells and triggering their differentiation and subsequent immune response. Farm exposure was associated with reduced DC numbers including both subtypes myeloid mDC and plasmacytoid pDC.[6] Farm milk consumption was linked with reduced circulating pDC numbers[6], whereas children growing up in farming environments had lower mDC2 levels.[7] Bacterial metabolites inhibited Th2-inducing DC while inducing Th1-promoting immunity together with reduced numbers of mDC2 and monocyte-derived DC (moDC).[7–9] LPS can modulate the stimulatory effect of DC to promote Treg and prevent Th2 and IgE response by inhibiting the migration of DC to the lymph node.[7] Dose and timing are hereby critical. While acute short-term stimulation with high-dose LPS results in inflammatory immune responses, chronic low-dose exposure can trigger LPS tolerance - a state of hyporesponsiveness of immune cells and anti-inflammatory signaling.[10] T cell responses were also modulated by farm exposure e.g. as farm children have induced Treg numbers and reduced T cell responses.[11–13] Besides protection via *in vivo* farm exposure, we have disentangled anti-inflammatory capacities of *ex vivo* farm-dust stimulation of PBMCs of asthmatic children, indicating a potential therapeutic role of these modulatory substances.[14,15] However, more specific immunological mechanisms including regulation of gene and protein expression within distinct immune cells upon *ex vivo* farm-dust stimulation are still not well defined. This study examined detailed regulation of *ex vivo* farm-dust stimulation in peripheral blood cells of children with manifest allergic asthma compared to healthy controls. By analyzing cellular frequencies and protein expression in peripheral blood of healthy and asthmatic children on single-cell levels via mass cytometry (CyTOF), multiplex gene expression (NanoString) in isolated DC and Tregs and functional experiments, we aimed to disentangle immunological mechanisms of environment-mediated protection of asthma on molecular, cellular, and functional levels.

Methods

Study population

Children at the age of 4-17-years were recruited at LMU children´s hospital within the cross-sectional CLARA/CLAUS cohort.[14,16,17] Allergic mild-to-moderate asthmatics were defined by doctors diagnosis of asthma according to GINA guidelines[18], at least 3 wheezing episodes, classical asthma symptoms and use of asthma medication, specific IgE-levels >0.35 IU/mL, and a significant reversible airflow obstruction indicated by lung function testing (ATS/ERS guidelines).[14,15,16,19] Written informed consent was given by the parents. The study approval was given by the local ethics board, LMU Munich, Germany, Nr. 379-08.

Cell cultivation and farm-dust stimulation

PBMCs were isolated within 24 hours of blood withdrawal and cultivated in X-Vivo at $5x10^6$ c/mL with (G) and without (U) 40 µg/mL dust extract collected by electrostatic dust collectors placed for 4 weeks in the cowshed of a German farm and extracted by a standardized cold extraction method.[14,15,20,21] Upon 24h cultivation at 37°C, 5% CO_2 cells were harvested. For mass cytometry, the collected cells were stored in liquid nitrogen.

NanoString

Gene expression was measured in N=48 samples. Dendritic cells (DC) and regulatory T cells (Treg) were isolated from 24h cultivated unstimulated and farm-dust stimulated PBMCs of 7 HC and 5 AA using the DC and Treg isolation kit (Miltenyi) and the AutoMACS. The subpopulation was representative to the total cohort, AA included in this approach had higher FEV1/FVC levels compared to the whole population.[15] RNA (100ng for DC, 50ng for Treg) was extracted and hybridized for 18h at 65°C. Gene expression was measured with the NanoString PanCancer Immune Profile Panel and raw data exported from nSolver Analysis Software v3.0. Bead counts were standardized on positive controls (standard curve). Genes with expressions below the detection limit, defined as three standard deviations above the mean of the negative controls were excluded from analysis. Gene expression was normalized to the expression of the 33 (DC) housekeeper genes included in the panel, that were expressed above the detection limit. In Tregs, normalization was based on two housekeeping genes, as the others were below the detection limit due to generally low expression in Tregs. Pathway analysis was performed using the annotations provided by NanoString. Group differences were assessed by t-tests.

Mass cytometry

A schematic representation of the CyTOF workflow is shown in Fig.1. Mass cytometric measurements were performed as described before.[15] Briefly, thawed PBMCs were stained with cisplatin, barcoded, and stained with surface metal-tagged antibodies. Upon fixation and permeabilization, samples were stained with intracellular antibodies (Suppl.Tab.1). Fixed cells were stained with $^{191/193}$Iridium and resuspended in calibration beads. Measurements were performed on a CyTOF-2.1 mass cytometer using Helios software (version 6.5.358): acquisition flow rate of 0.03 ml/min, default thresholding scheme, lower convolution threshold of 800 intensity units, active noise reduction, minimum and maximum event duration of 8 and 100 pushes, respectively. fcs-files were normalized with standalone MATLAB application Normalizer 0.3.

Manual cell identification

After removal of beads, dead cells, and doublets, live single cells (mean(SD): 269.647 (84.621)) were gated into known cell populations (Suppl.Fig.1). The inter-sample variability is shown in Suppl.Fig.2A. Overall, 87% (9.4M) of live single cells were assigned to at least one manually labeled gate, whereas 13% (1.4M cells) were not assigned and further characterized. Their marker expression is shown in Suppl.Tab.2. Of note, $CD4^+$ T cells were gated three times to differentiate between Treg and Th2-cells.

Semi-supervised cell identification

For cell clustering, we followed the approach of FlowSOM similar to the CyTOF workflow proposed by Nowicka et al.[22,23] We used cells from healthy unstimulated samples for cell discovery and transferred those to the other samples. The key elements were (Fig.1): (1) Removal of dead cells, (2) selection of unstimulated (U) HC samples and downsampling to 10,000 cells/sample, (3) transformation of the 22 lineage markers, (4) training of a Kohonen Map with 900 nodes on a toroidal space equipped with hexagonal topology, (5) dimension reduction (22 lineage markers to 6 dimensions), (6) consensus clustering of Kohonen codes, (7) manual naming and merging of clusters, and (8) projection of all cells to Kohonen map and transfer of cluster. This resulted in a unique cell-to-cluster mapping. Low frequent clusters were joined to highly similar clusters, ie: Cluster 10 (1.10%) was merged to cluster 6, cluster 8 (0.10%) to cluster 1, cluster 20 (0.30%) to cluster 17, and cluster 19 (<0.01%) to cluster 14.

Cell frequency analysis

Differences in cellular composition (manual and cluster cell populations) were studied on population level. For each population, differences in frequencies (defined as percentage parent

cells) were studied using a random effect linear regression model on *log*-transformed frequency using the *lme4*-package:

$$\log(abundancy) \sim I_{phenotype==AA} * I_{stimulus==G} + (1|sample)$$

As our study design has two conditions per blood sample (unstimulated and stimulated), we modeled this degree of similarity using random intercept for each sample. Due to fitting the regression model only within each cell population separately and an expected difference not covering the whole [0;1]-interval, log transformation was adequate and sufficient to use a linear regression approach.

Marker expression analysis

The marker expression profile of a cell population is best summarized by a function mapping the marker range to the proportion of cells with an expression greater than a specific value. Traditional analysis strategies compared summary statistics (eg. Mean/median) but neglected this inherent functional nature of data origin. We aimed to investigate if HC and AA responded differently to stimulation in terms of cellular activity and followed basic principles of functional data analysis. For each sample and cell population, we calculated the cumulative density function of a marker's intensity, subtracted it from one, calculated a stabilized ratio between stimulated and unstimulated functions, and analyzed these functions using an Anova test for functional data.[24]

Details on cell identification, cell frequency analysis, and marker expression analysis can be found in the provided code (Supplementary Code 1-3).

Monocyte-derived dendritic cells (moDC)

CD14-positive cells (monocytes) were isolated (CD14 microbeads, Miltenyi Biotec) from PBMCs of 5 children (2HC/3AA) with a purity of >90% validated by CD14-FITC staining (Flow cytometry). Monocytes were cultivated in RPMI+10%FCS with 100 U/mL Penicillin, 100 µg/mL Streptomycin and 2nM L-Glutamine supplemented with 500 U/mL IL-4, 500 U/mL GM-CSF for 6 days (37°C, 5% CO_2). On day 4, fresh medium with 250 U/mL GM-CSF was added. Cells were stimulated with 40µg/mL farm-dust extract at day 7. RNA and fresh cells were harvested 24h upon stimulation (37°C, 5% CO_2). The experiment was repeated in PBMCs of two healthy adult donors. For flow cytometry analysis, non-differentiated monocytes and moDC were stained with CD14-FITC and measured on a FACS Canto II.

RNA isolation, cDNA synthesis, and quantitative RT-PCR

Concentration and quality of extracted RNA (RNeasy Mini Kit) from PBMCs, moDC, isolated DC, and Treg were measured using Nanodrop and/or Bioanalyzer. RNA of isolated DC and

Treg was concentrated (RNA Clean & Concentrator™-5 Kit). cDNA synthetization was perfomed using the QuantiTect Reverse-Transcription Kit. Quantitative real-time PCR was conducted as described previously.[14] 18S gene expression was used for normalization. Primer sequences are shown in Suppl.Tab.3. Gene expression (qPCR) was assessed by two-sample t-tests for stimulation effects and differences between phenotypes using R (version 3.3.1)[25,26] and GraphPad prism 9. Statistical significance was defined by a p-value ≤ 0.05.

T cell proliferation assay

Treg suppression assays were performed using blood samples of n=4 (2HC/2AA) children. Upon PBMC isolation and cultivation overnight, CD4+CD25+ T cells (Treg) were isolated (CD4+CD25+ Treg isolation kit). The CD4+CD25- fraction was purified by negative selection to get effector T cells (Teff). Purity of isolated cell populations was tested by FACS. Teff were stained with 5µM CFSE for 5 minutes, washed three times with PBS/10%FCS (1100 rcf, 10min, 20°C), and incubated in 2mL RPMI for 1-2 hours at 37°C, 5% CO_2. CFSE-stained Teff ($5x10^5$c/mL) were incubated with Treg ($5x10^5$c/mL) at different ratios and in presence of the stimulative Treg suppression inspector (CD2/3/28 beads) with and without farm-dust stimulation (40µg/mL). Upon incubation for 120 h, proliferation of Teff was analyzed by CFSE levels (flow cytometry).

Results

Semi-supervised clustering of PBMCs of asthmatic and healthy children identified T cell populations distinguished by DC markers beyond classical major cell populations

Mass cytometry was applied in unstimulated and farm-dust stimulated PBMCs of HC and AA. Besides the analysis of manually gated cell populations, we performed a semi-supervised clustering approach aiming to identify differences in yet undefined cell populations. The 20 resulting clusters were labeled based on their distribution of lineage marker expression (Fig.2A) and by comparing the attribution of manually gated cell types within each cluster (Fig.2B). The distribution of the 16 remaining clusters, resulting after summarizing low frequent clusters to their closest related cluster is shown as UMAP and a minimum spanning tree (Fig.2C). The clusters of non-activated B cells/DC, monocytes/DC and activated B cells, were clustered together in a characteristic antigen-presenting cloud (yellow, orange, red). The T cell-associated clusters are grouped in the upper part, which can be separated by T-helper cell clusters (different greens) and cytotoxic T cells (blue), NK-cells (pink), and double-negative ($CD4^-$, $CD8^-$; DN) T cells (purple). Cytotoxic $CD8^+$ T cells were grouped in two clusters, that might represent central and effector memory $CD8^+$ T cells based on CCR7 expression. Clustering of certain $CD4^+$ T-helper subpopulations identified novel T cell subpopulations that are distinguished by the expression of DC-associated markers CD141, BDCA1/2, and CD11b. By comparing individual differences of cluster frequencies within each sample, a variability of cluster frequencies was detected between all groups and both stimulation conditions (Suppl.Fig.2B). In summary, we have successfully performed clustering of the mass cytometric data and identified known and new cell populations including T cell subpopulations that are differentiated by CD141, BDCA1/2 and CD11b.

Under unstimulated conditions children with manifest allergic asthma showed altered gene and protein immune profiles with increased inflammatory properties

Under unstimulated conditions, no differences in the frequency of manually gated and clustered PBMC subpopulations were detectable between HC and AA (Fig.3A,B; Table 1,2 “AA U vs HC U”). However, when we investigated gene and protein expression in distinct innate and adaptive cells, phenotypical differences were demonstrated on baseline level without stimulation. Gene expression was measured in a multiplex approach (NanoString) in isolated DC and Treg of HC and AA. These cell populations were already associated with childhood asthma and its environment-mediated protection.[6,12,13,27,28] In unstimulated DC, 58 genes were significantly differentially expressed between the phenotypes, with 32 genes showing decreased (blue) and 26 genes with increased expression (red) in AA (Fig.4A,B Suppl.Tab.4), with strongly enhanced IRF5 and decreased CD141 remaining significant after

adjusting for multiple testing for the 21 immune response categories included in this panel given by NanoString (Fig.4A, bold). Reduced baseline levels of CD4 in mDC, that is associated with immature DC were shown for AA on gene expression level and confirmed on protein level (Table 3). Although CD4 is generally used for T-helper cell gating, its expression in DC is known and confirmed by previous studies.[34-37]
In Tregs of AA, the transcription factor (TF) ATF2, which is activated by inflammatory signaling was significantly increased (Fig.4C, Suppl.Tab.4). On protein level, RORγ expression was significantly increased in effector Treg of AA, while CCR2 was highly decreased in several manually gated T cell subpopulations of AA. Besides, CD11b was highly upregulated in effector memory Th2-cells of AA (Table 3).

In summary, children with manifest allergic asthma expressed higher levels of pro-inflammatory IRF5, while CD141 and CD4 were less expressed in isolated DC of AA at unstimulated baseline conditions. In parallel, AA expressed higher levels of ATF2 (gene) and RORγ (protein) in Treg populations together with reduced CCR2 levels in all T cell subpopulations.

***Ex vivo* farm-dust stimulation downregulated properties of antigen presentation and innate inflammation**

Aiming to identify cellular changes upon farm-dust stimulation, the frequency of manually gated PBMC subpopulations was compared by mass cytometry with and without stimulation in AA and HC (Fig.3A,B, Table 1 “HC G vs HC U” and “AA G vs AA U”).

Farm-dust stimulated PBMCs of both HC and AA demonstrated a strong decrease in cell numbers of innate APCs namely monocytes and DC (Table 1). HLA-DR^{low} CD56^{high} NK-cells were induced in both phenotypes, whereas classical and HLA-DR^{low} NK-cells and HLA-DR^{low} monocytes were exclusively downregulated in AA. Moreover, the regulation of protein expression within NK-cells differed prominently between phenotypes. While several markers associated with catalytic function and regulation within NK-cells like GZMB, CD56, GATA3, and CD45RA were upregulated in HC upon farm-dust stimulation, these were downregulated or remained unchanged in AA (Table 4, 5). Significantly lower proportions of monocytes/DC-cluster in both phenotypes and lower frequencies of non-activated B cells/DC-cluster and NK-cells in AA were confirmed by semi-supervised clustering (Table 2).

Although total counts of both pDC and mDC, were reduced upon farm-dust stimulation, a shift in their composition was demonstrated in HC and AA. While the share of pDC in all DC increased, the proportion of mDC decreased (Suppl.Fig.3). Moreover, a significant

downregulation of HLA-DR was identified in monocytes of HC and mDC of both phenotypes (Table 4). While CCR7 was upregulated in all DC, especially in HC, this maturation marker was downregulated in pDC and mDC2 of AA.

Following dust stimulation, 378 and 255 genes were up- or downregulated in isolated DC in HC and AA, respectively (Fig.5A,B, Suppl.Tab.5). 127 and 85 remained significantly regulated after adjustment for multiple testing (Fig.5A,B bold). The majority of genes was downregulated (75.7% HC, 70.2% AA). Most significantly regulated genes in both phenotypes were surface CD molecules, innate and adaptive immunity-genes, cell-type-specific signaling, cytokines, chemokines, and basic cell function (Fig.5C,D). Transcription regulators, genes of the TNF superfamily, and genes associated with inflammatory response, T cell polarization, microglial cell activation, defense response to virus, and acute-phase response were only regulated in HC.

As DC have been identified as a central cell population affected by farm-dust stimulation *ex vivo*[14] and have shown key relevance in CyTOF, we further investigated their functional effect via farm-dust stimulation. $CD14^+$ monocytes isolated from PBMCs (Suppl.Fig.4A) of AA and HC and two healthy adults for confirmation were differentiated to moDC confirmed by successful downregulation of the monocyte marker CD14 and induction of a DC-like morphology (Suppl.Fig.4B,C). When stimulating these moDC with farm-dust, strong and highly significant anti-inflammatory regulation of NF-κB and MAPK signaling genes was demonstrated for moDC from both HC and AA (Fig.6). Specifically, as shown for PBMCs and isolated DC, farm-dust stimulation upregulated anti-inflammatory TNFAIP3 expression, while downregulated TLR4. Moreover, the anti-inflammatory co-stimulatory molecule CD274 was significantly induced in AA while pro-inflammatory CD209 was decreased in HC upon stimulation with farm-dust extract. Significant upregulation of the DC maturation marker CCR7 as shown by mass cytometry was confirmed in moDC of AA.

In conclusion, the number of innate APC (monocytes and mDC) and antigen presentation associated HLA-DR were highly reduced upon farm-dust stimulation in both HC and AA. NK-cells and their catalytic proteins were exclusively downregulated in AA, confirmed by semi-supervised clustering. Moreover, these data further demonstrated the anti-inflammatory role of DC in farm-dust-mediated asthma protection, as anti-inflammatory NF-κB/MAPK signaling and co-stimulatory molecules were induced upon *ex vivo* stimulation of moDC.

Ex vivo farm-dust stimulation reduced T cell activation and allergy-associated Th2/Th17 markers in children with manifest allergic asthma

On the adaptive part of the immune system, farm-dust stimulation had significant effects on the frequency of B cells by strong upregulation (Table 1). Especially the number of activated ($CD25^+$) B cells was strongly augmented (Suppl.Fig.5).

In T cells, a consistent downregulation of T cell receptors (TCR) associated with co-stimulation and activation including CD3, CD4, CD8, CD28, and CD44 was demonstrated in both phenotypes (Table 4). In all investigated T cell subpopulations (CD4+, CD8+, Th2, Treg, DN), the central memory phenotype was significantly increased, while effector memory cells were significantly reduced in both HC and AA (Table 1). The DC-defining lineage markers CD141, BDCA1/2, and CD11b that were associated with T cell activation and were demonstrated to distinguish specific T cell clusters were consistently downregulated in AA (Table 4, 5), indicating reduced T cell activation upon farm-dust stimulation especially in AA.

Exclusively in T cells of AA, the Th2 and Th17-associated TFs GATA3, RORγ as well as CD56 and CD45RA were downregulated. In contrast, GZMB, CCR7, and CD69 were only upregulated upon farm-dust stimulation in T cell populations of HC (Table 4, 5). Farm-dust stimulation increased Treg numbers exclusively in HC. However, Treg-associated CD25 and FOXP3 were induced in both phenotypes.

Semi-supervised clustering confirmed significantly upregulated clusters of activated B cells, CD4 Tcm CD25+ and CD4 Tcm CD25+ BDCA1+, while effector memory T cells were significantly downregulated (Table 2). The proportions of the cluster of DN T cells were only significantly increased in HC but not AA.

Comparing gene expression levels, 15 and 49 genes were significantly different upon stimulation in Tregs of HC and AA respectively (Fig.7A,B, Suppl.Tab.6, Suppl.Fig.6). Upon adjustment for multiple testing, only TXNIP remained significantly decreased in Treg of HC and AA together with eight other significantly downregulated genes in AA (Fig.7A,B; bold).

To further assess T cell function qualitatively, we performed T cell proliferation assays and assessed the effect of farm-dust stimulation on proliferation of effector T cells (Teff) and the suppressive capacity of Treg. The presence of Tregs inhibited proliferation of Teff induced by anti-CD2/3/28-beads in a dose-dependent manner. Although farm-dust stimulation did not result in further suppressive capacity of Tregs, highly decreased Teff proliferation was shown

upon farm-dust stimulation even in the absence of Treg especially in AA (Fig.8), indicating a Treg-independent inhibition of T cell activation by farm-dust stimulation.

In conclusion, farm-dust stimulation resulted in strong upregulation of B cells and central memory T cells, while effector memory T cell subpopulations were decreased. Th2 and Th17 TF were exclusively downregulated in stimulated T cells of AA. While Treg numbers were solely upregulated in HC, oxidative stress-related TXNIP was decreased in Treg of both HC and AA. Reduced activation of effector T cells as shown by downregulation of CD28, CD44, and TCR was further demonstrated by functional analysis as Teff proliferation was highly reduced independent of regulatory T cells when stimulated with farm-dust extracts.

Discussion

Strong environmental protection of asthma via farm-dust was repetitively shown in epidemiological studies and some attempts on understanding its functional mechanisms were made.[2,3,7,29] However, a detailed insight of immune regulation following *ex vivo* farm-dust stimulation on diverse immune cells and functional mechanisms is lacking.

Our novel approach now comprehensively assessed functional regulation of farm-dust stimulation in peripheral blood of healthy and asthmatic children covering a wide range of specific cell populations on gene, protein, and functional level. By combining mass cytometry, a state-of-the-art technology measuring lineage markers and specific targets on single-cell protein expression level (CyTOF) with multiplex gene expression (NanoString) in relevant isolated cells (DC/Treg) together with DC and T cell function, the detailed effects of *ex vivo* farm-dust stimulation on innate and adaptive immune responses were demonstrated. On the innate side, anti-inflammatory regulation and reduced antigen presentation as shown by the reduced number of monocytes and DC, a shift towards pDC, and downregulation of the MHC surface receptor HLA-DR was shown in both HC and AA upon farm-dust stimulation. Exclusively in AA, farm-dust stimulation resulted in reduced NK-cell numbers and cytolytic protein expression. On the adaptive side, farm-dust stimulation upregulated B cell numbers and central memory T cells, whereas the number of effector memory T cells was reduced in both phenotypes. Reduced activation of effector T cells was demonstrated by downregulated TCR and co-stimulatory CD28 and confirmed by functional proliferation assays. While Treg numbers were only induced in HC, stimulation with farm-dust downregulated GATA3 and RORγ in AA.

DC play an important role in asthma development and environment-mediated protection. Whereas AA have been associated with increased DC numbers[30–32], pDC were reported to be decreased in AA.[6] pDC are involved in response to viral infections, tolerance, and Treg control, whereas mDC are associated with antigen presentation and contribute to Th2-development and allergic response.[27] In line, the tolerogenic DC marker, Thrombomodulin (CD141) was significantly decreased in unstimulated DC of AA confirmed on both gene (NanoString) and protein (CyTOF) expression. Of note, treatment with thrombomodulin was associated with improved lung function and lower IgE levels by induction of tolerogenic DC in murine models.[33] In addition, CD4 was decreased in DC of AA on gene and confirmed on protein levels. Although CD4 is the main lineage marker of T-helper cells, its expression on DC, especially immature DC, was demonstrated in several studies.[34–37] A function in recruitment and proliferation of T cell and increased Fc receptor signaling is suggested.[34] Moreover, the transcription factor IRF5 was significantly increased in unstimulated DC of AA. IRF5 being involved in pro-inflammatory

regulation downstream of TLR4 and subsequent signaling was shown to be significantly increased in BAL of severe asthmatics.[38] Elevated inflammation in DC of AA could be counteracted by *ex vivo* farm-dust stimulation. Besides anti-inflammatory regulation, also the number of innate APC, namely DC and monocytes was highly reduced upon stimulation, in line with reported induction of apoptosis in these cells upon stimulation with microbial components.[39] Programmed cell death is an important process to regulate DC homeostasis, mediate immune tolerance, and prevent autoimmunity.[40,41] The reduction of APC via apoptosis may represent a key mechanism in environmental-mediated asthma protection, as the reduction of antigen processing and presentation via APC limits the associated T cell priming and antigen-specific immune responses.[40] While mDC, which are associated with antigen presentation and Th2-responses were especially reduced, the share of tolerogenic pDC was increased, in line with data showing that mDC are short-lived in contrast to pDC.[40,42] Besides DC, also monocytes were highly decreased in their frequency, especially those expressing high levels of HLA-DR. Protective farm exposure has been linked with lower levels of HLA-DR in monocytes *in vivo*.[43] In our study, the MHC-II receptor HLA-DR was strongly decreased in DC and monocytes, indicating a less responsive and suppressive phenotype. Monocytes are highly associated with LPS tolerance mechanisms through enhanced anergy of these cells and downregulation of HLA-DR to repeated or prolonged LPS stimulation.[11,44] In addition, due to their high plasticity under certain stimulatory conditions, rapid differentiation of this cell type is likely. Also, shifts within monocyte subsets have been reported like induction of memory and a suppressive phenotype upon exposure to farming environments.[11,45] Co-stimulatory signaling was also shifted towards anti-inflammation by farm-dust stimulation of moDC together with increased expression of DC maturation markers, confirmed by upregulated CCR7 on the DC surface measured by mass cytometry. This is in line with other farming studies where stimulation of human and murine moDC or PBMCs with farm-derived bacteria or cowshed farming extracts also resulted in the upregulation of these co-stimulatory molecules, yet these DC were unable to prime allergic immune responses.[8,21,46,47] These findings indicate that farm-dust stimulation highly reduces antigen presentation as shown by decreased DC numbers and downregulated HLA-DR expression and induces the maturation of tolerogenic DC and anti-inflammatory signaling.

Moreover in our study, farm-dust stimulation resulted in an anti-cytotoxic effect with reduced NK-cells, GZMB, and CD56 expression exclusively in AA. Cytotoxic NK cells were associated with the pathogenesis of allergic asthma, due to their Th2 promoting function, involvement of eosinophilic inflammation, elevated airway hyperresponsiveness, and enhanced and more severe asthma exacerbations.[48,49] On the other hand, regulatory functions were attributed to NK-cells, especially those with high CD56 expression, that have also been shown to be

reduced in allergic patients and are associated with increased DC maturation.[50] In fact, these HLA-DR^{low} CD56^{high} NK-cells showed a differential regulatory pattern with a highly significant increase in this cell type in both phenotypes upon farm-dust stimulation in our study.

Ex vivo farm-dust stimulation did not only affect innate immune responses but played also a role in the subsequent adaptive immunity. B cells were highly induced when cells were stimulated with farm-dust extracts. LPS, one of the main components of farm-dust extracts has been shown to induce proliferation of B-lymphocytes, however, it induces anergy and lowers CD8 T cell stimulative capacities.[51,52] Also, in farm children with *in vivo* exposure, higher levels of B cell activation factors (BAFF) were demonstrated to be positively correlated with memory B cells.[53] Stimulation with high-dose LPS (>0.1 ug/mL) was associated with Th1-polarization and the induction of anti-inflammatory IL-10.[51] IL-10 producing regulatory B cells (Bregs) were associated with microbial contact that in turn suppress immune responses.[54,55] In fact, TLR signaling that is highly induced upon farm-dust stimulation is important for the regulatory function of Bregs.[55] Since identification of particular subpopulations of B cells was not focus of this analysis and identifying markers were missing in our panel and further studies investigating the effect of protective farming environments on B cell phenotypes are needed in the future.

