Ca^{2+} and innate immune pathways are activated and differentially expressed in childhood asthma phenotypes

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ABSTRACT

Background: Asthma is the most common chronic disease in children. Underlying immunological mechanisms - in particular of different phenotypes - are still just partly understood. The objective of the study is the identification of distinct cellular pathways in allergic asthmatics (AA) and non-allergic asthmatics (NA) vs healthy controls (HC). **Methods:** Peripheral blood mononuclear cells (PBMCs) of steroid-naïve children (n(AA/NA/HC) = 35/13/34)) from the CLARA study (n=275) were stimulated (anti-CD3/CD28, LpA) or kept unstimulated. Gene-expression was investigated by transcriptomics and quantitative RT-PCR. Differentially regulated pathways between phenotypes were assessed after adjustment for sex and age (KEGG-pathways). Networks based on correlations of gene expression were built using force-directed graph drawing. **Results:** AA vs NA and asthmatics overall vs HC showed significantly different expression of Ca^{2+}- and innate immunity-associated pathways. PCR-analysis confirmed significantly increased Ca^{2+}-associated gene-regulation (*ORMDL3* and *ATP2A3*) in asthmatics vs HC, most prominent in AA. Innate immunity receptors (LY75, TLR7), relevant for virus infection, were also upregulated in AA and NA compared to HC. AA and NA could be differentiated by increased *ATP2A3* and *FPR2* in AA, decreased *CLEC4E* in AA and increased *IFIH1* expression in NA following anti-CD3/28-stimulation vs unstimulated (fold change). **Conclusions:** Ca^{2+}-regulation and innate immunity response pattern to viruses were activated in PBMCs of asthmatics. Asthma phenotypes were differentially characterized by distinct regulation of *ATP2A3* and expression of innate immune receptors (*FPR2*, *CLEC4E*, *IFIH1*). These genes may present promising targets for

56 future in depth investigation with the long-term goal of more phenotype-specific
57 therapeutic interventions in asthmatics.
58

Background

Asthma is a complex chronic pulmonary disease with the following main pathogenic properties: airway hyperresponsiveness and wheezing, airway remodelling and inflammation. The global prevalence of this most common chronic childhood disease has been increasing over decades, particularly in countries with Western lifestyle (1-3). The multidimensional interaction of genetic predisposition and environmental factors can lead to asthma onset already in childhood (4). Asthma can be clinically divided into two main phenotypes: allergic asthma (AA) with presentation of allergic symptoms and specific sensitization to common allergens, and non-allergic asthma (NA) without allergy (5). Underlying immune regulation within and between these two phenotypes has only been partly discovered. Whereas AA and NA were shown to have increased regulatory T-cell (Treg) numbers compared to healthy controls (HC), only Tregs from patients with AA were able to suppress T-cell proliferation and cytokine production sufficiently. Further, children with NA showed a Th17-shifted pro-inflammatory immune status (6).

The common symptoms of AA and NA such as wheezing and increased airway hyperresponsiveness and pulmonary obstruction point to shared immunological pathways besides the known Th2-shift. Yet, distinct characteristics such as allergic sensitization indicate also differential regulation between these two main phenotypes. To investigate the differences between asthmatics and healthy children as well as AA and NA with respect to underlying immunological pathways we analysed gene expression patterns of a well-characterized childhood asthma cohort using microarray technology. We aimed to identify novel and shared pathways and genes between AA and NA and compared to HC as potential future markers for allergic and

non-allergic childhood asthma in peripheral blood mononuclear cells (PBMCs) extending our prior findings on Tregs and Th17 (6). Furthermore, identification of novel phenotype-specific targets may contribute in the long-term to develop novel therapeutic options for specific asthma phenotypes.

Methods

Study population

The study population comprised 4-15 year old steroid-naïve mild to moderate AA (n=35), NA (n=13), and HC (n=34), being a subset of the CLARA study (n=275), Germany, based on sample availability and characteristics comparable to the whole population (as shown in (6), Fig. S1).

Asthmatic children were carefully characterized by clinical examination, pulmonary function test, FeNO, chest radiograph, full blood count and measurement of total and specific IgE (Allergy Screen®, Mediwiss Analytic GmbH; supporting information), and defined according to GINA guidelines (7). Inclusion criteria for AA/NA were: classical asthma symptoms and/or doctor´s diagnosis of asthma and/or history of asthma medication and a lung function indicating significant reversible airflow obstruction. The classification in allergic and non-allergic asthma was based on positive (spec. IgE≥0.35IU/ml) or negative specific allergic sensitization, in accordance with clinical symptoms. For inclusion asthmatic children had to be steroid-naïve, without intake of steroids (also inhaled steroids) for at least 14 days. HC had no allergies or chronic diseases, were in the same age-range, and showed similar sex distribution. Exclusion criteria for HC were clinical symptoms of allergy or positive specific IgE, even without occurrence of wheeze, cough, shortness of breath and normal lung function. Generally excluded were children with current infections, assessed by CrP and IL-6 levels in addition to clinical examination and temperature measurement (6). Informed oral and written consent was obtained from the parents for participation and blood collection. Approval was obtained from the local ethics board, LMU Munich, Munich, Germany.

Culture of PBMCs, RNA Isolation, microarrays, quantitative real-time qPCR and cytokine measurement

PBMCs were isolated within 24 hours after blood withdrawal and cultivated for 48 hours without stimulation, stimulated with plate-bound anti-CD3 (3µg/ml) plus soluble anti-CD28 (1µg/ml) for T-cell stimulation or lipid A (LpA, 0.1µg/ml) for innate immune stimulation at 37°C at a density of $5x10^6$ cells/ ml in X-Vivo (Lonza).

RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quality and quantity for microarray analysis was determined with Agilent Bioanalyzer chips (Bio-Rad Laboratories, Hercules, CA, USA).

Total RNA was labeled and hybridized to Affymetrix GeneChip® Human Gene 1.0 ST arrays according to the manufacturer's instructions as described before (6). The scanned arrays (n=108 arrays in 14 AA, 8 NA and 14 HC) were checked for quality control by MvA-, density- and RNA degradation plots. Computational analyses were performed using R (8). Background correction and normalization was performed by RMA normalization (9). Data were deposited in NCBI's Gene Expression Omnibus (GEO accession number GSE40889). Gene-specific PCR products were measured in n (AA/NA/HC) = (21/13/20) children by CFX96 TouchTM Real-time-PCR Detection System (Bio-Rad, Munich, Germany) for 40 cycles. The difference in CT values relative to *18S* was used in the calculation of deltaCT (ΔCT); a higher ΔCT-value refers to lower gene expression.

IFN-γ, IL-5, IL-10 and IL-17 cytokine secretion was analysed in supernatants by LUMINEX according to the manufactures instructions (BIORAD). The lowest value measured was 0.58 pg/ml for IFN-γ, 0.03 pg/ml for IL-5, 0.41 pg/ml for IL-10 and 0.02 pg/ml for IL-17.

Statistical analysis

Differences of phenotypes and/or subpopulations were assessed with Wilcoxon test for continuous variables and Fisher-test for categorical variables, respectively.

Pathway analysis by GlobalAncova

GlobalAncova (10) was used to identify pathways with differential expression between phenotypes. GlobalAncova provides a single p-value for each tested pathway, being a powerful alternative to single gene tests with consecutive significance level or false discovery rate (FDR) adjustment (11). Pathways were constructed as group of genes provided by KEGG pathways and grouping of genes related to different T cell subgroups, macrophages, dendritic and B cells. Phenotype differences were examined pairwise (AA vs HC, NA vs HC, AA vs NA), and stratified for stimulation condition. P-values according to different pathways were reported unadjusted. Increased rate of false positive discoveries was accepted as the results were used as screening for subsequent confirmation with RT-PCR.

Real-time PCR

ΔCT and fold-change values according to gene expression before (unstimulated, M) and after stimulation were analyzed by pairwise t-tests to identify differential expression in distinct phenotypes, stratified for stimulation condition. All analyses were adjusted for age and sex within a linear regression model. Gene networks were built on residuals of the forenamed regression models, that is, gene expression adjusted for age and sex. Spatial arrangement of the nodes was calculated via a force-directed graph drawing algorithm. In brief, the nodes repel each other to ensure

a readable schematic representation in the plane, combined with an additional force contracting nodes (genes) with positive correlation and further repel nodes with negative correlation.

Cytokine secretion

Cytokine secretion below the detection limit were set to 0.01 pg/ml and assessed for phenotype differences by pairwise t-tests following log2-transformation to ensure normality. Relation to gene expression is given by Spearman-correlation.

Results

Representative subsample selection

In this study, a subgroup of AA and/or NA compared to HC, largely comparable to the whole CLARA-Study as shown before (6), were assessed for differential immune regulation based on sample availability (Fig. S1). This group was used for identification of novel pathways (microarray, Table 1) and confirmation by RT-PCR as gold-standard (Table S1). The study characteristics showed significantly increased eosinophils, IgE and FeNO in AA compared to NA, trend-wise increased neutrophils were detected in NA as shown before (6). Both AA and NA had significantly decreased lung function compared to HC (indicated by higher bronchodilator-response or lower z-scores for FEV1, respectively). The groups were comparable in terms of age, sex, maternal asthma, maternal eczema, parental asthma, eczema and hay fever, smoking at home, siblings but not maternal hay fever. Of note, in the group of CLARA children with analysed qPCR data, mothers of NA had no hay fever (Table S1).

Calcium and innate immune pathway-associated genes ranked top

There were n=21465 annotated probes on the array. Analysis of n=36 children with 108 arrays (Fig. S1) showed significantly increased fold-change expression after LpA stimulation ($p<0.05$) (20%, 16%, 14% of the probes, for HC, AA, NA) and after anti-CD3/28-stimulation (45%, 47%, 41% for HC, AA, NA; data not shown).

