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New insights into immunological mechanisms behind the farm effect in childhood asthma: Modulation of *ex vivo* gene expression involved in NF-κB and MAPK signalling pathways

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1 Introduction

1.1 Asthma – definition and pathology

Asthma is a complex heterogeneous disease, usually characterised by chronic airway inflammation and associated with airway hyperresponsiveness [1]. Its predominant symptoms vary over time and intensity and include wheeze, cough, shortness of breath and chest tightness as well as variable expiratory airflow limitation [1]. Different phenotypes can be defined considering timing and severity of symptoms, presence or absence of atopy, responsiveness to triggers, and characteristics of airway inflammation [2, 3]. This dissertation investigates allergic asthma which is the most common phenotype, accounting for up to 80% in children and still 40-50% in adults [4]. Allergic asthma is usually defined as the presence of allergic sensitisation together with a correlation of allergen exposure and asthma symptoms [5]. It describes an exaggerated immune response producing immunoglobulin E (IgE) antibodies against normally harmless inhaled antigens, such as pollen, mould, pet dander and dust mite [6] and is frequently associated with related atopic disorders such as hay fever, atopic eczema and (food) allergies [7]. For reasons of simplicity, in the following, the term “allergic asthma” will often be referred to only as “asthma”.

Both, the innate and the adaptive immune system play crucial roles in the development of or the protection from asthma [8]. Innate immunity is conveyed by dendritic cells (DCs), mast cells, macrophages, neutrophils, basophils, and eosinophils. These cells mediate immediate immune response through a limited repertoire of germline-encoded receptors [9]. Structural airway cells including epithelial and endothelial cells as well as fibroblasts and fibrocytes also support innate defence [8]. B and T cells are part of the adaptive immune system and provide long term memory response to antigens. Natural killer cells (NK cells) seem to have properties of both arms of immunity [9].

In asthma, normal development of the respiratory and immune system is disturbed [7]. Cells of the innate and adaptive immune system are regulated differently in asthmatic patients: mast cells, eosinophils and lymphocytes are increased in asthmatic airways [10]. Furthermore, structural changes in pulmonary epithelial and endothelial cells, fibroblasts and smooth muscle cells contribute to inflammation, remodelling and hyperresponsiveness in asthma [11]. Primary sensitisation and induction of allergic asthma are shown in **Figure 1** [12].

Upon allergen perturbation pulmonary epithelial cells release cytokines and chemokines that drive attraction and maturation of immature DCs from the bone marrow. The allergen is taken up by pattern recognition receptors on DCs and processed to small peptides. Subsequently, DCs migrate to the draining lymph nodes and present the processed allergen to T cells via the major histocompatibility complexes (MHC) class I and II [12]. This causes T cell expansion and differentiation, particularly of the Th2 type. Th2 cells then migrate to the inflammatory area and release cytokines that mediate their survival (regulated by interleukin (IL) 4), class switching of B cells to produce IgE antibodies (IL-4 and IL-13), differentiation and maturation of mast cells (IL-3, IL-9 and IL-13), survival and maturation of eosinophils (IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF)) and basophil recruitment (IL-3 and GM-CSF) [12, 13]. Plasma cells synthesise and secrete allergen specific IgE antibodies that bind to basophils and mast cells. When an allergen is subsequently inhaled by a sensitised individual, it cross links these IgE antibodies on the cell surface and initiates rapid basophil and mast cell degranulation [12, 13]. The released cytokines (IL-4, IL-5, IL-6, and IL-13), biogenic amines (serotonin and histamine), proteoglycans, serglycin, proteases and lipid mediators (e.g. leukotrienes, prostaglandins) regulate inflammatory cell recruitment, increased vascular permeability and smooth muscle constriction [13]. This phase is called the “rapid” or “initial” phase of asthma and is responsible for the typical asthma symptoms. The “late-phase reaction” follows 4-18 hours later and involves leukocytes, especially eosinophils and T cells. This may induce chronic airway inflammation and remodelling which is often difficult to treat [14].

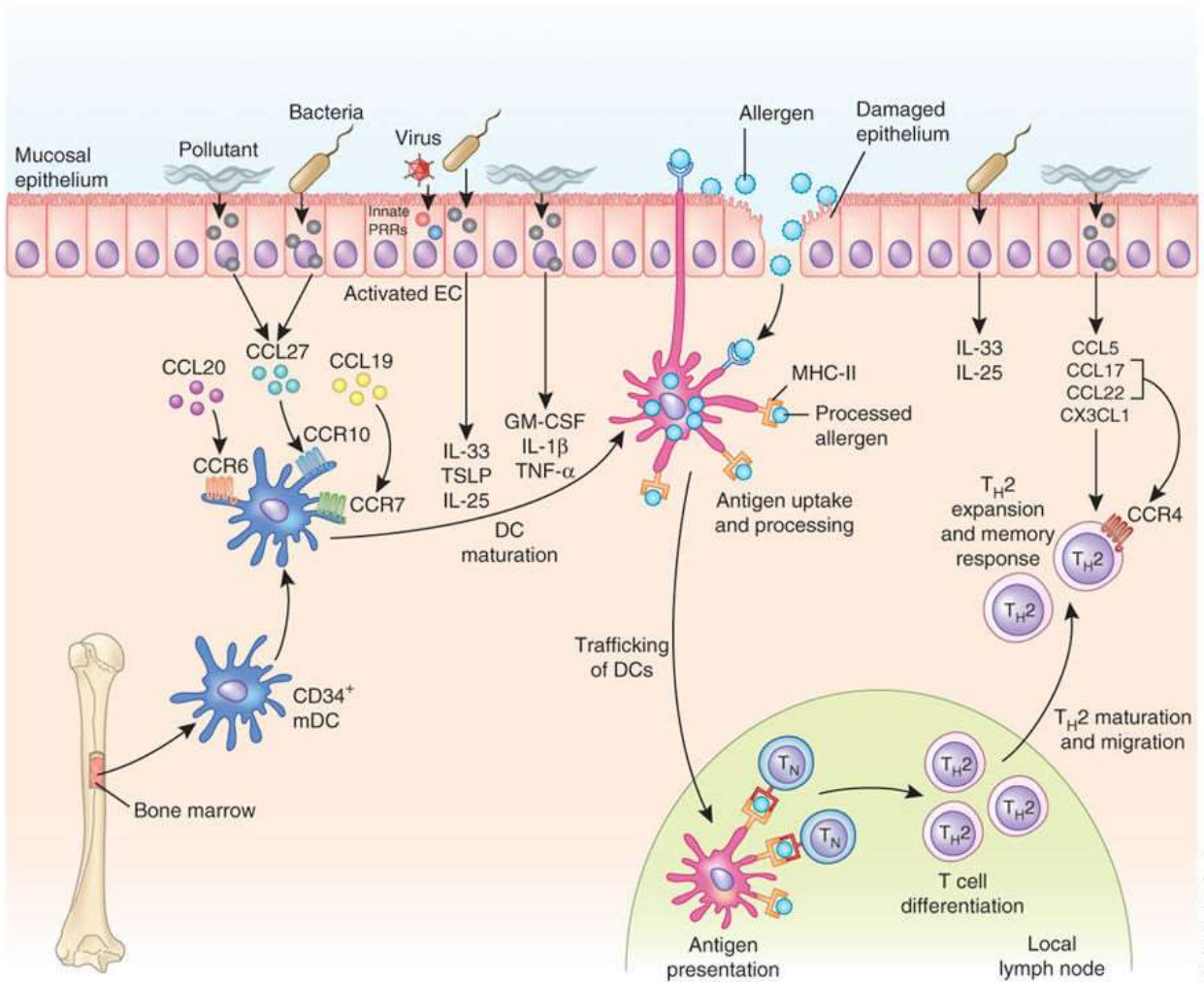

Figure 1: Primary sensitisation in the induction of allergic-type asthma [12]
Pattern recognition receptors (PRRs), epithelial cell (EC), dendritic cell (DC), cluster of differentiation 34 antigen-derived myeloid dendritic cell ($CD34^+$ mDC), major histocompatibility complex (MHC), C-C motif chemokine ligand (CCL), C-C motif chemokine receptor (CCR), C-X3-C motif chemokine ligand (CX3CL), interleukin (IL), thymic stromal lymphoprotein (TSLP), Granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor α (TNF-α), T helper 2 cell (T_H2). Figure available from [12], copyright permission granted by Copyright Clearance Center, Inc. on 18/09/2021.

1.2 The burden of asthma – prevalence and socio-economic effects

Asthma is the most common chronic disease in childhood and represents a major public health burden [15]. Globally, around 262 million people suffer from asthma [16]. In 2019, the Lancet Global Burden of Disease (GBD) Resource Centre estimated 416 000 deaths and 21.6 million disease-adjusted life years (DALY) attributable to asthma [16]. According to the Centers for Disease Control and Prevention (CDC), asthma, especially if poorly controlled, is a risk factor for school absenteeism and 49% of asthmatic children reported one or more asthma related missed school days in 2013 [17, 18]. In the same year, the United States spent an estimated amount of US$ 5.92 billion on asthma health care for school-aged

children [19]. These numbers outline the ongoing burden despite available therapeutic asthma medications and emphasise the need for preventive measures.

In Germany, representative data on childhood health are collected by the *Studie zur Gesundheit von Kindern und Jugendlichen in Deutschland* (KiGGS). KiGGS is a combined cross-sectional and longitudinal study conducted by the Robert Koch Institute since 2003. Data are collected by questionnaires, physical examinations, and laboratory tests. According to the KiGGS Wave 2 (2014-2017) 6% of all children and teenagers aged 0 to 17 years were diagnosed with asthma by a doctor. Boys were diagnosed more often than girls (7.5% vs. 4.5%). 3.5% reported asthma symptoms during the past 12 months (4.4% boys vs. 2.6% girls). Compared to the KiGGS baseline data collection (2003-2006) the prevalence remained constantly high [20].

Due to the lack of a standardised asthma definition and differences between study populations, survey periods and data collection methods used in epidemiological studies, worldwide comparison of asthma prevalence is difficult. The *International Study of Asthma and Allergies in Childhood* (ISAAC) aimed at allowing comparisons of asthma prevalence around the world by means of standardised questionnaires [21]. These were conducted in representative samples of two age groups of school children (6-7 and 13-14 years) [21]. In Phase One, a higher asthma prevalence was found in more affluent, English-speaking Western countries (approximately 15-20%) and in Latin America, and there was a Northwest-Southeast gradient in Europe and a lower prevalence in Asia and Africa [22]. While Phase Two was designed to identify determinants responsible for the observed differences in asthma prevalence [23], results from Phase One where mostly confirmed in Phase Three, but additional centres in Africa revealed a higher prevalence than previously expected [24]. **Figure 2** shows the prevalence of asthma diagnoses and asthma symptoms in children and young adults from 1965 to 2005 [25]. In most countries, the prevalence increased noticeably in the second half of the 20th century [25]. Since around the turn of the century, some countries seem to have reached a plateau or even a decrease, whereas others are still on the rise. Overall however, asthma prevalence is further increasing or remains stable [26].

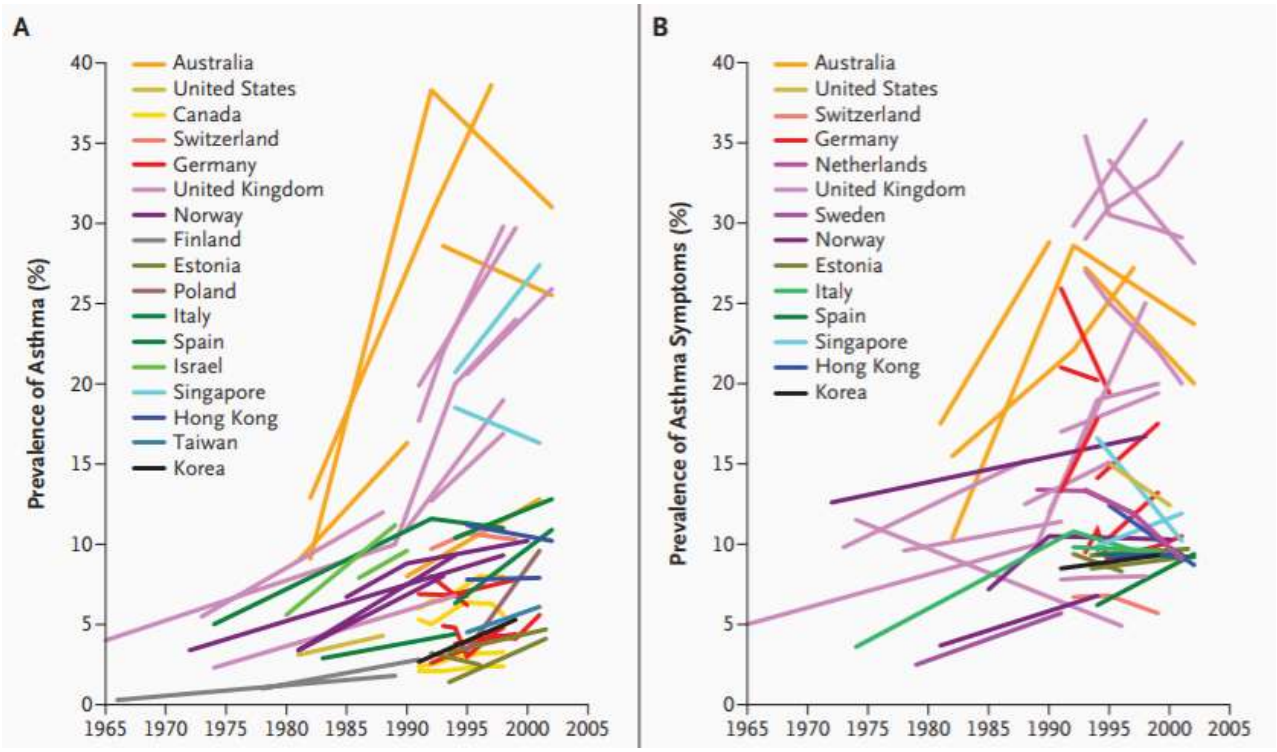

Figure 2: Changes in asthma prevalence in children and young adults [25]
A: Prevalence of diagnosed asthma, B: prevalence of asthma symptoms, Figure reproduced with permission from [25], Copyright Massachusetts Medical Society.

The causes for this temporal trend are still unclear, but the rapid changes are unlikely due to genetic factors but rather related to modifications in lifestyle and environmental exposures leading to asthma manifestation in genetically susceptible individuals. This theory is supported by epidemiological studies in populations with a similar genetic background but different living environments. Over the years there has been accumulating evidence for a lower prevalence of asthma and allergies among children growing up in rural compared to urban areas. Examples are found all over the world including the United States [27-29], Australia [30], Latin America [31], China [32-35] and several African countries such as Ethiopia [36], Kenya [37] and Ghana [38]. Likewise, a range of studies provided evidence for urbanisation and urban residence being important determinants of asthma prevalence in middle- and low-income countries [39]. Studies from Europe, Canada and Australia reported significantly lower rates of asthma and allergies in children living on traditional farms [40]. These epidemiological observations are possibly related to a complex interplay of risk and protective factors.

1.3 The protective farm effect

Farm exposure during childhood has repeatedly and consistently been associated with lower risks of asthma, hay fever and allergic sensitisation [29, 41-48]. Since more than two decades, the most effective protection is considered to be related to consumption of raw cow's milk and early contact to livestock [42, 49-51]. As shown in **Figure 3**, timing is crucial and exposure in or even before the first year of life conveys the strongest protective effects [45].

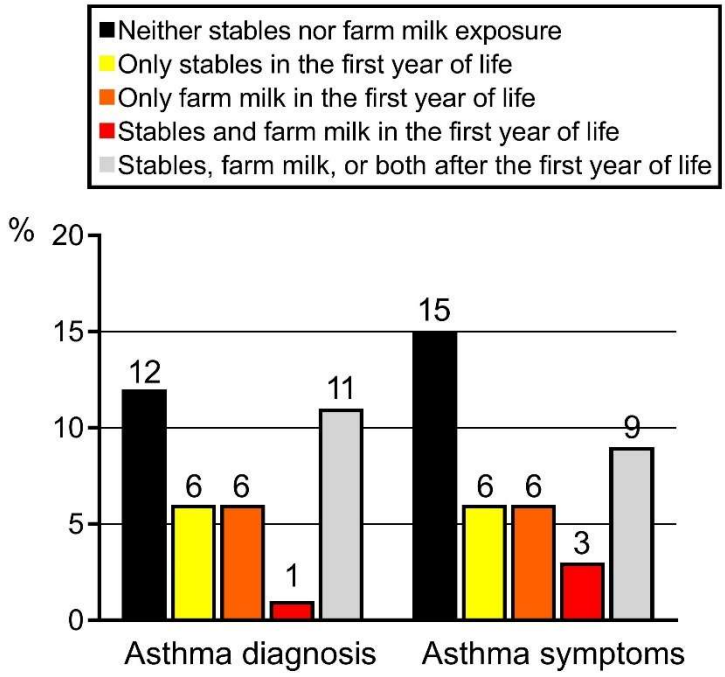

Figure 3: Lower prevalence of asthma diagnosis and symptoms associated with farm exposure
Data from a cross-sectional survey in rural areas of Austria, Germany, and Switzerland, Figure adapted from [45].

Exposure to increased endotoxin levels may account for at least some part of this protective effect [43, 52, 53]. The innate immune repertoire is shaped by the diverse and rich microbial environment found on traditional farms, and particularly inside stables. The European PASTURE/EFRAIM study investigated a prospective birth cohort recruited in rural areas in Germany, Austria, Switzerland, France, and Finland. The authors found lower rates of allergies, especially seasonal ones, in children of women who were regularly exposed to a farm environment during pregnancy [54]. The earlier the exposure, the stronger the protective effect [46] which may even last into adulthood [42].

Moreover, the phenomenon of the protective farm environment has become extremely apparent in studies comparing Amish and Hutterite communities in the United States. Both have similar ancestries and lifestyles regarding most factors associated with a modified asthma risk such as large sibship size, long duration of breast feeding and low rates of domestic and environmental pollution (including tobacco smoke). However, farming practices are distinct between the two communities: while the Amish live on small, traditional family-run dairy farms without almost any usage of additional technologies, the Hutterites practice highly industrialised large-scale farming. Asthma prevalence among the Amish was 5.2% versus 21.3% among the Hutterites, while median endotoxin levels were 6.8-fold higher within the Amish community [28]. Also, comparison of dust samples collected at the farms revealed a different microbial composition, with only the Amish dust conveying anti-inflammatory properties in an asthma mouse model [28]. Furthermore, farm living affects adaptive immunity, e.g. by protecting against IgE, IgG1 and IgG4 antibody development [42].

Differences in farming practices, livestock, and regional environmental circumstances influence the manifestation of the protective farm effect [55, 56]. In China for example, exposure to arable farming seems to be just as protective as livestock farming [34]. Also in China, keeping poultry is associated with protection against current wheeze and use of asthma medication [57] (and personal communication with Professor Gary Wong, Department of Paediatrics, The Chinese University of Hong Kong on 30/05/2018). These aspects outline the heterogeneity and complexity of the farm effect and related environmental influences on the immune system.

1.4 Linking the protective farm environment to gene regulation – Pathway modulation through environmental influences

High concordance rates in twins and typically positive family histories in atopic patients outline the important role of an individual's genetic background in asthma development [58, 59]. Various genome wide association studies (GWAS) identified certain susceptibility loci for asthma and allergies. For example, several single nucleotide polymorphisms (SNPs) on chromosome 17q21 show associations with asthma development [60, 61]. Identifying asthma related genes may help paving the way for targeted gene therapies that bear the potential for future treatment, cure or prevention options in genetically susceptible individuals [62].

As stated above, asthma heritability may only partly be explained by genetic variants. In fact, gene-environment interactions play a crucial role as various effects from (epi-)genetic background to microbial exposures, respiratory tract infections, nutrition habits and many more are part of the pathogenesis of asthma [8]. Immunological pathways involved in asthma pathogenesis can be influenced by environmental stimuli. This dissertation focuses on two of them: NF-κB and MAPK signalling pathways may convey protective or harmful properties depending on environmental influences, such as exposure to lipopolysaccharide (LPS) or smoking. Analysing several genes at once within a pathway provides the opportunity to better understand gene interactions and to identify key regulators in asthma protection.

1.4.1 NF-κB signalling – its role in asthma development and its potential in environmentally mediated protection

The nuclear factor “kappa-light-chain-enhancer” of activated B cells (NF-κB) family is one of the most important regulators of inflammation and innate and adaptive immune responses [63]. It controls the expression of genes responsible for increased production of inflammatory mediators including cytokines, chemokines and chemical mediators [64, 65] in asthma [66, 67] and other respiratory diseases such as chronic obstructive pulmonary disease (COPD) [68]. Its overexpression is involved in a range of chronic inflammatory conditions including asthma [66, 69-71], but also arthritis, atherosclerosis [72-74] and inflammatory bowel disease [75]**. In the following, genes investigated in the present study are highlighted in bold.**

The signalling occurs via two major branches, called the canonical and non-canonical pathway (see **Figure 4**). Environmental stimuli such as LPS, and other inflammatory stimuli like tumor necrosis factor-alpha (TNF-α), IL-1 or byproducts of viral and bacterial infections activate the canonical signalling via **MAP3K7** (also commonly known as transforming growth factor beta (TGF-β) activated kinase 1, TAK1). The stimuli may bind to Toll like receptor 4 (TLR4), upon which MyD88 interacts with IL-1 receptor-associated kinase family members, including **IRAK4** [76]. Upon activation, **IRAK4** phosphorylates and thereby activates IRAK1. The IRAK members then dissociate from MyD88 and interact with the RING-domain E3 ubiquitin ligase TRAF6. Together with other enzymes, TRAF6 promotes polyubiquitination of target proteins, including TRAF6 itself and NEMO leading to the recruitment of **MAP3K7** and related binding proteins [76].

MAP3K7 subsequently phosphorylates the β-subunit of an inhibitor of κB (IκB) kinase (IKK) complex, which consists of catalytic (IKKα and IKKβ) and regulatory (IKKγ, also called NF-κB essential modulator, NEMO) subunits. The IKK complex (mainly IKKβ) then phosphorylates IκB family members such as **NF-κB-inhibitor alpha** (NFKBIA, alias IκBα), the prototypical and best studied member of the IκB family. Another member of the IκB family is the IκB-like molecule p105. In non-stimulated cells, the IκB family retains NF-κB-dimers in the cytoplasm. **NFKBIA** for example associates with Rel family members (RelA or c-Rel) and p50 dimers. On the contrary, p105 associates with Rel (RelA or c-Rel) or p50. After cell stimulation and phosphorylation by IKK, **NFKBIA** and p105 are ubiquitinated and subsequently degraded via the proteasome. This exposes strong nuclear localisation signals on the previously bound NF-κB members inducing their nuclear translocation. In the nucleus, they bind to particular DNA elements (so called κB enhancers of target genes) in the form of RelA-p50, cREL-p50, p50-p50 and other dimeric complexes [77, 78]. NF-κB pathway activation eventually results in the induction of expression of pro-inflammatory genes such as **PTGS2** [79] and other pro-inflammatory mediators including chemokines, cytokines and adhesion molecules [80].

The non-canonical pathway on the other hand is regulated quite differently from the canonical pathway. It is triggered by certain members of the tumor necrosis factor receptor (TNFR) superfamily, including B cell activating factor of the TNF family (BAFF) or lymphotoxin-β via the activation of **MAP3K14** (also commonly known as NF-κB-inducing kinase, NIK). **MAP3K14** phosphorylates and thereby activates IKKα. IKKα thereupon phosphorylates specific p100 carboxy-terminal serine residues. This causes the selective degradation of the C-terminal IκB-like structure of p100 resulting in the release and nuclear translocation of the N-terminal p52 fragment bound to RelB [77, 78].

Cellular inhibitors of apoptosis proteins (cIAP) such as BIRC2 and **BIRC3** (also known as cIAP2) are crucial regulators of pro-survival NF-κB signalling [81]. While they enhance canonical pathway activation by ubiquitylation of **RIPK1**, they suppress constitutive activation of noncanonical NF-κB signalling [81, 82]. Hence, overexpression as well as loss of **BIRC3** and other inhibitor of apoptosis related targets can result in deregulated NF-κB activation [82].

The canonical cascade typically mediates the rapid and reversible inflammatory immune response, whereas the non-canonical cascade regulates the slower and irreversible developmental response [83]. Signalling via either branch is flexible, and interactions between the two occur in different cell types and situations [84].

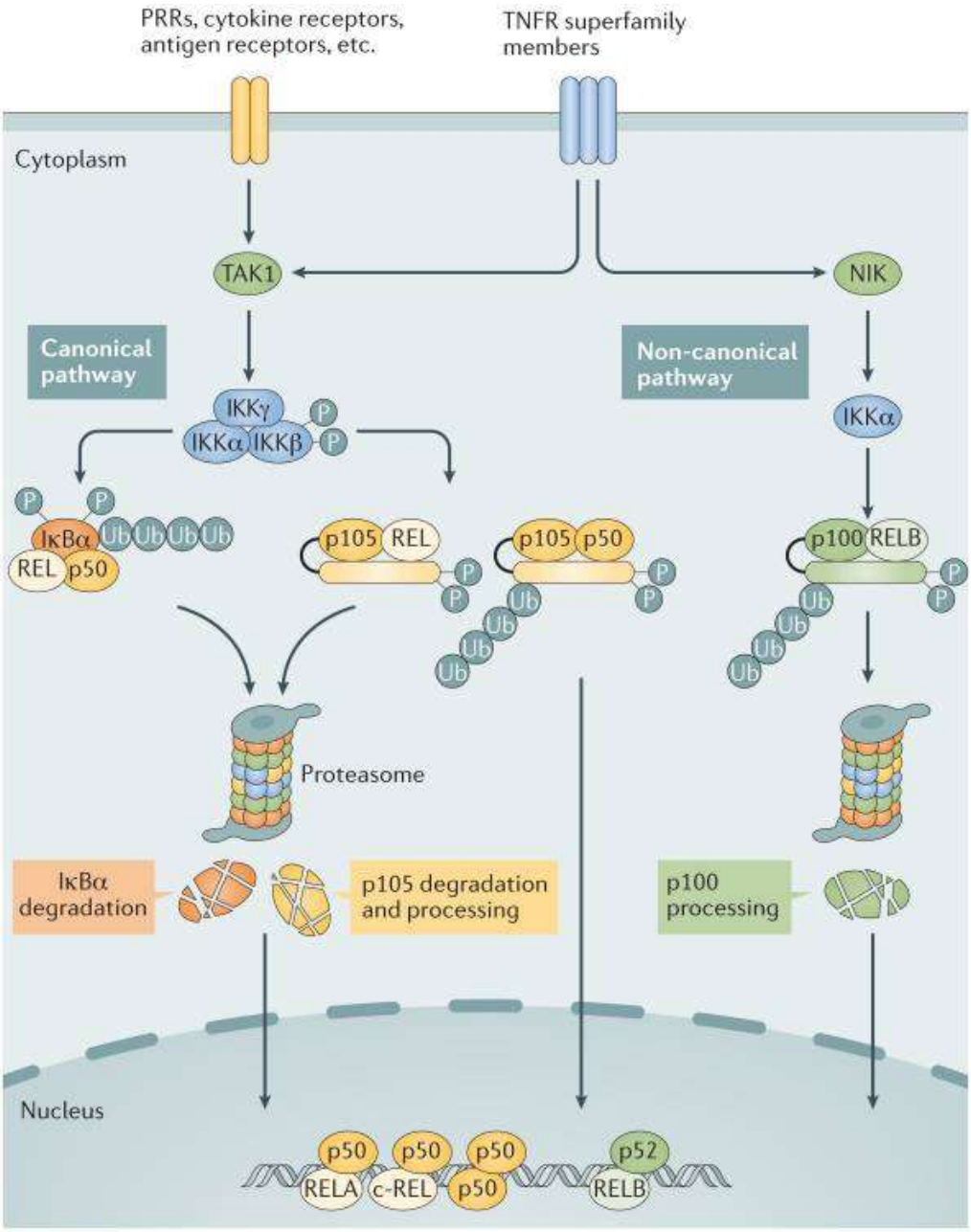

Figure 4: Overview of canonical and non-canonical NF-κB signalling [78]
Pattern recognition receptors (PPRs), tumor necrosis factor receptor (TNFR), TGFβ -activated kinase 1 (TAK1 = **MAP3K7**), NF-κB-inducing kinase (NIK = **MAP3K14**), IκB kinase (IKK), inhibitor of κB (IκBa = **NFKBIA**). Phosphorylation (p), ubiquitylation (Ub). Members of the NF-κB transcription factor family: p50, p100, p105, REL, RELA, RELB, c-REL. Figure available from [78], copyright permission granted by Copyright Clearance Center, Inc. on 18/09/2021.

NF-κB activity is increased in asthmatics and plays a key role in asthma development and severity [69-71]. Some *in vivo* studies showed an increased activation of NF-κB in inflammatory cells and airway tissue upon intranasal challenge with allergen, endotoxin, or microbial infection [68]. Allergen-induced airway inflammation was attenuated in recombinant mice deficient in certain intermediates of NF-κB signalling such as c-Rel and **NFKBIA** [68].

Tumor necrosis factor alpha induced protein 3 (TNFAIP3, commonly known as A20) is a key negative regulator of NF-κB. A murine allergic asthma model showed that farm dust exposure provided asthma protection via upregulation of TNFAIP3 in lung epithelial cells [85]. A study comparing Amish and Hutterite school children revealed that TNFAIP3 was more highly expressed among the Amish, while prevalence of asthma and allergic sensitisation was significantly lower [28]. In another murine asthma model, intranasal instillation of Amish farm dust extracts significantly inhibited airway eosinophilia and hyperreactivity and lowered specific IgE levels [28].

The present study built on previous findings from the Schaub research group underlying the role of TNFAIP3 as a central player in childhood asthma development and environmentally mediated protection [86, 87]. Urban asthmatic children showed significantly lower gene and protein expression levels of anti-inflammatory TNFAIP3 than healthy controls, whilst their expression of pro-inflammatory TLR4 was significantly higher. By stimulating peripheral blood mononuclear cells (PBMCs) *ex vivo* with German and Finnish farm dusts or LPS, TNFAIP3 expression in asthmatics was raised to the level of healthy controls. Also, other gene expression levels associated with NF-κB signalling shifted towards an anti-inflammatory state. In addition, cord blood samples of newborns that suffered from asthma at school age already expressed lower TNFAIP3 levels at birth. TNFAIP3 could therefore serve as a potential biomarker to predict asthma development [86, 87].

Table 1 gives an overview of the NF-κB related pathway genes investigated in the present study. Their relevance regarding environmentally mediated asthma protection by means of the farm effect is outlined in detail in the discussion (see section **5.2**). Of note, **MAP3K7** activates both NF-κB and MAPK signalling and is involved in innate and adaptive immune responses [88]. Its key function is to initiate immune responses downstream of TLRs, mainly through MyD88, the TLR/IL-1 superfamily adapter protein [89]. Considering its involvement in the MAPK pathway and given the similar regulatory effects compared to the other investigated pro-inflammatory MAPKs found in this study, it is analysed and discussed in closer context to MAPK related signalling. **Table 1** additionally gives an overview of

alternative gene names commonly used in scientific literature. In this dissertation, the gene symbols approved by the HUGO Gene Nomenclature Committee (HGNC) were used. The HGNC approves unique names and symbols for human loci, including protein coding genes, non-coding RNA genes and pseudogenes, to enable unambiguous scientific communication [90].

Gene symbol	Gene name	Also known as
MAP3K14	Mitogen activated protein kinase kinase kinase 14	NIK (NF-κB-inducing kinase)
RIPK1	Receptor interacting serine/threonine kinase 1	RIP
PTGS2	Prostaglandin-endoperoxide synthase 2	COX2 (cyclooxygenase 2)
IRAK4	Interleukin 1 receptor associated kinase 4	NY-REN-64
BIRC3	Baculoviral IAP repeat containing 3	cIAP2 (cellular inhibitor of apoptosis 2)
NFKBIA	NF-κB inhibitor alpha	IKBA, IκBα

Table 1: Gene symbols and common aliases, NF-κB pathway
[a] Approved by the HUGO Gene Nomenclature Committee (HGNC) [90]

Since not all NF-κB-related genes investigated in this study are displayed in **Figure 4**, **Figure 5** provides a more detailed overview of the NF-κB pathway. Genes investigated in this study are indicated by black rectangles. The figure shows the complex interactions and multiple pathway modulations by phosphorylation, ubiquitination, and other regulatory processes. It also illustrates that several of the investigated genes are involved at multiple sites of the pathway.

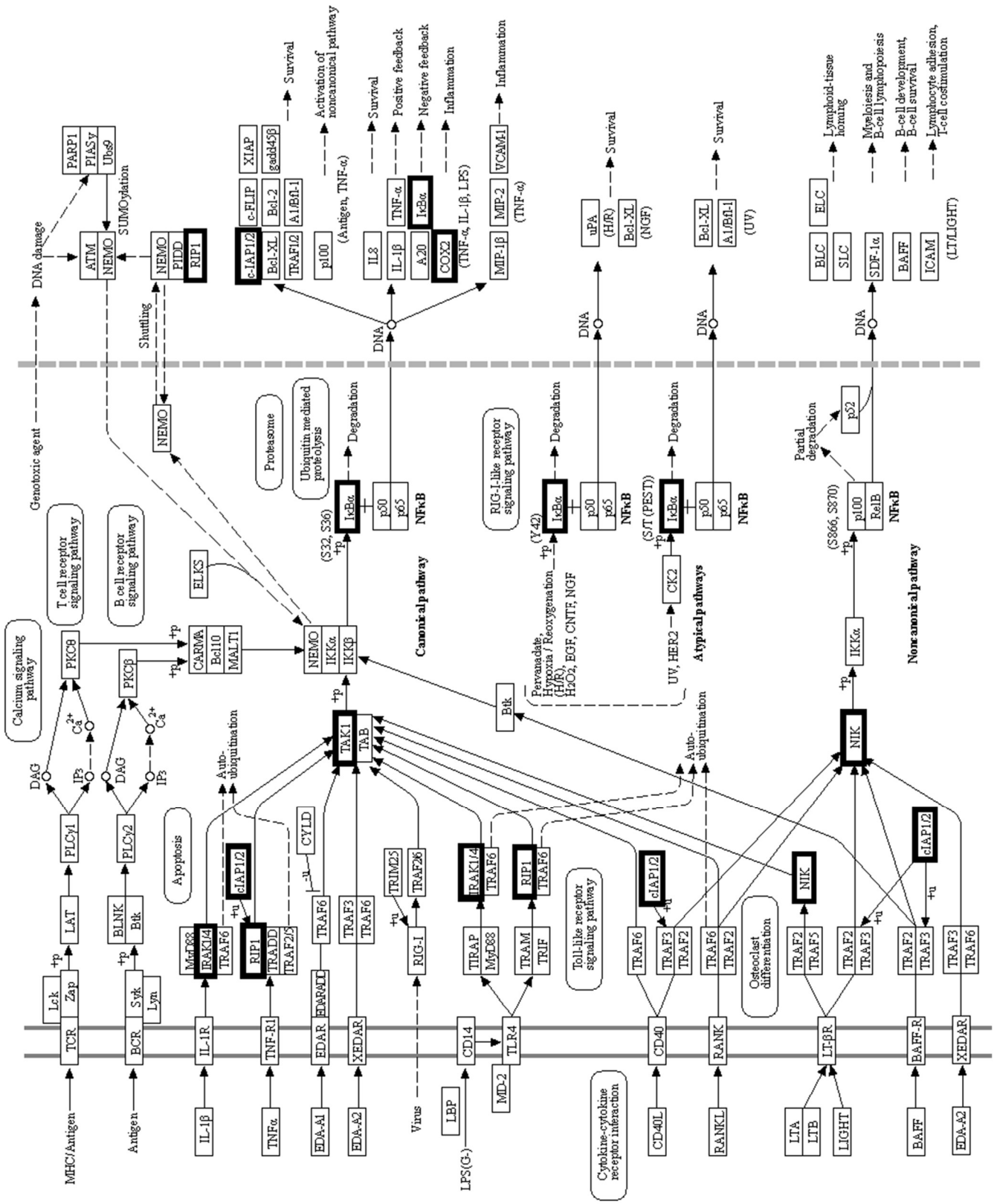

Figure 5: Detailed overview of the NF-κB signalling pathway and of investigated genes [91-94]
Black rectangles indicate the genes investigated in this study (modified by the author): mitogen-activated protein kinase kinase kinase 14 (**MAP3K14**, NIK), mitogen-activated protein kinase kinase kinase 7 (**MAP3K7**, TAK1), receptor interacting serine/threonine kinase 1 (**RIPK1**, RIP1), prostaglandin-endoperoxide synthase 2 (**PTGS2**, COX2), interleukin 1 receptor associated kinase 4 (**IRAK4**), baculoviral IAP repeat containing 3 (**BIRC3**, cIAP2), NF-κB inhibitor alpha (**NFKBIA**, IκBα). Lipopolysaccharide (LPS), gram-negative bacteria (G-), ubiquitylation (u), phosphorylation (p). For full list of abbreviations please see section **9**. Figure available and adapted from: [91-94]. Copyright permission granted by Kanehisa Laboratories on 21/09/2021.

1.4.2 MAPK signalling in asthma

Mitogen-activated protein kinases (MAPKs) are cytoplasmic serine/threonine protein kinases that are expressed in all eukaryotic cells [95]. They are involved in a variety of cellular functions such as cell differentiation, proliferation, metabolism, mobility, survival and apoptosis [95]. In humans, the MAPK extracellular signal-related kinases **MAPK1**/3 (also known as ERK2/1), **MAPK**8/**9**/10 (also known as c-Jun amino-terminal kinases (JNK1/2/3)), **MAPK14** proteins (also known as p38) and **MAPK7** (also known as ERK5) represent four distinctly regulated central hubs in the signalling cascade. Even though they differ in some characteristics their ultimate function is to transfer environmental stimuli to the nucleus through phosphorylation cascades [96]. These processes are controlled on several levels by feedback mechanisms including direct post-translational modification of various inflammatory targets and induction of *de novo* gene expression of inhibitors like MAPK phosphatases and sprouty proteins [97]. **DUSP1** is important in the complex regulation of MAPK signalling. It is the archetype of dual-specificity phosphatases (DUSP) and deactivates MAPKs through dephosphorylation of the tyrosine and threonine residues within the MAPK activation motif [98].

MAPKs are involved in immune responses and are proposed to play important roles in the pathogenesis of asthma [99]. Epithelial cells from asthmatic subjects show an increased expression of the phosphorylation of **MAPK1**/3 and **MAPK14** [100] in correlation with asthma severity [101]. This suggests that MAPK signalling is associated with airway hyperresponsiveness and asthma exacerbations [102]. T cells isolated from asthmatics express higher levels of phosphorylated **MAPK1**/3 both at baseline and in response to anti-CD3 antibodies [103]. In addition, enhanced activation of the MAPK signalling pathway seems to be involved in a stimulus-dependent manner in steroid-insensitive asthma, making it a promising target for asthma treatment and prevention [104]. In a murine model of asthma, the inhibition of **MAPK14** or NF-κB activation with respective inhibitors attenuated allergen-induced bronchial hyperresponsiveness and airway inflammation [105]. Also, a range of *in vitro* and *in vivo* studies have shown that respiratory medicines such as corticosteroids, β2-agonists and phosphodiesterase inhibitors induce the negative MAPK regulator **DUSP1** [106]. Yet, the role of MAPK signalling in the development of childhood asthma is still unclear.

MAPK1/3 [107] and **MAPK**8/**9** cascades activate NF-κB signalling in human airway epithelial cells and monocytes [108], indicating the potential for crosstalk between these pathways [109]. Also, as mentioned earlier, **MAP3K14**, **MAP3K7** are involved in both pathways.