Decreased CCR7^{+} Tcm and upregulated CCR7^{-} Tem phenotypes together with highly increased CCR7 expression in DC and T cells upon farm-dust stimulation might provide a potential mechanistic insight in the demonstrated reduced proliferation of effector T cells, as this chemokine receptor has been linked to suppression of effector T cell proliferation.[56,57] In contrast, *in vivo* farming studies did not reveal significant differences between the percentage of CD4^{+} T cells of peripheral blood leucocytes of children from the Amish people with very low asthma prevalence due to their protective environment of traditional farming compared to the Hutterite children.[11] Although asthma is associated with Th2-driven allergic responses, we did not find any significant baseline differences in Th2-cells between AA and HC. One explanation may be a stable disease status of mild AA and no acute phase-response. Yet, the DC-associated surface receptor CD11c, which was also associated with T cell activation[58], was expressed significantly higher on effector memory Th2-cells of AA. However, upon farm-dust stimulation, TFs of Th2 and Th17 immunity, namely GATA3 and RORγ were significantly decreased in AA, suggesting a beneficial shift from Th2/Th17 to Th1 balance by environmental exposure. Decreased T cell activating expression of TCR and CD28 may suggest an inhibited T cell and inflammatory activation upon farm-dust stimulation. CD28 was also significantly reduced in CD4+ T cells of the “asthma-protected” Amish children.[11] Reduced T cell activation upon farm-dust stimulation is further supported by reduced Teff proliferation upon farm-dust stimulation as shown in functional proliferation assays. Also, the DC-associated markers

BDCA1, CD11b, and CD11c that were shown to distinguish several CD4+ T cell clusters have been shown to regulate T cell activation[58–60] were downregulated in T cell subsets upon farm-dust stimulation.

Another cell population that is strongly associated with protective farming environments are Treg. In this study, we identified that only HC were able to upregulate these regulatory cells upon farm-dust stimulation, while they remained unchanged in AA. The upregulated ATF2 levels, a TF induced by inflammatory signaling including the MAPK pathway that is associated with Th2-mediated inflammation in unstimulated Treg of AA, reflect their higher inflammatory baseline levels compared to HC. Importantly, farm-dust stimulation was able to downregulate ATF2 in Tregs of AA. In addition, TXNIP, linked to oxidative stress pathways was downregulated upon stimulation in both phenotypes. Inhibition of this regulator was associated with anti-allergic and anti-inflammatory response in murine models, suggesting an anti-oxidative stress-related mechanism of farm-dust stimulation in Treg.[61]

In summary, in our study analyzing the asthma-protective effect of farm environments on cellular, molecular and functional levels, we were able to demonstrate the induction of immunological tolerance by downregulation of anti-inflammatory signaling, reduced antigen presentation, and decreased subsequent T cell activation. While farm-dust stimulation induced Treg numbers only in HC, allergy-associated markers of Th2, Th17, and NK-cell immune responses were exclusively decreased in asthma-affected children.

Of note, some facets require specific consideration. While CyTOF and NanoString offered a comprehensive analysis of numerous cell populations and markers, the sample size was limited, however comparable to other CyTOF studies. Moreover, significant findings and adjustments for multiple testing of the NanoString data make us confident about the statistical validity of our results. Moreover, the consistency of manually and clustered data in parallel with confirmation of results on gene and protein expression with different methods supports the biological importance of these novel data of mechanisms of *ex vivo* farm-dust stimulation. Regarding the number of decreased cell frequencies following stimulation, we can only speculate on the fate of these cells. Besides induction of apoptosis, a shift from one cell type into another is possible especially in cells with high plasticity as monocytes and DC. Based on our previous relevance of DC and Treg in farm-mediated asthma-protecion[12,13,27,62,63] and limited available cell numbers, we focused on these specific subpopulations for NanoString. Thus, further B cell isolation or markers for Bregs (CyTOF) and monocyte subpopulations were not included in this study. Certainly, we support and will further functionally assess these in further studies based on our novel findings of highly induced B cell numbers upon farm-dust stimulation.

We are confident that this comprehensive, in-depth analysis of the cellular and molecular immunological mechanisms underlying the protective farm effect is important for several reasons: i) Anti-inflammatory and anti-allergic properties of *ex vivo* farm-dust stimulation as shown by downregulated inflammatory signaling, decreased antigen presentation, reduced subsequent T cell activation and downregulation of Th2 and Th17 immunity may offer a solid base for future translational and intervention studies – of course following all regulatory steps required. ii) The newly identified markers that are regulated upon farm-dust stimulation including HLA-DR, CD28, GATA3, RORγ and DC-markers may also offer a "novel toolbox" for patient selection in *ex vivo* studies for subsequent application to translational studies. iii) The fact that farm-dust stimulation can modulate the immune response towards anti-inflammation and decreased antigen presentation and T cell activation even in manifest asthmatics opens novel avenues not only for early preventive strategies but importantly also for already symptomatic children. The reversibility of immune markers and cells may reflect an option for cellular plasticity during a certain time frame. Although we cannot define an exact window of possible intervention until later adolescence, at least during school-age modulation of pro-inflammatory immune activation still seems possible. Whether our *ex vivo* findings will also translate to an *in vivo* setting and is possible even in more severe asthmatic children certainly requires further translational and large follow-up studies.

Figure Legends

Figure 1: Schematic representation of the CyTOF workflow.
N=20 (10 HC/10 AA) children (4-17y, CLARA/CLAUS cohort) were recruited. Isolated PBMCs were cultivated for 24h upon unstimulated (U) and German farm-dust stimulated (G) conditions. PBMCs were stained with metal-ions labeled antibodies and analyzed by mass cytometry. The resulting FCS files were analyzed by manual gating and semi-supervised clustering.

Figure 2: Immune profiling clusters identified by semi-supervised clustering.
A) Heatmap showing the mean expression of each lineage marker (asinh-transformed) in each of the 20 clusters, identified by semi-supervised clustering in the unstimulated PBMC samples of the N=10 healthy children (CLARA/CLAUS cohort). Color-code represents the strength of marker expression. B) Comparison of the events assigned to a certain major cell population by manual gating (x-axis) to the corresponding cluster they were allocated to (y-axis). The values show the proportion of cells of a certain gate that have been allocated to the corresponding cluster. On the right, the frequency of each cluster related to all living cells is shown as a mean of all 40 samples. C) UMAP and minimum spanning tree of the identified clusters based on semi-supervised clustering of the N=10 unstimulated samples of healthy children.

Figure 3: Cellular immune profile of PBMCs of healthy and allergic asthmatic children under unstimulated and German farm-dust stimulated conditions.
The abundance of specific cell populations identified by A) manual gating and B) semi-supervised clustering in PBMCs shown as the percentage of the corresponding parent populations as mean +/- standard errors presented for the four groups (HC U, HC G, AA U, and AA G) measured in N=20 children (10 HC/10 AA) (CLARA/CLAUS cohort) cultivated under unstimulated (U) and German farm-dust (G) stimulated conditions for 24 hours. Major cell populations are shown as the percentage of all living cells. Significant differences of cellular frequencies between stimulated and unstimulated conditions are labeled in bold with *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Figure 4: Differentially expressed genes in isolated DC and Treg of allergic asthmatic children.
Volcano-plot presenting the most significant differentially expressed genes between healthy and asthmatic children as measured by NanoString in unstimulated isolated DC (Fig.4A) and Treg (Fig. 4C) of N=12 (7HC/5AA) children (CLARA/CLAUS cohort). Genes that are significantly downregulated in AA compared to HC are marked in blue, while significantly

upregulated genes are labeled in red (A, C). Bold written genes remain significant ($p<0.05$) after adjustment for multiple testing. In B) up- (red) and downregulated (blue) immune categories annotated by NanoString in AA compared to HC to which the significant differentially expressed genes of DC could be allocated.

Figure 5: Immunomodulatory effects of farm-dust stimulation on gene expression within DC.

Volcano-plot presenting the most significant differentially expressed genes between unstimulated (U) and German farm-dust stimulated (G) conditions measured by NanoString in DC of A) N=7 HC and B) N=5 AA children of the CLARA/CLAUS cohort. Genes that are significantly downregulated are marked in blue, while significantly upregulated genes are labeled in red. Bold written genes remain significant ($p<0.05$) after adjustment for multiple testing. C-D) Up- (red) and downregulated (blue) immune categories annotated by NanoString upon farm-dust stimulation to which the significant differentially expressed genes of Tregs could be allocated in C) HC and D) AA children. Immune categories containing genes that are exclusively significantly regulated in HC are labeled in bold.

Figure 6: Induction of anti-inflammatory signaling by farm-dust stimulation in moDC.

Up- (red) and downregulation (blue) of gene expression upon 24h farm-dust stimulation measured by qPCR in moDC of N=2 HC and N=3 AA children (CLARA/CLAUS cohort) and N=2 healthy adults. Saturation reflects the strength of the fold change (German farm dust vs. unstimulated conditions). P-values derive from t-tests (#$p<0.1$, *$p<0.05$, **$p<0.01$).

Figure 7: Immunomodulatory effects of farm-dust stimulation on gene expression within Tregs.

Volcano-plot presenting the most significant differentially expressed genes between unstimulated and German farm-dust stimulated (G) conditions measured by NanoString in Tregs of A) N=7 HC and B) N=5 AA children (CLARA/CLAUS cohort). Genes that are significantly downregulated are marked in blue, while significantly upregulated genes are labeled in red. Bold written genes remain significant ($p<0.05$) after adjustment for multiple testing.

Figure 8: Reduced T cell proliferation upon farm-dust stimulation.

T cell proliferation of CFSE-treated effector T cells upon 5 days of stimulation with anti-CD2/3/28-beads with and without the presence of Tregs and German farm-dust (G) in various ratios measured by flow cytometry in N=4 (2 HC/2 AA) children (CLARA/CLAUS cohort).

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Tables

Table 1: Frequencies of manually gated cell populations (CyTOF)

Population	HC U %Parent Mean	HC U %Parent SD	HC G (vs HC U) %Parent Mean	HC G (vs HC U) %Parent SD	AA U (vs HC U) %Parent Mean	AA U (vs HC U) %Parent SD	AA G (vs AA U) %Parent Mean	AA G (vs AA U) %Parent SD
Total								
B cells	2,59	±0,73	**5,24**	±2,06	3,93	±2,04	**6,24**	±2,74
activated B-cells	58,90	±7,30	**68,83**	±6,98	64,86	±14,04	**73,63**	±12,53
non-activated B-cells	41,10	±7,30	**31,17**	±6,98	35,14	±14,04	**26,37**	±12,53
HLADRlow monocytes	0,51	±0,14	0,50	±0,17	0,70	±0,43	**0,56**	±0,32
monocytes	1,77	±1,74	**0,55**	±0,49	2,53	±3,02	**0,42**	±0,18
NK cells	4,16	±1,80	3,81	±1,52	4,31	±2,24	**3,83**	±1,97
HLADRlow NK cells	2,66	±1,08	2,48	±0,93	2,88	±1,73	**2,42**	±1,50
HLADRlow NK cells CD56high	0,36	±0,13	**0,52**	±0,19	0,42	±0,24	**0,51**	±0,26
DC	0,49	±0,22	**0,24**	±0,09	0,63	±0,23	**0,27**	±0,08
pDC	42,74	±19,01	**66,30**	±17,23	39,76	±17,35	**69,67**	±9,03
mDC1	43,94	±16,36	**26,00**	±14,35	47,08	±14,30	**23,22**	±7,44
mDC2	13,32	±3,88	**7,70**	±3,67	13,16	±4,12	**7,10**	±2,29
CD4+ T-cells	44,67	±7,46	45,28	±7,93	39,07	±6,09	**41,65**	±5,87
CD4+ Central memory T-cells	88,07	±2,67	**89,82**	±2,76	86,12	±2,77	**87,81**	±3,55
CD4+ Effector memory T-cells	5,85	±3,37	**4,98**	±3,18	6,24	±2,05	**5,06**	±1,94
CD4+ Effector T-cells	0,08	±0,05	**0,07**	±0,05	0,09	±0,02	0,09	±0,06
CD4+ naive T-cells	5,99	±2,99	**5,13**	±2,32	7,56	±3,31	7,04	±3,61
Th2 cells	9,71	±1,05	9,48	±1,30	10,18	±1,15	10,06	±1,33
Th2 central memory	87,57	±2,64	**89,28**	±2,56	85,31	±3,01	**86,94**	±3,78
Th2 effector memory	5,54	±3,00	**4,82**	±2,77	5,74	±1,95	**4,67**	±1,89
Th2 effector	0,09	±0,04	0,09	±0,06	0,12	±0,03	0,11	±0,08
Th2 naive	6,79	±3,21	**5,82**	±2,51	8,83	±3,74	8,28	±4,01
Treg	28,44	±6,78	**33,53**	±11,19	28,46	±5,96	29,48	±5,09
Helios+ Tregs	24,96	±3,28	24,59	±3,63	26,66	±4,85	26,98	±5,41
Treg central memory	83,54	±2,78	**86,79**	±3,29	81,57	±2,31	**84,41**	±3,55
Treg effector memory	12,87	±3,97	**9,97**	±3,95	14,20	±2,59	**11,18**	±3,15
Tregs effector	0,08	±0,05	**0,06**	±0,05	0,10	±0,05	0,10	±0,05
Tregs naive	3,51	±1,93	3,17	±1,60	4,13	±2,07	4,32	±2,29
CD8+ T-cells	22,66	±3,30	23,31	±3,40	22,95	±4,21	23,88	±4,30
CD8+ Central memory T-cells	77,31	±5,74	**78,51**	±6,09	78,12	±6,30	**79,65**	±6,42
CD8+ Effector memory T-cells	17,48	±6,61	16,94	±6,48	15,52	±7,03	**14,49**	±6,90
CD8+ Effector T-cells	0,78	±0,44	0,77	±0,47	0,80	±0,40	0,77	±0,41
CD8+ Naive T-cells	4,43	±2,18	**3,78**	±1,55	5,57	±2,94	5,09	±3,08
DN T-cells	4,27	±2,06	4,20	±2,16	5,23	±3,34	5,37	±3,13
DN central memory	59,92	±5,29	**62,94**	±6,04	58,45	±6,34	**59,77**	±6,36
DN effector memory	34,41	±7,34	**31,77**	±7,28	35,20	±9,42	**33,38**	±10,12
DN effector	1,50	±0,57	1,31	±0,50	1,60	±0,54	1,53	±0,70
DN naive	4,17	±2,23	3,97	±1,64	4,76	±3,61	5,32	±4,64

p<.01
p<.05
ns
p<.05
p<.01

Table 2: Frequencies of clusters (CyTOF)

	HC U		HC G (vs HC U)		AA U (vs HC U)		AA G (vs AA U)	
Cluster	Mean	SD	Mean	SD	Mean	SD	Mean	SD
monocytes, DC	4,21	±3,25	**1,51**	±1,22	5,22	±4,97	**1,20**	±0,42
activated B-cells	5,62	±1,87	**7,98**	±2,84	7,63	±3,48	**9,40**	±3,83
non-activated B-cells, DC	6,60	±1,44	5,96	±1,24	6,76	±4,13	**5,85**	±4,02
DN	4,72	±2,05	**4,15**	±1,93	5,30	±2,85	5,35	±2,34
CD8 Tcm	15,45	±1,14	16,12	±1,54	16,29	±3,42	17,08	±3,32
CD8 effector memory	5,57	±2,58	5,58	±2,71	5,07	±2,38	5,02	±2,48
NK-cells	7,76	±2,83	7,33	±2,51	8,40	±4,48	**7,46**	±3,98
CD4 Tcm CD141-	6,96	±1,55	6,55	±1,86	6,24	±1,08	6,86	±0,90
CD4 Tcm CD141+	3,90	±1,06	3,68	±1,39	3,58	±1,42	3,77	±1,35
CD4 Tcm BDCA1+	7,04	±1,32	6,74	±1,85	6,42	±1,08	6,69	±0,95
CD4 Tcm BDCA1+ CD11b+	4,88	±1,01	4,63	±1,34	4,32	±0,43	4,38	±0,54
CD4 Tcm BDCA2+	5,25	±1,41	4,83	±1,49	4,91	±0,93	5,09	±0,90
CD4 Tcm CD11b+	6,88	±1,35	6,51	±1,73	6,08	±1,00	6,47	±0,96
CD4 effector memory	1,46	±0,39	**1,30**	±0,46	1,44	±0,57	**1,31**	±0,58
CD4 Tcm CD25+	7,96	±1,90	**9,92**	±4,47	7,09	±1,63	**8,26**	±1,42
CD4 Tcm CD25+ BDCA1+	5,74	±1,18	**7,21**	±2,84	5,24	±1,00	**5,82**	±0,99

p<.01
p<.05
ns
p<.05
p<.01

Table 3: Phenotypical differences of marker expression within manually gated cell populations (CyTOF)

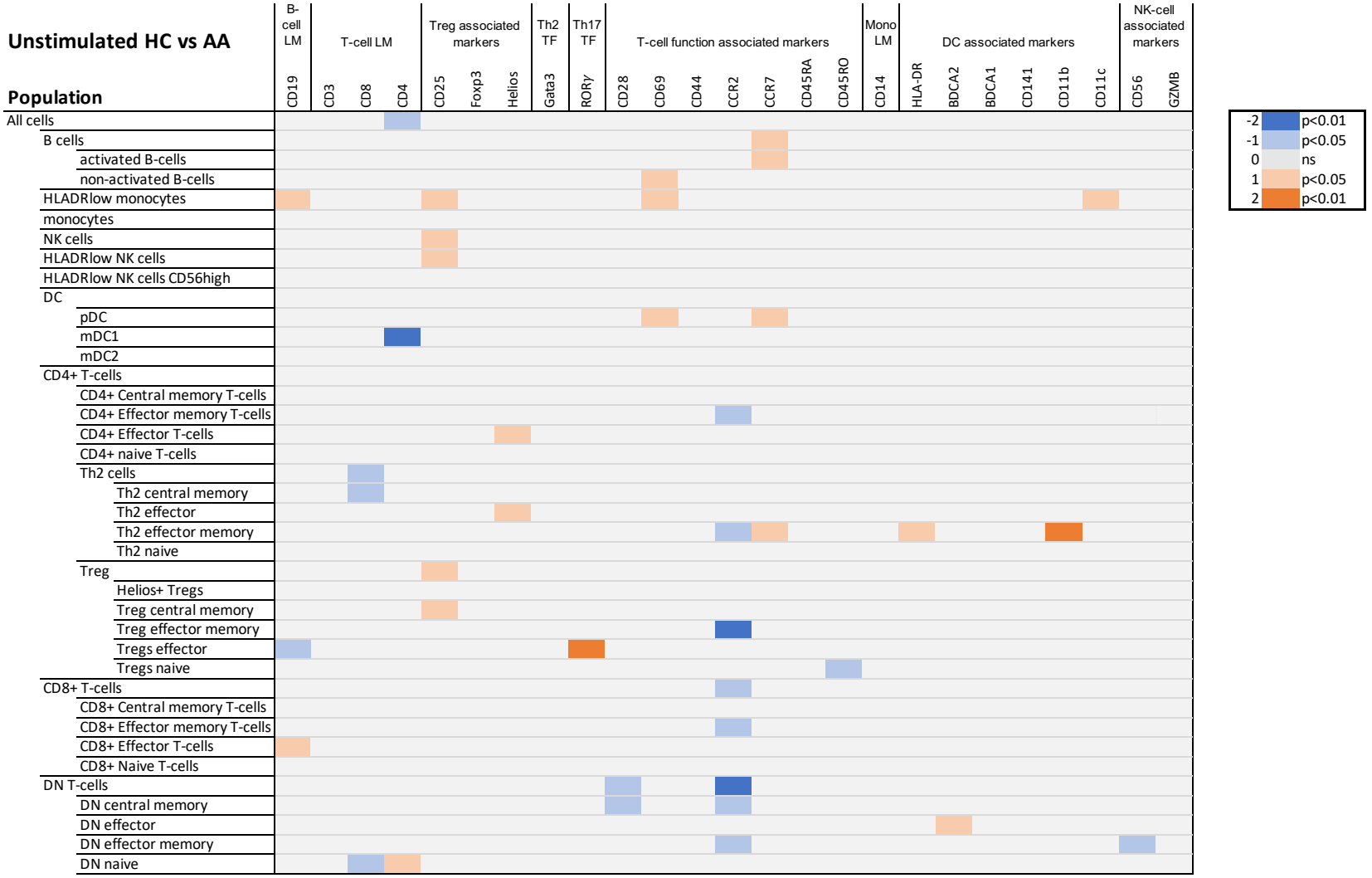
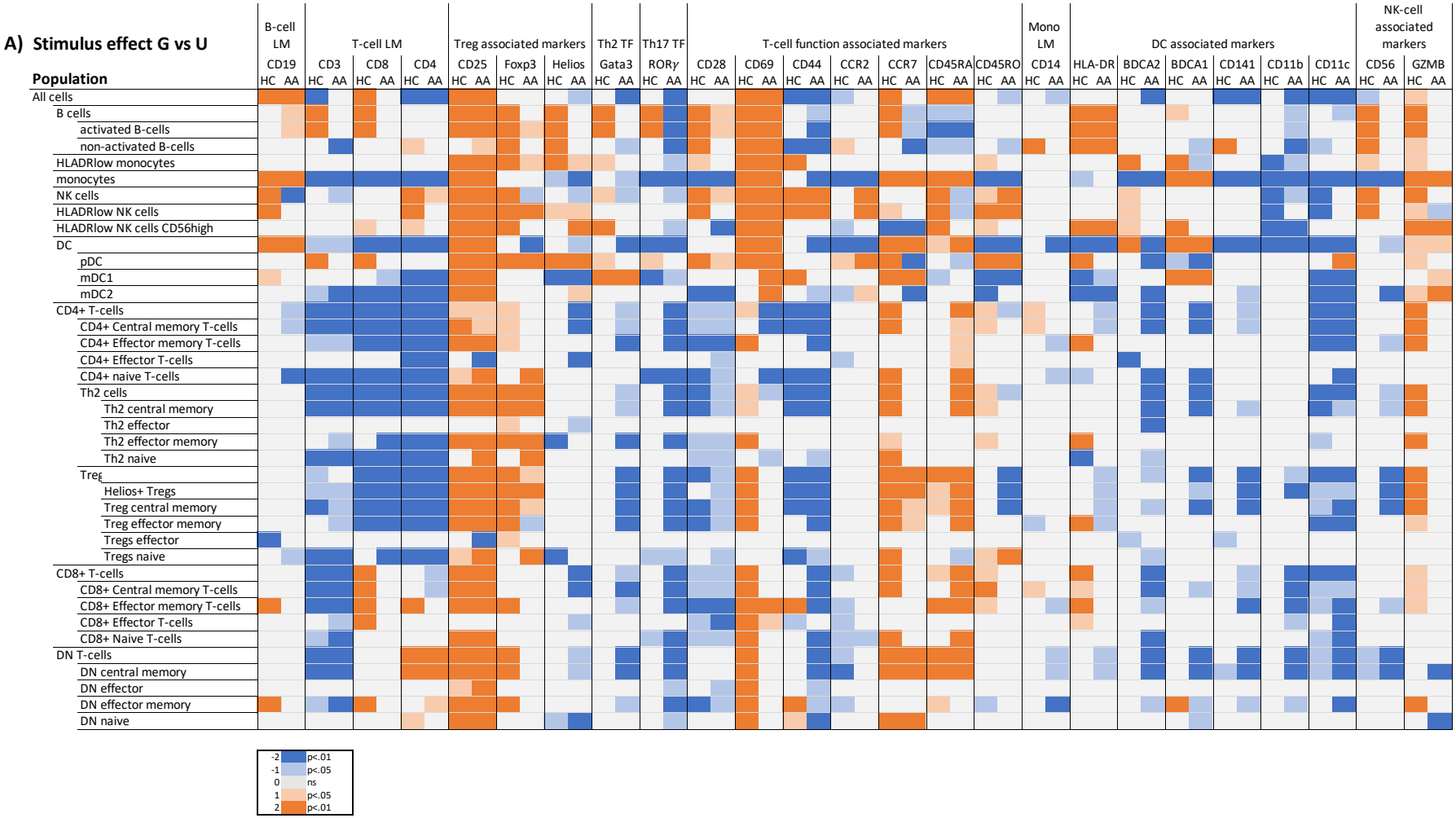


Table 4: Stimulatory effect of farm-dust stimulation on marker expression within manually gated cell populations (A) and clusters (B) (CyTOF)