KEGG pathway analysis identified differential gene expression between asthmatics (AA and NA) compared to HC, and between asthma phenotypes (AA vs NA). Pathways showing differential expression between all three phenotypes comprised

193 calcium signaling, MAPK signaling, TGF-β signaling and TLR signaling (Table 1).
194 Based on this differential expression in microarray analysis and biological findings
195 reported in the literature, twenty-three genes assignable to calcium signaling and
196 innate immunity were selected for investigation of RT-PCR in a second group of
197 children of the CLARA population for replication (Table S1). Selected genes
198 categorized to the calcium pathway were *ATP2A3*, *CALM2*, *INPP5B*, *ITPR2*, *ORAI1*,
199 *ORMDL3*, *S100A9*, *SPOCK2*, *STIM1*, and *STIM2*. Genes associated with innate
200 immunity were *CD209*, *CLEC4E*, *CLEC7A*, *DDX58*, *IFIH1*, *DHX58*, *FPR2*, *LY75*,
201 *NOD1*, *SEMA3A*, *TLR6*, *TLR7* and *TLR8*.
202
203 **Asthmatic children showed overexpression of Ca^{2+}- and innate- immunity**
204 **associated genes**
205 To confirm findings from the microarray experiment, signature genes within the
206 respective Ca^{2+} and innate immune pathways were selected based on literature. For
207 n=54 children (21 AA, 13 NA, 20 HC; Table S1) the panel of 23 genes (Table S2)
208 was measured by RT-PCR in unstimulated cells and after stimulation with LpA and
209 anti-CD3/28. Mean and standard deviations of raw data are given in Table S3. In the
210 following, results from the adjusted analysis are reported. Increased expression of
211 genes from the calcium pathway was identified for *ORMDL3* and *ATP2A3* in AA and
212 NA vs HC. In detail, *ORMDL3* was increased in AA compared to HC following LpA
213 and anti-CD3/28-stimulation, and in NA compared to HC unstimulated and following
214 LpA-stimulation. For *ATP2A3,* both asthma phenotypes showed an increased
215 expression compared to HC solely following LpA-stimulation. Additionally for *ORAI1,*
216 *S100A9, SPOCK2* and *STIM1* AA vs HC showed increased expression following
217 stimulation with LpA (*ORAI1, STIM1),* with anti-CD3/28 (S100A9) or following both

stimuli (*SPOCK2*) (Table 2, lower part ΔCT). Innate immunity genes *LY75* and *TLR7* expression were upregulated in both AA and NA vs HC. For *LY75*, expression was increased only following anti-CD3/28 stimulation, for *TLR7* in all conditions for AA, and following anti-CD3/28 for NA vs HC. *DDX58* and *DHX58* were increased only in AA vs HC, unstimulated and following anti-CD3/28-stimulation (*DDX58*) or following LpA-stimulation (*DHX58*). *CD209* and *TLR6* were increased in NA vs HC, both without stimulation (Table 2, lower part, Fig. 1). Significant differences between asthma phenotypes AA and NA were detected for *ATP2A3* and genes of the innate immune system *CLEC4E*, *IFIH1*, and *FPR2* regarding their individual response following stimulation compared to baseline gene expression (fold change, next paragraph) (Table 2, Fig. S2) and was not detectable for expression analysed as ΔCT.

Increased expression for both Ca- and innate immunity- associated genes following stimulation in asthma phenotypes

Following anti-CD3/28 stimulation, expression of calcium signaling genes *ITPR2*, *SPOCK2*, *STIM2* showed a stronger increase in NA vs HC, compared to the unstimulated level (fold change). For AA, *ATP2A3* fold change was higher as compared to NA (Table 2, Fig. S2). A phenotype-specific response to stimulation was detected for innate immune regulation (*FPR2*, *TLR6*, *IFIH1*, *LY75*, and *CLEC4E*) (Table 2, upper part).

Gene expression following anti-CD3/28 stimulation was stronger in NA compared to HC in the innate immunity-associated genes *IFIH1*- and *LY75* and calcium-associated gene *STIM2* and stronger in AA vs HC in the innate immune receptor *TLR6*. Between asthma phenotypes, NA showed a stronger increase for genes of

innate immunity, namely *CLEC4E* and *IFIH1,* and AA showed a stronger effect for the innate immunity gene *FPR2*. Reaction following LpA stimulation was stronger in *FPR2* expression for AA compared to HC (Table 2, upper part, Fig. S2). In summary, gene expression was strongly inducible by anti-CD3/CD28 and LpA stimulation in innate immunity genes.

Gene networks

Gene networks were built on correlation of gene expressions separately for all n=54 subjects (Fig. 2A; adjusted for phenotype, age, and sex) and individually for each phenotype (Fig. 2B - 2D; adjusted for age and sex). The visualisation simplifies the correlation structure, i.e. their relation or co-expression to each other as a whole by projection in a two-dimensional plane. Differential gene expression patterns between the phenotypes were no component in the network building process, as adjusted expression levels were used. However, the results reported in the previous paragraphs were additionally included in the graphs. Differentially expressed genes between phenotypes were highlighted using larger circle symbols annotated with the information which phenotype shows higher expression (a=AA>HC, b=NA>HC).

When all individuals were included in the network building, *TLR6* and *TLR7* were positioned central (Fig. 2A), indicating a balanced correlation to other genes. There was no co-expression of high absolute expression, which was indicated by connecting lines in the graph. Expression levels were higher for NA vs HC (*TRL6*, unstimulated) and higher for AA vs HC (*TLR7* unstimulated). In unstimulated cells, a group of genes (*NOD1*, *DHX58, STIM1, ATP2A3)* around *ORMDL3* and *CD209,* which were both upregulated in AA vs HC, were located more in the periphery. Their compactness as a group and peripheral position indicates above-average co-

268 expression, compared to other genes or stimulation conditions, respectively.
269 Following LpA-stimulation *ORMDL3*, *SPOCK2* and *TLR7/STIM1* in central position
270 spanned a subnetwork (left side in Fig. 2A), which consisted exclusively of LpA-
271 stimulated gene expressions. *TLR7* expression may represent the connection
272 between this subnetwork and the other part of the whole network being centrally
273 located in the subnetwork but not highly significantly correlated with other gene
274 expression. In comparison with the distributed placement of gene expressions before
275 and following anti-CD3/28 stimulation, this indicates an induction of correlation
276 through LpA-stimulation for genes contained in the subnetwork. Another prominent
277 role within the network had *FPR2*, building a cluster for all three stimulation
278 conditions (top right in Fig 2A). Its negative correlation to whole gene groups (dotted
279 lines) may potentially point to its counter regulatory role.
280 Within HC, strong co-regulation was visible for gene expression after LpA stimulation,
281 in particular for *INPP5B* with *CLEC4E* and *CLEC7A* and *TLR* genes (left part of Fig.
282 2B, green) and unstimulated around *SEMA3A*, *ITPR2* and *STIM2* (upper part, Fig.
283 2B, blue). *FPR2* expression without stimulation showed a distinct role, and was
284 negatively correlated with a cluster of genes following anti-CD3/28 stimulation
285 (bottom of Fig. 2B, *IFIH1* to *TLR7*; top of Fig. 2B). As this negative correlation was
286 observed beyond the same stimulation condition, a co-regulatory network, which
287 depends on the base level of *FPR2* in healthy children, is indicated.
288 Within AA, the role of *FPR2* as described for HC (unstimulated expression negatively
289 correlated to gene sub-networks) is still visible, but less pronounced to a specific
290 group of genes. All genes with a differential expression in AA were located in the
291 same (upper) half of the graph (Fig. 2C), which reflects a moderate positive (more
292 distant placement) to strong positive (compact placement) co-regulation of genes.

293 This also coincides with the observation that AA showed only significant
294 overexpression of genes. *ORMDL3* expression in AA showed higher correlations as
295 compared to HC (placed closer), especially after stimulation, which indicates a
296 phenotype-specific response to stimulation (Fig. 2C, LpA, green and anti-CD3/28,
297 red). Toll like receptor genes (*TLR6*, *TLR7*) were located in the centre of differentially
298 regulated genes in AA. Additionally *S100A9* is the only gene which is centrally
299 located in the network and expressed significantly different, which might indicate an
300 independent expression regulation beside the calcium pathway in AA.
301 For NA, the correlation of differentially expressed genes was less prominent as
302 compared to the pattern in AA, i.e. affected genes were distributed over the whole
303 network (Fig. 2D, large circles). Strong co-regulation was identified after LpA
304 stimulation for *LY75, CLEC4E, ITPR2*, and *STIM2*, which did not differ in their
305 expression level from other phenotypes (Fig. 2D, left part). After anti-CD3/CD28
306 stimulation, *STIM2* and *FPR2* were placed far out at the edges, indicating no or
307 negative correlation to the majority of the other genes (right, in red), and strong
308 negative correlation of *FPR2* to the cluster of LpA-stimulated genes. In summary,
309 stimulation-induced gene expression is essential for its position in the network.
310
311 **Correlation of TLR7 and IL-17 cytokine secretion**
312 Within individuals with RT-PCR data (n=54), cytokine secretion was available for
313 n=50 (20 AA, 10 NA, 20 HC).
314 For IL-17 following stimulation with LpA, AA and NA showed higher cytokine
315 secretion compared to HC (p=0.047 AA; p=0.015 NA). Again, following LpA
316 stimulation IL-5 showed higher secretion levels in AA (p=0.016) and NA (p=0.002)
317 compared to HC and in NA for IL-10 (p=0.004), as previously reported for the whole

318 cohort (6). There were no phenotype differences between unstimulated and following
319 anti-CD3/CD328 stimulation detectable. Of note, for IFN-γ, IL-5 and IL-10 no
320 significant differences between phenotypes in parallel to relevant correlations of
321 cytokine- and gene expression were detected.
322 A significant positive rank correlation of *TLR7* gene expression and IL-17 secretion
323 was detected after LpA stimulation over pooled phenotypes (n=50, cor=0.34,
324 p=0.017), and in particular within AA (n=20, cor=0.53, p=0.016) (Fig. 2E), but not
325 within HC.

326

327

Discussion

Asthma is the most common chronic disease in children worldwide. The identification of specific biomarkers for AA and NA during disease manifestation can not only offer the opportunity for distinct clinical characterization, but also provide the chance for future prediction of the disease course based on biomarkers. Finally, identification of novel biomarkers can lead to novel targets for more specific endotype- based therapy in the long run. Importantly, in adults different asthma phenotypes were already associated with distinct responses to treatment (12). In the present study in children, we aimed to identify genes and molecular networks characterizing steroid naive AA and NA by using transcriptomics and confirmation by RT-PCR. In a microarray-based screening of unstimulated and stimulated PBMCs of children with different phenotypes (HC, AA and NA), the calcium signaling pathway and innate immunity associated pathways turned out to be regulated significantly different between the phenotypes. For more in depth analysis of these pathways, we performed specific gene expression analysis using RT-PCR and identified two calcium- associated genes, namely *ATP2A3* (sarco endoplasmatic reticulum Ca^{2+} ATPase 3) and *ORMDL3 (*Orosomucoid-1 like 3)*,* as well as TLR7 *(Toll like receptor 7)* and *LY75* (lymphocyte antigen 75, associated with inflammation) both being increased in asthmatic children independent of their allergic status.

Furthermore, the Ca^{2+} signaling associated gene *ATP2A3* and the innate immunity genes *CLEC4E, IFIH1* and *FPR2* were significantly different upon stimulation (fold change) between NA and AA. Identification of differently expressed genes is one step forward in identifying asthma phenotypes by screening tests.

Increased expression of the calcium signaling genes *ORMDL3* and *ATP2A3* in childhood asthma

Since the discovery of the relevance of *ORMDL3* SNPs for childhood asthma (13-16), the Ca^{2+}- signaling pathway has been studied in more depth. In this study, the calcium associated genes *ATP2A3/SERCA3* and *ORMDL3* were upregulated in asthmatic children compared to HC, independent of their allergic status. This points to an allergy-independent upregulation of these two Ca^{2+}- signaling genes in asthmatic children. Of note, apart from *ATP2A3* and *ORMDL3* no other analysed Ca^{2+} pathway-associated gene was significantly up-regulated in NA and no difference was identified between AA and NA, regarding the absolute level of gene expression.

Our correlation-based network analysis showed an independent regulation of *ATP2A3* and *ORMDL3* as they were less correlated with other Ca^{2+}-associated genes. Thus, *ATP2A3/SERCA3* and *ORMDL3* may act independently of other Ca^{2+}-associated genes in asthma patients. Due to sample limitations, we were not able to further study the function or the quantity of the respective proteins in this study.

