DR KATJA LANDGRAF-RAUF (Orcid ID : 0000-0002-3649-7990)

Article type : Original

IRF-1 SNPs influence the risk for childhood allergic asthma: a critical role for pro-inflammatory immune regulation

Short title: *IRF-1* SNPs influence childhood asthma and inflammation

Katja Landgraf-Rauf, PhD[a,b], Andreas Boeck, PhD[a], Diana Raedler, PhD[a] , Elisabeth Klucker, MD[a], Vanessa Vogelsang[a], Susanne Schmidt, MD[a], Sonja Kunze, PhD[c,d], Claudia Weissenbacher, MD[e]; Anke Graessel, PhD[f], Carsten Schmidt-Weber, PhD[f] Erika von Mutius, MD[a, b] Michaela Schedel, PhD[g], Bianca Schaub, MD[a, b]

[a] University Children´s Hospital Munich, Department of Pulmonary & Allergy, LMU Munich, Germany

[b] Comprehensive Pneumology Center Munich (CPC-M), Member of the German Center for Lung Research

[c] Institute of Epidemiology II, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany

[d] Research Unit of Molecular Epidemiology, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany

[e] University Children´s Hospital Munich, Department of Endocrinology, LMU Munich, Germany

[f] ZAUM - Center of Allergy and Environment, Technische Universität and Helmholtz Center Munich, Munich, Germany

[g] Department of Pediatrics, Division of Cell Biology, National Jewish Health, Denver USA

Corresponding author

Bianca Schaub, MD, Prof.

University Children´s Hospital Munich

Lindwurmstr. 4

80337 Munich, Germany

Ph: +49-89-4400-57856

Fax: +49-89-4400-54764

Email: Bianca.Schaub@med.uni-muenchen

Declaration of all sources of funding:

This study was supported by:

The German research foundation as part of the trans-regional collaborative research program TR22 "allergic immune responses of the lung", grant DFG SFB TR22/A22 (DR, AB, EK, BS), grant DFG SFB/Z03 (SK) GSK grant (DR), FöFoLe Reg.Nr. 839 (DR).

ABSTRACT

Background: Allergic and non-allergic childhood asthma has been characterized by distinct immune mechanisms. While *interferon regulating factor* 1 (*IRF-1*) polymorphisms (*SNPs*) influence atopy risk, the effect of SNPs on asthma phenotype-specific immune mechanisms is unclear. We assessed whether *IRF-1* SNPs modify distinct immune regulatory pathways in allergic and non-allergic childhood asthma (AA/NA).

Methods: In the CLARA study, asthma was characterized by doctor´s diagnosis and AA vs NA by positive or negative specific IgE. Children were genotyped for four tagging SNPs within *IRF-1* (n=172). mRNA expression was measured with qRT-PCR. Gene expression was analyzed depending on genetic variants within *IRF-1* and phenotype including haplotype estimation and an allelic risk-score.

Results: Carrying the risk alleles of *IRF-1* in rs10035166, rs2706384 or rs2070721 was associated with increased risk for AA. Carrying the non-risk allele in rs17622656 was associated with lower risk for AA but not NA. In AA carrying the risk alleles, an increased pro-inflammatory expression of *ICAM3, IRF-8, XBP-1, IFN-γ, RGS13, RORC,* and *TSC2* was observed. *NOD2* expression was decreased in AA with risk alleles in rs2706384 and rs10035166 and with risk-haplotype. Further, AA with risk-haplotype showed increased IL-13 secretion. NA with risk allele in rs2070721 compared to non-risk allele in rs17622656 showed significantly upregulated calcium, innate, mTOR, neutrophil and inflammatory associated genes.

Conclusion: *IRF-1* polymorphisms influence the risk for childhood allergic asthma being associated with increased pro-inflammatory gene-regulation. Thus, it is critical to implement *IRF-1* genetics in immune assessment for childhood asthma phenotypes.

Key Words

Asthma, Ca-signalling, childhood, IRF-1, pro-inflammatory, single-nucleotide-polymorphism

ABBREVIATIONS

AA	Allergic asthma, Allergic asthmatic(s)
BAL	Bronchoalveolar lavage
Ca^{2+}	Calcium
CLARA	Clinical Asthma Research Association
CLEC4E	C-type lectin domain family 4 member E
CT	Cycle threshold
DC	Dendritic cell(s)
ER	Endoplasmatic reticulum
FCRL5	Fc receptor like 5
FENO	Fractional exhaled nitric oxide
HC	Healthy control(s)
HT	Heterozygous allele
HWE	Hardy-Weinberg equilibrium
ICAM3	Intercellular adhesion molecule 3
IL	Interleukin
ILT4	Immunologlobulin like transcript 4
IP3	Inositoltriphosphate 3
IRF	Interferon regulating factor
ISRE	Interferon stimulated regulatory elements
LD	linkage disequilibrium
LpA	Lipid A
MAF	minor allele frequency

NA	Non-allergic asthma, Non-allergic asthmatic(s)
NCBI	National center for Biotechnology Information
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
PBMCs	Peripheral blood mononuclear cells
RHEB	Ras homolog enriched in brain
RGS13	Regulator of G-protein signaling 13
TCR	T-cell receptor
Th	T helper cell
TF	Transcription factor
Treg	regulatory T-cell(s)
WT	Wild type
XBP-1	X-Box binding protein 1