A) Stimulus effect G vs U



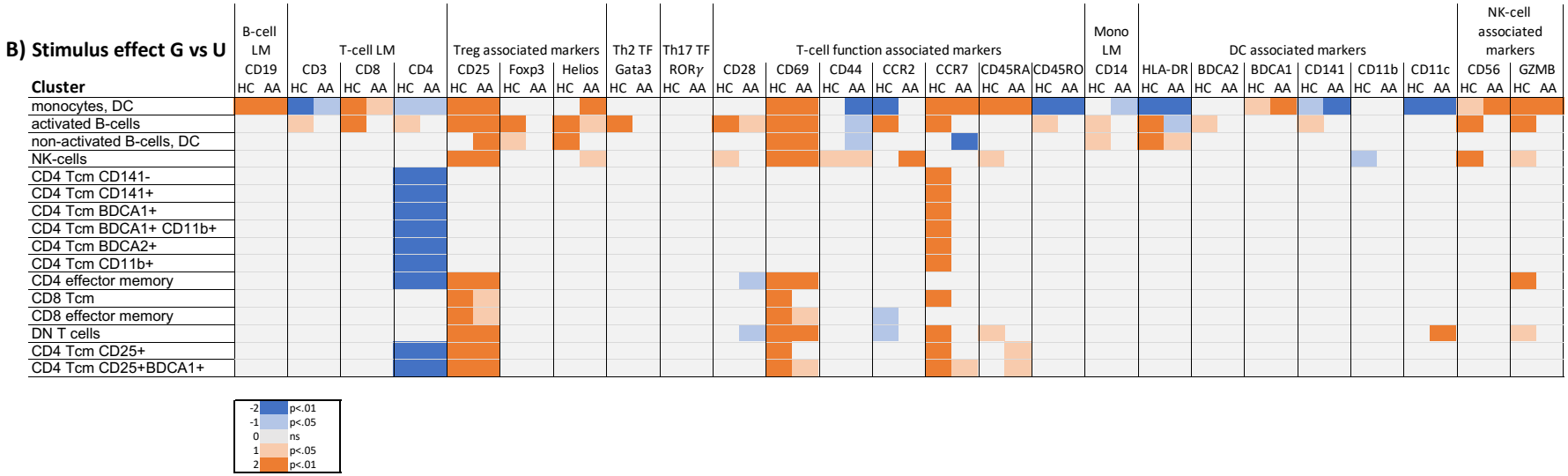
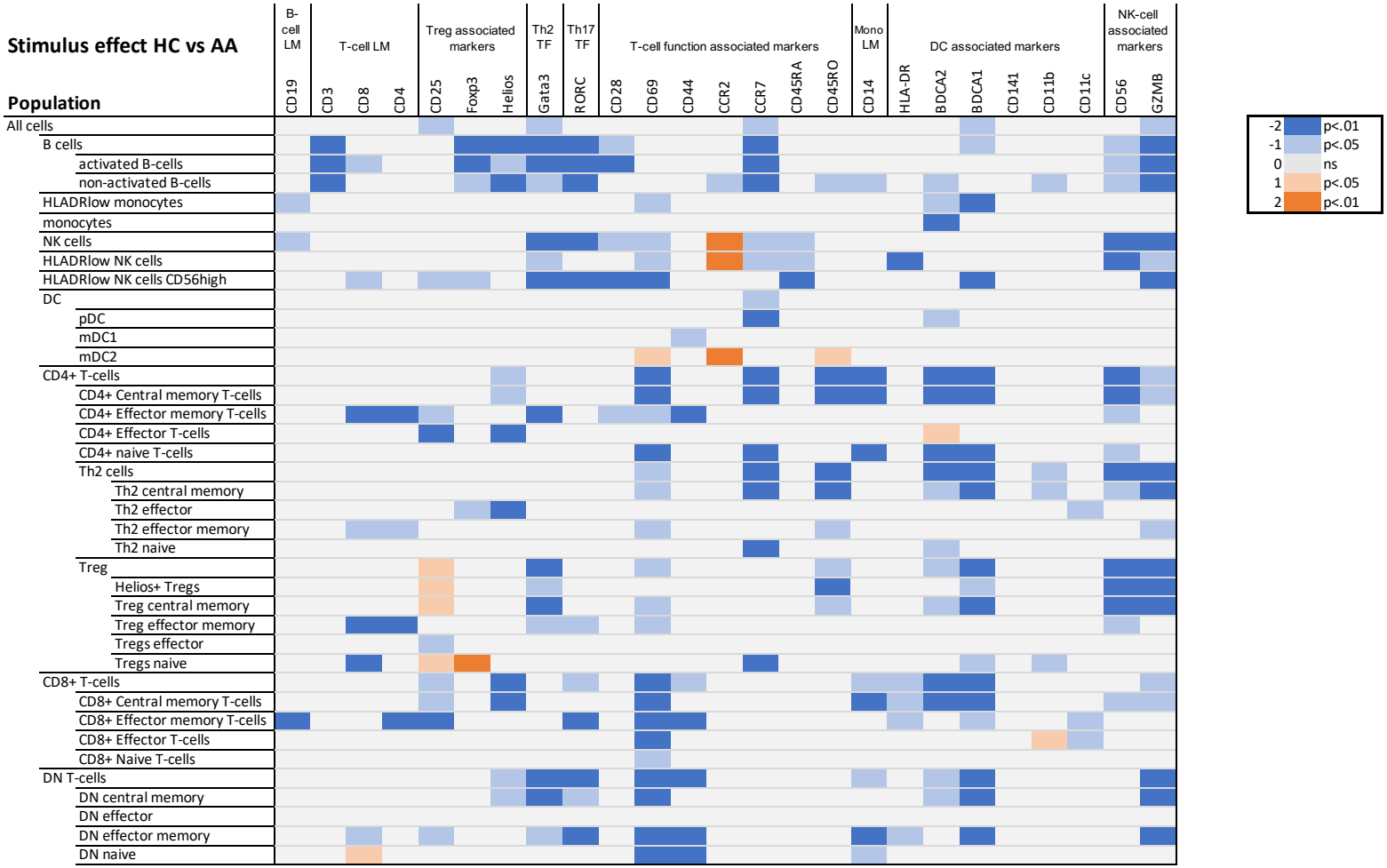

B) Stimulus effect G vs U
Cluster
B-cell LM
CD19
T-cell LM
CD3
CD8
CD4
Treg associated markers
CD25
Foxp3
Helios
Th2 TF
Gata3
Th17 TF
RORγ
T-cell function associated markers
CD28
CD69
CD44
CCR2
CCR7
CD45RA
CD45RO
Mono LM
CD14
DC associated markers
HLA-DR
BDCA2
BDCA1
CD141
CD11b
CD11c
NK-cell associated markers
CD56
GZMB
HC AA
monocytes, DC
activated B-cells
non-activated B-cells, DC
NK-cells
CD4 Tcm CD141-
CD4 Tcm CD141+
CD4 Tcm BDCA1+
CD4 Tcm BDCA1+ CD11b+
CD4 Tcm BDCA2+
CD4 Tcm CD11b+
CD4 effector memory
CD8 Tcm
CD8 effector memory
DN T cells
CD4 Tcm CD25+
CD4 Tcm CD25+BDCA1+
-2 p<.01
-1 p<.05
0 ns
1 p<.05
2 p<.01

Table 5: Phenotypical differences of the stimulatory effect of farm-dust stimulation on marker expression within manually gated cell populations (CyTOF)



Figures

Figure 1

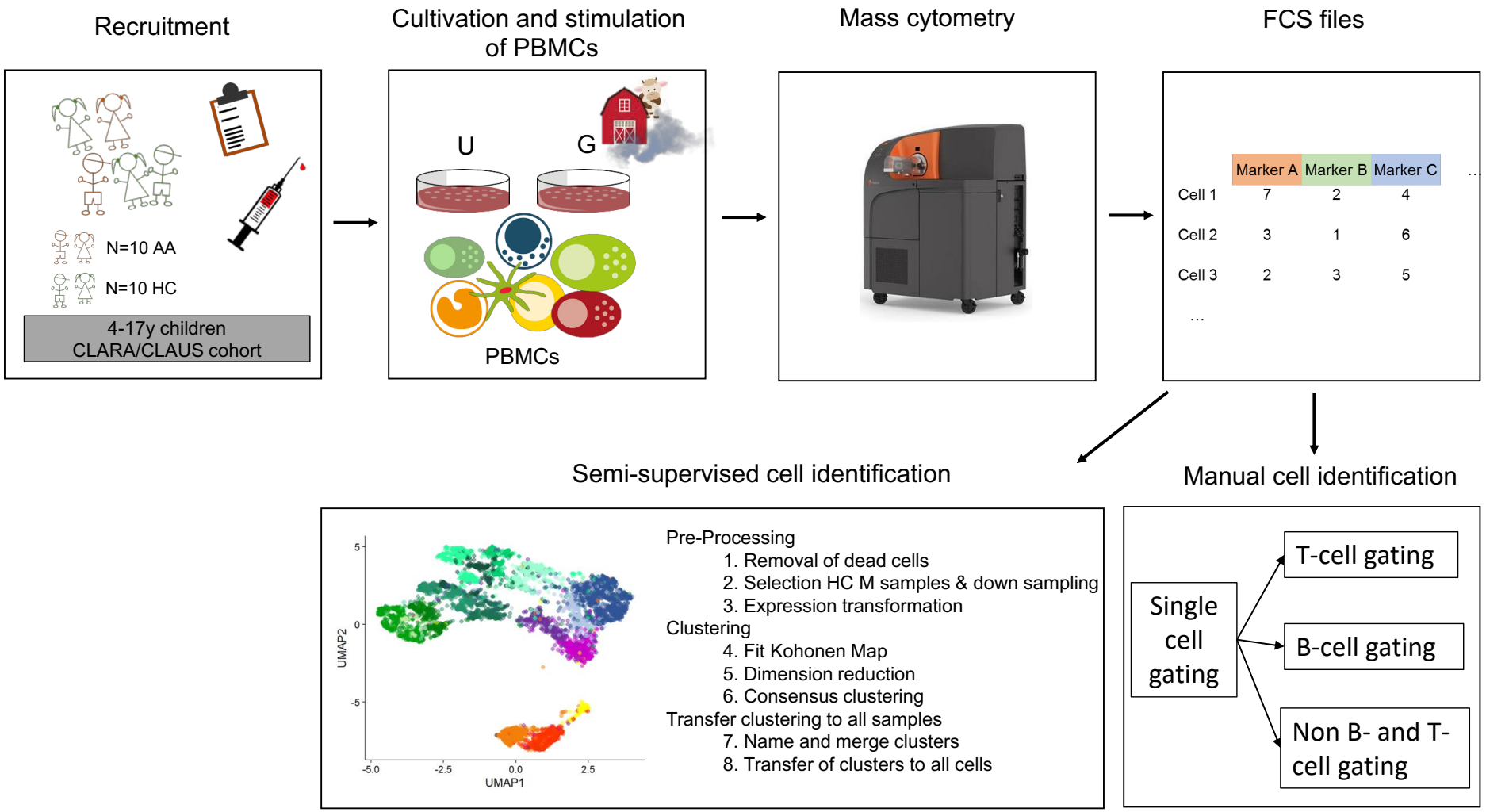
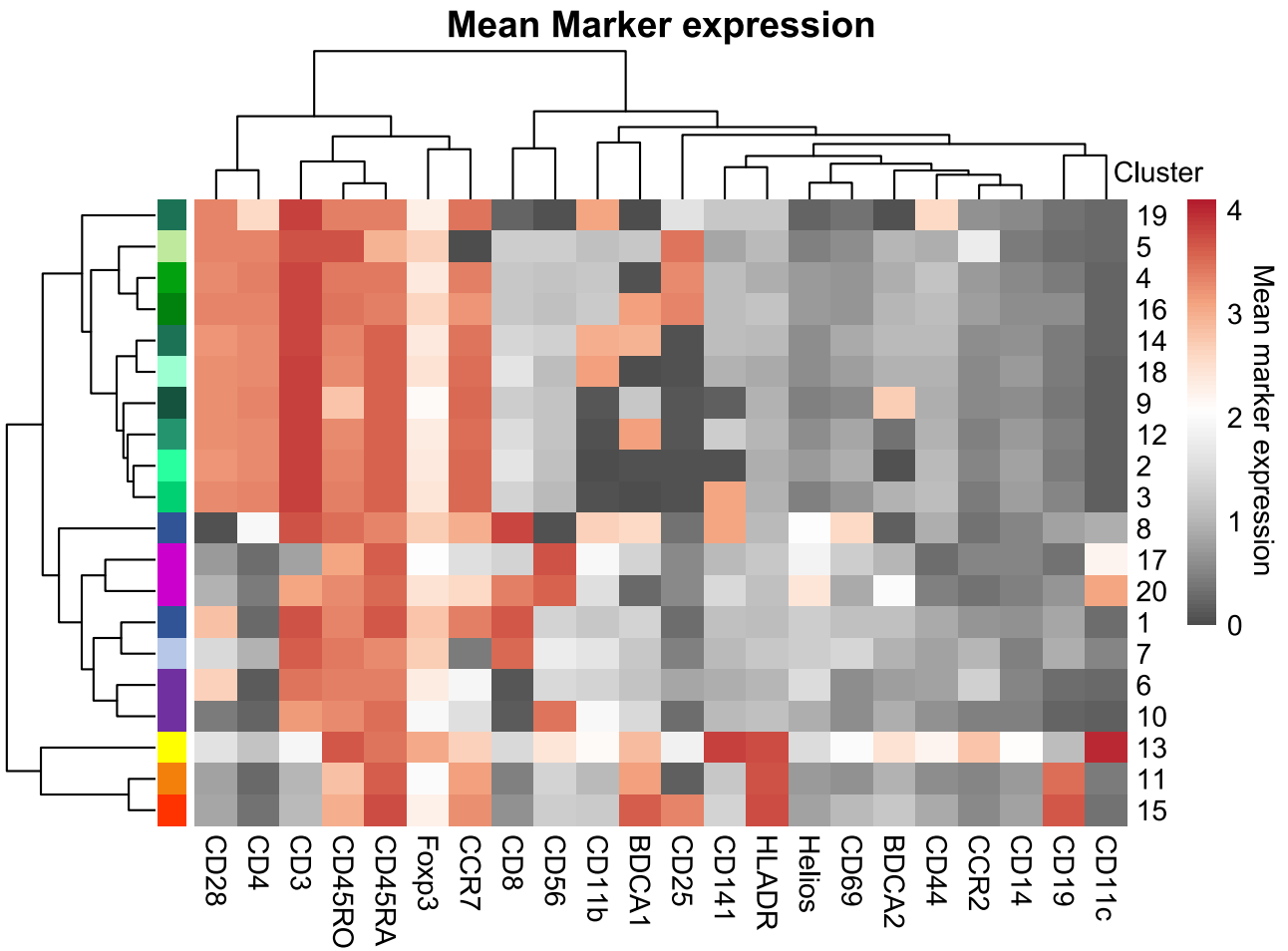
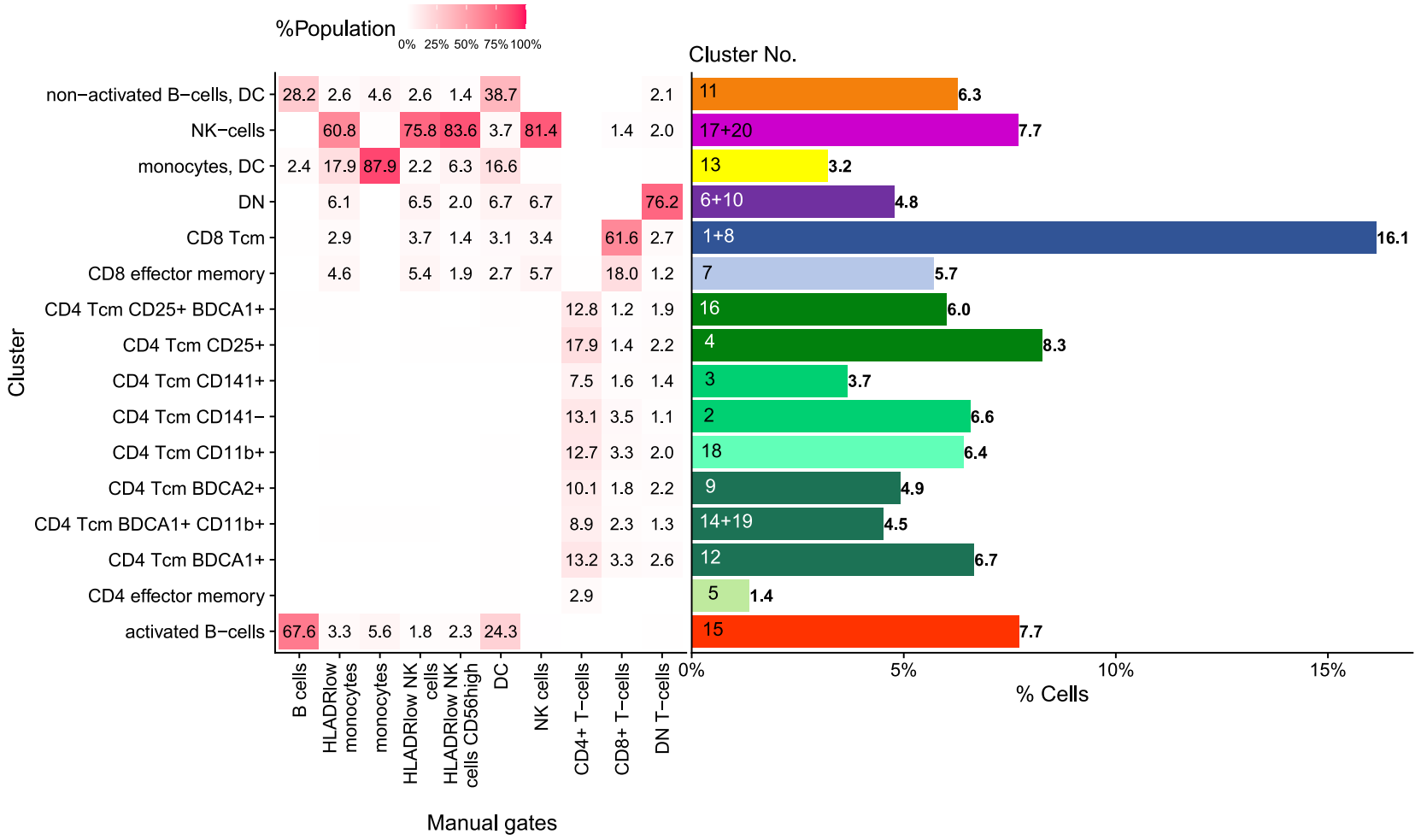


Figure 2

A


Mean Marker expression
Cluster
19
5
4
16
14
18
9
12
2
3
8
17
20
1
7
6
10
13
11
15
CD28
CD4
CD3
CD45RO
CD45RA
Foxp3
CCR7
CD8
CD56
CD11b
BDCA1
CD25
CD141
HLADR
Helios
CD69
BDCA2
CD44
CCR2
CD14
CD19
CD11c
Mean marker expression
4
3
2
1
0

B


%Population
0% 25% 50% 75% 100%
Cluster No.
non−activated B−cells, DC 28.2 2.6 4.6 2.6 1.4 38.7 2.1 11 6.3
NK−cells 60.8 75.8 83.6 3.7 81.4 1.4 2.0 17+20 7.7
monocytes, DC 2.4 17.9 87.9 2.2 6.3 16.6 13 3.2
DN 6.1 6.5 2.0 6.7 6.7 76.2 6+10 4.8
CD8 Tcm 2.9 3.7 1.4 3.1 3.4 61.6 2.7 1+8 16.1
CD8 effector memory 4.6 5.4 1.9 2.7 5.7 18.0 1.2 7 5.7
CD4 Tcm CD25+ BDCA1+ 12.8 1.2 1.9 16 6.0
CD4 Tcm CD25+ 17.9 1.4 2.2 4 8.3
CD4 Tcm CD141+ 7.5 1.6 1.4 3 3.7
CD4 Tcm CD141− 13.1 3.5 1.1 2 6.6
CD4 Tcm CD11b+ 12.7 3.3 2.0 18 6.4
CD4 Tcm BDCA2+ 10.1 1.8 2.2 9 4.9
CD4 Tcm BDCA1+ CD11b+ 8.9 2.3 1.3 14+19 4.5
CD4 Tcm BDCA1+ 13.2 3.3 2.6 12 6.7
CD4 effector memory 2.9 5 1.4
activated B−cells 67.6 3.3 5.6 1.8 2.3 24.3 15 7.7
Cluster
B cells
HLADRlow monocytes
monocytes
HLADRlow NK cells
HLADRlow NK cells CD56high
DC
NK cells
CD4+ T−cells
CD8+ T−cells
DN T−cells
Manual gates
0%
5%
10%
15%
% Cells

C

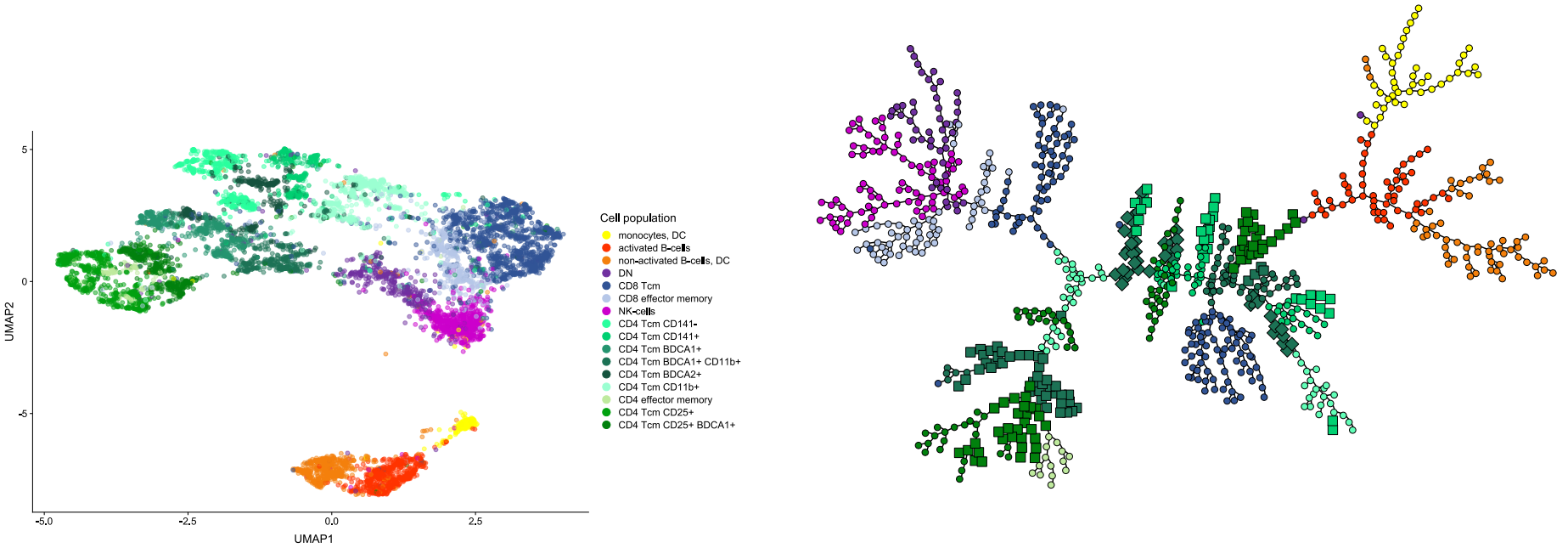
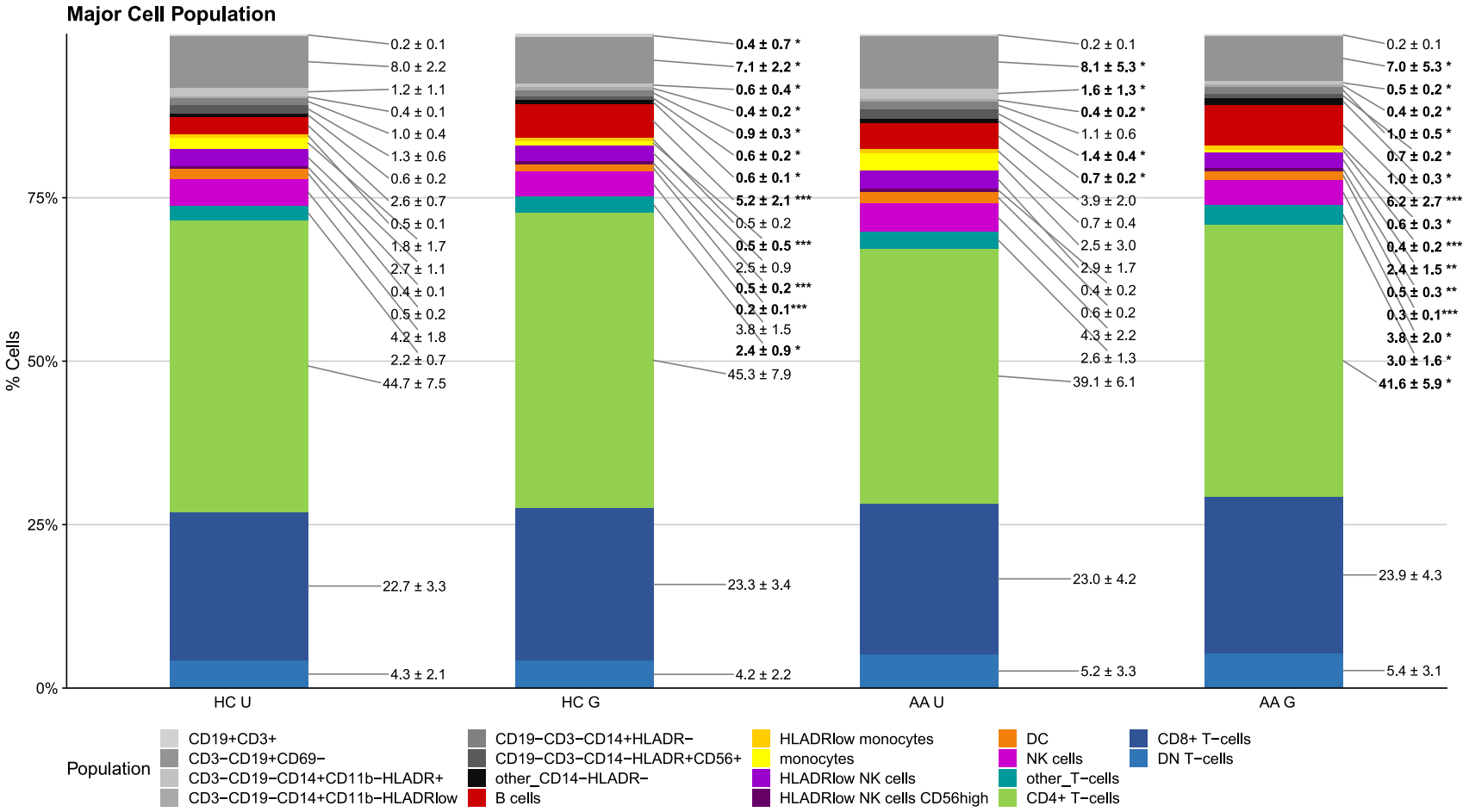

UMAP2
5
0
-5
-5.0
-2.5
0.0
2.5
UMAP1
Cell population
monocytes, DC
activated B-cells
non-activated B-cells, DC
DN
CD8 Tcm
CD8 effector memory
NK-cells
CD4 Tcm CD141-
CD4 Tcm CD141+
CD4 Tcm BDCA1+
CD4 Tcm BDCA1+ CD11b+
CD4 Tcm BDCA2+
CD4 Tcm CD11b+
CD4 effector memory
CD4 Tcm CD25+
CD4 Tcm CD25+ BDCA1+