In line with **Table 1** (see section **1.4.1**), **Table 2** shows the MAPK gene symbols used in this dissertation and common aliases. Again, the detailed functions of the genes investigated in this dissertation are outlined in section **5.2**.

Gene symbol[a]	Gene name	Also known as
MAP3K7	Mitogen-activated protein kinase kinase kinase 7	TAK1 (TGF-beta activated kinase 1)
MAPK1	Mitogen-activated protein kinase 1	ERK2 (extracellular signal-regulated kinase 2)
MAPK9	Mitogen-activated protein kinase 9	JNK2 (c-Jun N-terminal kinase 2)
MAPK14	Mitogen-activated protein kinase 14	p38
MAPK7	Mitogen-activated protein kinase 7	ERK5 (extracellular signal-regulated kinase 5), BMK1 (big MAP kinase 1)
DUSP1	Dual specificity phosphatase 1	MKP-1 (Mitogen-activated protein kinase phosphatase 1)

Table 2: Gene symbols and common aliases, MAPK pathway
[a] Approved by the HUGO Gene Nomenclature Committee (HGNC) [90]

Figure 6 gives an overview of the MAPK pathway. Genes investigated in this study are marked by black rectangles.

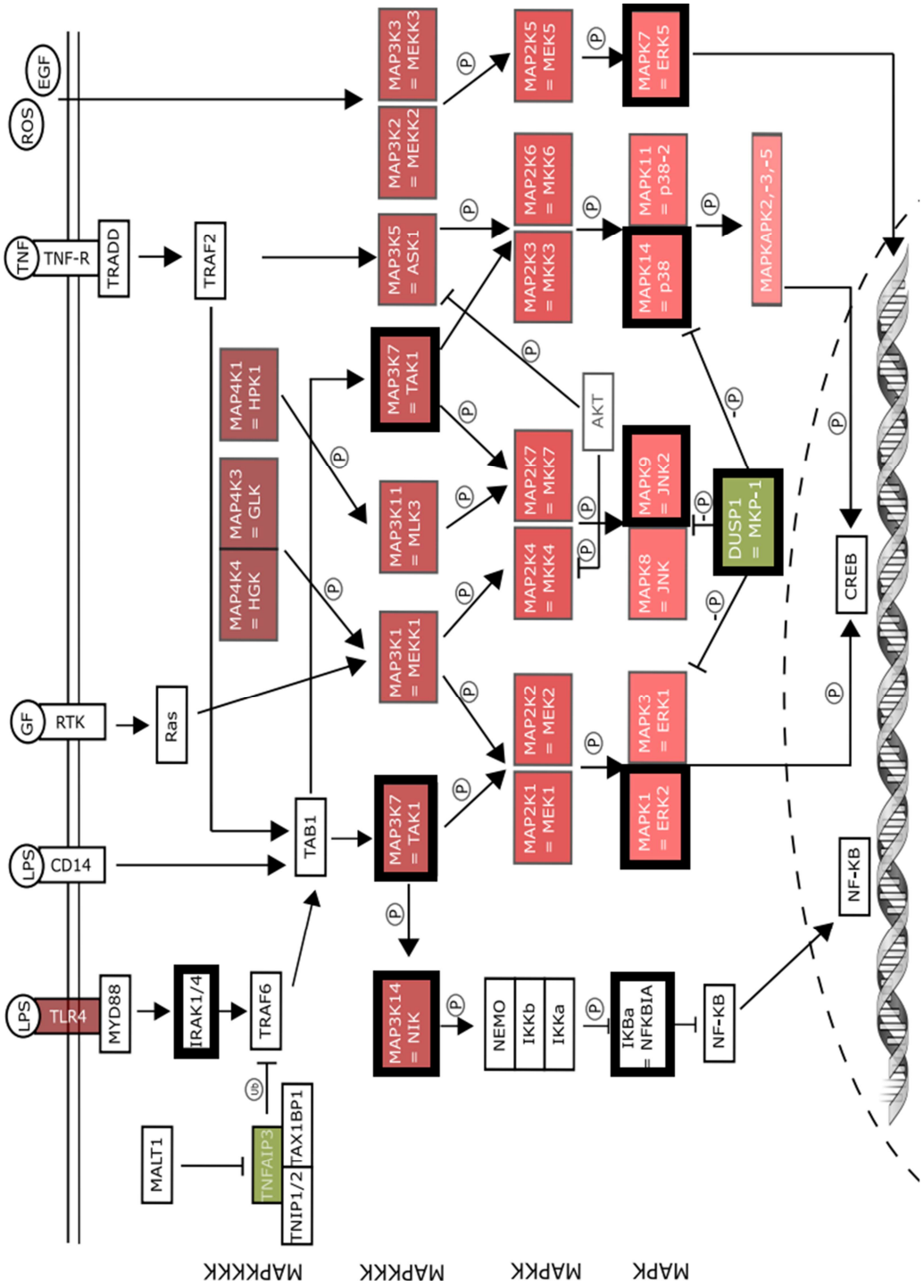

Figure 6: LPS stimulated MAPK signalling [110]
Overview of MAPK signalling pathway and part of its interaction with the NF-kB pathway. Pro-inflammatory targets like TLR4 and MAPK are labelled in red, anti-inflammatory **DUSP1** and TNFAIP3 labelled in green. Black rectangles indicate the genes investigated in this study (modified by the author): interleukin-1 receptor-associated kinase 4 (**IRAK4),** mitogen-activated protein kinase kinase kinase 14 (**MAP3K14**), mitogen-activated protein kinase kinase kinase 7 (**MAP3K7**), mitogen-activated protein kinase 1 (**MAPK1**), mitogen-activated protein kinase 9 (**MAPK9**), mitogen-activated protein kinase 14 (**MAPK14**), mitogen-activated protein kinase 7 (**MAPK7**), dual specificity phosphatase 1 (**DUSP1**), NF-κB inhibitor alpha (**NFKBIA**). Lipopolysaccharide (LPS), growth factor (GF), Tumor necrosis factor (TNF), reactive oxygen species (ROS), epidermal growth factor (EGF), ubiquitylation (Ub), phosphorylation (P). For full list of abbreviations please see section **9**. Figure designed by Johanna Theodorou, available and adapted from [110] (Supplementary Figure S2), copyright permission granted by Johanna Theodorou on 18/07/2021 and by Copyright Clearance Center, Inc. on 18/09/2021.

2 Objectives

Many studies have focused on treatment of asthma and related atopic disorders and a step-by-step guide for treatment is well established in clinical practice. Therapeutic methods however are mostly symptomatic and causal approaches besides immunotherapy do not really exist. The current burden of asthma and its increasing prevalence call for new therapeutic as well as preventive strategies. While there is no doubt on the existence of the protective farm effect, its underlying immunological mechanisms are only partly understood. In order to translate asthma protection by means of the farm effect into everyday life (e.g. by using modulated dust-extracts for treatment or prevention purposes), genes and pathways susceptible to the immunomodulating properties of farm dust need to be identified. Enhancing immune tolerance may thus serve as a new strategy to treat or even prevent asthma and related allergic conditions.

As explained above, the NF-κB and MAPK cascades are key pathways in immunity and are proposed to play important roles in the pathogenesis of asthma and allergies. The aim of this research project was to investigate their potential contribution in the environmentally mediated protection from childhood asthma. Additionally, this study investigated three dust samples from Chinese farms. Thus, peripheral blood mononuclear cells (PBMCs) were stimulated with six different asthma protective dusts from different environmental areas around the world to assess the following key questions: Do the baseline expression levels of genes involved in NF-κB and MAPK signalling differ in PBMCs between steroid-naïve asthmatic and healthy children? And can NF-κB and MAPK related gene expressions be modulated by exposure to different farm dust extracts *ex vivo*?

The hypotheses were as follows:

1. **NF-κB and MAPK pathway genes show different baseline expression levels in PBMCs of allergic asthmatic versus healthy children with generally higher pro-inflammatory expression profiles among asthmatics.**
2. ***Ex vivo* exposure to "asthma protective" farm dust extracts and LPS reduces expression of pro-inflammatory genes, while it induces expression of anti-inflammatory genes and genes involved in innate immunity.**

3 Materials and methods

The materials and methods were used and applied according to the standard operating procedures of the research group “Allergy and Immunology” headed by Professor Bianca Schaub. Parts of the materials and methods used in the present study were already described by previous members of the research group [86, 111, 112]. These were adapted as follows.

3.1 Materials

3.1.1 Chemicals and reagents

Substance	**Provider**
100 bp DNA ladder	New England BioLabs, Ipswich, Massachusetts, USA
2-Mercaptoethanol	Sigma-Aldrich, St. Louis, Missouri, USA
ACK (Ammonium-Chloride-Potassium) Lysing Buffer	Lonza, Basel, Switzerland
Anti-CD28 antibody	Thermo Fisher Scientific, eBioscience™, Waltham, Massachusetts, USA
Anti-CD3 antibody (OKT3)	Thermo Fisher Scientific, eBioscience™, Waltham, Massachusetts, USA
Aqua ad iniectabilia	Braun, Melsungen, Germany
Biozym LE Agarose	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Boric acid	Sigma-Aldrich, St. Louis, Missouri, USA
Bromphenol blue	Carl Roth, Karlsruhe, Germany
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (PIC)	Merck KGaA, Darmstadt, Germany
diH_2O (deionised water)	Milli-Q® water purification system, Merck KGaA, Darmstadt, Germany

EDTA 0.5 M (Ethylenediaminetetraacetic acid)	Sigma-Aldrich, St. Louis, Missouri, USA
Ethanol 100%	Sigma-Aldrich, St. Louis, Missouri, USA
Ethidiumbromide (10 mg/ml)	Bio-Rad, Hercules, California, USA
Ficoll-Paque™ PLUS solution	GE Healthcare, Chicago, Illinois, USA
Glycerol	Sigma-Aldrich, St. Louis, Missouri, USA
Liquid nitrogen	Linde Gas, Pullach, Germany
LPS of the Escherichia coli serotype O111:B4	Sigma-Aldrich, St. Louis, Missouri, USA
Nuclease-free water	Qiagen, Venlo, Netherlands
PBS (Phosphate-Buffered Saline)	Thermo Fisher Scientific, Gibco™, Waltham, Massachusetts, USA
Primers	Thermo Fisher Scientific, Invitrogen™, Waltham, Massachusetts, USA
RIPA Lysis and Extraction Buffer	Thermo Fisher Scientific, Waltham, Massachusetts, USA
RPMI 1640 Medium + GlutaMax™	Thermo Fisher Scientific, Gibco™, Waltham, Massachusetts, USA
Sodium chloride, NaCl	Carl Roth, Karlsruhe, Germany
Ssco Advanced™ Universal SYBR® Green Supermix	Bio-Rad, Hercules, California, USA
Trizma®-Base	Merck KGaA, Darmstadt, Germany
Trypan Blue solution 0.4%	Sigma-Aldrich, St. Louis, Missouri, USA
X-VIVO™ 15 Medium	Lonza, Basel, Switzerland
Xylene cyanol	Merck KGaA, Darmstadt, Germany

3.1.2 Buffers and solutions

Substance	Composition
5X TBE buffer	54 g Trizma®-Base 27.5 g boric acid 20 ml 0.5 M EDTA (pH 8.0) 1 l H_2O bidest
DNA ladder	10 µl 100 bp DNA ladder 80 µl 0.5x TBE-Buffer 10 µl loading dye diluted solution
Ethidiumbromide (500 µg/ml)	100 µl ethidiumbromide 1.9 ml H_2O
Loading dye stock solution	0.25 g bromphenol blue 0.25 g xylene cyanol 30% glycerol 70 ml dH_2O
Loading dye diluted solution	5 ml loading dye stock solution 13.5 ml glycerol 31.5 ml dH_2O

3.1.3 Safety and reagent kits

Kit	Provider
AllergyScreen®	MEDIWISS Analytic GmbH, Moers, Germany
Cryo-Protection® Safety Kit	Tempshield®, Mount Desert, Maine, USA
RNeasy® Mini Kit	Qiagen, Venlo, Netherlands
QuantiTect® Reverse Transcription Kit	Qiagen, Venlo, Netherlands

3.1.4 Disposables

Disposable	Provider
24-well flat bottom cell culture plates, Primaria™	Corning, Corning, New York, USA
96-well skirted PCR plates, low-profile, Hard-Shell®	Bio-Rad, Hercules, California, USA
Adhesive seals, Microseal® 'B'	Bio-Rad, Hercules, California, USA
Aluminum foil	Quickpack, Renningen, Germany
Blood collecting tubes Vacutainer®	BD, Franklin Lakes, New Jersey, USA
Blood collection set, Vacutainer® Safety-Lok™	BD, Franklin Lakes, New Jersey, USA
Borosilicate glass tubes, pyrogen free	Lonza, Basel, Switzerland
Conical centrifuge tubes Falcon® (50 ml, 15 ml)	Corning, Corning, New York, USA
Cryogenic vial (2.0 ml) Corning®	Corning, Corning, New York, USA
Filter tips 100 ml, low binding SafeSeal®	Biozym Scientific, Hessisch Oldendorf, Germany
Filter tips 100-1000 µl SafeGuard	Peqlab, Erlangen, Germany
Leucosep™ centrifuge tubes (12 ml)	Greiner Bio-One, Kremsmünster, Austria
Microcentifuge tubes (1.5 ml)	Carl Roth, Karlsruhe, Germany
Microcentrifuge tubes Safe-Lock (2.0 ml)	Eppendorf, Hamburg, Germany
Microcentrifuge tubes Multiply® -µStrip Pro 8 (0.2 ml)	Sarstedt, Nümbrecht, Germany
Pasteur pipettes, sterile	VWR International, Radnor, Pennsylvania, USA
Parafilm® M laboratory film, PM-996	Bemis Company, Neenah, Wisconsin, USA
Serological pipettes (2 ml, 5 ml, 10 ml, 50 ml)	Sarstedt, Nümbrecht, Germany

S-monovette, EDTA, 1.2 ml	Sarstedt, Nümbrecht, Germany
S-monovette, Serum, 2.7 ml	Sarstedt, Nümbrecht, Germany

3.1.5 Laboratory equipment

Equipment	**Provider**
Balance Explorer® Analytical	OHAUS, Parsippany, New Jersey, USA
Bio-Plex® 200 System	Bio-Rad, Hercules, USA
Bucket 5 l	Jokey SE, Wipperfürth, Germany
CFX96 Touch™ Real-time PCR Detection System	Bio-Rad, Hercules, California, USA
Centrifuge 5417 R	Eppendorf, Hamburg, Germany
Centrifuge 5810 R	Eppendorf, Hamburg, Germany
Centrifuge Jouan CR412	Jouan, Thermo Fisher Scientific™, Waltham, Massachusetts, USA
Closure clips, Spectra/Por®	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Cryo/freezer boxes	SP Bel-Art, Wayne, USA
Dialysis membrane, ZelluTrans, 45 mm	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
DURAN® Beakers (400 ml, 1 l, 3 l)	Schott AG, Mitterteich, Germany
Electrophoresis power supply	VWR International, Radnor, USA
Freeze Dryer Lyovac® GT2	GEA, Düsseldorf, Germany
Gel iX Imager	Intas Science Images Instruments, Göttingen, Germany
Headspace vial 20 ml	Macherey Nagel GmbH & Co. KG, Düren, Germany
Incubator HERAcell® 240	Heraeus, Hanau, Germany

Magnetic stirrer IKA RCT	IKA®-Werke GmbH & Co. KG, Staufen, Germany
Magnetic stirring bars	IKA®-Werke GmbH & Co. KG, Staufen, Germany
Measuring cylinder 500 ml	Vitlab GmbH, Grossostheim, Germany
Measuring funnel	Vitlab GmbH, Grossostheim, Germany
Micro Centrifuge II	neoLab Migge, Heidelberg, Germany
Microplate centrifuge Perfect-Spin P	Peqlab Biotechnologies, Erlangen, Germany
Microscope Axiovert 40C	Zeiss, Göttingen, Germany
Neubauer counting chamber, depth 0.1 mm	Glaswarenfabrik Karl Hecht GmbH & Co. KG, Assistent®, Sondheim von der Rhön, Germany
Owl™ D3-14 wide gel electrophoresis system	Thermo Fisher Scientific, Thermo Scientific™, Waltham, USA
PCR Thermocycler PeqSTAR 96 universal	Peqlab Biotechnologies, Erlangen, Germany
Pipette controller Easypet®	Eppendorf, Hamburg, Germany
Pipette Eppendorf Research® plus 0.1-2.5 µl	Eppendorf, Hamburg, Germany
Pipette Eppendorf Research® plus 0.5-10 µl	Eppendorf, Hamburg, Germany
Pipette Eppendorf Research® plus 10-100 µl	Eppendorf, Hamburg, Germany
Pipette Eppendorf Research® plus 2-20 µl	Eppendorf, Hamburg, Germany
8-channel pipettor Research® plus 10-100 µl	Eppendorf, Hamburg, Germany
Round-bottom flasks DURAN®	Schott AG, Mainz, Germany

Scalpel, sterile	neoLab Migge GmbH, Heidelberg, Germany
Spectrophotometer NanoDrop™ 2000	Thermo Fisher Scientific, Thermo Scientific™, Waltham, Massachusetts, USA
Sterile Vacuum Filter Units, Merck Millipore Stericup™	Thermo Fisher Scientific, Schwerte, Germany
Tweezers	Unknown
Vacuum Pump Millivac™	Millipore®, Merck Millipore, Darmstadt, Germany
Vortex shaker VF2	Janke&Kunkel, IKA®, Staufen, Germany

3.1.6 Software

Software	**Provider**
EndNote X8	ISI ResearchSoft, Berkeley, California, USA
Ensembl Genome Browser	http://www.ensembl.org/
GraphPad Prism 9	GraphPad Software, San Diego, California, USA
GraphPad QuickCalcs Web site	http://www.graphpad.com/quickcalcs/contingency1/ (accessed 01-06/2021)
National Center for Biotechnology Information (NCBI)	http://www.ncbi.nlm.nih.gov/
Primer-BLAST designing tool	https://www.ncbi.nlm.nih.gov/tools/primer-blast/, National Center for Biotechnology (NCBI), Bethesda, Maryland, USA

R software Version 3.6.0	R Core Team (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. www.R-project.org
SPSS Version 25	SPSS IBM Inc., Armonk, New York, USA
Vector NTI software Version 10 Advance 11.5	Thermo Fisher Scientific, Invitrogen™, Waltham, Massachusetts, USA

3.1.7 Primer sequences

Primers were designed using the NCBI primer designing tool *Primer-BLAST.* For selection criteria please see section **3.10**.

Gene	Forward sequence (5' → 3' on plus strand)	Reverse sequence (5' → 3' on minus strand)
18S	AGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC
MAP3K14	CTTGGTTGGGGAGATCGGC	TCCTTGGCTTTGGGGAGTTC
RIPK1	CCGACATTTCCTGGCATTGA	ACTGCATTCTCTTCACAACTGC
IRAK4	TCTCTTGCTTGGATGGTACTC	AATTGATGCCATTAGCTGCA
PTGS2	TGGCTACAAAAGCTGGGAAG	ACTCACCTTTGACACCCAAG
BIRC3	GCGGGTTTTTATTATGTGGGTAA	TGACGGATGAACTCCTGTCC
NFKBIA	AATGCTCAGGAGCCCTGTAA	CTGTTGACATCAGCCCCACA
MAP3K7	TGTAGAGCTTCGGCAGTTATCC	ATAAAGAGCCCCCTTCAGCA
MAPK1	GATCTCAAGATCTGTGACTTTGG	CACATATTCTGTCAGGAACCC
MAPK9	CATAGAGATTTGAAGCCTAGCA	TGACCAGATATCAACGTTCTC
MAPK14	ACCAGACAGTTGATATTTGGTCAG	TGGGGTTCCAACGAGTCTTA
MAPK7	ACCAGTCTTTCGACATGGG	GACTCAATATCGGCAGGGT
DUSP1	CTCAAAGGAGGATACGAAGCG	CCCTGATCGTAGAGTGGGGT

Table 3: Primer sequences

3.2 Recruitment

3.2.1 Ethics approval of the study, entry criteria and asthma questionnaire

Within the scope of the Clinical Asthma Research Association Study (CLARA/CLAUS-Study) allergic asthmatic and healthy children aged 4-14 years were recruited. The study is registered at the German Clinical Trials Register (DRKS, https://www.drks.de/drks_web/, trial ID: DRKS00004635). It is approved by the Ethics Committee of LMU Munich (Ethics

Committee No.: 379-08). Allergic asthmatics were recruited in the *Christiane-Herzog* outpatient department of the *Dr. von Haunersches Children Hospital* and in the pediatric practice *Kinderarztpraxis am Arabellapark,* Munich. Healthy control subjects were mainly recruited in the surgery outpatient clinic of the *Dr. von Haunersches Children Hospital*. Informed oral and written consent were obtained from the parents and exclusion criteria were inquired before performing blood collection. Questions were based on the systematic and standardised ISAAC questionnaire [113, 114] and recorded a detailed history of asthma, atopy, and allergic diseases, use of medication, family history and important socio-demographic data (see appendix, section **10.4**). **Table 4** gives an overview of inclusion and exclusion criteria for healthy controls and allergic asthmatics respectively.

Allergic asthma was diagnosed after thorough clinical examination and in consideration of laboratory and lung function data according to the German *Nationale Versorgungsleitlinie* (NVL) *Asthma* [115] and the international *Global Initiative for Asthma* (GINA) guidelines [1].

The author recruited 25 children within this research project, 19 of them met the inclusion criteria. Six children were excluded based on in- and exclusion criteria. To obtain a total sample size of 58 children (34 healthy and 24 allergic asthmatics), an additional 39 samples of children recruited prior to this study were selected. The selection was based on a case-control design and was therefore not random.

3.2.2 Lung function

Spirometry and body plethysmography were performed in all asthmatic children by the outpatient clinic or practice staff. At least two doctors made a diagnosis whether the children had significant reversible airflow obstruction according to the American Thoracic Society (ATS) and the European Respiratory Society (ERS) guidelines [116, 117]. Healthy controls did not undergo lung function testing during this study. From previous analyses from the CLARA study population normal lung function data were documented in healthy controls [118], and selection criteria were kept identical.

	Healthy controls (HC)	Allergic asthmatics (AA)

Inclusion criteria	• Age 4 - 14
	• None of the following exclusion criteria applicable
		• Age 4 - 14
		• At least one positive characteristic out of the following:
		  ○ At least three episodes of typical asthma symptoms (e.g. wheezing, cough) or at least three episodes of obstructive bronchitis in the past
		  ○ A doctor's diagnosis of asthma and/or a history of taking asthma medication
		  ○ Lung function showing significant reversible airflow obstruction according to ATS/ERS guidelines [117]
		• Specific IgE ≥ 0.35 kU/l in accordance with allergic symptoms

Exclusion criteria	• Preterm birth (i.e. before 37 completed weeks of gestation) • Any respiratory disease • Allergies (any reported allergic reactions and corresponding specific IgE ≥ 0.35 kU/l) • Any chronic disease • Any autoimmune disorder • Feverish infection within the past 14 days • CRP levels > 0.5 mg/dl • Intake of steroids, antibiotics, probiotics, or any (other) immunomodulating medication within the last 14 days prior to recruitment	• Preterm birth (i.e. before 37 completed weeks of gestation) • Any other chronic respiratory disease than asthma or bronchitis • Any other chronic diseases • Any autoimmune disorder • Feverish infection within the past 14 days • CRP levels > 0.5 mg/dl • Intake of steroids, antibiotics, probiotics, or any (other) immunomodulating medication within the last 14 days prior to recruitment • Immunotherapy for allergy

Table 4: Inclusion and exclusion criteria of healthy controls and allergic asthmatics
Inclusion and exclusion criteria for healthy controls (HC) on the left and allergic asthmatics (AA) of the right. American Thoracic Society (ATS), European Respiratory Society (ERS).

3.2.3 Blood sampling

A maximum volume of 30 ml blood was taken by either a doctor or the author of this thesis: three sodium-heparin blood tubes, three ethylenediaminetetraacetic acid (EDTA) tubes, and two serum tubes. All blood tubes were labelled with study number and date before further processing. One EDTA and one serum tube were analysed by the laboratory department of the *Dr. von Haunersches Children Hospital* for blood count and quantitative determination of C-reactive protein (CRP) and total IgE. The remaining two EDTA tubes and one serum tube were centrifuged within one hour after blood sampling at 1100 x g for 10 minutes at 20

°C. 200 to 500 µl serum supernatant was collected and stored at -20 °C for later specific IgE measurement. 500 µl supernatant of the EDTA tubes was filled in two screw cap micro tubes respectively. The remaining blood in the EDTA and serum tubes as well as the two micro tubes was frozen at -80 °C for potential later analyses and future research projects.

3.2.4 Allergy test

The medical technical assistant performed immunoblot assays for the quantitative determination of circulating allergen specific IgE in each blood sample taken using AllergyScreen® (MEDIWISS Analytic GmbH, Moers, Germany). The allergens tested in this study were: alder pollen, almond, Alternaria alternata, apple, Aspergillus fumigatus, birch pollen, carotte, casein, cat epithelium, celery, Cladosporium, codfish, Dermatophagoides farinae, Dermatophagoides pteronyssinus, Dermatophagoides microceras, dog hair, crab, egg white, egg yolk, grass pollen mixture, guinea pig, hamster, hazelnut, hazel pollen, horse epithelium, kiwi, latex, milk protein, mugwort pollen, oak pollen, peanut, Penicillium notatum, plantain pollen, potato, rabbit, rye flour, sesame, soybean, timothy grass pollen, tomato, walnut and wheat flour. Specific IgE ≥ 0.35 kU/l were considered positive. Healthy children that had at least one specific IgE ≥ 0.35 kU/l with corresponding allergic symptoms were excluded from the analysis.

3.3 Farm dust collection and extraction

Farm dust samples were collected prior to this project. German, Finnish and the three different Chinese dust samples were collected within the scope of the TRILATERAL study in 2013 and 2014 using electrostatic dust fall collectors (EDCs). TRILATERAL is a multi-centre, DFG (*Deutsche Forschungsgemeinschaft*) -financed project between Munich in Germany, Kuopio in Finland, Hong Kong, and Guangzhou in China. EDCs are electrostatic cloths that serve as a reliable and effective passive collecting method for airborne dust samples and have been evaluated in different farm environments [87, 119-122]. German and Finnish dust samples were obtained from traditional cowsheds in Icking, Bavaria, Germany (G, dust ID: PB838) and Kitee, Finland (Fi, dust ID: TN01). Chinese dust sample 1 was collected in living rooms in rural Conghua, China (Ch1, dust ID: CH_5109). Chinese dust samples 2 and 3 were collected inside chicken stables in rural Conghua, China (Ch2,

dust ID: TLCH64144; Ch3, dust ID: TLCH78158). Amish dust samples (Am) were not collected by EDCs but were scraped off the walls in a cowshed in Middlebury, Indiana, USA. After collection, dust samples were stored and frozen at -80 °C and sent to Munich for extraction.

The German, Finnish, Amish and one Chinese farm dusts (Ch1) had already been extracted in previous projects and were ready for application. Since previous studies of the research group showed weaker effects of Ch1 on gene expression compared to the other dusts [86], another two additional Chinese dusts were investigated in this project. These new Chinese dusts (Ch2 and Ch3) had not been extracted or tested before.

The dust extraction was performed according to the cold extraction protocol implemented by Professor Otto Holst, Leibniz Research Center, Borstel, Germany [123]. Under sterile conditions the four EDCs (two EDCs per dust sample) were each placed in a pyrogen-free glass beaker filled with 400 ml of 9% concentrated sodium chloride. The beakers were placed on magnetic fields and constantly stirred by magnetic stir bars. This was done for six hours at room temperature and then overnight at 4 °C. On day two, the EDCs were carefully transferred into 50 ml falcons and wrung using a pair of tweezers. The outgoing dust solution was retransferred into the glass beakers. The dust solutions were then centrifuged at 6000 x g for 30 minutes at 4 °C, acceleration 9, deceleration 9. The supernatant was transferred into new glass beakers. Four buckets were filled with 5 l deioinised water (Milli-Q® water, Merck KGaA) and the magnetic stir bars were placed inside. The dust solution of each EDC was transferred into two dialysis membranes (ZelluTrans, Carl Roth) respectively which were sealed with clips. The buckets were placed on magnetic fields and magnetically stirred at 4 °C. The water was changed four times with a minimum time interval of four hours. The dust solution was then transferred into sterile cups and filtered using a vacuum pump (Millivac™, Merck Millipore). After filtration, the filter was carefully cut out and stored at -80 °C for possible microbiome analysis. The dust solution was transferred into four glass flasks which were subsequently frozen in liquid nitrogen. The flasks were then connected to the freeze dryer (Lyovac GT2®, GEA) and freeze dried for two days. This process was repeated twice whereby smaller flasks were chosen every time. Chinese dust sample 2 (Ch2) had a dust yield of 0.4 g, Chinese dust sample 3 and (Ch3) had a dust yield of 0.9 g.

All dusts were weighed using a high precision scale (Explorer Analytica®, OHAUS) and split in 100 µl aliquots each containing a 1 mg/ml dust stock solution (dissolved in phosphate buffered saline, PBS). The aliquots were labelled and stored at -20 °C.

3.4 Analysis of dust composition

Endotoxin (LPS) concentrations of German, Finnish, and Chinese dust 1 samples were analysed prior to this project. This was done using the endpoint chromogenic Limulus Amoebocyte Lysate (LAL) assay (Limulus Amoebocyte Lysate QCL1000™ Assay + ß-Glucan Blocker Kit, Lonza, Basel, Switzerland). Endotoxin concentrations in German and Finnish dust samples were far higher than the maximum test standards and significantly higher compared to the Chinese dust sample 1 (mean endotoxin values 1082 EU/ml for G, 887 EU/ml for Fi, and 0.17 EU/ml for Ch1) [86]. Endotoxin concentrations of the Amish dust sample were not measured due to logistic reasons. Endotoxin concentrations of the newly extracted Chinese dust samples 2 and 3 were not measured within the present study but are planned to undergo further analysis in following projects of the research group.

Also prior to this project, bacterial 16S ribosomal RNA (rRNA) was sequenced in German and Finnish dust samples to further characterise the composition. Most sequences belonged to gram-negative Proteobacteria (90.1%). Gram-positive Firmicutes accounted for 8.6% and Actinobacteria for 1% [86, 87].

3.5 PBMC isolation

The sodium-heparin tubes were usually processed within one hour after blood withdrawal, with a maximum time span of 24 hours. This time span was tested in previous projects of the research group and was considered appropriate for the genes investigated in this study. Peripheral blood mononuclear cells (PBMCs) were isolated according to the Qiagen supplementary protocol of the RNeasy® Mini Kit (Qiagen). Whole blood was diluted with PBS (Thermo Fisher Scientific, Gibco™) in a 2:1 dilution and isolated via Ficoll-Paque density-gradient (Ficoll-Paque™ PLUS, GE Healthcare). Centrifugation was performed at 1400 x g for 10 minutes at 20 °C, acceleration 9, deceleration 0 (i.e. no brake). The white blood cell layer was carefully taken up with a Pasteur pipette and filled into new tubes. RPMI 1640 cell culture medium (Thermo Fisher Scientific, Gibco™) was used to wash PBMCs, and the tubes were centrifuged again at 1100 x g for 10 minutes at 20 °C, acceleration 9, deceleration 9. The supernatant was discarded, and the pellet was resuspended in 10 ml of RPMI 1640. 50 µl of the PBMC-RPMI mixture were mixed with 50 µl of ACK lysing buffer (Lonza) and placed on ice for 7.5 minutes. 50 µl of 0.4% trypan blue solution (Sigma-Aldrich) were added and mixed by pipetting up and down. Subsequently, 10 µl of the mixture were

pipetted onto the Neubauer counting chamber (Karl Hecht, Assistent®) and PBMCs were counted through a light microscope (Axiovert 40C, Zeiss). In the meanwhile, the rest of the 10 ml PBMC-RPMI mix was centrifuged at 1100 x g for 10 minutes at 20 °C, and the supernatant was discarded. The total number of PBMCs was calculated and the cell pellet was resuspended in the corresponding amount of X-Vivo™ 15 cell medium (Lonza) to obtain a final concentration of 5 x 10^6 cells/ml.

3.6 PBMC stimulation

Cell culture plates with 24 wells (Corning) were labelled with study number, date, and stimuli. One well was coated with 550 µl PBS and 1.6 µl anti-CD3 (OKT3, Thermo Fisher Scientific, eBioscience™, final concentration of 3 µl/ml). The coating was usually done the day before PBMC stimulation at room temperature. If blood withdrawal and PBMC stimulation were performed the same day, the coated plate was incubated for at least two hours at 37 °C. 500 µl of cell suspension (2.5 x 10^6 PBMCs) were pipetted into a well and the different stimuli were added. The final concentration of the dust stock solutions (German, Finnish, Amish and three different Chinese dusts) was 40 µg/ml. This concentration was based on previous experiments of the research group that evaluated ideal stimulatory conditions for this project. Positive controls were anti-CD3 and anti-CD28 antibodies (anti-CD3/28, Thermo Fisher Scientific, eBioscience™; final concentration of anti-CD3: 3 µg/ml, final concentration of anti-CD28: 1 µg/ml) and LPS (final concentration of 0.1 µg/ml). Anti-CD3/28 is a potent T cell stimulus. It mimics stimulation by antigen-presenting cells, thereby enhancing T cell proliferation. LPS is the major component of the cell wall of gram-negative bacteria and stimulates innate immune cells. Concentration of LPS was based on use in previous work of the research group as well as other farm studies [49, 86, 87, 124]. One well served as negative control and contained 50 µl additional X-Vivo™ 15 cell medium (Lonza) instead of an additional stimulus. The cell culture plate was then incubated at 37 °C and CO_2 concentration of 5% for 24 hours (HERAcell® 240, Heraeus).

3.7 Cell harvest

After 24 hours the PBMCs were harvested. 120 µl cell suspension of each well was filled into Eppendorf tubes (one tube per well) for protein extraction (see below). The remaining cell suspension was filled into 1.5 ml Eppendorf tubes and centrifuged at 1100 x g for 10 minutes at 20 °C. In the meantime, 1000 µl PBS were added to the wells to wash off remaining cells. After centrifugation, the supernatant was pipetted into three Eppendorf tubes (per well) each containing around 110 µl and stored at -80 °C for cytokine assays. The 1000 µl PBS were then transferred from the wells to the corresponding 1.5 ml Eppendorf tubes to resuspend the cell pellet. The tubes where then centrifuged again at 1100 x g for 10 minutes. The supernatant was discarded. RLT-2-mercaptoethanol mix (100 µl RLT buffer containing 10 µl 2-mercaptoethanol, contained in the RNeasy® Mini Kit, Qiagen) was vortexed and the cell pellets were each resuspended in 350 µl RLT-2-mercaptoethanol mix. All samples were stored at -80 °C.

For protein extraction, the Eppendorf tubes containing 120 µl cell suspension were centrifuged at 3700 x g for 10 minutes at 4 °C. The supernatant was carefully removed, and each cell pellet was lysed with 100 µl of RIPA-PIC mix (4% solution made of 32 µl cOmplete™ EDTA free Protease Inhibitor Cocktail, Sigma-Aldrich, and 800 µl RIPA-lysis and extraction buffer, Thermo Fisher Scientific). The tubes were vortexed shortly and placed on ice for 30 minutes. Centrifugation was performed at maximum speed (20200 x g) for 15 minutes (again at 4 °C). The supernatant was then carefully pipetted into a new tube and stored at -80 °C.

3.8 RNA extraction

RNA was isolated using RNeasy® Mini Kit (Qiagen). After thawing frozen cell lysates, 350 µl of 70% ethanol were added onto the RNA lysates in a one-to-one dilution and mixed well to promote selective RNA binding to the RNeasy membrane. The sample was applied to the RNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for 15 sec at 8000 x g at 20 °C. The flow through was discarded. After adding 700 µl buffer RW1 (contained in the RNeasy® Mini Kit, Qiagen) to the RNeasy spin column, centrifugation was performed as in the previous step and the flow through was discarded again. 500 µl RPE buffer (Qiagen) were added, and the tubes were centrifuged at 8000 x g for 2 minutes at 20 °C. Again, the flow through was discarded. The RNeasy spin column was placed in a new 2 ml collection

tube and centrifuged at maximum speed for 1 minute to dry the membrane. The RNeasy spin column was then placed in a new 1.5 ml collection tube and 30-50 ml of RNase-free water were directly added to the spin column membrane. Another centrifugation step was performed for at 8000 x g for 1 minute to elute the RNA.

The exact RNA concentration was measured photometrically using Nanodrop2000 (Thermo Fisher Scientific). The RNA concentration was based on absorbance at 260 nm. The absorbance ratios A260/230 and A260/A280 were used to assess sample purity. Samples were considered “pure” when the A260/A230 ratio was between 2.0 and 2.2, and the A260/A280 ratio was between 2.0 and 2.1. Extracted RNA samples were stored at -80 °C.

3.9 cDNA synthesis

To use the samples for polymerase chain reaction, complementary DNA (cDNA) had to be synthesised. This was done using the QuantiTect® Reverse Transcription Kit (Qiagen). Whenever possible, a final cDNA concentration of 50 ng/ml was aimed at. To eliminate genomic DNA, 2 µl DNA wipeout buffer (contained in QuantiTect® Reverse Transcription Kit, Qiagen) were pipetted into a tube labelled with study number and stimulatory condition. Depending on the RNA concentration measured by Nanodrop2000, x µl RNA and 12 - x µl of RNAse free water (contained in QuantiTect® Reverse Transcription Kit, Qiagen) were added to the corresponding tubes. This mix was then incubated at 42 °C in the PCR Thermocycler PeqSTAR 96 universal (Peqlab Biotechnologies) for 2 minutes. After adding the reverse transcriptase mix (1 µl reverse transcriptase, 4 µl QRT buffer and 1 µl RT primer mix, all contained in the QuantiTect® Reverse Transcription Kit, Qiagen) the samples were incubated at 42 °C for another 15 minutes. Finally, the samples were heated to 95 °C for 3 minutes to inactivate the reverse transcriptase. All cDNA samples were stored at -20 °C.

3.10 Primer design, dilution and validation

Primers were designed using the Primer Blast designing tool (National Center for Biotechnology Information, NCBI) and according to the standard operating procedure of the research group of Professor Schaub at the *Dr. von Haunersches Children Hospital*. The primer sequence of the housekeeping gene 18S was already evaluated in previous experiments of the research group and was available for this project. All other primers were newly designed by the author. Primers that met the following criteria were chosen preferentially:

- Primer length between 18-27 (better 18-22) base pairs
- Primer melting temperature (T_m) between 52 °C and 58 °C and similar between forward and reverse sequences, i.e. T_m difference < 0.5 °C
- Guanine and cytosine percentage between 40 and 60 % and similar between forward and reverse sequences
- At least one guanine or cytosine within the last five bases from the 3' end with a maximum of three guanines or cytosines
- Obligatory location behind the ATG gene sequence since messenger RNA (mRNA) transcription starts hereafter
- Energy by which hairpins or dimers are formed by the primers no more than ± 2 kcal/mol to assure good annealing efficiency
- No more than four di-nucleotide repeats di-nucleotides (e.g. ATATATAT)
- Maximum number of runs accepted four base pairs (e.g. GGGG)
- Location of forward and reverse primers on different exons with large introns in between to avoid amplification of genomic DNA residues
- Maximum length of final PCR product ~200 base pairs

Primer sequences were ordered from Thermo Fisher Scientific, Invitrogen™ and stored at 4 °C until needed. Depending on the amount of nmol of each primer sequence, an according amount of distilled water was added to the primer tube to obtain a 1 mM concentration. The primer-water mix was then vortexed for 10-15 seconds and spun down. Forward and reverse primer sequences were joined and further diluted with distilled water to obtain a final concentration of 1 µM. Aliquots were stored at -20 °C. Quality control was ensured by analysing the melting curves and performing gel electrophoresis (see section **3.11** and **3.12**).