Introduction

Asthma is the most common chronic airway inflammatory disease in children(1) and is characterized by reversible airflow obstruction and bronchial hyperresponsiveness, being influenced by environmental factors, genetic predisposition and subsequent immune modulation(2). We have demonstrated that children with allergic (AA) and non-allergic asthma (NA) were characterized by distinct immune regulatory mechanisms(3). However, modifications of immune regulatory mechanisms by genetic background have not been fully elucidated. One important candidate is interferon regulating factor 1 (IRF-1), as Schedel *et al*. have shown, that genetic variants in *IRF-1* influence the development of childhood atopy and IgE-regulation(4). Furthermore, *IRF-1* levels in AA were reduced upon rhinovirus stimulation compared to healthy controls(5). The IRF transcription factors (TF) were originally identified in the context of type I interferon regulation(6). IRF-1 is expressed at low levels in most resting immune cells and regulates IFN-β after viral infection by binding enhancer elements in the IFN-β promotor. High expression of *IRF-1* is induced by virus, LPS and cytokines (e.g. IL-1, IL-12, IFN-β, IFN-γ, TNF). IRF-1 can increase expression of IFN-γ–inducible genes and influences target genes towards induction of IL-4, IL-12, IL-23, caspase1, iNOS and NK cells. It regulates T-cell differentiation after antigen stimulation(7-9). After T-cell receptor (TCR) activation of the precursor T-cell, IRF-1 promotes the Th1 response by repressing IL-4 transcription via binding the Interferon stimulated regulatory elements (ISRE) of the *IL-4* gene(6, 7, 10, 11). Thus, it is an important candidate potentially influencing immune regulation.

In this study we now aimed to investigate, whether human *IRF-1* genetic variants modify specific immune regulatory pathways in children with allergic and non-allergic asthma compared to healthy controls.

METHODS

Study population

The CLARA study population comprised 4-15 year old steroid-naïve AA, NA and HC (healthy control) children (n=273), described previously(3). Genotyping, cytokine analyses and RT-PCR was performed in a subgroup of children (N= max 172), comparable to the whole study population. Asthmatic children were characterized by clinical examination, body plethysmography, fractional exhaled nitric oxide (FeNO), full blood count, total and specific IgE (Allergy Screen®, Mediwiss Analytic GmbH), and defined according to GINA-guidelines(12, 13). Inclusion-criteria for AA/NA were recurrent obstructive bronchitis 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 AA/NA was based on positive (spec. IgE≥0.35IU/ml) or negative allergic-sensitization, in accordance with clinical symptoms. HC had no allergies and were in the same age-range. Exclusion-criteria for all children comprised other chronic-, pulmonary-, autoimmune-diseases, immunodeficiency, intake of steroids, antibiotics, probiotics and infection within 14 days before blood-withdrawal.

SNP-selection and genotyping

Schedel *et al.* previously identified 39 common genetic variants in the *IRF-1* gene, with a minor allele frequency (MAF) of at least 10%. By linkage-disequilibrium *(*LD) analysis, 5 major LD blocks were identified with R^2>0.8, each represented by a tagging-SNP(4). The study-population was genotyped for four of these tagging-SNP, which were significantly associated with changes in total IgE-levels(4). Genomic DNA was extracted from whole blood (Flexigene DNA-Kit (Qiagen Hilden, Germany). Samples were genotyped using matrix-assisted laser desorption/ionization time-of-flight-mass-spectrometry (Sequenom, Inc., San Diego, CA). Deviations from Hardy-Weinberg-Equilibrium were assessed for quality control of genotyping procedures.

Isolation and culture of PBMCs and quantitative RT-PCR

Peripheral blood mononuclear cells (PBMCs) were isolated within 24h after blood-withdrawal, cultured unstimulated (M) or stimulated with plate-bound anti-CD3 (3µg/ml) plus soluble anti-CD28 (1µg/ml) or lipid-A (LpA, 0.1µg/ml). Cell-pellets were used for RNA-isolation and supernatants for cytokine-measurements. RNA, isolated with RNeasy Mini-Kit was processed (1µg) with reverse transcriptase (Qiagen, Hilden, Germany). Gene-specific PCR-products were measured by CFX96 TouchTM Real-time-PCR Detection-System (Bio-Rad, Munich, Germany) for 40 cycles (details in supplement).

Statistical analyses

Clinical/ laboratory characteristics of the genotyped population were described with 25-, 50- and 75th percent quartiles. Differences in distribution between phenotypes and *IRF-1* genotypes were assessed with Kruskal-Wallis-Tests (three-group-comparison) and Wilcoxon-Test (two-group-comparison). Independence of asthma phenotypes and *IRF-1* genotype was tested with Fisher's exact test. The genotype effect on gene-expression was determined by pairwise t-tests, with stratified and unstratified phenotype-analysis. Each SNP was analyzed separately. Correlations were reported as Pearson- or Spearman-rank-correlations as indicated. Haplotype-frequencies and association-modeling was conducted with R-package haplo.stats, using an EM-algorithm to derive haplotype estimates based on SNP-data with unknown phase. An individual can have multiple possible haplotypes for which probabilities are estimated. In the regression model these probabilities are used as weights. In turn, the given haplotype frequencies are the sum of weights for each haplotype(14). The risk-score counts all risk-alleles. An individual`s score ranges between zero (all alleles homozygous non-risk) and eight (all alleles homozygous risk). In this explorative-study adjustment for multiple testing was not performed. All statistical analyses were conducted with R3.1.