ATP2A3 is an endoplasmatic reticulum (ER) Ca^{2+}-pump which pumps calcium from the cytosol back to the ER to restore Ca^{2+} after cell activation. Three tissue specific SERCA proteins are known, SERCA1-3 encoded by *ATP2A1-3*. *ATP2A2*, which is specific for muscle cells, was studied in smooth muscle cells of asthma patients, where its expression was decreased followed by hyperproliferation. *ATP2A3* was not detectable in smooth muscle cells (17). In contrast, we detected elevated expression of *ATP2A3* in PBMC of asthmatics in this study. The Ca^{2+} homoeostasis seems to be imbalanced in favour of decreasing the Ca^{2+} level in the cytosol of PBMCs induced by *ATP2A3.* For functional relevance, knowledge about intracellular Ca^{2+} levels

would be important. Yet this requires a different study design as measurement takes place seconds after stimulation.

Orosomucoid-1 like 3 (*ORMDL3*) exerts two main cellular functions. It negatively regulates the *de novo* sphingolipid (SL) synthesis and is important for cellular Ca^{2+} homeostasis. Single nucleotide polymorphism (SNP)- dependent overexpression of *ORMDL* was associated with childhood asthma (13-15). Increased *ORMDL* leads to an impaired *de novo* synthesis of sphingolipids. It was shown that an impaired *de novo* SL synthesis leads to asthma like symptoms such as airway hyperreactivity in murine studies (18). ORMDL is also involved in the regulation of the unfolded protein response (UPR). The UPR is induced by decreased levels of ER Ca^{2+}, a cellular stress signal, followed by increased expression of *ORMDL*. It triggers inflammation through activation of NF-kB and c-Jun N-terminal kinase (JNK) and leads therefore to inflammatory processes (19, 20).

In our study *ORMDL3,* which inhibits *SERCA* and the SERCA encoding gene *ATP2A3* were both upregulated in PBMCs from asthmatics. The upregulation of *ATP2A3* could be a counter-regulation to overcome *ORMDL3* overexpression in order to reduce cellular stress and inflammatory signaling in asthma patients.

The additional upregulation of *ORAI1, S100A9, SPOCK2* and *STIM1* in AA, besides *ORMDL3* and *ATP2A3*, points to a more prominent Ca^{2+}signal dysregulation in AA than in NA. Allergy might trigger the Ca^{2+} signaling- associated dysregulation in addition to asthma.

Upregulation of TLR7 and LY75 expression as shared pattern in asthma independent of allergy

As calcium signaling is strongly associated with innate immunity, we assessed whether genes of innate immune regulation were upregulated in a similar fashion. Indeed, *LY75* and *TLR7* expression was upregulated in asthmatic children independent of their allergic status. Although *TLR7* and *TLR8* are both endosomal receptors for virus RNA, they were independently regulated in our cohort. The expression of the *TLR7-gene,* whose protein detects ssRNA was strongly upregulated in asthmatic children (AA and NA). The role of this RNA receptor is still under debate in asthma research. Different studies were able to detect an impaired *TLR7* function in PBMCs of allergic asthmatics (21, 22). Also increased receptor function was detected in the PBMCs of patients with exacerbations (23). Surprisingly, these studies did not observe different expression of *TLR7* between HC and asthmatics in PBMCs in contrast to findings from epithelium of severe asthmatics, where *TLR7*-expression was shown to be decreased (24). In our cohort, *TLR7* was investigated before and following innate and T-cell stimulation. The positive correlation of TLR7 and IL-17 following innate LpA-stimulation points to a pro-inflammatory regulation in AA, but not HC. Related to this, a recent report of Ye at al indicates that TLR7 signaling can manipulate Th17 cell regulation in the context of inflammatory disease (25). Certainly, further functional analysis of TLR7 regulation in childhood asthma is required to truly identify the role of TLR7 signaling in early disease development. *TLR8,* whose protein also detects ssRNA, was not differently expressed in asthmatics, compared to HC. It seems that the propensity for defence against ssRNA viruses may be more pronounced in asthma patients. As viruses of upper respiratory tract infection such as influenza virus or rhinoviruses (both ssRNA)

425 are associated with severity and course of asthma, increased expression of ssRNA
426 receptors following anti-CD3/28-stimulation may indicate either previous exposure to
427 viruses or a propensity for immediate response to viruses in children with asthma
428 (26). A recently published study could show a decrease of inflammation in an allergic
429 asthmatic non-human primate model by a *TLR7/8* ligand (27), which underlines the
430 importance of ssRNA receptors in asthma research. Additionally, gene expression of
431 dsRNA virus receptors (*DHX58* and *DDX58)* was upregulated in PBMCs of AA
432 children but not in NA in our study. This supports the concept of a contribution of
433 virus infections to asthma pathogenesis. Further studies are necessary to investigate
434 whether overexpression of virus receptors results in permanent inflammation
435 contributing to a “shift” towards asthma or whether prolonged immune activation after
436 termination of a virus infection is involved in asthma pathogenesis. We speculate that
437 the children are more susceptible to virus infections which may lead to persistent
438 inflammation as upregulation of more receptors induces even more stimulation. Our
439 data indicate that this may be relevant for virus-specific receptors.
440
441 **AA showed different stimulation patterns compared to NA**
442 For *AT2A3*, *CLEC4E, IFIH1,* and *FPR2,* we *found s*ignificant differences in fold
443 change (FC) after anti-CD3/28 stimulation between AA and NA. The innate immunity
444 receptors *CLEC4E* and *IFIH1* were less responsive to stimulation in AA than in NA,
445 which may reflect a decreased capacity to react following stimulation in AA compared
446 to NA but not HC. This needs to be further evaluated as these genes were not
447 differently expressed between AA and NA in regard to ΔCT. In our study we detected
448 an increased expression of the Lipoxin A4 receptor *FPR2* with stimulation in AA but
449 not NA. Other studies showed an upregulation in severe asthmatics on natural killer

450 (NK) and innate lymphoid cells (ILC) (28), but decreased in sputum and and
451 bronchoalveolar lavage (BAL) fluid (29). An increase in the short chain fatty acid
452 receptor (SCFA) *FPR2* in AA, but not in NA, points to a more pronounced influence
453 of SCFA in AA than in NA. The differentially regulated response to anti-CD3/28
454 stimulation between AA and NA might be a basis for the distinction of these two
455 phenotypes. Of note, cell stimulation is a prerequisite for elucidation of the
456 differences. Thus, despite this important distinction, use as a biomarker for
457 phenotype definition in clinical practice is more challenging.
458 In conclusion, we identified increased *ATP2A3, ORMDL3, TLR7* and *LY75* in
459 asthmatic children independent of allergy, while *ATP2A3* and *FPR2* were increased
460 in AA and *CLEC4E* decreased in AA following stimulation, all *s*ignificantly different
461 between NA and AA.
462 The strength of this study is the transcriptomic analysis with confirmatory analysis by
463 RT-PCR in a representative subgroup of the well-defined CLARA asthma children
464 cohort with clear in- and exclusion criteria. All asthmatic children are steroid-naive,
465 which gives us the opportunity to analyse immune regulatory pathways without
466 influence of different medications. Of note, due to analysis of PBMCs, interpretation
467 regarding the source of distinct cell populations is not feasible.
468 We are limited in our case numbers regarding non-allergic asthmatics as they
469 present a minority of asthma patients in childhood and due to material availability we
470 could not perform western blots.
471 In summary, this study characterizes asthmatic children by differentially expressed
472 genes and NA vs AA by different expression of the Ca^{2+} pathway and innate
473 immunity receptors upon stimulation. This study thus represents an important step
474 forward to a endotypic characterization of different asthma phenotypes in children,

potentially relevant for development of non-invasive, easy to use diagnostic tools for the future. Current asthma medication in childhood, including corticosteroids, short and long acting beta-2 adrenergic agonists, already target Ca^{2+} signaling- associated processes, however with only partial success. Thus, future functional studies on Ca^{2+}-regulation in both asthma phenotypes, before and following Ca^{2+}-pathway regulating treatments, may contribute to disentangle specific groups of children, which may profit from distinct treatment regimens. The characterization of innate immunity receptor profiles in asthma patients could be an additional step forward to better understand responses to different viral triggers for both allergic and non-allergic asthmatic children.

ACKNOWLEDGEMENTS

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TABLES

Table 1: Regulation of KEGG pathways and T cell group-characteristic genes.

Regulatory pathways were assessed for differential expression between phenotypes via global tests providing a single p-value for each set of genes. Analysis was conducted stratified for stimulation on the microarray data (n=14/8/14 for AA/NA/HC). Grouping of genes was according to KEGG, annotated pathways from the Kyoto Encyclopedia of Genes and Genomes. T cell groups, characteristic genes for the respective T cell subset. MAPK, mitogen-activated protein kinase signaling pathway; TGF-β, Transforming growth factor beta signaling pathway; NK cyto, Natural killer cell mediated cytotoxicity; Cyto-cyto, Cytokine-cytokine receptor interaction; TLR, toll-like receptor signaling pathway; Comp, Complement and coagulation cascades; TCR, T cell receptor signaling;

Analysis / KEGG	Nr. of genes	PBMCs								
		AA vs HC (14 vs 14)			NA vs HC (8 vs 14)			NA vs AA (8 vs 14)		
		unstimulated	LpA	anti-CD3/28	unstimulated	LpA	anti-CD3/28	unstimulated	LpA	anti-CD3/28
Apoptosis	85	**0.039**	**0.004**	0.143	0.158	0.133	0.210	0.085	0.203	0.182
B	11	0.144	0.269	**0.015**	0.079	0.092	0.152	0.253	0.343	0.126
B cell receptor signaling	76	0.103	0.072	0.204	0.064	**0.046**	0.146	**0.016**	**0.007**	0.466
Calcium signaling	175	**0.019**	**0.013**	0.439	0.187	**0.031**	0.173	0.084	**0.005**	0.192
Chemokine signaling	189	0.298	**0.049**	0.840	0.486	0.057	0.569	0.432	**0.037**	0.344
Complement and coagulation cascades	69	0.467	0.145	0.557	0.328	0.079	0.211	0.139	0.102	0.089
Cytokine-cytokine receptor interaction	248	0.380	**0.040**	0.670	0.390	0.064	0.406	0.412	0.128	0.176
Dendritic cells	10	0.322	0.352	0.796	0.121	0.143	0.155	0.218	0.068	0.116
Jak-STAT signaling	146	**0.039**	**0.013**	0.299	0.151	0.092	0.120	0.192	0.163	0.100
Macrophage regulation	7	0.286	0.139	0.835	**0.008**	**0.029**	0.236	**0.050**	**0.006**	0.239
MAPK signaling	265	0.082	**0.003**	0.429	0.110	**0.047**	0.206	0.087	**0.031**	0.090

mTOR signaling	53	**0.005**	**0.012**	**0.031**	0.133	0.051	0.075	**0.048**	0.075	**0.011**
Natural killer cell mediated cytotoxicity	111	**0.020**	0.075	0.347	0.068	0.121	0.169	**0.040**	**0.023**	0.267
NK	12	0.512	0.438	0.283	0.155	0.208	0.417	0.211	0.464	0.797
NOD-like receptor signaling	54	0.063	**0.032**	0.482	0.053	**0.015**	0.297	0.079	0.162	0.279
nTh	4	0.090	0.103	0.586	0.703	0.326	0.740	0.244	0.837	0.231
p53 signaling	64	0.197	0.372	0.434	0.063	0.752	0.234	0.483	0.582	0.072
T cell receptor signaling	110	**0.014**	**0.038**	0.206	0.108	0.089	0.178	0.136	0.063	0.656
Tfh	12	0.430	0.312	0.557	0.572	0.169	0.272	0.720	0.167	0.249
TGF-β signaling	86	0.122	**0.000**	0.327	**0.013**	0.109	0.225	0.227	**0.050**	0.380
Th1	14	0.176	0.358	0.505	0.097	0.505	0.555	0.494	0.498	0.938
Th17	13	0.175	0.434	0.662	0.103	0.509	0.177	0.128	0.540	0.070
Th2	12	0.258	**0.045**	0.491	0.313	**0.020**	0.127	0.338	0.710	0.097
Th22	12	0.212	0.264	0.322	0.069	0.362	0.429	0.077	0.080	**0.049**
Th9	5	0.650	0.649	0.247	0.112	0.169	0.698	0.136	0.258	0.400
Toll-like receptor signaling	93	0.180	**0.016**	0.297	0.177	**0.010**	0.264	**0.057**	**0.035**	**0.028**
Treg	16	**0.033**	0.105	0.270	0.431	0.522	0.322	**0.032**	0.797	0.864