3.11 Quantitative real-time PCR

Quantitative real-time polymerase chain reaction (qPCR) serves as a method of specific DNA amplification that also quantifies the amount of original cDNA used in the sample. 18S was used as a housekeeping gene. Housekeeping genes are expressed independently of regulatory processes, individuum, cell type, and cell state and therefore reflect the baseline cell activity [125]. They hence serve as reference genes and are necessary for data normalisation. No-template-control (NTC) wells were used to indicate possible contaminations by replacing the cDNA template with the same amount of nuclease-free water. All experimental conditions were performed in duplicates. Pipetting was performed quickly and on ice. A total volume of 10 µl consisting of Mastermix, primer dilution, cDNA and nuclease-free water was pipetted into each well:

- 5 µl Mastermix (Sso Advanced™ Universal SYBR® Green Supermix, Bio-Rad)
- 1.8 µl cDNA mix consisting of 0.12 µl of cDNA (concentration 50 ng/ml) and 1.68 µl nuclease-free water
    - If the cDNA concentration was lower than 50 ng/ml, appropriate amounts of cDNA and nuclease-free water were added in order to always obtain the final same amount of 6 ng cDNA per well.
- 3.2 µl of the final primer dilution (1 µM)
- 1.8 µl nuclease-free water were added into each NTC well instead of the cDNA mix.

After pipetting, optical foil (Microseal®, Bio-Rad) was used to seal the plate. A short centrifugation step was performed at 500 x g to remove air bubbles. The plate was then placed in the iCycler (CFX96 Touch™ Real-time PCR Detection System, Bio-Rad) and the qPCR thermal cycling protocol (see **Table 5**) was started.

The melting curve generated by the iCycler was analysed with the associated computer program (BioRad CFX Manager™ software version 2.1) to detect unspecific or unwanted amplification, e.g. due to contamination. A precise melting curve had a high and narrow peak and a uniform look between the duplicates. In case of unspecific melting curves qPCR was repeated. The cycle threshold (Ct) indicates the number of cycles required at which the fluorescence signal of qPCR product crosses a threshold of detection, i.e. exceeds background signal. Ct values are inversely related to the amount of starting template, i.e. the higher the amount of template, the lower the Ct value. When Ct values differed > 0.5 between the duplicates, qPCR was also repeated.

Cycle	Repetition	Temperature	Duration
Cycle 1 Initial denaturation	1x	95.0 °C	2 min
Cycle 2 Denaturation Annealing + Elongation	40x	95.0 °C 62.5 °C	20 sec 30 sec
Cycle 3 Elongation	1x	72.0 °C	2 min
Cycle 4	1x	95.0 °C	30 sec
Cycle 5	1x	55.0 °C	30 sec
Cycle 6	80x	55.0 °C	5 sec
Cycle 7	1x	20.0 °C	Hold

Table 5: qPCR thermal cycling protocol

3.12 Gel electrophoresis

In gel electrophoresis, mixtures of DNA, RNA, and/or proteins are separated according to molecular size. An electrical field pushes the molecules through small pores in the gel at a speed that is inversely correlated to the molecules' lengths. The gel was made by solving 6 g agarose in 200 ml of 0.5% buffer (900 ml H_2O bidest and 100 ml 5X TBE). The solution was boiled until it was clear and then cooled down to 80 °C. Next, 70 µl ethidium bromide were added and the gel was cast into a 30 x 27 x 11 cm gel chamber equipped with several combs to form pockets. After the gel cooled down, the chamber was filled with 800 ml buffer. 2 µl loading dye solution were added to the qPCR products and the negative controls which were then pipetted into the gel's pockets. To quantify product size a ladder containing DNA fragments of known size was also pipetted into the pockets. An electrical field was applied by connecting the gel chamber to 120 V voltage and 400 mA current. After running for 40 minutes a photograph of the gel was taken in a UV light chamber showing different bands representing the different molecule sizes. This method was applied for additional qPCR quality control. It was performed for all primers used in this project and on a few random samples throughout the project. Whenever more than one band was detected, the qPCR was repeated.

3.13 Statistical analyses

Clinical data and laboratory parameters were entered in SPSS software version 25. GraphPad Prism 9 and *R* software version 3.6.0 were used for statistical analyses. Graphs were designed by the author using GraphPad Prism 9.

D'Agostino-Pearson normality test was used to test whether the data in each group was distributed normally. Accordingly, differences in population as well as clinical and laboratory characteristics were analysed with Welch's t-tests or Mann-Whitney-U tests for continuous variables. Fisher's exact test was used for contingency tables. Paired t-test and Wilcoxon matched-pairs signed-rank test were used to analyse stimulatory effects on gene expression within one phenotype (see section **4.5**). Gene-gene correlations were analysed by Spearman correlation.

For population characteristics that were significantly different between the phenotypes, confounder analysis was performed by regrouping samples according to the significant findings regardless of the phenotype (see section **4.5.1**). This was conducted whenever mRNA expression levels were significantly different between HC and AA. Confounder adjustment was performed by multiple linear regression.

P-values < 0.05 were considered statistically significant. P-values < 0.1 were considered to show non-significant tendencies. All study population analyses as well as analyses of differences in gene expression data were performed by the author using GraphPad Prism 9.

Mean Ct values of technical replicates were calculated by the laboratory's biostatistician using *R*. For further analyses and visualisation, gene expression data were displayed as ΔCt values. ΔCt describes the difference between the Ct value of the housekeeper and any given sample. It thereby indicates the relative gene expression level using the formula:

$$\Delta Ct = Ct\ value\ gene_x - Ct\ value\ 18S,$$

whereby $gene_x$ stands for the investigated gene and 18S for the housekeeping gene. ΔCt values for individual samples were also calculated by the laboratory's biostatistician using *R.* Median ΔCt values and interquartile ranges shown in this study were calculated by the author using GraphPad Prism 9.

The fold change gives information about the stimulatory effect in reference to both the unstimulated condition (i.e. baseline mRNA expression) and the housekeeping gene 18S. The fold change is therefore expressed as $2^{-\Delta\Delta Ct}$. $\Delta\Delta$Ct values were calculated as follows:

$\Delta\Delta$Ct = ΔCt value gene$_x$ at baseline (i.e. unstimulated) - ΔCt value gene$_x$ stimulated

Log_2 scales were applied for statistical analyses and for visualisation in heat maps (see results, section **4.6**). Fold changes and log_2 (fold change $2^{-\Delta\Delta Ct}$) ratios were calculated by the author using GraphPad Prism 9 and cross checked by the biostatistician using *R*.

As the experiments performed in the present study where exploratory, adjustment for multiple testing was not performed. Significant findings are therefore observational and further studies are required for validation.

3.14 Declaration of the author's contribution

25 children were recruited by the author with some support of another doctoral candidate (Alexander Neuner) and the questionnaires were answered by the parents. In these children, blood withdrawal was performed by the author or a doctor. 19 of them met the inclusion criteria and were used for gene analysis in the study. Allergy tests and lung function tests were conducted by technical assistants. Phenotypes were checked by the author and the final classification was supervised by at least two doctors. After initial instruction and support by the technical assistant, PBMC isolation, stimulation, cell harvest, cDNA synthesis, qPCR and gel electrophoresis were performed independently by the author in the 19 newly recruited samples. RNA extraction was mostly performed by the medical laboratory assistant and in part by the author. As a total of 58 children were included in this project, the remaining 39 samples were chosen from previous projects. Hence, in these samples the processing steps prior to qPCR were already completed. In some cases, cDNA had to be newly synthesised by the author. qPCR of all included samples was performed independently by the author. The author chose the investigated genes based on previous findings of the research group, literature research and advice from postdoctoral researchers. All primers besides the housekeeper 18S were newly designed, processed and tested by the author. Two of the Chinese dusts (Ch2 and Ch3) were extracted and processed by the author with support of the medical laboratory assistant. ΔCt values for individual samples were calculated by the laboratory's biostatistician using *R*. Further calculations, including statistical analyses of participants' characteristics, differences in ΔCt values and $\log_2$ (fold change $2^{-\Delta\Delta Ct}$) ratios, and confounder and correlation analyses were conducted by the author with minor support from the biostatistician who also cross-checked the results. The author interpreted the data and drew scientific conclusions.

4 Results

4.1 Subjects' charactereristics

Subjects' characteristics are shown in **Table 6**. Answers were available for most questions and only a few subjects did not answer all questions. Healthy controls (HC) and allergic asthmatics (AA) shared a lot of characteristics in the recorded socio-demographic and family history data. Sex distribution did not differ significantly but there were fewer females among AA. Age, body height and weight did not differ statistically between the two groups. As allergic asthma is often associated with other atopic diseases, hay fever, atopic dermatitis and food allergy were common among AA. The percentage of positive family history for maternal diagnosis of asthma, hay fever and eczema and paternal diagnosis of eczema was generally higher among AA, but not significantly different. Education level of the parents was indicated by the total number of completed school years and was slightly but not significantly higher among AA. There were no significant differences between exposure to passive smoking at home and duration of breastfeeding. Significantly more HC had older siblings than AA. Moreover, HC had generally more siblings than AA (data not displayed in **Table 6**). Also, significantly more HC reported to have "any indoor pets currently" and "any indoor pets during the first or second year of life".

Confounder analysis was performed by analysing all significantly different categories other than related atopic diseases, i.e. "older siblings", "any indoor pets currently" and "any indoor pets during the first or second year of life". This was done for all significantly different findings regarding mRNA expression levels between the phenotypes HC and AA (see section **4.5.1**).

	HC n = 34	AA n = 24	p-value
Female sex, n (%)	17 (50%)	6 (25%)	0.064[b]
Median age, y (IQR)	10 (7-12)	10 (7-11.8)	0.498[a]
Median height, cm (IQR)	147 (129-153)	143.1 (123.6-150.2)	0.757[a]
Median weight, kg (IQR)	36 (26-49.5)	36.2 (24.4-45.6)	0.856[a]
Doctor's diagnosis of hay fever ever, n (%)	0 (0%)	13 (51.2%)	**< 0.001[b]**
Doctor's diagnosis of atopy ever, n (%)	0 (0%)	10 (41.7%)	**< 0.001[b]**
Food allergy, n (%)	0 (0%)	6 (25%)	**0.003[b]**
Maternal diagnosis of asthma, n (%)	3[d] (9.1%)	4 (16.7%)	0.439[b]
Maternal diagnosis of hay fever, n (%)	5[d] (15.2%)	8 (33.3%)	0.124[b]
Maternal diagnosis of eczema, n (%)	1[d] (2.9%)	2 (8.3%)	0.578[b]
Paternal diagnosis of asthma, n (%)	6[d] (18.2%)	3 (12.5%)	0.720[b]
Paternal diagnosis of hay fever, n (%)	9[d] (27.3%)	6 (25.0%)	1.000[b]
Paternal diagnosis of eczema, n (%)	2[d] (6.1%)	3 (12.5%)	0.640[b]
Highest education level of the mother, i.e. high school or higher	19 (55.9%)	19 (79.2%)	0.094[b]
Highest education level of the father, i.e. high school or higher	21[d] (63.7%)	20 (83.3%)	0.139[b]
Exposure to passive smoking at home, n (%)	2 (5.9 %)	1 (4.2%)	1.000[b]
Breastfeeding "at all", n (%)	28 (82.4%)	22 (91.7%)	0.449[b]
Median duration of breastfeeding in months (IQR)	9 (4.3-12)	8.5 (6-14)	0.428[c]
Older siblings	20[d] (60.6%)	3[d] (13%)	**< 0.001[b]**
Regular attending to kindergarten (age 0-6)	32 (94.1%)	24 (100%)	0.580[b]
Exposure to indoor mold and/or dampness ever	12 (35.3%)	10 (41.7%)	0.432[b]
Any indoor pets currently, n (%)	13[d] (39.4%)	3 (12.5%)	**0.037[b]**
Any indoor pets during the first or second year of life, n (%)	11[f] (35.5%)	2[e] (9.1%)	**0.050[b]**
Any indoor pets at birth, n (%)	10[f] (32.3%)	2[e] (9.1%)	0.093[b]

Table 6: Population characteristics

Healthy controls (HC), allergic asthmatics (AA), table shows number of children (n) and percentages (%), interquartile ranges (IQR), Welch's t-test ([a]), Fisher's exact test ([b]), Mann-Whitney-U test ([c]), one answer missing ([d]), two answers missing ([e]), three answers missing ([f]). Significant p-values ($p < 0.5$) are highlighted in bold.

Blood values relevant to asthma and the immune response were analysed further and are displayed in **Table 7**. AA had significantly higher absolute leukocyte counts than HC but still within a normal range. AA had a significantly higher proportion of eosinophils and significantly lower proportions of neutrophils and monocytes than HC. Percentages of lymphocytes and basophils were similar between the two groups. Before stimulation, PBMCs of each sample were counted through a light microscope and stimulated in a final concentration of 2.5 x 10^6 cells/ml (see methods, section **3.6**). Absolute leukocyte counts for qPCR should therefore be equal between the two groups. PBMCs include lymphocytes (T cells, B cells, and NK cells), dendritic cells, and monocytes. Erythrocytes and granulocytes, i.e. eosinophils, neutrophils and basophils are removed by density gradient (see methods, section **3.5**) [126]. To analyse whether gene expression levels were confounded by differences in cell composition, Spearman correlation analysis was therefore only performed for monocytes for all significantly different mRNA expression levels between HC and AA (see **Table 11** in section **4.5.1**). As there was no significant correlation between monocyte counts and gene expression levels, adjusting was not necessary.

	HC, n = 34	**AA, n = 24**	**p-value**
Leucocytes, G/l (IQR)	5.8 (4.6-7)	7.3 (5.9-8.8)	**0.007[b]**
Eosinophils, % (IQR)	2.5 (2-4)	8.5 (6-10.8)	**< 0.001[b]**
Neutrophils, % (IQR)	46 (42.1-54.5)	41.5 (35.3-49.8)	**0.035[a]**
Lymphocytes, % (IQR)	40 (35-46.3)	40.5 (37-47)	0.520[a]
Basophils, % (IQR)	1 (0.8-1)	1 (1-1)	0.604[b]
Monocytes, % (IQR)	8 (7-9)	7 (6-8)	**0.042[b]**

Table 7: Differential blood count in healthy and allergic asthmatic children
Healthy controls (HC), allergic asthmatics (AA), number of samples included (n), table sows median values with interquartile ranges (IQR), Welch's t-test ([a]), Mann-Whitney-U-test ([b]). Significant p-values ($p < 0.5$) are highlighted in bold.

Total IgE levels were measured in 16 HC and 19 AA and were significantly higher in AA.

	HC	AA	p-value
Total IgE, IU/ml (IQR)	94 (32.6-264.5)	305 (94-856.2)	**0.015[b]**
Specific IgE ≥ 0.35 IU/ml			
Perennial allergens (%)	0 (0%)	21 (87.5%)	**< 0.001[a]**
Seasonal allergens (%)	0 (0%)	15 (62.5%)	**< 0.001[a]**
Food allergens (%)	1 (2.9%)	10 (41.7%)	**< 0.001[a]**

Table 8: Total and specific IgE in healthy and allergic asthmatic children
Total and specific IgE levels measured in 16 healthy controls (HC) and 19 allergic asthmatics (AA). Inter quartile ranges (IQR), Fisher's exact test ([a]), Mann-Whitney-U-test ([b]). Significant p-values ($p < 0.5$) are highlighted in bold.

NF-κB and MAPK cascades are also activated upon inflammatory signals such as systemic and local infections. Hence, participants that had declared any kind of infectious symptoms (including the common cold) within the previous two weeks were excluded from the study. To provide an objective parameter, CRP levels were measured and any child with a CRP > 0.5 mg/dl was excluded from the study. There were no significant differences in CRP levels between HC and AA ($p = 0.740$).

4.2 cDNA availability, technical exclusion and final sample sizes

cDNA was available for almost all genes and stimulatory conditions. In a few cases, due to low RNA quantity and use in previous projects, the amount of cDNA was not sufficient for qPCR. Additionally, a few samples were excluded due to technical errors (e.g. Ct > 0.5 between duplicates). **Table 9** gives an overview of missing samples out of all measured samples for the respective gene and stimulatory condition.

The Chinese dusts Ch2 and Ch3 were used for stimulation in the 19 newly recruited subjects. These 19 children (10 HC and 9 AA) matched well, and HC and AA did not differ significantly in any of the criteria listed in **Table 6** other than related atopic disorders. Laboratory parameters were also similar to those of the entire study population.

Most genes were analysed in all subjects. **MAPK7** and **DUSP1** were tested in fewer subjects (see **Table 9**), as they were introduced later during this research project based on further literature research. **MAPK7** and **DUSP1** were tested in the 19 newly recruited subjects. To increase the sample size for these genes, **MAPK7** was additionally tested in 13 of the previously recruited subjects (5 HC and 8 AA) and **DUSP1** was tested in 1 additional AA. Again, population characteristics of these subsamples were similar to the entire study population.

	U	anti-CD3/28	LPS	G	Fi	Am	Ch1	Ch2	Ch3
18S	0/58	0/58	0/58	0/58	0/58	0/58	0/58	0/19	0/19
MAP3K14	0/58	0/58	1/58	2/58	2/58	1/58	1/58	0/19	0/19
RIPK1	0/58	0/58	0/58	2/58	0/58	1/58	1/58	0/19	0/19
IRAK4	0/58	0/59	0/58	2/58	0/58	1/58	1/58	0/19	0/19
PTGS2	0/58	0/58	0/58	1/58	0/58	0/58	1/58	0/19	0/19
BIRC3	0/58	0/58	0/58	2/58	1/58	0/58	0/58	0/19	0/19
NFKBIA	0/58	0/58	0/58	2/58	0/58	0/58	0/58	0/19	0/19
MAP3K7	0/58	0/58	0/58	2/58	1/58	0/59	1/58	0/19	0/19
MAPK1	0/58	0/58	0/58	1/58	1/58	0/58	1/58	0/19	0/19
MAPK9	0/58	0/58	0/58	2/58	0/58	0/58	1/58	0/19	0/19
MAPK14	0/58	0/58	1/58	2/58	1/58	1/58	1/58	0/19	0/19
MAPK7	0/32	0/32	0/32	0/32	0/32	0/32	0/32	0/19	0/19
DUSP1	0/20	1/20	0/20	1/20	0/20	0/20	0/20	0/19	0/19

Table 9: Number of missing samples out of all measured samples
Genes listed in rows, stimulatory conditions listed in columns. Unstimulated condition (U), anti-CD3/28 (anti-CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3).

4.3 Differences in baseline inflammatory gene expression profiles

Figure 7-18 show the relative baseline mRNA expression levels (i.e. no stimulus added) of all genes investigated for both HC and AA. D'Agostino-Pearson normality test was used to test whether the data in each group was distributed normally. Welch's t-test was applied for normally distributed data, and Mann-Whitney-U-test was applied for not normally distributed data. The graph description indicates which test was used. For more clarity, median ΔCt values and interquartile ranges can be found in the appendix (see section **10.1**)

Non-canonical NF-κB related expression levels of pro-inflammatory **MAP3K14** did not differ significantly between HC and AA.

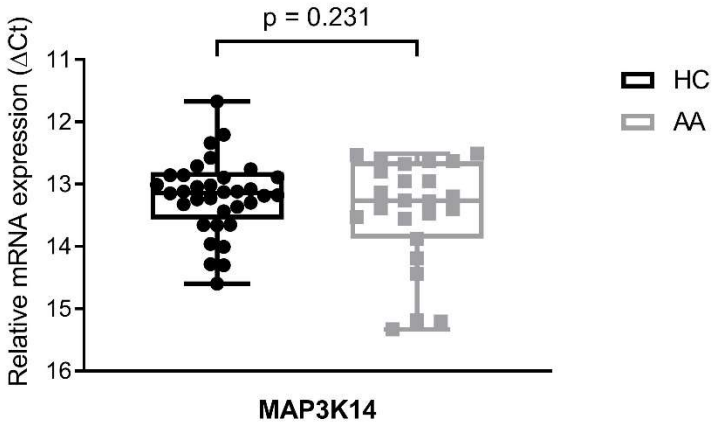

Figure 7: Baseline mRNA expression levels of MAP3K14
Relative mRNA expression levels of MAP3K14 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Boxes show medians and interquartile ranges, whiskers range from minimum to maximum values. Welch's t-test.

mRNA expression of the innate immunity gene **RIPK1** was significantly lower among AA. Two HC had much lower **RIPK1** expression than the remaining HC. Neither any distinct clinical or laboratory feature nor a technical error could be identified that might explain the low expression in these two children. Within AA, it seemed as if there were two relatively distinct subgroups with one of them showing lower and one showing higher expression of **RIPK1**. These two subgroups were analysed for clinical and laboratory data. However, no apparent feature could be recognised to date that might explain this difference.

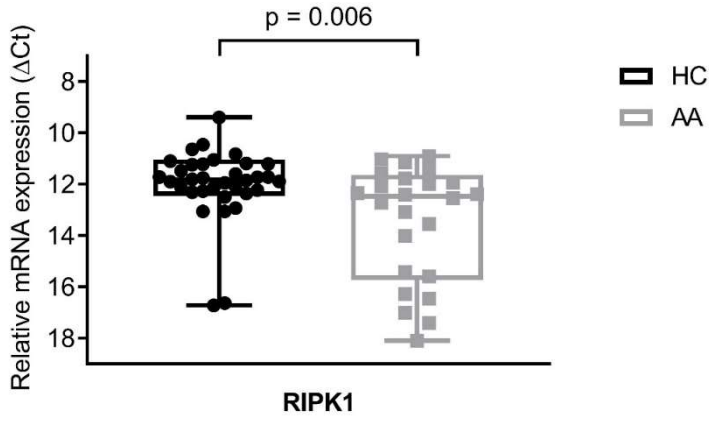

Figure 8: Baseline mRNA expression levels of RIPK1
Relative mRNA expression levels of RIPK1 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Boxes show medians and interquartile ranges, whiskers range from minimum to maximum values. Mann-Whitney-U-test.

mRNA expression of the innate immunity gene **IRAK4** was also significantly lower among AA.

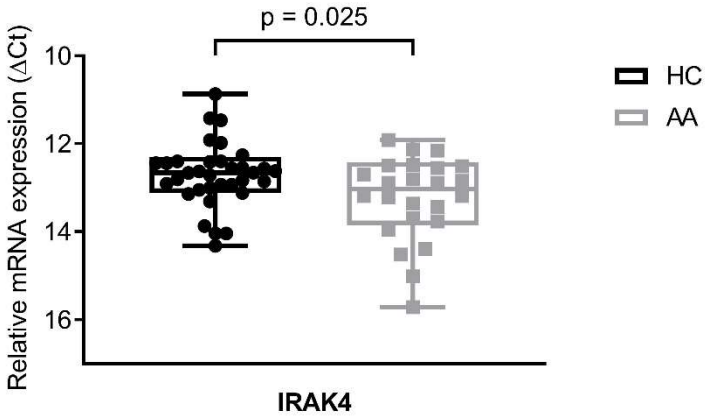

Figure 9: Baseline mRNA expression levels of IRAK4
Relative mRNA expression levels of IRAK4 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Boxes show medians and interquartile ranges, whiskers range from minimum to maximum values. Welch's t-test.

Pro-inflammatory **PTGS2** expression was significantly lower among AA. HC showed greater variations than AA.

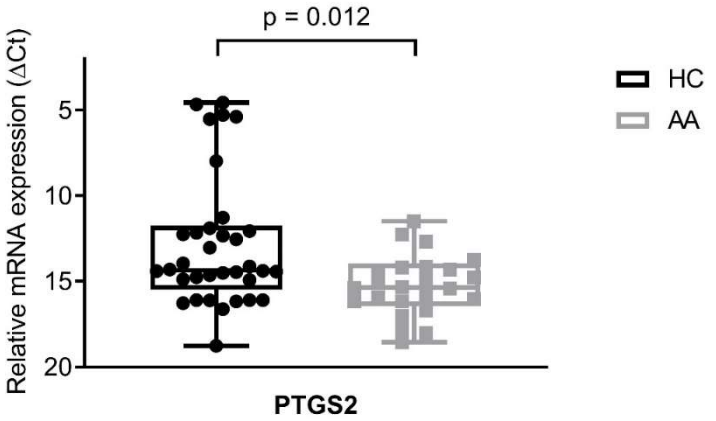

Figure 10: Baseline mRNA expression levels of PTGS2
Relative mRNA expression levels of PTGS2 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Boxes show medians and interquartile ranges, whiskers range from minimum to maximum values. Mann-Whitney-U-test.

Anti-apoptotic **BIRC3** expression was slightly but significantly lower among AA.

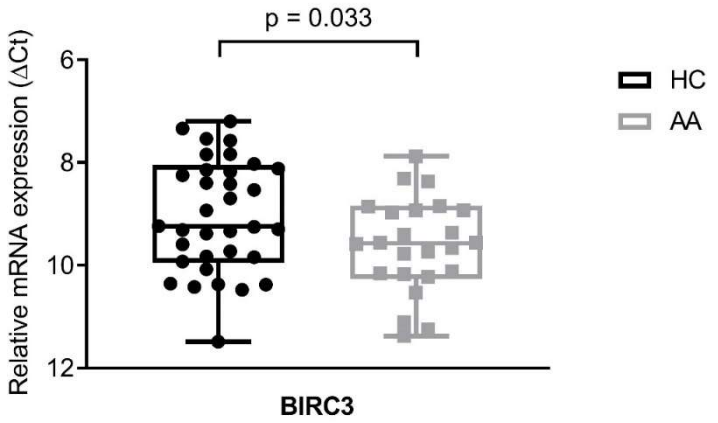

Figure 11: Baseline mRNA expression levels of BIRC3
Relative mRNA expression levels of BIRC3 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Boxes show medians and interquartile ranges, whiskers range from minimum to maximum values. Welch's t-test.

Anti-inflammatory **NFKBIA** expression was also significantly lower among AA.

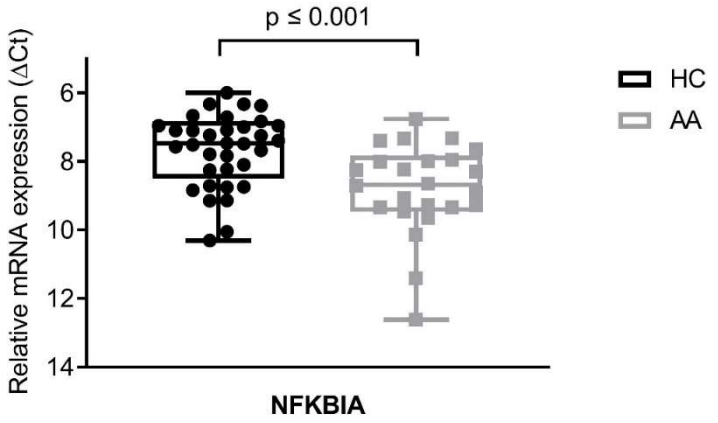

Figure 12: Baseline mRNA expression levels of NFKBIA
Relative mRNA expression levels of NFKBIA in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Boxes show medians and interquartile ranges, whiskers range from minimum to maximum values. Mann-Whitney-U-test.

Pro-inflammatory **MAP3K7** expression did not differ significantly between HC and AA.

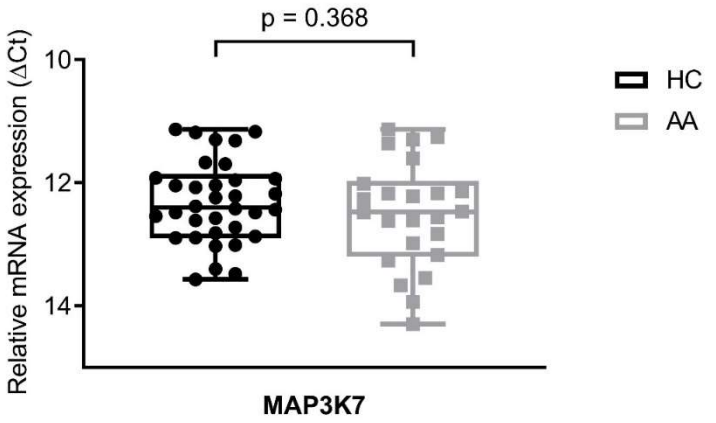

Figure 13: Baseline mRNA expression levels of MAP3K7
Relative mRNA expression levels of MAP3K7 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Boxes show medians and interquartile ranges, whiskers range from minimum to maximum values. Welch's t-test.

Pro-inflammatory **MAPK1** expression did also no differ significantly between HC and AA. Two AA had very low **MAPK1** expression levels. Neither any distinct clinical or laboratory feature nor a technical error could be identified that might explain the low expression in these two children.

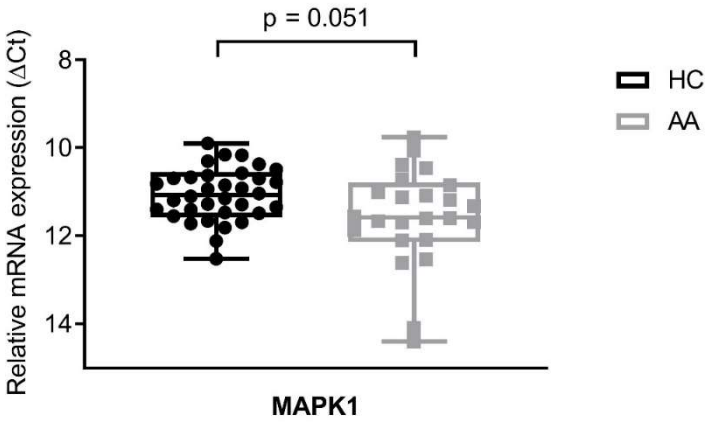

Figure 14: Baseline mRNA expression levels of MAPK1
Relative mRNA expression levels of MAPK1 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Boxes show medians and interquartile ranges, whiskers range from minimum to maximum values. Mann-Whitney-U-test.

Similarly, pro-inflammatory **MAPK9** expression did not differ significantly between HC and AA. The same two AA that had very low baseline expressions of **MAPK1** also showed very low expressions of **MAPK9**. Again, neither any distinct clinical or laboratory feature nor a technical error could be identified that might explain the low expression in these two children.

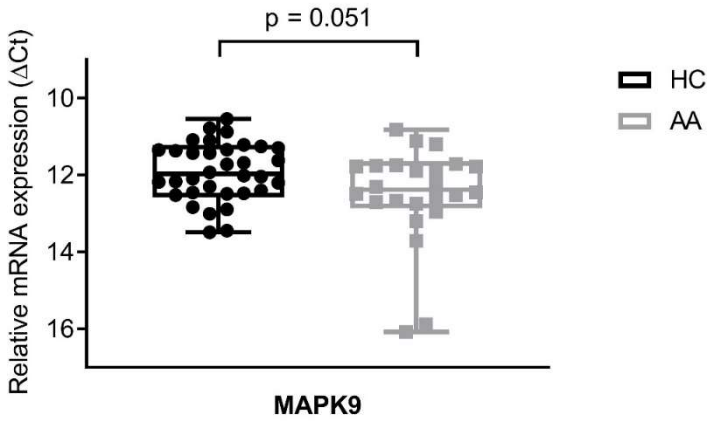

Figure 15: Baseline mRNA expression levels of MAPK9
Relative mRNA expression levels of MAPK9 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Boxes show medians and interquartile ranges, whiskers range from minimum to maximum values. Mann-Whitney-U-test.

Pro-inflammatory **MAPK14** expression did not differ significantly between HC and AA.

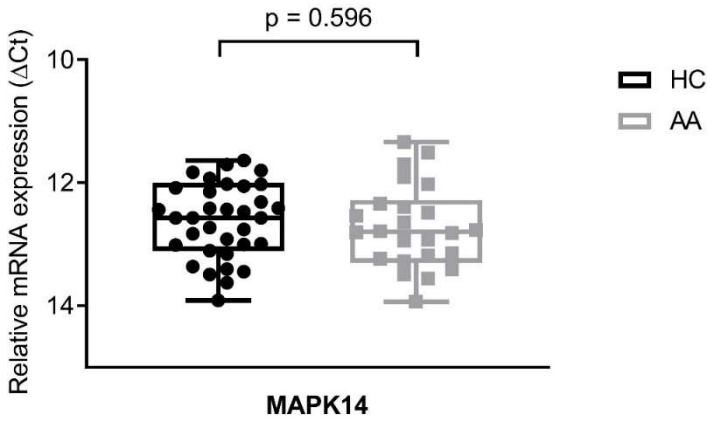

Figure 16: Baseline mRNA expression levels of MAPK14
Relative mRNA expression levels of MAPK14 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Boxes show medians and interquartile ranges, whiskers range from minimum to maximum values. Welch's t-test.

Pro-inflammatory **MAPK7** expression did also not differ significantly between HC and AA.

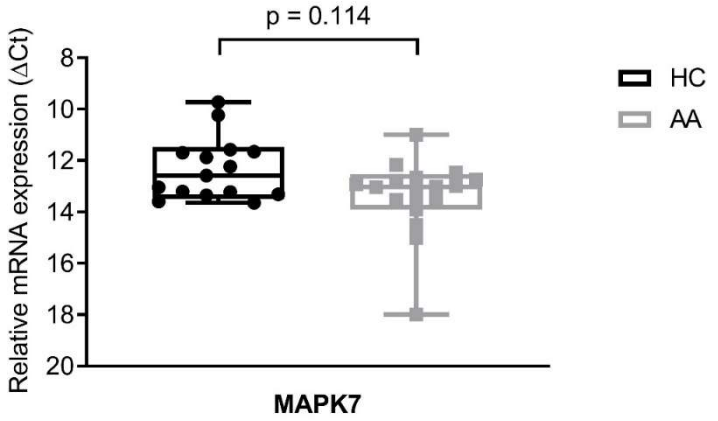

Figure 17: Baseline mRNA expression levels of MAPK7
Relative mRNA expression levels of MAPK7 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Boxes show medians and interquartile ranges, whiskers range from minimum to maximum values. Mann-Whitney-U-test.

Expression levels of anti-inflammatory **DUSP1**, a key negative regulator of MAPK related signalling, were significantly lower among AA.

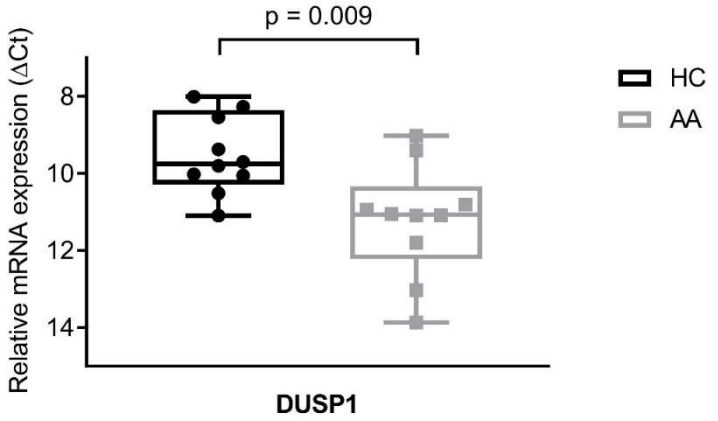

Figure 18: Baseline mRNA expression levels of DUSP1
Relative mRNA expression levels of DUSP1 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Boxes show medians and interquartile ranges, whiskers range from minimum to maximum values. Welch's t-test.

4.3.1 Summary of differences in baseline gene expressions

Overall, AA showed significantly lower baseline expression levels of innate and pro-inflammatory NF-κB pathway genes, namely **RIPK1, IRAK4** and **PTGS2** as well as of the anti-apoptotic gene **BIRC3** and the anti-inflammatory gene **NFKBIA**, a key negative regulator of NF-κB signalling. Expression of pro-inflammatory **MAP3K14**, a central player of the non-canonical NF-κB pathway, and of pro-inflammatory **MAP3K7**, a gene involved in both NF-κB and MAPK pathways, did not differ significantly between HC and AA. Pro-inflammatory **MAPK1** and **MAPK9** were slightly, though not significantly lower expressed in AA. Pro-inflammatory **MAPK14** and **MAPK7** did not differ significantly between the two groups. Expression levels of **DUSP1**, a key negative regulator in MAPK signalling, were significantly lower among AA. **Table 10** provides a summary of all investigated genes. The arrows in the last column indicate whether baseline mRNA expression level in AA was lower (↓) or similar to (↔) HC.

Gene	HC ΔCT median (IQR)	AA ΔCT median (IQR)	p-value	Expression AA vs. HC
MAP3K14	13.14 (12.88-13.49)	13.27 (12.71-13.80)	0.231[a]	↔
RIPK1	11.83 (11.22-12.28)	12.47 (11.81-15.54)	**0.006**[b]	↓
IRAK4	12.65 (12.40-13.01)	13.03 (12.52-13.74)	**0.025**[a]	↓
PTGS2	14.35 (12.01-15.21)	15.36 (14.21-16.17)	**0.0121**[b]	↓
BIRC3	9.25 (8.14-9.86)	9.57 (8.93-10.17)	**0.0332**[a]	↓
NFKBIA	7.47 (6.95-8.37)	8.67 (7.96-9.33)	**0.006**[b]	↓
MAP3K7	12.40 (11.93-12.83)	12.47 (12.05-13.12)	0.368[a]	↔
MAPK1	11.07 (10.66-11.47)	11.58 (10.89-12.03)	0.051[b]	↔
MAPK9	11.97 (11.33-12.46)	12.38 (11.76-12.74)	0.051[b]	↔
MAPK14	12.57 (12.08-13.04)	12.80 (12.36-13.23)	0.596[a]	↔
MAPK7	12.59 (11.65-13.31)	13.02 (12.71-13.72)	0.114[b]	↔
DUSP1	9.75 (8.47-10.17)	11.07 (10.46-12.11)	**0.009**[a]	↓

Table 10: Summary of differences in baseline mRNA expression levels between HC and AA
Gene expression normalised to 18S, healthy controls (HC) and allergic asthmatics (AA), ΔCt values with interquartile ranges (IQR), ↓ indicates significantly lower baseline gene expression in AA, ↔ indicates no significant difference in gene expression between HC and AA. Welch's t-test ([a]), Mann-Whitney-U-test ([b]). Significant p-values ($p < 0.5$) are highlighted in bold.

4.4 Gene-gene correlation analyses

Several of the investigated genes are involved at multiple sites of the pathways (compare **Figure 5**) and as described in sections **1.4.1** and **1.4.2** some genes are involved in both, NF-κB and MAPK related signalling. To detect possible differences in co-expression patterns and to allow for comprehensive discussion and cross-over interpretation, gene expression correlations (in the following also referred to as gene-gene correlation analyses) were performed by Spearman correlation.

Figure 19-21 illustrate differences in gene-gene correlations between the two phenotypes both at baseline and upon stimulation. To facilitate pattern recognition, data are displayed in heat maps where red indicates positive correlation (Spearman correlation coefficient $r_s > 0$), and blue indicates negative correlation ($r_s < 0$). Gene-gene correlations of all children (i.e. regardless of clinical phenotype) are displayed in **Figure 36-38** which can be found in the appendix (see section **10.1.2**). Stimulatory conditions were abbreviated as follows: unstimulated condition (U), anti-CD3 and anti-CD28 antibodies (anti-CD3/28), lipopolysaccharide (LPS), German farm dust (G), Amish farm dust (Am), Finnish farm dust (Fi), Chinese farm dusts 1, 2 and 3 (Ch1, Ch2, Ch3). Differences between the "asthma protective" farm dust extracts are discussed in detail in section **5.3**.