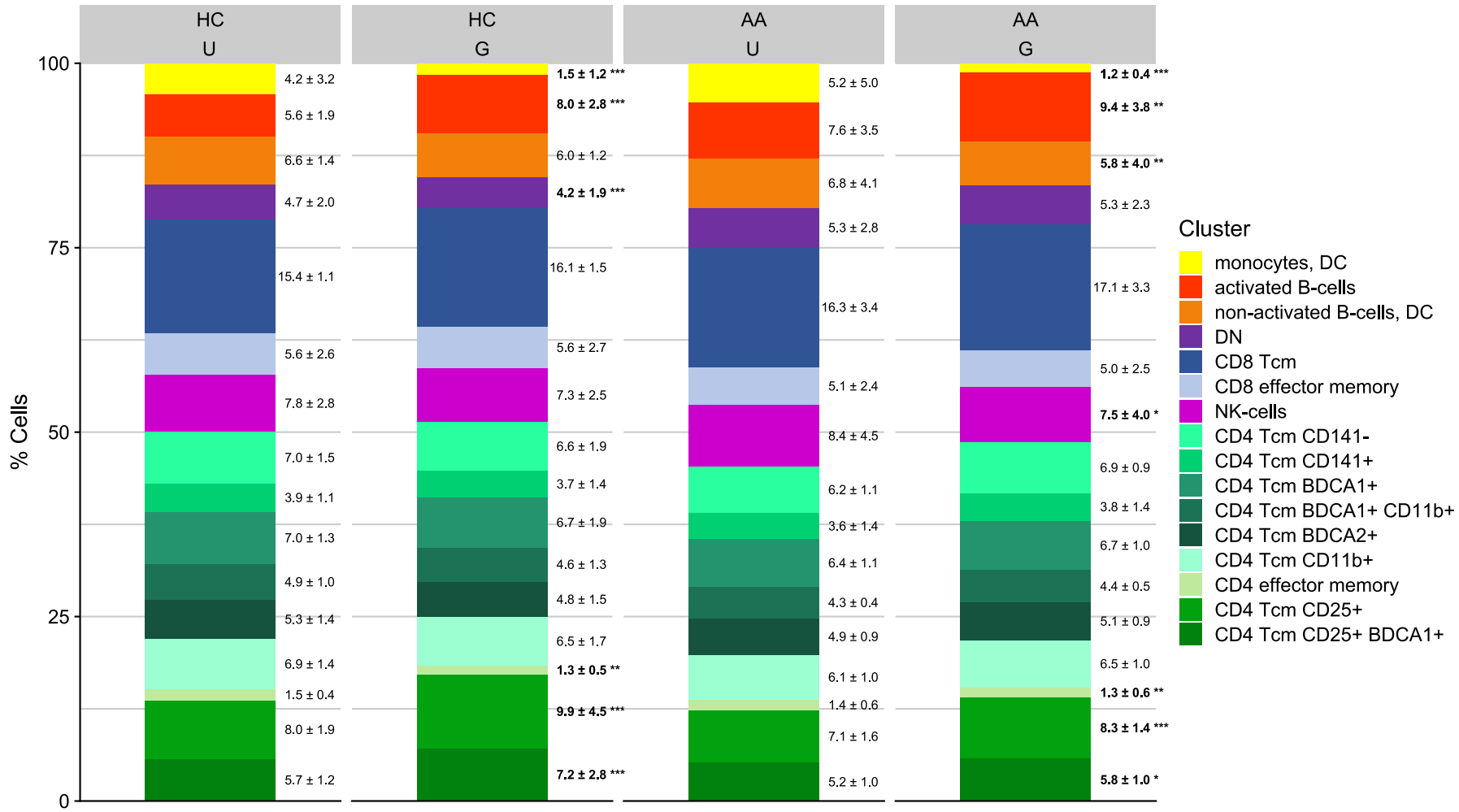
Figure 3

A



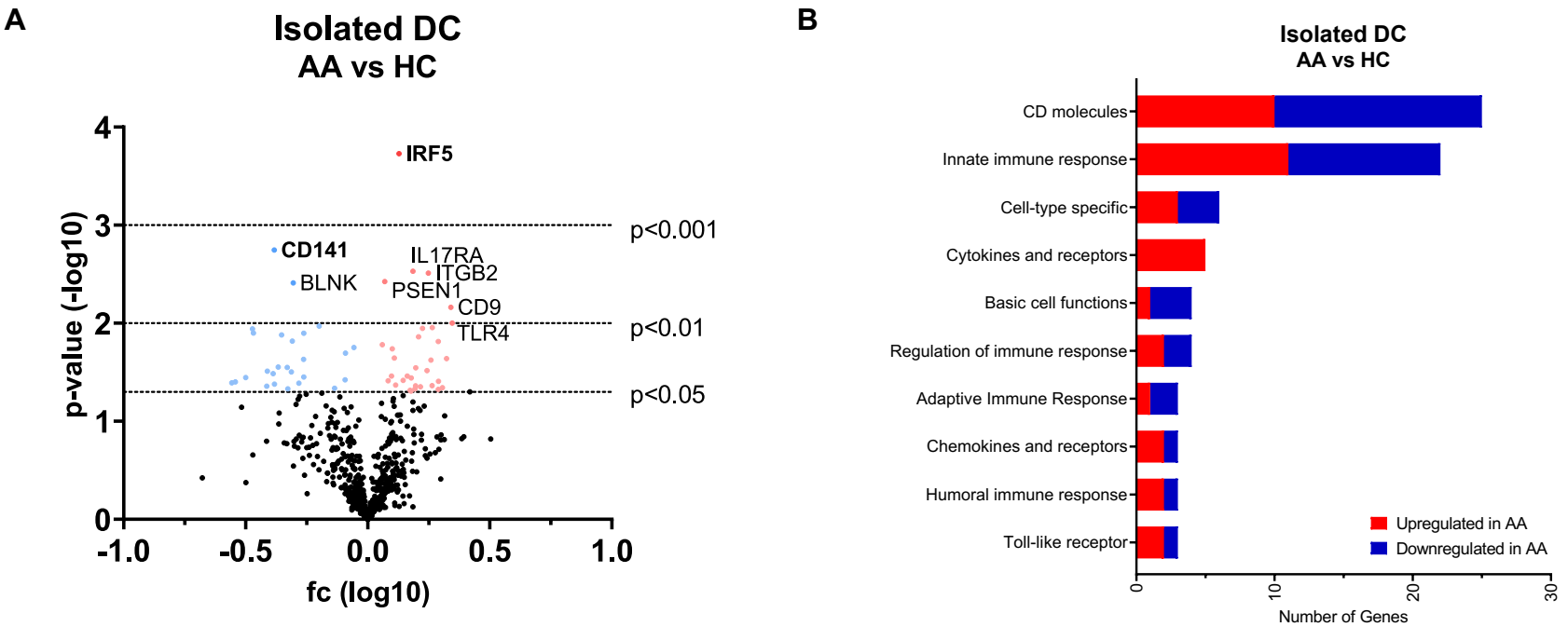
In **bold** HC G/AA G: significant differences German farm-dust stimulated (G) compared to unstimulated conditions (U); *p<0.05,**p<0.01,***p<0.001
AA U: significant differences AA U compared to HC U

B



In **bold** HC G/AA G: significant differences German farm-dust stimulated (G) compared to unstimulated conditions (U); *p<0.05,**p<0.01,***p<0.001

Figure 4


A
Isolated DC
AA vs HC
p-value (-log10)
fc (log10)
IRF5
CD141
BLNK
IL17RA
ITGB2
PSEN1
CD9
TLR4
p<0.001
p<0.01
p<0.05
B
Isolated DC
AA vs HC
CD molecules
Innate immune response
Cell-type specific
Cytokines and receptors
Basic cell functions
Regulation of immune response
Adaptive Immune Response
Chemokines and receptors
Humoral immune response
Toll-like receptor
Upregulated in AA
Downregulated in AA
Number of Genes

C

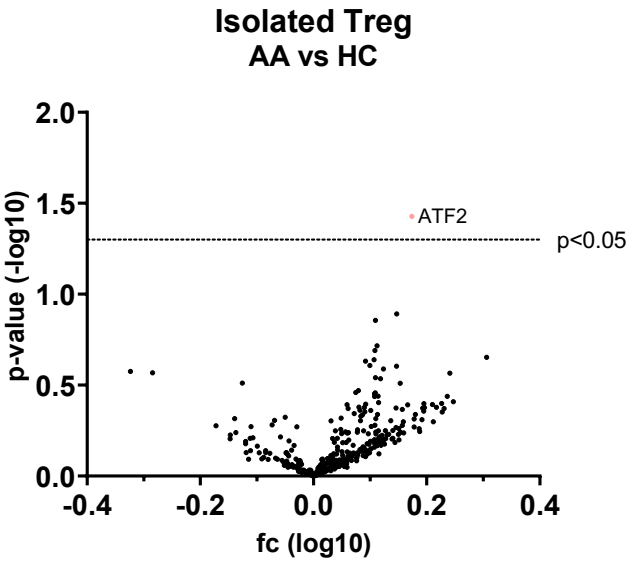
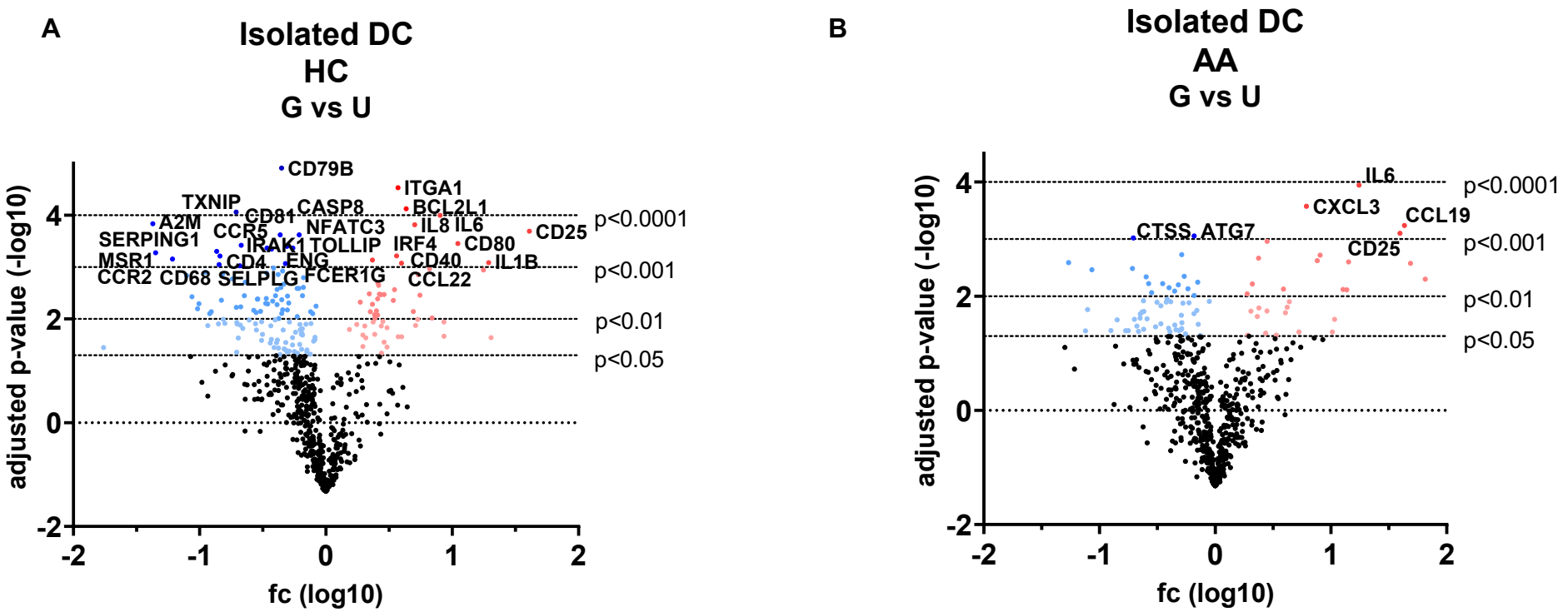
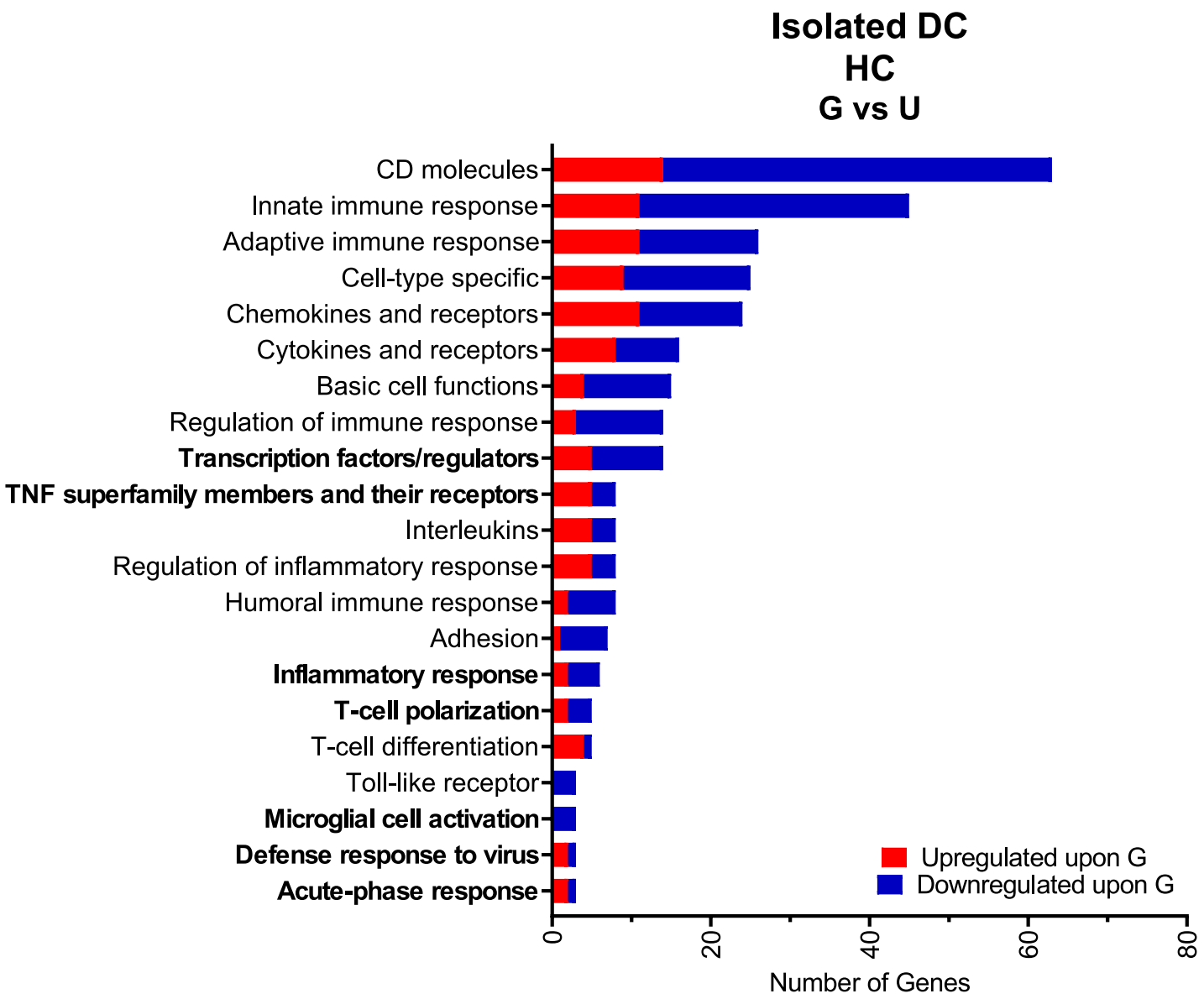

Isolated Treg
AA vs HC
2.0
1.5
1.0
0.5
0.0
p-value (-log10)
ATF2
p<0.05
-0.4
-0.2
0.0
0.2
0.4
fc (log10)

Figure 5


A
Isolated DC
HC
G vs U
adjusted p-value (-log10)
fc (log10)
CD79B
ITGA1
TXNIP
CASP8
BCL2L1
CD81
A2M
CCR5
NFATC3
IL8
IL6
CD25
SERPING1
IRAK1
TOLLIP
IRF4
CD80
MSR1
CD4
ENG
CD40
IL1B
CCR2
CD68
SELPLG
FCER1G
CCL22
p<0.0001
p<0.001
p<0.01
p<0.05
B
Isolated DC
AA
G vs U
adjusted p-value (-log10)
fc (log10)
IL6
CXCL3
CCL19
CTSS
ATG7
CD25
p<0.0001
p<0.001
p<0.01
p<0.05

C


Isolated DC
HC
G vs U
CD molecules
Innate immune response
Adaptive immune response
Cell-type specific
Chemokines and receptors
Cytokines and receptors
Basic cell functions
Regulation of immune response
Transcription factors/regulators
TNF superfamily members and their receptors
Interleukins
Regulation of inflammatory response
Humoral immune response
Adhesion
Inflammatory response
T-cell polarization
T-cell differentiation
Toll-like receptor
Microglial cell activation
Defense response to virus
Acute-phase response
Upregulated upon G
Downregulated upon G
0
20
40
60
80
Number of Genes

D

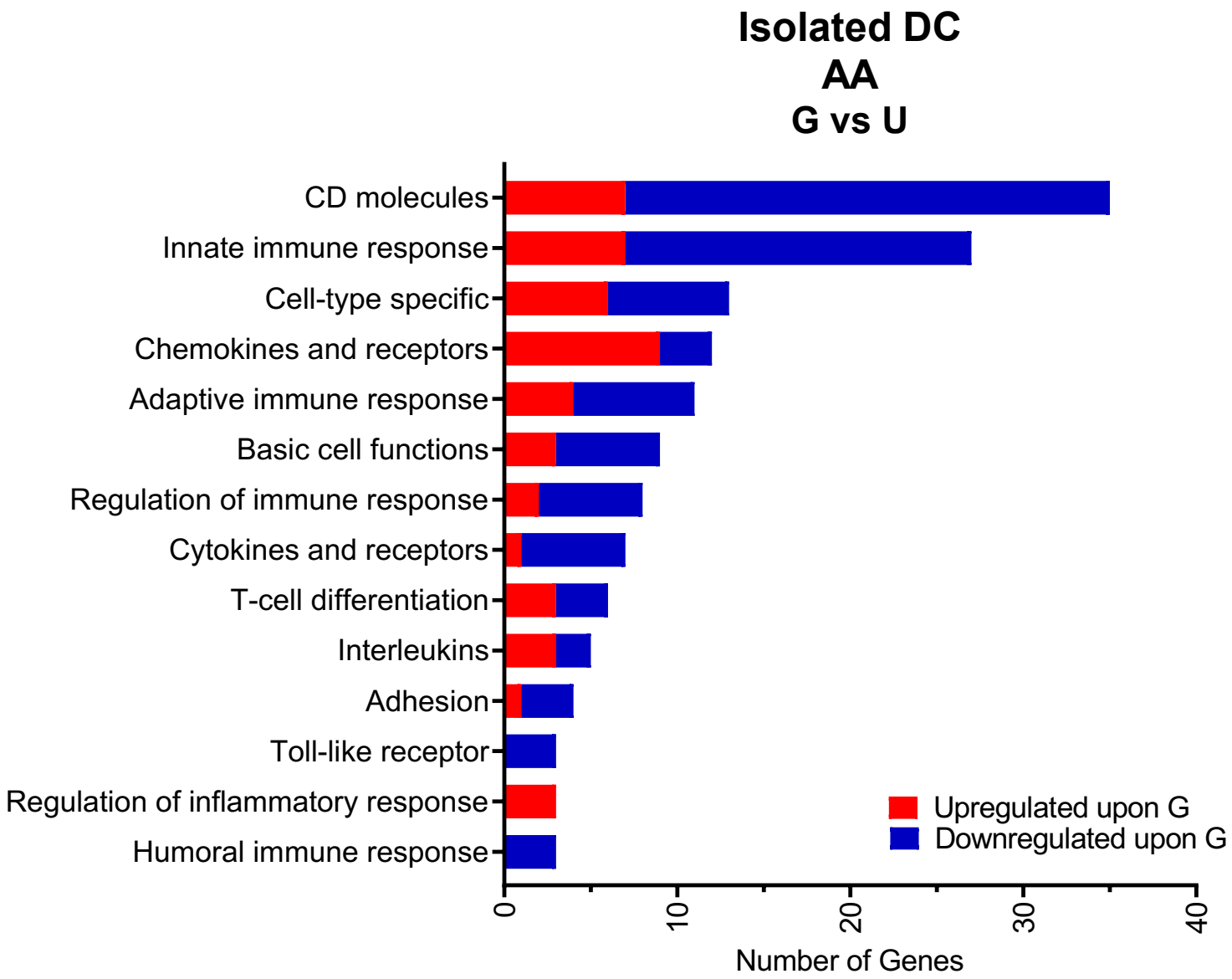

Isolated DC
AA
G vs U
CD molecules
Innate immune response
Cell-type specific
Chemokines and receptors
Adaptive immune response
Basic cell functions
Regulation of immune response
Cytokines and receptors
T-cell differentiation
Interleukins
Adhesion
Toll-like receptor
Regulation of inflammatory response
Humoral immune response
Upregulated upon G
Downregulated upon G
0
10
20
30
40
Number of Genes

Figure 6

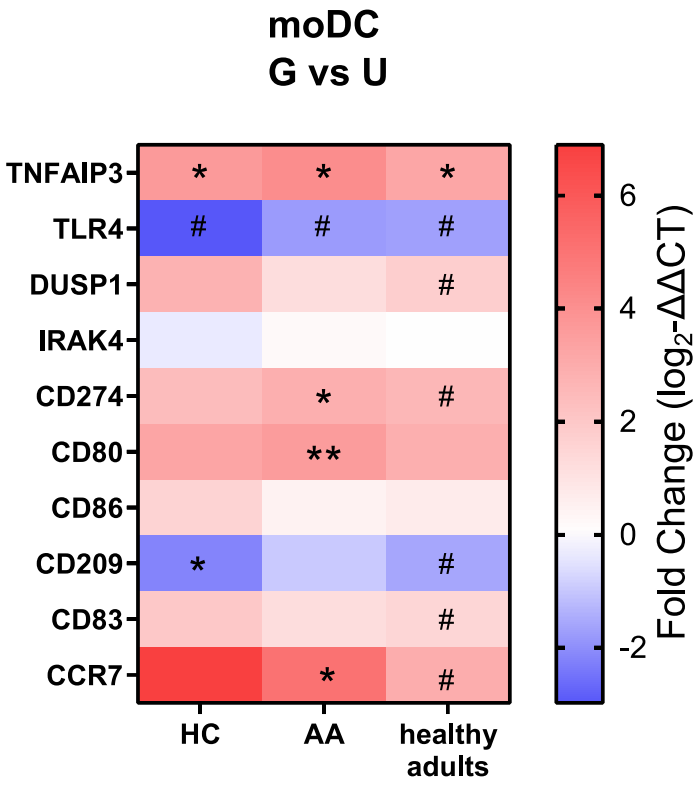

moDC
G vs U
TNFAIP3
TLR4
DUSP1
IRAK4
CD274
CD80
CD86
CD209
CD83
CCR7
HC
AA
healthy adults
6
4
2
0
-2
Fold Change (log2-ΔΔCT)

Figure 7

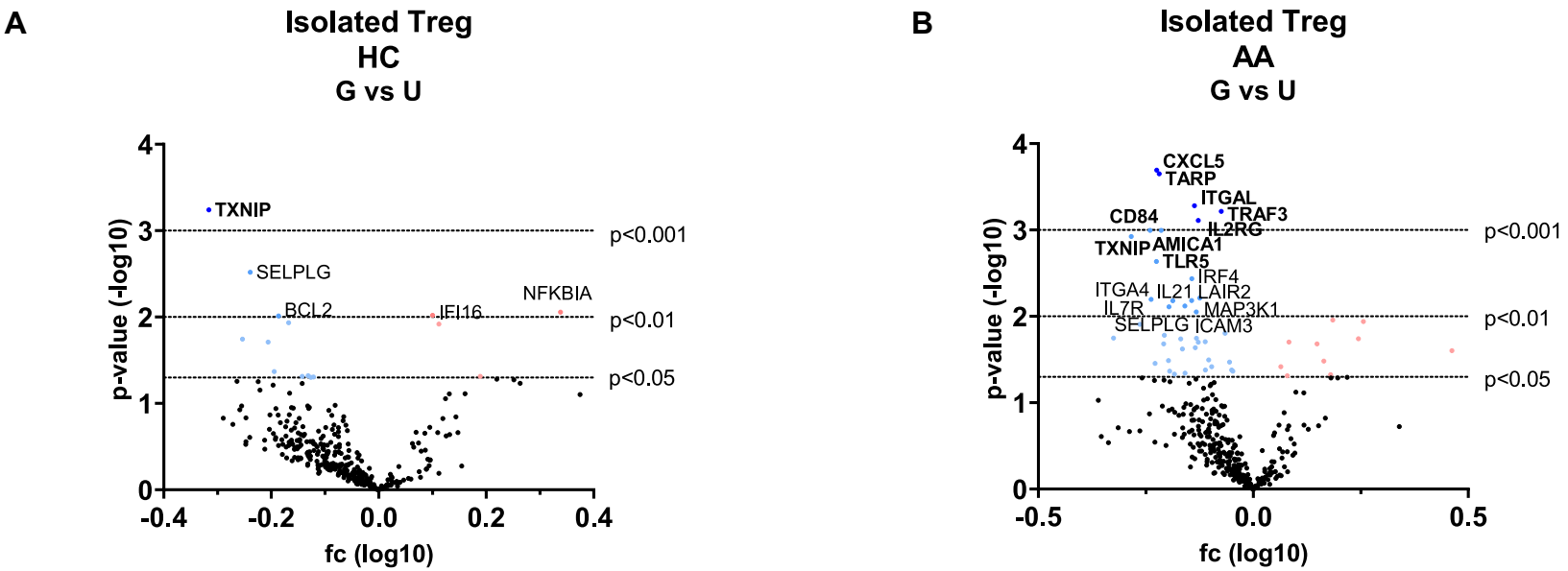
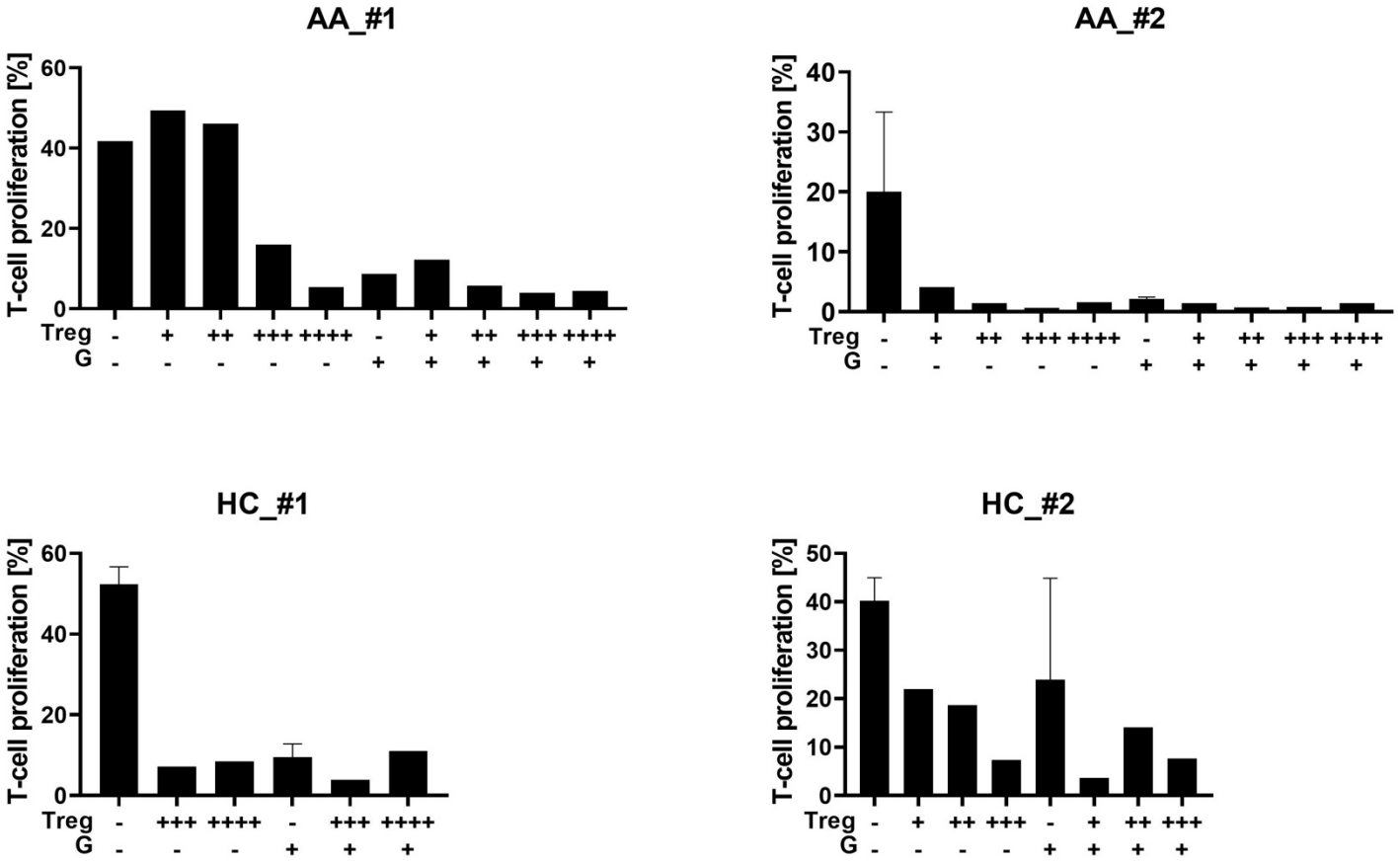

A
Isolated Treg
HC
G vs U
p-value (-log10)
fc (log10)
TXNIP
SELPLG
BCL2
IFI16
NFKBIA
p<0.001
p<0.01
p<0.05
B
Isolated Treg
AA
G vs U
p-value (-log10)
fc (log10)
CXCL5
TARP
ITGAL
CD84
TRAF3
IL2RG
TXNIP
AMICA1
TLR5
IRF4
ITGA4
IL21
LAIR2
IL7R
MAP3K1
SELPLG
ICAM3
p<0.001
p<0.01
p<0.05

Figure 8


AA_#1
T-cell proliferation [%]
60
40
20
0
Treg - + ++ +++ ++++ - + ++ +++ ++++
G - - - - - + + + + +
AA_#2
T-cell proliferation [%]
40
30
20
10
0
Treg - + ++ +++ ++++ - + ++ +++ ++++
G - - - - - + + + + +
HC_#1
T-cell proliferation [%]
60
40
20
0
Treg - +++ ++++ - +++ ++++
G - - - + + +
HC_#2
T-cell proliferation [%]
50
40
30
20
10
0
Treg - + ++ +++ - + ++ +++
G - - - - + + + +

Supplementary Files

Farm-dust mediated protection of childhood asthma: Identification of unique cellular and molecular regulatory mechanisms

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Methods

Details on statistical methods

This section provides more details on the data analysis workflow. It will list a sequence of steps and will provide some reasoning for the main parts. There were three complex steps in this project calling for a more specific description: Semi-supervised cell identification, analysis of cell frequencies, and marker expression analysis.