Table 2: Differential gene expression between phenotypes

RT-qPCR (RNA form PBMCs cultivated for 48 hours) data were assessed for differential expression between phenotypes stratified for stimulation using two-sample t-tests within the linear regression framework and were adjusted for age and sex. Included in the table are comparisons with an unadjusted p-value below 0.05. ↑ reflects increased expression in the noted group, ↓ decreased expression and — reflects unchanged expression in the noted group in stimulated compared to unstimulated cells. M reflects unstimulated cells, anti-CD3/28-stimulation and LpA Lipid A-stimulation, respectively.

One example: ↑ NA, —HC in context of NA> HC stands for a positive stimulation effect in NA, which is significantly higher than the effect in HC, where the latter itself is not significantly different from the basal, unstimulated expression level.

	AA > HC	**NA > HC**	**AA< NA**	**AA > NA**
	Stimulation effect (fold change)			
Calcium signaling		ITPR2 (↑ NA, —HC: anti-CD3/28) SPOCK2 (↑NA, —HC: anti-CD3/28) STIM2 (— NA — HC: anti-CD3/28)		ATP2A3 (↑ AA, — NA: anti-CD3/28)
Innate immunity	FPR2 (↑ AA, ↑HC: LpA) TLR6 (— AA, ↓ HC: anti-CD3/28)	IFIH1 (↑NA, —HC: anti-CD3/28) LY75 (↑ NA, ↑HC: anti-CD3/28)	CLEC4E (—NA, ↓ AA: anti-CD3/28) IFIH1 (↑NA,— AA: anti-CD3/28)	FPR2 (↑AA,—NA: anti-CD3/28)
	Expression level (ΔCT)			
Calcium signaling	ATP2A3 (LpA) ORAI1 (LpA) ORMDL3 (anti-CD3/28, LpA) S100A9 (anti-CD3/28) SPOCK2 (anti-CD3/28, LpA) STIM1 (LpA)	ATP2A3 (LpA) ORMDL3 (M, LpA)		
Innate immunity	DDX58 (M, anti-CD3/28) DHX58 (LpA) LY75 (anti-CD3/28) TLR7 (M, anti-CD3/28, LpA)	CD209 (M) LY75 (anti-CD3/28) TLR6 (M) TLR7 (anti-CD3/28)		

Figure Legends

Figure 1: Differential gene expression of asthma phenotypes.
RT-qPCR (PBMCs cultivated for 48 hours) data were assessed for differential expression between phenotypes stratified for stimulation using two-sample t-tests within the linear regression framework and were adjusted for age and sex (n=21/13/20 for AA/NA/HC). Results were shown by mean difference in ΔCT with 95% confidence interval. Significantly different comparisons ($p < 0.05$) were marked with asterisks (*) below. For both confidence intervals and asterisks colour indicates stimulation (red for anti-CD3/28; green for LpA, blue for unstimulated). Negative values on y-axis (above zero-line) reflect a higher gene expression of the phenotype named in the upper line (x-axis) and vice versa.

Figure 2A-D: Gene network based on correlations.
Network on adjusted (phenotype, age, sex) correlations. Unstimulated (blue), anti-CD3/28 (red), LpA (green). Bigger size of nodes indicates differential expression between phenotypes, a = AA>HC , b = NA>HC and refers to the results given in Table 2.
Pairwise correlation is indicated by solid lines (positive) and dotted lines (negative). 2.5% of highest and 2.5% of lowest correlations were shown, corresponding to thresholds of 0.79 and -0.38.
Spatial arrangement was calculated according to a force-directed-graph algorithm on gene expressions, which places genes according to their correlation gradually from close (highly positively correlated) to distant (negatively correlated). Networks were independently calculated on all individuals and stratified per phenotype:

A) Based on all N=54 children, correlations adjusted for sex, age and phenotype.
B) Only within n=20 HC.
C) Only within n=21 AA.
D) Only within n=13 NA.

Figure 2E: Correlation of gene expression and cytokine secretion.
Scatter-plot showing IL-17 cytokine secretion versus TLR7 gene expression for AA (n=20), NA (n=10), and HC (n=20). Rank correlation was significant for AA after LpA stimulation (top-left, cor=0.53, p=0.016) and unstimulated within HC (bottom-right, cor=0.34, p=0.017). Blue lines represent parametric regression line for log2-transformed cytokine levels.

Figure 3: Summary of main study results
In microarray analysis significant differences in gene expression of innate immune and calcium pathway- associated genes were identified and confirmed by PCR analysis. Increased gene expression was exemplified by arrows labelled with AA and/or NA**.**

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Conflict of Interest:

KLR, AB, VV, DS, DPdC, EK, UM have nothing to disclose.

EM reports grants from European Commission, during the conduct of the study; personal fees from PharmaVentures, from OM Pharma, Decision Resources, Novartis Pharma SAS, The Chinese University of Hongkong, University of Copenhagen, HAL Allergie GmbH, Ökosoziales Forum Oberösterreich, Mundipharma, American Thoracic Society, AbbVie Deutschland GmbH & Co. KG, University of Tampere, European Commission, Massachassuetts Medical Society, American Academy of Allergy, Asthma and Immunology, University of Helsinki, and University of Turku, Peptinnovate, outside the submitted work.

TB reports grants from DFG TR22 during the conduct of the study; Outside the submitted work: patens from 2015 "T-Cell Specific Genome Editing" TUM151201PEP, 2009 “Genetic Sterility” PAT053760, 2007 “Targeting recombinase activity by trans-splicing” PCT/EP2008/004958, 2003 “siRNA-Selektionsverfahren” PCT/EP04/050669, all without royalties at the moment; personal fees from

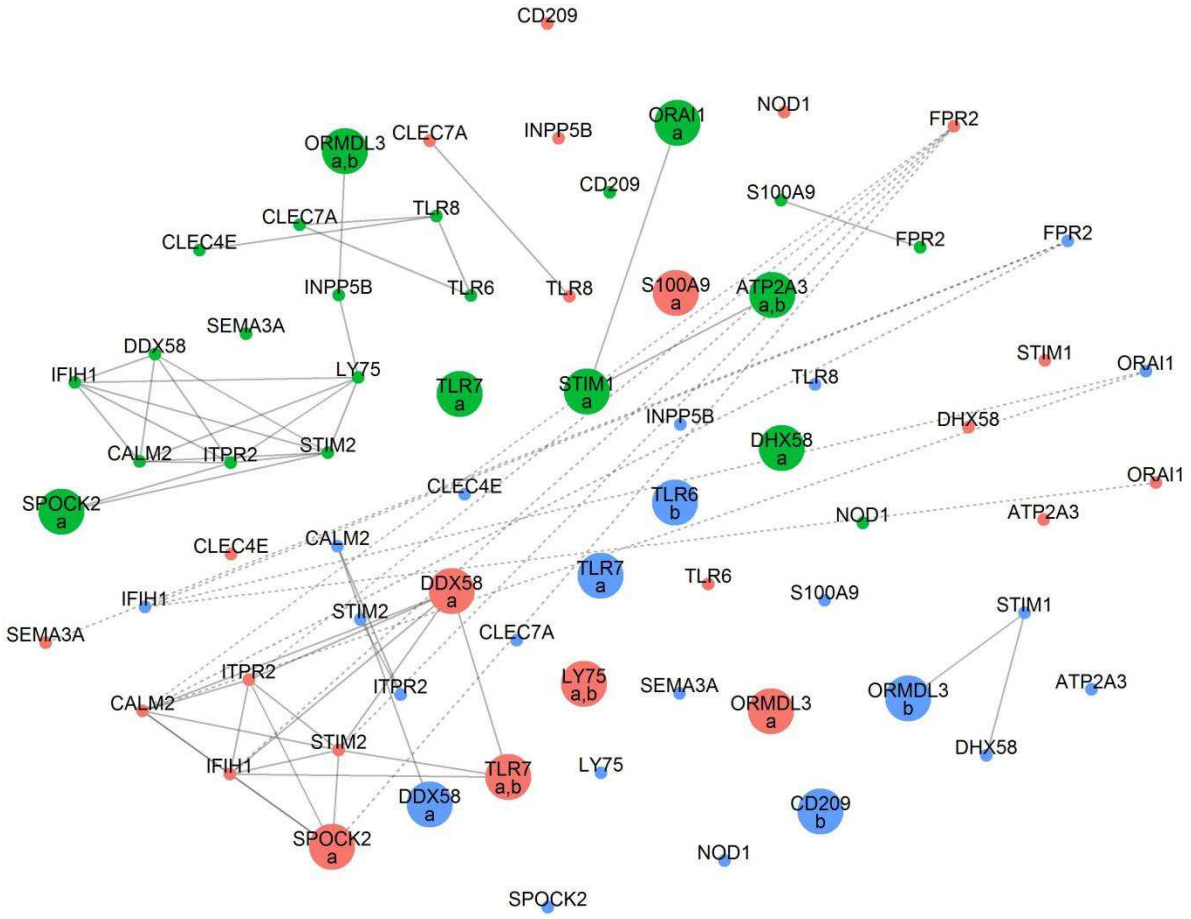
DFG/SNF/Hertie Foundation/SMSG/SGV/ProPferd/CTI and Biotech stocks within ETFs.

BS reports grants from DFG, BMBF during the conduct of the study and outside the submitted work.