Overall, gene expressions correlated highly and mostly significantly with each other. Under unstimulated conditions, MAPK related genes correlated stronger with one another (indicated by more intense red colour coding) among AA. On the contrary, correlation of the innate immunity gene **RIPK1** with the other investigated genes was weaker (pale red) among AA compared with HC. Anti-inflammatory **DUSP1** correlated strongly and mostly significantly with the other genes in HC but not in AA.

Upon stimulation with the potent T cell stimulus anti-CD3/28, **DUSP1** correlated negatively among AA but not among HC. This may indicate that AA may not be able to upregulate **DUSP1** even upon exposure to such a strong and potent T cell stimulus. However, the negative correlation was not statistically significant. **MAP3K14**, a key regulator involved in non-canonical NF-κB signalling correlated much less with the other genes in both groups. Interestingly, after stimulation with anti-CD3/28, pro-inflammatory **PTGS2** expression hardly correlated with the other genes among HC, whereas it strongly correlated among AA.

After exposure to LPS, correlation of pro-inflammatory MAPK related genes, as well as anti-inflammatory **NFKBIA**, anti-apoptotic **BIRC3** and the pro-inflammatory and innate immunity NF-κB related genes **PTGS2** and **IRAK4** correlated stronger among AA compared with HC.

In contrast, non-canonical **MAP3K14** and the innate immunity gene **RIPK1** correlated stronger with the other investigated genes in HC. Anti-inflammatory **DUSP1** only correlated significantly with **MAP3K14** among HC and with **MAPK7** among AA.

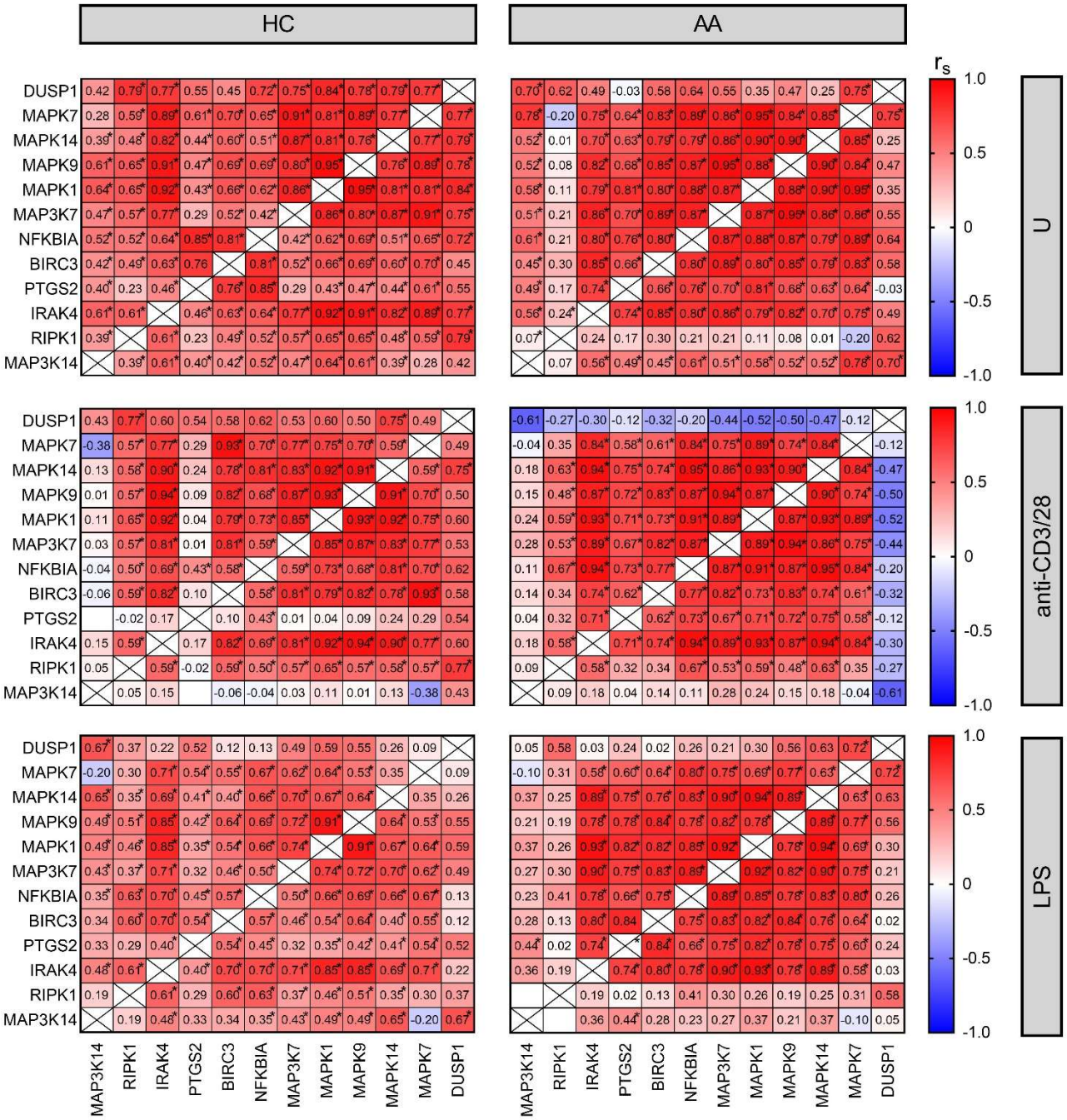

Figure 19: Gene-gene correlations between HC and AA at baseline (U) and after stimulation with anti-CD3/28 and LPS

Heat maps indicating gene-gene correlations for healthy controls (HC) and allergic asthmatics (AA). Unstimulated condition (U), anti-CD3/28 (anti CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide). Spearman correlation coefficient (r_s). Positive regulation is colour coded in red ($r_s > 0$), negative correlation is colour coded in blue ($r_s < 0$). Significant findings are marked with an asterisk (*).

Following stimulation with German farm dust, correlation patterns were similar between the two phenotypes (see **Figure 20**). Yet again, MAPK related genes correlated stronger with each other and **RIPK1** correlated weaker with the other investigated genes among AA. Stimulation with Finnish and Amish farm dust revealed similar correlation patterns. Of note, however, **DUSP1** correlated strongly and mostly significantly with the other genes upon stimulation with G and Am in both phenotypes, but weaker upon stimulation wit Fi. Also, **MAP3K14** correlated weaker with the other genes upon stimulation with Fi compared with G and Am in both phenotypes.

The correlation patterns shown in **Figure 21** reveal different gene expression patterns upon stimulation with the three Chinese farm dusts. Following stimulation with Ch1, the investigated genes correlated strongly and mostly significantly with each other among AA. On the contrary, apart from **IRAK4** and a few MAPK, correlations were weaker among HC. Interestingly, upon stimulation with Ch2, correlation patterns between the phenotypes were almost reversed compared to stimulation with Ch1. In HC, non-canonical **MAP3K14** expression correlated negatively, though not significantly, with all other investigated genes after stimulation with Ch2. In AA, **MAP3K14** expression was only negatively correlated with **PTGS2**, while there was a strong positive correlation with the innate immunity genes **RIPK1** and **IRAK4**. Correlation of anti-inflammatory **DUSP1** and pro-inflammatory **PTGS2** with the other investigated genes was weak in both phenotypes after stimulation with Ch2. Exposure to Ch3 induced a correlation pattern that was to some extent in between correlation patterns of Ch1 and Ch2.

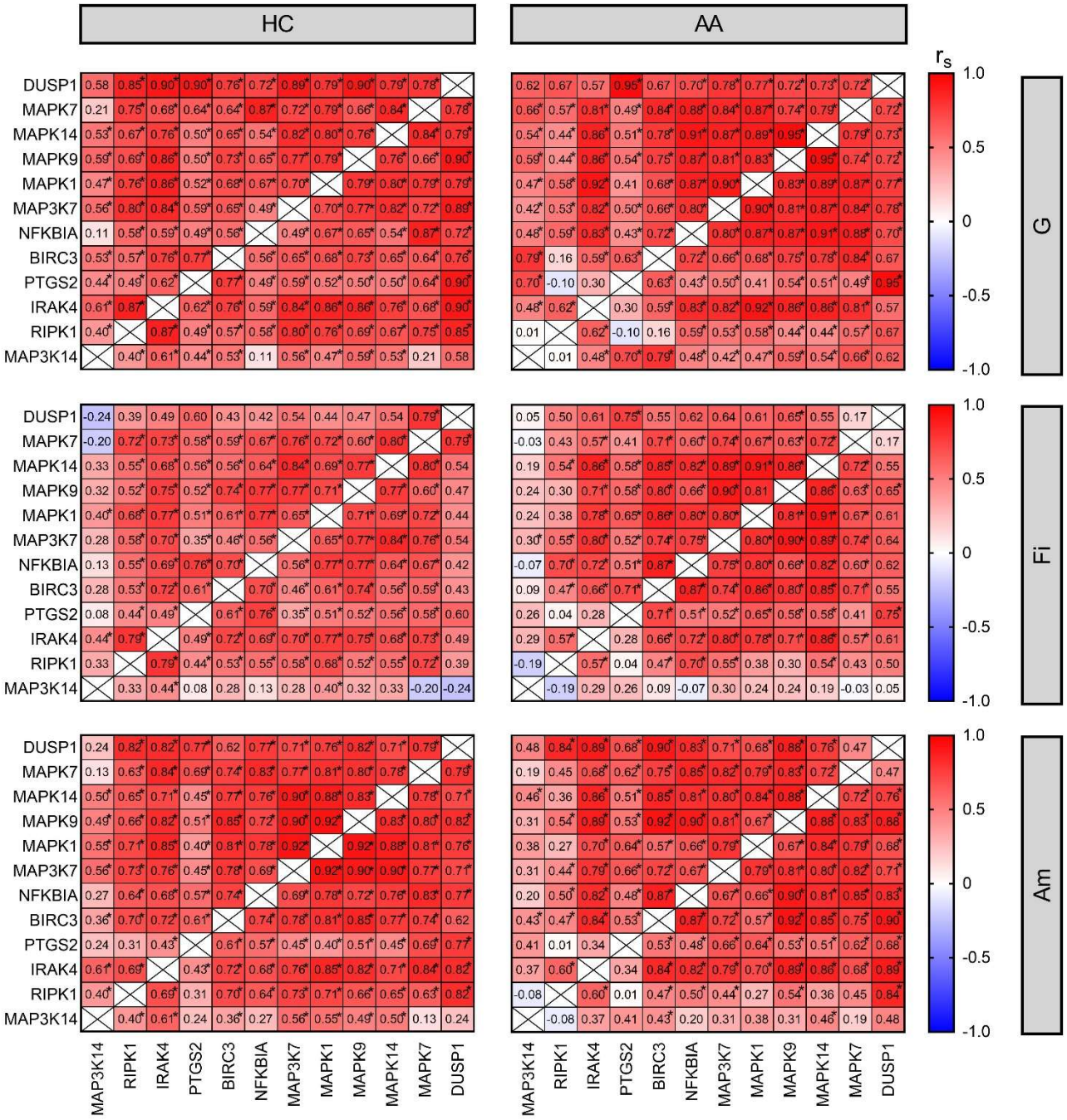

Figure 20: Gene-gene correlations between HC and AA after stimulation with German, Finnish and Amish farm dust extracts

Heat maps indicating gene-gene correlations for healthy controls (HC) and allergic asthmatics (AA). G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust). Spearman correlation coefficient (r_s). Positive regulation is colour coded in red ($r_s > 0$), negative correlation is colour coded in blue ($r_s < 0$). Significant findings are marked with an asterisk (*).

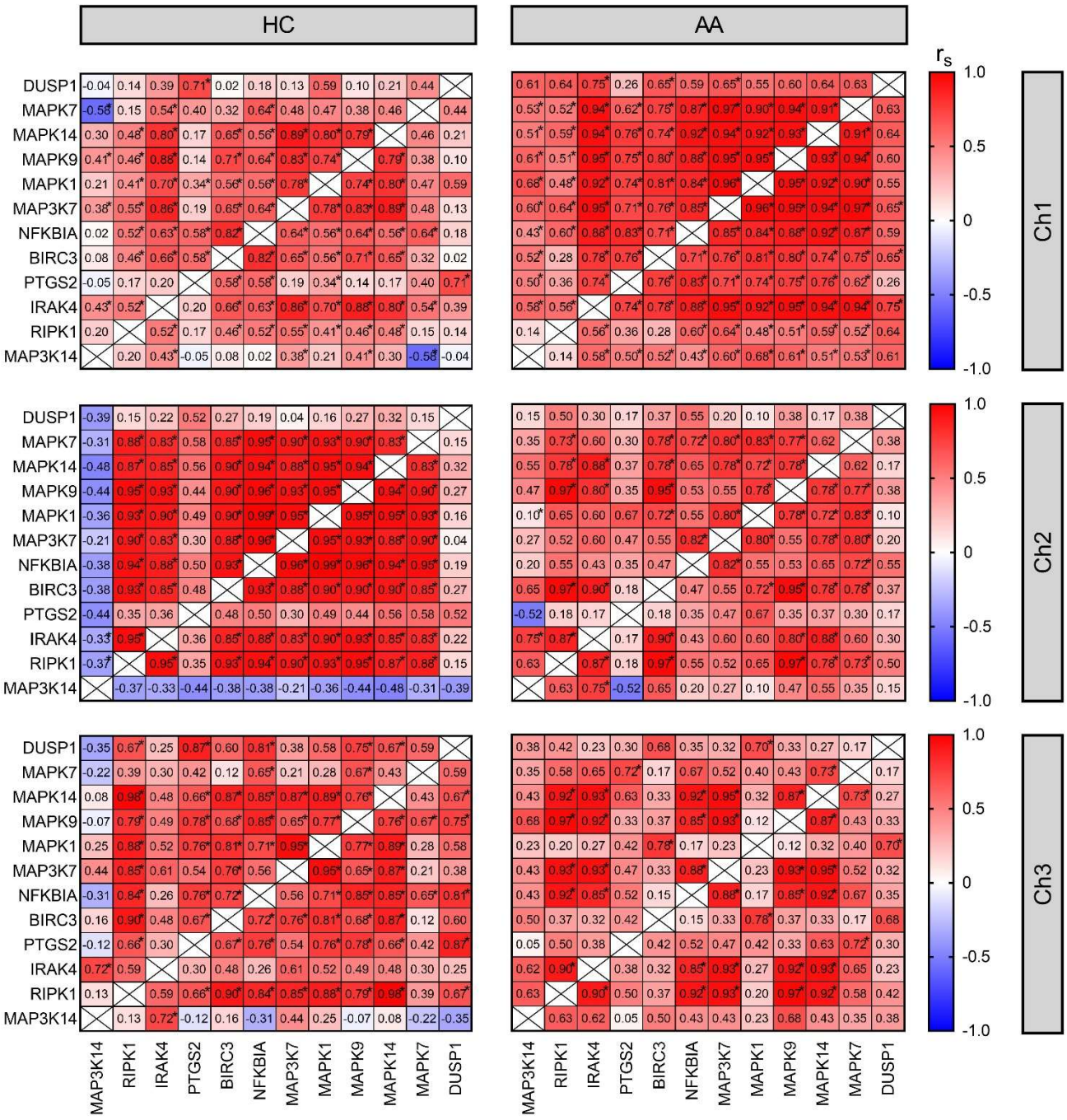

Figure 21: Gene-gene correlations between HC and AA after stimulation with Chinese farm dust extracts

Heat maps indicating gene-gene correlations for healthy controls (HC) and allergic asthmatics (AA). Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Spearman correlation coefficient (r_s). Positive regulation is colour coded in red ($r_s > 0$), negative correlation is colour coded in blue ($r_s < 0$). Significant findings are marked with an asterisk (*).

4.5 Effect of farm dust stimulation on gene expression

The effects of *ex vivo* PBMC stimulation with “asthma protective” farm dust extracts on gene expression are shown in detail in the following section. Each graph illustrates changes in gene expression upon stimulation. Boxplots indicate median ΔCt values and interquartile ranges. Whiskers are extended to the 5th (lower) and 95th (upper) percentile. Values beyond this range are indicated as black circles for HC and grey squares for AA. The red dotted line indicates the median expression level of HC under unstimulated conditions, thereby indicating “healthy baseline expression levels”. For more clarity, each graph is accompanied by a table outlining the number of samples included, median ΔCt values and interquartile ranges. The tables can be found in the appendix (see section **10.1**).

Differences between phenotypes are indicated on top of each graph and are analysed by Welch’s t-test for normally and by Mann-Whitney-U-test for not normally distributed data. Asterisks on top of the box plots indicate tendencies and significant differences upon stimulation for each phenotype in relation to their expression under unstimulated condition. When no asterisk is shown on top of a box plot, the stimulus effect was not significant. To maintain an overview these cases were not additionally labelled with “ns”. Differences upon stimulation were analysed by paired t-test for normally distributed and Wilcoxon matched-pairs signed rank test for not normally distributed data.

In HC, **MAP3K14** was significantly upregulated upon stimulation with anti-CD3/28. LPS induced a slight downregulation almost reaching statistical significance (p = 0.0504). None of the dust extracts caused significant changes in **MAP3K14** expression in HC. In AA, **MAP3K14** was significantly upregulated upon stimulation with anti-CD3/28 and Ch2. Am and Ch1 also showed a tendency towards upregulation, whereas LPS had no significant impact on **MAP3K14** expression in AA.

There were no significant differences between HC and AA when comparing **MAP3K14** expression levels both at baseline (see section **4.3**) and upon stimulation.

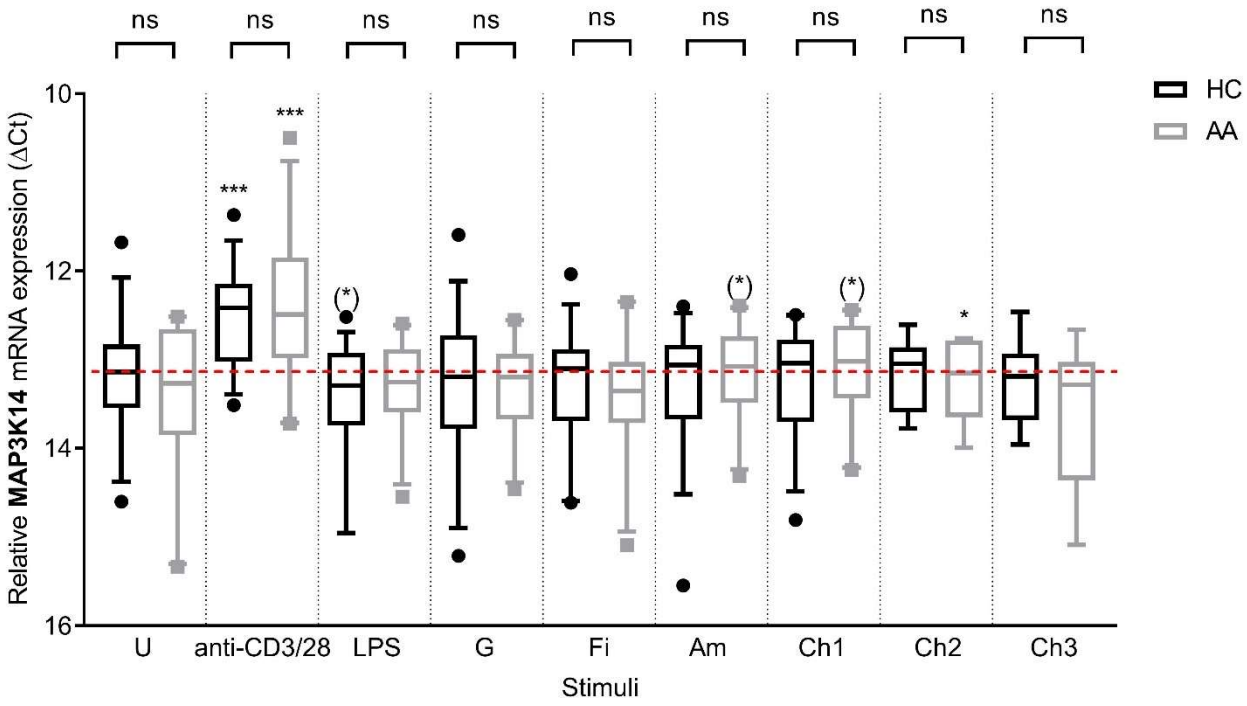

Figure 22: Changes in MAP3K14 mRNA expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of MAP3K14 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Unstimulated condition (U), anti-CD3/28 (anti-CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Boxes show medians and interquartile ranges, whiskers indicate 5-95 percentiles, values beyond are indicated as circles for HC and squares for AA. The red dotted line indicates the median expression level of HC under unstimulated condition. **Differences between phenotypes are indicated on top of the graph** and are analysed by Welch's t-test for normally and by Mann-Whitney-U-test for not normally distributed data. **Asterisks on top of the box plots indicate tendencies and significant differences upon stimulation for each phenotype in relation to their expression under unstimulated condition**. Differences upon stimulation are analysed by paired t-test for normally distributed data and Wilcoxon matched-pairs signed rank test for not normally distributed data. P-value not significant (ns), (*) $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

RIPK1 expression was significantly upregulated upon stimulation with the potent T-cell stimulus anti-CD3/28 in AA, but not in HC. LPS, G, Fi, Ch2 and Ch3 had no significant effects in both groups. Upon simulation with Am, **RIPK1** was significantly upregulated among AA, now reaching an expression level similar to HC under unstimulated condition. On the contrary, **RIPK1** was slightly, but not significantly, downregulated among HC upon stimulation with Am. Ch1 induced significant **RIPK1** downregulation in HC, whereas it had no significant impact in AA.

AA started at significantly lower **RIPK1** baseline expression levels than HC (see section **4.3**). However, after stimulation with anti-CD3/28 and dust extracts, this difference was no longer significant. When comparing **RIPK1** expression levels after stimulation with LPS, AA still had lower levels, yet also not significant.

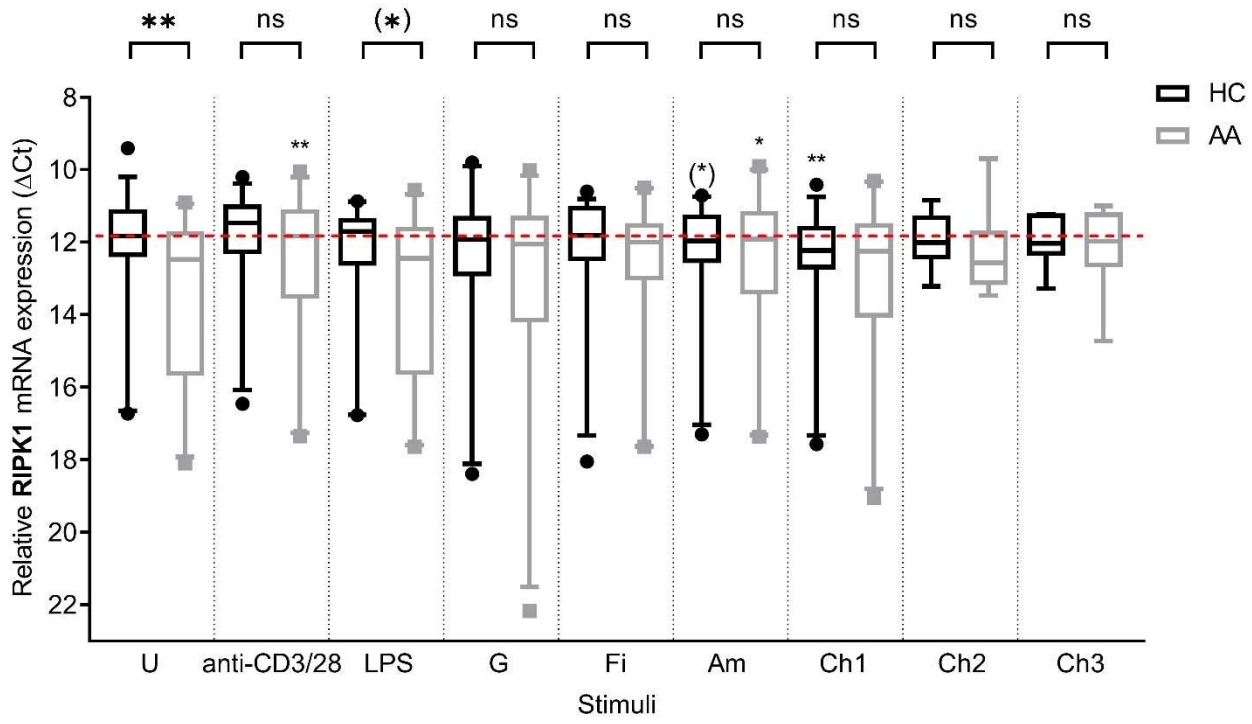

Figure 23: Changes in RIPK1 mRNA expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of RIPK1 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Unstimulated condition (U), anti-CD3/28 (anti-CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Boxes show medians and interquartile ranges, whiskers indicate 5-95 percentiles, values beyond are indicated as circles for HC and squares for AA. The red dotted line indicates the median expression level of HC under unstimulated condition. **Differences between phenotypes are indicated on top of the graph** and are analysed by Welch's t-test for normally and by Mann-Whitney-U-test for not normally distributed data. **Asterisks on top of the box plots indicate tendencies and significant differences upon stimulation for each phenotype in relation to their expression under unstimulated condition**. Differences upon stimulation are analysed by paired t-test for normally distributed data and Wilcoxon matched-pairs signed rank test for not normally distributed data. P-value not significant (ns), (*) $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

IRAK4 was significantly upregulated upon stimulation with anti-CD3/28 among AA, but not HC. However, neither LPS nor any of the dusts caused significant changes in **IRAK4** expression among AA. In HC, **IRAK4** was significantly downregulated following LPS and Am stimulation. Ch3 also showed a tendency towards downregulation, although not significant.

AA started at significantly lower **IRAK4** baseline expression levels than HC (see section **4.3**). However, after stimulation with anti-CD3/28, LPS and dust extracts, this difference was no longer significant.

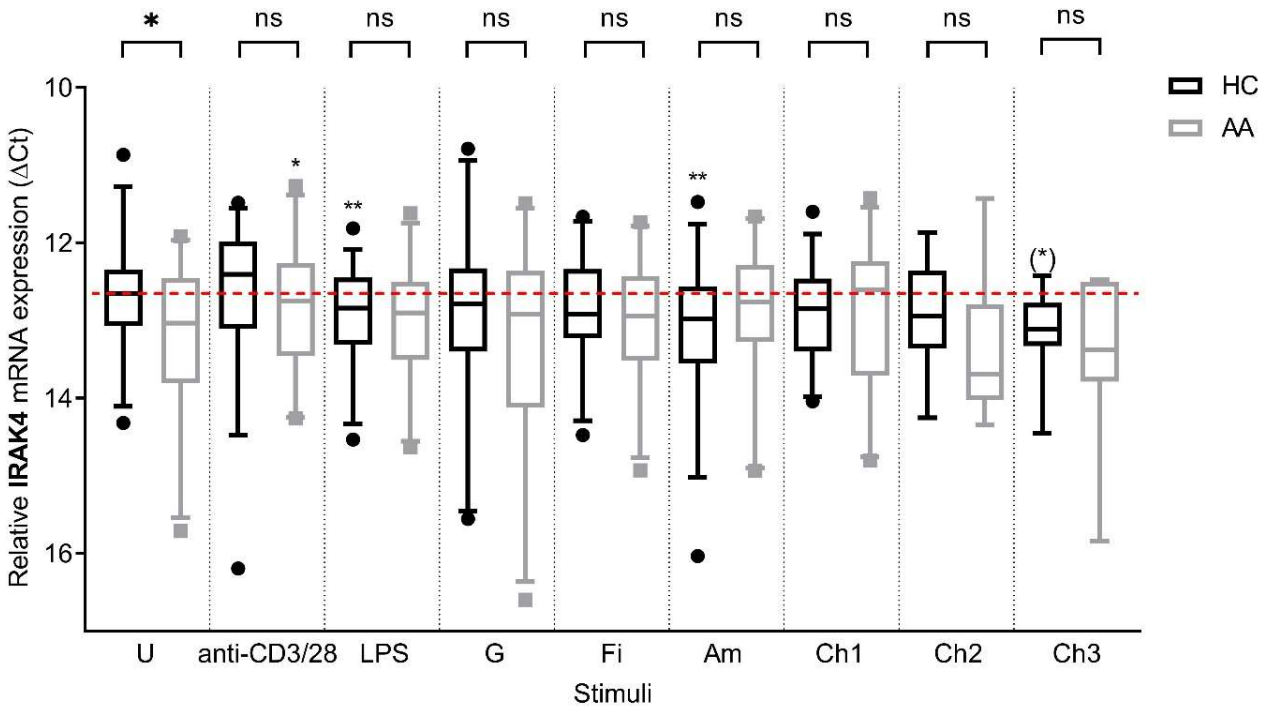

Figure 24: Changes in IRAK4 mRNA expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of MAP3K14 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Unstimulated condition (U), anti-CD3/28 (anti-CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Boxes show medians and interquartile ranges, whiskers indicate 5-95 percentiles, values beyond are indicated as circles for HC and squares for AA. The red dotted line indicates the median expression level of HC under unstimulated condition. **Differences between phenotypes are indicated on top of the graph** and are analysed by Welch's t-test for normally and by Mann-Whitney-U-test for not normally distributed data. **Asterisks on top of the box plots indicate tendencies and significant differences upon stimulation for each phenotype in relation to their expression under unstimulated condition**. Differences upon stimulation are analysed by paired t-test for normally distributed data and Wilcoxon matched-pairs signed rank test for not normally distributed data. P-value not significant (ns), (*) $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Of all genes investigated, changes in **PTGS2** expression were the most pronounced upon stimulation. All stimuli except for Ch1 induced a strong and significant upregulation in both

groups. Among HC, Ch1 caused a slight though not significant upregulation, whereas it had no considerable effect on **PTGS2** expression among AA.

AA started with a significantly lower baseline expression of **PTGS2** than HC (see section **4.3**). Since stimulation induced a strong upregulation of **PTGS2** in both groups, these differences remained significant after stimulation for almost all stimuli. Only after stimulation with Fi the difference was less pronounced but still lower among AA.

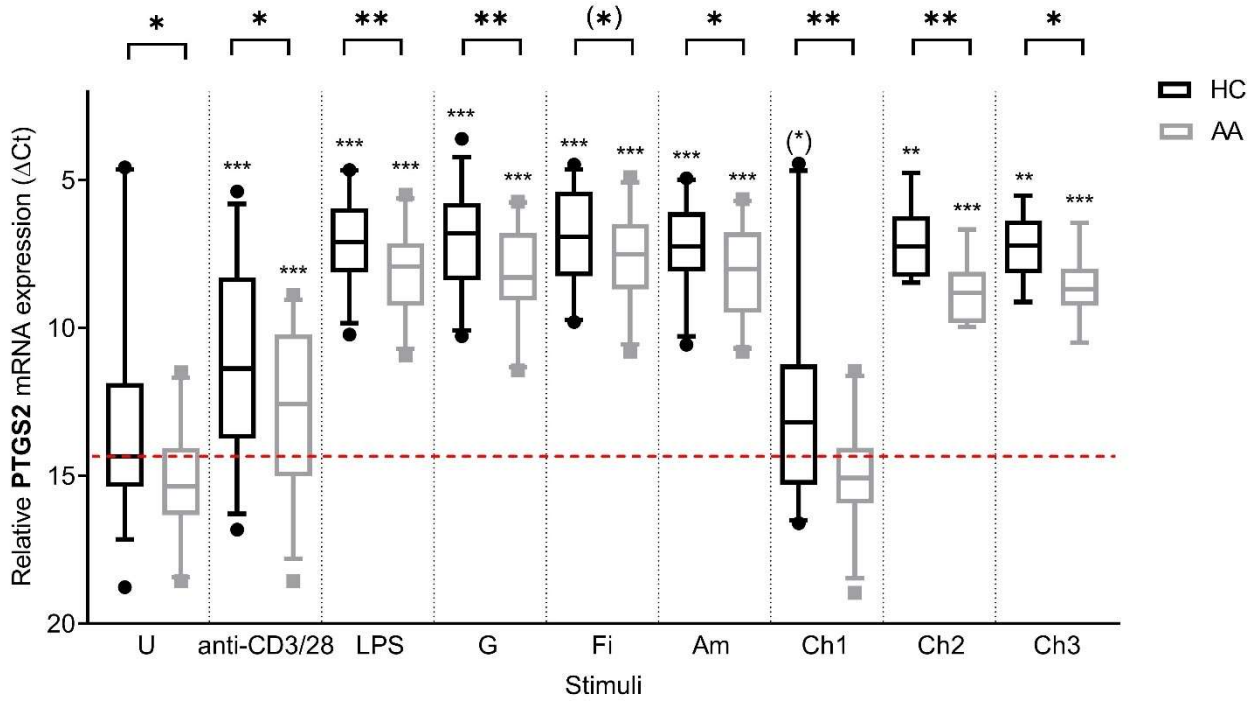

Figure 25: Changes in PTGS2 mRNA expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of PTGS2 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Unstimulated condition (U), anti-CD3/28 (anti-CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Boxes show medians and interquartile ranges, whiskers indicate 5-95 percentiles, values beyond are indicated as circles for HC and squares for AA. The red dotted line indicates the median expression level of HC under unstimulated condition. **Differences between phenotypes are indicated on top of the graph** and are analysed by Welch's t-test for normally and by Mann-Whitney-U-test for not normally distributed data. **Asterisks on top of the box plots indicate tendencies and significant differences upon stimulation for each phenotype in relation to their expression under unstimulated condition**. Differences upon stimulation are analysed by paired t-test for normally distributed data and Wilcoxon matched-pairs signed rank test for not normally distributed data. P-value not significant (ns), (*) $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Upon stimulation, changes in **BIRC3** expression were similar to **PTGS2**. All stimuli except for Ch1 induced significant upregulation of **BIRC3** in both groups. Upon Ch2 stimulation, AA reached a similar gene expression level as unstimulated HC.

AA started with a significantly lower baseline expression of **BIRC3** than HC (see section **4.3**). Even though stimulation induced a strong upregulation of **BIRC3** in both groups, these differences were no longer significant after stimulation.

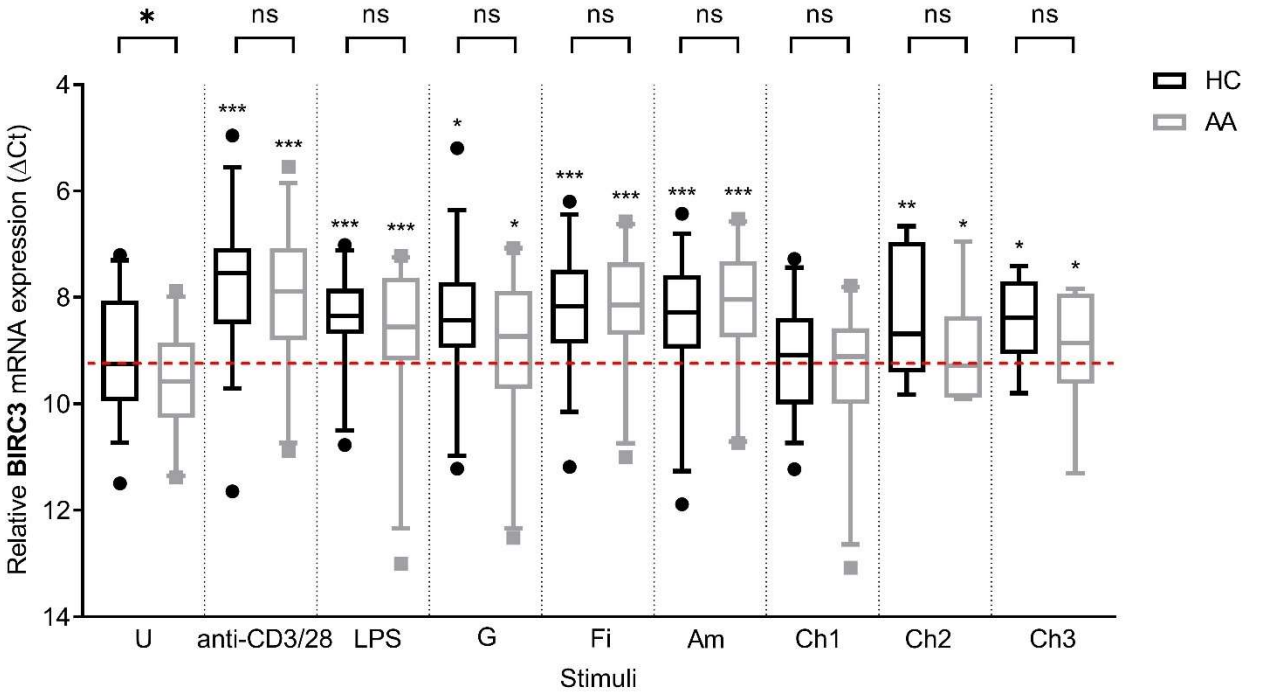

Figure 26: Changes in BIRC3 mRNA expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of BIRC3 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Unstimulated condition (U), anti-CD3/28 (anti-CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Boxes show medians and interquartile ranges, whiskers indicate 5-95 percentiles, values beyond are indicated as circles for HC and squares for AA. The red dotted line indicates the median expression level of HC under unstimulated condition. **Differences between phenotypes are indicated on top of the graph** and are analysed by Welch's t-test for normally and by Mann-Whitney-U-test for not normally distributed data. **Asterisks on top of the box plots indicate tendencies and significant differences upon stimulation for each phenotype in relation to their expression under unstimulated condition**. Differences upon stimulation are analysed by paired t-test for normally distributed data and Wilcoxon matched-pairs signed rank test for not normally distributed data. P-value not significant (ns), (*) $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In HC, **NFKBIA** was significantly upregulated upon stimulation with anti-CD3/28 and LPS. G and Am induced a tendency towards upregulation. On the contrary, Fi, Ch1, Ch2 and C3 downregulated **NFKBIA** expression with Fi, Ch1 and Ch3 reaching statistical significance (however only marginal difference in **NFKBIA** expression after stimulation with Fi). In AA, all stimuli except for Ch1, which had no considerable impact on gene expression, induced upregulation of **NFKBIA**, mostly reaching statistical significance. Upon stimulation with LPS, Fi, Am and Ch3, AA expressed similar **NFKBIA** levels as unstimulated HC.

AA started with a significantly lower baseline expression of **NFKBIA** than HC (see section **4.3**). Since most stimuli induced a strong upregulation of **NFKBIA** in AA and some induced a downregulation in HC, these differences were no longer significant after stimulation. As the effect of stimulation with German dust was weaker, the difference in **NFKBIA** expression levels after stimulation with German dust remained significant.