Semi-supervised cell identification

Aim of this step is to assign each cell to a cluster of similar cells, which was done by clustering the cells. The input was a matrix with a row for each cell (about 11.5M rows) and a column for each marker used for cell identification (n=22). The process is shown in pseudocode 1 and described in more detail in the following:

The first step in this pipeline is to reduce the number of rows from nearly 11.5M to 900 by training a self-organizing map (SOM) to the data (1). Pre-processing consisted of (1a) filtering to unstimulated cells of HC's as we intended to define cell populations based on cellular characteristics on these, (1b) random down-sampling to 1,000 cells per sample to increase computational efficiency without losing too many details, and (1c) transforming marker intensities. Then we defined the hyperparameters of our SOM (1d): we have chosen a hexagonal topology (more interaction with neighbors) and a grid size of 30 in both dimensions. The grid size was chosen a bit higher than usual (n=10) to be more sensitive towards rare cell populations. Finally (1e), we fitted this SOM to our data. This means that each cell was sequentially assigned to a grid point based on its similarity to the other cells being assigned previously to this grid point and its neighboring grid points. After repeating this for every cell, the grid points (aka codes) were used as representatives of its assigned cells.

The second big step is clustering these SOM codes (2). Before clustering, we reduced the SOM codes dimensions from 22 to 6 using an UMAP transformation (2a). This reduced noise in data and counteracted the problem of Euclidean distance in higher dimensions. We used a consensus clustering approach with 100 repetitions and a random sampling of 5/6 features for every repetition (2b). After assessing consensus cumulative distribution functions, delta area plots, and tracking plots (all provided by the *ConsensusClusterPlus*-package) we decided to choose the 20-cluster solution. Finally, we compared clusters based on their mean marker expression (Figure 3A) and merged low frequent clusters to the most similar cluster. This resulted in 16 semi-supervised clusters.

Lastly, we extended this clustering to all cells available (3). This was done in two steps: (3a) Project each cell onto the SOM –i.e. each cell was mapped to its most similar SOM code– and (3b) assign each cell to the cluster of the mapped SOM code.

Summing things up, we used cells of unstimulated HCs to define SOM, used the resulting SOM codes to fit a clustering, and then allocated each cell (unstimulated and stimulated, HC and AA) to the cluster with the most similar SOM code.

Pseudocode 1: Fitting SOM and consensus cluster

```
Input  Marker intensity matrix (n_cell x n_marker)
Output Cell to cluster assignment
  1. Fit SOM to data
       a. Filter cell matrix from unstimulated HC
       b. Down sample to … cells per sample
       c. Data transformation: arcsinh with cofactor 5
       d. Define SOM object
       e. Fit SOM object to data
  2. Define clusters on SOM codes
       a. Reduce dimensions of UMAP codes using UMAP
       b. Fit consensus clustering
       c. Manual merging of low frequent clusters
  3. Transfer results to all samples
       a. Predict each cell on the SOM codes
       b. Assign each cell to the cluster of its predicted SOM code
```

Analysis of cell frequencies

For manual gating and cluster gating, cell population frequencies were analyzed in the same way. At first, the population's frequency was calculated by

$$frequency_{population} = \frac{number\ of\ cells_{population}}{number\ of\ cells_{parent}}.$$

For manual gating, a population's parent is the population of the previous gating step. I.e. the parent population of activated B cells is all B cells. For cluster gating, the parent population equals the whole set of cells.

As we made comparisons only within a cell population (no big differences in frequencies, not close to 0 and 1 in one population), it is appropriate to *log2*-transform the calculated frequencies and use a linear regression approach. Similar to previous CyTOF findings, we saw higher individual differences than phenotype differences in our data. Along with the study design of measuring unstimulated and stimulated cells for each participant/ID, we decided to model the frequency with a linear mixed-effects model (from the *lme4*-package) with a random intercept for each ID:

$$log_2(frequency) \sim Phenotype * Stimulation + (1|ID)$$

After fitting this model for each cell population (manual gated and cluster gated), we used the *multcomp*-package to test our set of hypotheses:

$$H_{0,A}: Frequency\ unstimulated\ HC == Frequency\ stimulated\ HC$$

$$H_{0,B}: Frequency\ unstimulated\ HC == Frequency\ unstimulated\ AA$$

$$H_{0,C}: Frequency\ unstimulated\ AA == Frequency\ stimulated\ AA$$

Additionally to comparing the mean of frequencies across phenotypes and stimulation, we calculated F-tests to identify differences in variance across groups. Null hypotheses were the same as above, just looking at variance instead of mean.

Analysis of marker expression/intensity

Previous work on analyzing marker expression values was mainly comparing the mean or median expression across groups of interest. However, we believe that this concentration on a single measure of location is not sufficient to describe cellular activation as a continuous process. Therefore, we studied the marker expression from a more functional point of view.

We looked at the empirical cumulative density function of marker and compared those across our study groups.

In detail, we defined for each cell population, marker, stimulation condition, and sample a curve describing a sample's cellular activity with respect to the chosen marker. The following steps were done for each cell population and marker separately:

This curve is defined with help of the empirical cumulative density function (ecdf) of the marker:

$$curve_{ID}(stimulation) = 1 - ecdf(marker)$$

We then compared the curves of the unstimulated curves with an anova for functional data from the *fda.usc*-package to identify differences at baseline between HC and AA.

Next, we wanted to answer the question of whether stimulation had the same effect for HC and AA regarding their cellular activity. Therefore, we calculated a stabilized ratio of the stimulated and unstimulated curve of a sample.

$$ratio_{ID} = \frac{curve_{ID}(stimulated) + 0.1}{curve_{ID}(unstimulated) + 0.1}$$

We added a constant of 0.1 to numerator and denominator to avoid division by zero and not to move the estimate too far away as compared to adding one.

We then made again use of a functional anova to compare *log2*-transformed of HC and AA. A log2-ratio curve greater than zero indicates that call activity increases after stimulation as an increased proportion of cells have higher marker intensities throughout the whole marker range. If the ratio curve of AA lies above the ratio curve of HC, then AA have after stimulation a relatively higher cellular activity.

This process was visualized in Pseudocode 2.

Pseudocode 2: Functional marker expression analysis at baseline and stimulation effect

```
INPUT  Matrix with marker intensities and unique row to sample and
cell population assignment.
OUTPUT
   1. For each cell population, marker, sample, stimulation calculate:
         a. ecdf of marker intensity
         b. Curve defined as 1-ecdf
   2. For each cell population, marker compare curves of unstimulated
      HC to unstimulated AA samples (functional anova)
   3. For each cell population, marker, sample calculate the ratio of
      curves with the stimulated curve as numerator and unstimulated
      curve as denominator
   4. For each cell population, marker compare ratio curves of HC and
      AA samples (functional anova)
```

Supplementary Figure Legends

Supplementary Figure 1: Gating Strategy of mass cytometry data.

Gating strategy used for manual gating based on marker expression of lineage markers measured by mass cytometry in PBMCs of N=20 children (10 HC/10 AA). The exclusion of beads, dead and doublet cells (A) was performed for each sample individually, while all other gates including B cell (B), T cell (C) and Non B- and T cell gating (D) were gated on one healthy, unstimulated sample and transferred to all other samples.

Supplementary Figure 2: Cellular immune profile of PBMCs of healthy and allergic asthmatic children.

Major cell populations shown as percent of living cells identified by A) manual gating and B) semi-supervised clustering in PBMCs of N=20 children (10 HC/10 AA) (CLARA/CLAUS cohort) cultivated under unstimulated (U) and German-farm dust (G) stimulated conditions for 24 hours. A) Each individual sample is presented separated for the two stimulation conditions (U and G).

Supplementary Figure 3: Cellular immune profile of manually gated DC of healthy and allergic asthmatic children under unstimulated and German-farm dust stimulated conditions.

Share of mDC1, mDC2 and pDC identified by manual gating in PBMCs shown as percentage of all DCs as mean +/- standard errors presented for the four groups (HC U, HC G, AA U and AA G) measured in N=20 children (10 HC/10 AA) (CLARA/CLAUS cohort) cultivated under unstimulated (U) and German-farm dust (G) stimulated conditions for 24 hours.

Supplementary Figure 4: Differentiation of monocytes to monocyte-derived DC (moDC) shown in one representative sample.

A) Purity of CD14 expression upon CD14-isolation using magnetic beads (AutoMACS) measured by flow cytometry in unstained and CD14-FITC stained monocytes. B) Morphological appearance of moDC upon 7 days of differentiation imaged by light microscopy in a 1:20 and 1:40 magnification. C) Histogram showing downregulated CD14 expression measured by flow cytometry in undifferentiated monocytes (blue) compared to differentiated moDC (red) upon 7 days of stimulation with GM-CSF and IL-4.

Supplementary Figure 5: Cellular immune profile of manually gated B cells of healthy and allergic asthmatic children under unstimulated and German-farm dust stimulated conditions.

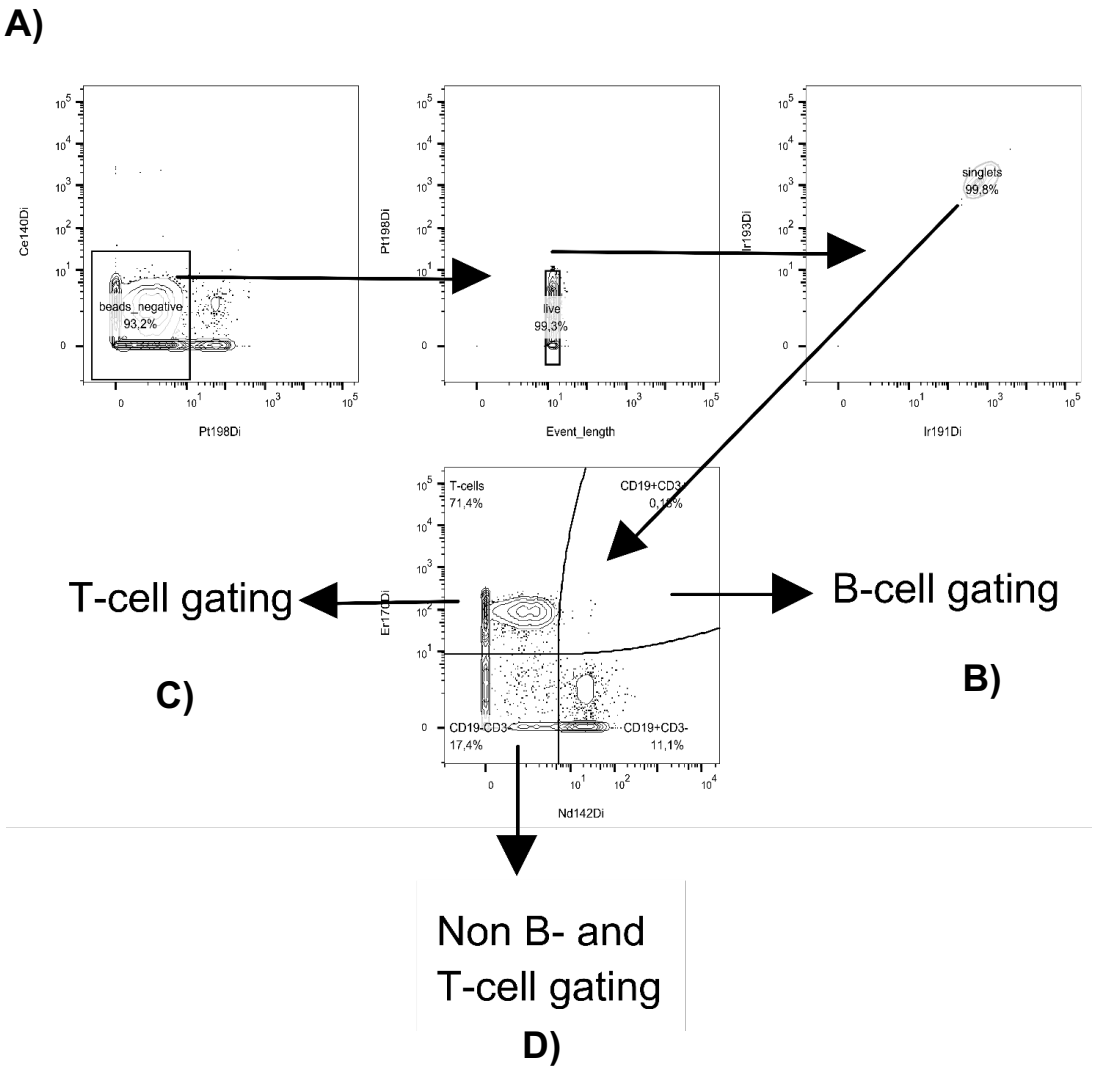
Share of activated (CD25+) and non-activated (CD25-) B cells identified by manual gating in PBMCs shown as percentage of all B cells as mean +/- standard errors presented for the four groups (HC U, HC G, AA U and AA G) measured in N=20 children (10 HC/10 AA) (CLARA/CLAUS cohort) cultivated under unstimulated (U) and German-farm dust (G) stimulated conditions for 24 hours.

Supplementary Figure 6: Immunomodulatory effects of farm dust stimulation on gene expression within Tregs.

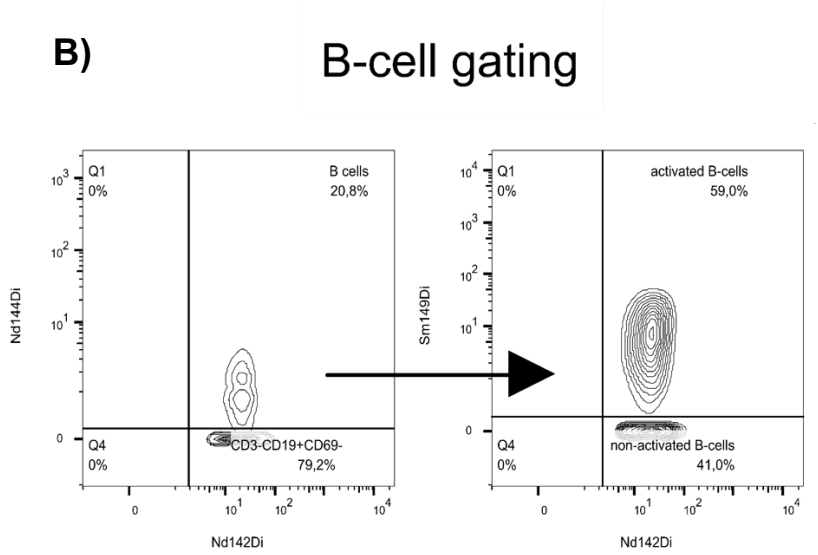
Up- (red) and downregulated (blue) immune regulation categories annotated by NanoString upon farm-dust stimulation to which the significant differentially expressed genes of Tregs could be allocated in A) HC and B) AA children measured by NanoString in Tregs of A) N=7 HC and B) N=5 AA children (CLARA/CLAUS cohort). Immune categories containing genes that are regulated exclusively in AA are written in bold.

Supplementary Figures

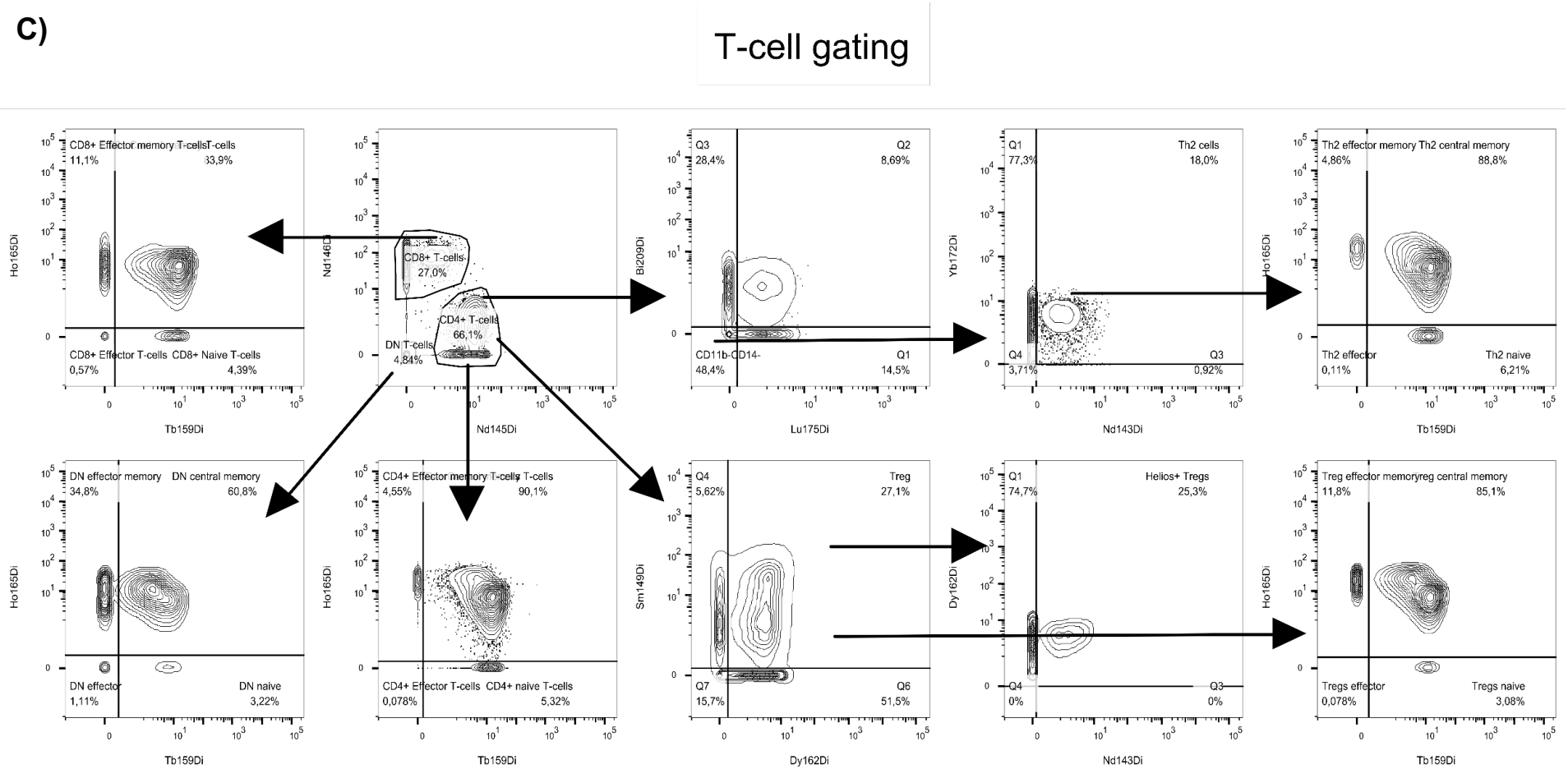
Supplementary Figure 1



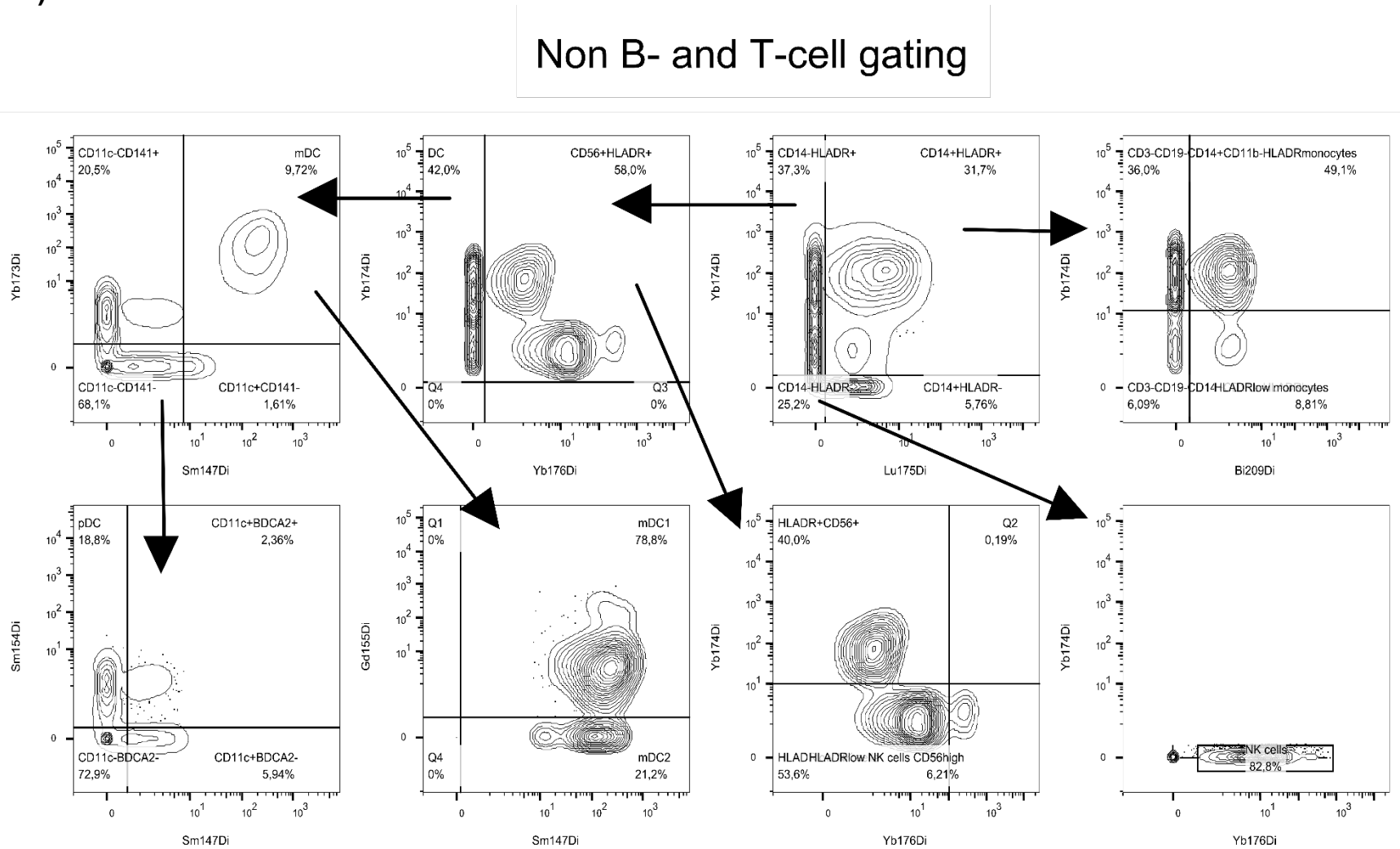
B)


B-cell gating
Q1
0%
B cells
20,8%
Q4
0%
CD3-CD19+CD69-
79,2%
Nd144Di
Nd142Di
Q1
0%
activated B-cells
59,0%
Q4
0%
non-activated B-cells
41,0%
Sm149Di
Nd142Di

C)