Figure 1


calcium
ATP2A3
CALM2
INPP5B
ITPR2
ORAI1
ORMDL3
S100A9
SPOCK2
STIM1
STIM2
innate
CD209
CLEC4E
CLEC7A
DDX58
DHX58
FPR2
IFIH1
LY75
NOD1
SEMA3A
TLR6
TLR7
TLR8
Phenotype differences in expression (Δct)
AA - HC
NA - AA
NA - HC
stimulus
anti-CD3/28
LpA
M

Figure 2A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FPR2
ORAI1 a
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ORMDL3 a,b
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CLEC4E
FPR2
INPP5B
TLR6
TLR8
S100A9 a
ATP2A3 a,b
SEMA3A
DDX58
STIM1
ORAI1
IFIH1
LY75
TLR7 a
STIM1 a
TLR8
INPP5B
DHX58
CALM2
ITPR2
STIM2
DHX58 a
ORAI1
CLEC4E
SPOCK2 a
TLR6 b
NOD1
ATP2A3
CLEC4E
CALM2
TLR7 a
TLR6
IFIH1
DDX58 a
S100A9
STIM1
STIM2
SEMA3A
CLEC7A
ITPR2
LY75 a,b
SEMA3A
ATP2A3
ITPR2
ORMDL3 a
ORMDL3 b
CALM2
STIM2
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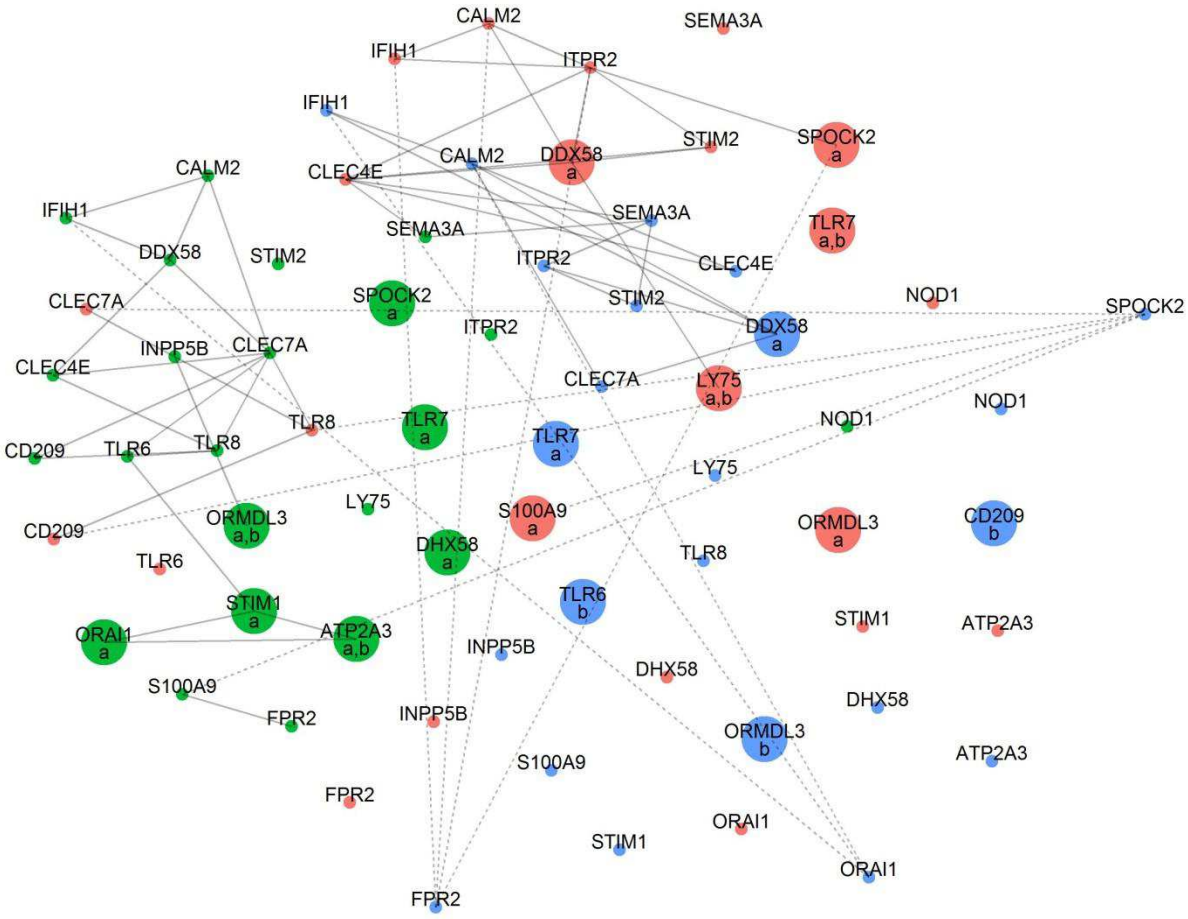
IFIH1
TLR7 a,b
LY75
DDX58 a
CD209 b
SPOCK2 a
NOD1
SPOCK2

Figure 2B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ITPR2
IFIH1
STIM2
SPOCK2 a
CALM2
DDX58 a
CALM2
CLEC4E
SEMA3A
TLR7 a,b
IFIH1
SEMA3A
DDX58
STIM2
ITPR2
CLEC4E
CLEC7A
SPOCK2 a
STIM2
NOD1
SPOCK2
ITPR2
DDX58 a
INPP5B
CLEC7A
CLEC4E
CLEC7A
LY75 a,b
NOD1
TLR8
TLR7 a
NOD1
CD209
TLR6
TLR8
TLR7 a
LY75
LY75
S100A9 a
ORMDL3 a,b
ORMDL3 a
CD209 b
CD209
DHX58 a
TLR8
TLR6
TLR6 b
STIM1 a
STIM1
ATP2A3
ORAI1 a
ATP2A3 a,b
INPP5B
DHX58
S100A9
DHX58
FPR2
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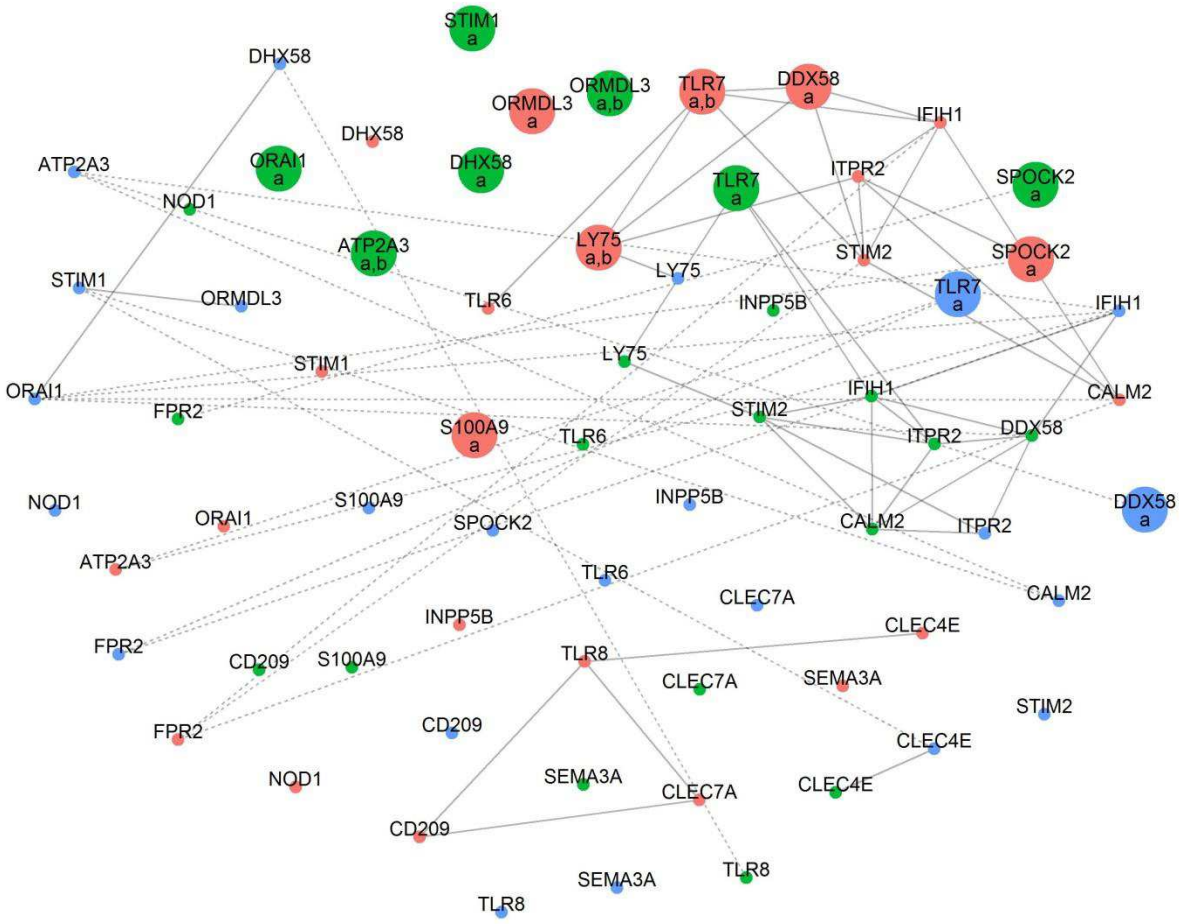
FPR2
ORAI1
STIM1
ORAI1
FPR2

Figure 2C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DHX58 a
TLR7 a
ITPR2
SPOCK2 a
NOD1
ATP2A3 a,b
LY75 a,b
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SPOCK2 a
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STIM2
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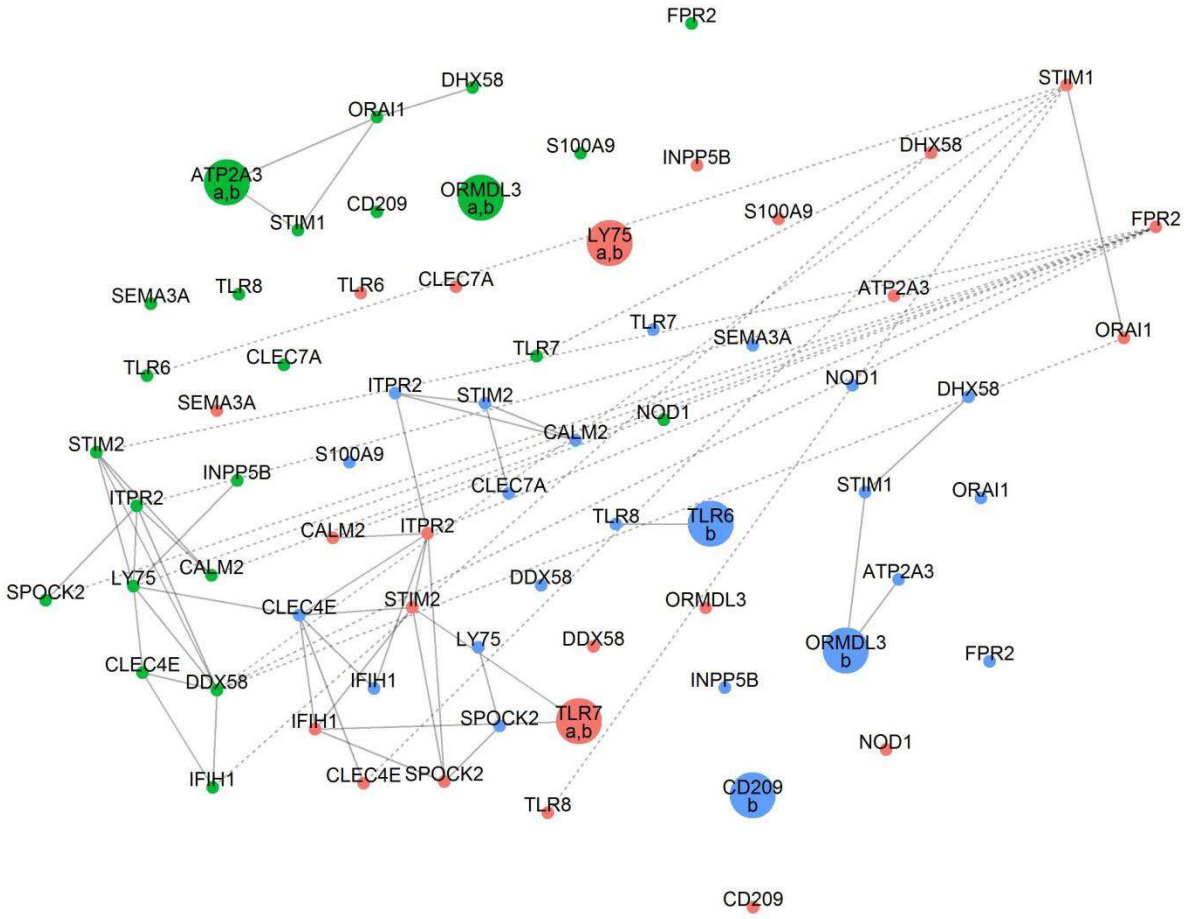
CD209
TLR8
SEMA3A
TLR8