RESULTS

Clinical characteristics of genotyped study population

Children with allergic (AA) and/or non-allergic asthma (NA) compared to healthy controls (HC) significantly differed in clinical and laboratory parameters as previously reported(3). AA had increased eosinophils, IgE and FeNO compared to NA and HC. Trendwise increased neutrophils were detected in NA ($p \leq 0.1$, Tab.1). Both AA and NA had significantly lower z-scores for FEV1 and FEV1/FVC ratio and significantly higher bronchodilator-response compared to HCs. The groups were comparable in terms of epidemiological characteristics.

Description of *IRF-1* polymorphisms

SNP characteristics were displayed in OR Tab.E1. Success rate for genotyping was between 98-100%. None of the alleles significantly deviated from HWE. The MAF of analyzed tagging SNPs was comparable with the European population indicated by National center for Biotechnology Information (NCBI) (15-18) (OR.Tab.E1). Changes of TF-binding patterns described in supplement (OR.Tab.E7).

Association of *IRF-1* genotypes with childhood asthma

The association of the four *IRF-1* SNPs with asthma prevalence was shown in Tab.2. Homozygous carriers of the three *IRF-1* SNPs rs2706384, rs2070721, rs10035166 had a higher risk being AA compared to HC. These alleles are called “risk-alleles” subsequently. Homozygous carriers of rs17622656 were significantly less prevalent in AA compared to HC, subsequently called “protective-alleles”. Homozygous carriers of rs17622656 were significantly more prevalent in the NA compared to AA. There were no significant differences between HC and NA children. Fig.1 shows the

distribution of children with different risk-scores. Relating the risk-score to the proportion of AA resulted in a highly significant OR (Fig.1).

Influence of *IRF-1* genotype on immune-regulation

We analyzed potential underlying immune regulation by gene-expression and cytokine secretion (all details on cytokines in supplement, including method, results E2A, E2B, E2C and discussion). Due to our prior findings(3), gene selection comprised genes involved in innate immunity, calcium- and mTOR-signaling, pro-/anti-inflammatory immune responses, Treg/Th17-regulation and neutrophil-associated genes.

Significant regulation of gene-expression in at least two different *IRF-1* polymorphic-alleles within AA compared to homozygous WT carriers or heterozygous plus homozygous carriers of the WT allele were seen for *NOD2* (partly down and up- regulation)*, RGS13, RORC, IRF-8, IFN-γ, ICAM-3, FCRL5* and *XBP-1* (up-regulated, Tab.3, details OR.Tab.E3A.). Analyzing haplotype-specific gene-expression comparing the risk-associated haplotype over all four SNPs with the protection-associated haplotype ATAT showed significantly decreased *NOD2*- and increased *FCRL5, RGS13, RORC, IRF-8, IFN-γ* and *XBP-1*-expression (Tab.3, details OR. Tab.E4). *FCRL5, XBP-1* and *ICAM3, XBP-1* gene-expression was significantly and strongly correlated in AA (OR.Tab.E5.).

Downregulated *NOD2*-expression in children carrying the risk-allele compared to homozygous WT or heterozygous plus homozygous WT-allele-carriers was also found in HC (OR.Tab.E3B). Haplotype-analysis in HC with risk-haplotype showed significant up-regulation of *NOD1* (change in ΔCT 1.67) and down-regulation of *TLR4 (*ΔCT 2.43), *TLR6* (ΔCT 2.13) and downregulated *ILT-4* (ΔCT 1.74) and *ORAI1*-expression (ΔCT 1.51) compared to the non-risk-haplotype.

Including all significant different expressed genes between phenotypes, correlation analysis of gene-expression and high risk-score (all *IRF-1* alleles are homozygous risk for AA) for the *IRF-1* loci showed upregulated *FCRL5* and *XBP-1-expressoin* and down-regulated *NOD2*-expression within AA. Expression of *RGS13, ICAM3, IFN-γ and IRF-8* was upregulated in both AA and NA with high risk-score. In HC, *SLC25A3, INPP5B* and *NOD1* expression was upregulated and *ILT4* expression was down-regulated. There was no significant correlated gene-expression shared between AA and HC. In NA, 14 genes were correlated with high asthma risk-score (all *IRF-1* alleles are homozygous risk) (Fig.2A, details OR.Tab.E6). Fig.2B shows scatter plots of unstimulated (M) gene-expression depending on increasing risk-score for AA and HC with significant different slopes for HC compared to AA.

Gene regulation in NA children with *IRF-1* SNP

NA with risk-allele in rs2070721 showed upregulated gene-expression in calcium-, innate-, m-TOR-, neutrophil- and inflammatory-associated genes. Conversely, NA with non-risk-allele rs17622656 showed significant downregulation of these pathways, thus expressing the opposite gene regulation pattern (OR. Tab. E3C).