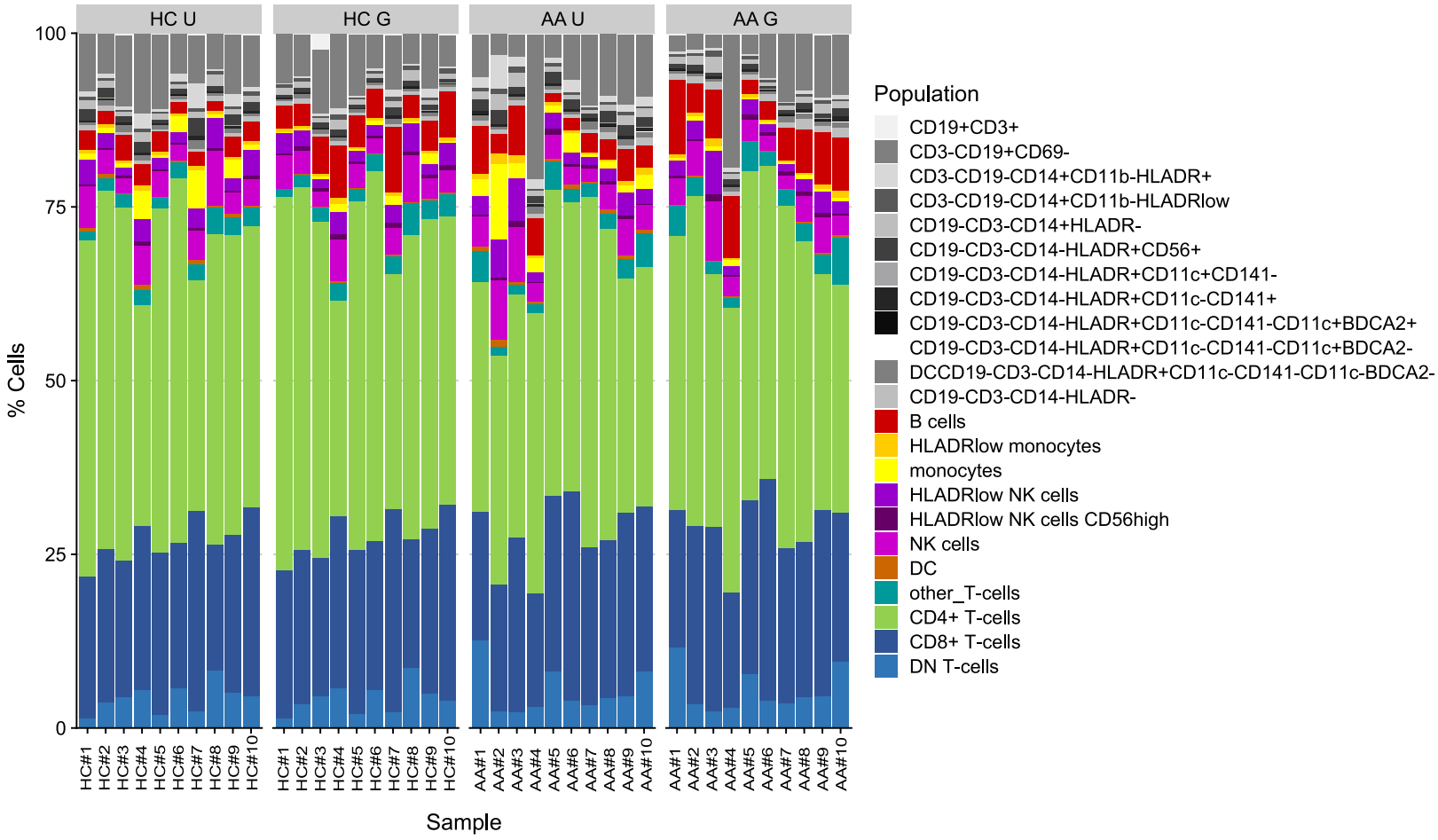

T-cell gating
CD8+ Effector memory T-cellsT-cells
11,1%
33,9%
Ho165Di
Tb159Di
CD8+ Effector T-cells CD8+ Naive T-cells
0,57%
4,39%
Nd146Di
CD8+ T-cells
27,0%
CD4+ T-cells
66,1%
DN T-cells
4,84%
Nd145Di
Q3
28,4%
Q2
8,69%
Bi209Di
CD11b-CD14-
48,4%
Q1
14,5%
Lu175Di
Q1
77,3%
Th2 cells
18,0%
Yb172Di
Q4
3,71%
Q3
0,92%
Nd143Di
Th2 effector memory Th2 central memory
4,86%
88,8%
Th2 effector
0,11%
Th2 naive
6,21%
DN effector memory DN central memory
34,8%
60,8%
DN effector
1,11%
DN naive
3,22%
CD4+ Effector memory T-cells T-cells
4,55%
90,1%
CD4+ Effector T-cells CD4+ naive T-cells
0,078%
5,32%
Q4
5,62%
Treg
27,1%
Sm149Di
Q7
15,7%
Q6
51,5%
Dy162Di
Q1
74,7%
Helios+ Tregs
25,3%
Q4
0%
Q3
0%
Treg effector memoryreg central memory
11,8%
85,1%
Tregs effector
0,078%
Tregs naive
3,08%

D)

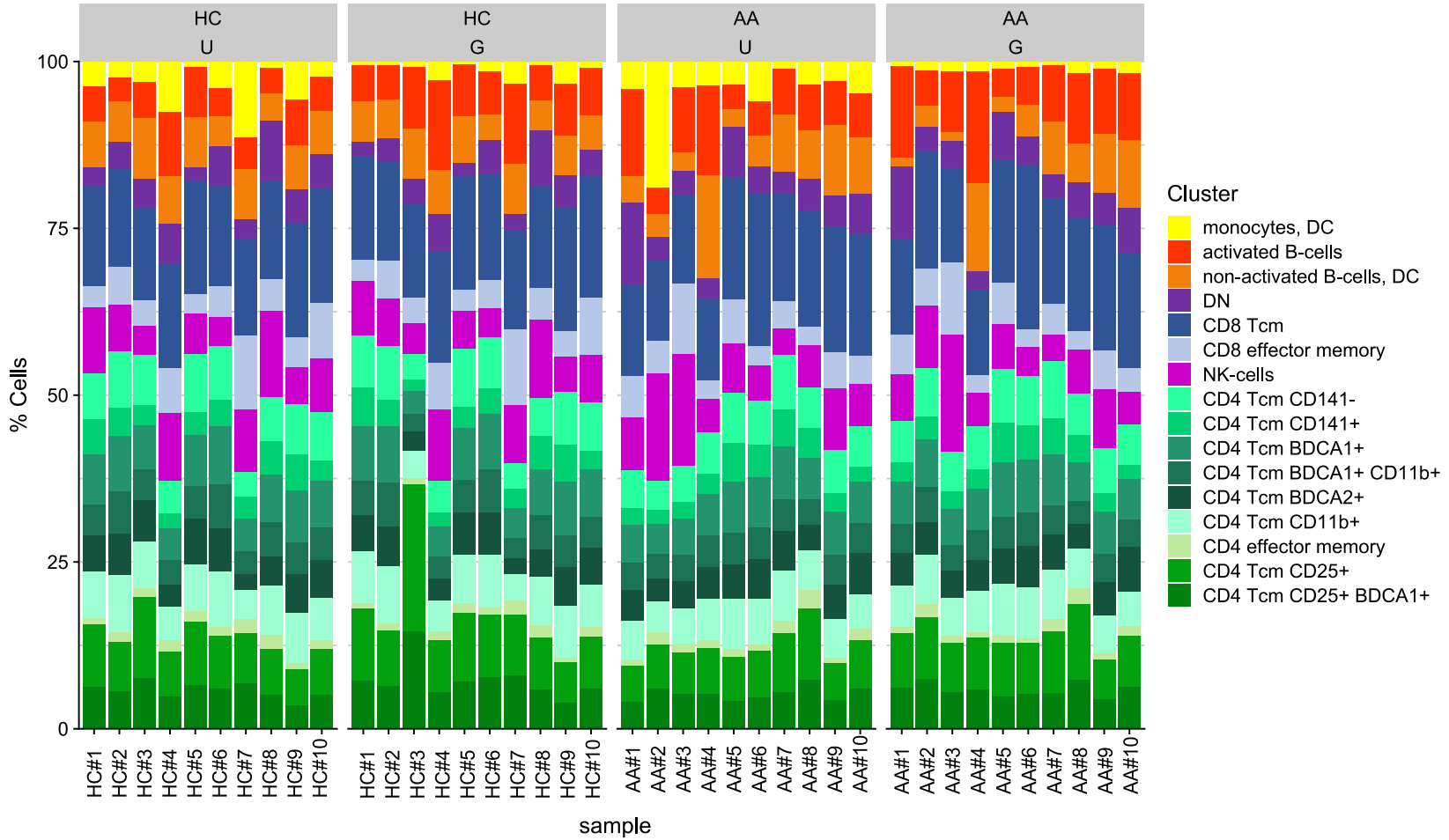

Non B- and T-cell gating
CD11c-CD141+
20,5%
mDC
9,72%
CD11c-CD141-
68,1%
CD11c+CD141-
1,61%
Yb173Di
Sm147Di
DC
42,0%
CD56+HLADR+
58,0%
Q4
0%
Q3
0%
Yb174Di
Yb176Di
CD14-HLADR+
37,3%
CD14+HLADR+
31,7%
CD14-HLADR-
25,2%
CD14+HLADR-
5,76%
Yb174Di
Lu175Di
CD3-CD19-CD14+CD11b-HLADRmonocytes
36,0%
49,1%
CD3-CD19-CD14HLADRlow monocytes
6,09%
8,81%
Yb174Di
Bi209Di
pDC
18,8%
CD11c+BDCA2+
2,36%
CD11c-BDCA2-
72,9%
CD11c+BDCA2-
5,94%
Sm154Di
Sm147Di
Q1
0%
mDC1
78,8%
Q4
0%
mDC2
21,2%
Gd155Di
Sm147Di
HLADR+CD56+
40,0%
Q2
0,19%
HLADHLADRlow NK cells CD56high
53,6%
6,21%
Yb174Di
Yb176Di
NK cells
82,8%
Yb174Di
Yb176Di

Supplementary Figure 2:

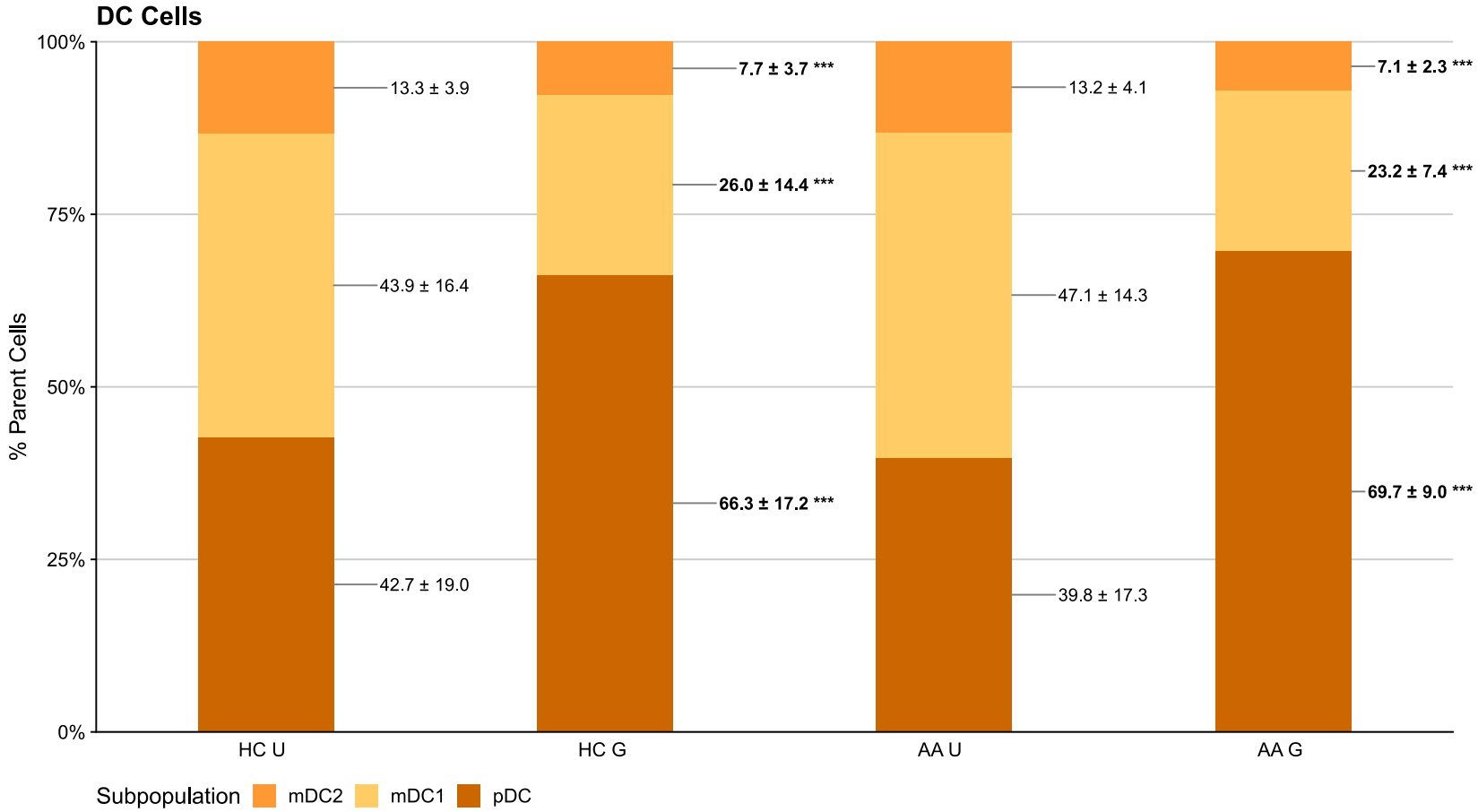
A



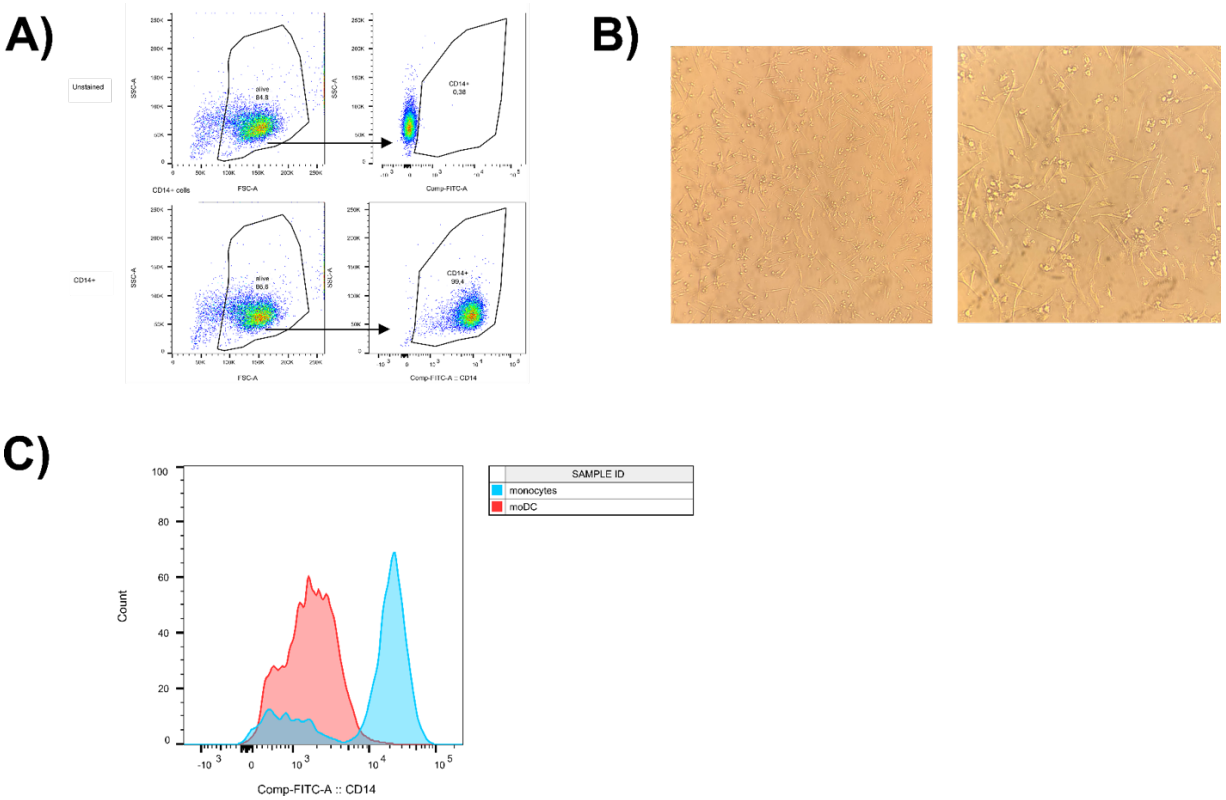
B


HC
U
HC
G
AA
U
AA
G
100
75
50
25
0
% Cells
HC#1
HC#2
HC#3
HC#4
HC#5
HC#6
HC#7
HC#8
HC#9
HC#10
AA#1
AA#2
AA#3
AA#4
AA#5
AA#6
AA#7
AA#8
AA#9
AA#10
sample
Cluster
monocytes, DC
activated B-cells
non-activated B-cells, DC
DN
CD8 Tcm
CD8 effector memory
NK-cells
CD4 Tcm CD141-
CD4 Tcm CD141+
CD4 Tcm BDCA1+
CD4 Tcm BDCA1+ CD11b+
CD4 Tcm BDCA2+
CD4 Tcm CD11b+
CD4 effector memory
CD4 Tcm CD25+
CD4 Tcm CD25+ BDCA1+

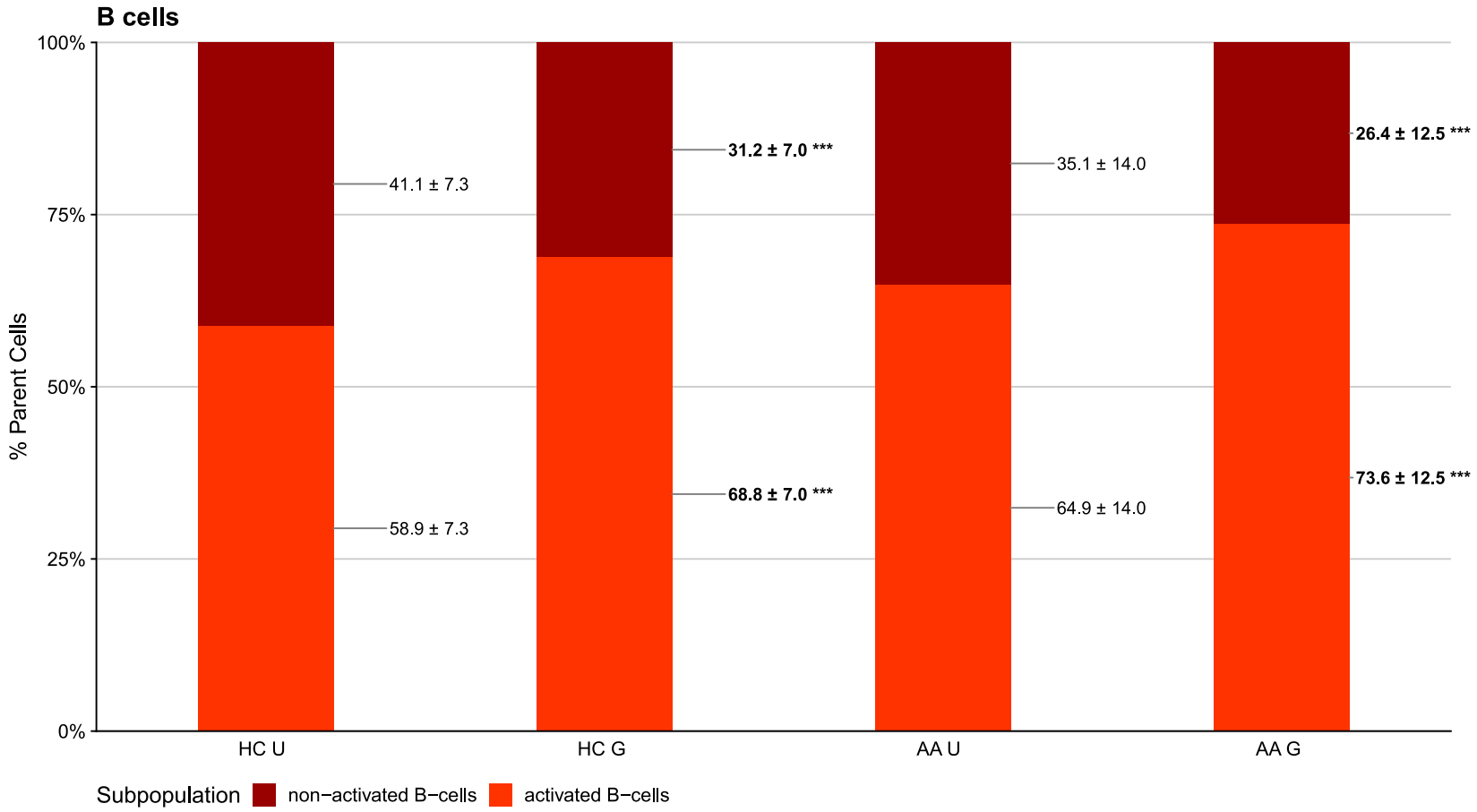
Supplementary Figure 3:



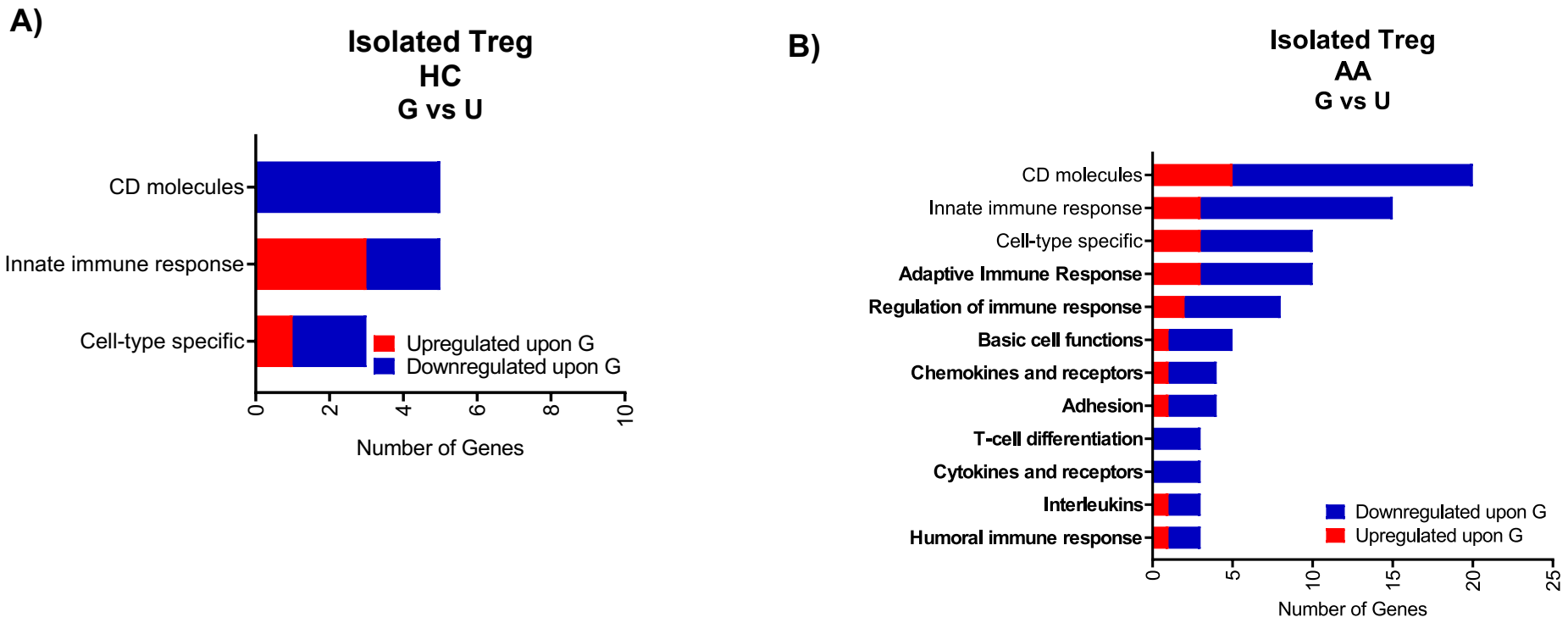
Supplementary Figure 4:



Supplementary Figure 5:



Supplementary Figure 6:



Supplementary Tables

Supplementary Table 1: Antibodies used for mass cytometry

	Target	Clone	Mass number	Metal	Source	Dilution
Lineage marker	CD11b (ITGAM)	ICRF44	209	Bi	Fluidigm	1:200
	CD28	CD28.2	160	Gd	Fluidigm	1:400
	CD69	FN50	144	Nd	Fluidigm	1:200
	CD44	BJ18	166	Er	Fluidigm	1:1600
	CCR7 (CD197)	G043H7	159	Tb	Fluidigm	1:200
	CD14	M5E2	175	Lu	Fluidigm	1:1600
	CD19	HIB19	142	Nd	Fluidigm	1:3200
	CCR2 (CD192)	K036C2	153	Eu	Fluidigm	1:3200
	CD25 (IL-2R)	2A3	149	Sm	Fluidigm	1:800
	CD3	UCHT1	170	Er	Fluidigm	1:1600
	CD4	RPA-T4	145	Nd	Fluidigm	1:1600
	CD45RA	HI100	169	Tm	Fluidigm	1:400
	CD45RO	UCHL1	165	Ho	Fluidigm	1:100
	CD56 (NCAM1)	NCAM16.2	176	Yb	Fluidigm	1:3200

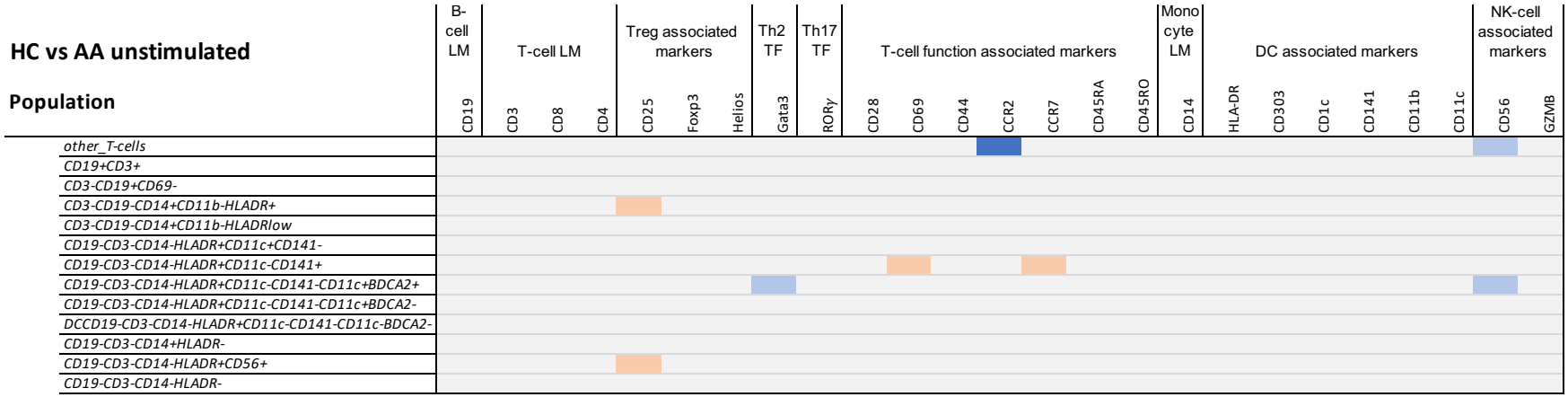
	CD8	RPA-T8	146	Nd	Fluidigm	1:3200
	HLA-DR	L243	174	Yb	Fluidigm	1:1600
	Helios (IKZF2)	22F6	143	Nd	Biolegend	1:3200
	FOXP3	PCH101	162	Dy	Fluidigm	1:200
	CD11c (ITGAX)	Bu15	159	Tb	Fluidigm	1:800
	BDCA2 (CD303, CLEC4C)	polyclonal	154	Sm	R&D	1:200
	CD141 (THBD)	1A4	173	Yb	Fluidigm	1:400
	BDCA1 (CD1c)	polyclonal	155	Gd	R&D	1:200
TF	GATA3	634919	172	Yb	R&D	1:100
	RORγ	600214	168	Er	Fluidigm	1:200
	GZMB	GB11	171	Yb	MCF	1:400

Supplementary Table 2: Frequencies (A) of minor gated subpopulations and their marker expression at baseline (B) and the fold-change upon farm-dust stimulation (C)

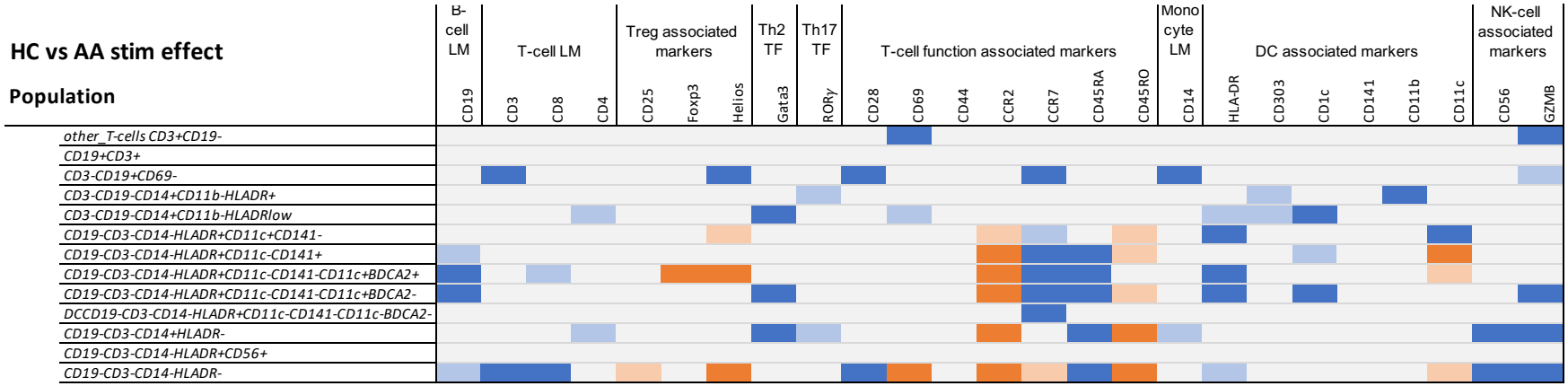
A)

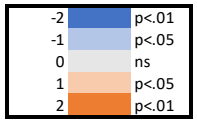
Population	HC U %Parent Mean	HC U %Parent SD	HC G (vs HC U) %Parent Mean	HC G (vs HC U) %Parent SD	AA U (vs HC U) %Parent Mean	AA U (vs HC U) %Parent SD	AA G (vs AA U) %Parent Mean	AA G (vs AA U) %Parent SD
other_T-cells	2,17	±0,73	2,42	±0,93	2,56	**±1,34**	**3,03**	**±1,60**
CD19+CD3+	0,20	±0,10	0,42	±0,71	0,18	**±0,05**	0,20	**±0,09**
CD3-CD19+CD69-	7,97	±2,24	**7,07**	±2,15	**8,07**	**±5,27**	**6,98**	±5,29
CD3-CD19-CD14+CD11b-HLADR+	1,24	±1,07	**0,56**	**±0,40**	**1,60**	±1,25	**0,47**	**±0,15**
CD3-CD19-CD14+CD11b-HLADRlow	0,35	±0,11	**0,45**	**±0,18**	**0,42**	**±0,21**	**0,38**	±0,20
CD19-CD3-CD14-HLADR+CD11c+CD141-	0,02	±0,01	**0,01**	**±0,01**	**0,03**	±0,01	**0,03**	±0,01
CD19-CD3-CD14-HLADR+CD11c-CD141+	0,34	±0,11	**0,30**	**±0,09**	**0,41**	±0,10	**0,38**	±0,11
CD19-CD3-CD14-HLADR+CD11c-CD141-CD11c+BDCA2+	0,02	±0,01	0,02	±0,01	**0,04**	**±0,02**	**0,05**	**±0,03**
CD19-CD3-CD14-HLADR+CD11c-CD141-CD11c+BDCA2-	0,05	±0,01	**0,04**	±0,01	**0,06**	±0,02	**0,08**	**±0,02**
DCCD19-CD3-CD14-HLADR+CD11c-CD141-CD11c-BDCA2-	0,60	±0,28	**0,43**	**±0,14**	**0,66**	±0,28	**0,51**	**±0,18**
CD19-CD3-CD14+HLADR-	1,01	±0,35	**0,94**	±0,31	1,14	**±0,58**	**1,04**	**±0,49**
CD19-CD3-CD14-HLADR+CD56+	1,29	±0,61	**0,64**	**±0,23**	**1,44**	**±0,41**	**0,69**	**±0,20**
CD19-CD3-CD14-HLADR-	0,59	±0,16	**0,55**	**±0,13**	**0,72**	**±0,24**	**1,00**	**±0,29**

B) **HC vs AA unstimulated**



C) **HC vs AA stim effect**





Supplementary Table 3: Primers used for qPCR analysis

Gene	Primer sequence (forward primer, 5´- 3´)
18S	AGTCCCTGCCCTTTGTACACA
TNFAIP3	GCCCAGGAATGCTACAGATACCC
TLR4	CTCAACCAAGAACCTGGACCTG
DUSP1	CTCAAAGGAGGATACGAAGCG
IRAK4	TCTCTTGCTTGGATGGTACTC
CD274	ACCACCACCAATTCCAAGAGAG
CD80	CTGGCTGGTCTTTCTCACTTCTGTTC
CD86	GCGGCTTTTATCTTCACCTTTC
CD209	GCTCGTCGTAATCAAAAGTGCTG
CD83	ACAGAGCGGAGATTGTCCTG
CCR7	AGCGTCATGGACCTGGGGAAA

Supplementary Table 4: Phenotype differences (AA vs HC) in Tregs and DCs under unstimulated conditions (p-value < 0.05)

	Cell type	Gene	Diff. (AA/HC)	p-value	p-value adjusted for multiple testing	Immune Response Category	Annotations
	Treg	ATF2	0,577	0,037 (*)	0,784		Innate immune response
Genes downregulated in allergic asthmatic children (AA)	DC	CD141 (THBD)	-1,272	0,002 (**)	0,038 (*)	Leukocyte Functions	CD molecules, Leukocyte migration
	DC	BLNK	-1,015	0,004 (**)	0,082		Humoral immune response
	DC	MEF2C	-0,661	0,011 (*)	0,225		Humoral immune response
	DC	TLR9	-1,570	0,011 (*)	0,240	TLR	CD molecules, Innate immune response, Toll-like receptor
	DC	CCL20	-1,555	0,013 (*)	0,265	Chemokines	Chemokines and receptors
	DC	APP	-0,871	0,013 (*)	0,266		Innate immune response
	DC	TLR7	-1,173	0,013 (*)	0,277	Microglial Functions, TLR	Microglial cell activation, Innate immune response, Toll-like receptor
	DC	MAPKAPK2	-1,027	0,015 (*)	0,320		Innate immune response
	DC	ABCF1	-0,188	0,018 (*)	0,371		
	DC	EDC3	-0,303	0,020 (*)	0,425		Innate immune response
	DC	ETS1	-0,873	0,023 (*)	0,492		
	DC	SELL	-1,218	0,028 (*)	0,588	Regulation	CD molecules, Regulation of immune response
	DC	IRF7	-1,097	0,028 (*)	0,595		Innate immune response

	DC	CXCR3	-1,368	0,031 (*)	0,648	Chemokines, Regulation	CD molecules, Chemokines and receptors, Regulation of inflammatory response
	DC	TNFRSF17	-1,040	0,031 (*)	0,661	Cell Functions, TNF Superfamily	Basic cell functions, Cell Type specific, CD molecules, TNF superfamily members and their receptors
	DC	LTB	-1,287	0,033 (*)	0,686	Cytokines, TNF Superfamily	Cytokines and receptors, TNF superfamily members and their receptors
	DC	CD4	-0,871	0,035 (*)	0,743		Adaptive immune response, CD molecules, Innate immune response
	DC	CLEC4C	-1,661	0,036 (*)	0,754		CD molecules, Innate immune response
	DC	TP53	-0,308	0,038 (*)	0,794	T cell Functions	T cell proliferation
	DC	LILRA4	-1,802	0,040 (*)	0,833		CD molecules, Innate immune response
	DC	IL1B	-1,850	0,041 (*)	0,852	Cytokines, Interleukins	Acute-phase response, Cytokines and receptors, Inflammatory response, Innate immune response, Interleukins
	DC	CARD11	-0,938	0,041 (*)	0,860	Regulation	Regulation of immune response
	DC	IL6	-1,270	0,042 (*)	0,877	Cytokines	Cell Type specific, CD molecules, Cytokines and receptors
	DC	CCR2	-1,374	0,044 (*)	0,924	Cytokines	Cell Type specific, CD molecules, Cytokines and receptors, Inflammatory response
	DC	PYCARD	-0,452	0,046 (*)	0,968		Innate immune response

	DC	IRF8	-1,089	0,047 (*)	0,981	Chemokines, Regulation	Cytokines and receptors, Transcription factors, Transcriptional regulators
Genes upregulated in AA	DC	IRF5	0,426	0,0002 (***)	0,004 (**)	Senescence	Senescence initiators interferon related
	DC	IL17RA	0,614	0,003 (**)	0,062	Interleukins	Interleukins
	DC	ITGB2	0,824	0,003 (**)	0,065	Adhesion, Regulation	Adhesion, CD molecules, Regulation of immune response
	DC	PSEN1	0,232	0,004 (**)	0,079	T cell Functions	T cell activation
	DC	CD9	1,132	0,007 (**)	0,145		CD molecules
	DC	TLR4	1,148	0,010 (*)	0,210	Microglial Functions, TLR	CD molecules, Innate immune response, Microglial cell activation, Toll-like receptor
	DC	LILRB3	0,878	0,011 (*)	0,233	Regulation	CD molecules, Regulation of immune response
	DC	CSF1R	0,746	0,011 (*)	0,237		CD molecules, Innate immune response
	DC	CTSS	0,692	0,014 (*)	0,290		Adaptive immune response
	DC	TREM2	0,961	0,015 (*)	0,324		Humoral immune response
	DC	ATG16L1	0,199	0,017 (*)	0,348	Transporter Functions	Autophagic vacuole formation, Genes responsible for protein transport
	DC	LAMP2	0,335	0,018 (*)	0,384		CD molecules
	DC	ATG7	0,362	0,023 (*)	0,476	Transporter Functions	Genes responsible for protein transport, Protein ubiquitination
	DC	IL1R2	1,073	0,023 (*)	0,482		CD molecules, Innate immune response

	DC	LRP1	0,860	0,024 (*)	0,499		CD molecules
	DC	HAVCR2	0,653	0,028 (*)	0,597		Innate immune response
	DC	CD63	0,807	0,030 (*)	0,640		CD molecules
	DC	CLEC7A	0,538	0,035 (*)	0,726		Innate immune response
	DC	LCP1	0,324	0,035 (*)	0,726	T cell Functions	T cell activation
	DC	CD68	0,593	0,036 (*)	0,758	Cell Functions	Basic cell functions, Cell Type specific, CD molecules
	DC	HAMP	0,482	0,038 (*)	0,806	Cell Functions, Cytotoxicity	Adaptive immune response, Basic cell functions, Cell Type specific, Cytotoxicity, Innate immune response
	DC	CD33	0,277	0,039 (*)	0,811		CD molecules
	DC	FCGR2A	0,962	0,039 (*)	0,822		CD molecules, Innate immune response
	DC	LY96	0,380	0,043 (*)	0,897		Innate immune response
	DC	LGALS3	0,875	0,043 (*)	0,910		Innate immune response
	DC	AICDA	0,652	0,044 (*)	0,915	B cell Functions	B cell differentiation
	DC	A2M	0,717	0,044 (*)	0,934	Chemokines	Chemokines and receptors, Innate immune response
	DC	ITGAX	1,018	0,046 (*)	0,956	Adhesion	Adhesion, CD molecules, Innate immune response
	DC	CEBPB	0,644	0,047 (*)	0,980		Acute-phase response
	DC	MSR1	0,960	0,047 (*)	0,994	Cell Functions	Basic cell functions, Cell Type specific, CD molecules
	DC	IGF2R	0,576	0,048 (*)	1,015	Senescence	CD molecules, Senescence initiators
	DC	CD84	0,589	0,049 (*)	1,036		CD molecules

Supplementary Table 5: Stimulatory effect (G vs M) in Treg of allergic asthmatic (AA) and healthy (HC) children (p-value < 0.05)

		Cell type	Gene	fc	p-value	p-value adjusted for multiple testing	fc	p-value	Adj. p-value	Immune Response Category	Annotations
				HC			**AA**				
in HC and AA	Downregulated	Treg	TXNIP	-1,050	0,001 (**)	0,012 (*)	-0,943	0,001 (**)	0,025 (*)		Innate immune response
		Treg	SELPLG	-0,795	0,003 (**)	0,064	-0,652	0,008 (**)	0,163		CD molecules
		Treg	JAK2	-0,436	0,048 (*)	1,002	-0,321	0,038 (*)	0,806	Cytokines, Regulation	Cytokines and receptors, Innate immune response, Positive regulation of immune response
		Treg	LAIR2	-0,403	0,050 (*)	1,041	-0,414	0,006 (**)	0,129	Cell Functions	Basic cell functions, Cell Type specific, CD molecules
		Treg	CD84	-0,420	0,050 (*)	1,049	-0,797	0,001 (**)	0,021 (*)		CD molecules
	Upregulated	Treg	NFKBIA	1,123	0,009 (**)	0,184	0,812	0,018 (*)	0,381		Innate immune response
in HC	Downregulated	Treg	BCL2	-0,619	0,010 (**)	0,204	-0,138	0,469	9,850	Cell Cycle	Cell Type specific, G1/S transition of mitotic cell cycle
		Treg	ST6GAL1	-0,557	0,012 (*)	0,244	-0,235	0,111	2,338		Humoral immune response

		Treg	CT45A1	-0,842	0,018 (*)	0,378	-0,144	0,664	13,938		
		Treg	SLAMF 1	-0,683	0,020 (*)	0,410	-0,126	0,705	14,814		CD molecules, Adaptive immune response
		Treg	KLRB1	-0,646	0,043 (*)	0,900	-0,859	0,052	1,088	Chemokines, NK Cell Functions	CD molecules, Chemokines and receptors, Inflammatory response, NK cell functions
		Treg	IL10RA	-0,473	0,049 (*)	1,023	-0,444	0,053	1,122	Interleukins	Immunosuppression, Interleukins
	Upregulated	Treg	IFI16	0,333	0,010 (**)	0,201	-0,117	0,567	11,908	Cytokines, T cell Functions	Cell Type specific, Cytokines and receptors, Chronic inflammatory response, Th2 orientation, T cell proliferation
		Treg	TANK	0,372	0,012 (*)	0,254	-0,027	0,770	16,166		Innate immune response
		Treg	IRF7	0,627	0,049 (*)	1,021	-0,106	0,833	17,488		Innate immune response
in AA	Downregulated	Treg	CXCL5	-0,497	0,219	4,606	-0,746	<0,0001 (***)	0,004 (**)	Chemokines	Cell Type specific, Chemokines and receptors
		Treg	TARP	-0,334	0,407	8,543	-0,727	<0,0001 (***)	0,005 (**)	Cell Functions	Basic cell functions, Cell Type specific
		Treg	ITGAL	-0,353	0,299	6,276	-0,454	0,001 (**)	0,011 (*)	Adhesion, Regulation	Adhesion, CD molecules, Regulation of immune response
		Treg	TRAF3	0,216	0,321	6,734	-0,247	0,001 (**)	0,013 (*)		Innate immune response

		Treg	IL2RG	-0,085	0,669	14,040	-0,425	0,001 (**)	0,016 (*)	Cytokines	Adaptive immune response, CD molecules, Cytokines and receptors
		Treg	AMICA1	-0,551	0,076	1,604	-0,712	0,001 (**)	0,021 (*)	Regulation	Regulation of immune response
		Treg	TLR5	0,439	0,230	4,831	-0,748	0,002 (**)	0,049 (*)	TLR	Innate immune response, Toll-like receptor
		Treg	IRF4	-0,121	0,396	8,321	-0,476	0,004 (**)	0,077	Regulation, T cell Functions	Regulators of T cell activation, Transcription factors, Transcriptional regulators, T cell differentiation
		Treg									
		Treg	IL7R	-0,268	0,366	7,679	-0,790	0,006 (**)	0,134	Interleukins	Adaptive immune response, Humoral immune response, Interleukins
		Treg	IL21	-0,144	0,763	16,018	-0,476	0,007 (**)	0,138	Cytokines, T cell Functions, Regulation	Adaptive immune response, Anti-inflammatory cytokines, B cell activation, Cytokines and receptors, Innate immune response, Interleukins, Th1 & Th2 differentiation, T cell differentiation, T cell polarization, T cell regulators
		Treg	ITGA4	-0,653	0,062	1,292	-0,622	0,007 (**)	0,138	Adhesion, Regulation	Adhesion, CD molecules, Regulation of immune response
		Treg	MAP3K 1	-0,539	0,112	2,355	-0,529	0,008 (**)	0,160		Innate immune response
		Treg									
		Treg	ICAM3	-0,290	0,137	2,886	-0,441	0,009 (**)	0,187	Adhesion, Regulation	Adhesion, CD molecules, Regulation of immune response

		Treg	CLU	-0,216	0,723	15,187	-0,877	0,012 (*)	0,260		Innate immune response
		Treg	CD3E	-0,217	0,143	3,001	-0,218	0,016 (*)	0,331	T cell Functions	Adaptive immune response, Cell Type specific, CD molecules, T cell anergy, T cell differentiation, T cell proliferation
		Treg	CCR6	-0,235	0,530	11,136	-0,688	0,017 (*)	0,348		CD molecules, Humoral immune response, Innate immune response
		Treg	HAMP	0,312	0,527	11,074	-1,080	0,018 (*)	0,376	Cell Functions, Cytotoxicity	Adaptive immune response, Basic cell functions, Cell Type specific, Cytotoxicity, Innate immune response
		Treg	CD99	-0,194	0,207	4,337	-0,439	0,018 (*)	0,377		CD molecules
		Treg	MFGE8	-0,439	0,107	2,249	-0,561	0,018 (*)	0,384	Transporter Functions	Receptors involved in phagocytosis
		Treg	TRAF2	0,078	0,700	14,693	-0,371	0,020 (*)	0,414		Innate immune response
		Treg	SMAD3	-0,117	0,701	14,727	-0,425	0,020 (*)	0,418	Regulation	Regulation of immune response
		Treg	LAMP2	-0,059	0,882	18,522	-0,692	0,021 (*)	0,438		CD molecules
		Treg	NFATC3	-0,151	0,583	12,236	-0,448	0,023 (*)	0,483	Regulation	Transcriptional regulators
		Treg	CD79B	-0,746	0,056	1,178	-0,548	0,024 (*)	0,502	B cell Functions	Adaptive immune response, B cell activation, CD molecules

		Treg	TMUB2	-0,026	0,914	19,198	-0,344	0,032 (*)	0,671		
		Treg	IL16	-0,249	0,242	5,076	-0,655	0,033 (*)	0,684	Chemokines	Adaptive immune response, CD molecules, Chemokines and receptors
		Treg	ANP32B	-0,270	0,105	2,213	-0,184	0,034 (*)	0,712	Cell Functions	Basic cell functions, Cell Type specific
		Treg	BLK	-0,489	0,273	5,730	-0,759	0,035 (*)	0,736	B cell Functions	B cell receptor signaling pathway, Cell Type specific
		Treg	ATF2	-0,049	0,740	15,546	-0,370	0,042 (*)	0,878		Innate immune response
		Treg	CD46	-0,249	0,191	4,003	-0,167	0,042 (*)	0,878		CD molecules, Innate immune response
		Treg	ANXA1	-0,201	0,519	10,904	-0,647	0,043 (*)	0,905	Transporter Functions	Phagocytosis recognition and engulfment
		Treg	RORA	-0,003	0,985	20,683	-0,158	0,043 (*)	0,907	Regulation	Transcription factors
		Treg	CXCL6	-0,443	0,379	7,952	-0,527	0,046 (*)	0,957	Chemokines, Regulation	Chemokines and receptors, Regulation of inflammatory response
		Treg	FCGR3A	-0,821	0,147	3,092	-0,609	0,047 (*)	0,982	Regulation	CD molecules, Regulation of immune response
		Treg	CD25 (IL2RA)	-0,001	0,997	20,941	0,615	0,011 (*)	0,231	Interleukins	Innate immune response, Interleukins
	Upre-gulated	Treg	CD7	0,835	0,053	1,120	0,851	0,012 (*)	0,242	Regulation, T cell Functions	Adaptive immune response, CD molecules, Regulators of T cell activation, Regulators of Th1 and Th2 development

		Treg	KIT	-0,315	0,548	11,501	0,275	0,020 (*)	0,417	Cell Functions	Basic cell functions, Cell Type specific, CD molecules
		Treg	MAPK11	-0,198	0,671	14,090	0,493	0,021 (*)	0,438		Innate immune response
		Treg	CXCL13	1,244	0,079	1,657	1,535	0,025 (*)	0,524	Chemokines	Adaptive immune response, Cell Type specific, Chronic inflammatory response, Humoral immune response, Chemokines and receptors
		Treg	NCAM1	-0,112	0,824	17,311	0,545	0,033 (*)	0,692		CD molecules
		Treg	CD58	-0,192	0,417	8,760	0,213	0,038 (*)	0,804		Adaptive immune response, CD molecules
		Treg	BCL6	0,730	0,052	1,098	0,598	0,047 (*)	0,995	Regulation	Cell Type specific, Regulation of immune response
		Treg	ICOS	0,230	0,216	4,540	0,263	0,049 (*)	1,026	Adhesion, Regulation	Adhesion, CD molecules, Regulation of immune response

Supplementary Table 6: Stimulatory effect (G vs M) in DC of allergic asthmatic (AA) and healthy (HC) children (adjusted p-value < 0.05)

		Cell type	Gene	fc	p-value	Adj. p-value	fc	p-value	Adj. p-value	Immune Response Category	Annotations
				HC			**AA**				
in HC and AA	Downregulated	DC	TXNIP	-2,361	<0,0001	<0,0001	-1,823	<0,0001	0,009		Innate immune response
		DC	A2M	-4,556	<0,0001	<0,0001	-4,211	<0,0001	0,003	Chemokines	Chemokines and receptors, Innate immune response
		DC	CD81	-1,206	<0,0001	<0,0001	-1,046	0,002	0,040	Regulation	Adaptive immune response, CD molecules, Regulation of immune response
		DC	NFATC3	-1,025	<0,0001	<0,0001	-0,755	0,001	0,013	Regulation	Transcriptional regulators
		DC	ENG	-1,550	<0,0001	<0,0001	-1,169	<0,0001	0,008		Innate immune response
		DC	IRAK1	-1,433	<0,0001	<0,0001	-1,164	0,002	0,032		Innate immune response
		DC	MSR1	-4,480	<0,0001	0,001	-3,663	0,001	0,017	Cell Functions	Basic cell functions, Cell Type specific, CD molecules
		DC	CD4	-2,789	<0,0001	0,001	-2,320	0,001	0,025		Adaptive immune response, CD molecules, Innate immune response
		DC	CD68	-2,811	<0,0001	0,001	-2,375	<0,0001	0,003	Cell Functions	Basic cell functions, Cell Type specific, CD molecules
		DC	IRF8	-3,169	<0,0001	0,001	-2,158	0,001	0,024	Chemokines, Regulation	Cytokines and receptors, Transcription factors, Transcriptional regulators
		DC	CLEC7A	-2,715	<0,0001	0,001	-2,495	0,002	0,040		Innate immune response

		DC	CLEC4C	-3,668	<0,0001	0,001	-2,572	0,002	0,040		CD molecules, Innate immune response
		DC	REPS1	-0,596	<0,0001	0,002	-0,528	0,001	0,018	Cell Functions	Basic cell functions, Cell Type specific
		DC	LTBR	-1,183	<0,0001	0,002	-0,892	<0,0001	0,005	Chemokines	Chemokines and receptors
		DC	TREM2	-3,522	<0,0001	0,004	-3,539	<0,0001	0,003		Humoral immune response
		DC	LY96	-1,792	<0,0001	0,004	-1,559	0,001	0,015		Innate immune response
		DC	CTSS	-1,804	<0,0001	0,004	-2,353	<0,0001	0,001		Adaptive immune response
		DC	GUSB	-1,164	<0,0001	0,004	-0,855	0,002	0,046	Cell Functions	Basic cell functions, Cell Type specific
		DC	PECAM1	-2,752	<0,0001	0,004	-2,102	0,001	0,019	Transporter Functions	CD molecules, Receptors involved in phagocytosis
		DC	LILRB3	-1,400	<0,0001	0,005	-1,061	<0,0001	0,006	Regulation	CD molecules, Regulation of immune response
		DC	IL17RA	-1,534	<0,0001	0,005	-1,379	0,001	0,011	Interleukins	Interleukins
		DC	ST6GAL1	-1,286	<0,0001	0,005	-1,124	0,002	0,039		Humoral immune response
		DC	LY9	-2,241	<0,0001	0,006	-2,151	0,001	0,021		Adaptive immune response, CD molecules
		DC	CSF3R	-2,358	<0,0001	0,006	-2,055	0,002	0,038	Cytokines	Cell Type specific, CD molecules, Cytokines and receptors
		DC	PTPRC	-1,836	<0,0001	0,008	-2,062	0,001	0,012	B cell Functions, T cell Functions	B cell proliferation, CD molecules, T cell differentiation
		DC	CD37	-1,239	<0,0001	0,010	-0,784	<0,0001	0,007		Adaptive immune response, CD molecules
		DC	CD164	-1,338	<0,0001	0,010	-0,927	0,001	0,013		CD molecules