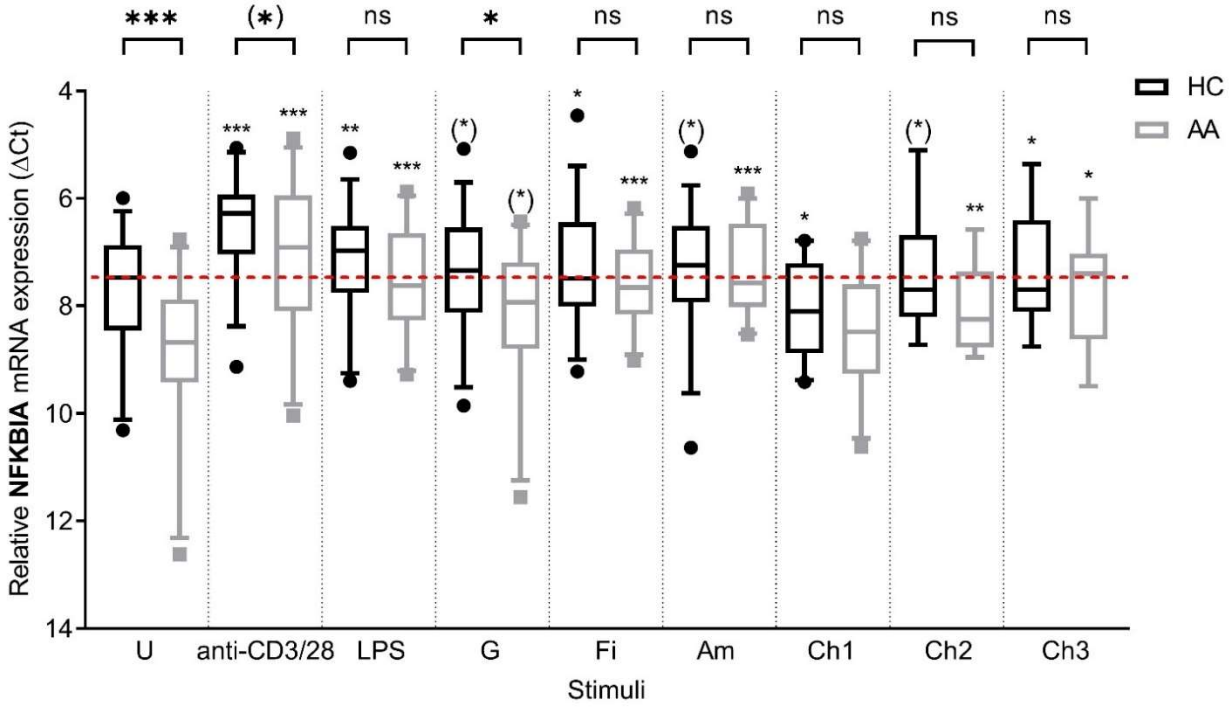

Figure 27: Changes in NFKBIA mRNA expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of NFKBIA in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Unstimulated condition (U), anti-CD3/28 (anti-CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Boxes show medians and interquartile ranges, whiskers indicate 5-95 percentiles, values beyond are indicated as circles for HC and squares for AA. The red dotted line indicates the median expression level of HC under unstimulated condition. **Differences between phenotypes are indicated on top of the graph** and are analysed by Welch's t-test for normally and by Mann-Whitney-U-test for not normally distributed data. **Asterisks on top of the box plots indicate tendencies and significant differences upon stimulation for each phenotype in relation to their expression under unstimulated condition**. Differences upon stimulation are analysed by paired t-test for normally distributed data and Wilcoxon matched-pairs signed rank test for not normally distributed data. P-value not significant (ns), (*) $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

MAP3K7 expression significantly increased upon anti-CD3/28 exposure in both HC and AA. LPS induced a slight yet significant downregulation of **MAP3K7** in HC. G and Ch1 induced a stronger and also significant downregulation in HC. Likewise, Am and Ch3 showed a downwards tendency, whereas Fi and Ch2 had no considerable effects among HC. In AA, neither LPS nor any of the dusts induced significant changes in **MAP3K7** expression.

There were no significant differences between HC and AA when comparing **MAP3K7** expression levels both at baseline (see section **4.3**) and upon stimulation.

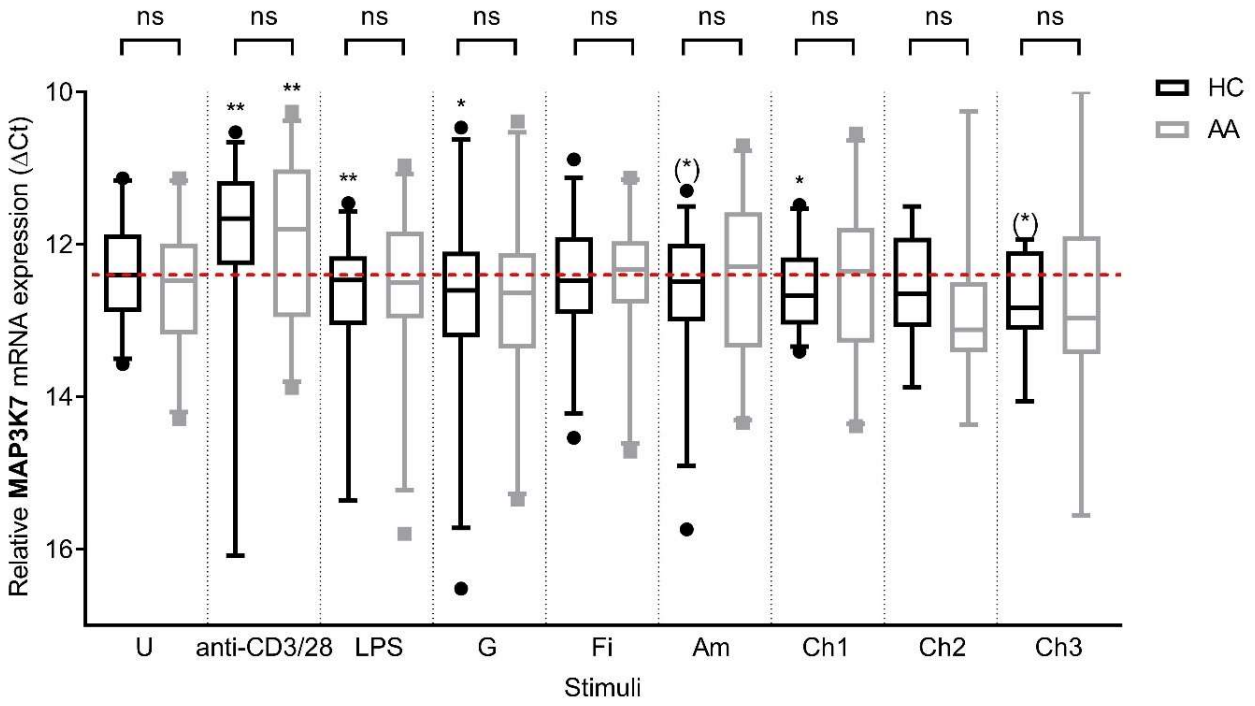

Figure 28: Changes in MAP3K7 mRNA expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of MAP3K7 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Unstimulated condition (U), anti-CD3/28 (anti-CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Boxes show medians and interquartile ranges, whiskers indicate 5-95 percentiles, values beyond are indicated as circles for HC and squares for AA. The red dotted line indicates the median expression level of HC under unstimulated condition. **Differences between phenotypes are indicated on top of the graph** and are analysed by Welch's t-test for normally and by Mann-Whitney-U-test for not normally distributed data. **Asterisks on top of the box plots indicate tendencies and significant differences upon stimulation for each phenotype in relation to their expression under unstimulated condition**. Differences upon stimulation are analysed by paired t-test for normally distributed data and Wilcoxon matched-pairs signed rank test for not normally distributed data. P-value not significant (ns), (*) $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

MAPK1 expression significantly increased upon stimulation with anti-CD3/28 among AA and slightly among HC. Upon stimulation with LPS, G, Fi, Am and Ch3, **MAPK1** expression significantly decreased among HC. Neither LPS nor any of the dusts showed significant effects on **MAPK1** expression among AA.

Neither baseline expression (see section **4.3**) nor expression levels after stimulation were significantly different between HC and AA.

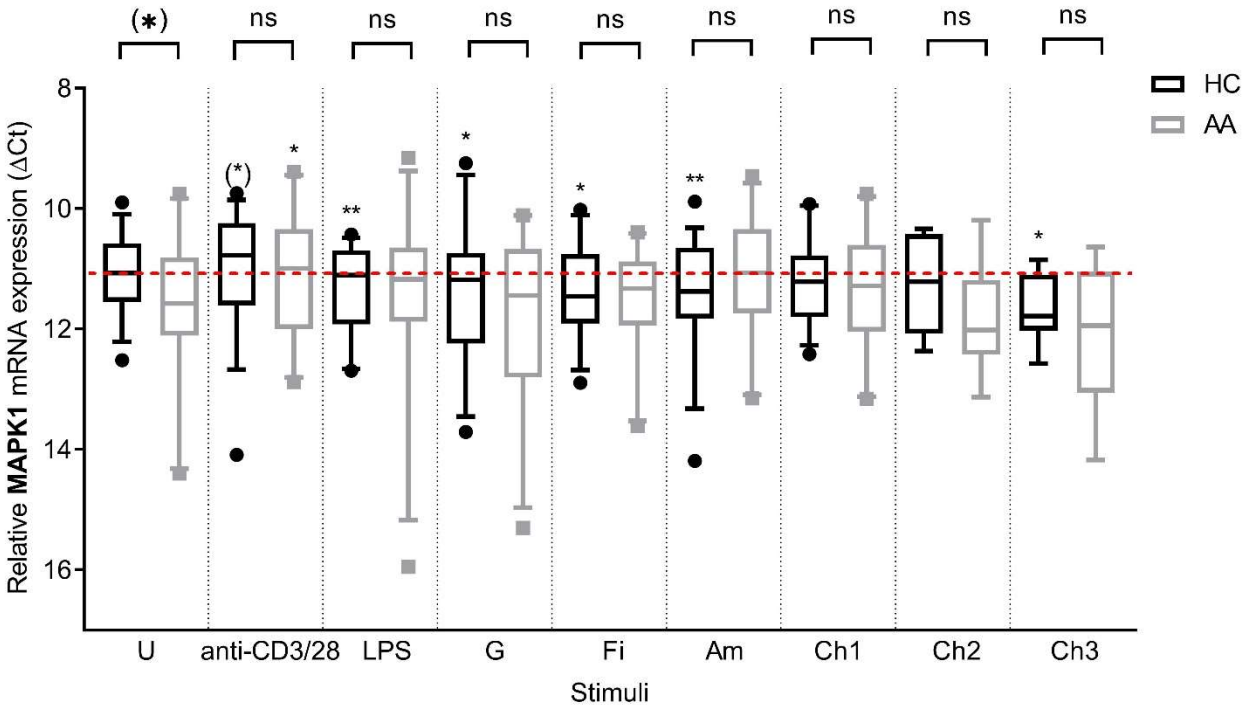

Figure 29: Changes in MAPK1 mRNA expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of MAP3K14 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Unstimulated condition (U), anti-CD3/28 (anti-CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Boxes show medians and interquartile ranges, whiskers indicate 5-95 percentiles, values beyond are indicated as circles for HC and squares for AA. The red dotted line indicates the median expression level of HC under unstimulated condition. **Differences between phenotypes are indicated on top of the graph** and are analysed by Welch's t-test for normally and by Mann-Whitney-U-test for not normally distributed data. **Asterisks on top of the box plots indicate tendencies and significant differences upon stimulation for each phenotype in relation to their expression under unstimulated condition**. Differences upon stimulation are analysed by paired t-test for normally distributed data and Wilcoxon matched-pairs signed rank test for not normally distributed data. P-value not significant (ns), (*) $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

MAPK9 expression significantly increased upon stimulation with anti-CD3/28 in both groups. In HC, LPS and all dusts except for Ch2, which showed no significant effect, induced a significant decrease in **MAPK9** expression. In AA on the contrary, neither LPS nor any of the dusts showed significant effects on **MAPK9** expression.

Neither baseline expression (see section **4.3**) nor expression levels after stimulation were significantly different between HC and AA.

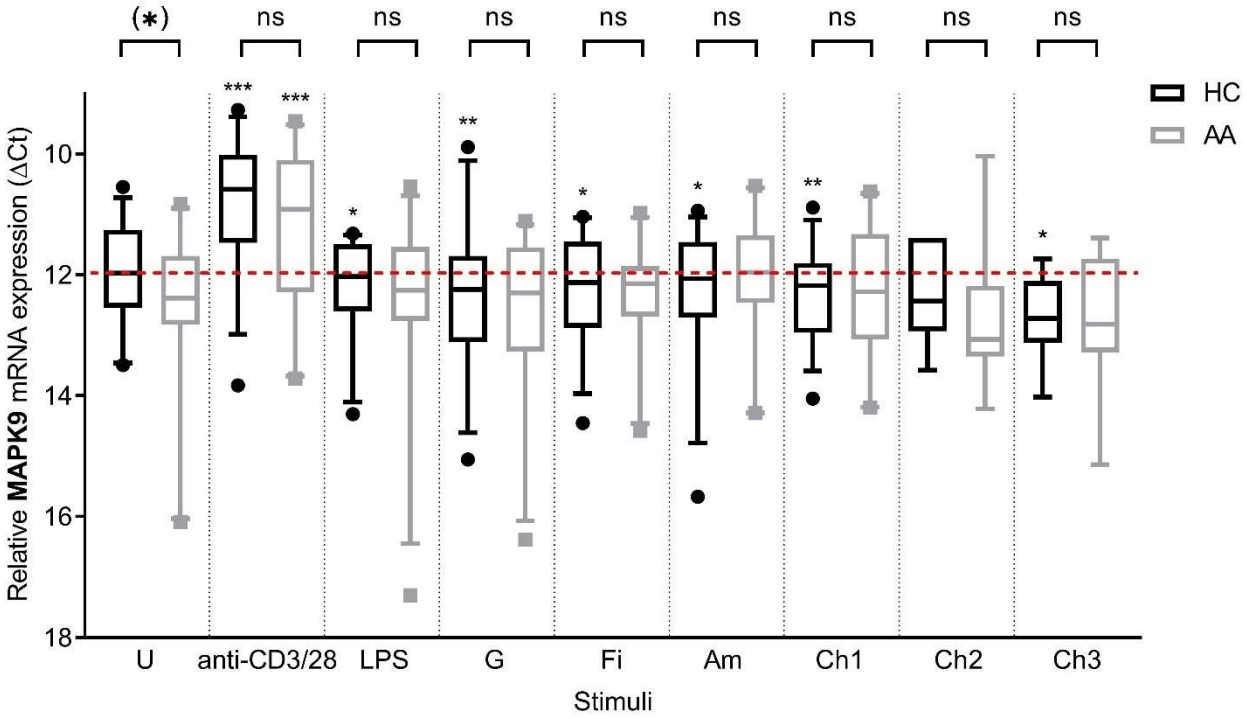

Figure 30: Changes in MAPK9 mRNA expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of MAPK9 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Unstimulated condition (U), anti-CD3/28 (anti-CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Boxes show medians and interquartile ranges, whiskers indicate 5-95 percentiles, values beyond are indicated as circles for HC and squares for AA. The red dotted line indicates the median expression level of HC under unstimulated condition. **Differences between phenotypes are indicated on top of the graph** and are analysed by Welch's t-test for normally and by Mann-Whitney-U-test for not normally distributed data. **Asterisks on top of the box plots indicate tendencies and significant differences upon stimulation for each phenotype in relation to their expression under unstimulated condition**. Differences upon stimulation are analysed by paired t-test for normally distributed data and Wilcoxon matched-pairs signed rank test for not normally distributed data. P-value not significant (ns), (*) $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Expression of **MAPK14** was slightly though not significantly upregulated upon stimulation with anti-CD3/28 in HC, but not in AA. In HC, LPS and all dusts except for Ch2, which showed no significant effect, induced a significant decrease in **MAPK14** expression. In AA, only G induced a significant decrease in **MAPK14** expression, whereas neither LPS nor any of the other dusts had significant effects.

Neither baseline expression (see section **4.3**) nor expression levels after stimulation were significantly different between HC and AA. After stimulation with Am, **MAPK14** expression was slightly though not significantly lower among HC.

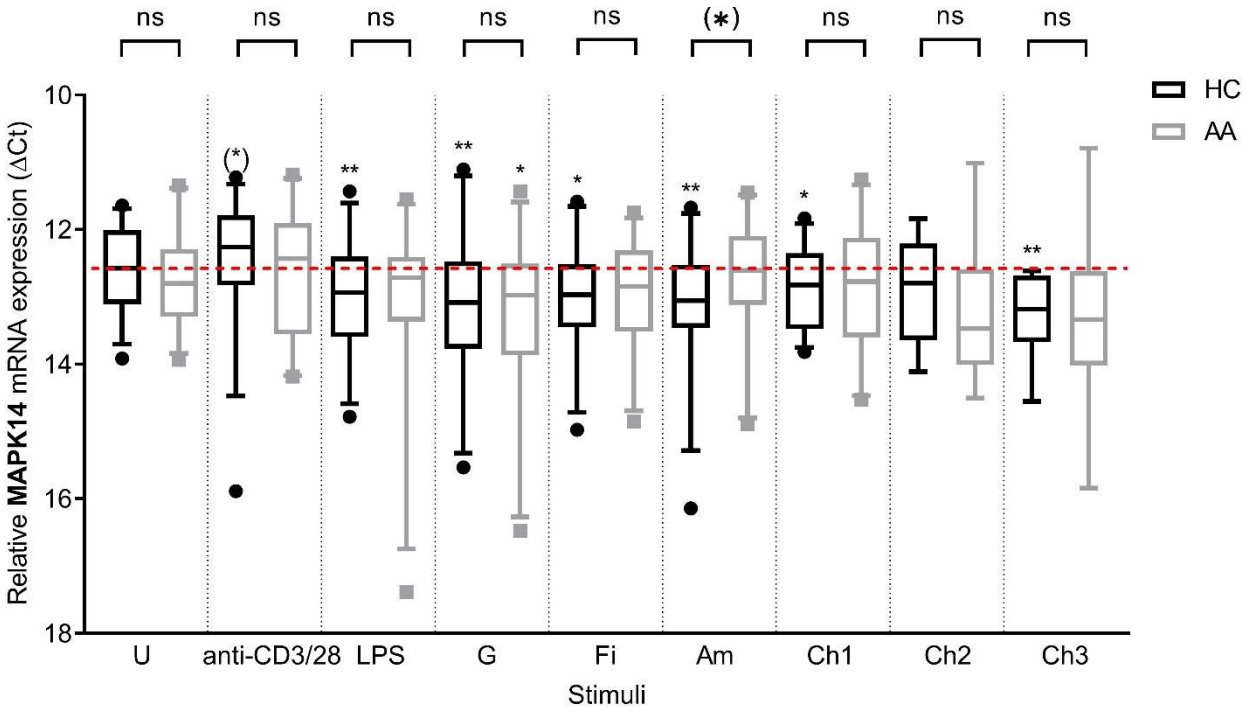

Figure 31: Changes in MAPK14 mRNA expression levels upon stimulation (∆Ct values)
Relative mRNA expression levels of MAPK14 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ∆Ct. Unstimulated condition (U), anti-CD3/28 (anti-CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Boxes show medians and interquartile ranges, whiskers indicate 5-95 percentiles, values beyond are indicated as circles for HC and squares for AA. The red dotted line indicates the median expression level of HC under unstimulated condition. **Differences between phenotypes are indicated on top of the graph** and are analysed by Welch's t-test for normally and by Mann-Whitney-U-test for not normally distributed data. **Asterisks on top of the box plots indicate tendencies and significant differences upon stimulation for each phenotype in relation to their expression under unstimulated condition**. Differences upon stimulation are analysed by paired t-test for normally distributed data and Wilcoxon matched-pairs signed rank test for not normally distributed data. P-value not significant (ns), (*) $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

MAPK7 expression significantly increased upon stimulation with anti-CD3/28 in both groups. In HC, LPS, Am, Ch1 and Ch3 induced a significant downregulation, whereas the other dusts showed no significant effect. In AA, neither LPS nor any of the other dusts had a significant impact on **MAPK7** expression.

Neither baseline expression (see section **4.3**) nor expression levels after stimulation with LPS or farm dust were significantly different between HC and AA. After stimulation with anti-CD3/28, **MAPK7** expression was significantly higher among HC. However, when adjusting for older siblings this finding was no longer significant (p = 0.158, for detailed analysis please see section **4.5.1**).

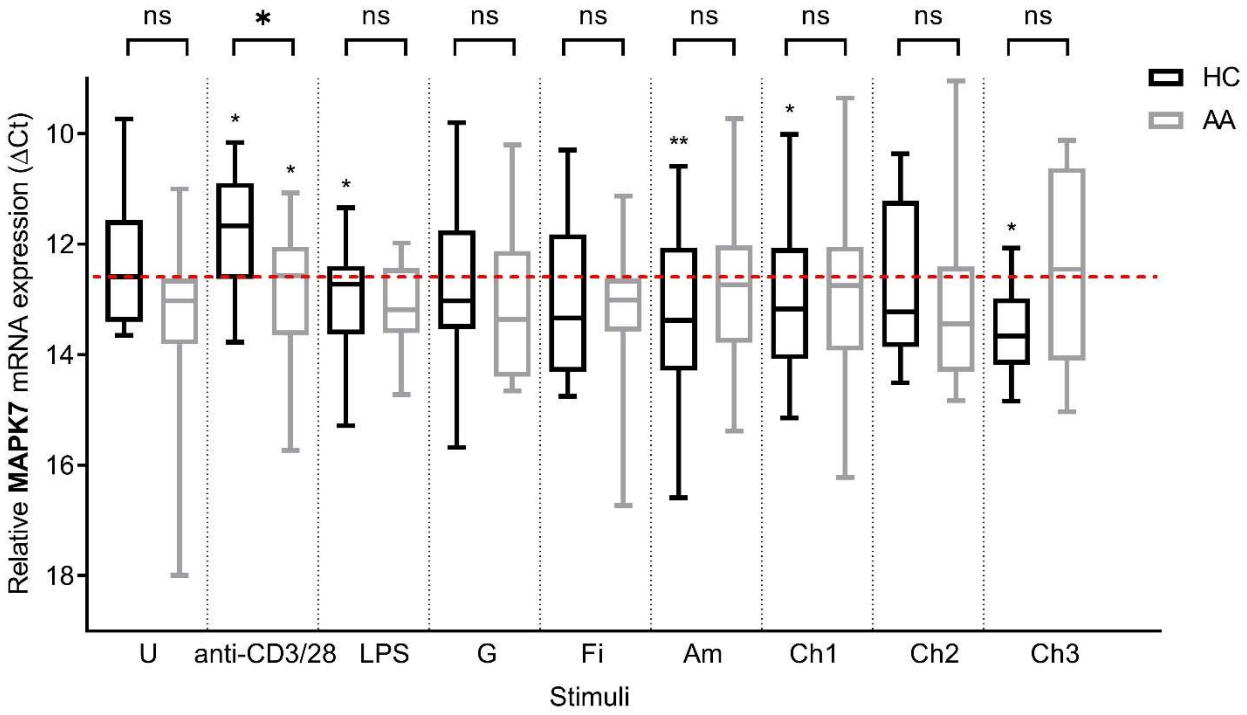

Figure 32: Changes in MAPK7 mRNA expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of MAPK7 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Unstimulated condition (U), anti-CD3/28 (anti-CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Boxes show medians and interquartile ranges, whiskers indicate 5-95 percentiles, values beyond are indicated as circles for HC and squares for AA. The red dotted line indicates the median expression level of HC under unstimulated condition. **Differences between phenotypes are indicated on top of the graph** and are analysed by Welch's t-test for normally and by Mann-Whitney-U-test for not normally distributed data. **Asterisks on top of the box plots indicate tendencies and significant differences upon stimulation for each phenotype in relation to their expression under unstimulated condition**. Differences upon stimulation are analysed by paired t-test for normally distributed data and Wilcoxon matched-pairs signed rank test for not normally distributed data. P-value not significant (ns), (*) p < 0.1, * p < 0.05, ** p < 0.01, *** p < 0.001.

Upon stimulation with anti-CD3/28, **DUSP1** expression was significantly downregulated in HC, but not in AA. In HC, neither LPS nor any of the dusts had a significant impact on **DUSP1** expression. In AA, **DUSP1** expression was significantly upregulated upon LPS stimulation and slightly, though not significantly upregulated upon stimulation with G, Fi, Ch1 and Ch3.

AA started with a significantly lower baseline expression of **DUSP1** than HC (see section **4.3**). Since most stimuli induced an upregulation of **DUSP1** in AA, these differences were no longer significant after stimulation for most stimuli except for Ch2 which had no considerable effect on **DUSP1** expression in either of the groups. As anti-CD3/28 induced a strong downregulation of **DUSP1** in HC, the difference was also no longer significant.

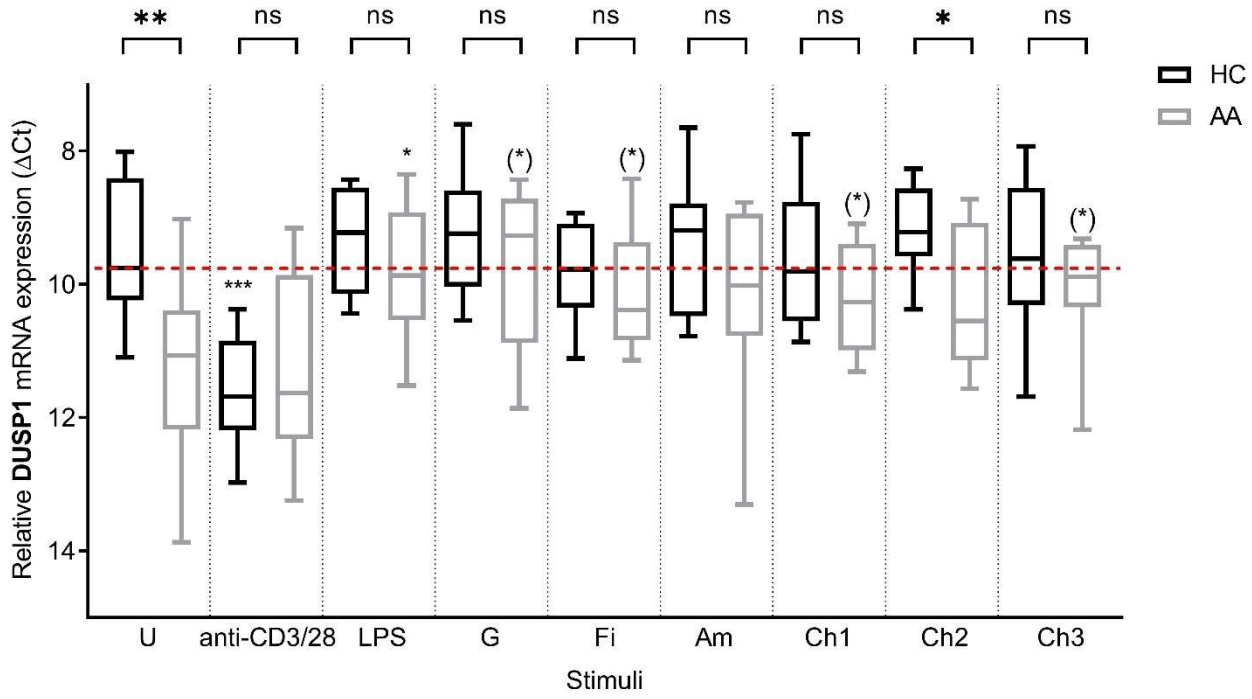

Figure 33: Changes in DUSP1 mRNA expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of DUSP1 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt. Unstimulated condition (U), anti-CD3/28 (anti-CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Boxes show medians and interquartile ranges, whiskers indicate 5-95 percentiles, values beyond are indicated as circles for HC and squares for AA. The red dotted line indicates the median expression level of HC under unstimulated condition. **Differences between phenotypes are indicated on top of the graph** and are analysed by Welch's t-test for normally and by Mann-Whitney-U-test for not normally distributed data. **Asterisks on top of the box plots indicate tendencies and significant differences upon stimulation for each phenotype in relation to their expression under unstimulated condition**. Differences upon stimulation are analysed by paired t-test for normally distributed data and Wilcoxon matched-pairs signed rank test for not normally distributed data. P-value not significant (ns), (*) $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.5.1 Confounder analysis

As stated in section **4.1,** significantly different characteristics between HC and AA were investigated for possible confounding effects. All significantly different categories other than related atopic diseases were analysed, namely “older siblings”, “any indoor pets currently” and “any indoor pets during the first or second year of life” (compare **Table 6**). This was done for all significantly different findings regarding mRNA expression levels between HC and AA summarised in **Table 11**.

Gene	Stimulus	HC ΔCT median (IQR)	AA ΔCT median (ICR)	p-value
RIPK1	U	11.83 (11.22-12.28)	12.47 (11.81-15.54)	**0.006**[b]
IRAK4	U	12.65 (12.40-13.01)	13.03 (12.52-13.74)	**0.025**[a]
PTGS2	U	14.35 (12.01-15.21)	15.36 (14.21-16.17)	**0.012**[b]
PTGS2	anti-CD3/28	11.37 (8.450-13.59)	12.58 (10.38-14.86)	**0.045**[a]
PTGS2	LPS	7.10 (6.11-7.96)	7.92 (7.29-9.10)	**0.007**[a]
PTGS2	G	6.80 (5.93-8.22)	8.29 (6.94-8.91)	**0.007**[a]
PTGS2	Am	7.23 (6.24-7.92)	8.00 (6.92-9.32)	**0.046**[a]
PTGS2	Ch1	13.19 (11.39-15.14)	15.07 (14.21-15.78)	**0.003**[b]
PTGS2	Ch2	7.243 (6.38-8.11)	8.81 (8.25-9.67)	**0.004**[a]
PTGS2	Ch3	7.21 (6.52-7.99)	8.68 (8.15-9.09)	**0.014**[a]
BIRC3	U	9.25 (8.14-9.86)	9.57 (8.93-1.17)	**0.033**[a]
NFKBIA	U	7.47 (6.95-8.37)	8.68 (7.96-9.33)	**< 0.001**[b]
NFKBIA	G	7.34 (6.63-8.04)	7.93 (7.28-8.71)	**0.034**[b]
MAPK7	anti-CD3/28	11.67 (10.98-12.54)	12.57 (12.13-13.56)	**0.017**[a]
DUSP1	U	9.75 (8.47-10.17)	11.07 (10.46-12.11)	**0.009**[a]
DUSP1	Ch2	9.21 (8.63-9.51)	10.55 (9.14-11.07)	**0.021**[a]

Table 11: Significant differences in mRNA expression levels between HC and AA
Relative mRNA expression levels in PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt with interquartile ranges (IQR) in brackets. Unstimulated condition (U), anti-CD3/28 (anti-CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Only significantly different findings between phenotypes are displayed. Differences are analysed by Welch’s t-test ([a]) for normally distributed data and by Mann-Whitney-U-test ([b]) for not normally distributed data.

When grouping according to the presence or absence of older siblings regardless of the phenotype there were only significant differences in mRNA expression levels of **MAPK7** after stimulation with anti-CD3/28 with children having older siblings expressing significantly higher levels of **MAPK7** ($p = 0.017$). To further illustrate this observation, **Table 12** shows ΔCt values of **MAPK7** after exposure to anti-CD3/28 stratified for older siblings. After adjusting for older siblings by multiple linear regression, the difference was no longer significant but showed the same direction (estimate, i.e. mean difference of ΔCt between phenotypes = 0.659, $p = 0.158$).

	HC ΔCT median (IQR)	**AA ΔCT median (IQR)**
Older siblings	11.42 (10.44-12.31)	12.46 (12.36-12.57)
No older siblings	12.40 (11.60-13.00)	12.86 (11.90-13.65)

Table 12: Analysis of MAPK7 expression levels after stimulation with anti-CD3/28 stratified for older siblings
Relative mRNA expression levels in PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt with interquartile ranges (IQR) in brackets.

There were no significant differences in gene expression levels for the categories with/without “any indoor pets currently” and with/without “any indoor pets during the first or second year of life”. Hence, adjusting for these categories was not necessary.

4.5.2 Summary of gene expression changes upon stimulation

Table 13 and **Table 14** give a summary of gene expression changes (ΔCt values) upon stimulation for HC and AA. Significant upregulation is marked with two red arrows, tendencies ($p < 0.1$) towards upregulation with one red arrow. Significant downregulation is marked with two blue arrows, tendencies towards downregulation with one blue arrow. No considerable effect on gene expression is marked with ↔. When comparing the two phenotypes it becomes clear that exposure to farm dust has different effects on regulation of NF-κB- and MAPK-related gene expressions. The differences are discussed in more detail in section **4.6** and **5.2**.

	anti-CD3/28	LPS	G	Fi	Am	Ch1	Ch2	Ch3
MAP3K14	↑↑	↓	↔	↔	↔	↔	↔	↔
RIPK1	↔	↔	↔	↔	↓	↓↓	↔	↔
IRAK4	↔	↓↓	↔	↔	↓↓	↔	↔	↓
PTGS2	↑↑	↑↑	↑↑	↑↑	↑↑	↑	↑↑	↑↑
BIRC3	↑↑	↑↑	↑↑	↑↑	↑↑	↔	↑↑	↑↑
NFKBIA	↑↑	↑↑	↑	↓↓	↑	↓↓	↓	↓↓
MAP3K7	↑↑	↓↓	↓↓	↔	↓	↓↓	↔	↓
MAPK1	↑	↓↓	↓↓	↓↓	↓↓	↔	↔	↓↓
MAPK9	↑↑	↓↓	↓↓	↓↓	↓↓	↓↓	↔	↓↓
MAPK14	↑	↓↓	↓↓	↓↓	↓↓	↓↓	↔	↓↓
MAPK7	↑↑	↓↓	↔	↔	↓↓	↓↓	↔	↓↓
DUSP1	↓↓	↔	↔	↔	↔	↔	↔	↔

Table 13: Summary of stimulatory effects (ΔCt values) in HC
Summary of ΔCt values normalised to 18S for healthy controls (HC). Up- and down regulation is colour coded with red indicating up- and a blue indicating downregulation. Significant changes (i.e. $p < 0.05$) upon stimulation are marked by two arrows (↑↑/↓↓), tendencies (i.e. $p < 0.1$) are marked by one arrow (↑/↓). ↔ indicates no considerable stimulatory effect on gene expression levels. Unstimulated condition (U), anti-CD3/28 (anti-CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3).

	anti-CD3/28	LPS	G	Fi	Am	Ch1	Ch2	Ch3
MAP3K14	↑↑	↔	↔	↔	↑	↑	↑↑	↔
RIPK1	↑↑	↔	↔	↔	↑↑	↔	↔	↔
IRAK4	↑↑	↔	↔	↔	↔	↔	↔	↔
PTGS2	↑↑	↑↑	↑↑	↑↑	↑↑	↔	↑↑	↑↑
BIRC3	↑↑	↑↑	↑↑	↑↑	↑↑	↔	↑↑	↑↑
NFKBIA	↑↑	↑↑	↑	↑↑	↑↑	↔	↑↑	↑↑
MAP3K7	↑↑	↔	↔	↔	↔	↔	↔	↔
MAPK1	↑↑	↔	↔	↔	↔	↔	↔	↔
MAPK9	↑↑	↔	↔	↔	↔	↔	↔	↔
MAPK14	↔	↔	↓↓	↔	↔	↔	↔	↔
MAPK7	↑↑	↔	↔	↔	↔	↔	↔	↔
DUSP1	↔	↑↑	↑	↑	↔	↑	↔	↑

Table 14: Summary of stimulatory effects (ΔCt values) in AA
Summary of ΔCt values normalised to 18S for allergic asthmatics (AA). For detailed description please see **Table 13**.

4.6 Different regulatory patterns of NF-κB and MAPK pathway related genes expressed as fold changes

The fold change describes the x-fold increase or decrease in gene expression levels upon stimulation in relation to the housekeeping gene (18S) as well as to baseline expression levels, i.e. the unstimulated condition (U). Hence, they were slightly different compared to ΔCt values shown in section **4.3** and **0**. Fold changes were calculated as $2^{-\Delta\Delta Ct}$. In **Figure 34** and **Figure 35**, the $\log_2$ scale was applied for visualisation in heat maps. Gene up- and downregulation was colour coded with red indicating an upregulation and blue indicating a downregulation. The colour gradient indicates the degree of change.

Figure 34 shows that in HC farm dust exposure generally led to a decrease (although not always statistically significant) in genes that were pro-inflammatory and involved in innate immune responses (**MAP3K14**, **RIPK1**, **IRAK4**). For these same genes, the impact of dust exposure was much less pronounced in AA. In fact, in AA, dust exposure even caused an increase in **MAP3K14** and **RIPK1** expression. However, as stated in section **4.3** AA had generally lower baseline gene expression levels of **RIPK1**. **PTGS2** expression was strongly upregulated upon all stimuli except for Ch1 in HC and AA. In both groups, anti-apoptotic **BIRC3** and anti-inflammatory **NFKBIA** expressions levels were upregulated upon exposure to farm dust. The colour coding illustrates that this modulatory effect was generally stronger among AA compared with HC. Again, Ch1 dust had no considerable influence in AA, but slightly downregulated **NFKBIA** expression in HC.

As shown in **Figure 35**, farm dust exposure had similar effects on MAPK signalling compared to NF-κB signalling. Pro-inflammatory and innate immunity genes (**MAP3K7**, **MAPK1**, **MAPK9**, **MAPK14**, **MAPK7**) were downregulated in HC, mostly reaching statistical significance. In AA, **MAP3K7**, **MAPK1**, **MAPK9** and **MAPK7** were less affected, with a slight tendency towards upregulation. **MAPK14** was significantly downregulated upon stimulation with German dust in AA. LPS exposure strongly induced anti-inflammatory **DUSP1** expression in AA, while it had no considerable impact on HC. Likewise, stimulation with G, Fi, Ch1 and Ch3 induced tendencies towards upregulation of **DUSP1** in AA but showed hardly any effects in HC.

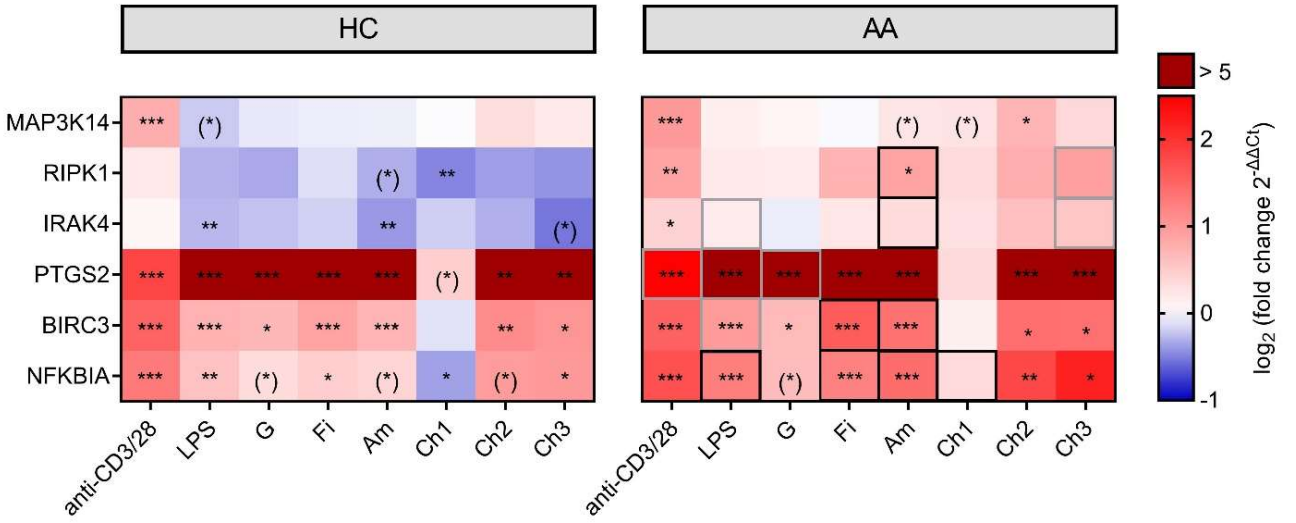

Figure 34: Stimulatory effects on NF-κB related gene expressions indicated as log_2 (fold change $2^{-\Delta\Delta Ct}$) Healthy controls (HC), allergic asthmatics (AA), unstimulated condition (U), anti-CD3/28 (anti CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Up- and down regulation is colour coded with a red- (up)/blue (down)-gradient. *Significant changes upon stimulation for each phenotype are marked by asterisks: (*) p < 0.1, * p < 0.05, ** p < 0.01, *** p < 0.001. Significantly different regulations between phenotypes (HC vs. AA) are marked with black (p < 0.05) or grey (p < 0.1) rectangles*.