Discussion

Previously, we identified distinct immune-regulatory mechanisms in AA and NA children, namely decreased expression of genes associated with innate immunity and increased pro-inflammatory IL-1β and IL-17-shifted neutrophilic inflammation. In this study we now identified the known atopy-associated *IRF-1* polymorphic-alleles in rs2706384, rs2070721 and rs10035166 being associated with an increased risk for AA in children. Analysis of SNP- and haplotype-dependent effects on gene-expression in AA, NA and HC showed distinct regulatory pattern towards a pro-inflammatory status with risk-alleles, especially in AA. This included inflammatory genes (*IFN-γ, IRF-8, FCRL5*), mTOR regulation (*RHEB*), a Th17-associated gene (*RORC*), the innate receptor *NOD2,* TFs (*XBP-1, RGS13*) and calcium-signaling (*ICAM3*). Correlation analysis of gene-expression with high risk-score showed significant correlation with increased *FCRL5* and *XBP-1* and decreased *NOD2* expression to be specific for AA. HC children had decreased *ILT4,* which was previously associated with asthma.

Increased risk for childhood-AA with *IRF-1* polymorphisms

A cross-sectional cohort study of German children identified genetic variants in *IRF-1* to be associated with atopy and increased IgE(4). Also, in a Japanese population, *IRF-1* polymorphisms in intron 1 and intron 7 were associated with atopic asthma in children(19). IRF-1 in combination with IRF-4 was previously shown to regulate Th9 cells(20).

In our cohort, the *IRF-1* polymorphic-alleles rs2706384, rs2070721 and rs10035166 were associated with increased risk for AA but not for NA. The polymorphic-allele rs17622656 was associated with protection from AA, in concordance with the decreased risk for atopy and atopic asthma described by Schedel et al.(4). Of note, other factors beyond *IRF*-1 risk-alleles contribute to asthma, as also risk-allele carriers were HC and *vice versa* children with WT-allele for *IRF-1* were AA.

Aiming to disentangle underlying immune regulatory pathways of children with *IRF-1* polymorphic-allele with asthma, we measured cytokine-secretion (supplement) and gene-expression for inflammatory signaling pathways, mTOR regulation, Treg/Th17 associated genes, innate receptors and regulators and calcium-signaling molecules in AA, NA and HC children.

Pro-inflammatory gene-expression in AA carriers of *IRF-1* risk polymorphisms

Overall, upregulation of *FCRL5, XBP-1, RGS13, ICAM-3, RORC, SLC25A3, IFN-γ and IRF-8* in AA with risk-alleles was confirmed in AA with risk-haplotype. Specifically, upregulation of pro-inflammatory genes such as *IRF-8, IFN-γ* and of the

TF *XBP-1* and *RGS13* indicate a pro-inflammatory immune status in AA, potentially leading to exuberant immune activation without sufficient control.

Aiming to further disentangle the influence of *IRF-1* polymorphic-alleles in AA, correlation analysis of gene-expression and risk-score showed significant correlations for increased *XBP-1* and *FCRL5* and decreased *NOD2* with higher *IRF-1* risk-score, yet only in AA. This was consistent with increased regulation in haplotype and SNP-specific analysis, thus emphasizing on their relevance for AA. *FCRL5* and *XBP-1* expression was significantly upregulated exclusively in AA children and not in HC with the same high risk-score.

The TF X-Box binding protein 1 (XBP-1) is upregulated in the endoplasmatic reticulum (ER) upon cellular stress and induces pro-inflammatory signals in immune cells(21, 22). Thus, increased *XBP-1*-expression in AA with *IRF-1* risk-alleles may indicate induction of a pro-inflammatory status. Increased *Fc-receptor-like-5* (*FCRL5*)- expression in AA carrying the risk gene variants may point to reduced B-cell regulation as this gene encodes for a membrane receptor found to bind IgG, thus negatively regulating B-cell stimulation. Yet, this is surprising as B-cell activation factor was previously upregulated in serum and sputum of asthma patients(23, 24). As we could not additionally assess B-cells in depth, we cannot conclude a direct influence of *FCRL5* expression on B-cell regulation in this cohort.

Reduced *NOD2*-expression in AA associated with high *IRF-1* risk-score was unexpected as NOD2 induces innate immune regulation(25) and increased *NOD2*-expression was detected in sputum cells of neutrophilic asthmatics. However, we had previously identified decreased innate immune induction in AA(3), thus being consistent with decreased NOD2-expression. Increased *IRF-8, ICAM3, RORC* and *XBP-1* were specific for AA risk-allele-carriers, but not HC, supported by highly

significant correlations of *ICAM3* and *XBP-1* in AA. Although these proteins were currently not described to be associated with asthma, they may represent interesting candidates for future in depth studies, as well as NOD2.

Remarkably, HC with highest risk-score had increased gene-expression of *SLC25A3, INPP5B, NOD1* and decreased expression of the *ILT4*. Additionally, *ILT4* is the only gene whose risk-score was correlated with gene-expression and which slopes were additionally significantly different between AA and HC. Decreased *ILT4* may act as a potential asthma-protective factor. Consistently, higher *ILT4* expression on dendritic cells (DCs) was described in asthmatics(26). *ILT4* was originally described to inhibit co-stimulating molecules and to induce regulatory T-cells (Treg)(27, 28). We previously identified increased Treg in AA compared to HC(3), which might be a compensatory mechanism for chronic inflammation of AA. This finding fits well with increased expression of *ILT4* with high risk-score for AA. Increased expression of *SLC25A3*, a phosphate-carrier-protein identified as being expressed in human activated $CD4^+$T-cells(29), may fulfil a protective role in high-risk HC as SLC25A3 mediates phosphate uptake and could influence Ca-signalling(30). An overshooting adaptive immune response might be attenuated at the onset of asthma development, but this needs to be proven by further experiments.