		DC	DUSP6	-2,020	0,001	0,011	-1,280	0,001	0,013		Innate immune response
		DC	BTLA	-0,960	0,001	0,011	-0,609	0,001	0,020	Cell Functions	Basic cell functions, CD molecules
		DC	CSF1R	-2,345	0,001	0,012	-1,912	<0,0001	0,006		CD molecules, Innate immune response
		DC	IL1R2	-2,661	0,001	0,012	-2,997	0,002	0,040		CD molecules, Innate immune response
		DC	ITGB2	-2,335	0,001	0,013	-2,049	0,001	0,019	Adhesion, Regulation	Adhesion, CD molecules, Regulation of immune response
		DC	HLA-DMA	-0,524	0,001	0,013	-0,487	0,001	0,031	Antigen Processing, Cytotoxicity, Regulation	Adaptive immune response, Antigen processing and presentation, Cytotoxicity, Regulation of immune response
		DC	TLR4	-3,106	0,001	0,014	-2,816	0,001	0,026	Microglial Functions, TLR	CD molecules, Innate immune response, Microglial cell activation, Toll-like receptor
		DC	ITGAL	-1,829	0,001	0,016	-1,562	0,001	0,031	Adhesion, Regulation	Adhesion, CD molecules, Regulation of immune response
		DC	CD63	-1,276	0,001	0,026	-1,671	0,001	0,013		CD molecules
		DC	LRP1	-1,696	0,001	0,026	-1,713	0,002	0,034		CD molecules
		DC	ATG7	-0,878	0,002	0,032	-0,606	<0,0001	0,001	Transporter Functions	Genes responsible for protein transport, Protein ubiquitination
		DC	CASP1	-1,248	0,002	0,037	-0,975	0,002	0,039		Innate immune response
		DC	C2	-0,966	0,002	0,042	-1,405	0,001	0,013	Complement	Innate immune response
	Up-regulate	DC	ITGA1	1,895	<0,0001	<0,0001	2,129	0,001	0,013	Adhesion, T cell Functions	Adhesion, CD molecules, T cell anergy

		DC	IL6	3,002	<0,0001	<0,0001	4,131	<0,0001	<0,0001	Cytokines	Cell Type specific, CD molecules, Cytokines and receptors
		DC	CD25 (IL2RA)	5,349	<0,0001	<0,0001	5,301	<0,0001	0,001	Interleukins	Innate immune response, Interleukins
		DC	CD80	3,471	<0,0001	<0,0001	3,424	0,001	0,025	B cell Functions, Regulation, T cell Functions	Adaptive immune response, B cell activation, CD molecules, Regulators of T cell activation, T cell differentiation
		DC	IRF4	1,222	<0,0001	0,001	1,485	0,001	0,018	Regulation, T cell Functions	Regulators of T cell activation, Transcription factors, Transcriptional regulators, T cell differentiation
		DC	IL1B	4,280	<0,0001	0,001	6,027	<0,0001	0,005	Cytokines, Interleukins	Acute-phase response, Cytokines and receptors, Inflammatory response, Innate immune response, Interleukins
		DC	CCL22	1,986	<0,0001	0,001	2,928	<0,0001	0,002	Chemokines, Pathogen Defense	Cell Type specific, Chemokines and receptors, Defense response to virus
		DC	IL1A	2,722	<0,0001	0,001	3,668	<0,0001	0,008	Chemokines, Interleukins	Chemokines and receptors, Interleukins
		DC	CCL17	4,142	<0,0001	0,001	5,603	<0,0001	0,003	Chemokines	Cell Type specific, Chemokines and receptors, Inflammatory response
		DC	CXCL1	2,428	<0,0001	0,001	3,068	0,002	0,043	Chemokines, Regulation	Chemokines and receptors, Regulation of inflammatory response
		DC	PNMA1	1,364	<0,0001	0,002	1,489	<0,0001	0,001		Inflammatory response to antigenic stimulus

		DC	NFKBIA	1,387	<0,0001	0,002	1,504	0,002	0,043		Innate immune response
		DC	BATF	1,801	<0,0001	0,003	1,755	0,002	0,048	Cell Functions	Basic cell functions, Cell Type specific
		DC	SLAMF1	2,470	<0,0001	0,003	3,011	<0,0001	0,002		CD molecules, Adaptive immune response
		DC	CASP3	1,361	<0,0001	0,004	0,915	<0,0001	0,009	Cell Cycle, Regulation	Cell cycle checkpoint and cell cycle arrest, Co-Regulators of autophagy and apoptosis/cell cycle, Negative regulation of cell cycle
		DC	MAPK8	0,903	<0,0001	0,005	0,930	0,002	0,048		Innate immune response
		DC	CD86	1,326	<0,0001	0,007	1,206	0,001	0,023	B cell Functions, Regulation, T cell Functions	Adaptive immune response, CD molecules, Defense response to virus, Regulators of T cell activation, Th1 & Th2 differentiation, T cell differentiation
		DC	CXCL2	2,296	<0,0001	0,007	2,403	0,002	0,042	Chemokines, Regulation	Regulation of inflammatory response, Chemokines and receptors
		DC	CD200	1,278	<0,0001	0,008	1,447	0,002	0,044	Regulation	CD molecules, Regulation of immune response
		DC	CD70	2,792	<0,0001	0,010	2,066	0,001	0,016	Cytokines, TNF Superfamily, T cell Functions	Adaptive immune response, CD molecules, Cytokines and receptors, TNF superfamily members and their receptors
		DC	NFKB1	1,291	<0,0001	0,010	1,256	0,001	0,016		Innate immune response
		DC	FLT3	0,811	0,001	0,013	1,242	<0,0001	0,002	Cell Functions	Basic cell functions, Cell Type specific

		DC	NFKB2	1,019	0,001	0,014	1,023	0,001	0,018		Innate immune response
		DC	PVR	1,607	0,001	0,015	2,013	0,001	0,020	Regulation	CD molecules, Regulation of immune response
		DC	TNFAIP3	0,969	0,001	0,020	1,065	<0,0001	0,006	TNF Superfamily	Innate immune response, TNF superfamily members and their receptors
		DC	CCL20	1,991	0,001	0,022	3,828	<0,0001	0,003	Chemokines	Chemokines and receptors
		DC	CCL19	4,344	0,001	0,023	5,433	<0,0001	0,001	Chemokines, Regulation	Chemokines and receptors, Anti-inflammatory cytokines, Regulation of inflammatory response
in HC	Downregulated	DC	CD79B	-1,167	<0,0001	<0,0001	-0,473	0,038	0,797	B cell Functions	Adaptive immune response, B cell activation, CD molecules
		DC	CASP8	-0,705	<0,0001	<0,0001	-0,475	0,005	0,098		Innate immune response
		DC	CCR5	-2,227	<0,0001	<0,0001	-1,682	0,009	0,186	Cytokines, T cell Functions	CD molecules, Cytokines and receptors, T cell polarization
		DC	TOLLIP	-0,872	<0,0001	<0,0001	-0,793	0,005	0,105		Innate immune response
		DC	SERPING1	-2,875	<0,0001	<0,0001	-2,861	0,004	0,075		Innate immune response
		DC	CCR2	-4,035	<0,0001	0,001	-2,363	0,006	0,133	Cytokines	Cell Type specific, CD molecules, Cytokines and receptors, Inflammatory response
		DC	FCER1G	-1,067	<0,0001	0,001	-0,861	0,010	0,203		Inflammatory response
		DC	SELPLG	-2,265	<0,0001	0,001	-1,139	0,019	0,395		CD molecules
		DC	SYK	-1,377	<0,0001	0,001	-0,987	0,003	0,060	Macrophage Functions	Macrophage activation, Adaptive immune response, Innate immune response

		DC	TGFB1	-1,141	<0,0001	0,001	-1,104	0,004	0,075	Interleukins, Regulation	Immunosuppression, Interleukins, Negative regulation of immune response
		DC	CX3CR1	-1,554	<0,0001	0,001	-0,781	0,011	0,241	Chemokines, Microglial Functions	Adaptive immune response, Chemokines and receptors, Microglial cell activation
		DC	CD48	-1,259	<0,0001	0,001	-1,013	0,002	0,050		CD molecules
		DC	MICA	-0,592	<0,0001	0,002	-0,247	0,058	1,220	Regulation	Immune response to tumor cell, Regulation of immune response
		DC	CMKLR1	-2,457	<0,0001	0,002	-1,267	0,026	0,539	Chemokines	Chemokines and receptors
		DC	CYBB	-2,124	<0,0001	0,002	-2,277	0,007	0,155	Chemokines	Cell Type specific, CD molecules, Chemokines and receptors
		DC	BLK	-1,106	<0,0001	0,002	-0,292	0,247	5,187	B cell Functions	B cell receptor signaling pathway, Cell Type specific
		DC	TIRAP	-0,762	<0,0001	0,003	-0,349	0,035	0,726		Innate immune response
		DC	CD22	-0,714	<0,0001	0,003	-0,551	0,170	3,579		Adaptive immune response, CD molecules
		DC	IFNAR2	-1,287	<0,0001	0,003	-0,618	0,007	0,140	Pathogen Defense	Defense response to virus
		DC	TNFRSF17	-2,147	<0,0001	0,004	-1,351	0,002	0,051	Cell Functions, TNF Superfamily	Basic cell functions, Cell Type specific, CD molecules, TNF superfamily members and their receptors
		DC	PPIA	-0,619	<0,0001	0,004	-0,160	0,142	2,977		
		DC	PYCARD	-1,327	<0,0001	0,004	-0,972	0,006	0,118		Innate immune response

		DC	ICAM2	-1,011	<0,0001	0,004	-1,040	0,004	0,078	Adhesion, Regulation	Adhesion, CD molecules, Regulation of immune response
		DC	SMPD3	-1,572	<0,0001	0,004	-0,905	0,028	0,584	Cell Functions	Basic cell functions, Cell Type specific
		DC	CXCR4	-0,979	<0,0001	0,005	-0,227	0,326	6,846	Chemokines, T cell Functions	Adaptive immune response, CD molecules, Chemokines and receptors, T cell polarization
		DC	CARD9	-1,586	<0,0001	0,005	-1,284	0,002	0,051		Innate immune response
		DC	CCR1	-3,342	<0,0001	0,005	-2,260	0,006	0,130	Chemokines, Cytokines, Regulation, T cell Functions	CD molecules, Chemokines and receptors, Cytokines and receptors, Regulation of inflammatory response, T cell polarization
		DC	TFE3	-0,260	<0,0001	0,006	-0,083	0,435	9,125		Humoral immune response
		DC	LILRA4	-3,380	<0,0001	0,006	-1,876	0,005	0,103		CD molecules, Innate immune response
		DC	CD99	-1,141	<0,0001	0,007	-0,946	0,006	0,118		CD molecules
		DC	CD38	-1,015	<0,0001	0,007	-0,945	0,066	1,396	B cell Functions, Regulation	Adaptive immune response, Cell Type specific, CD molecules, Response to drug, Positive regulation of B cell proliferation
		DC	TLR7	-1,900	<0,0001	0,007	-1,116	0,006	0,130	Microglial Functions, TLR	Microglial cell activation, Innate immune response, Toll-like receptor
		DC	FUT7	-1,723	<0,0001	0,007	-1,019	0,032	0,679	Cell Functions	Basic cell functions, Cell Type specific

		DC	APOE	-3,015	<0,0001	0,007	-4,041	0,009	0,189	Transporter Functions	Cell Type specific, Lipid transporter activity
		DC	IKBKB	-0,404	<0,0001	0,007	-0,112	0,263	5,526		CD molecules
		DC	LAIR2	-1,161	<0,0001	0,008	-0,490	0,011	0,239	Cell Functions	Basic cell functions, Cell Type specific, CD molecules
		DC	FCGR3A	-3,057	<0,0001	0,008	-1,583	0,045	0,943	Regulation	CD molecules, Regulation of immune response
		DC	STAT6	-0,349	<0,0001	0,008	-0,141	0,027	0,573	Chemokines, Regulation, T cell Functions	Adaptive immune response, Cytokines and receptors, Cell Type specific, Th2 orientation, Transcription factors, Transcriptional regulators
		DC	NFATC1	-0,757	<0,0001	0,009	-0,118	0,350	7,342	Regulation	Transcription factors, Transcriptional regulators
		DC	ITGAX	-2,700	<0,0001	0,010	-1,924	0,010	0,216	Adhesion	Adhesion, CD molecules, Innate immune response
		DC	BLNK	-1,693	0,001	0,011	-1,058	0,005	0,106		Humoral immune response
		DC	SDHA	-0,396	0,001	0,012	-0,025	0,843	17,696		
		DC	C3AR1	-1,852	0,001	0,012	-1,384	0,010	0,211	Regulation	Regulation of inflammatory response
		DC	TLR9	-2,829	0,001	0,012	-1,557	0,020	0,411	TLR	CD molecules, Innate immune response, Toll-like receptor
		DC	TNFRSF1A	-0,688	0,001	0,013	-0,367	0,004	0,091	Chemokines, TNF Superfamily	CD molecules, Chemokines and receptors, TNF superfamily members and their receptors
		DC	ANXA1	-2,252	0,001	0,013	-1,694	0,009	0,192	Transporter Functions	Phagocytosis recognition and engulfment
		DC	NOD2	-1,451	0,001	0,014	-1,112	0,015	0,320	Cytokines	Innate immune response, Cytokines and receptors

		DC	TNFSF 13B	-2,188	0,001	0,015	-1,114	0,029	0,619	Regulation, TNF Superfamily	CD molecules, Regulation of immune response, TNF superfamily members and their receptors
		DC	HLA-DMB	-0,624	0,001	0,015	-0,677	0,003	0,059	Antigen Processing, Regulation	Adaptive immune response, Antigen processing and presentation, Positive regulation of immune response
		DC	GPI	-0,676	0,001	0,016	-0,643	0,014	0,297		
		DC	SIGIRR	-1,128	0,001	0,016	-0,502	0,022	0,453		Innate immune response
		DC	CD97	-0,802	0,001	0,018	-0,675	0,064	1,353		CD molecules, Inflammatory response
		DC	PDGFC	-0,631	0,001	0,019	-0,363	0,196	4,119	Cell Functions	Basic cell functions
		DC	PRKCD	-0,650	0,001	0,020	-0,470	0,045	0,943	Senescence	Senescence initiators
		DC	HAVCR 2	-1,673	0,001	0,020	-1,682	0,003	0,063		Innate immune response
		DC	IGF2R	-0,616	0,001	0,020	-1,354	0,003	0,065	Senescence	CD molecules, Senescence initiators
		DC	MAP2K 2	-0,288	0,001	0,023	-0,128	0,093	1,946		Innate immune response
		DC	STAT2	-0,878	0,001	0,023	-0,796	0,005	0,101	Chemokines , Regulation	Cytokines and receptors, Transcription factors, Transcriptional regulators
		DC	CD1B	-2,305	0,001	0,023	-1,831	0,014	0,289	Cell Functions	Basic cell functions, Cell Type specific, CD molecules
		DC	CEBPB	-1,435	0,001	0,025	-0,937	0,003	0,059		Acute-phase response
		DC	MAPK1 4	-0,294	0,001	0,025	-0,156	0,030	0,640		Innate immune response
		DC	TRAF3	-0,327	0,001	0,027	-0,003	0,974	20,450		Innate immune response

		DC	IRF5	-1,057	0,001	0,028	-0,910	0,051	1,063	Senescence	Senescence initiators interferon related
		DC	IFIT2	-1,301	0,001	0,029	-1,192	0,039	0,827	Chemokines	Chemokines and receptors
		DC	CKLF	-1,465	0,001	0,029	-1,269	0,012	0,252	Chemokines	Chemokines and receptors, Inflammatory response
		DC	PAX5	-0,692	0,001	0,031	-0,132	0,493	10,355		Humoral immune response
		DC	CCL24	-5,850	0,002	0,036	-2,905	0,038	0,788	Chemokines, Regulation	Chemokines and receptors, Regulation of inflammatory response
		DC	CD33	-1,364	0,002	0,036	-1,065	0,011	0,226		CD molecules
		DC	SMAD3	-1,100	0,002	0,038	-0,661	0,028	0,588	Regulation	Regulation of immune response
		DC	PSMB8	-0,640	0,002	0,038	-0,602	0,007	0,143	Chemokines	Chemokines and receptors
		DC	FLT3LG	-0,501	0,002	0,040	-0,020	0,836	17,560		CD molecules
		DC	IL16	-1,052	0,002	0,040	-0,596	0,013	0,263	Chemokines	Adaptive immune response, CD molecules, Chemokines and receptors
		DC	ITGA5	-0,833	0,002	0,043	0,127	0,110	2,300	Adhesion	Adhesion, CD molecules, Innate immune response
		DC	POU2F2	-0,503	0,002	0,043	-0,486	0,079	1,653		Humoral immune response
		DC	FOS	-2,346	0,002	0,044	-1,915	0,004	0,089	Senescence	Senescence pathway
		DC	IKBKE	-1,015	0,002	0,044	-0,921	0,004	0,094		Innate immune response
		DC	IFNAR1	-0,748	0,002	0,046	-0,372	0,060	1,262	Interleukins	Innate immune response, Interleukins
		DC	FYN	-0,410	0,002	0,048	-0,412	0,015	0,312	Leukocyte Functions	Leukocyte migration
		DC	ICAM4	-1,208	0,002	0,048	-0,298	0,206	4,335	Adhesion, Regulation	Adhesion, CD molecules, Regulation of immune response
	Upregulated	DC	BCL2L1	2,111	<0,0001	<0,0001	1,574	0,005	0,095		Innate immune response
		DC	IL8	2,334	<0,0001	<0,0001	3,089	0,003	0,058	Cytokines	Adaptive immune response, CD molecules, Cytokines and receptors

		DC	CD40	1,860	<0,0001	0,001	1,674	0,010	0,217	Regulation	Adaptive immune response, CD molecules, Regulation of immune response
		DC	TUBB	1,138	<0,0001	0,003	0,966	0,002	0,050		
		DC	IL15RA	1,405	<0,0001	0,003	1,771	0,010	0,201	Interleukins, Regulation	Adaptive immune response, Interleukins, Positive regulation of immune response
		DC	CCL3L1	1,527	<0,0001	0,003	2,452	0,004	0,079	Cytokines	Cytokines and receptors
		DC	CD274	1,444	<0,0001	0,003	1,881	0,006	0,134	T cell Functions	CD molecules, T cell polarization
		DC	SOCS1	1,770	<0,0001	0,004	2,051	0,004	0,084	T cell Functions	T cell differentiation
		DC	TNFRSF9	1,221	<0,0001	0,005	1,719	0,003	0,056	TNF Superfamily	CD molecules, TNF superfamily members and their receptors
		DC	ICAM1	1,169	<0,0001	0,007	1,211	0,006	0,118	Cell Functions	Basic cell functions, Cell Type specific
		DC	CCL3	1,360	<0,0001	0,008	2,174	0,006	0,130	Chemokines, Regulation	Chemokines and receptors, Humoral immune response, Regulation of inflammatory response, T cell polarization
		DC	TARP	2,410	<0,0001	0,010	1,718	0,014	0,295	Cell Functions	Basic cell functions, Cell Type specific
		DC	CXCL6	1,498	0,001	0,011	0,613	0,085	1,778	Chemokines, Regulation	Chemokines and receptors, Regulation of inflammatory response
		DC	MAPK11	1,239	0,001	0,011	0,784	0,083	1,744		Innate immune response
		DC	IL23A	3,105	0,001	0,011	2,228	0,003	0,066	Chemokines	Chemokines and receptors
		DC	EBI3	2,356	0,001	0,013	2,842	0,003	0,054		Innate immune response

		DC	STAT4	1,453	0,001	0,014	1,059	0,022	0,469	Chemokines, Regulation, T cell Functions	Adaptive immune response, Cytokines and receptors, Cell Type specific, Transcription factors, Transcriptional regulators, Th1 orientation
		DC	AGK	0,617	0,001	0,016	0,821	0,013	0,282		
		DC	RUNX3	1,280	0,001	0,020	1,175	0,003	0,067	Regulation	Transcription factors
		DC	CXCL13	3,102	0,001	0,022	2,005	0,057	1,197	Chemokines	Adaptive immune response, Cell Type specific, Chronic inflammatory response, Humoral immune response, Chemokines and receptors
		DC	PTGS2	1,901	0,001	0,022	2,028	0,025	0,524	Cytokines	Acute-phase response, Cytokines and receptors
		DC	TNFRSF11A	1,047	0,001	0,023	0,799	0,010	0,212	TNF Superfamily	Adaptive immune response, CD molecules, TNF superfamily members and their receptors
		DC	CFB	1,530	0,001	0,028	1,293	0,026	0,548		Innate immune response
		DC	TNFRSF4	0,970	0,002	0,034	0,798	0,177	3,726	TNF Superfamily	CD molecules, TNF superfamily members and their receptors
		DC	CDKN1A	1,607	0,002	0,035	1,394	0,024	0,496	Cell Cycle, Regulation, Senescence	Cell cycle checkpoint and cell cycle arrest, Regulation of cell cycle, Senescence pathway, Senescence initiators interferon related
		DC	IL21R	1,460	0,002	0,045	1,247	0,009	0,192	Cytokines, Interleukins	Adaptive immune response, Cytokines and receptors, Interleukins
in AA	Downregulated	DC	CD1D	-2,027	0,003	0,054	-1,985	<0,0001	0,005	T cell Functions	CD molecules, T cell differentiation
		DC	MYD88	-0,720	0,003	0,068	-0,972	0,001	0,022	TLR	Innate immune response, Toll-like receptor
		DC	TLR8	-1,501	0,003	0,069	-1,340	0,002	0,036	TLR	CD molecules, Innate immune response, Toll-like receptor

		DC	CD14	-1,626	0,004	0,074	-0,958	0,001	0,029		CD molecules, Innate immune response
		DC	ITGAE	-1,035	0,004	0,090	-1,327	<0,0001	0,007	Adhesion	Adhesion, CD molecules
		DC	G6PD	-1,044	0,004	0,091	-0,963	0,002	0,037	Transporter Functions	Phagyocytosis signal transduction
		DC	BST1	-2,042	0,005	0,101	-1,511	<0,0001	0,006		CD molecules, Humoral immune response
		DC	LGALS 3	-1,768	0,008	0,159	-2,205	0,002	0,041		Innate immune response
		DC	CD209	-2,137	0,009	0,182	-2,250	0,002	0,033	Cell Functions	Basic cell functions, Cell Type specific, CD molecules
		DC	IL6ST	-0,467	0,012	0,258	-0,505	<0,0001	0,006	Cytokines	Acute-phase response, CD molecules, Cytokines and receptors
		DC	CCL5	-0,711	0,016	0,346	-0,962	<0,0001	0,002	Chemokines , Cytokines	Cytokines and receptors, Chronic inflammatory response, Chemokines and receptors, Acute-phase response, Inflammatory response, Innate immune response
		DC	AMICA1	-1,796	0,017	0,348	-1,959	0,001	0,025	Regulation	Regulation of immune response
		DC	IL12RB 1	-0,709	0,025	0,529	-0,432	0,002	0,049	Cytokines, Interleukins, NK Cell Functions, T cell Functions	Adaptive immune response, Anti-inflammatory cytokines, Cytokines and receptors, Interleukins, NK cell activation, Th1 orientation, Th1 & Th2 differentiation, T cell differentiation, T cell proliferation
		DC	BAX	-0,167	0,042	0,889	-0,169	0,001	0,012	Cell Cycle, Regulation	Co-Regulators of autophagy and apoptosis/cell cycle
		DC	FN1	-2,129	0,069	1,449	-3,724	0,002	0,040	Cytokines	Cell Type specific, Cytokines and receptors

		DC	ISG15	-0,732	0,179	3,762	-1,299	0,002	0,042		Innate immune response
		DC	LAMP1	-0,317	0,266	5,586	-0,604	<0,0001	0,010	Transporter Functions	Autophagy induction by intracellular pathogens, CD molecules, Genes linking autophagosome to lysosome
	Upregulated	DC	CXCL3	1,681	0,003	0,067	2,617	<0,0001	<0,0001	Chemokines, Regulation	Chemokines and receptors, Regulation of inflammatory response
		DC	ADORA 2A	1,309	0,003	0,072	1,957	<0,0001	0,008	Cell Functions	Basic cell functions
		DC	SERPIN B2	1,929	0,004	0,077	3,778	<0,0001	0,008	Senescence	Senescence initiators interferon related
		DC	CXCL5	1,417	0,021	0,441	3,362	0,002	0,042	Chemokines	Cell Type specific, Chemokines and receptors

Acknowledgments

I would like to thank the people who have supported me in the various processes of this work.

First and foremost, I would like to thank my supervisor Prof. med. Bianca Schaub. I would like to thank her for the opportunity to conduct the research on this exciting topic in her research group, her constant supervision and support, the in-depth discussions, and the contagious enthusiasm throughout my doctoral studies.

Many thanks to the members of my thesis advisory committee, Prof. Dr. med. Markus Ege and Prof. Dr. med. Anne Krug for their valuable comments, constructive feedback, and their time and commitment.

Moreover, I would like to thank Prof. Dr. Christoph Klein for the opportunity to do my Ph.D. at the KUBUS comprehensive childhood research center (CCRC) of the Dr. von Hauner Children's Hospital, and his group for technical support.

My special thanks to all the members of AG Schaub who put a smile on my face every working day, their open ears at all times, and their exceptional collegiality. Special thanks to Dr. Andreas Böck and Michael Salvermoser for their great statistical support; Tatjana Netz and Isolde Schleich for their technical support and Kristina Laubhahn, Sarah Basse, Alexander Neuner, Jana Eckert, Elisabeth Nowak, and Sarah Geiger for always being there for me and making the lab a feel-good place.

A great thanks also to Dr. med. Katharina Rehbach and Dr. Monika Twardziok for the great cooperation and the helpful discussions.

I would like to thank Prof. Dr. Erika v. Mutius and her whole research group for the great cooperation and helpful advice.

In addition, I would like to thank the study doctors, Dr. med. Meike Köhler, Dr. med. Christina Schauberger and especially Dr. med. Kathrin Zeber for the great cooperation and their commitment. A big thank you also to all the staff of the CHA and the TCH who supported the process of recruitment.

Special thanks to all participating families, without whom these studies would not have been possible.

Last but not least, a huge thank you to my husband, my family, and friends for their everlasting support and patience. Thanks to my son Leonidas for patiently listening to immunology books and publications.