Figure 2D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ORAI1
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a,b
ORMDL3
a,b
CD209
STIM1
S100A9
FPR2
LY75
a,b
SEMA3A
TLR8
TLR6
CLEC7A
ATP2A3
TLR7
SEMA3A
ORAI1
TLR7
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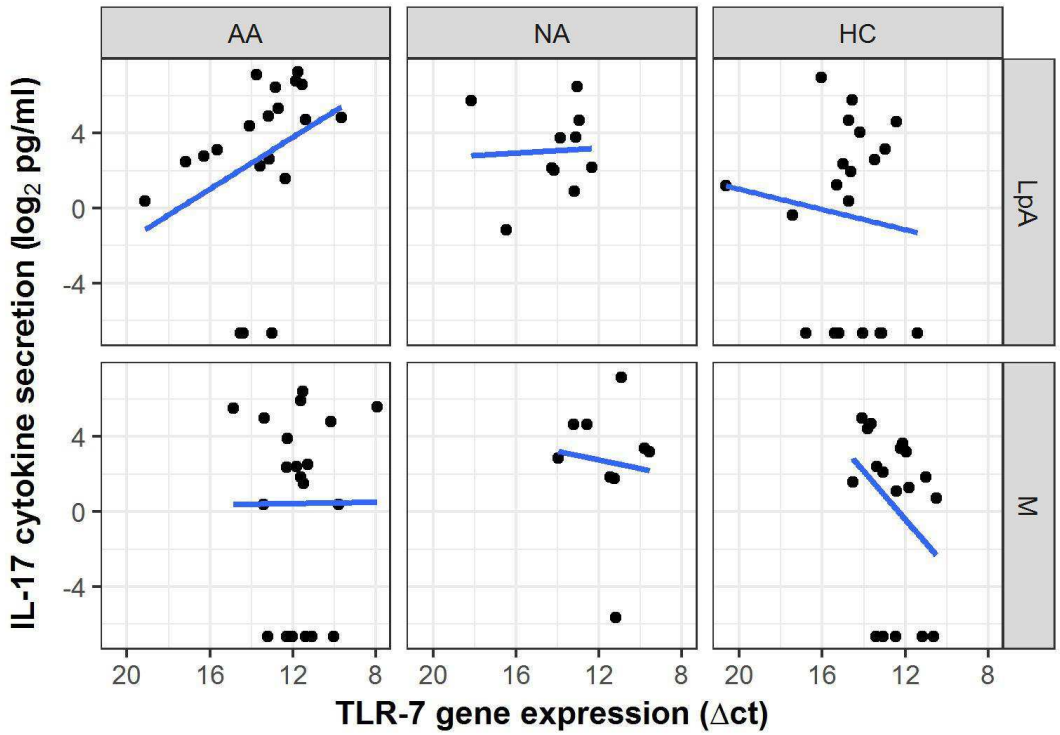
CD209
b
TLR8
CD209

Figure 2E


AA
NA
HC
LpA
M
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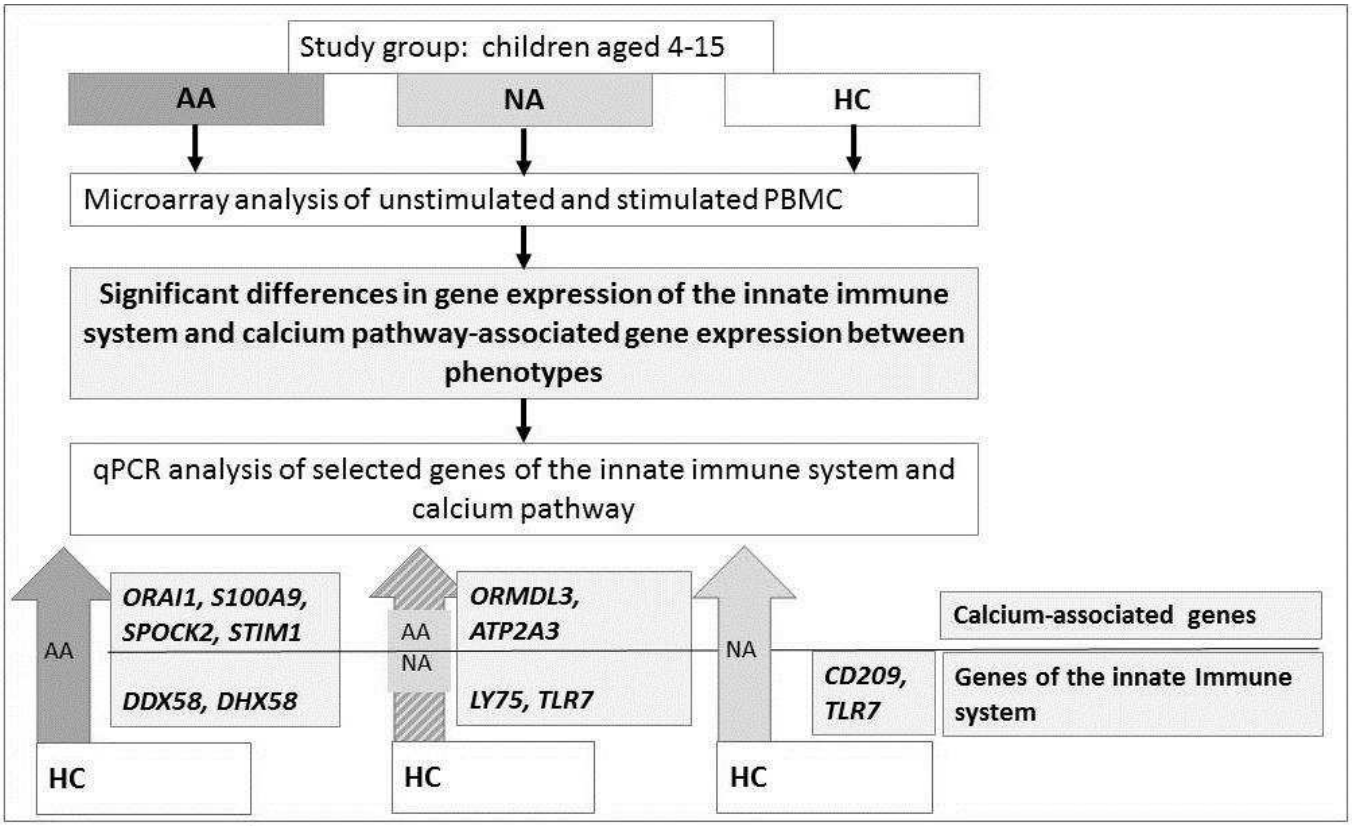
0
-4
20
16
12
8
TLR-7 gene expression (Δct)

Figure 3


Study group: children aged 4-15
AA
NA
HC
Microarray analysis of unstimulated and stimulated PBMC
Significant differences in gene expression of the innate immune system and calcium pathway-associated gene expression between phenotypes
qPCR analysis of selected genes of the innate immune system and calcium pathway
AA
ORAI1, S100A9, SPOCK2, STIM1
DDX58, DHX58
HC
AA
NA
ORMDL3, ATP2A3
LY75, TLR7
HC
NA
CD209, TLR7
HC
Calcium-associated genes
Genes of the innate Immune system

Supporting Information

Ca^{2+} and innate immune pathways are activated and differentially expressed in childhood asthma phenotypes

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Exclusion criteria for CLARA study

Only steroid-naïve children, without intake of steroids (also inhaled steroids) for at least 14 days, were included. Children with early transient wheeze in the first three years of life or episodic wheeze, without any wheeze, cough or any pulmonary symptoms in the last year before inclusion and without significant reversible airflow obstruction were not included.

Children with clinical symptoms of allergy and a positive specific IgE, but without wheeze, cough, shortness of breath and normal lung function were excluded. Also, children with current infections, assessed by CrP and IL-6 levels in addition to clinical examination and temperature measurement, were not included (1).

RNA Isolation, microarrays and quantitative real time RT-PCR

RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quality and quantity for microarray analysis was determined with Agilent Bioanalyzer chips (Bio-Rad Laboratories, Hercules, CA, USA).

Total RNA was labeled and hybridized to Affymetrix GeneChip® Human Gene 1.0 ST arrays according to the manufacturer's instructions as described before (1). The scanned arrays (n=108 arrays in 14 AA, 8 NA and 14 HC) were checked for quality control by MvA-, density- and RNA degradation plots. Computational analyses were performed using R (2). Background correction and normalization was performed by RMA normalization (3). Data were deposited in NCBI's Gene Expression Omnibus (GEO accession number GSE40889). Gene-specific PCR products were measured in n (AA/NA/HC) = (21/13/20) children by CFX96 TouchTM Real-time-PCR Detection

System (Bio-Rad, Munich, Germany) for 40 cycles. The difference in CT values relative to *18S* was used in the calculation of deltaCT (ΔCT); a higher ΔCT-value refers to lower gene expression.

Figure S1: Illustration of sample selection and experiments**.** For each phenotype, healthy controls (HC), allergic asthmatics (AA) and non-allergic allergic asthmatics (NA), microarray- and qPCR experiments were performed. qPCR measurements represent a replication in different groups of children for HC and AA. For NA, qPCR analysis was performed in n=13 children, of which n=5 new children were added.