NAs showed upregulation of distinct immune regulatory pathways with risk allele in rs2070721

The alleles associated with higher risk for AA did not change the risk for NA but do influence gene expression. Collectively, NA carriers of the polymorphic allele in rs17622656 showed down regulation of several genes, while *vice versa* NA with the polymorphic-allele rs2070721 showed upregulation of selected genes. Specifically,

this comprised genes of the calcium, innate immunity pathway, inflammatory and neutrophil-associated genes.

Specific facets of NA and AA carriers of *IRF-1* variants

IRF-1 regulates a number of immune-pathways beyond T-cell-differentiation. We showed consistently increased pro-inflammatory expression in carriers of *IRF-1*-risk-alleles. Thus, this study points to a clear contribution of *IRF-1*-regulation for inducing inflammation during early asthma-manifestation in AA. Of note *in silico* analyses predict allele-specific changes in TF-binding for each of the *IRF-1* variants, which may contribute to observed downstream differences in gene-expression of various genes in this study.

Yet, additional mechanisms beyond IRF-1 are involved, as children without *IRF-1*-risk-alleles do also develop asthma in our cohort.

The strength of this study is a well-defined asthma-cohort including exclusively steroid-naïve asthmatics with strict in- and exclusion-criteria. Of note, an unequal distribution of NA vs AA is prevalent in this study as shown before(3). Yet, in this group significant findings of increased gene-expression with polymorphic-allele rs2070721 are prevalent, indicating an underestimation of effect size. Our findings of immune-regulation of primarily pro-inflammatory pathways influenced by *IRF-1* gene-variants are relevant for future studies and require in depth mechanistic work-up. Due to a limited amount of blood we did not perform further *in vitro* functional studies. Finally, a better insight into *IRF-1*-regulation in childhood-asthma may contribute to a specific understanding of pro-inflammatory regulation in AA and NA in childhood.

Acknowledgement

We thank all medical doctors, nurses and lung function technicians of the pulmonary department of children´s hospital for support regarding recruitment and Tatjana Netz for her excellent technical support and Monika Twardziok for critical review.

AUTHOR CONTRIBUTIONS

DR, VV, SK, AB and MS performed experiments and/or analyzed the data. EK, SS, CW, AG, CSW, MS provided samples and data for the study. BS, EM designed the study and KLR and BS wrote the manuscript.

Literature

1. Asher MI, Montefort S, Bjorksten B, Lai CK, Strachan DP, Weiland SK, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. Lancet (London, England). 2006;368(9537):733-43.
2. Agache I, Akdis C, Jutel M, Virchow JC. Untangling asthma phenotypes and endotypes. Allergy. 2012;67(7):835-46.
3. Raedler D, Ballenberger N, Klucker E, Bock A, Otto R, Prazeres da Costa O, et al. Identification of novel immune phenotypes for allergic and nonallergic childhood asthma. The Journal of allergy and clinical immunology. 2015;135(1):81-91.
4. Schedel M, Pinto LA, Schaub B, Rosenstiel P, Cherkasov D, Cameron L, et al. IRF-1 gene variations influence IgE regulation and atopy. American journal of respiratory and critical care medicine. 2008;177(6):613-21.
5. Pritchard AL, White OJ, Burel JG, Carroll ML, Phipps S, Upham JW. Asthma is associated with multiple alterations in anti-viral innate signalling pathways. PloS one. 2014;9(9):e106501.
6. Lohoff M, Mak TW. Roles of interferon-regulatory factors in T-helper-cell differentiation. Nature reviews Immunology. 2005;5(2):125-35.
7. Savitsky D, Tamura T, Yanai H, Taniguchi T. Regulation of immunity and oncogenesis by the IRF transcription factor family. Cancer immunology, immunotherapy : CII. 2010;59(4):489-510.
8. Lohoff M, Ferrick D, Mittrucker HW, Duncan GS, Bischof S, Rollinghoff M, et al. Interferon regulatory factor-1 is required for a T helper 1 immune response in vivo. Immunity. 1997;6(6):681-9.
9. Coccia EM, Stellacci E, Marziali G, Weiss G, Battistini A. IFN-gamma and IL-4 differently regulate inducible NO synthase gene expression through IRF-1 modulation. International immunology. 2000;12(7):977-85.
10. Elser B, Lohoff M, Kock S, Giaisi M, Kirchhoff S, Krammer PH, et al. IFN-gamma represses IL-4 expression via IRF-1 and IRF-2. Immunity. 2002;17(6):703-12.
11. McElligott DL, Phillips JA, Stillman CA, Koch RJ, Mosier DE, Hobbs MV. CD4+ T cells from IRF-1-deficient mice exhibit altered patterns of cytokine expression and cell subset homeostasis. Journal of immunology. 1997;159(9):4180-6.
12. Von Mutius E. Presentation of new GINA guidelines for paediatrics. Clinical and Experimental Allergy. 2000;30(6; SUPP/1):6-10.
13. Horak F, Doberer D, Eber E, Horak E, Pohl W, Riedler J, et al. Diagnosis and management of asthma - Statement on the 2015 GINA Guidelines. Wiener klinische Wochenschrift. 2016;[Epub ahead of print].
14. Lake SL, Lyon H, Tantisira K, Silverman EK, Weiss ST, Laird NM, et al. Estimation and tests of haplotype-environment interaction when linkage phase is ambiguous. Human heredity. 2003;55(1):56-65.
15. The National Center for Biotechnology Information USNLoM, Bethesda MD USA. The National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda MD USA http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2706384 2016 [July 2016].
16. The National Center for Biotechnology Information USNLoM, Bethesda MD USA. The National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda MD USA, http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=2070721 2016 [July 2016].
17. The National Center for Biotechnology Information USNLoM, Bethesda MD USA. The National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda MD USA, http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=10035166 2016 [
18. The National Center for Biotechnology Information USNLoM, Bethesda MD USA. The National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda MD USA, http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=17622656 2016 [