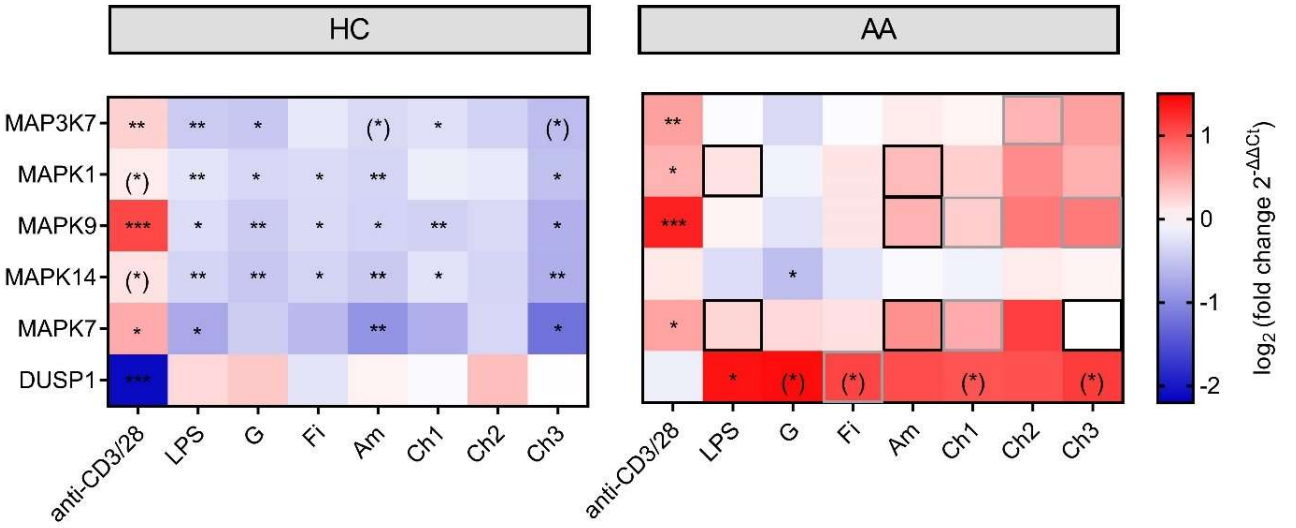

Figure 35: Stimulatory effects on MAPK related gene expressions indicated as log_2 (fold change $2^{-\Delta\Delta Ct}$) Healthy controls (HC), allergic asthmatics (AA), unstimulated condition (U), anti-CD3/28 (anti CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Up- and down regulation is colour coded with a red- (up)/blue (down)-gradient. *Significant changes upon stimulation for each phenotype are marked by asterisks: (*) p < 0.1, * p < 0.05, ** p < 0.01, *** p < 0.001. Significantly different regulations between phenotypes (HC vs. AA) are marked with black (p < 0.05) or grey (p < 0.1) rectangles*.

4.7 Summary of the key findings

1. Baseline expression levels of pro-inflammatory genes (**PTGS2**) and genes involved in regulation of innate immunity (**RIPK1**, **IRAK4**) were significantly lower in allergic asthmatics compared with healthy controls.
2. Baseline expression levels of anti-apoptotic **BIRC3**, a positive regulator of canonical and a negative regulator of non-canonical NF-κB signalling, were significantly lower in allergic asthmatics compared with healthy controls.
3. Baseline expression levels of anti-inflammatory NF-κB and MAPK related genes (**NFKBIA** and **DUSP1**) were also significantly lower in allergic asthmatics compared with healthy controls.
4. Expression levels of genes involved in NF-κB and MAPK signalling pathways could be modulated by *ex vivo* exposure to “asthma protective” farm dust extracts or LPS.
   4.1. In healthy controls, pro-inflammatory and innate immunity gene expression levels (**MAP3K14**, **RIPK1**, **IRAK4**, **MAP3K7**, **MAPK1**, **MAPK9**, **MAPK14**, **MAPK7**) were downregulated upon stimulation with farm dust extracts or LPS.
   4.2. In allergic asthmatics, farm dust or LPS exposure had only little impact on pro-inflammatory and innate immunity gene expression levels.
      4.2.1. Only **MAPK14** was significantly downregulated upon stimulation with German farm dust.
      4.2.2. **MAP3K14** was slightly upregulated upon stimulation with Amish and Chinese farm dusts 1 and 2.
      4.2.3. **RIPK1** was significantly upregulated upon stimulation with Amish farm dust, now reaching a “healthy baseline expression level”.
   4.3. In both phenotypes, anti-apoptotic **BIRC3** and anti-inflammatory **NFKBIA** were (mostly) significantly upregulated upon stimulation with farm dust extracts or LPS.
   4.4. In healthy controls, who already started with higher baseline expression levels, **DUSP1** expression levels did not change significantly upon stimulation with farm dust extracts or LPS.
   4.5. In allergic asthmatics, anti-inflammatory **DUSP1** was also significantly upregulated upon stimulation with LPS and showed tendencies towards upregulation upon stimulation with German, Finnish and Chinese farm dusts 1 and 2.
5. Thus, *ex vivo* PBMC stimulation with farm dust extracts or LPS may help restoring impaired asthmatic gene expression levels to a „healthy level”.

5 Discussion

For over 20 years, epidemiological studies have provided striking evidence for the existence of the so-called farm effect which describes the protection from asthma and allergies in children growing up on traditional farms. However, its underlying immunological mechanisms are still poorly understood. The purpose of this study was to further disentangle its (patho)physiology and to identify genes that are susceptible to farm dust exposure.

In the following chapters, similarities and differences between the investigated subjects' characteristics will be discussed first in section **5.1**, followed by the interpretation of the effect of farm dust exposure on NF-κB and MAPK related genes in section **5.2**. Differences between the farm dust extracts are discussed in section **5.3**. Eventually, strengths and limitations of the study are discussed in section **5.4.**

5.1 Similarities and differences in socio-demographic and clinical characteristics between phenotypes

34 healthy children and 24 allergic asthmatics were investigated in this study. Overall, the groups were similar regarding socio-demographic and family history data. The proportion of female sex was lower among AA compared with HC, yet this difference was not statistically significant. A large number of studies revealed differences in sex distribution in childhood asthma with higher incidence rates among boys, an observation which seems to switch after puberty [127, 128]. Asthma is frequently associated with related atopic disorders such as food allergy, atopic dermatitis, and hay fever [129-132] and all three conditions were common amongst AA in the present study population. Mothers of AA had generally higher education levels than mothers of HC, an observation which is well in line with previous studies reporting higher rates of atopic diseases and allergic sensitisation in children from educated parents [133, 134].

Besides related atopic disorders, significant differences were only found in the categories "older siblings", "any indoor pets currently" and "any indoor pets during the first or second year of life" When comparing sibship size and order, HC had both more and older siblings than AA. Ever since Strachan proposed the hygiene hypothesis in 1989 based on his observation that children growing up in larger families had lower rates of hay fever and eczema [135], this has been an interesting research field. Some studies found lower asthma

rates in younger siblings [136], whereas others found no difference in birth order but lower rates in larger families [137, 138]. Possible explanations for these observations are related to immune maturation through protective or harmful effects of infections, vaccinations, hygiene, use of antibiotics etc. Other concepts suggest endocrine effects and in utero programming to be responsible for the sibling effect. Identifying causal factors might help preventing at least 30% of asthma, hay fever and eczema [138]. Confounder analysis revealed significant differences for older siblings in **MAPK7** expression after stimulation with anti-CD3/28 with children having older siblings expressing higher **MAPK7** levels. Hence, adjustment for this gene and this stimulatory condition was performed.

Significantly more HC reported to have a pet at home. As allergies to animal fur are common amongst allergic asthmatics, the difference was not surprising. 11 AAs were sensitised to cat epithelium and 3 to dog hair. Hence, this observation might be influenced by reverse causation since atopic families may prefer to not keep pets [139]. Moreover, a range of retro- and prospective studies have reported a reduced risk of developing atopy in children exposed to indoor furry pets during the first years of life. This association is thought to be mediated through an increased microbial exposure [139]. As confounder analysis revealed no significant differences in gene expression levels, adjustment for keeping indoor pets was not necessary.

Analysis of the blood samples revealed that AA had significantly higher leukocyte counts than HC. This is in line with a study investigating systemic inflammation markers in newly diagnosed adult asthmatics that were either treatment-naïve or on rescue inhaler only. The authors found higher total leukocyte counts, higher CRP-levels and an increased erythrocyte sedimentation rate in asthmatics compared with healthy controls [140]. In the present study on the contrary, any child with infectious symptoms or a CRP > 0.5 mg/dl was excluded regardless of the phenotype to ensure that differences in gene expression were not due to general inflammation or infectious diseases. Also, both groups were within the normal range of total leukocyte counts. As PBMCs were stimulated in a final concentration of 2.5 x 10^6 cells/ml (see methods, section **3.6**), no further adjustment was performed.

AA had significantly higher proportions of eosinophils than HC. This is not surprising since eosinophils are the predominant leukocytes in asthma and their degree of involvement may correlate with severity of symptoms, airway obstruction and hyperresponsiveness and even predict exacerbations [5, 141, 142]. Interestingly, HC had significantly higher proportions of neutrophils than AA. Since both cell types, eosinophils and neutrophils were removed during

PBMC isolation, these differences should have no considerable impact on gene expression data. As already stated in section **4.1**, PBMCs consist of lymphocytes, dendritic cells, and monocytes [126]. AA had slightly lower proportions of monocytes than HC, but the difference was of marginal statistical and probably no clinical significance. In addition, monocyte counts did not correlate significantly with mRNA expression levels. The overall advantages and disadvantages of using PBMCs as sample material are discussed in section **5.4**.

Total IgE was much higher among AA reflecting the allergic phenotype. Also, unlike HC, AA frequently had elevated specific IgE to perennial, seasonal, and food allergens.

5.2 Immunomodulatory capacity of farm dust – effects on mRNA expression levels

The Schaub research group previously demonstrated the anti-inflammatory capacity of dust from German and Finnish farms by inhibiting pro-inflammatory NF-κB gene expression and upregulating anti-inflammatory TNFAIP3 [86, 87]. In the present study, additional genes of the NF-κB pathway and some genes of the MAPK pathway were investigated to further unravel the immunological mechanisms underlying the farm effect. In the following sections, the discussion of the findings starts with the interpretation of **MAP3K14** and the non-canonical cascade and continues with the pro-inflammatory and innate immunity genes of NF-κB and MAPK signalling. Eventually, the effects of farm dust stimulation on the anti-inflammatory genes are discussed. Of note, **BIRC3** plays contrary roles in canonical and non-canonical NF-κB signalling: while it enhances the canonical pathway, it suppresses the non-canonical pathway [81, 82]. Given its anti-apoptotic properties and in view of the similar regulatory pattern observed in this study (see **Figure 34**) it is discussed together with the anti-inflammatory genes **NFKBIA** and **DUSP1**.

5.2.1 Few effects of farm dust stimulation on MAP3K14, a central hub of the non-canonical NF-κB pathway

Most of the NF-κB pathway genes investigated in this study are part of the canonical cascade and are important for innate immunity. As the protective microbial farm environment is believed to shape innate immunity, they were of special interest for this research project. Yet, the two cascades do not work independently of each other, and the role of the non-

canonical pathway in the context of environmentally mediated protection from asthma is so far unclear. Hence, **MAP3K14** as a central hub of the non-canonical pathway [143] was also investigated.

The steady level of **MAP3K14** is typically low due to continuous ubiquitination-dependent degradation. Upon non-canonical pathway activation, **MAP3K14** is no longer degraded and can carry out its functions including the development of lymphoid organs and the survival and maturation of B cells. Aberrant activation of non-canonical NF-κB signalling can promote auto-immunity and inflammation [78]. Upregulated **MAP3K14** has been shown in several tumours such as multiple myeloma, T cell leukemia and Hodgkin Reed Sternberg cells [143]. In contrast to these findings, mice deficient in **MAP3K14** develop a spontaneous, progressive hyper eosinophilic syndrome-like condition with tissue and blood eosinophilia accompanied by organ damage and premature death [144]. The non-canonical NF-κB cascade is therefore involved in both normal immune response and inflammatory disease. So far, it is unclear whether it plays a role in the development of asthma.

In this study, baseline expression of **MAP3K14** did not differ significantly between PBMCs of HC and AA. Anti-CD3/28 induced a strong and significant increase of **MAP3K14** in both phenotypes. It mimics potent T cell stimulation thereby enhancing T cell proliferation and served as a positive control. Upon LPS stimulsecation, **MAP3K14** was slightly and almost significantly downregulated in HC, whereas there was no considerable effect in AA. Ch2 significantly upregulated **MAP3K14** in AA, and Amish and Chinese dust 1 showed tendencies towards upregulation. Overall, these effects were relatively small and none of the other dusts had considerable impact on **MAP3K14** expression.

Previous studies came to different conclusions regarding the activation of the non-canonical NF-κB pathway. While some suggested that LPS was not associated with its activation, since **MAP3K14** expression did not change upon stimulation with LPS [145, 146], others proposed the opposite [147, 148]. A study in human colonic epithelial cells found that LPS exposure induced phospho-**MAP3K14** with no change in total **MAP3K14** expression [149]. It would therefore be interesting to investigate phosphorylation patterns of **MAP3K14** on protein level as well as expression patterns over time.

Taken together, the findings of the present study did not show a consistent effect of farm dust exposure on MAP3K14 expression. However, as differences between HC and AA were identified, parts of the non-canonical NF-κB pathway may be regulated differently in asthmatics. Further studies investigating several non-canonical

mediators at once and especially studies investigating phosphorylation patterns will help understanding the effects of LPS and "asthma protective" farm dust exposure on the non-canonical NF-κB pathway and possible differences in asthmatic children.

5.2.2 Lower baseline expression levels of genes involved in innate immunity among allergic asthmatics and different regulations upon stimulation with farm dust extracts

Baseline expression levels of the innate immunity genes **RIPK1** and **IRAK4** were significantly lower among AA. This is in accordance with findings from the PARSIFAL (*Prevention of Allergy Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle*) study where **RIPK1** expression was negatively associated with the occurrence of asthma. The study group investigated essential marker genes of the innate and adaptive immune system in leukocytes of 316 Swiss farm and non-farm children and found an increased expression of genes of the innate immune system, including **RIPK1**, in farm children [150]. Several TLR genes and other genes related to innate immunity have been shown to be related to protective farm exposure with children growing up on traditional farm showing higher expression levels [54, 56, 151]. Interestingly, **IRAK4** was the only investigated gene in the PARSIFAL study that was decreased in farm children even though other members of the IRAK family such as IRAK1 and IRAK2 were increased [150].

In HC, expression levels of **RIPK1** and **IRAK4** decreased upon stimulation with LPS and farm dusts. In AA, **RIPK1** expression significantly increased upon stimulation with Amish dust, now reaching the baseline level of HC under unstimulated conditions. In contrast, neither LPS nor any of the dusts caused significant changes in **IRAK4** expression among asthmatics. This observation is in accordance with previous work from the research group showing that exposure to German farm dust induced significant downregulation of **IRAK4** in dendritic cells of HC whilst it had hardly any effect among AA [87]. Previous work of the research group also revealed significant downregulation of TLR4 expression upon stimulation with LPS and farm dust in both phenotypes and significant downregulation of MyD88 in AA and to a lesser extent in HC [86]. Following binding of LPS (and other stimuli) to TLR4, MyD88 interacts with **IRAK4** (and other members of the IRAK family), inducing the phosphorylation and activation of IRAK1 and the activation of canonical NF-κB signalling [76]. Interestingly, the recently mentioned studies of the research group revealed significantly higher baseline mRNA expression levels of pro-inflammatory TLR4 and MyD88

[86, 87], whereas in the present study expression of **IRAK4** was significantly lower among AA as outlined above.

Overall, the present study showed that baseline expression levels of the innate immunity genes RIPK1 and IRAK4 were significantly lower among AA. Exposure of PBMCs to farm dust extracts or LPS decreased their expression levels in HC. Following stimulation with Amish dust extract, RIPK1 expression significantly increased, now reaching the baseline level of HC under unstimulated conditions. This is a strong indicator of the anti-inflammatory capacity of the farm dust. IRAK4 expression on the contrary hardly changed upon stimulation in AA. More research is needed to understand different regulatory patters of RIPK1, IRAK4 and other innate immunity genes between asthmatics and healthy controls and their potential role in mediating protection via the environmentally mediated farm-effect.

5.2.3 Lower baseline expression of pro-inflammatory PTGS2 in allergic asthmatics and strong upregulation upon stimulation in both groups

Unexpectedly, this study revealed significantly lower baseline expression levels of pro-inflammatory **PTGS2** amongst AA. This finding is in contrast to another study investigating **PTGS2** expression in PBMCs of healthy and allergic children that showed significantly higher baseline levels in allergic children [152]. **PTGS2** expression has been shown to be elevated in airway smooth muscle cells of asthmatics [153]. Likewise, mRNA expression levels of **PTGS2** are increased in bronchial smooth muscle tissues of ovalbumin-challenged mice [154] and increased **PTGS2** protein expression is found in lungs of ovalbumin sensitised guinea pigs [155].

In the present study, all stimuli except for Ch1 induced significant upregulation of **PTGS2** in both groups. Ch1 showed a tendency towards upregulation among HC, but not AA.

Metabolites generated via **PTGS2**, such as prostaglandin E_2 (PGE_2) and prostaglandin D_2 (PGD_2) are essential mediators in inflammation and asthma [156, 157]. However, the role of **PTGS2** in asthma pathogenesis seems complex as some prostanoids have pro-allergic inflammatory properties, whilst others have anti-inflammatory effects. Since bronchoconstriction and -dilation are regulated by prostanoids, it is likely that the bronchial tone is mediated by a certain balance between PGE_2 and PGD_2 [158, 159].

In a pleurisy model in rats, **PTGS2** showed pro-inflammatory properties during early phase of inflammation with increased levels of pro-inflammatory PGE_2 but anti-inflammatory properties during later phase of inflammation with reduced levels of PGE_2 and increased levels of anti-inflammatory PGD_2 and PGJ_2 [160]. Also, not only may NF-κB activation result in increased expression of **PTGS2**, but also may **PTGS2** activate NF-κB. The transcriptional activity of NF-κB may be regulated depending on the prostaglandins produced by **PTGS2** [161].

Consequently, it is not possible to draw a conclusion from these PTGS2 mRNA expression data alone. As sample material for metabolite and cytokine assays was acquired in the present study, it would be interesting to investigate the effect of farm dust on metabolites induced by PTGS2. An even better understanding of the role of PTGS2 in asthma may be obtained by functional studies in airway tissues from asthmatic patients [159].

5.2.4 Similar baseline gene expression levels of pro-inflammatory MAPK signalling genes but different regulations upon farm dust stimulation

Baseline expression levels of **MAP3K7**, **MAPK1**, **MAPK9**, **MAPK14** and **MAPK7** did not differ significantly between HC and AA. They were slightly lower for **MAPK1** and **MAPK9** among AA (both $p = 0.051$), but this difference was mainly due to very low baseline expression levels in only two AA.

Stimulation induced distinct differences in regulatory patterns between the two groups. Upon stimulation with farm dust or LPS, **MAP3K7**, **MAPK1**, **MAPK9**, **MAPK14** and **MAPK7** were downregulated in HC, thereby mostly reaching statistical significance. This was not the case in AA where stimulation had no considerable impact. Only **MAPK14** was significantly downregulated in AA when stimulated with German farm dust. In fact, German farm dust was the only stimulatory condition upon which MAPK related gene expressions were downregulated in AA (even though not significant for **MAP3K7**, **MAPK1**, **MAPK9** and **MAPK7**). LPS and the other farm dusts extracts had no significant effects on MAPK related gene expression levels but showed a slight tendency towards upregulation (see **Figure 35**). These observations are likely due to differences in dust composition (see discussion, section **5.3**).

Taken together, this study showed a strong downregulation of mRNA expression of pro-inflammatory MAP3K7, MAPK1, MAPK9, MAPK14 and MAPK7 in PBMCs of HC upon stimulation with different farm dust extracts or LPS, whereas only little effect was observed in AA. These results suggest that even though the baseline expression of pro-inflammatory MAPK signalling is similar in asthmatics, its function might be altered. The effects on gene expression imply that MAPK signalling is regulated differently in mRNA among AA upon stimulation with farm dust. One could argue that the anti-inflammatory capacity of farm dust in downregulating pro-inflammatory MAPK signalling is prevailed in healthy children but altered in allergic asthmatics.

5.2.5 Lower baseline expression levels of anti-inflammatory NF-κB- and MAPK pathway related genes in allergic asthmatics and upregulation upon stimulation with farm dust

AA expressed significantly lower baseline levels of anti-apoptotic BIRC3 suggesting that there might be more apoptosis taking place in asthmatic children. BIRC3 is involved in suppressing apoptosis [162] and was shown to provide resistance to asthma-related inflammation in nasal airway epithelial cells from asthmatic and control donors [163]. Aberrant apoptosis is one of the pathomechanisms aggravating asthma symptoms and can be observed in some airway biopsies of asthmatic patients, particularly when they suffer from severe asthma. In fact, airway epithelial cell loss is even evident in asthmatic children [164]. Polymorphisms of **BIRC3** are associated with decreased susceptibility to asthma and reduced eosinophil and neutrophil loads [165]. Expression of **BIRC3** has been shown to correlate strongly with sputum eosinophil percentage, indicating that **BIRC3** may be involved in persisting eosinophilic inflammation in asthmatic patients [166]. High, inflammatory dosages of LPS have been shown to potently upregulate **BIRC3** in macrophages whereas **BIRC3**$^{-/-}$ macrophages are prone to apoptosis in a LPS-induced pro-inflammatory setting [167]. Consequently, **BIRC3** is viewed as a key player in maintaining a normal innate immune inflammatory response including the prevention of inflammation and the preservation of cell viability [167].

Expression levels of the anti-inflammatory NF-κB-related pathway gene NFKBIA were also significantly lower among AA. This is in line with previous work of the research group that found significantly lower baseline mRNA and protein expression levels of anti-inflammatory TNFAIP3 in AA [86, 87].

Several **NFKBIA** polymorphisms have been associated with the development of atopic asthma [66]. In a novel network approach based on gene expression data and molecular interaction networks **NFKBIA** and **BIRC3** were suggested to play important roles in childhood asthma [168]. **NFKBIA** is a major negative regulator of NF-κB and a central hub in transcriptional responses of common lung diseases in children, including respiratory syncytial virus infection, bronchopulmonary dysplasia, and asthma [169]. **Significantly lower baseline expression among AA may indicate that the NF-κB pathway may be more active among asthmatics.** However, this finding is in contrast to a study from 2015, where mRNA expression of **NFKBIA** was significantly higher in PBMCs of mild and moderate-to-severe asthmatic children compared with healthy controls [170].

The present study also revealed significantly lower baseline expression levels of DUSP1, a central negative regulator of MAPK signalling, among AA. Due to the strong difference found in this relatively small sample size (n for **DUSP1** expression data = 20), **DUSP1** expression was measured in additional children from the CLARA/CLAUS cohort and the significantly lower levels among AA were confirmed [110]. In addition, following the findings of this study, **DUSP1** expression was investigated in the Chinese cohort of the cross-sectional TRILATERAL study. Here, **DUSP1** expression was also lower in asthmatic children, yet the difference was not statistically significant. When only looking at children from rural Conghua, the difference was slightly stronger compared to Hong Kong but still not significant [110].

Taken together, this study showed that baseline mRNA expression levels of anti-apoptotic BIRC3 and of key negative regulators of NF-κB (namely NFKBIA) and MAPK (namely DUSP1) related signalling were significantly lower among AA.

When looking at data derived from fold changes (see **Figure 34**) **BIRC3 and NFKBIA expression levels were mostly significantly upregulated upon stimulation with farm dust extracts or LPS in both groups.** Of note, ∆Ct values showed a downregulation of **NFKBIA** expression following stimulation with Finnish and Chinese farm dusts in HC. However, when taking into account baseline expression levels (i.e. under unstimulated condition) **NFKBIA** was also upregulated upon these stimuli. In AA, LPS, Finnish, Amish and the Chinese farm dust 3 induced strong and significant upregulation of **NFKBIA**, now reaching an expression level similar to HC under unstimulated conditions. German farm dust also induced a tendency towards upregulation. This finding is well in line with previous findings of the research group showing significant upregulation of **NFKBIA** mRNA

expression in isolated dendritic cells upon stimulation with German farm dust in HC and AA [87].

Upon stimulation with LPS, **DUSP1** was significantly upregulated in AA, now reaching an expression level comparable to baseline expression of **DUSP1** in HC. Also, German, Finnish and the Chinese farm dusts 1 and 3 showed tendencies towards **DUSP1** upregulation in AA. Neither LPS nor any of the farm dusts had a significant impact on **DUSP1** expression in HC. **One could argue that HC may not need upregulation of anti-inflammatory DUSP1 since they already started at higher baseline expression levels than AA.** Interestingly, anti-CD3/28 caused a very strong and significant decrease in gene expression in HC while it had no significant impact on AA. Further research is needed to explain this difference in **DUSP1** response to the potent T cell stimulus.

Taken together, these findings are a strong indicator for an immunoregulatory protective mechanism of the farm dust extracts. Upregulating anti-apoptotic BIRC3 and anti-inflammatory NFKBIA and DUSP1 in allergic asthmatic children to "healthy baseline levels" indicate that farm dust exposure may not only be important in asthma prevention but may also convey beneficial immunomodulating capacities in children with manifest allergic asthma. Exposure to farm dust may therefore work as a therapeutic approach in controlling exaggerated NF-κB and MAPK activation.

5.3 Farm dust composition and differences between the Chinese farm dusts

Previous projects of the research group showed that German (G), Finnish (Fi) and Amish (Am) farm dust extracts had very similar effects on NF-κB related gene expressions, whereas effects of Chinese farm dust 1 (Ch1) differed a lot [86]. These observations were replicated in the present study and become particularly apparent when looking at **Figure 34**. Interestingly, in MAPK related genes, the effects of Ch1 were similar to those of the other dusts (see **Figure 35**). The Chinese farm dusts 2 (Ch2) and 3 (Ch3), which were newly extracted in this project, had similar effects on gene expression in both pathways compared with the European and Amish farm dusts. When looking at MAPK signalling alone, the effects of Ch2 stimulation were weaker compared to Ch1 and Ch3. Different regulatory gene expression patterns between HC and AA were highlighted by gene-gene correlation analyses in section **4.4**. Overall, genes correlated highly and mostly significantly with each other in both phenotypes upon stimulation with European and Amish farm dusts. However,

correlation patterns were more distinct between HC and AA upon stimulation with the Chinese dust extracts. These observations indicate that farm dust exposure induces gene expression changes at several sites of the investigated pathways and thus outline the complex interplay of NF-κB and MAPK related genes.

The European and Amish dust extracts were collected from traditional dairy farms. In China, poultry farms are far more common than dairy farms and keeping poultry is associated with protection against current wheeze and use of asthma medication [57] (and personal communication with Professor Gary Wong, Department of Paediatrics, The Chinese University of Hong Kong on 30/05/2018). Whereas Chinese dust 1 was collected in living rooms in rural Conghua, the Chinese dusts 2 and 3 were collected from inside chicken stables on poultry farms. The different locations are likely to lead to differences in dust compositions (including different endotoxin concentrations) and hence different stimulatory effects. Differences in endotoxin levels and in the microbial composition of farm dust influence the results of the experiments, as was shown in studies comparing asthma in Amish and Hutterite farm children [28]. As outlined in section **3.4,** endotoxin concentrations were much higher in German and Finnish dust samples compared with Ch1 [86]. It is reasonable to assume that endotoxin concentrations in Ch2 and Ch3 were also higher than in Ch1. Of note, farm dust may also contain harmful components. Hence, before farm dust may be applied as preventive or therapeutic measure in the future, detailed analyses and evaluations of farm dust components are crucial to ensure its safe use.

Taken together, as the aim of this study was not to identify certain protective microbial components, the dust composition was not specifically analysed in this project. In order to unravel the environmental influence, it would however be extremely interesting to look closer at the different components.

5.4 Strengths and limitations of the study

5.4.1 Discussion of the study population

The study subjects were a subsample of the larger CLARA/CLAUS cohort and included 4-14-year-old children with mild to moderate asthma and healthy controls. Detailed questionnaires and thorough examinations enabled reliable data acquisition. However, due to the cross-sectional study design variations of asthma symptoms over time were not captured. A main strength of this study was the recruitment and analysis of mostly new onset

allergic asthmatics. Since all allergic asthmatics recruited in this study were steroid-naïve (i.e. no use of inhaled corticosteroids within the last 14 days prior to recruitment), the measured gene expression levels were not affected by anti-inflammatory therapy. Clinical examination by at least one pediatric pulmonologist together with reversible pulmonary obstruction in lung function, increased specific and/or total IgE levels and recorded asthma and allergic symptoms provided strong and reliable indicators to make an asthma diagnosis.

Sample selection was based on a case-control design and was therefore not random. As the two groups shared similar socio-demographic characteristics, any changes observed in gene expression were likely to be mostly dependent on the presence or absence of allergic asthma. However, since childhood asthma is frequently associated with other atopic diseases such as hay fever, atopic dermatitis and food allergy and since all three conditions were common amongst AA (see **Table 6**), differences in gene expression profiles may not be solely due to the presence or absence of allergic asthma. Significant differences other than related atopic disorders were having older siblings and keeping indoor pets. For these categories, confounder analysis and, where necessary, adjustment was performed.

Even though the sample size was relatively small, significant changes in gene expression levels were clearly identified indicating differences between in NF-κB and MAPK related genes in HC and AA and the strong immunomodulatory capacity of stimulation with farm dusts.

In summary, thorough clinical, technical and laboratory examination ensured precise phenotype classification thereby providing a solid and reliable base for this study. Considering the identification of significant findings, the total sample size of 34 healthy and 24 allergic asthmatic children seemed appropriate.

5.4.2 Advantages and disadvantages of using PBMCs as sample material

PBMC stimulation is a widely used method to investigate the impact of external stimuli on gene expression. PBMCs are easily accessible in children and are therefore a conceivable option for possible future diagnostic or screening approaches. However, technical aspects regarding blood sampling as well as cell and RNA isolation methods may affect gene expression. For example, physical issues such as temperature during processing or time to analysis have shown to be particularly important [171]. In this study, PBMCs were isolated directly after blood withdrawal with a maximum delay of 24 hours and each sample was

processed individually according to the same protocols to ensure stable experimental conditions. It could not be completely ruled out that technical aspects affected gene expression. However, due to the fast and uniform processing of all samples it is likely that any influence would be similar for both phenotypes.

Allergic asthma is mainly restricted to inflammation in airways and lung tissue, but elevated levels of peripheral eosinophils in asthmatic patients correlate with the clinical severity and lung function [142], thereby indicating systemic involvement. Gene expression was analysed in PBMCs regardless of cell types. Since PBMC populations are heterogeneous, comparing these mixed cell populations may be confounded by changes in cell types. In the present study, there were some differences in the proportions of leukocyte composition between the phenotypes. As already described in previous chapters, PBMCs include lymphocytes (T cells, B cells, and NK cells), dendritic cells, and monocytes. Erythrocytes and granulocytes, i.e. eosinophils, neutrophils and basophils were removed by density gradient [126]. Hence, confounder analysis was performed for monocytes as their proportion differed slightly but significantly between HC and AA. As correlation of monocyte counts with gene expression levels revealed no significant findings, adjusting for differences in cell composition was not necessary.

Following the findings of the present study, the research group investigated MAPK related gene expression in several PBMC subpopulations, namely DCs, monocytes, NK cells, B cells and several T cell populations including CD4+ T cells, CD8+ T cells, T helper 2 cells and regulatory T cells. As shown for PBMCs, anti-inflammatory **DUSP1** gene expression was increased upon stimulation with German farm dust in isolated DCs, yet not reaching statistical significance. In addition, pro-inflammatory **MAPK1**/3 and **MAPK14** gene and protein expression levels were downregulated in nearly all investigated cell populations, though not always statistically significant, in both HC and AA [110].

In addition to blood analyses, investigating induced sputum might provide further information about local differences in gene modulatory effects. Of course, airway biopsies may provide even more reliable information but given the invasiveness of the procedure, this method is rarely applied in children due to ethical concerns.

Taken together, PBMCs serve as a sensible and feasible sample material to investigate gene expression in young children. In addition, the aim of this study was not to provide an in-depth analysis of different immune cells involved, but to identify overall changes in gene expression in peripheral blood. Comparing frequencies of

distinct cell populations e.g. by mass cytometry can ensure a similar PBMC composition between phenotypes, which has indeed been shown by other projects of the research group [110].

5.4.3 Further technical aspects – time frame and stimulatory agents

Gene expression levels were investigated 24 hours after stimulation, as kinetic experiments conducted prior to this study showed optimal immune-modulatory effects of farm dust after stimulation for 24 hours (data not shown).

In the context of endotoxin tolerance, several studies showed that prolonged LPS stimulation (i.e. 16-48 hours) reduced inflammatory responses including TLR4-induced pathways and inhibited NF-κB and MAPK activation [172]. In fact, the effect has been demonstrated for several genes investigated in the present study, as prolonged LPS stimulation has been reported to reduce **IRAK4** activity, **NFKBIA** degradation, and activation of NF-κB and various MAPKs including **MAPK1**/3, **MAPK9** and **MAPK14** [172]. **It may therefore be assumed that the time of investigation was suitable to observe changes in gene expression.**

PBMCs were stimulated with consistent concentrations of farm dust extracts. The dosage was chosen based on previously established concentrations by the research group, where several concentrations and time points had been examined in PBMCs by qPCR for NF-κB related signalling genes (data not shown). 40 µg/mL dust was chosen as optimal concentration in order to detect the selection of genes of NF-κB related signalling, with the strongest capacity to induce and/or inhibit specific genes. To allow for comparability of the data, the same concentration was chosen for MAPK signalling genes. To further mimic exposure to farm environments it could also be useful to repeat stimulation in certain time intervals and with varying dust concentrations.

5.4.4 Analysing mRNA expression levels as the final readout of this study

qPCR was the main method used in this project and provided reliable information about differences in mRNA expression levels between HC and AA both at baseline and upon stimulation. Since the investigated genes are targets of various modifications including posttranscriptional processes (e.g. phosphorylation, ubiquitination, methylation, acetylation)

further methods are required in order to draw conclusions about differences downstream of mRNA expression. Since some of the differences in mRNA expression levels found in the present study were strong and promising, the research group thereupon investigated protein expression levels in a selection of genes.

Interestingly, different regulation levels were detected upon stimulation with farm dusts or LPS in HC and AA: In HC, **DUSP1** mRNA expression was only slightly affected by stimulation, leading to a weak reduction of MAPK phosphorylation on protein level. In AA on the contrary, stimulation caused a significant increase in anti-inflammatory **DUSP1** mRNA expression, followed by an enhanced dephosphorylation activity and subsequently decreased levels of pro-inflammatory p**MAPK1**/3 and p**MAPK14** protein expression. The weak changes observed on MAPK mRNA levels (compare **Figure 35)** in AA may be due to negative feedback loops, as pMAPK restrict *de novo* gene synthesis [110].

These findings complement the present study and add valuable information about immunological processes involved in the protective farm effect downstream of mRNA expression. Further studies exploring feedback and temporal dynamics and total protein levels in the closely regulated NF-κB and MAPK pathways will help understanding possible phenotypical differences. In addition, material for metabolite analyses was acquired during this study and cytokine assays will provide an even more comprehensive picture of downstream immune modulation through exposure to "asthma protective" farm dusts.

5.5 Closing remarks and outlook

This study aimed at identifying possible differences in NF-κB and MAPK related gene expressions between healthy and allergic asthmatic children both at baseline and upon stimulation with LPS and “asthma protective” farm dusts, thereby investigating their potential contribution in the environmentally mediated protection from childhood asthma. This was done by assessing gene expression levels by qPCR in PBMCs of 34 healthy and 24 allergic asthmatic children.

Regarding the hypotheses, the conclusions were as follows:

Hypothesis 1: NF-κB and MAPK pathway genes show different baseline expression levels in PBMCs of allergic asthmatic versus healthy children with generally higher pro-inflammatory expression profiles among asthmatics.

Conclusion: Several innate immunity and anti-inflammatory genes were significantly lower expressed among AA. In addition, gene-gene correlation analyses revealed different patterns in NF-κB and MAPK signalling genes with mostly stronger correlations among AA. However, it cannot be concluded based on these data alone that AA had generally higher pro-inflammatory expression profiles than HC. Yet, the findings add valuable information to further understand differences in gene expressions relevant in childhood asthma.

Hypothesis 2: *Ex vivo* exposure to “asthma protective” farm dust extracts and LPS reduces expression of pro-inflammatory genes, while it induces expression of anti-inflammatory genes and genes involved in innate immunity.

Conclusion: Overall, the data acquired in present study revealed a strong immunomodulatory capacity of farm dust exposure on mRNA level. Different gene-gene correlation patterns following stimulation indicated that parts of the investigated pathways may respond differently to environmental stimuli in AA. **The effects observed in children with clinically manifest allergic asthma indicate that farm dust exposure may not only be beneficial in asthma prevention but possibly also in asthma treatment. By upregulating anti-inflammatory NF-κB and MAPK signalling, farm dust may help restoring impaired asthmatic gene expression to a “healthy level”.** (Phospho)protein analyses performed after this study have complemented some of the findings (see section **5.4.4**) and further projects including cytokine assays are planned. Together, these findings will give valuable insights in the effect of farm dust exposure on immune modulation further downstream of mRNA level.

One of the many unanswered questions in asthma and immunology research is why some children that are sensitised to an allergen (i.e. increased specific IgE) do not develop allergic and asthmatic symptoms, while others do. As parent-reported, survey-based recording of presence or absence of allergic reactions might risk incomplete or incorrect detection of symptoms, healthy children with elevated specific IgE were excluded in the present study even if no allergic reactions were reported. Investigating gene expression patterns in these children as a third group might provide valuable information about immune regulation at an “intermediate level”. Also, as these children are more susceptible to developing allergic symptoms at some point, they might represent a target group for preventive measures.

In recent years, a range of studies outlined the importance of the skin, lung, and gut microbiomes in regulation of immune tolerance [173]. Asthma is associated with dysbiosis of intestinal and airway microbiota and the crosstalk between the two compartments is referred to as the gut-lung axis [174]. There is an increasing amount of evidence that the human microbiota, influenced by environmental stimuli, is crucial in determining trajectories of health and immune development [175].

The ongoing burden of childhood asthma calls for new treatment options as well as preventive strategies. It has been recognised for a long time that farm exposure protects against asthma and related atopic diseases. The present study identified new mediators which may play important roles in conveying the protective effect of farm exposure. After all, the key challenge will be to functionally transfer the protective farm effect to non-farming environments [176]. This was approached in a complex analysis of three large birth cohort studies revealing a lower risk of asthma in children with farm-like home bacterial microbiota composition even if they did not grow up on farms themselves. By developing the so-called „Farm home resembling microbiota index” (FaRMI) the authors developed a way to assess the asthma protective potential of any indoor dust microbiota composition [176]. In the long term, these findings may help paving the way for new asthma treatment and preventive measures by means of asthma protective farm dust.

6 Summary

Asthma is the most common chronic disease in childhood and affects around 262 million people worldwide [15, 16]. Even though asthma treatment is well established in clinical practice, the current burden of asthma and its increasing prevalence outline the need for new therapeutic and preventive approaches. For over 20 years, epidemiological studies have provided striking evidence for the existence of the so-called farm effect which describes a significantly lower prevalence of asthma and allergies amongst children growing up on traditional farms [42]. However, its underlying immunological mechanisms are still poorly understood. NF-κB and MAPK cascades are key pathways in immunity and are proposed to play important roles in the pathogenesis of asthma and allergies [69-71, 99]. This study built on previous findings from the research group underlying the role of TNFAIP3, a central negative regulator of NF-κB signalling, as a key player in development of childhood asthma and environmentally mediated protection [86, 87]. The aim was to identify additional NF-κB and MAPK related pathway genes relevant in childhood asthma and susceptible to the immunomodulating properties of farm dust exposure. 58 children aged 4-14 years recruited within the scope of the CLARA/CLAUS cohort were investigated. PBMCs of 24 steroid-naïve allergic asthmatic children and 34 healthy controls were isolated from whole blood samples and *ex vivo* gene expression was assessed by means of qPCR both at baseline and upon stimulation with six different “asthma protective” dusts from farms in Germany, Finland, the Amish community in the United States and three different locations in rural China, as well as with and without LPS. Allergic asthmatics had significantly lower baseline expression levels of the innate immunity genes **RIPK1**, **IRAK4** and **BIRC3**, which is also relevant for anti-apoptotic signalling, as well as of anti-inflammatory **NFKBIA**, a key negative regulator of NF-κB signalling, and of **DUSP1**, a key negative regulator of MAPK signalling. Stimulation with farm dust extracts or LPS revealed their strong immunomodulatory capacity on mRNA level. While pro-inflammatory genes and genes involved in innate immunity were strongly and mostly significantly downregulated in healthy children, their expression levels were mostly not significantly affected in allergic asthmatics indicating that their regulation might be altered. However, anti-apoptotic **BIRC3** and anti-inflammatory **NFKBIA** and **DUSP1** were significantly upregulated upon stimulation in both phenotypes with allergic asthmatic children now reaching “healthy expression levels”. Thus, these findings are in accordance with previous work of the research group and support the thesis that farm dust exposure may convey beneficial immunomodulating capacities in

children with manifest allergic asthma. Exposure to farm dust may therefore possibly work as a therapeutic approach in controlling exaggerated NF-κB and MAPK activation. After all, transferring beneficial farm exposure to everyday life remains a key challenge. The present study gave new insights in NF-κB and MAPK related gene expressions in allergic asthmatic children and their modulation through farm dust stimulation. Downregulation of pro-inflammatory and upregulation of anti-inflammatory gene expression may be part of the tolerance mechanisms responsible for asthma protection through farm exposure. In the long term, these findings shall contribute to develop new strategies in the treatment and prevention of childhood asthma.

7 Zusammenfassung

Asthma bronchiale ist die häufigste chronische Erkrankung im Kindesalter und betrifft etwa 262 Millionen Menschen weltweit [15, 177]. Obwohl die Therapie im klinischen Alltag unter Verwendung eines Stufenschemas gut etabliert ist, wird der Bedarf an neuen therapeutischen und präventiven Maßnahmen durch die große Belastung sowie die steigende Prävalenz von Asthma deutlich. Seit über 20 Jahren haben epidemiologische Studien bemerkenswerte Beweise für den sogenannten Bauernhofeffekt geliefert. Dieser beschreibt das signifikant seltenere Auftreten von Asthma und Allergien bei Kindern, die auf traditionellen Bauernhöfen aufwachsen [42]. Allerdings ist bislang nur wenig über die zugrundeliegenden immunologischen Mechanismen bekannt. Die NF-κB und MAPK Signalwege spielen eine wichtige Rolle in der Regulation des Immunsystems sowie in der Pathogenese von Asthma und Allergien [69-71, 99]. Diese Arbeit baute auf Vorstudien der Arbeitsgruppe auf, in denen gezeigt wurde, dass TNFAIP3, ein zentraler Inhibitor von NF-κB, eine Schlüsselrolle in der Entwicklung von sowie dem Umwelt-vermitteltem Schutz vor Asthma im Kindesalter einnimmt [86, 87]. Das Ziel dieser Arbeit war es, weitere Gene der NF-κB und MAPK Signalwege zu identifizieren, die von Bedeutung in der Entwicklung von Asthma sind und darüber hinaus durch Exposition gegenüber Bauernhofstäuben moduliert werden können. Hierzu wurden 58 Kinder im Alter von 4-14 Jahren, welche im Rahmen der CLARA/CLAUS Kohorte rekrutiert wurden, untersucht. Aus Vollblutproben von 24 steroid-naiven Kindern mit allergischem Asthma sowie 34 gesunden Kontrollen wurden PBMCs isoliert. Anschließend wurde die *ex vivo* Genexpression unter unstimulierten sowie stimulierten Bedingungen mittels qPCR untersucht. Zur Stimulation wurden sechs verschiedene „asthmaprotektive“ Stäube von Bauernhöfen in Deutschland, Finnland, den Amisch in den USA und von drei verschiedenen Orten im ländlichen Raum Chinas sowie LPS verwendet. Kinder mit allergischem Asthma zeigten signifikant niedrigere Expressionen der angeborenen Immunantwort-Gene **RIPK1**, **IRAK4** und **BIRC3**, welches ebenfalls eine wichtige Rolle im Schutz vor Apoptose spielt. Zudem waren **NFKBIA**, ein wichtiger Inhibitor des NF-κB Signalwegs sowie **DUSP1**, ein wichtiger Inhibitor des MAPK Signalwegs, ebenfalls signifikant niedriger in Kindern mit Asthma exprimiert. Durch Stimulation mit Bauernhofstäuben oder LPS wurde die ausgeprägte immunomodulatorische Eigenschaft der verwendeten Stimuli deutlich. Während die Expression proinflammatorischer Gene sowie von Genen der angeborenen Immunantwort in gesunden Kindern überwiegend signifikant reduziert werden konnte, war dieser Effekt bei asthmakranken Kindern deutlich

schwächer ausgeprägt. Diese Unterschiede lassen vermuten, dass Teile der NF-κB und MAPK Signalwege bei Kindern mit Asthma anders reguliert sind als bei gesunden Kindern. Im Gegensatz dazu wurde in beiden Gruppen die Expression des anti-apoptotischen Gens **BIRC3** sowie der antiinflammatorischen Gene **NFKBIA** und **DUSP1** durch Stimulation mit Bauernhofstäuben oder LPS signifikant hoch reguliert. Hierdurch konnten bei Kindern mit Asthma sogar „Expressionsniveaus auf dem Level von gesunden Kindern“ erreicht werden. Diese Ergebnisse decken sich mit vorigen Schlussfolgerungen der Arbeitsgruppe und unterstützen die These, dass Exposition gegenüber Bauernhofstäuben auch in Kindern mit manifestem allergischem Asthma noch positive immunmodulatorische Effekte haben könnte. Auf diese Weise könnte eine Bauernhofstaubexposition dabei helfen, eine übermäßige Aktivierung der NF-κB und MAPK Signalwege zu unterdrücken. Letztendlich ist es nach wie vor eine große Herausforderung, die positiven Effekte der Bauernhofumgebung in den Alltag jenseits des Bauernhofs zu überführen. Diese Arbeit gab neue Einblicke in unterschiedliche Genregulationen der NF-κB und MAPK Signalwege in Kindern mit allergischem Asthma und ihre Modulation durch Bauernhofstäube. Die Reduktion proinflammatorischer sowie die Induktion antiinflammatorischer Gene könnten zu den Toleranzmechanismen beitragen, die den asthmaprotektiven Bauernhofeffekt vermitteln. Langfristig sollen diese Ergebnisse dazu beitragen, neue Behandlungs- sowie Präventionsstrategien für Asthma im Kindesalter zu entwickeln.

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9 Abbreviations

AA	allergic asthmatics
Am	Amish farm dust
anti-CD3/28	anti-CD3 and anti-CD28 antibodies
ATS	American Thoracic Society
BAFF	B cell activating factor of the TNF family
BIRC3	baculoviral IAP repeat containing 3
CDC	Centers for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
Ch1	Chinese farm dust 1
Ch2	Chinese farm dust 2
Ch3	Chinese farm dust 3
cIAP2	cellular inhibitor of apoptosis 2
CLARA/CLAUS	Clinical Asthma Research Association Study
COPD	chronic obstructive pulmonary disease
COX2	cyclooxygenase 2
CRP	C-reactive protein
Ct	cycle threshold
DALY	disease-adjusted life years
DCs	dendritic cells
DFG	Deutsche Forschungsgemeinschaft
DNA	deoxyribonucleic acid
DRKS	Deutsches Register Klinischer Studien, German Clinical Trials Register
DUSP	dual-specificity phosphatase
EDCs	electrostatic dust fall collectors
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-related kinase
ERS	European Respiratory Society
FaRMI	farm home resembling microbiota index
Fi	Finnish farm dust

G	German farm dust
GBD	Global Burden of Disease
GINA	Global Initiative for Asthma
GM-CSF	granulocyte-macrophage colony-stimulating factor
GWAS	genome wide association studies
HC	healthy controls
HGNC	HUGO Gene Nomenclature Committee
IgE	Immunoglobulin E
IgG	Immunglobulin G
IKK	IκB kinase
IL	interleukin
IRAK4	interleukin 1 receptor associated kinase 4
ISAAC	International Study of Asthma and Allergies in Childhood
IκB	inhibitor of κB
IQR	interquartile range
JNK	c-jun terminal kinase
KiGGS	Studie zur Gesundheit von Kindern und Jugendlichen in Deutschland
LAL	Limuls Amoebocyte Lysate
LPS	lipopolysasccharide
MAP3K14	mitogen-activated protein kinase kinase kinase 14
MAP3K7	mitogen-activated protein kinase kinase kinase 7
MAPK1	mitogen-activated protein kinase 1
MAPK14	mitogen-activated protein kinase 14
MAPK7	mitogen-activated protein kinase 7
MAPK9	mitogen-activated protein kinase 9
MAPKs	mitogen-activated protein kinases
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
MyD88	myeloid differentiation primary response 88
NCBI	National Center for Biotechnology
NEMO	NF-κB essential modulator

NFKBIA	NF-κB-inhibitor alpha
NF-κB	nuclear factor “kappa-light-chain-enhancer” of activated B cells
NIK	NF-κB-inducing kinase
NK cells	natural killer cells
NTC	negative control
NVL	Nationale Versorgungsleitlinie
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PGD2	prostaglandin D2
PGE2	prostaglandin E2
PTGS2	prostaglandin-endoperoxide synthase 2
qPCR	quantitative real-time polymerase chain reaction
RING	Really Interesting New Gene
RIPK1	receptor interacting serine/ threonine kinase 1
RNA	ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute medium (Cell culture medium)
rRNA	ribosomal ribonucleic acid
r_s	Spearman correlation coefficient
SNPs	single nucleotide polymorphisms
TAK1	transforming growth factor-β-activated kinase 1
TGF-β	transforming growth factor beta
TLR4	toll like receptor 4
Tm	melting temperature
TNFAIP3	tumor necrosis factor alpha induced protein 3
TNFR	tumor necrosis factor receptor
TNF-α	tumor necrosis factor alpha
TRAF6	tumor necrosis factor receptor associated factor 6
U	unstimulated condition
x g	times gravity

Full list of abbreviations used in Figure 5 (available and adapted from [91-94])**:**

ATM, TEL1, serine-protein kinase ATM [EC:2.7.11.1]; BCL10, B-cell CLL/lymphoma 10; BCL2, apoptosis regulator Bcl-2; BCL2A1, hematopoietic Bcl-2-related protein A1; BCL2L1, bcl-xL, Bcl-2-like 1 (apoptosis regulator Bcl-X); **BIRC**2/**3**, baculoviral IAP repeat-containing protein 2/3; BLNK, B-cell linker protein; BTK, Bruton agammaglobulinemia tyrosine kinase [EC:2.7.10.2]; CARD10, caspase recruitment domain-containing protein 10; CARD11, caspase recruitment domain-containing protein 11; CARD14, caspase recruitment domain-containing protein 14; CCL13, C-C motif chemokine 13; CCL19, ELC, C-C motif chemokine 19; CCL21, SLC, C-C motif chemokine 21; CCL4, C-C motif chemokine 4; CD14, monocyte differentiation antigen CD14; CFLAR, FLIP, CASP8 and FADD-like apoptosis regulator; CSNK2A, casein kinase II subunit alpha [EC:2.7.11.1]; CSNK2B, casein kinase II subunit beta; CXCL1/2/3, GRO, C-X-C motif chemokine 1/2/3; CXCL12, C-X-C motif chemokine 12; CYLD, USLP2, ubiquitin carboxyl-terminal hydrolase CYLD [EC:3.4.19.12]; DDX58, RIG-I, ATP-dependent RNA helicase DDX58 [EC:3.6.4.13]; EDA, ectodysplasin-A; EDA2R, TNFRSF27, XEDAR, tumor necrosis factor receptor superfamily member 27; EDAR, tumor necrosis factor receptor superfamily member EDAR; EDARADD, ectodysplasin-A receptor-associated adapter protein; ERC1, CAST2, ELKS, ELKS/RAB6-interacting/CAST family member 1; GADD45, growth arrest and DNA-damage-inducible protein; ICAM1, CD54, intercellular adhesion molecule 1; IGH, immunoglobulin heavy chain; IKBA, **NFKBIA**, NF-kappa-B inhibitor alpha; PARP1, poly [ADP-ribose] polymerase 1 [EC:2.4.2.30]; IKBKA, IKKA, CHUK, inhibitor of nuclear factor kappa-B kinase subunit alpha [EC:2.7.11.10]; IKBKB, IKKB, inhibitor of nuclear factor kappa-B kinase subunit beta [EC:2.7.11.10]; IKBKG, IKKG, NEMO, inhibitor of nuclear factor kappa-B kinase subunit gamma; IL1B, interleukin 1 beta; IL1R1, CD121a, interleukin 1 receptor type I; IL8, CXCL8, interleukin 8; IRAK1, interleukin-1 receptor-associated kinase 1 [EC:2.7.11.1]; **IRAK4**, interleukin-1 receptor-associated kinase 4 [EC:2.7.11.1]; LAT, linker for activation of T cells; LBP, lipopolysaccharide-binding protein; LCK, lymphocyte cell-specific protein tyrosine kinase [EC:2.7.10.2]; LRDD, PIDD, leucine-rich repeats and death domain-containing protein; LTA, TNFB, lymphotoxin alpha (TNF superfamily, member 1); LTB, TNFC, lymphotoxin beta (TNF superfamily, member 3); LY96, MD-2, lymphocyte antigen 96; LYN, tyrosine-protein kinase Lyn [EC:2.7.10.2]; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1 [EC:3.4.22.-]; **MAP3K14**, NIK, mitogen-activated protein kinase kinase kinase 14 [EC:2.7.11.25]; **MAP3K7**, TAK1, mitogen-activated protein kinase kinase kinase 7 [EC:2.7.11.25]; MAP3K7IP1, TAB1, TAK1-binding protein 1; MAP3K7IP2, TAB2, TAK1-binding protein 2; MAP3K7IP3, TAB3, TAK1-binding protein 3; MYD88, myeloid differentiation primary response protein MyD88; NFKB1, nuclear factor NF-kappa-B p105 subunit; NFKB2, nuclear factor of kappa light polypeptide gene enhancer in B-cells 2; PIAS4, E3 SUMO-protein ligase PIAS4 [EC:2.3.2.27]; PLAU, urokinase plasminogen activator [EC:3.4.21.73]; PLCG1, phosphatidylinositol phospholipase C, gamma-1 [EC:3.1.4.11]; PLCG2, phosphatidylinositol phospholipase C, gamma-2 [EC:3.1.4.11]; PRKCB, classical protein kinase C beta type [EC:2.7.11.13]; PRKCQ, novel protein kinase C theta type [EC:2.7.11.13]; **PTGS2**, COX2, prostaglandin-endoperoxide synthase 2 [EC:1.14.99.1]; RELA, transcription factor p65; RELB, transcription factor RelB; **RIPK1**, RIP1, receptor-interacting serine/threonine-protein kinase 1 [EC:2.7.11.1]; SYK, spleen tyrosine kinase [EC:2.7.10.2]; TIRAP, toll-interleukin 1 receptor (TIR) domain-containing adaptor protein; TIRP, TRAM, TRIF-related adaptor molecule; TLR4, CD284, toll-like receptor 4; TNF, TNFA, tumor necrosis factor superfamily, member 2; TNFAIP3, A20, OTUD7C, tumor necrosis factor, alpha-induced protein 3 [EC:3.4.19.12]; TNFRSF11A, RANK, CD265, tumor necrosis factor receptor superfamily member 11A; TNFRSF13C, BAFFR, CD268, tumor necrosis factor receptor superfamily member 13C; TNFRSF1A, TNFR1, CD120a, tumor necrosis factor receptor superfamily member 1A; TNFRSF3, LTBR, lymphotoxin beta receptor TNFR superfamily member 3; TNFRSF5, CD40, tumor necrosis factor receptor superfamily member 5; TNFSF11, RANKL, CD254, tumor necrosis factor ligand superfamily member 11; TNFSF13B, TNFSF20, CD257, tumor necrosis factor ligand superfamily member 13B; TNFSF14, LIGHT, CD258, tumor necrosis factor ligand superfamily member 14; TNFSF5, CD40L, CD154, tumor necrosis factor ligand superfamily member 5; TRADD, tumor necrosis factor receptor type 1-associated DEATH domain protein; TRAF1, TNF receptor-associated factor 1; TRAF2, TNF receptor-associated factor 2 [EC:2.3.2.27]; TRAF3, TNF receptor-associated factor 3; TRAF5, TNF receptor-associated factor 5; TRAF6, TNF receptor-associated factor 6 [EC:2.3.2.27]; TRAV, T cell receptor alpha chain V region; TRBV, T-cell receptor beta chain V region; TRIF, toll-like receptor adapter molecule 1; TRIM25, EFP, tripartite motif-containing protein 25 [EC:2.3.2.27]; UBE2I, UBC9, ubiquitin-conjugating enzyme E2 I; VCAM1, CD106, vascular cell adhesion molecule 1; XIAP, BIRC4, E3 ubiquitin-protein ligase XIAP [EC:2.3.2.27]; ZAP70, tyrosine-protein kinase ZAP-70 [EC:2.7.10.2]. Chemical compounds: Calcium cation, Ca2+; Calcium (2+), Calcium ion; DAG, Diacylglycerol, Diglyceride; D-myo-Inositol 1,4,5-trisphosphate, 1D-myo-Inositol 1,4,5-trisphosphate; Inositol 1,4,5-trisphosphat.

Full list of abbreviations used in Figure 6 (available and adapted from [90, 110]):

CD14, cluster of differentiation 14; CREB, cAMP responsive element binding protein; **DUSP1** = MKP-1, dual specificity phosphatase 1; EGF, epidermal growth factor; GF, growth factor; IKBa = **NFKBIA**, NF-κB inhibitor alpha; IKKa, inhibitor of nuclear factor kappa-B kinase subunit alpha; IKKb, inhibitor of nuclear factor kappa B kinase subunit beta; **IRAK**1/**4**, interleukin 1 receptor associated kinase 1/4; LPS, lipopolysaccharide; MALT1, mucosa associated lymphoid tissue lymphoma translocation gene 1; MAP2K1 = MEK1, mitogen-activated protein kinase kinase 1; MAP2K2 = MEK2, mitogen-activated protein kinase kinase 2; MAP2K3 = MKK3, mitogen-activated protein kinase kinase 3; MAP2K4 = MKK4, mitogen-activated protein kinase kinase 4; MAP2K5 = MEK5, mitogen-activated protein kinase kinase 5; MAP2K6 = MKK6, mitogen-activated protein kinase kinase 6; MAP2K7 = MKK7, mitogen-activated protein kinase kinase 7; MAP3K1 = MEKK1, mitogen-activated protein kinase kinase kinase 1; MAP3K11 = MLK3, mitogen-activated protein kinase kinase kinase 11; **MAP3K14** = NIK, mitogen-activated protein kinase kinase kinase 14; MAP3K2 = MEKK2, mitogen-activated protein kinase kinase kinase 2; MAP3K3 = MEKK3, mitogen-activated protein kinase kinase kinase 3; MAP3K5 = ASK1, mitogen-activated protein kinase kinase kinase 5; **MAP3K7** = TAK1, mitogen-activated protein kinase kinase kinase 7; MAP4K1 = HPK1, mitogen-activated protein kinase kinase kinase kinase 1; MAP4K3 = GLK, mitogen-activated protein kinase kinase kinase kinase 3;MAP4K4 = HGK, mitogen-activated protein kinase kinase kinase kinase 4; **MAPK1** = ERK2, mitogen-activated protein kinase 1; MAPK11 = p38-2, mitogen-activated protein kinase 11; **MAPK14** = p38, mitogen-activated protein kinase 14; MAPK3 = ERK1, mitogen-activated protein kinase 3; **MAPK7** = ERK5, mitogen-activated protein kinase 7; MAPK8 = JNK, mitogen-activated protein kinase 8; **MAPK9** = JNK2, mitogen-activated protein kinase 9; MAPKAPK2, -3, -5, mitogen-activated protein kinase-activated protein kinase 2, -3, -5 ; MYD88, myeloid differentiation primary response 88; NEMO, NF-κB essential modulator; NF-KB, nuclear factor kappa-light-chain-enhancer of activated B cells; Ras, rat sarcoma; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; TAB1, TGF-beta activated kinase 1 (MAP3K7) binding protein 1; P, phosphorylation; TAX1BP1, Tax1 binding protein 1; TLR4, toll-like receptor 4; TNF, tumor nectrosis factor; TNFAIP3, TNF alpha induced protein 3; TNF-R, tumor necrosis factor receptor; TNIP1/2, TNFAIP3 interacting protein 1/2; TRADD, TNFRSF1A (Tumor necrosis factor receptor superfamily member 1A)-associated via death domain; TRAF2, TNF receptor associated factor 2; TRAF6, TNF receptor associated factor 6, Ub, ubiquitylation.

10 Appendix

10.1 Supplementary tables and figures

10.1.1 Supplementary tables – Changes in gene expression upon stimulation

The following tables contain the data for the respective graphs shown in section **4.3** and **4.5**.

Stimulus	HC, n	HC ΔCt median (IQR)	HC U vs. stimulus p-value	AA, n	AA ΔCt median (IQR)	AA U vs. stimulus p-value	HC vs. AA p-value
U	34	13.14 (12.88-13.49)	-	24	13.27 (12.71-13.80)	-	0.231[c]
anti-CD3/28	34	12.42 (12.19-12.97)	**< 0.001[a]**	24	12.49 (11.90-12.93)	**< 0.001[a]**	0.777[c]
LPS	34	13.29 (12.98-13.69)	0.050[b]	23	13.25 (12.93-13.54)	0.964[b]	0.513[d]
G	33	13.19 (12.77-13.73)	0.456[a]	23	13.20 (12.99-13.62)	0.610[a]	0.856[c]
Fi	34	13.10 (12.93-13.64)	0.470[a]	22	13.35 (13.08-13.66)	0.775[b]	0.382[d]
Am	34	13.06 (12.89-13.62)	0.589[b]	23	13.08 (12.79-13.43)	0.095[a]	0.524[d]
Ch1	34	13.04 (12.83-13.65)	0.787[b]	23	13.02 (12.67-13.39)	0.060[b]	0.453[d]
Ch2	10	13.05 (12.91-13.54)	0.132[a]	9	13.15 (12.83-13.60)	**0.046[a]**	0.666[c]
Ch3	10	13.19 (12.98-13.63)	0.307[a]	9	13.28 (13.08-14.30)	0.339[a]	0.291[c]

Table 15: Changes in MAP3K14 expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of MAP3K14 in PBMCs of healthy controls (HC) and allergic asthmatics (AA). Number of included samples (n). Gene expression normalised to 18S and displayed as ΔCt with interquartile ranges (IQR) in brackets. Unstimulated condition (U), anti-CD3/28 (anti-CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide), G (German farm dust), Fi (Finnish farm dust), Am (Amish farm dust), Ch1 (Chinese farm dust 1), Ch2 (Chinese farm dust 2), Ch3 (Chinese farm dust 3). Differences upon stimulation are analysed by paired t-test ([a]) for normally distributed data and Wilcoxon matched-pairs signed rank test ([b]) for not normally distributed data. Differences between phenotypes are indicated in the last column and are analysed by Welch's t-test ([c]) for normally and by Mann-Whitney-U-test ([d]) for not normally distributed data.

Stimulus	HC, n	HC ΔCt median (IQR)	HC U vs. stimulus p-value	AA, n	AA ΔCt median (IQR)	AA U vs. stimulus p-value	HC vs. AA p-value
U	34	11.83 (11.22-12.28)	-	24	12.47 (11.81-15.54)	-	**0.006[d]**
anti-CD3/28	24	11.47 (11.08-12.19)	0.125[b]	24	11.82 (11.22-13.42)	**0.002[b]**	0.113[d]
LPS	34	11.70 (11.46-12.51)	0.417[b]	24	12.11 (11.64-15.89)	0.349[a]	0.065[d]
G	33	11.93 (11.40-12.81)	0.166[b]	23	12.05 (11.39-14.08)	0.731[b]	0.239[d]
Fi	34	11.81 (11.13-12.39)	0.488[b]	24	12.00 (11.59-12.92)	0.160[b]	0.410[d]
Am	34	11.96 (11.36-12.44)	0.078[b]	23	11.91 (11.27-13.31)	**0.020[a]**	0.955[d]
Ch1	34	12.22 (11.68-12.62)	**0.007[b]**	23	12.25 (11.60-13.95)	0.368[a]	0.693[d]
Ch2	10	12.00 (11.39-12.34)	0.275[b]	9	12.56 (11.80-13.05)	0.734[b]	0.133[d]
Ch3	10	12.03 (11.33-12.24)	0.232[b]	9	11.97 (11.30-12.56)	0.250[b]	0.905[d]

Table 16: Changes in RIPK1 expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of RIPK1 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt with interquartile ranges (IQR) in brackets. For detailed description please see **Table 15**.

Stimulus	HC, n	HC ΔCt median (IQR)	HC U vs. stimulus p-value	AA, n	AA ΔCt median (IQR)	AA U vs. stimulus p-value	HC vs. AA p-value
U	34	12.65 (12.40-13.01)	-	24	13.03 (12.52-13.74)	-	**0.025[c]**
anti-CD3/28	34	12.40 (12.04-13.05)	0.191[b]	24	12.75 (12.32-13.40)	**0.026[a]**	0.235[d]
LPS	34	12.84 (12.51-13.25)	**0.003[a]**	24	12.90 (12.56-13.45)	0.239[a]	0.734[c]
G	33	12.79 (12.39-13.34)	0.131[a]	23	12.91 (12.42-14.05)	0.810[a]	0.209[c]
Fi	34	12.92 (12.40-13.16)	0.176[a]	24	12.94 (12.49-13.46)	0.294[a]	0.538[c]
Am	34	12.98 (12.62-13.49)	**0.002[b]**	23	12.76 (12.45-13.22)	0.205[a]	0.240[d]
Ch1	34	12.85 (12.52-13.34)	0.100[a]	23	12.60 (12.30-13.65)	0.258[a]	0.768[c]
Ch2	10	12.94 (12.42-13.30)	0.239[a]	9	13.69 (12.85-13.96)	0.295[a]	0.261[c]
Ch3	10	13.12 (12.83-13.27)	0.084[b]	9	13.38 (12.56-13.72)	0.250[b]	0.604[c]

Table 17: Changes in IRAK4 expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of IRAK4 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt with interquartile ranges (IQR) in brackets. For detailed description please see **Table 15**.

Stimulus	HC, n	HC ΔCt median (IQR)	HC U vs. stimulus p-value	AA, n	AA ΔCt median (IQR)	AA U vs. stimulus p-value	HC vs. AA p-value
U	34	14.35 (12.01-15.21)	-	24	15.36 (14.21-16.17)	-	**0.012[d]**
anti-CD3/28	34	11.37 (8.450-13.59)	**< 0.001[b]**	24	12.58 (10.38-14.86)	**< 0.001[a]**	**0.045[c]**
LPS	34	7.10 (6.11-7.96)	**< 0.001[b]**	24	7.92 (7.29-9.10)	**< 0.001[a]**	**0.007[c]**
G	34	6.80 (5.93-8.22)	**< 0.001[b]**	23	8.29 (6.94-8.91)	**< 0.001[a]**	**0.007[c]**
Fi	34	6.92 (5.55-8.09)	**< 0.001[b]**	24	7.51 (6.64-8.58)	**< 0.001[a]**	0.051[c]
Am	34	7.23 (6.24-7.92)	**< 0.001[b]**	24	8.00 (6.92-9.32)	**< 0.001[a]**	**0.046[c]**
Ch1	34	13.19 (11.39-15.14)	0.078[b]	23	15.07 (14.21-15.78)	0.212[a]	**0.003[d]**
Ch2	10	7.243 (6.38-8.11)	**0.002[b]**	9	8.81 (8.25-9.67)	**< 0.001[a]**	**0.004[c]**
Ch3	10	7.21 (6.52-7.99)	**0.002[b]**	9	8.68 (8.15-9.09)	**< 0.001[a]**	**0.014[c]**

Table 18: Changes in PTGS2 expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of PTGS2 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt with interquartile ranges (IQR) in brackets. For detailed description please see **Table 15**.

Stimulus	HC, n	HC ΔCt median (IQR)	HC U vs. stimulus p-value	AA, n	AA ΔCt median (IQR)	AA U vs. stimulus p-value	HC vs. AA p-value
U	34	9.25 (8.14-9.86)	-	24	9.57 (8.93-1.17)	-	**0.033[c]**
anti-CD3/28	34	7.54 (7-16-8.41)	**< 0.001[b]**	24	7.89 (7.16-8.72)	**< 0.001[a]**	0.327[d]
LPS	34	8.34 (7.92-8.60)	**< 0.001[b]**	24	8.55 (7.71-9.09)	**< 0.001[b]**	0.438[d]
G	33	8.43 (7.80-8.86)	**0.038[a]**	23	8.73 (7.96-9.63)	**0.0365[a]**	0.158[c]
Fi	34	8.16 (7.56-8.78)	**< 0.001[b]**	23	8.14 (7.43-8.61)	**< 0.001[b]**	0.396[d]
Am	34	8.28 (7.67-8.87)	**< 0.001[b]**	24	8.04 (7.40-8.66)	**< 0.001[a]**	0.401[d]
Ch1	34	9.08 (8.48-9.92)	0.408[a]	24	9.12 (8.67-9.91)	0.375[b]	0.567[d]
Ch2	10	8.68 (7.04-9.32)	**0.004[a]**	9	9.29 (8.44-9.79)	**0.019[a]**	0.214[c]
Ch3	10	8.38 (7.78-8.98)	**0.011[a]**	9	8.86 (8.01-9.53)	**0.011[a]**	0.229[c]

Table 19: Changes in BIRC3 expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of BIRC3 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt with interquartile ranges (IQR) in brackets. For detailed description please see **Table 15**.

Stimulus	HC, n	HC ΔCt median (IQR)	HC U vs. stimulus p-value	AA, n	AA ΔCt median (IQR)	AA U vs. stimulus p-value	HC vs. AA p-value
U	34	7.47 (6.95-8.37)	-	24	8.68 (7.96-9.33)	-	**< 0.001[d]**
anti-CD3/28	34	6.28 (6.02-6.95)	**< 0.001[a]**	24	6.91 (6.03-8.01)	**< 0.001[b]**	0.077[c]
LPS	34	6.97 (6.60-7.67)	**0.002[a]**	24	7.619 (6.734-8.18)	**< 0.001[b]**	0.108[c]
G	33	7.34 (6.63-8.04)	0.097[a]	23	7.93 (7.28-8.71)	0.056[b]	**0.034[d]**
Fi	34	7.50 (6.53-7.92)	**0.043[a]**	24	7.66 (7.04-8.07)	**< 0.001[b]**	0.182[c]
Am	34	7.24 (6.60-7.84)	0.057[b]	24	7.57 (6.55-7.93)	**< 0.001[b]**	0.611[d]
Ch1	34	8.10 (7.29-8.79)	**0.015*[b]**	24	8.48 (7.69-9.17)	0.509[b]	0.178[d]
Ch2	10	7.70 (6.77-8.11)	0.078[a]	9	8.24 (7.45-8.68)	**0.004[b]**	0.130[c]
Ch3	10	7.69 (6.49-8.02)	**0.022[a]**	9	7.40 (7.11-8.53)	**0.020[b]**	0.511[c]

Table 20: Changes in NFKBIA expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of NFKBIA in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt with interquartile ranges (IQR) in brackets. For detailed description please see **Table 15**.

Stimulus	HC, n	HC ΔCt median (IQR)	HC U vs. stimulus p-value	AA, n	AA ΔCt median (IQR)	AA U vs. stimulus p-value	HC vs. AA p-value
U	34	12.40 (11.93-12.83)	-	24	12.47 (12.05-13.12)	-	0.368[c]
anti-CD3/28	34	11.66 (11.23-12.21)	**0.002[b]**	24	11.80 (11.08-12.90)	**0.002[a]**	0.633[d]
LPS	34	12.47 (12.22-13.00)	**0.007[b]**	24	12.50 (11.89-12.91)	0.726[b]	0.515[d]
G	33	12.61 (12.16-13.16)	**0.017[b]**	23	12.64 (12.17-13.31)	0.253[a]	0.895[d]
Fi	34	12.47 (11.97-12.85)	0.184[a]	23	12.33 (12.02-12.72)	0.888[a]	0.819[c]
Am	34	12.49 (12.05-12.95)	0.081[b]	24	12.29 (11.64-13.29)	0.645[a]	0.286[d]
Ch1	34	12.67 (12.23-12.99)	**0.029[a]**	23	12.35 (11.84-13.23)	0.821[a]	0.667[c]
Ch2	10	12.65 (11.97-13.02)	0.243[a]	9	13.12 (12.56-13.35)	> 0.999[b]	0.133[d]
Ch3	10	12.83 (12.15-13.06)	0.063[a]	9	12.97 (11.95-13.38)	0.426[a]	0.987[c]

Table 21: Changes in MAP3K7 expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of MAP3K7 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt with interquartile ranges (IQR) in brackets. For detailed description please see **Table 15**.

Stimulus	HC, n	HC ΔCt median (IQR)	HC U vs. stimulus p-value	AA, n	AA ΔCt median (IQR)	AA U vs. stimulus p-value	HC vs. AA p-value
U	34	11.07 (10.66-11.47)	-	24	11.58 (10.89-12.03)	-	0.051[d]
anti-CD3/28	34	10.78 (10.32-11.53)	0.070[b]	24	10.99 (10.42-11.92)	**0.029[b]**	0.367[d]
LPS	34	11.10 (10.77-11.84)	**0.008[a]**	24	11.18 (10.73-11.80)	0.603[b]	0.931[d]
G	34	11.18 (10.82-12.16)	**0.023[a]**	23	11.44 (10.75-12.72)	0.360[b]	0.524[d]
Fi	34	11.46 (10.83-11.83)	**0.019[a]**	23	11.33 (10.96-11.86)	0.482[b]	0.994[d]
Am	34	11.37 (10.73-11.75)	**0.008[b]**	24	11.07 (10.42-11.66)	0.290[b]	0.319[d]
Ch1	34	11.21 (10.86-11.72)	0.161[a]	23	11.28 (10.68-11.97)	0.731[b]	0.620[c]
Ch2	10	11.21 (10.50-12.00)	0.522[a]	9	12.02 (11.27-12.35)	> 0.999[b]	0.162[c]
Ch3	10	11.79 (11.18-11.95)	**0.033[a]**	9	11.94 (11.13-12.98)	0.496[b]	0.356[c]

Table 22: Changes in MAPK1 expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of RIPK1 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt with interquartile ranges (IQR) in brackets. For detailed description please see **Table 15**.

Stimulus	HC, n	HC ΔCt median (IQR)	HC U vs. stimulus p-value	AA, n	AA ΔCt median (IQR)	AA U vs. stimulus p-value	HC vs. AA p-value
U	34	11.97 (11.33-12.46)	-	24	12.38 (11.76-12.74)	-	0.051[d]
anti-CD3/28	34	10.58 (10.09-11.39)	**< 0.001[b]**	24	10.91 (10.18-12.20)	**< 0.001[b]**	0.268[d]
LPS	34	12.03 (11.57-12.52)	**0.012[b]**	24	12.25 (11.61-12.68)	0.565[b]	0.515[d]
G	33	12.24 (11.77-13.03)	**0.009[a]**	23	12.30 (11.62-13.19)	0.111[b]	0.563[d]
Fi	34	12.13 (11.53-12.80)	**0.028[a]**	24	12.15 (11.94-12.61)	0.491[b]	0.585[c]
Am	34	12.06 (11.53-12.62)	**0.022[b]**	24	11.92 (11.42-12.39)	0.439[b]	0.393[d]
Ch1	34	12.18 (11.88-12.88)	**0.002[a]**	23	12.28 (11.40-12.99)	0.988[b]	0.807[c]
Ch2	10	12.43 (11.47-12.85)	0.284[a]	9	13.06 (12.26-13.27)	> 0.999[b]	0.182[d]
Ch3	10	12.72 (12.18-13.04)	**0.012[a]**	9	12.82 (11.81-13.20	0.359[b]	0.942[c]

Table 23: Changes in MAPK9 expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of MAPK9 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt with interquartile ranges (IQR) in brackets. For detailed description please see **Table 15**.

Stimulus	HC, n	HC ΔCt median (IQR)	HC U vs. stimulus p-value	AA, n	AA ΔCt median (IQR)	AA U vs. stimulus p-value	HC vs. AA p-value
U	34	12.57 (12.08-13.04)	-	24	12.80 (12.36-13.23)	-	0.596[c]
anti-CD3/28	34	12.26 (11.86-12.76)	0.081[b]	24	12.43 (11.97-13.49	0.476[a]	0.536[d]
LPS	34	12.93 (12.47-13.52)	**0.003[a]**	23	12.71 (12.48-13.30)	0.170[b]	0.567[d]
G	33	13.08 (12.54-13.70)	**0.003[a]**	23	12.97 (12.57-13.80)	**0.016[b]**	0.895[d]
Fi	34	12.96 (12.59-13.38)	**0.012[a]**	23	12.84 (12.38-13.44)	0.117[a]	0.937[c]
Am	34	13.06 (12.59-13.39)	**0.002[b]**	23	12.60 (12.16-13.05)	0.779[a]	0.075[d]
Ch1	34	12.82 (12.42-13.41)	**0.025[a]**	23	12.77 (12.19-13.53)	0.581[a]	0.911[c]
Ch2	10	12.80 (12.28-13.57)	0.183[a]	9	13.47 (12.66-13.94)	0.787[a]	0.473[c]
Ch3	10	13.18 (12.75-13.60)	**0.008[a]**	9	13.33 (12.69-13.95)	0.887[a]	0.975[c]

Table 24: Changes in MAPK14 expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of MAPK14 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt with interquartile ranges (IQR) in brackets. For detailed description please see **Table 15**.

Stimulus	HC, n	HC ΔCt median (IQR)	HC U vs. stimulus p-value	AA, n	AA ΔCt median (IQR)	AA U vs. stimulus p-value	HC vs. AA p-value
U	15	12.59 (11.65-13.31)	-	17	13.02 (12.71-13.72)	-	0.114[d]
anti-CD3/28	15	11.67 (10.98-12.54)	**0.019[a]**	17	12.57 (12.13-13.56)	**0.027[b]**	**0.017[c]**
LPS	15	12.72 (12.48-13.54)	**0.012[a]**	17	13.18 (12.52-13.52)	0.963[b]	0.829[c]
G	15	13.02 (11.83-13.44)	0.141[a]	17	13.36 (12.21-14.31)	0.378[b]	0.429[c]
Fi	15	13.33 (11.91-14.22)	0.195[a]	17	13.01 (12.71-13.49)	0.747[b]	0.823[d]
Am	15	13.38 (12.15-14.20)	**0.005[a]**	17	12.73 (12.11-13.69)	0.431[b]	0.357[c]
Ch1	15	13.17 (12.16-13.99)	**0.043[a]**	17	12.74 (12.13-13.83)	0.854[b]	0.811[c]
Ch2	10	13.22 (11.30-13.77)	0.501[a]	9	13.44 (12.49-14.22)	0.734[b]	0.549[d]
Ch3	10	13.66 (13.06-14.09)	**0.048[a]**	9	12.45 (10.71-14.01)	0.129[b]	0.133[d]

Table 25: Changes in MAPK7 expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of MAK7 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt with interquartile ranges (IQR) in brackets. For detailed description please see **Table 15**.

Stimulus	HC, n	HC ΔCt median (IQR)	HC U vs. stimulus p-value	AA, n	AA ΔCt median (IQR)	AA U vs. stimulus p-value	HC vs. AA p-value
U	10	9.75 (8.47-10.17)	-	10	11.07 (10.46-12.11)	-	**0.009[c]**
anti-CD3/28	10	11.68 (10.91-12.11)	**< 0.001[a]**	10	11.62 (9.928-12.25)	0.745[a]	0.572[c]
LPS	10	9.22 (8.62-10.08)	0.379[a]	10	9.87 (8.99-10.46)	**0.050[a]**	0.204[c]
G	10	9.24 (8.66-9.96)	0.345[a]	9	9.268 (8.782-10.810)	0.053[a]	0.257[c]
Fi	10	9.77 (9.16-10.28)	0.509[a]	10	10.38 (9.43-10.77)	0.080[a]	0.367[c]
Am	10	9.19 (8.85-10.40)	0.790[a]	10	10.01 (9.007-10.70)	0.275[b]	0.315[d]
Ch1	10	9.81 (8.83-10.48)	0.843	10	10.27 (9.458-10.92)	0.099[a]	0.133[c]
Ch2	10	9.21 (8.63-9.51)	0.259[a]	9	10.55 (9.14-11.07)	0.201[a]	**0.021[c]**
Ch3	10	9.61 (8.62-10.24)	0.979[a]	9	9.89 (9.48-10.27)	0.098[b]	0.315[d]

Table 26: Changes in DUSP1 expression levels upon stimulation (ΔCt values)
Relative mRNA expression levels of DUSP1 in unstimulated PBMCs of healthy controls (HC) and allergic asthmatics (AA). Gene expression normalised to 18S and displayed as ΔCt with interquartile ranges (IQR) in brackets. For detailed description please see **Table 15**.

10.1.2 Supplementary figures – Gene-gene correlation analyses of all children

Figure 36-38 display gene-gene correlations in all children regardless of the clinical phenotype. Overall, the investigated genes correlated highly and mostly significantly with each other both at baseline and after stimulation.

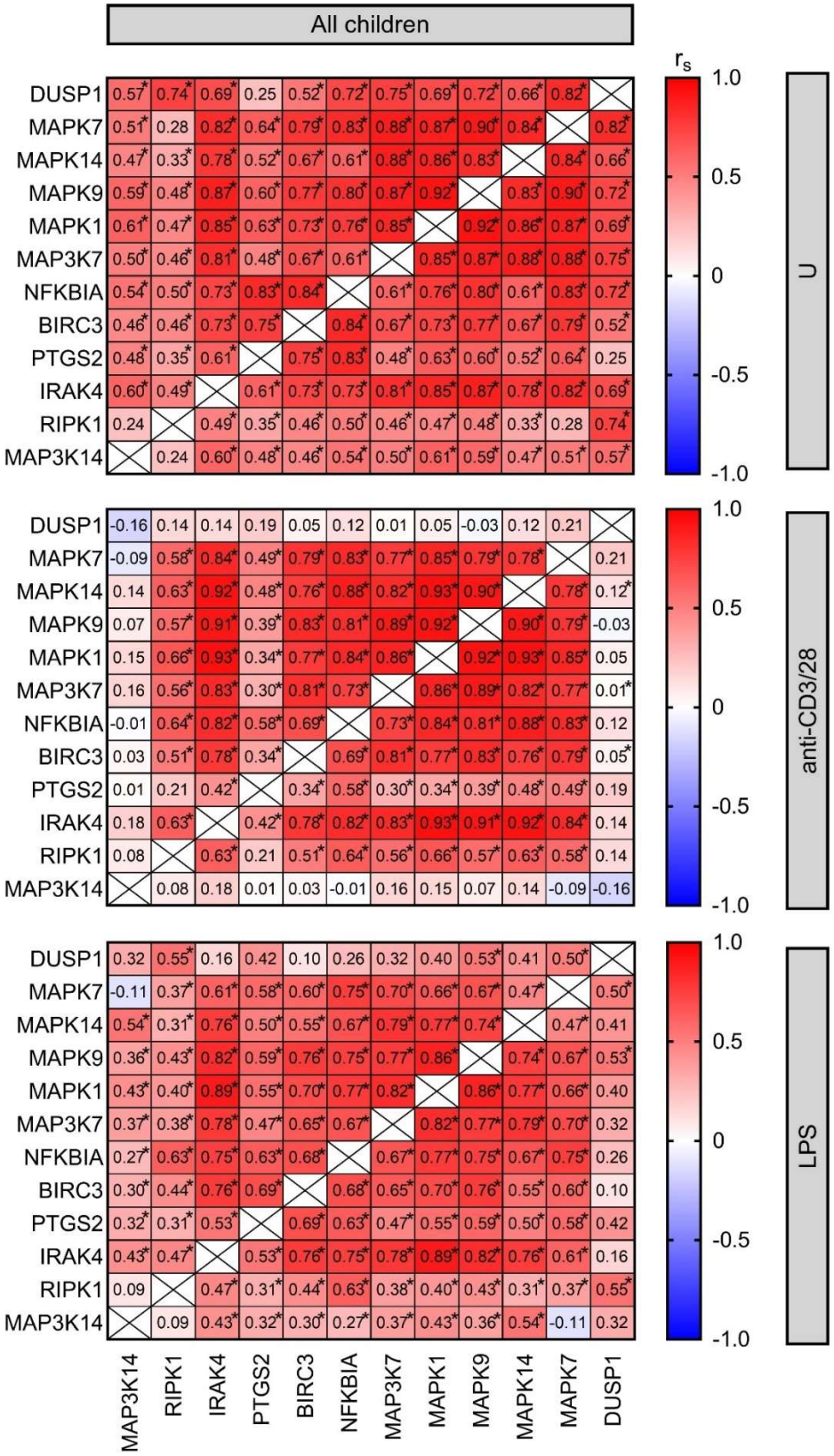

Figure 36: Gene-gene correlations of all children at baseline (U) and after stimulation with anti-CD3/28 and LPS

Heat maps indicating gene-gene correlations of all children (i.e. healthy controls and allergic asthmatics). Unstimulated condition (U), anti-CD3/28 (anti CD3 and anti-CD28 antibodies), LPS (lipopolysaccharide). Spearman correlation coefficient (r_s). Positive regulation is colour coded in red (rs > 0), negative correlation is colour coded in blue (r_s < 0). Significant findings are marked with an asterisk (*).

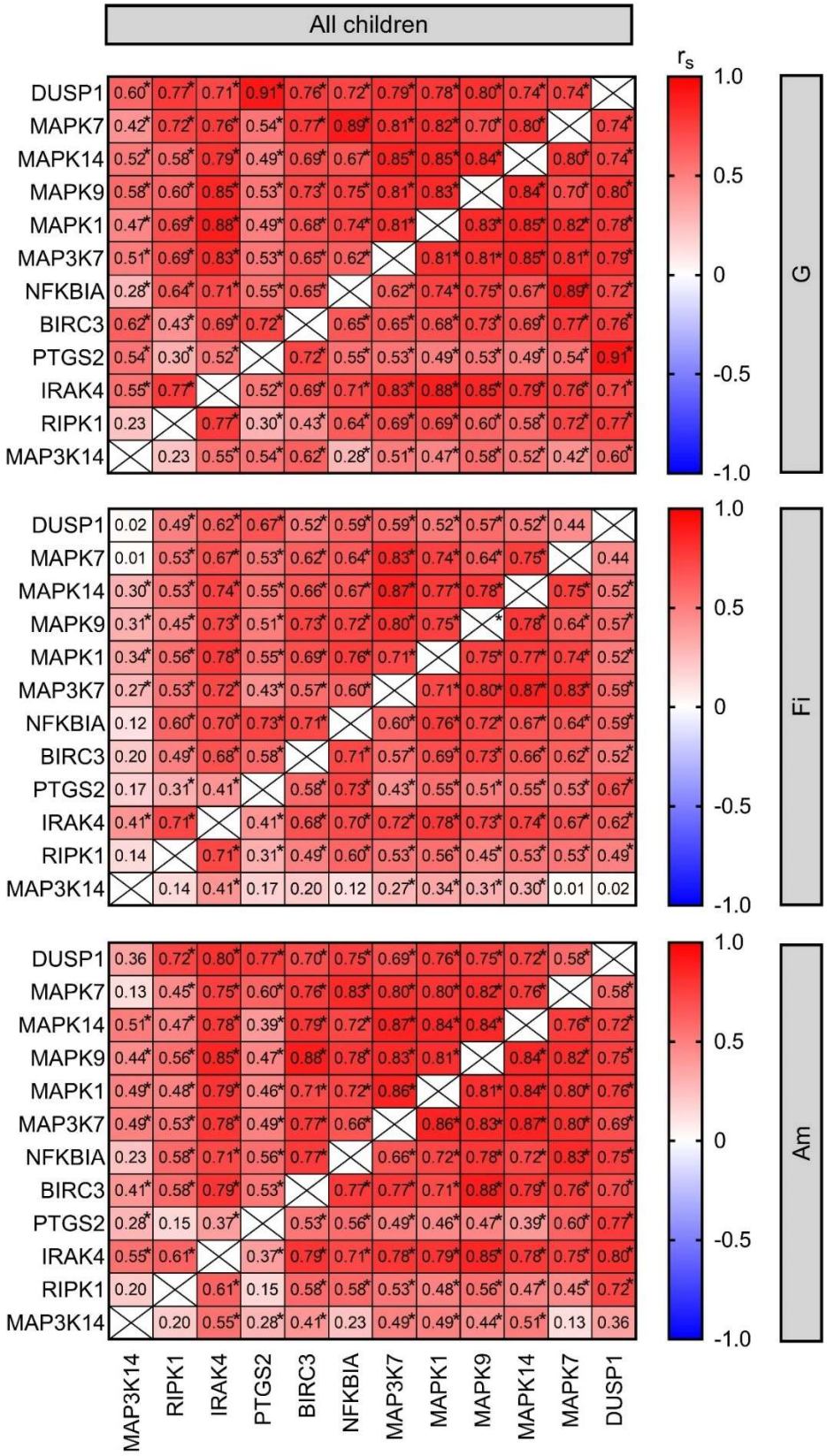

Figure 37: Gene-gene correlations of all children after stimulation with German, Finnish and Amish farm dust extracts

Heat maps indicating gene-gene correlations of all children (i.e. healthy controls and allergic asthmatics). German farm dust (G), Finnish farm dust (fi), Amish farm dust (Am). Spearman correlation coefficient (r_s). Positive regulation is colour coded in red (rs > 0), negative correlation is colour coded in blue (r_s < 0). Significant findings are marked with an asterisk (*).

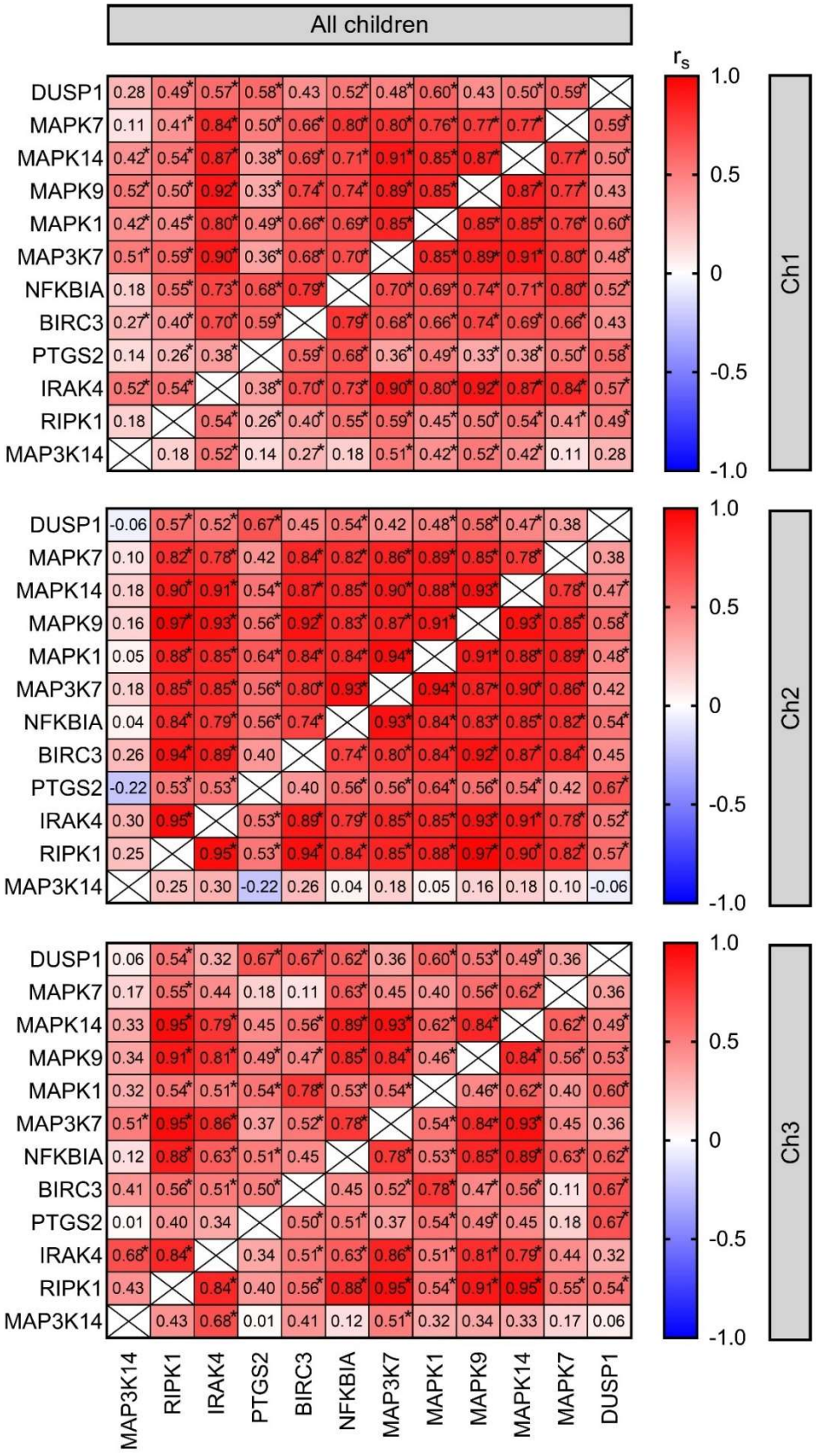

Figure 38: Gene-gene correlations of all children after stimulation with Chinese farm dust extracts
Heat maps indicating gene-gene correlations of all children (i.e. healthy controls and allergic asthmatics). Chinese farm dust 1 (Ch1), Chinese farm dust 2 (Ch2), Chinese farm dust 3 (Ch3). Spearman correlation coefficient (r_s). Positive regulation is colour coded in red (rs > 0), negative correlation is colour coded in blue (r_s < 0). Significant findings are marked with an asterisk (*).

10.2 List of tables

10.3 List of figures

10.4 CLARA/CLAUS questionnaire

Datum:_____ Studiennummer:_____

Bei Studieneinschluss erhobene Daten:	
Körpergröße (in cm)	
Gewicht (in kg)	

Asthmastudie CLAUS

Fragebogen für die Eltern

Wir freuen uns, dass Sie an unserer Asthmastudie teilnehmen. Bitte kreuzen Sie die folgenden Fragen an. Ihre Antworten werden vertraulich behandelt. Wenn Sie eine Frage nicht beantworten möchten, lassen Sie sie bitte aus.

Wir danken Ihnen herzlich für Ihre Mitarbeit!

Wir beginnen mit Fragen zu pfeifenden und keuchenden Atemgeräuschen. Mit pfeifenden Atemgeräuschen meinen wir ein pfeifendes Geräusch, das aus dem Brustkorb kommt, aber nicht geräuschvolles Atmen durch die Nase.

1. Hat Ihr Kind jemals pfeifende bzw. keuchende Atemgeräusche gehabt?

Ja ..... ☐

Falls Ja,

wann sind diese zum ersten Mal aufgetreten:_____

Nein ..... ☐ ⇒ weiter mit Frage 12

2. Hatte Ihr Kind in den letzten 12 Monaten pfeifende bzw. keuchende Atemgeräusche?

Ja..... ☐

Nein..... ☐ ⇒ weiter mit Frage 12

3. Wie oft hatte Ihr Kind in den letzten 12 Monaten pfeifende bzw. keuchende Atemgeräusche?

Gar nicht ..... ☐

1-3 mal..... ☐

4-12mal ..... ☐

Mehr als 12 mal ..... ☐

4. Hatte Ihr Kind <u>in den letzten 12 Monaten</u> jemals <u>Atemnot</u>, als die pfeifenden/ keuchenden Atemgeräusche auftraten?

Ja ..... ☐

Nein ..... ☐

5. Wie häufig ist Ihr Kind <u>in den letzten 12 Monaten nachts</u> wegen pfeifender oder keuchender Atemgeräusche aufgewacht?

Seltener als einmal pro Monat ..... ☐

Einmal pro Monat ..... ☐

Mindestens zweimal pro Monat ..... ☐

6. Wodurch wurden bei Ihrem Kind die <u>pfeifenden / keuchenden Atemgeräusche</u> ausgelöst?

	Ja	Nein
Anstrengung	☐	☐
Erkältung.....	☐	☐
Kontakt mit Tieren.....	☐	☐
Kontakt mit Hausstaub	☐	☐
Kontakt mit Gras	☐	☐
Sonstiges.....	☐	☐

_____

7. Wie häufig hatte Ihr Kind <u>in den letzten 12 Monaten</u> pfeifende oder keuchende Atemgeräusche, <u>ohne dass es erkältet war</u>?

Nie..... ☐

Seltener als einmal pro Monat ..... ☐

Einmal pro Monat ..... ☐

Mindestens zweimal pro Monat ..... ☐

8. Ist das Kind zwischen diesen Episoden völlig beschwerdefrei?

Ja .... ☐ ⇒ weiter mit Frage 12

Nein..... ☐

9. Hat Ihr Kind zwischen diesen Episoden folgende Beschwerden bei Anstrengung?

	Ja	Nein
Husten	☐	☐
Pfeifende Atemgeräusche	☐	☐
Atemnot	☐	☐

Sonstiges:_____

Bei Temperaturwechsel/Nebel?

	Ja	Nein
Husten	☐	☐
Pfeifende Atemgeräusche	☐	☐
Atemnot	☐	☐

Sonstiges:_____

Nachts?

	Ja	Nein
Husten	☐	☐
Pfeifende Atemgeräusche	☐	☐
Atemnot	☐	☐

Sonstiges:_____

Sonstige Beschwerden?

:_____

10. Hat Ihr Kind jemals <u>in den letzten 12 Monaten</u> von einem Arzt Medikamente gegen pfeifende oder keuchende Atemgeräusche, oder Giemen oder Atemnot verschrieben bekommen?
(Gemeint sind damit nicht nur Medikamente zum Schlucken, sondern auch Inhalationen oder Sprays)

Ja	☐	
Nein	☐	⇒ weiter mit Frage 12

11. Welche Medikamente waren dies?

Bitte geben Sie den Markennamen möglichst genau an! Und sofern Sie es wissen die Dosis sowie den Zeitraum, in dem das Medikament eingenommen wurde.

1. _____
2. _____
3. _____

12. Hat Ihr Kind jemals <u>in den letzten 12 Monaten</u> von einem Arzt Medikamente aus einem anderen Grund verschrieben bekommen?

(Gemeint sind damit nicht nur Medikamente zum Schlucken, sondern auch Inhalationen oder Sprays)

Ja ..... ☐

Nein ..... ☐ ⇒ weiter mit Frage 14

13. Welche Medikamente waren dies?

Bitte geben Sie den Markennamen möglichst genau an! Und sofern sie es wissen die Dosis sowie den Zeitraum in dem das Medikament eingenommen wurde.

1. _____
2. _____
3. _____

Es folgen Fragen zu Beschwerden der Nase und der Augen

14. Hat Ihr Kind **jemals** Niesanfälle oder eine laufende, verstopfte oder juckende Nase, obwohl es **nicht erkältet** war?

Ja ..... ☐

Falls Ja, wann ist dies zum ersten Mal aufgetreten:

Nein ..... ☐ ⇒ weiter mit Frage 18

15. Hatte Ihr Kind **in den letzten 12 Monaten** Niesanfälle oder eine laufende, verstopfte oder juckende Nase, obwohl es **nicht erkältet** war?

Ja ..... ☐

Nein..... ☐ ⇒ weiter mit Frage 18

16. Hatte Ihr Kind in den letzten 12 Monaten gleichzeitig mit diesen Nasenbeschwerden juckende oder tränende Augen?

Ja ..... ☐

Nein..... ☐

17. Wann in den letzten 12 Monaten traten diese Nasen-Beschwerden auf?

Mehrere Antworten sind möglich.

Januar	☐	Mai	☐	September	☐
Februar	☐	Juni	☐	Oktober	☐
März	☐	Juli	☐	November	☐
April	☐	August	☐	Dezember	☐

18. Ist von einem Arzt bei Ihrem Kind schon einmal Heuschnupfen oder eine allergische Rhinitis bzw. Rhinokonjunktivitis festgestellt worden?

Ja ..... ☐

Nein..... ☐

Es folgen Fragen zu Hauterkrankungen

19. **Hatte Ihr Kind jemals eine Neurodermitis/atopische Dermatitis/ atopisches Ekzem**

Ja ..... ☐

Falls Ja, wann ist diese zum ersten Mal aufgetreten:

Nein ..... ☐ ⇒ weiter mit Frage 29

20. **Wurde bei Ihrem Kind die Diagnose einer Neurodermitis/atopischen Dermatitis/ atopisches Ekzem von einem Arzt gestellt?**

Ja ..... ☐

Nein..... ☐

21. **Hatte Ihr Kind <u>in den letzten 12 Monaten</u> eine Neurodermitis/atopische Dermatitis/ atopisches Ekzem**

Ja ..... ☐

Nein ..... ☐

22. War der Hautausschlag je an einer der folgenden Stellen?

	Ja	Nein
Gesicht	☐	☐
Hals	☐	☐
Ellenbeugen / Kniekehlen	☐	☐
Hand- / Fußgelenke	☐	☐
Brust/Rücken	☐	☐

23. Hat sich die Lokalisation des Ausschlages im Laufe der Zeit geändert?

Ja ☐

Falls Ja, wo war er zu Beginn? Wo befindet er sich heute?

Zu Beginn:

	Ja	Nein
Gesicht	☐	☐
Hals	☐	☐
Ellenbeugen / Kniekehlen	☐	☐
Hand- / Fußgelenke	☐	☐
Brust/Rücken	☐	☐

Heute:

	Ja	Nein
Gesicht	☐	☐
Hals	☐	☐
Ellenbeugen / Kniekehlen	☐	☐
Hand- / Fußgelenke	☐	☐
Brust/Rücken	☐	☐

Nein ☐

24. Wenn Sie die Zeiten, in denen Ihr Kind diesen Hautausschlag hatte, zusammenzählen: Wie lange haben Sie diesen Hautausschlag insgesamt beobachtet?

Für insgesamt weniger als 3 Monate .... ☐
Für insgesamt 3-6 Monate ..... ☐
Für insgesamt 6-12 Monate ..... ☐
Für länger als 12 Monate ..... ☐

25. Ist der Hautausschlag wieder völlig verschwunden, oder „kommt und geht" der Hautausschlag?

Der Hautausschlag ist vollständig
Verschwunden ..... ☐
Der Hautausschlag „kommt und geht".. ☐
Der Hautausschlag ist noch da..... ☐

26. Wie alt war Ihr Kind, als der Hautausschlag vollständig verschwunden ist?

_____ Monate

27. Wie häufig ist Ihr Kind <u>nachts</u> wegen Juckreiz aufgewacht?

Seltener als einmal pro Monat oder nie ..... ☐
Einmal pro Monat ..... ☐
Mindestens zweimal pro Monat ..... ☐

28. Haben Sie die Haut Ihres Kindes <u>in den letzten 12 Monaten</u> mit einer cortisonhaltigen Creme / Salbe oder einer Tacrolimus- bzw. Pimecrolimus-haltigen Salbe (Protopic, Elidel) behandelt?

Ja..... ☐
Nein..... ☐

Es folgen Fragen zu Nahrungsunverträglichkeiten oder –allergien

29. Hat Ihr Kind eine Nahrungsmittelallergie?

Ja ..... ☐
Nein ..... ☐ ⇒ weiter mit Frage 32

30. Wie äußert sich diese Nahrungsmittelallergie?

Ausschlag/rote Flecken um den Mund herum ..... ☐
Ausschlag/rote Flecken an anderen Körperstellen .... ☐
Schwellung der Lippen ..... ☐
Juckreiz ..... ☐
Durchfall ..... ☐
Erbrechen..... ☐
Verschlechterung der Neurodermitis..... ☐
Pfeifende Atemgeräusche ..... ☐
Atemnot ..... ☐
Kreislaufreaktion/Blutdruckabfall ..... ☐
Sonstiges:

31. Auf welche Nahrungsmittel reagiert Ihr Kind?

	Ja	Nein
Milch und Milchprodukte	☐	☐
Hühnereier	☐	☐
Fisch	☐	☐
Weizenmehl oder andere Getreideprodukte	☐	☐
Nüsse	☐	☐
Soja	☐	☐
Zitrusfrüchte	☐	☐
Anderes Obst oder Gemüse	☐	☐
Andere Nahrungsmittel	☐	☐

Welche? _____

32. A) Haben Sie Ihr Kind gestillt?

Ja ☐ Wie lange haben Sie Ihr Kind gestillt?.....

Nein ☐.....

33. Hat Ihr Kind jemals Hypoallergene Nahrung bekommen?

(z.B. Alete H.A., Aptamil H.A., Beba H.A., Hipp H.A., Humana H.A., Milumil H.A.)

Ja..... ☐

Falls Ja,

Zur Vorbeugung einer Nahrungsmittelallergie ..... ☐

Aufgrund einer manifesten Nahrungsmittelallergie .. ☐

Welches Hypoallergne Nahrung haben sie verwendet:

(Bitte geben Sie den Namen möglichst genau an)

Nein ☐

Es folgen Fragen zu anderen Erkrankungen

34. Wurde bei Ihrem Kind jemals <u>von einem Arzt/einer Ärztin</u> eine spastische Bronchitis, obstruktive Bronchitis oder asthmatische Bronchitis diagnostiziert?

Nein, nie ..... ☐

Ja, einmal..... ☐

Ja, mehrmals ..... ☐

35. Wurde bei Ihrem Kind in den letzten 12 Monaten <u>von einem Arzt/einer Ärztin</u> eine der folgenden Diagnosen gestellt?

	Ja	Nein
Asthma.....	☐	☐
Neurodermitis, atopische Dermatitis oder endogenes Ekzem.....	☐	☐
Allergische Rhinitis/Heuschnupfen	☐	☐

36. Hatte Ihr Kind bisher eine der folgenden Erkrankungen <u>im ersten Lebensjahr</u>?

	Ja	Nein
Mittelohrentzündung	☐	☐
Pseudokrupp	☐	☐
Lungenentzündung	☐	☐
Bronchitis	☐	☐
Bronchiolitis	☐	☐
Keuchhusten	☐	☐
Andere Infektionen	☐	☐

Welche? _____

Waren stationäre Aufenthalte im Krankenhaus notwendig.... ☐.....☐

Warum? _____

37. Hatte Ihr Kind bisher eine der folgenden Erkrankungen <u>nach dem ersten Lebensjahr</u>?

	Ja	Nein
Mittelohrentzündung	☐	☐
Pseudokrupp	☐	☐
Lungenentzündung	☐	☐
Bronchitis	☐	☐
Bronchiolitis	☐	☐
Keuchhusten	☐	☐
Andere Infektionen	☐	☐

Welche? _____

Waren stationäre Aufenthalte im Krankenhaus notwendig.... ☐.....☐

Warum? _____

_____

38. Hatten Sie den Eindruck, dass Ihr Kind im Säuglings- oder Kleinkindalter vermehrt geschwitzt hat?

Ja.....	☐
Nein	☐

Fragen zu Verhalten und Erkrankungen der Mutter in der Schwangerschaft

39. Hatten Sie in der Schwangerschaft eine Infektion, die die Einnahme eines Antibiotikums erfordert hat?

Ja ..... ☐

Nein ..... ☐

Falls Ja,

Welche Art der Infektion:

Welches Antibiotikum haben Sie eingenommen:

40. Waren stationäre Aufenthalte im Krankenhaus, außer für die Geburt notwendig?

Ja ..... ☐

Nein ..... ☐

Falls Ja,

Warum:

41. Haben Sie in der Schwangerschaft Medikamente eingenommen?

	Ja	Nein
3. Paracetamol.....	☐	☐
4. Andere Medikamente:		

Angaben zur Wohnungs- und Lebenssituation

42.

A) Wie viele <u>jüngere</u> Geschwister hat Ihr Kind?

Bitte auch Stiefgeschwister mitzählen, die in Ihrer Familie leben!

Schwestern..... Brüder.....

B) Wie viele <u>ältere</u> Geschwister hat Ihr Kind?
Bitte auch Stiefgeschwister mitzählen, die in Ihrer Familie leben!

Schwestern.....Brüder.....

43. Wird oder wurde Ihr Kind <u>regelmäßig</u> zusammen mit anderen Kindern durch eine Tagesmutter oder bei den Großeltern betreut? Die eigenen Geschwister sind dabei nicht gemeint.

Ja, ... ☐

Im 3. bis 6. Lebensjahr	Im 1. oder 2. Lebensjahr	Bei Geburt
☐	☐.....	☐

Mit wie vielen anderen Kindern: _____

Nein ..... ☐

44. Wird oder wurde Ihr Kind <u>regelmäßig</u> zusammen mit anderen Kindern in einer Kinderkrippe oder im Kindergarten betreut? Die eigenen Geschwister sind dabei nicht gemeint.

Ja, ... ☐

Im 3. bis 6. Lebensjahr	Im 1. oder 2. Lebensjahr	Bei Geburt
☐	☐.....	☐

Mit wie vielen anderen Kindern? _____

Nein ☐

45. Welche der folgenden Haustiere haben/hatten Sie innerhalb der Wohnung?
Mehrere Antworten sind möglich.

	Zur Zeit	Im 1. oder 2. Lebensjahr	Bei Geburt
Keine	☐.....	☐	☐
Hund	☐	☐	☐
Katze	☐.....	☐	☐

A) Darf oder durfte sich eine Katze <u>im Zimmer</u>, in dem Ihr Kind schläft aufhalten?

Ja ☐

Nein ☐

B) Darf oder durfte sich eine Katze <u>im Bett</u> Ihres Kindes aufhalten?

Ja ☐

Nein ☐

C) Darf oder durfte sich ein Hund <u>im Zimmer</u>, in dem Ihr Kind schläft aufhalten?

Ja ☐

Nein ☐

D) Darf oder durfte sich ein Hund <u>im Bett</u> Ihres Kindes aufhalten?

Ja ☐

Nein ☐

46. Hat/hatte Ihr Kind sonst regelmäßig (ca. 1x/Woche) Kontakt zu folgenden Tieren (z.B. in der Wohnung von Freunden/ Verwandten)? *Mehrere Antworten sind möglich.*

	Zur Zeit	1. oder 2. Lebensjahr	Bei Geburt	Nein
Hund	☐	☐	☐	☐
Katze	☐	☐	☐	☐

47. Gab es in Ihrer Wohnung <u>jemals</u> Feuchtigkeitsflecken bzw. Schimmelbefall an Wänden oder Decken?

Feuchtigkeitsflecken in Bad oder Küche sind dabei nicht gemeint, sondern nur in Räumen wie Wohnzimmer, Schlafzimmer oder Kinderzimmer.

	Ja	Nein
Feuchtigkeitsflecken, aber <u>ohne</u> Schimmelbefall	☐	☐

Zur Zeit	Im 1. oder 2. Lebensjahr	Bei Geburt
☐	☐	☐

	Ja ..... Nein
Feuchtigkeitsflecken mit Schimmelbefall.....	☐..... ☐

Zur Zeit	Im 1. oder 2. Lebensjahr	Bei Geburt
☐.....	☐	☐

Es folgen Fragen zu Ihrer Familie

48. Hat ein Arzt bei der Mutter des Kindes jemals eine der folgenden Erkrankungen diagnostiziert?

☐ Asthma

☐ Heuschnupfen

☐ Ekzem

☐ Autoimmunerkrankung, wie z.B. Diabetes, rheumatoide Arthritis.

☐ Schilddrüsenerkrankung, bitte angeben welche _____

☐ Darmerkrankung (M. Crohn, ulzerative Kolitis)

☐ Weitere: _____

☐ Nein

49. Falls die Mutter an einer allergischen Erkrankung (Heuschnupfen, allergisches Asthma bronchiale, Neurodermitis/atopische Dermatitis/endogenes Ekzem) leidet, war diese aktiv während der Schwangerschaft?

Ja ☐

Nein ☐

50. Hat ein Arzt bei dem Vater des Kindes jemals eine der folgenden Erkrankungen diagnostiziert?

☐ Asthma

☐ Heuschnupfen

☐ Ekzem

☐ Autoimmunerkrankung, wie z.B. Diabetes, rheumatoide Arthritis.

☐ Schilddrüsenerkrankung, bitte angeben welche _____

☐ Darmerkrankung (M. Crohn, ulzerative Kolitis)

☐ Weitere: _____

☐ Nein

51. Hat ein Arzt <u>bei Verwandten</u> jemals eine allergische Erkrankung diagnostiziert?

Asthma?

Ja ..... ☐

Nein ..... ☐

Falls Ja, bei wem:

Heuschnupfen?

Ja ..... ☐

Nein ..... ☐

Falls Ja, bei wem:

Neurodermitis/atopische Dermatitis/endogenes Ekzem?

Ja ..... ☐

Nein ..... ☐

Falls Ja, bei wem:

Es folgen Fragen zum Rauchverhalten

52. Rauchen Sie oder Ihre Familie in Ihrer Wohnung/Haus?

Ja ..... ☐

Nein ..... ☐

53. Haben sie in der Schwangerschaft geraucht?

Ja ..... ☐

Falls ja, wie viele Zigaretten am Tag (durchschnittlich):

Nein ☐

54. Haben Sie und Ihre Familie <u>in den letzten 12 Monaten</u> mit dem Rauchen in der Wohnung aufgehört bzw. das Rauchen innerhalb der Wohnräume eingeschränkt?

Ja ..... ☐

Nein ..... ☐

Es wurde nie geraucht ..... ☐ ⇒ weiter mit Frage 56

55. Wie viele Zigaretten werden durchschnittlich am Tag <u>in Ihrer Wohnung</u> (damit meinen wir auch die Küche) geraucht? Zigaretten, die auf dem Balkon oder der Terrasse geraucht werden, brauchen nicht mitgezählt zu werden. Wie viele davon von... (keine=0)

Mutter	_____	pro Tag
Partner	_____	pro Tag
Andere Personen	_____	pro Tag
Insgesamt	_____	pro Tag

Es folgen noch allgemeine Fragen

56. Wurde Ihr Kind in Deutschland geboren?

☐ Ja
☐ Nein Es wurde in .....geboren.
Es kam mit .....Jahren nach Deutschland?

57. Welche Staatsangehörigkeit hat Ihr Kind?

☐ Deutsch
☐ Andere → Welche? _____

58. In welchem Land wurde die (leibliche) **Mutter des Kindes** geboren?

☐ Deutschland
☐ Türkei
☐ Andere → Welche? _____

59. In welchem Land wurde der (leibliche) **Vater des Kindes** geboren?

☐ Deutschland
☐ Türkei
☐ Andere → Welche? _____

60. Welches ist der **höchste** Schulabschluss der Mutter bzw. des Vaters des Kindes?

	Mutter	**Vater**
Keine abgeschlossen Schulausbildung	☐	☐
Hauptschule, Volksschule	☐	☐
Realschule, Mittlere Reife	☐	☐
Gymnasium, Abitur, Fachabitur	☐	☐
Hochschule, Fachhochschule, Universität	☐	☐
Andere Ausbildung	☐	☐

Welche _____ .

Haben Sie noch weitere Kommentare zum Fragebogen oder allgemein?

_____

_____

_____

Wir danken Ihnen herzlich für das Ausfüllen des Fragebogens!

Bei Fragen können Sie sich jederzeit gerne an uns wenden.

Studienleitung:

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11 Publications, Posters and Presentations

11.1 Publications

Theodorou J, **Nowak E**, Böck A, Salvermoser M, Zeber K, Kulig P, Tsang M, Wong C, Wong GWK, Roponen M, Kumbrink J, Alhamdan F, Michel F, Garn H, Tosevski V, Schaub B, Mitogen activated protein kinase (MAPK) signalling in childhood asthma development and environment-mediated protection. Pediatric Allergy and Immunology, online ahead of print 08/2021, DOI: 10.1111/pai.13657

Nowak E, Schaub B, Aktuelle Daten zur Prävention von Asthma und Allergie im Kindesalter. pädiatrische praxis 2019; 92, 1-12, Mediengruppe Oberfranken – Fachverlage GmbH & Co. KG, 09/2019

Nowak E, Schaub B, Prevention of allergies. In: Implementing precision medicine in best practices of chronic airway disease. Elsevier S&T Books, 09/2018

Nowak E, Neuner A, Landgraf-Rauf K, Schaub B, Asthma und Allergieprävention. Pädiatrie up2date 2017; 2:143-159, 06/2017

11.2 Posters and Presentations

Krusche J, **Nowak E**, Böck A, Hengst M, Roponen M, Kumbrink J, Alhamdan F, Potaczek DP, Garn H, Schaub B, DUSP1 plays a pivotal role in MAPK signalling in childhood asthma development and environment-mediated protection. EAACI Congress Lisbon, Portugal, 06/2019 (Abstract)

Krusche J, Böck A, Nicklas T, **Nowak E**, Rehbach K, Twardziok M, Roponen M, Schaub B, Role of IL-18 activation in childhood asthma development and environment-mediated protection. EAACI Congress Munich, Germany, 05/2018 (Poster)

Krusche J, **Nowak E**, Protection against childhood asthma: Importance of NF-κB and MAPK signalling. TRILATERAL meeting, Munich, Germany, 05/2018 (Presentation)

Nowak E, Protection against childhood asthma: Importance of NF-κB and MAPK signalling. Final seminar of the *FöFoLe* doctoral study programme. Herrsching, Germany, 04/2018 (Presentation)

Nowak E, Protection against asthma in childhood: Importance of NF-κB and MAPK pathway. *Allergy on the Isle*, doctoral candidates' workshop of the German Society of Allergology and Clinical Immunology (DGAKI). Frauenchiemsee, Germany, 01/2018 (Presentation)

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13 Affidavit

Eidesstattliche Versicherung

Nowak, Elisabeth Dorothea Susanne Victoria

Name, Vorname

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel

New insights into immunological mechanisms behind the farm effect in childhood asthma: Modulation of *ex vivo* gene expression involved in NF-κB and MAPK signalling pathways

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, 01.10.2021

Ort, Datum

Elisabeth Nowak

Unterschrift Doktorandin

14 Curriculum vitae