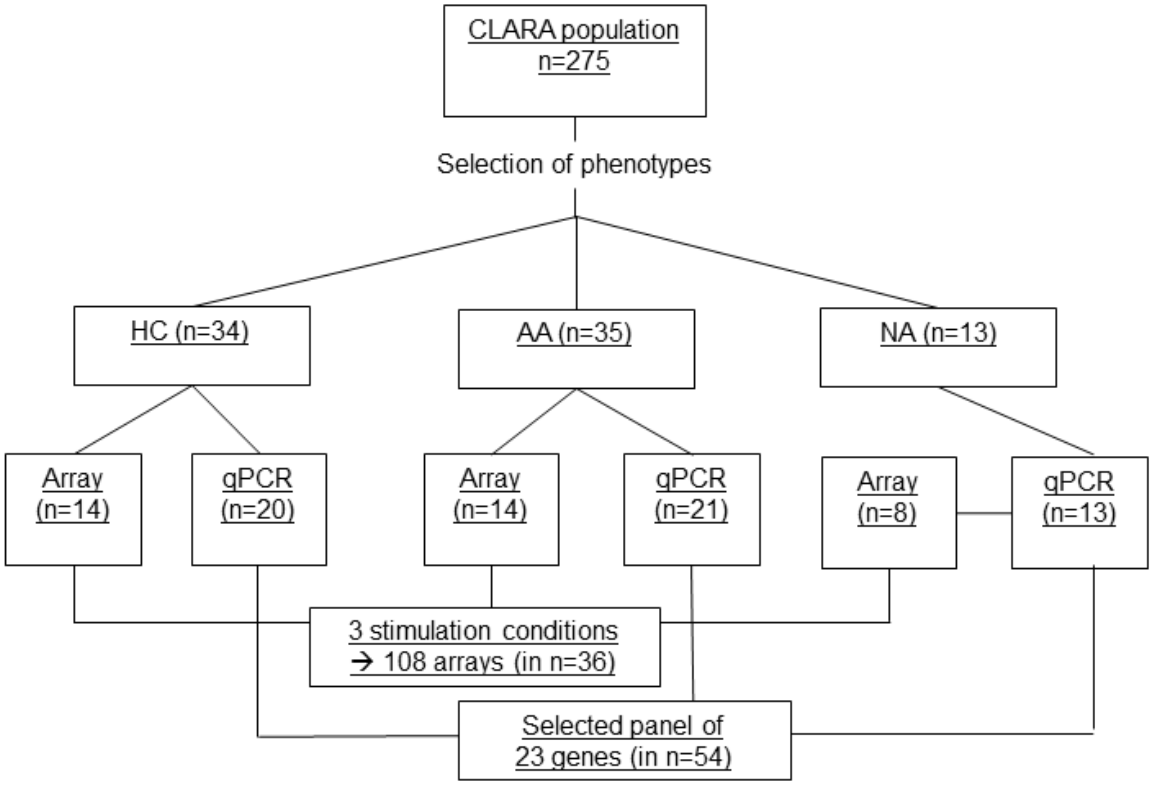

Figure S2: Comparisons of stimulation effect in gene expression between phenotypes (qPCR data). Significant fold changes were shown by lines (—) located above (pairwise two-sample t-tests). Effects following stimulation were shown by mean of fold change on log-2 scale with 95% confidence interval. Significant ($p < 0.05$) effects of stimulation with anti-CD3CD28 / LpA (color coded) within phenotypes compared to unstimulated expression are indicated by asterisks (*) at the bottom of the figure (one sample t-test for mean = 0).

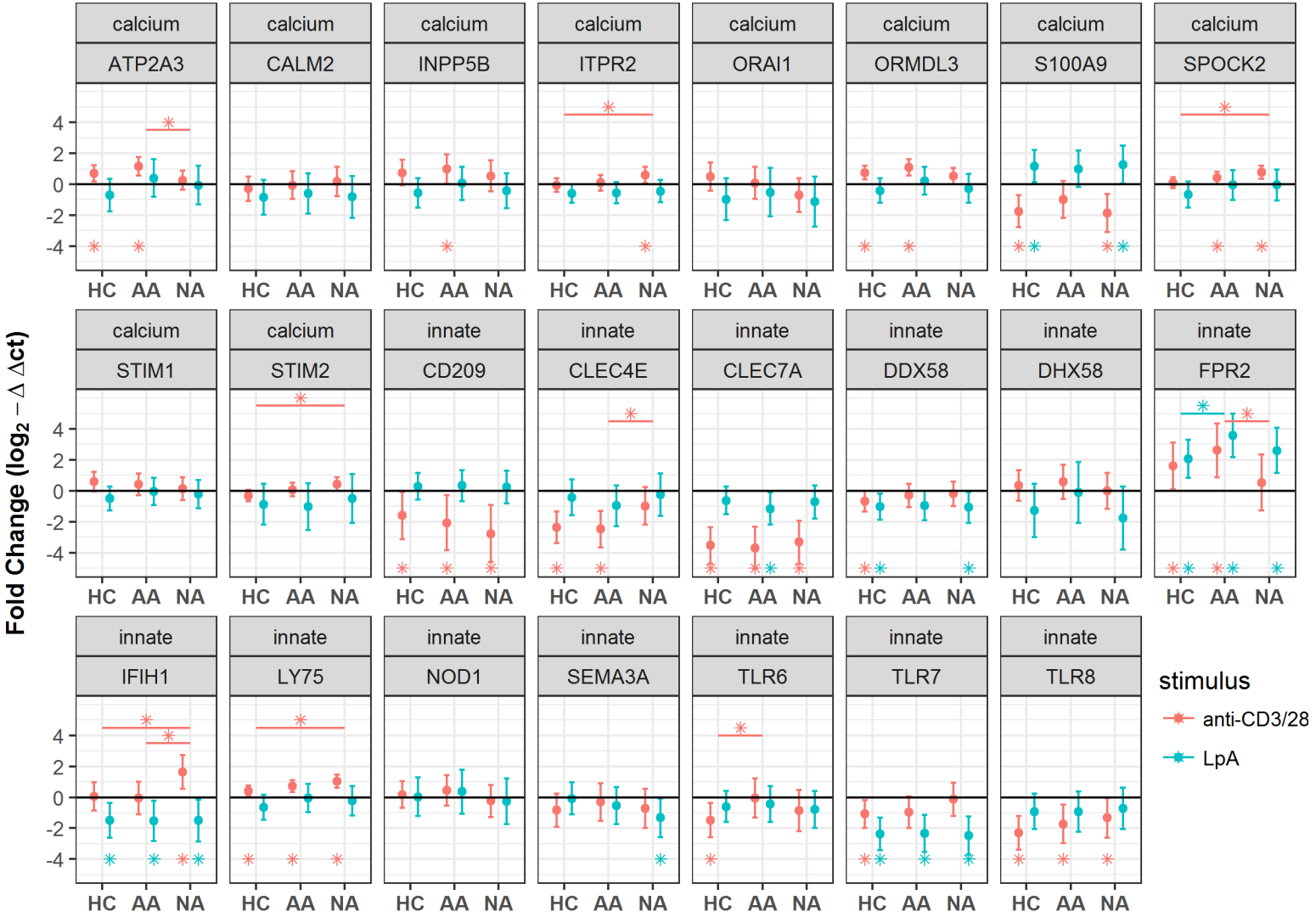

Table S1: Characteristics of subpopulation with RT-PCR measurements.

Median (med); [Q25] 25% percentile; Kruskal-Wallis-tests (KW) as overall three-group comparison and Wilcoxon-test (W) for two-group comparisons on continuous data; Fisher-test (F) was used on contingency tables (categorical data).

Parameter	N	HC				AA				NAA					HC-AA	HC-NAA	AA-NAA
		N	med	[Q25]	[Q75]	N	med	[Q25]	[Q75]	N	med	[Q25]	[Q75]	p (KW)	p(W)	p(W)	p(W)
Age	54	20	9.4	7.0	10.7	21	8.8	6.6	10.2	13	7.5	6.5	9.8	0.529	0.689	0.265	0.484
Eosinophils [%]	54	20	2.5	1.8	3.0	21	9.0	6.0	11.0	13	3.0	2.0	5.0	**<.001**	**<.001**	0.220	**0.002**
IgE [IU/ml]	53	19	39.1	20.0	89.7	21	328.0	186.0	765.0	13	42.9	13.8	141.0	**<.001**	**<.001**	0.985	**<.001**
FeNO	23	2	9.0	8.8	9.3	14	36.9	27.7	87.4	7	10.4	6.3	15.9	**0.008**	0.067	0.889	**0.004**
Bronchodilator response ((FEV1 post -pre)/pre)*100	33	4	5.4	3.9	6.0	19	19.6	12.0	24.3	10	11.7	9.3	15.6	**0.003**	**0.002**	**0.002**	0.077
zFEV1	41	7	0.7	-0.5	1.4	21	-1.6	-2.3	-0.6	13	-0.2	-0.8	0.1	**0.002**	**0.002**	0.183	**0.012**
		N(HC)		**% within HC**		**N(AA)**		**% within AA**		**N(NAA)**		**% within NA**			**p(F)**	**p(F)**	**p(F)**
Female	54	20		45.0		21		23.8		13		46.2			0.197	1.000	0.262
Maternal asthma	54	20		10.0		21		14.3		13		7.7			1.000	1.000	1.000
Maternal eczema	54	20		15.0		21		9.5		13		7.7			0.663	1.000	1.000

Maternal hay fever	54	20	35.0	21	28.6	13	0.0		0.744	**0.027**	0.062
Paternal asthma	54	20	15.0	21	14.3	13	0.0		1.000	0.261	0.270
Paternal eczema	54	20	0.0	21	4.8	13	7.7		1.000	0.394	1.000
Paternal hay fever	54	20	30.0	21	23.8	13	15.4		0.734	0.431	0.682
Smoking at home	54	20	20.0	21	9.5	13	30.8		0.410	0.681	0.173
Two siblings or more	54	20	35.0	21	19.0	13	15.4		0.306	0.263	1.000

Table S2: **Overview of function of selected genes for qRT-PCR**

Genes associated with the Calcium pathway	Encoded protein	Function	References
ATP2A3	ATPase sarcoplasmic/ endoplasmic reticulum Ca2+ transporting 3 *alias* SERCA 3 (sarco-endoplasmatic reticulum Ca2+ - ATPase)	Ca^{2+} pump from the cytoplasm back to the ER inhibited by ORMDL Diminished SERCA expression in airway smooth muscle cells contributes to airway remodeling in bronchial asthma	(4-6)
CALM2	Calmodulin 2	Membrane receptor for elevated extracellular calcium Upon activation, CALMKII (calmodulin-dependent kinase II) activation via IP_3 regulates with STIM/ STIM2 and ORAI1 store operated calcium entry (SOCE) → Ca^{2+} release activated Ca^{2+} channel (CARC) STIM1 and calmodulin interact with Orai1 to induce Ca^{2+}-dependent inactivation of CRAC channels.	(7-9)
INPP5B	inositol polyphosphate-5-phosphatase B	Involved in cellular calcium signalling Negative regulator of IgE-Ag-initiated mast cell degranulation	(10)
ITPR2	Inositol 1,4,5-trisphosphate receptor, type 2	Ca^{2+} channel in ER Upon activation, it initiates calcium release from the ER stores into the cytoplasm	(11)
ORAI1	Calcium release-activated calcium channel protein 1	pore subunit of a store-operated calcium channel (SOCC) activated upon depletion of Ca^{2+} stores main source of Ca^{2+} influx in T cells B-cells lacking STIM2/ORAI not able to produce IL-10	(7, 12-15)
ORMDL3	orosomucoid 1-like 3	regulates ER-mediated Ca^{2+}-signaling and cellular stress Associated with Asthma in GWAS with Chromosome 17q21	(16, 17)
S100A9	S100 calcium binding protein A9	early Calcium ion binding form complex with S100A8 to calprotectin Activates inflammasome and NF-kB Pathway Described in severe asthma, Increased during the early airway response in asthma (rat lung model of asthma); expressed abundantly in the cytosol of neutrophils, monocytes Amplifies tissue destruction associated with neutrophil activation in several autoimmune diseases; Expressed with S100A8 in neutrophils, macrophages and epithelia cells in inflammation Induced by IL-1beta	(18-21)