19. Nakao F, Ihara K, Kusuhara K, Sasaki Y, Kinukawa N, Takabayashi A, et al. Association of IFN-gamma and IFN regulatory factor 1 polymorphisms with childhood atopic asthma. The Journal of allergy and clinical immunology. 2001;107(3):499-504.
20. Campos Carrascosa L, Klein M, Kitagawa Y, Luckel C, Marini F, Konig A, et al. Reciprocal regulation of the Il9 locus by counteracting activities of transcription factors IRF1 and IRF4. Nature communications. 2017;8:15366.
21. Martinon F, Glimcher LH. Regulation of innate immunity by signaling pathways emerging from the endoplasmic reticulum. Current opinion in immunology. 2011;23(1):35-40.
22. Bettigole SE, Glimcher LH. Endoplasmic reticulum stress in immunity. Annual review of immunology. 2015;33:107-38.
23. Kang JS, Yoon YD, Ahn JH, Kim SC, Kim KH, Kim HM, et al. B cell-activating factor is a novel diagnosis parameter for asthma. International archives of allergy and immunology. 2006;141(2):181-8.
24. Jee HM, Choi BS, Kim KW, Sohn MH, Han MY, Kim KE. Increased B cell-activating factor (BAFF) level in the sputum of children with asthma. Korean journal of pediatrics. 2010;53(8):795-800.
25. Caruso R, Warner N, Inohara N, Nunez G. NOD1 and NOD2: signaling, host defense, and inflammatory disease. Immunity. 2014;41(6):898-908.
26. Martikainen MV, Kaario H, Karvonen A, Schroder PC, Renz H, Kaulek V, et al. Farm exposures are associated with lower percentage of circulating myeloid dendritic cell subtype 2 at age 6. Allergy. 2015;70(10):1278-87.
27. Manavalan JS, Rossi PC, Vlad G, Piazza F, Yarilina A, Cortesini R, et al. High expression of ILT3 and ILT4 is a general feature of tolerogenic dendritic cells. Transplant immunology. 2003;11(3-4):245-58.
28. Kornete M, Piccirillo CA. Functional crosstalk between dendritic cells and Foxp3(+) regulatory T cells in the maintenance of immune tolerance. Frontiers in immunology. 2012;3:165.
29. Graessel A, Hauck SM, von Toerne C, Kloppmann E, Goldberg T, Koppensteiner H, et al. A Combined Omics Approach to Generate the Surface Atlas of Human Naive CD4+ T Cells during Early T-Cell Receptor Activation. Molecular & cellular proteomics : MCP. 2015;14(8):2085-102.
30. Basso E, Petronilli V, Forte MA, Bernardi P. Phosphate is essential for inhibition of the mitochondrial permeability transition pore by cyclosporin A and by cyclophilin D ablation. The Journal of biological chemistry. 2008;283(39):26307-11.

Tab.1: Clinical characteristics of the genotyped study population

Parameter	N	HC				AA				NA				
		N	med	Q25	Q75	N	med	Q25	Q75	N	med	Q25	Q75	p (KW)
Age	172	82	10.34	7.16	12.34	77	9.37	6.44	11.43	13	7.58	6.52	8.18	**0.015**
Eosinophils [%]	166	76	2.00	1.00	3.00	77	8.00	4.00	10.00	13	4.00	3.00	6.00	**<0.001**
Neutrophils [%]	166	76	3.37	2.77	4.44	77	3.33	2.74	4.26	13	4.10	3.76	5.89	**0.092**
IgE [IU/ml]	169	80	38.65	16.90	88.50	76	331.00	180.25	785.25	13	63.50	29.30	108.00	**<0.001**
FeNO	57	8	8.15	5.28	10.23	40	34.55	24.13	69.48	9	7.90	4.70	11.40	**<0.001**
Bronchodilator response	88	11	4.88	1.94	6.12	66	14.08	9.43	21.65	11	12.30	10.06	16.53	**<0.001**
zFEV1	116	28	-0.13	-0.54	0.85	75	-1.09	-1.56	-0.31	13	-0.44	-1.33	-0.08	**<0.001**
zFEV1/FVC	116	28	0.38	-0.33	0.79	75	-1.58	-2.15	-0.79	13	-1.48	-1.72	-0.66	**<0.001**

HC: healthy controls; AA: allergic asthmatics; NA: non-allergic asthmatics; N: number of children; med: median, Q25, Q75: 25,75 percent quartiles

Total N may differ slightly due to available data; Eosinophils, percentage of blood eosinophils; Neutrophils, percentage of blood neutrophils; IgE, total serum immunoglobulin E; FEV1, volume in one second, Bronchodilator response, ((FEV1 post bronchodilation-FEV1pre bronchodilation)/FEV1 pre bronchodilation)*100; Bronchodilator response: ((FEV1 post bronchodilation-FEV1pre bronchodilation)/FEV1 pre bronchodilation)*100; zFEV1: z-score for FEV1; zFEV1/FVC: z-score for FEV1/FVC; p-value from Kruskal-Wallis (KW) test; data presented as Median (IQR=med) or n (%)

Tab.2. Association between *IRF-1* genotype and childhood asthma

IRF-1 SNP	N	Alleles	HC		AA		NA			SNP vs HT/WT		SNP/HT vs WT		Allele count	
			%	N	%	N	%	N		Odds ratio (95% CI)	p	Odds ratio (95% CI)	p	Odds ratio (95% CI)	p
rs2706384	170	TT	56.8	46	32.5	25	61.5	8	AA vs HC	**4.28** (1.34;13.65)	**0.011**	**2.73** (1.43;5.23)	**0.002**	**2.42** (1.48;4.09)	**0.001**
		TG	38.3	31	49.4	38	23.1	3	NA vs HC	3.50 (0.57;21.41)	0.192	0.82 (0.25;2.73)	1.000	1.16 (0.43;2.85)	0.757
		GG	4.9	4	18.2	14	15.4	2	NA vs AA	0.82 (0.16;4.11)	1.000	0.30 (0.09;1.01)	0.062	0.50 (0.19;1.20)	0.144
rs2070721	171	AA	35.4	29	23.4	18	46.2	6	AA vs HC	**3.47** (1.43;8.41)	**0.007**	1.79 (0.89;3.59)	0.118	**1.93** (1.20;3.16)	**0.007**
		AC	54.9	45	49.4	38	30.8	4	NA vs HC	2.78 (0.63;12.22)	0.172	0.64 (0.20; 2.08)	0.540	1.06 (0.42;2.60)	0.896
		CC	9.8	8	27.3	21	23.1	3	NA vs AA	0.80 (0.20;3.19)	1.000	0.36 (0.11;1.20)	0.100	0.60 (0.25;1.35)	0.224
rs10035166	168	TT	67.1	53	48.1	37	61.5	8	AA vs HC	**3.78** (1.00;14.32)	**0.045**	**2.20** (1.15;4.21)	**0.023**	**2.03** (1.22;3.47)	**0.008**
		TC	29.1	23	39.0	30	30.8	4	NA vs HC	2.11 (0.20;22.00)	0.462	1.27 (0.38;4.28)	0.756	1.32 (0.46;3.38)	0.580
		CC	3.8	3	13.0	10	7.7	1	NA vs AA	0.56 (0.07; 4.77)	1.000	0.58 (0.17;1.93)	0.550	0.65 (0.23;1.56)	0.409
rs17622656	168	GG	26.2	21	54.5	42	33.3	4	AA vs HC	0.28 (0.07;1.07)	0.080	**0.30** (0.15;0.58)	<**0.001**	**0.35** (0.20;0.61)	<**0.001**
		GA	61.3	49	41.6	32	41.7	5	NA vs HC	2.33 (0.54;10.10)	0.366	0.71 (0.19;2.61)	0.729	1.15 (0.43;3.04)	0.781
		AA	12.5	10	3.9	3	25.0	3	NA vs AA	**8.22** (1.44;47.01)	**0.030**	2.40 (0.67;8.64)	0.221	**2.81** (1.09;7.76)	**0.035**

HC: healthy control; AA: allergic asthmatic; NA: non-allergic asthmatic; WT: wild type allele; HT: heterozygous; SNP: single nucleotide polymorphism; SNP vs HT/WT: recessive model; WT vs HT/SNP: dominant model.

Tab.3: Association between *IRF-1* SNPs and haplotypes with gene expression in AA

	Gene regulation with *IRF-1* SNP				Risk-haplotype
Gene	**rs2706384**	**rs2070721**	**rs10035166**	**rs17622656**	**GCCG**
NOD1	LpA↑	LpA↑			
NOD2	CD3/28↓	CD3/28↑	CD3/28↓	M↓CD3/28↑	CD3/28↓
CCR2	CD3/28↓LpA↑	CD3/28↓	M↑		
S100A8		LpA↓			
SPOCK2		M↓			
ICAM3	M, LpA↑	M↑	M↑		
FCRL5		M↑	LpA, M↑		M↑
TSC2				LpA↓	LpA↑
RHEB			CD3/28, M↑	LpA↓	LpA↑
IL-9	M↓				
XBP-1	M, LpA↑	M↑	M↑		M↑
IL-37			LpA↑		
IRF-8	LpA↑	LpA, M↑	LpA, M↑		LpA↑
IFN-γ		LpA, M↑	LpA, M↑	LpA↓	LpA, M↑
FOXP3	LpA↑				
RORC		M↑	CD3/28, M↑	LpA↓	M, CD3/28↑
CD93	CD3/28↓				
TREM1				CD3/28↑	
RGS13		LpA, M↑	M↑	LpA↓	LpA↑
GMFG			LpA, M↑		
ETS2				LpA↓	
TLR4	CD3/28↑				
FRP2				LpA↓	LpA↑
EVI2A	M↓				
CD80	CD3/28↓				
SLC25A3	M ↓	LpA ↓		M↓	LpA, M↑