SPOCK2	SPARC/osteonectin, cwcv and kazal like domains proteoglycan 2 alias Testican 2	extracellular Ca^{2+} binding proteoglycans Associated with early trans differentiation of human lung aleovar Type 2 epithelia cells (high expression in lung development) Described in bronchopulmonary dysplasia	(22, 23)
STIM1	Stromal interaction molecules	STIM1 and STIM2 detect depletion of the ER's calcium stores and inflict changes on the intracellular $[Ca^{2+}]_i$. share the same function but differ in sensitivity (STIM2 higher sensitivity) regulates store and basal calcium concentrations in cell both interact with ORAI1 They control B-cell Function through IL-10 production Important for T cell activation and function of Tregs	(13-15, 24-26)
STIM2			
Genes associated with innate immunity	Encoded protein	Function	
CD209	DC-Sign (dendritic cell specific intracellular adhesion molecule grabbing non-integrin)	C-type lectin pathogen receptor on DC and basophils/ Important for DC T-cell interaction Increased expression in DC of allergic patients in lung, Down regulated with DERP interaction, induce Th2- polarization	(27-31)
CLEC4E	C-type lectin domain family 4 member A Alias DCIR- DC Immunoreceptor Alias Mincle (Macrophage inducible Ca^{2+}-dependent lectin)	Pattern recognition receptor (PRR) for glycolipids expressed DC and basophils Found to induce Tregs in allergic airway disease model Suppresses cDCs that impairs inflammation and T-cell immunity.	(31-33)
CLEC7A	DECTIN 1	glucan PRR expressed on DC, basophils decreased upon allergen-specific immunotherapy Suggested to be involved in HDM allergen recognition IL-5, IL-13 and IL-17 induction upon HDM stimulation (mouse model)	(31, 34)
DDX58	DExD/H-box helicase 58 alias RIG-1 (retinoic acid inducible gene I RLR)	Expressed in all cells types Sense double stranded RNA (viruses) activation of MAP kinase, IRF and NFκB pathway RLR SNPs not been associated with allergic diseases or asthma Viral dsRNA binding to MDA5 and TLR-3 induce airway inflammation and hyperresponsiveness Interaction of MDA5 and LGP2	(35, 36) (37) (38, 39)
IFIH1	interferon induced with helicase C domain 1 alias MDA5		
DHX58	DExH-box helicase 58		

	alias RLR-3/ LGP2		
FPR2	formylpeptide receptor 2	Receptor for lipoxinA4 Increased expression in severe asthmatics on NK and ILC expressed on human neutrophils, eosinophils, monocytes, macrophages, T cells, synovial fibroblasts, and intestinal and airway epithelium and ILC Important for the trafficking of monocyte derived DC to migrate in small airway in the mouse inflamed lung model by down regulating CCR2 on mdDC Reduced in sputum and BAL cells of children with severe asthma	(40-43)
LY75	Lymphocyte antigen 75 alias CD205, CLEC13B, DEC-205	Endocytic receptor for apoptotic and virus infected cells → ensuing processed via MHCI and MHCII Important for self-tolerance in thymus	(44-46)
NOD1	Nucleotide-binding oligomerization domain-containing protein 1	PRR for intracellular bacteria bacterial infection-mediated activation of NOD1,2 in triggering allergic asthma via the activation of eosinophils interacting with bronchial epithelial cells at inflammatory airway. NOD1 and NOD2 regulate the interaction between human eosinophils and bronchial epithelial cells in allergic asthma The polymorphisms of NOD1 and NOD2 have also been shown to be associated with Th2-mediated atopic diseases such as allergic asthma	(47, 48)
SEMA3A	Semaphorin3A	Originally described as factor for the development of neuronal pattern affects diverse signaling pathways such as MAPK, PI3K, STAT and small GTPases through a receptor complex containing neuropilin-1 (Nrp1) as its direct binding partner decreased in atopic dermatitis and allergic rhinitis in nasal epithelium of rhinitis mouse model Potential therapeutic target for asthma regulation of immune responses and the maintenance of self-tolerance, SEMA3A and NP1 expressions on BAL cells and lung homogenates were significantly elevated in asthmatic mice	(49-51) (52)
TLR6	Toll-like receptor 6	Receptor for Gram+ bacteria and fungi together with TLR2 TLR6 located on chromosome 4p14, region associated with atopy in GWAS Decreased expression on PBMC of asthmatics, correlating with severity TLR2 signaling pathway dependent hypersensitivity to inhaled allergens	(53) (54)
TLR7 *TLR8*	Toll-like receptor 7 and 8	endosomal receptor for ssRNA (TLR7) and dsRNA (TLR8) linked to neutrophilic activation and viral asthma exacerbations TLR7 stimulation seems to prevent Th2-mediated airway inflammation in animal models of asthma Potential therapeutic target R848 or S28463 a TLR7/8 agonist suppress airway hyperresponsiveness when administered either systemically or intranasal (mouse).	(55) (56, 57)

Table S3: Mean value and standard deviation (SD) of delta CT gene expression (unstimulated, LpA- or anti-CD3/CD28-stimulated for 48 hours). N(HC) = 20, N(AA) =21, N(NA)=13. It was indicated which group differences are significant; p-values correspond to the linear regression model with adjustment for age and sex. If no comparison was significant for the respective gene, this was labelled with n.s.

	Unstimulated (M)							LpA-stimulation							Anti CD3/CD28-stimulation						
	HC		AA		NA			HC		AA		NA			HC		AA		NA		
	Mean	SD	Mean	SD	Mean	SD	p-value	Mean	SD	Mean	SD	Mean	SD	p-value	Mean	SD	Mean	SD	Mean	SD	p-value
ATP2A3	12.60	1.11	12.89	1.56	12.06	0.91	n.s.	13.52	1.88	12.78	1.51	12.30	1.15	**AA>HC p=0.009 NA>HC p=0.006**	12.08	0.79	11.99	1.16	11.99	0.69	n.s.
CALM2	5.84	1.77	4.94	1.20	5.33	1.94	n.s.	6.80	2.58	5.62	1.90	6.11	2.04	n.s.	5.74	1.90	4.39	1.65	4.64	1.74	n.s.
INPP5B	13.25	1.41	13.35	1.46	12.68	1.22	n.s.	13.76	1.65	13.41	1.63	13.18	1.17	n.s.	12.46	0.85	12.30	1.79	12.07	0.61	n.s.
ITPR2	9.24	0.84	8.76	0.84	9.08	1.47	n.s.	9.80	1.50	9.23	1.38	9.46	1.53	n.s.	9.28	1.26	8.64	1.15	8.46	1.38	n.s.
ORAI1	12.79	2.33	12.11	1.69	11.78	1.18	n.s.	13.33	2.20	12.23	2.30	12.58	1.74	**AA>HC p=0.002**	12.28	1.44	11.97	2.06	12.39	1.45	n.s.
ORMDL3	12.68	0.64	12.32	0.91	11.98	0.99	**NA>HC p=0.003**	13.13	1.45	12.16	1.23	12.31	1.06	**AA>HC p=0.002 NA>HC p=0.036**	12.09	0.68	11.49	0.81	11.67	0.96	**AA>HC p=0.001**
S100A9	7.32	1.54	7.34	2.68	6.55	1.75	n.s.	6.07	2.08	6.20	2.60	5.14	1.70	n.s.	9.39	1.67	8.67	3.13	8.59	1.57	**AA>HC p=0.024**
SPOCK2	9.74	0.65	9.35	0.76	9.66	1.06	n.s.	10.49	1.69	9.48	1.37	9.75	1.32	**AA>HC**	9.59	0.90	8.90	0.88	8.89	1.05	**AA>HC**

														p=0.042							**p=0.045**
STIM1	12.90	1.39	12.65	0.67	12.41	1.07	n.s.	13.26	1.52	12.73	1.09	12.67	0.97	**AA>HC p=0.042**	12.07	0.96	11.92	1.09	12.10	0.55	n.s.
STIM2	10.45	0.80	10.15	0.81	10.38	1.24	n.s.	11.44	1.84	11.33	3.51	11.01	1.49	n.s.	10.75	1.03	10.05	0.97	9.93	1.32	n.s.
CD209	13.21	1.35	13.34	2.03	12.37	0.80	**NAA>HC p=0.015**	12.91	1.81	13.01	2.04	12.17	1.30	n.s.	15.42	2.13	16.09	3.16	15.63	2.28	n.s.
CLEC4E	9.00	2.12	7.80	1.79	8.24	2.11	n.s.	9.13	2.77	8.29	2.10	8.09	2.56	n.s.	11.43	2.87	10.26	2.68	9.07	2.68	n.s.
CLEC7A	11.72	3.01	11.35	1.96	10.73	1.02	n.s.	12.30	2.61	12.59	2.76	11.47	1.30	n.s.	15.80	3.23	15.72	2.85	14.52	1.83	n.s.
DDX58	10.38	1.60	9.46	1.31	10.06	1.42	**AA>HC p=0.048**	11.15	1.78	10.01	1.73	10.77	1.51	n.s.	11.13	1.32	9.79	1.71	10.20	1.33	**AA>HC p=0.009**
DHX58	14.20	2.19	13.64	2.05	13.36	2.27	n.s.	15.42	2.92	13.68	2.16	15.04	2.97	**AA>HC p=0.003**	13.97	2.83	13.22	2.57	13.38	2.13	n.s.
IFIH1	9.65	2.14	8.32	2.23	9.14	2.21	n.s.	10.91	2.06	9.50	2.75	10.34	3.12	n.s.	9.60	2.00	8.34	2.59	7.39	3.38	n.s.
FPR2	15.86	2.13	16.36	2.24	15.07	1.95	n.s.	13.72	2.29	12.62	2.09	12.50	1.66	n.s.	15.48	2.95	14.91	2.71	15.54	2.62	n.s.
LY75	10.13	0.72	9.82	0.92	9.83	1.22	n.s.	10.89	1.81	9.99	1.48	10.08	1.47	n.s.	9.80	1.02	9.22	0.97	8.87	1.26	**AA>HC p=0.021** **NAA>HC p=0.007**
NOD1	19.14	1.80	19.09	1.19	18.10	2.49	n.s.	19.65	2.08	19.57	1.57	19.21	1.48	n.s.	19.29	1.13	18.87	1.10	18.79	1.15	n.s.
SEMA3A	16.53	2.56	15.93	1.50	15.16	2.67	n.s.	16.99	1.98	17.17	2.00	17.05	2.36	n.s.	17.76	2.43	16.81	1.30	16.41	1.72	n.s.

TLR6	12.67	0.83	12.26	0.97	11.72	2.28	**NA>HC p=0.046**	13.19	1.66	12.59	1.08	12.47	1.17	n.s.	14.80	3.16	13.30	1.06	13.41	0.70	n.s.
TLR7	12.71	1.30	11.66	1.47	11.90	1.50	**AA>HC p=0.014**	14.77	2.00	13.56	2.14	14.10	1.58	**AA>HC p=0.010**	13.73	1.30	12.54	1.57	11.99	1.96	**AA>HC p=0.034 NAA>HC p=0.019**
TLR8	10.83	1.60	10.74	1.28	10.50	1.12	n.s.	11.43	2.33	11.23	2.36	10.91	1.65	n.s.	13.78	2.13	13.39	2.38	12.50	1.25	n.s.

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