AA: allergic asthmatic ; GCCG Significant (p≤0.05) ↑ gene-expression up-regulated in homozygous carriers of the SNP; ↓gene-expression down-regulated in homozygous carriers of the SNP
GCCG: risk-haplotype of *IRF-1* SNPs; ↓/↑, decreased/increased gene-expression in risk-haplotype of *IRF-1* SNPs and phenotype; only significant data shown (p≤0.05), linear regression model, relating gene-expression to estimated haplotypes. Shown is up-/down regulation of pairwise comparisons between haplotypes using t-tests based on the fitted model, genes in bold indicates genes which are regulated with risk-haplotype

Figure legends

Figure 1: Distribution of AA and HC stratified for risk-score

Black bar shows the proportion of AA, grey bars for HC. Absolut numbers of AA and HC are given below and on top of the bars, respectively. Relating the risk-score to proportion of AA yields an OR of 1.33 (p<0.001).

Figure 2A: Venn diagram showing genes with significant correlations to *IRF-1* risk-score

Different ellipses indicate gene names significantly correlated with high risk-score. AA: allergic asthmatics, HC: healthy controls, NA: non-allergic asthmatic, ↓/↑ decreased/increased gene-expression compared to non risk-score.

Figure 2B: Gene-expression of HC and AA vs Risk-score:
Scatter plot of unstimulated (M) gene-expression vs risk-score. Black points show AA, gray points HC. Overlaid lines were derived from linear regression model relating expression to risk-score. Slopes of AA and HC differ significantly ($p<0.05$). For AA, slopes of *FCRL5* and *XBP-1* and for HC slope of *ILT4* were different to zero ($p<0.05$).

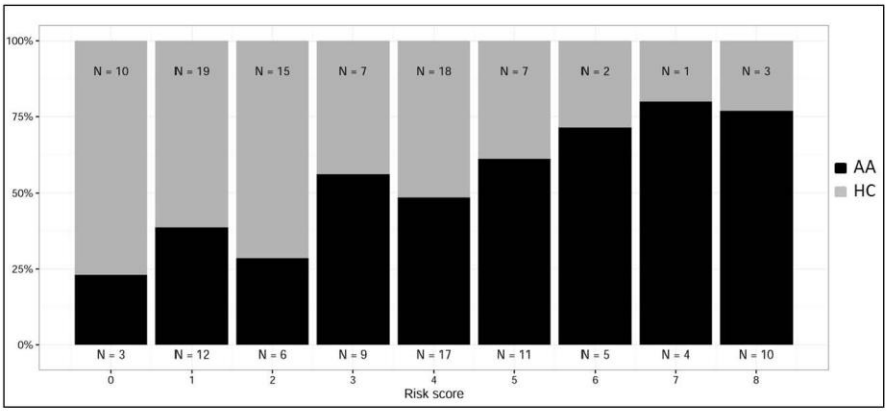

Fig.1: Distribution of AA and HC stratified for risk score

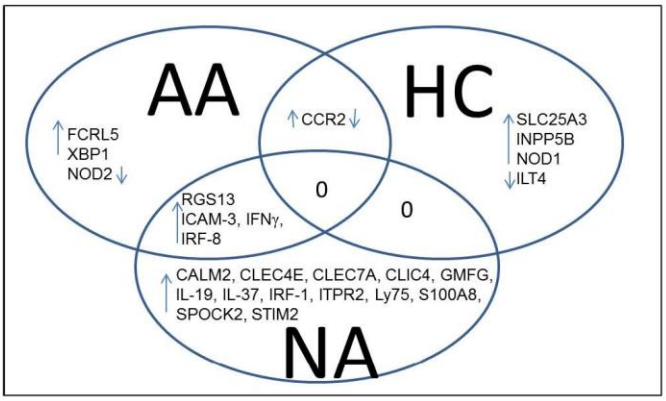

Fig.2A: Venn diagram showing genes with significant correlations to *IRF-1* risk score

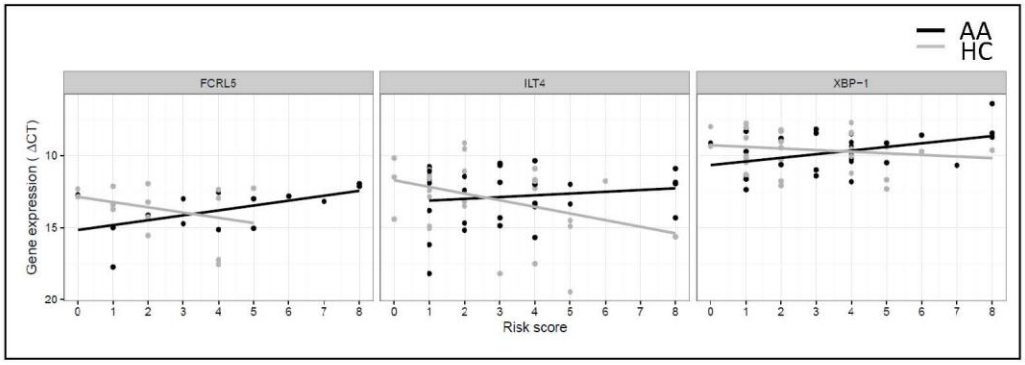

Fig.2B: Gene expression of *FCRL5, ILT4* and *XBP-1* in HC and AA with increasing risk-score: