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Vorstand: Prof. Dr. Dr. Christoph Klein

The role of TNFAIP3 and NF-κB signalling in childhood asthma development and environment-mediated protection

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Katharina Johanne Rehbach
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Berichterstatterin:	Prof. Dr. Bianca Schaub
Mitberichterstatter:	Prof. Dr. Jürgen Behr Prof. Dr. Katja Radon Prof. Dr. Jürgen Kleinschmidt
Dekan:	Prof. Dr. med. dent. Reinhard Hickel
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Table of content

1 Introduction

1.1 Childhood asthma – the burden of a chronic disease

Asthma is the leading chronic respiratory disease in children and adolescents, with a present prevalence varying from 10 to 30% in Western Europe and English-speaking countries[1]. Clinical features of asthma are characterised by respiratory symptoms such as wheeze, chest tightness, shortness of breath and cough together with variable expiratory airflow limitation[2]. These symptoms are widely assumed to be the result of chronic airway inflammation with early structural airway 'remodelling' and airway hyperresponsiveness (AHR) to certain external trigger factors.

Current asthma management aims at effective symptom control and prevention of adverse disease outcomes. It involves two key treatments: (i) Inhaled corticosteroids (ICS), which target inflammatory processes, and (ii) β_2-agonists (bronchodilators) that dilate the airways through bronchial smooth muscle relaxation. In paediatric asthma, leukotriene receptor antagonists (LTRA) complement therapy, and in patients with severe asthma, long-acting muscarinic antagonists (LAMA) and biological agents such as anti-immunoglobulin E antibody treatment are also considered[2,3]. More recently, novel biological therapies targeting the inhibition of the cytokines interleukin (IL)-4, IL-13 and IL-5 have been applied in paediatric patients as well. Non-pharmacological treatment of allergic asthma further includes allergen-specific immunotherapy (IT).

Available therapeutic regimens provide symptom relief in most asthma patients, but cannot fully avert a progressive decline in lung function as a consequence of airway remodelling[4]. Furthermore, therapeutic options for patients with incomplete symptom control despite exhausted and high-dose standard therapy are still limited[5,6]. The heterogeneity in clinical presentation of asthma, with varying clinical course and different individual treatment responses is a reflection of the complexity of the underlying disease pathobiology[5,7]. Although several risk factors, including genetic determinants and environmental exposures, have been identified, the exact aetiology of asthma is still unknown[8,9]. Immunological pathways and inflammatory processes that provoke initiation, exacerbation and progression of the disease remain poorly understood.

Clinical and epidemiological approaches in clustering specific observable clinical features and pathological characteristics have led to the definition of certain asthma 'phenotypes'[10-12] and 'endotypes'[13], forming the basis for the development of new targeted treatment strategies for a subset of patients. Yet only a few of the identified phenotypes have been largely agreed upon, and particularly in pre-school children the natural course of the disease remains difficult to predict[10,14-16]. While clinical remission may be expected for some phenotypes during adolescence, persistence of symptoms beyond childhood and also relapse in adulthood is not uncommon[17-19]. These more severe forms of asthma have been associated with inadequate corticosteroid responsiveness and with allergic disease and sensitisation, as defined by elevated immunoglobulin E (IgE) levels[20]. Although anti-IgE treatment

(omalizumab) is already available, further personalised therapies targeting other known molecular pathways in severe paediatric asthma still await approval or have yet to be designed[21].

Severe and uncontrolled disease results in a substantial reduction in children's health-related quality of life by limiting physical activity and social interaction. Persistent symptoms predispose children to develop chronic obstructive pulmonary disease (COPD) later in life[22]. Thus, paediatric asthma in many respects represents a major burden for those affected and for society as a whole[23]. It affects children and adolescents in their most productive years[24], and due to direct and indirect costs, it represents a considerable economic burden for the global health care system[25-27].

1.2 Environmental factors and the development of allergic asthma

1.2.1 Westernisation, urbanisation and the hygiene hypothesis

Today, 'allergic asthma', based on allergen-specific IgE levels (i.e. allergic sensitisation) and allergy-related characteristic respiratory symptoms[28], is considered to be the most common asthma phenotype not only in the paediatric population[29].

Although few reports of allergic disease had already been documented in antiquity[30], there had been no awareness of this condition until the first description of hay fever in 1870, and another hundred years went by before asthma was recognised as a 'common' disease[31]. However, the subsequent increase in allergic disease and asthma prevalence in affluent Western societies during the last 40 years of the 20th century was dramatic, reaching epidemic dimensions[32,33]. Given the dynamics and rapidity of this trend, epidemiological research focussed on the changes in environmental factors and social lifestyle that coincided with that period. Increasing relocation to urban environments and the accompanying adoption of a modern, 'westernised' lifestyle have early been identified to have substantially contributed to the global changes and geographical variations in prevalence observed across countries[34-36] as well as within the same country[37-39].

In 1989, David Strachan postulated the idea that the reduced number of infections in early childhood and improved public hygiene in the developed world were responsible for the significant increase in atopic diseases[40]. He had previously demonstrated that a large number of older siblings, an indirect marker for a higher rate of cross-infections within the household, was associated with a reduced prevalence of hay fever and atopic dermatitis. To this day, this so-called 'hygiene hypothesis' has provided the theoretical framework for further research on atopic diseases, inspiring numerous scientists worldwide. Subsequent studies have consistently confirmed lower rates of atopy[41-45], allergic disease[46] and also asthma-related symptoms[47] in children raised in larger and less affluent families. Similar effects have been reported in children with day-care attendance during infancy[48-50], indicating that relevant protection is mediated by any exposure to many other children also outside the domestic environment (**Figure 1**).

Despite clear data from mouse models[51], epidemiological studies assessing the role of infections as the purported mediator for the protective sibling and day-care attendance effect have provided inconsistent

results and could not fully support the original statement of the 'hygiene hypothesis'[52]. In fact, viral respiratory tract infections in particular have been proven to increase the risk of subsequent asthma development in predisposed children[53,54]. Robust evidence only exists for a protective effect of parasitic, i.e. helminth infections on allergic diseases[55,56], while findings on exposure to mycobacteria[57-59] or orofecally transmitted organisms such as Hepatitis A virus[60] or Helicobacter pylori[61-63] among others[64-68] remain contradictory.

In view of these findings, the 'old friends hypothesis' has been formulated as an amendment to the 'hygiene hypothesis'[69]. Most relevant protective organisms thus represent archaic microorganisms ('old friends'), which co-evolved with the human race in earlier eras and are still found in rural environments. The abundant presence of these microbes as (pseudo)commensals and subliminal rather than clinically apparent infections has been proposed as providing the beneficial immunoregulatory effects. The disruption of man's symbiotic relationship with these environmental and commensal microbiota, as well as microbial deprivation as a consequence of lifestyle changes in the course of urbanisation, has resulted in dysregulated immunity in susceptible subjects[69] (**Figure 1**).

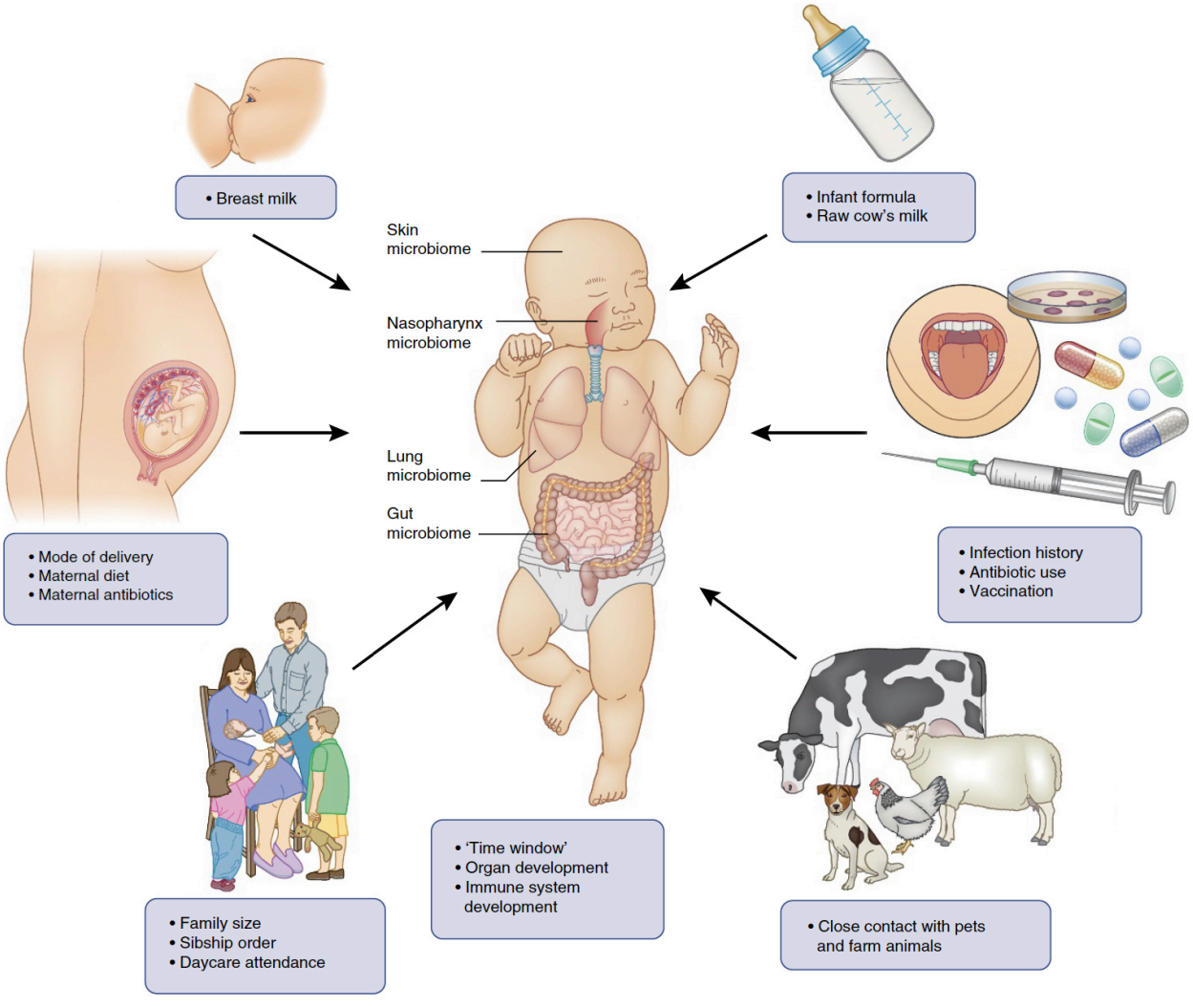

Figure 1. Protective and risk factors for the development of allergic disease in early life.
Most of these factors may affect the microbiota of the skin, nasopharynx, lung and gut, particularly during a critical time window in the early postnatal period. Figure and caption adapted from Ref. 33. © Springer Nature.

1.2.2 The farm effect – protection through external microbial exposure early in life

In this respect, increasing rates of atopy and asthma have been identified particularly in urban areas compared to rural ones, with a high consistency in studies from both high-income[70-73] and low- and middle-income countries[74-76]. Increased air pollution in metropolitan areas could not explain these regional differences[77-80], but a protective factor of rural living could be identified, namely that of being raised in a farming environment[81,80].

At the beginning of the 21st century, reports from all over the world documented remarkably lower prevalence rates of atopy and asthma among children that had grown up on traditional farms when compared to their peers from non-farming families. First landmark publications about this protective 'farm effect' on childhood atopy and asthma came from Switzerland, Germany, Austria and Finland[80-83], setting the framework for numerous further studies conducted in European[80,83-97], and non-European environments[98-108]. Cross-sectional multicentre surveys followed[109-112] and provided further evidence for this hypothesis on a large scale. Today, the protective 'farm effect' on childhood atopy[113] and asthma[114] has evolved to be a commonly accepted epidemiological concept.

A certain 'window of opportunity' for farm-related exposure is widely considered to be critical to its beneficial effects[33], supporting the notion that there is a sensitive early phase in infancy in which immune maturation and potential modulation occurs[115] (**Figure 1**). In this respect, regular exposure including animal contact and farm milk consumption during the period from pregnancy until the first year of life[109,107,85,108] has been associated with the strongest protection against asthma and allergic disease, and continual long-term or lifelong exposure seems to provide additive effects[106]. It has recently been shown in a birth cohort study that beneficial effects on lung function persist even into adulthood[116].

Neither genetic variations[117,118] nor common lifestyle factors[109,119-121] in which farm and non-farm populations apparently differ have proved likely to account for the reduced risk of allergic diseases among farm-children. Instead, distinct characteristics of the farming environment have been observed, thus relating protection only to certain types of farm-associated exposure[122]. Features of traditional farming, which comprise animal husbandry[82,83,104], particularly (dairy) cattle farming[112,123] and grain cultivation[112] and associated regular exposure to animal sheds and barns as well as to grain or hay as animal feed[112,109,107], have been identified to confer protection especially in European farming environments. These findings have been corroborated in a recent study among children from two farming populations in the United States, the Amish and the Hutterites, that engage in different farming practices. Traditional farming of the Amish, as opposed to the Hutterite's modern, industrialised practice, was associated with significantly lower prevalence of atopy and asthma in Amish children[118]. Furthermore, the consumption of unprocessed cow's milk[124,125,109,122,88,105] has been described as an independent protective factor, suggesting that not only inhalative environmental exposures may play a role in mediating the protective effect[126] (**Figure 1**).

Exposure to farm animals and stables is associated with increased concentrations of plant material from fodder[127], but also a variety of microbial sources. Inspired by the 'hygiene hypothesis', further research focussed on the external microbial exposure encountered in the farming environments. In order to allow quantification and qualitative measurement of children's daily exposure, samples of settled dust indoors[128] from children's mattresses and room floors and of airborne dust from stables and barns were collected and screened for microbes and their nonviable, but bioactive compounds such as fungal products[129] and bacterial cell-wall components[130-132].

Lipopolysaccharide (LPS), or endotoxin, a major part of the outer membrane of Gram-negative bacteria and abundantly present particularly in livestock farming settings[118,132-134] was used early as a generic marker to reflect environmental microbial load. Various studies conducted in farming[135,108] as well as non-farming households[131,135-137] have shown an inverse relation of early-life exposure to high levels of endotoxin and atopy or allergic asthma in children, while the risk for nonatopic-wheeze in infants and toddlers appears to be increased[138,139].

In addition, the evolution of molecular methods involving sequencing of bacteria-specific 16S rRNA has made it possible to identify distinct bacterial genera and species in the dust samples. Specific micro-organisms including Eurotium species from the fungal taxon, and many Gram-positive bacteria like Listeria monocytogenes, Bacillus species[140], Staphylococcus species[141], Lactobacillus species and Gram-negative bacteria such as Acinetobacter species[142] among others[143,144] were consistently associated with protection from atopic diseases. Current knowledge suggests that a unique composition of many of these micro-organisms, the diversity of microbial sources[145], as represented in farm dust samples, can best explain the protective effect of the farming environment against allergic diseases and asthma in particular[146,143].

1.3 Immunological mechanisms in the pathogenesis of asthma and the farm effect

The epidemiological 'farm effect' comprising all its associated protective exposures has been unambiguously proven in a global context. The strong evidence provided by epidemiological studies on the farming environment is encouraging, as it suggests that causes for the rapid rise in childhood asthma prevalence may both be identified and prevented. However, epidemiology is unable to draw causal conclusions from any effects observed. Immunological research is needed to identify the relevant mechanisms underlying the asthma-protective 'farm effect', thus providing further insights into pathophysiological processes involved in the development of asthma.

1.3.1 Basic principles of the innate and adaptive immune system

In order to be able to place mechanisms of asthma immunopathology in the context of basic immunological concepts, the fundamental principles of the human immune system will be briefly outlined hereafter. Due to the limited scope of this work, the following explanations will focus on immunological aspects that are especially important for a clear understanding of the present study.

Innate and adaptive immunity are the two types of immunity operating in humans to protect them from acute infections as well as chronic inflammatory disease. Although the division into the two subtypes has proven artificial in many respects, their distinction makes it easier to understand the complex immunological network involved in the pathogenesis of asthma.

1.3.1.1 Innate immunity

The innate immune system is the human's first line of defence against potentially infectious threats such as bacteria, viruses, fungi and certain parasites. It is programmed to detect features of these invading pathogens as well as structures of altered host cells, to discriminate between self and foreign molecules and to activate adaptive immune responses to the latter[147].

Apart from humoral components such as complement proteins, innate immune protection largely depends on cellular defence mechanisms provided by cells of both haematopoietic and non-haematopoietic origin[148]. The major haematopoietic cells within innate immunity include neutrophils, macrophages and dendritic cells (DCs) as phagocytic cells, but also mast cells, eosinophils, basophils, innate lymphoid cells (ILCs), and natural killer T (NKT) cells (**Figure 2**). Non-haematopoietic innate immune cells, in turn, are represented by epithelial cells lining the skin and the mucosal surface barriers of the respiratory and the gastrointestinal tract[148].

These innate immune cells express a limited number of evolutionary conserved 'pattern recognition receptors' (PRRs) on the cell membrane or in the cytoplasm which recognise invariant molecular motifs of microbes, e.g. nucleic acids, secretory products, cell-wall-components such as LPS, called pathogen-associated molecular patterns (PAMPs)[149,150]. Likewise, non-infectious endogenous molecules which are released from damaged host cells, termed damage-associated molecular patterns (DAMPs) can trigger PRRs[151,152]. Five subgroups of these germline-encoded innate receptors have commonly been described. They consist of the membrane-bound Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) as well as the intracellular NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and the AIM2-like receptors (ALRs)[149]. Some PRRs require adaptor proteins in order to integrate, sort and transmit signals. The TIR-containing adaptor (TIRAP) and the myeloid differentiation primary response 88 (MyD88) protein are examples of such adaptors utilised by the TLR-family.

When encountering PAMPs and DAMPs, most PRRs (and their adaptors) trigger complex downstream signalling cascades that lead to the activation of transcription factors such as nuclear factor 'kappa-light-chain-enhancer' of activated B cells (NF-κB) and interferon regulatory factors (IRFs.) Their target genes encode proteins involved in inflammatory processes such as cytokines, interferons and chemokines, which in turn initiate innate and adaptive immune responses. Ligand binding may also trigger direct defence mechanisms such as the induction of phagocytosis, cell death, autophagy, and cytokine processing.

Several mechanisms are involved to tightly control and coordinate signalling pathways at multiple checkpoints, in order to allow effective clearance of pathogens and altered cells while preventing excessive inflammatory responses at the same time.

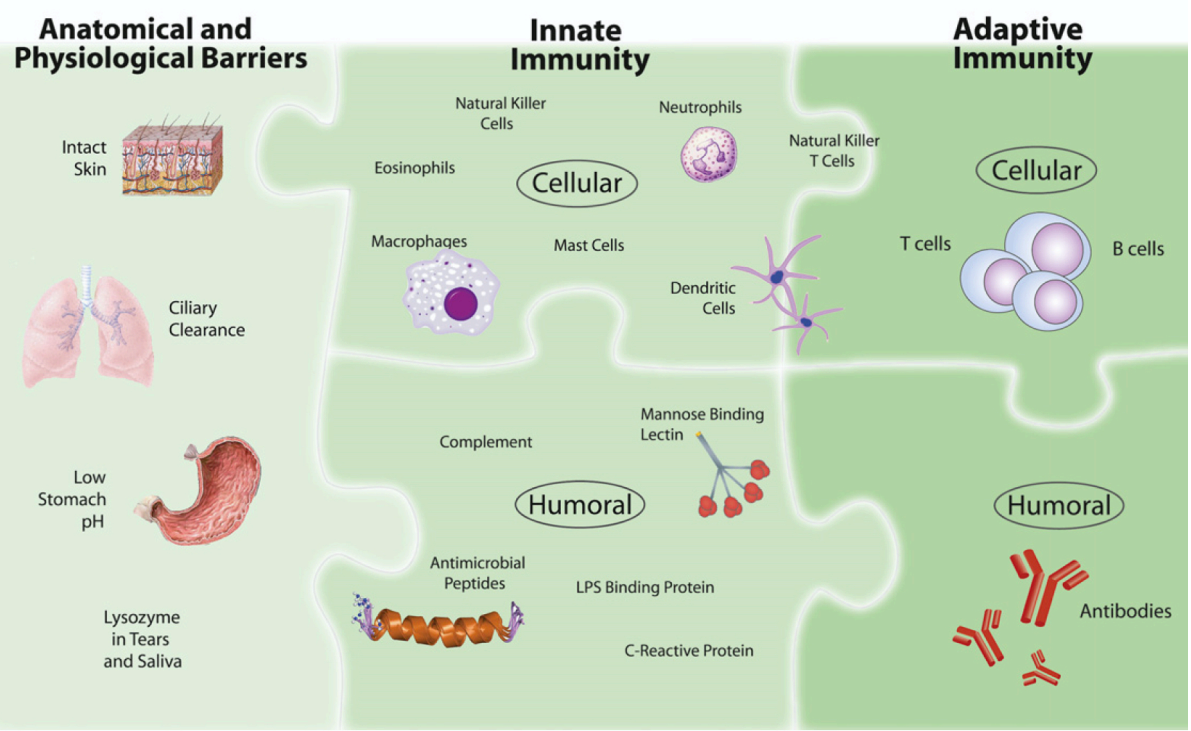

Figure 2. Integrated and simplified view of the human immune system.
Some elements, such as natural killer T (NKT) cells and dendritic cells (DCs), are difficult to categorize, since they act at the interface of innate and adaptive immunity. Figure from Ref. 148. © Elsevier Inc.

1.3.1.2 Adaptive immunity

In contrast to the innate immune system, immune responses of adaptive immunity are slower, yet highly sophisticated and target-specific processes. They are mediated by two types of lymphocytes, T and B cells. While T cells, once activated by antigen-presenting cells (APCs), mainly govern the cell-mediated part of adaptive immune responses, B cells represent - through antibody-production – the humoral part of adaptive immunity (**Figure 2**). Following differentiation into plasma cells, B cells secrete highly specific antibodies which bind to antigens from foreign agents like bacteria and viruses, flagging them for subsequent elimination. The unique memory function of adaptive immunity is featured by memory T and B cells, which are able to mount an accelerated immune response upon re-exposure to the same antigen[153].

Both T cells and B cells express a diverse repertoire of unique antigen-binding receptors on their cell surface, called T cell receptors (TCRs) and B cell receptors (BCRs), respectively. Unlike the PRRs of the innate immune system, these receptors are highly variable structures, generated through somatic

DNA rearrangement processes, allowing the recognition of a broad range of potential ligand molecules or fragments, the so-called antigens[147].

In order to recognise these specific antigens, T cells need to interact with antigen-presenting cells (APCs) of the innate immune system (mostly DCs and macrophages). These professional APCs have the capacity to take up foreign antigens, process them into fragments (i.e. peptides) and present these peptides to T cells through surface proteins termed 'major histocompatibility complex' (MHC). MHC are classified as either class I proteins, which are expressed on all nucleated cells and present endogenous cytoplasmic proteins, or class II molecules which are found only on certain APCs within the immune system. While cytotoxic CD8 expressing ($CD8^+$) T cells are activated by the interaction of their TCR with the complex of antigen-peptides bound to MHC I molecules, $CD4^+$ helper T cells are activated through TCR recognition of peptide/MHC II-complexes[147]. $CD8^+$ T cells primarily kill host cells infected with intracellular pathogens, such as viruses, or tumour cells expressing foreign antigens. $CD4^+$ helper T (Th) cells, in turn, are essential for activating, enhancing but also limiting both innate and adaptive immune responses. Besides the antigen-T cell receptor signal (primary signal), signalling through co-stimulatory molecules on APCs is required as a second signal for effective activation and differentiation of naive T cells[154,155]. In response to activation, $CD4^+$ T cells proliferate and, depending on the cytokine milieu, develop into different subsets of helper T (Th) cells, with Th1, Th2 and Th17 cells as well as regulatory T (Treg) cells being the most common. They are distinguished by distinct effector functions[156].

Th1 cells mainly produce the cytokines IFN-γ and interleukin (IL)-2, which primarily promote and enhance cell-mediated responses, involving the activation of macrophages and cytotoxic $CD8^+$ T cells for the destruction of intracellular foreign agents and virally infected cells. In contrast, Th2 cells are characterised by the production of IL-4, IL-5 and IL-13, which stimulate B cells to produce, among others, immunoglobulin E (IgE) antibodies, and promote the recruitment and activation of mast cells and eosinophils that are essential for an effective immune response against extracellular parasites. While the Th17 subset of Th cells, which produce cytokines of the IL-17 family, is known to be pro-inflammatory[157], Treg cells have been identified as important anti-inflammatory cells, suppressing immune responses and controlling aberrant reactivity against self-antigens[158].

Innate and adaptive immunity are essentially complementary systems; they act synergistically and communicate with each other. Defects in both systems may lead to ineffective or misdirected immune responses as observed in autoimmune disease, chronic inflammatory disorders, allergic disease and many more.

1.3.2 The pathogenesis of asthma and what we have learnt from the farming studies

Chronic airway inflammation, which is known as the cardinal feature in asthma, is sustained by a complex immunological network linking processes of innate and adaptive immunity. Studies on the environmental determinants that influence both the development of asthma and protection from it have led to substantial revision in the common understanding of the underlying pathobiology and related immunological pathways.

1.3.2.1 Mechanisms of adaptive immunity

Asthma has long been considered the prototype of adaptive Th2 cell-induced inflammatory disease, with the presence of high serum IgE being the hallmark especially for allergic asthma[159]. In individuals with the atopic phenotype of asthma, high levels of allergen-specific IgE are indeed a reflection of an inappropriate Th2-biased immune response to common environmental allergens[160,161], and main clinical features of asthma can be traced back to excessive Th2-dominated immunity. Recently published data on beneficial effects of targeted therapies suppressing Th2-specific cytokines continue to underscore the central role of Th2 cells in mediating airway inflammation in allergic asthma[162,163]. Th2 cytokines drive B cell-mediated allergen-specific IgE production and induce accumulation of mucosal mast-cells (mainly via IL-4), basophils and eosinophils (via IL-5) in the airways (**Figure 3**), leading to increased mucus production, airway hyperreactivity (AHR) with airway narrowing also to nonspecific stimuli, and structural changes of the airway wall ('remodelling')[164]. These inflammatory processes that occur in the lungs are amplified by vasoactive mediators such as histamine, as well as chemokines and cytokines released from mast-cells and basophils upon IgE-crosslinking with their high affinity receptors FcεRI (**Figure 3**).

Further evolution of the Th2 concept was reflected in the 'Th1/Th2 paradigm' of asthma, which proposed an imbalance between T helper cell subsets with excessive Th2-type immune responses driving airway inflammation in the absence of counterbalancing Th1-type immunity[165,166]. In a variety of human studies, reduced levels of the Th1-specific cytokine IFN-γ at birth and failure to upregulate Th1 immunity during infancy[167] have been consistently associated with increased risk of atopic disease[168-172] and chronic wheeze[173] in childhood.

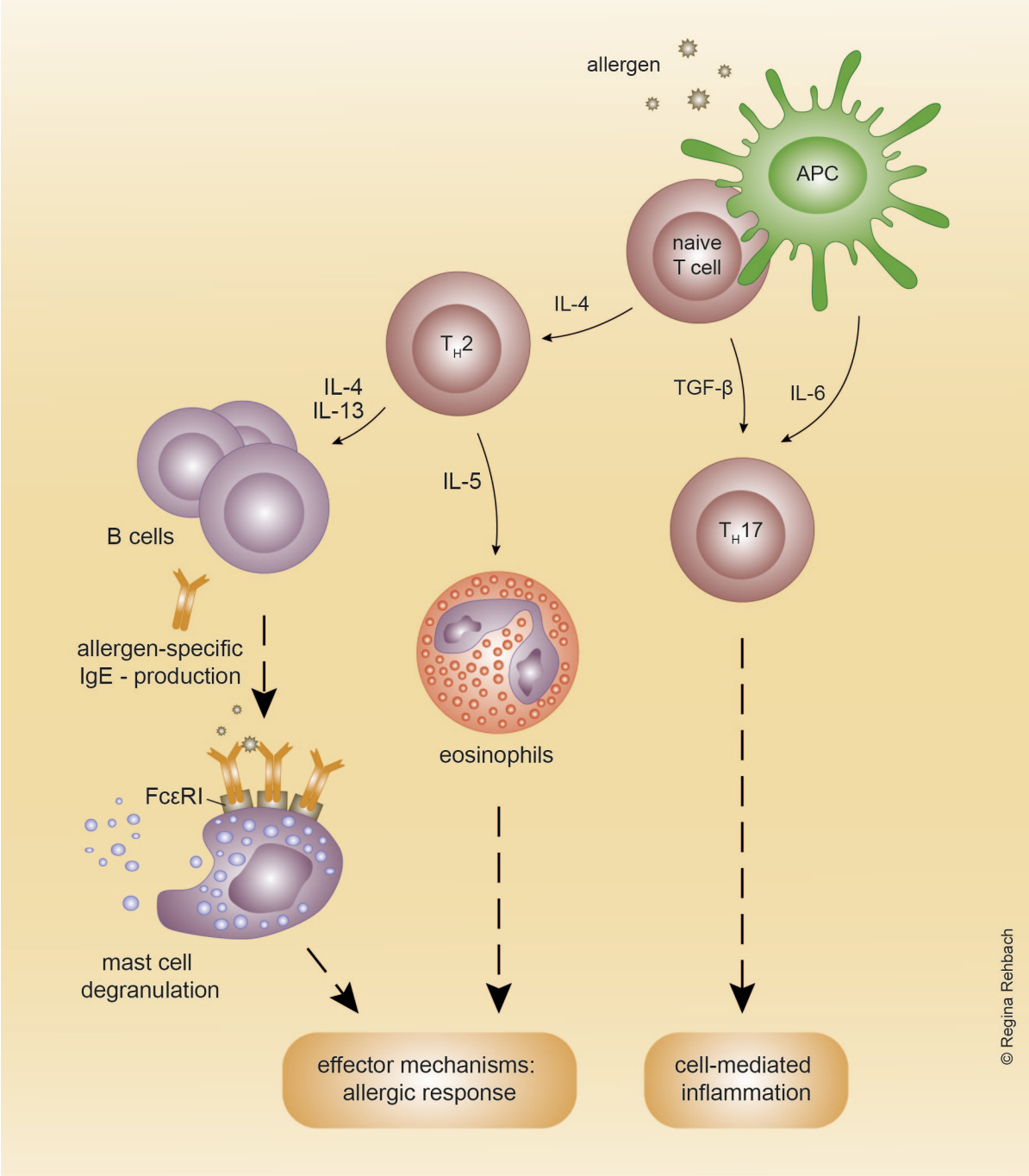

Figure 3. Th2- and Th17-induced immune processes in (allergic) asthma.
Upon allergen sensitisation, APCs interact with naive T cells in the local lymph nodes of the airways and promote their differentiation into different T cell subsets (only T_H2 and T_H17 shown here). Following maturation, T_H2 and T_H17 cells produce cytokines which activate humoral and cellular processes to promote the development of asthma. The T_H2 cytokines IL-4, IL-5 and IL-13 stimulate allergen-specific IgE production and induce migration and activation of mucosal mast-cells, basophils and eosinophils. Release of mediators such as leukotrienes and histamine from these cells induces allergic inflammatory responses as well as airway epithelial and smooth-muscle cell changes. *APC*, antigen-presenting cell; T_H, helper T cell; *TGF-β*, transforming growth factor beta; *FcεRI*, high-affinity receptor for IgE. Figure designed by Regina Rehbach.

Th2 cell sensitisation to common allergens and resulting aberrant immune responses in predisposed children are largely determined by environmental factors encountered during early childhood[174]. As Th1 cells are known to inhibit the proliferation and differentiation of Th2 cells[156], the atopy-and asthma-protective effect of early childhood microbial stimulation was attributed to mechanisms of immune deviation towards Th1-type immunity counteracting allergen-induced Th2 cell development[109] (**Figure 4**). On this score, microbial exposure particularly encountered in farming environments has been shown to enhance Th1-associated immune responses in human *in vivo*[175,176] and *in vitro* studies[142] while decreasing Th2-dependent markers[177]. However, animal models assessing the effects of different farm-derived agents on asthma development did not reveal signatures of Th1-polarisation while invariably confirming the inhibition of Th2-mediated allergic inflammation and experimental features of asthma[178,179]. Findings from mechanistic studies on the binary Th1-Th2 paradigm in mouse models of asthma further challenged the concept of Th1/Th2 immune deviation as a unifying explanation for both asthma development and protection[180,181].

Considering the fact that only a minority of children with atopy (i.e. Th2-dependent excessive IgE-mediated reaction) also develop asthma[182], and that Th2-skewed chronic helminth infections can even provide protection from allergic disease, general thinking shifted towards a more complex understanding of immune regulation in asthma than initially proposed[55]. Additional cell types and mechanisms were included in the pathophysiological network.

Th2 responses to environmental antigens were shown to be controlled by distinct populations of IL-10 and TGF-β producing T cells, the regulatory T (Treg) cells[183]. In fact, decreased percentages of distinct Treg phenotypes were detected in blood and bronchoalveolar lavage (BAL) fluid samples of children with asthma and severely affected adult patients, and their suppressive activity was found impaired[184,185]. Concurrently, specific farming exposures were associated with an increased number and enhanced function of Treg cells or their respective cytokines (IL-10 and TGF-β) in children's blood samples[186-188]. Likewise, it was shown that chronic parasitic and bacterial infections reduced experimental allergic airway inflammation through the induction of Treg and IL-10-secreting B cells[189-192]. The development of allergic disease was thus explained by a deficient regulatory network, with a loss of respiratory tolerance in the absence of microbial instruction[174] (**Figure 4**).

More recently, it has been proposed that Th17 cells are involved in this regulatory network by counteracting anti-inflammatory activities of Treg cells[193]. Th17 responses have been associated with neutrophilic inflammation in severe asthma[194] and have proved to induce airway remodelling in chronic disease[193] (**Figure 3**). These findings further confirm the assumption that additional, non-Th2-related pathways are engaged in the pathogenesis of different forms of asthma.

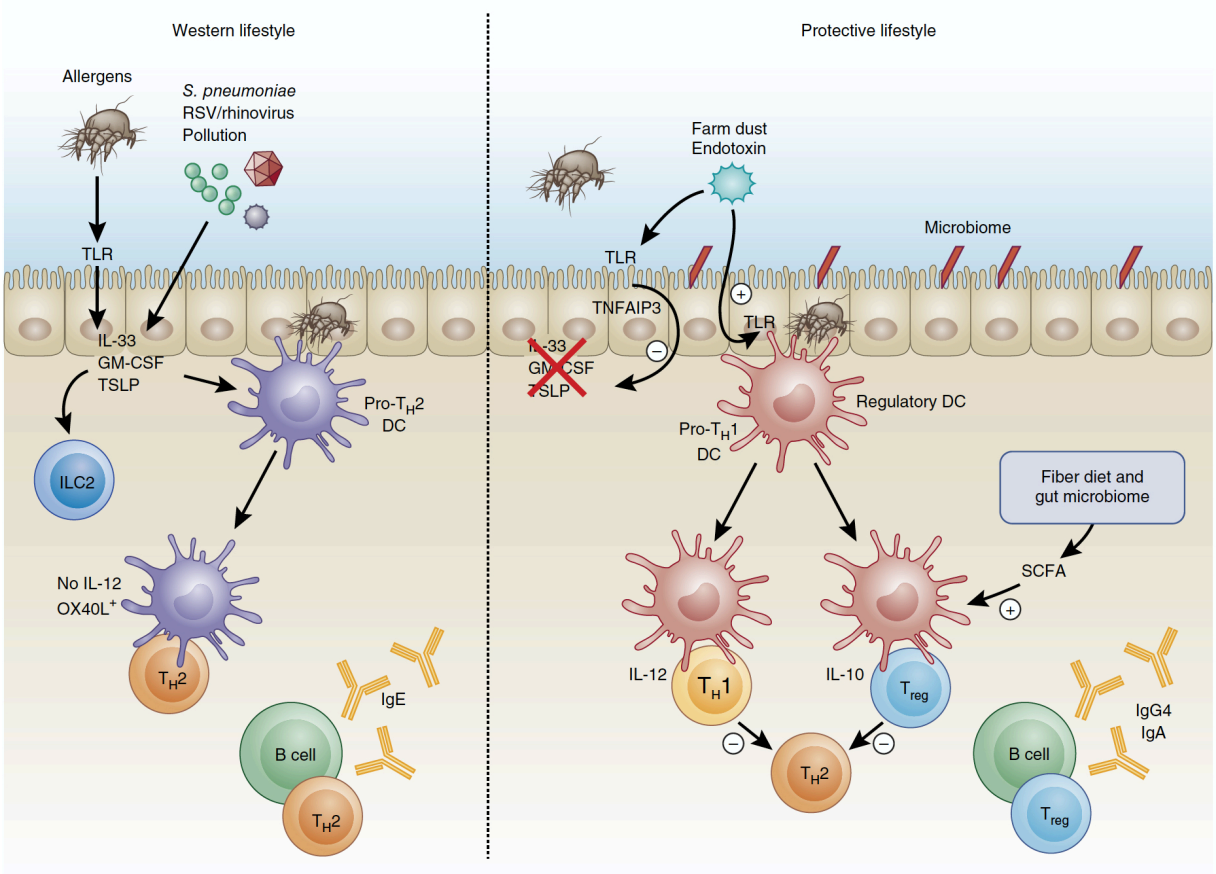

Figure 4. Immune processes potentially underlying the impact of environmental conditions on the development of asthma.
In a Western-lifestyle environment, allergen exposure triggers Toll-like receptors (TLRs) on epithelial cells, leading to the release of cytokines such as IL-33 and GM-CSF, which boost the T_H2-promoting capacity of DCs. These cytokines also promote an innate immune response to allergens involving ILC2s, basophils and eosinophils. In a protective environment, chronic exposure to environmental farm dust and microbial-derived signals such as endotoxin or metabolites from the lung or gut microbiome may induce increased formation and differentiation of T_{reg} and T_H1 cells which suppress T_H2 immune responses. Such exposure may further induce the regulatory enzyme TNFAIP3 in lung epithelial cells to inhibit the production of pro-inflammatory cytokines. *RSV*, respiratory syncytial virus; *GM-CSF*, granulocyte-macrophage colony-stimulating factor; *TSLP*, thymic stromal lymphopoietin; *SCFA*, short-chain fatty acids; *DC*, dendritic cell; T_{reg}, regulatory T cell; T_H, helper T cell. Figure and caption adapted from Ref. 33. © Springer Nature.

1.3.2.2 Mechanisms of innate immunity

Studies on the mechanistic underpinnings of the farm effect on asthma have led to an immunological paradigm shift, with increased appreciation of the essential role of innate immunity, and particularly of antigen-presenting dendritic cells (DCs) in the pathogenesis of asthma (**Figure 4**).

Both inflammatory and regulatory T cell responses to allergens require activation by innate immune cells. In allergic asthma, maladaptive Th2- and Th17-mediated immune responses to intrinsically innocuous allergens are initiated by pulmonary DCs, which first encounter inhaled antigens at the mucosal surface of the respiratory tract[195]. Upon antigen ligation and uptake via PRRs, DCs migrate to

the draining bronchial lymph nodes to activate naive T cells by releasing cytokines and expressing co-stimulatory molecules. In this way, DCs determine the outcome of subsequent adaptive immune responses[196], involving the induction of both airway inflammation and tolerance to foreign antigens[197]. Distinct functional properties have been ascribed to different types of DCs. Based on phenotype characteristics, two major dendritic cell subsets have been identified in humans - plasmacytoid DCs (pDCs) and myeloid DCs (mDCs, also referred to as conventional DCs (cDCs)), which are further subdivided into mDCs type 1 and type 2[198]. Although the evidence for the distinct division of labour among human DCs is not as clear, mDCs have been shown to critically contribute to the development of allergic airway inflammation in mice[199,200], whereas pDCs suppress inflammation by inducing Treg cell responses[200-203]. Supporting this notion, decreased numbers of pDCs have been associated with subsequent wheeze or asthma in children[204,205]. Conversely, mDC subsets were found in decreased numbers in asthma-protected farm children[206,207] and exposure to farm-derived dust extracts was shown to modulate DC biology and function both in humans and in mice[178,179,208]. On this score, a decreased T cell stimulatory capacity of human monocyte derived DCs was observed when cultured in the presence of stable dust[178]. Prolonged *in vitro* exposure to farm dust extracts and derived products was shown to reduce the allergy-inducing capacities of murine DCs through an IL-10 dependent mechanism[208,179].

Another crucial mediator in both protection and disease is represented by structural components of the lung, airway epithelial cells (ECs). It has recently been demonstrated that ECs, acting in close crosstalk with DCs, constitute an integral part of the innate immunity network at the interface between host and environment[209] (**Figure 4**). Like professional APCs, they express pattern recognition receptors from the TLR-family on their cell surface[210]. In response to TLR-stimulation, ECs release cytokines, chemokines and lipid mediators which control the activation of DCs and programme them to mount Th2-mediated immune responses upon allergen exposure[211].

Airway EC-derived cytokines may also control activation of innate lymphoid cells (ILCs), a most recently identified innate immune cell type[212] (**Figure 4**). Being a major source of the Th2 cell-associated cytokines IL-5 and IL-13, type 2 ILCs (ILC2), in particular, are increasingly emerging as important contributors of allergic airway inflammation[213]. Interaction with different types of DCs has been shown to either promote[214] or suppress[215] type 2 inflammatory response. ILC2 numbers were consistently found to be elevated in human asthmatic subjects, both in sputum and in peripheral blood[216-218]. Research studying the interaction of ILCs with the microbiota at mucosal surface barriers is now beginning to unravel their involvement in host-protective regulatory functions[219] in the context of asthma development as well[220].

Recent data further suggest that, apart from ILCs, DCs and ECs, basophils contribute to the development of Th2-biased immune responses to allergens[221-223], although their precise role in driving

disease processes of asthma remains to be defined. Innate immunity is also represented by natural killer T (NKT) cells, whose involvement has been described both in the pathogenesis of asthma[224] and the protection from it through early life microbial exposure[225].

As demonstrated above, a variety of cellular mechanisms are considered to be involved in the pathogenesis of asthma and its environmentally mediated protection, but their relative contributions are still unclear. Although immunological research based on the 'hygiene hypothesis' and the 'farm effect' on asthma has significantly enhanced the current understanding of the disease, the overall picture remains fragmented and a unifying mechanistic concept has yet to be defined.

1.4 Molecular mechanisms and pathways analysed in this work

Allergic asthma constitutes inappropriate, i.e. inflammatory immune responses to innocuous environmental stimuli such as common allergens. Children growing up in farming environments are constantly exposed to high concentrations of these stimuli and a vast number of microbial agents, which harbour evolutionary conserved structural components such as endotoxin (LPS). Environmental LPS and many allergens are recognised by cells of the innate immune system, specifically DCs and macrophages but also airway ECs, through Toll-like receptor 4 (TLR4), which is expressed on their cell surface. TLR4 in complex with myeloid-differentiation protein 2 (MD-2) engages LPS. LPS binding with the help of CD14 initiates dimerisation of the TLR4-MD2 complex, which triggers the recruitment of Toll/interleukin-1 receptor (TIR) domain-containing adaptor proteins[226] (**Figure 5**). Intracellular TLR4 signal transmission then takes place via two distinct pathways, the MyD88-dependent and the TRIF-dependent pathway, both ultimately culminating in the activation of the transcription factor NF-κB. NF-κB represents a family of five transcription factors that regulate the transcription of genes involved in a variety of inflammatory processes[227]. In the presence of membrane-bound CD14, the TLR4-MD2-LPS complex is internalised into endosomes, where it recruits the adaptors TRAM and TRIF, driving downstream signalling via the induction of type I interferons through IRF3 and NF-κB (TRIF-dependent pathway)[228]. Engagement with TIRAP (MAL) and MyD88 adaptors mainly triggers the early phase of NF-κB-dependent induction of pro-inflammatory cytokines such as IL-6, IL-12 and TNF-α (MyD88-dependent pathway)[226,229] (**Figure 5**).

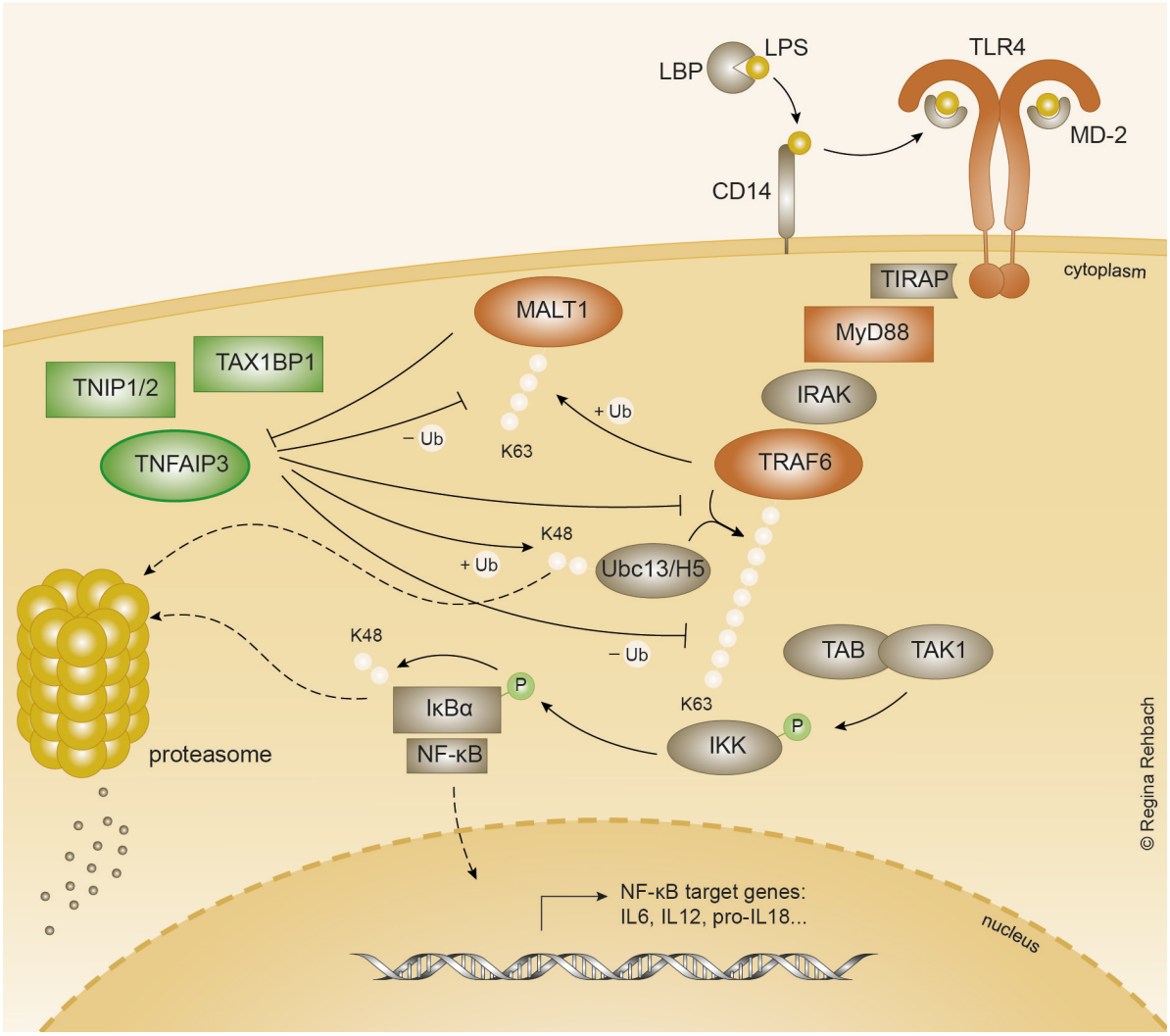

Figure 5. TLR4-dependent NF-κB signalling pathway and regulatory activities of TNFAIP3.
Upon LPS binding with Toll-like receptor (TLR) 4 in complex with MD-2, several adaptor proteins including TIRAP, MyD88 and TRAF6 are recruited to the receptor. TRAF6 interacts with the ubiquitin conjugating enzymes Ubc13 and UbcH5c, leading to the K63-linked polyubiquitination of TRAF6, which promotes recruiting of the TAK1 kinase complex and of the IκB kinase (IKK) complex. TAK1 phosphorylates (P) and activates IKK, eventually resulting in proteasomal degradation of IκB proteins, release of NF-κB and subsequent translocation to the nucleus, where transcription of target genes is achieved. The enzyme TNFAIP3 inhibits NF-κB signalling by disrupting the interaction of TRAF6 with Ubc13 and UbcH5c, K48-linked polyubiquitination and proteasomal degradation of Ubc13/H5c and by removing K63-linked polyubiquitin chains from TRAF6. TNFAIP3 activity is regulated by the binding proteins TAX1BP1 and TNIP1/2, which directly enhance TNFAIP3's inhibitory effects on NF-κB. The paracaspase MALT1 negatively regulates TNFAIP3's function. Anti-inflammatory regulators analysed in this thesis are highlighted in green, while pro-inflammatory pathway molecules are shown in (rust) red. Molecules with enzymatic functions are represented in an elliptical shape. Figure content and caption based on Ref. 251 as well as Ref. 272. Figure designed by Regina Rehbach. All rights reserved.
Abbreviations: *LPS*, lipopolysaccharide; *LBP*, LPS binding protein; *TLR4*, Toll-like receptor 4; *MD-2*, Myeloid-differentiation protein 2; *TIRAP*, TIR domain containing adaptor protein; *MyD88*, Myeloid differentiation primary response 88; *IRAK*, Interleukin-1 receptor-associated kinase; *TRAF6*, TNF receptor-associated factor 6; *IκB*, Inhibitor of kappa B; *IKK*, IκB kinase; *NF-κB*, Nuclear factor 'kappa-light-chain-enhancer' of activated B cells; *TNFAIP3*, Tumor necrosis factor alpha induced protein 3; *TNIP*, TNFAIP3-interacting protein; *TAX1BP1*, Tax1-binding protein 1; *MALT1*, Mucosa-associated lymphoid tissue lymphoma translocation protein 1; *Ub*, ubiquitin; *K63 (48),* lysine (K) residue at position 63 (48) in the ubiquitin molecule.

Signalling through TLRs and the adaptor MyD88 is essential for effective DC activation[230], and a prerequisite for the development and control of adaptive T cell responses[231,232]. Inappropriate TLR4 activation through direct binding of distinct aeroallergens has been associated with allergic responses[233]. Some genetic variants of TLR4 were found to modify the risk for atopy and asthma development in children[117,234,235]. Findings from experimental mouse models of asthma also indicate a crucial role for TLR-dependent signalling in suppressing allergic airway inflammation[236-239] particularly upon farm dust exposure[118]. This is paralleled by human *in vivo* studies showing that different farming exposures correlate with the expression of innate immunity receptor genes, especially TLR4 and related signalling pathway genes[118,122,188,240,241].

MyD88 signalling adaptor is shared by almost all TLR family members and facilitates TLR-signal transduction by coupling TLRs with their downstream signalling molecules. It has been shown to be particularly essential for driving an effective immune response upon LPS stimulation[242]. MyD88 is also required for the induction of the proinflammatory cytokine IL-18[243], which has been associated with allergic airway inflammation by inducing eosinophil accumulation[244] and the production of Th2-specific cytokines and histamine[245].
MyD88-dependent TLR signalling involves the recruitment of kinases from the Interleukin-1 receptor-associated kinase (IRAK) family, which in turn interact with TNF receptor-associated factor 6 (TRAF6), thus activating its E3 ubiquitin ligase activity (**Figure 5**). TRAF6 associates with the E2 ubiquitin conjugating enzymes Ubc13 and UbcH5c allowing K63-linked polyubiquitination of TRAF6[246], which ultimately leads to the activation of the ubiquitin-dependent kinase TAK1[247] and the 'inhibitor of kappa B (IκB) kinase' (IKK) complex. Following phosphorylation by IKK eventually leads to proteasomal degradation of IκB proteins, the release of NF-κB and subsequent translocation to the nucleus, where the transcription of target genes is achieved (**Figure 5**).

NF-κB signalling has been shown to be critically involved in a large number of inflammatory diseases including asthma[227,248]. Excessive NF-κB activation appears when negative regulation of TLR-induced signalling is affected[249]. Tumor necrosis factor α-induced protein 3 (TNFAIP3), also referred to as A20, has been identified as one central negative regulator of TLR-mediated and NF-κB-dependent inflammatory responses[250]. TNFAIP3 is constitutively expressed at high levels in T and B cells, whereas only limited amounts are observed in most other types of immune as well as non-immune cells under resting conditions[251]. Once expression is induced by NF-κB[252], TNFAIP3 exerts its enzymatic activity by modulating various ubiquitination processes[253,254] critical to the activation of NF-κB[255]. Through direct[256] or indirect interactions, TNFAIP3 engages with key signalling molecules thus attenuating NF-κB-dependent signalling in the form of a negative feedback loop. One essential mechanism by which TNFAIP3 restricts LPS-induced NF-κB activation in the TLR4 pathway is the inhibition of polyubiquitin chain assembly to TRAF6 through interfering with Ubc13 and UbcH5c[254] (**Figure 5**).

Various studies have consistently linked cell-type specific TNFAIP3 deficiency to severe inflammatory conditions in mice[257-259]. The essential role of TNFAIP3 in driving particularly severe Th17-mediated forms of allergic airway inflammation has been highlighted most recently[260]. In the context of human asthma, reduced *TNFAIP3* gene expression was found in bronchial ECs of asthma patients when compared to healthy subjects[261,262]. In a genome wide association study (GWAS)[117], a risk allele for asthma has been detected for a distinct TNFAIP3 gene polymorphism (SNP) inside the protein coding sequence[263], and the same SNP has been significantly implicated in a gene-by-environment interaction with farming[261]. The same study has identified TNFAIP3 as a key player in mediating the protective effect of LPS and farm dust exposure in an animal model of allergic asthma[261]. More recently, *in vivo* farming exposure has been associated with increased TNFAIP3 gene expression in children from the Amish farming population[118].

TNFAIP3 expression and activity is regulated by a number of mechanisms and molecular pathways[264]. Interaction with the binding proteins Tax1-binding protein 1 (TAX1BP1)[265] and TNFAIP3-interacting protein 2 (TNIP2), also referred to as ABIN-2[266,267], directly mediates and enhances TNFAIP3's inhibitory effects on NF-κB. TAX1BP1 was shown to recruit TNFAIP3 to polyubiquitinated TRAF6, thereby promoting its deubiquitination and inactivation[268,269,254]. Its essential role in controlling NF-κB-mediated inflammation has been illustrated in a study using TAX1BP1 knockout mice[269].
TNIP2, a member of the TNIP-family, is known as another important regulatory protein, which functions as an adaptor between ubiquitinated signalling proteins and TNFAIP3[266]. Recent findings from animal models suggest that TNIP2 critically contributes to the pathogenesis of allergic airway inflammation[270].
In contrast to TAX1BP1 and TNIP2, the paracaspase 'Mucosa-associated lymphoid tissue lymphoma translocation protein 1' (MALT1) negatively regulates TNFAIP3's function by cleaving TNFAIP3 upon T cell stimulation[271]. MALT1 itself is a substrate targeted by TNFAIP3, which catalyses its deubiquitination[272] (**Figure 5**). MALT1 was proposed as playing a crucial role in mediating FcεRI-dependent cytokine production in mast cells, thus linking it to IgE-induced chronic allergic inflammation[273].

LPS-induced TLR4-mediated signalling activates professional APCs, particularly DCs, which then prime the adaptive T cell response (**Figure 6**).

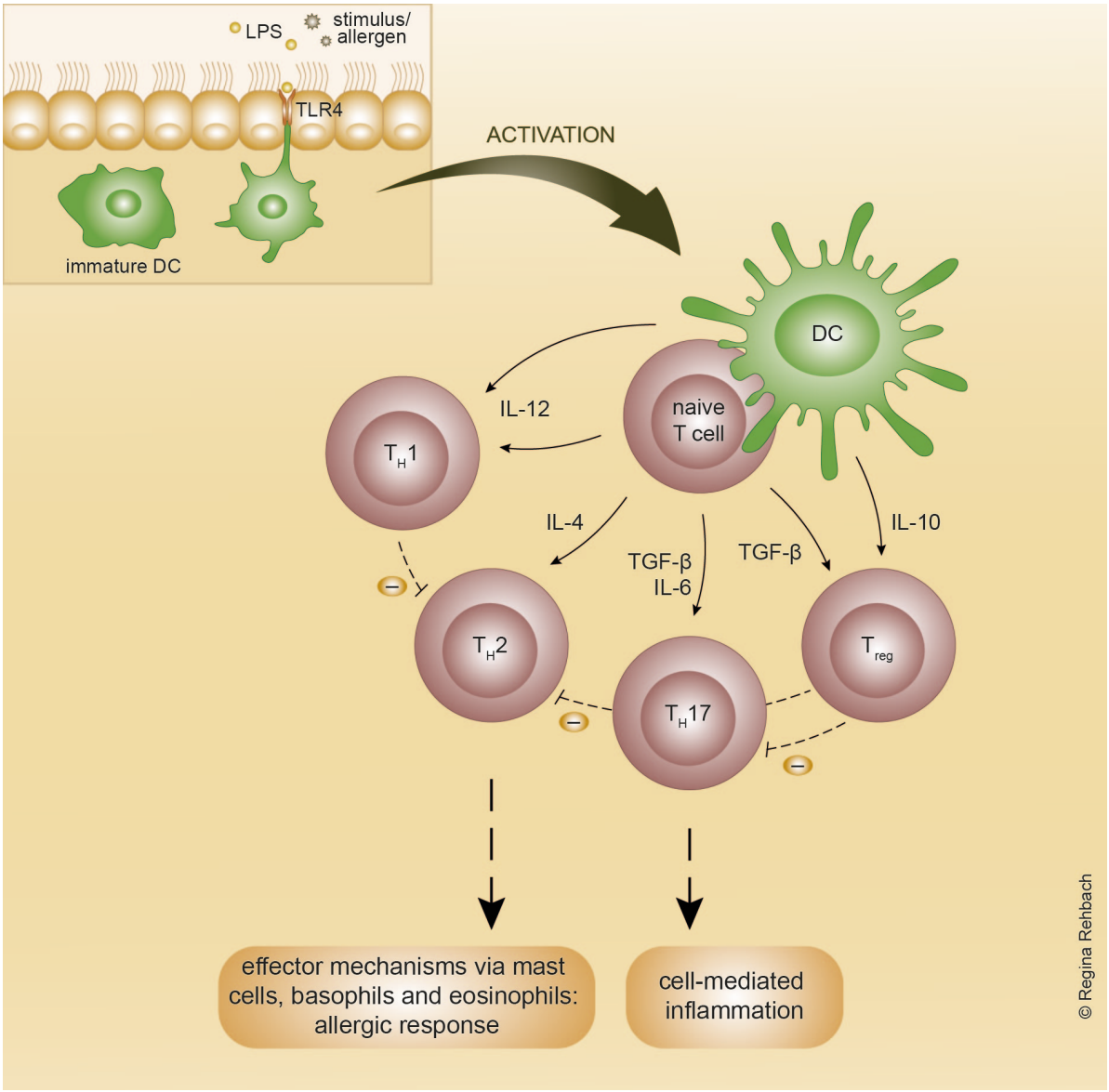

Figure 6. Dendritic cell activation and control of T cell responses.
Environmental antigens such as LPS and allergens are recognized and taken up by DCs across the mucosal barrier of the airways. Once activated, DCs mature and migrate to the draining lymph nodes where they interact with naive T cells and promote their differentiation into different effector T cell subsets. The combination of signals transmitted by cytokines and co-regulatory molecules during DC-T cell interaction determines the differentiation and expansion of certain T cell subsets, which may either promote or suppress the development of the allergic response. *TGF-β*, transforming growth factor beta. Figure designed by Regina Rehbach. All rights reserved.

Upon activation, DCs undergo cellular differentiation by expressing co-stimulatory molecules of the B7 family (B7-1, B7-2, B7-H1, among others) on their cell surface[274] - an important step in reaching functional maturity (**Figure 7**). CD80 (B7-1) and CD86 (B7-2) are mainly expressed on APCs such as macrophages, B cells and DCs in particular, but are also found on T cells[274]. The CD86 molecule is constitutively expressed at low levels, whereas CD80 is only present when its expression is induced upon activation[274]. During T cell priming, CD80 and CD86 on APCs both interact with CD28 on T cells, thus allowing effective activation upon antigen-TCR encounter[155] (**Figure 7**). The absence of such co-stimulation has been associated with the induction of tolerance[275,276].

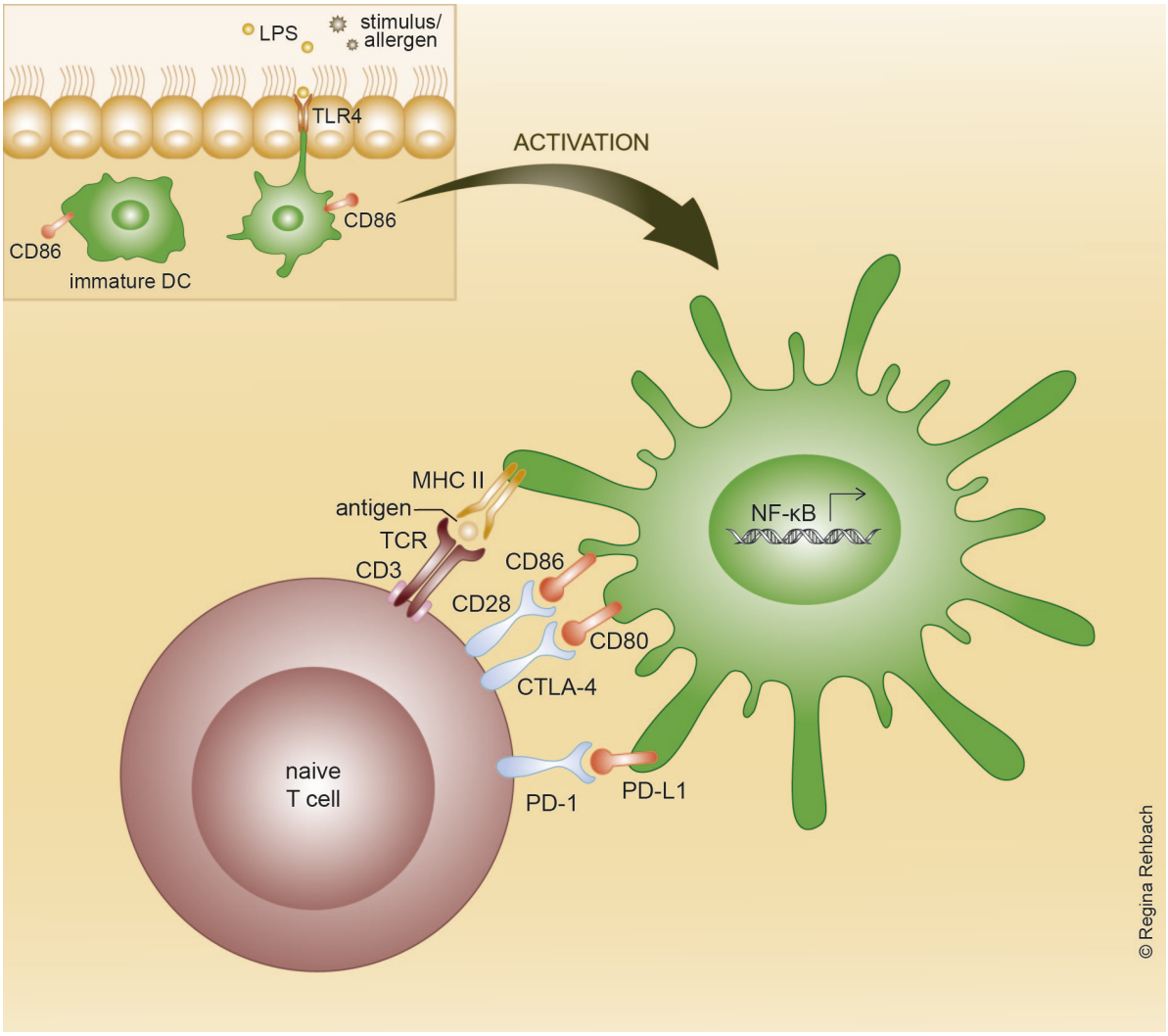

Figure 7. DC-T cell interaction following antigen encounter.
Stimulation via TLRs leads to the activation of DCs. Mature DCs interact with naive T cells via the T cell receptor (TCR), major histocompatibility complex class II (MHC II) and a distinct repertoire of co-regulatory molecules. The CD80 (B7-1) and CD86 (B7-2) molecules on DCs both bind to CD28 as well as to CTLA-4 on T cells. PD-L1 (B7-H1) can interact with both the PD-1 receptor and the CD80 molecule on activated T cells. Crosslinking of CD80 and CD86 with CD28 transmits stimulatory signals to T cells, while CTLA-4 as well as PD-L1 binding provides inhibitory signals. *CTLA-4*, cytotoxic T lymphocyte-associated protein 4; *PD-L1*, programmed death ligand 1; *PD-1*, programmed death receptor 1. Figure designed by Regina Rehbach. All rights reserved.

Recent studies in mice bearing TNFAIP3-deleted DCs have demonstrated that DC activation and maturation is controlled by TNFAIP3[257,277]. CD80 and CD86 expression was markedly increased on TNFAIP3-deficient DCs, and the cells exhibited exaggerated NF-κB-dependent cytokine production, resulting in aberrant adaptive immune responses[277]. In the context of asthma, the CD86 co-stimulatory molecule in particular has been related to allergen-induced Th2 cell immune responses and airway inflammation in mice[278,279]. A polymorphism in the human CD86 gene locus[280] and dysregulated CD86 expression have been associated with allergic asthma and atopy in human studies[281-284]. It has recently been demonstrated that asthma-protective LPS and dust exposure *in vivo* suppressed the maturation of murine DCs, and that TNFAIP3 mediated this effect to some extent[261].

The co-stimulatory repertoire of DCs is further represented by the Programmed Death Ligand 1 (PD-L1) surface protein (B7-H1, encoded by the *CD274* gene)[285,286], which functions as a binding partner to both the PD-1 receptor and the CD80 (B7-1) molecule on activated T cells[287] (**Figure 7**). Similar to CD86, PD-L1 is constitutively expressed on resting APCs and T cells and upregulated during activation[288]. Unlike CD86-mediated signalling, which mainly governs T cell activation, crosslinking of PD-L1 with either PD-1 or CD80 provides inhibitory bidirectional signals between T cells and APCs[289,290]. Abnormal PD-L1 expression in humans has been related to several autoimmune diseases, which underscores its role in controlling T cell mediated immune responses and the induction of tolerance[291]. However, its contribution to the pathogenesis of allergic asthma has been less clear. Experimental studies in mice have yielded contradictory findings on the role of PD-L1/PD-1 interaction in regulating features of allergen-induced airway inflammation and AHR[202,292]. A more recent study has linked PD-L1 to atopic sensitisation and asthma in humans[293].

1.5 Aims of the study

The prevalence of asthma has been increasing worldwide in recent decades, making it one of the most common chronic diseases among children particularly in Western countries. Despite substantial efforts in research, its aetiology and pathobiology remain poorly understood. Previous knowledge about mechanistic processes in asthma mainly relied on the concept of a dysregulated Th2 cell-dominated adaptive immune response, resulting in chronic inflammation. Current efforts increasingly integrate innate immune pathways and cellular networks, providing the framework for novel targeted therapies. However, available therapeutic options cannot provide cure and remain ineffective in some forms of asthma, leaving a considerable group of patients with residual and severely uncontrolled disease.

Primary prevention of asthma is still a vision, although effective natural protection through early life farm exposure has been proven in epidemiological studies worldwide. From an immunological perspective, the underlying mechanisms of this association have not yet been clearly identified. A more comprehensive understanding of the immunobiology behind the asthma-protective 'farm effect' will provide the scaffold for disentangling the complexity of asthma pathophysiology, and ultimately developing new strategies of primary disease prevention and therapy.

There is now mounting evidence suggesting that continuous exposure to microbial products, such as LPS, from farming environments induces critical regulatory pathways within innate immunity to maintain immune homoeostasis in a child's developing immune system. In this context, the enzyme TNFAIP3, which attenuates NF-κB dependent inflammation, has recently evolved to be a key regulator. TNFAIP3 in bronchial epithelial cells has been shown to critically mediate asthma-protective effects of *in vivo* farm dust exposure in adult mice. Although these data strongly point towards a unifying molecular mechanism most relevant to both disease prevention and therapy, equivalent immunological studies in humans are missing. Moreover, a large body of evidence suggests that

immunoregulatory processes crucial to asthma development occur during infancy, and do not only involve the airway epithelium.

Therefore, the experimental approach of the present study aimed to assess immune regulation in primary immune cells of children. The aim of this thesis was to unravel the role of the anti-inflammatory regulatory molecule TNFAIP3 and related signalling pathways in both the inception and environmentally mediated prevention of allergic asthma in children.

In particular, the following objectives were pursued:

(i) To investigate if TNFAIP3 gene and protein expression and expression of genes related to NF-κB signalling and dendritic cell maturation was dysregulated in peripheral blood immune cells (PBMCs) of steroid-naive asthmatic children during asthma manifestation.

(ii) To study whether *in vitro* exposure to dust extracts from different 'asthma-protective' farming environments, as well as LPS, altered expression of TNFAIP3 and related genes in children's PBMCs. To analyse in particular whether such stimulation could counterbalance potentially maladapted pathways in asthmatic subjects.
In this context, to specifically study whether distinct farm dust extracts featured differential immunoregulatory properties upon *in vitro* exposure.

(iii) To assess whether continuous *in vivo* farm exposure early in life modulated TNFAIP3 pathway gene expression in farm children's immune cells by the age of 4.5, 6 and 10 years.
To specifically assess immune responses in farm children's immune cells upon acute *in vitro* farm dust and LPS exposure.

2 Materials and methods

2.1 Materials

2.1.1 Reagents and chemicals

ACK (Ammonium-Chloride-Potassium) Lysing Buffer	Lonza, Basel, Switzerland
Anti-A20/TNFAIP3 antibody 4625S	Cell Signaling Technology, Cambridge, UK
Anti-rabbit IgG, HRP-linked antibody 7074S	Cell Signaling Technology, Cambridge, UK
Anti-β-Actin antibody (C4) HRP sc-47778	Santa Cruz Biotechnologies, Santa Cruz, California, USA
APS (Ammonium persulphate)	Carl Roth, Karlsruhe, Germany
Bromphenol blue	Carl Roth, Karlsruhe, Germany
BSA (Bovine Serum Albumin), heat shock fraction, pH 7, ≥ 98%	Sigma-Aldrich, St. Louis, Missouri, USA
cOmplete™ Mini, EDTA-free protease inhibitor cocktail	Roche, Basel, Switzerland
diH_2O (deionised water)	Milli-Q® water purification system, Milli-Q®, Merck, Darmstadt, Germany
ECL Substrate (SuperSignal™ West Dura Extended Duration Substrate)	Thermo Fisher Scientific, Thermo Scientific™, Waltham, Massachusetts, USA
EDTA (Ethylenediaminetetraacetic acid)	Carl Roth, Karlsruhe, Germany
Ethanol 100%	Sigma-Aldrich, St. Louis, Missouri, USA
Ficoll-Paque™ PLUS solution	GE Healthcare, Piscataway, New Jersey, USA
Glycerol	Sigma-Aldrich, St. Louis, Missouri, USA
Glycine	Carl Roth, Karlsruhe, Germany
HCl (Hydrochloric acid) 37 %	Carl Roth, Karlsruhe, Germany
HPLC-grade water	Milli-Q®, Merck, Darmstadt, Germany
LPS from E. coli serotype O111:B4	Sigma-Aldrich, St. Louis, Missouri, USA
Methanol	Sigma-Aldrich, St. Louis, Missouri, USA
Milk, powdered, blotting grade, low fat	Carl Roth, Karlsruhe, Germany
NaCl (Sodium chloride)	Carl Roth, Karlsruhe, Germany
NaF (Sodium fluoride)	Sigma-Aldrich, St. Louis, Missouri, USA
NP-40 Detergent	Sigma-Aldrich, St. Louis, Missouri, USA
Nuclease-free water	Qiagen, Venlo, the Netherlands
PageRuler™ Prestained Protein Ladder, 10 to 180 kDa	Thermo Fisher Scientific, Waltham, Massachusetts, USA
PBS (Phosphate-Buffered Saline)	Thermo Fisher Scientific, Gibco™, Waltham, Massachusetts, USA

Primers for use in qPCR	Thermo Fisher Scientific, invitrogen™, Waltham, Massachusetts, USA
RLT lysis buffer	Qiagen, Venlo, the Netherlands
Roti®-PVDF membrane, 0.45µm pore size	Carl Roth, Karlsruhe, Germany
Roti®-Quant 5X conc. Bradford staining solution	Carl Roth, Karlsruhe, Germany
Rotiphorese® NF-Acrylamide/Bis-solution 30 % (29:1)	Carl Roth, Karlsruhe, Germany
RPMI 1640 Medium + GlutaMax™	Thermo Fisher Scientific, Gibco™, Waltham, Massachusetts, USA
SDS 20% (Sodium dodecyl sulphate, 20% solution)	Carl Roth, Karlsruhe, Germany
TEMED (Tetramethylethylenediamine)	Carl Roth, Karlsruhe, Germany
Tris base	Carl Roth, Karlsruhe, Germany
Tris-HCl	Carl Roth, Karlsruhe, Germany
Trypan Blue solution 0.4%	Sigma-Aldrich, St. Louis, Missouri, USA
Tween® 20 (Polysorbate) Detergent	Sigma-Aldrich, St. Louis, Missouri, USA
Whatman® filter paper	Wagner & Munz, München, Germany
X-VIVO™ 15 Medium	Lonza, Basel, Switzerland
2-Mercaptoethanol	Sigma-Aldrich, St. Louis, Missouri, USA
2-Propanol	Sigma-Aldrich, St. Louis, Missouri, USA

2.1.2 Solutions and buffers

APS 10%	1.0 g APS add diH_2O up to 10 ml total volume
BSA stock solution (400 µg/ml)	20 mg BSA add diH_2O up to 50 ml total volume
BSA 5% (w/v)	2.5 g BSA add diH_2O up to 50 ml total volume
5X Laemmli sample buffer	5 ml 1.5 M Tris, pH 6.8 3 ml SDS 20% 15 ml glycerol 0.9 mg bromphenol blue add diH_2O up to 50 ml total volume add 2-mercaptoethanol
Milk 5% (w/v)	2.5 g milk powder add diH_2O up to 50 ml total volume
RIPA buffer	150 mM NaCl 25.0 mM Tris HCl, pH 7.5 1.0 mM EDTA 1% NP-40

	5% glycerol 2.5 mM pyrophosphate 50 mM NAF add diH_2O up to 50 ml total volume
10X Running buffer	30.3 g Tris 144.1 g glycine 50.0 ml of SDS 20% add diH_2O up to 1 L total volume
1X Running buffer	Dilute 10X running buffer to a 1X working solution, using diH_2O. 1X running buffer contains 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3
10X Transfer buffer	30.3 g Tris 144.1 g glycine add diH_2O up to 1 L total volume
1X Transfer buffer	Dilute 10X transfer buffer to a 1X working solution, using 800 ml of diH_2O, 100 ml of methanol, 100 ml of 10X transfer buffer stock solution. 1X transfer buffer contains 25 mM Tris, 192 mM glycine, 10% methanol.
10X Tris buffered saline (TBS)	60.6 g Tris 87.6 g NaCl dissolve in 800 ml of diH_2O adjust pH to 7.6 using concentrated HCl add diH_2O up to 1 L total volume
1X TBS, 0.1% Tween 20 (TBS-T)	Dilute 10X TBS to a 1X working solution, using diH_2O. For 1X TBS-T, add 1 ml of Tween 20% to 1 L of 1X TBS. 1X TBS-T working solution contains 50 mM Tris, 150 mM NaCl, 0.1% Tween® 20, pH 7.6
1.5 M Tris, pH 8.8	181.7 g Tris add diH_2O up to 1 L total volume adjust pH to 8.8 using concentrated HCl
0.5 M Tris, pH 6.8	60.6 g Tris add diH_2O up to 1 L total volume adjust pH to 6.8 using concentrated HCl

2.1.3 Reagent systems (kits)

Limulus Amoebocyte Lysate (LAL) QCL-1000™ Assay + ß-Glucan Blocker Kit	Lonza, Basel, Switzerland
QuantiTect® Reverse Transcription Kit	Qiagen, Venlo, the Netherlands
RNeasy® Mini Kit	Qiagen, Venlo, the Netherlands
SsoAdvanced™ Universal SYBR® Green Supermix	Bio-Rad, Hercules, California, USA

2.1.4 Consumables

Adhesive seals, Microseal® 'B'	Bio-Rad, Hercules, California, USA
Blood Collection Set, Vacutainer® Safety-Lok™	BD, Franklin Lakes, New Jersey, USA
Blood collection tubes Vacutainer®	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, New York, USA
Filter tips 0.1-20 µl Biosphere®	Sarstedt, Nümbrecht, Germany
Filter tips 100 µl, low binding SafeSeal®	Biozym Scientific, Hessisch Oldendorf, Germany
Filter tips 100-1000 µl Biosphere®	Sarstedt, Nümbrecht, Germany
Filter tips 2-100 µl Biosphere®	Sarstedt, Nümbrecht, Germany
Filter tips, 1000 µl for use with the QIAcube®	Qiagen, Venlo, the Netherlands
LeucoSep™ centrifuge tubes (12 ml)	Greiner Bio-One, Kremsmünster, Austria
Microcentrifuge 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
Reagent reservoirs, sterile	Lonza, Basel, Switzerland
Rotor adapters (10 x 24) for use with the QIAcube®	Qiagen, Venlo, the Netherlands
Serological pipettes (2 ml, 5 ml, 10 ml, 50 ml)	Sarstedt, Nümbrecht, Germany
Syringe filters Millex® (0.22 µm pore size, sterile)	Millipore®, Merck, Darmstadt, Germany
Syringes 10 ml	BD, Franklin Lakes, New Jersey, USA
24-well flat bottom cell culture plate, Primaria™	Corning, Corning, New York, USA
96-well microplates	Greiner Bio-One, Kremsmünster, Austria
96-well skirted PCR plates, low-profile, Hard-Shell®	Bio-Rad, Hercules, California, USA

2.1.5 Laboratory equipment

Balance Explorer® Analytical	OHAUS, Parsippany, New Jersey, USA
Centrifuge 5417 R	Eppendorf, Hamburg, Germany
Centrifuge 5810 R	Eppendorf, Hamburg, Germany
Centrifuge Jouan CR412	Thermo Fisher Scientific, Thermo Scientific™, Waltham, Massachusetts, USA
CFX96 Touch™ Real-time PCR Detection System	Bio-Rad, Hercules, California, USA
ChemiDoc ™ MP Imaging System	Bio-Rad, Hercules, California, USA
Electrophoresis chamber and gel casting equipment (Mini-PROTEAN® Tetra handcast system)	Bio-Rad, Hercules, California, USA
Freeze Dryer Lyovac® GT 2	GEA, Düsseldorf, Germany
Incubator HERAcell® 240	Heraeus, Hanau, Germany
Micro Centrifuge II	neoLab Migge, Heidelberg, Germany
Microplate centrifuge PerfectSpin P	Peqlab Biotechnologies, Erlangen, Germany
Microplate reader Synergy H1	BioTek, Winooski, Vermont, USA
Microscope Axiovert 40C	Zeiss, Göttingen, Germany
Milli-Q® water purification system	Milli-Q®, Merck, Darmstadt, Germany
Neubauer counting chamber, depth 0.1 mm	Karl Hecht, Assistent®, Sondheim/Rhön, Germany
Orbital und horizontal shaker Stuart®	Cole-Parmer, Vernon Hills, Illinois, 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 2-20 µl	Eppendorf, Hamburg, Germany
Pipette Eppendorf Research® plus 10-100 µl	Eppendorf, Hamburg, Germany
8-channel pipettor Research® plus 10-100 µl	Eppendorf, Hamburg, Germany
Power supply PowerPac™ Basic	Bio-Rad, Hercules, California, USA
QIAcube® robotic workstation	Qiagen, Venlo, the Netherlands
Round-bottom flask	Schott, Duran®, Mainz, Germany
Spectrophotometer NanoDrop™ 2000	Thermo Fisher Scientific, Thermo Scientific™, Waltham, Massachusetts, USA
Stirrer Stuart®	Cole-Parmer, Vernon Hills, Illinois, USA
Thermomixer Comfort 5355	Eppendorf, Hamburg, Germany
Vacuum pump Millivac™	Millipore®, Merck, Darmstadt, Germany
Vortex shaker VF2	Janke & Kunkel, IKA®, Staufen, Germany
Wet/Tank blotting system (Mini Trans-Blot® Cell)	Bio-Rad, Hercules, California, USA

2.1.6 Software

Bio-Rad CFX Manager™ Version 2.1	Bio-Rad, Hercules, California, USA
EndNote X9	ISI ResearchSoft, Berkeley, California, USA
Ensembl Genome Browser	http://www.ensembl.org/index.html
Gen5™ microplate data analysis software	BioTek, Winooski, Vermont, USA
GraphPad Prism 8	Graphpad Software, San Diego, California, USA
Image lab™ software 6.0	Bio-Rad, Hercules, California, USA
ImageJ software	National Institutes of Health, Bethesda, Maryland, USA
Primer-BLAST designing tool	https://www.ncbi.nlm.nih.gov/tools/primer-blast/ National Center for Biotechnology (NCBI), Bethesda, Maryland, USA
R software Version 3.3.1	http://www.R-project.org/
SPSS Version 25	IBM, Armonk, New York, USA
Vector NTI software Version 10 Advance 11.5	Thermo Fisher Scientific, invitrogen™, Waltham, Massachusetts, USA

2.1.7 Primer sequences

Gene	**Forward primer sequence** (5′→ 3′ on plus strand)	**Reverse primer sequence** (5′→ 3′ on minus strand)
18S	AGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC
TLR4	CTCAACCAAGAACCTGGACCTG	TTGATAGTCCAGAAAAGGCTCCC
MYD88	TGCCTTCATCTGCTATTGCCCC	AGTCGCTTCTGATGGGCACCTG
TRAF6	TGATGTAGAGTTTGACCCACCCCTG	TGGAAGGGACGCTGGCATTG
TNFAIP3	GCCCAGGAATGCTACAGATACCC	CAGCCTTGGACGGGGATTTC
TNIP2	CAAAGGAATGTGGGGGAGAGAAGTC	TTCGTCCCTGCTGGCGTTGTAG
TAX1BP1	GCAGCAGAGGCAGATTTTGACATAG	TCACTTGTTCTTTCCATTTCAGCTCC
MALT1	CTCAGCCCCCCAGGAATAAAG	GCTCTCTGGGATGTCGCAAAC
CD80	CTGGCTGGTCTTTCTCACTTCTGTTC	ACTCGTATGTGCCCTCGTCAGATG
CD86	GCGGCTTTTATCTTCACCTTTC	AAAACACGCTGGGCTTCATC
CD274	ACCACCACCAATTCCAAGAGAG	CACATCCATCATTCTCCCTTTTC
PDCD1	AGGGTGACAGAGAGAAGGGCAG	TCCACAGAGAACACAGGCACG

2.2 Methods

2.2.1 Author's contribution statement

The author herself was responsible for recruitment of participants and sample acquisition within the CLARA/CLAUS study protocol. This involved organisation and realisation of recruitment activities in schools and paediatrician's offices in Munich, including questionnaire-based interviews, data entry, clinical assessment, blood collection and sample processing. Support was supplied by the study doctor during clinical workup. All laboratory experiments outlined below were performed independently and exclusively by the author. Statistical analyses and interpretation of data shown in this thesis were carried out by the author herself, with partial support provided by the biostatistician of the research group.
Since the 4.5- as well as the 6-year follow-up of the PASTURE/EFRAIM birth cohort study had been conducted prior to the start of the present project, clinical workup and some sample processing steps were not performed by the author herself but by (former) members of the research group. Wherever this was the case, it is indicated by an asterisk (*) in the respective sections below.

Details regarding the two study setups, recruitment and sample selection criteria, as well as clinical workup, will be outlined hereinafter.

2.2.2 Study populations, sample selection criteria and clinical workup

For the immunological experiments demonstrated in this work, blood samples of children from two different study populations were analysed. Firstly, a nested case-control design was applied for a sample of newly recruited allergic asthmatic (AA) and healthy control (HC) children (n = 48) as part of the CLARA/CLAUS study[294]. Secondly, a nested sample of farm (F) and non-farm (NF) children from the prospective birth cohort study PASTURE/EFRAIM was selected for analysis at ages 4.5 and 6 years (n = 37 and n = 63, respectively).

2.2.2.1 CLARA/CLAUS study

The cross-sectional CLARA (CLinical Asthma Research Association) /CLAUS study was initiated in January 2009 at the Dr. von Hauner Children´s Hospital, Munich, and includes healthy and asthmatic 4- to 14-year-old children[294,295]. More than six hundred children have been enrolled so far. Within CLARA, a total number of 275 children was recruited from 2010 to 2014, and another 334 children have been enrolled since 2014 within the ongoing cohort called CLAUS. The study is registered at the DRKS - German Clinical Trials Register (study ID DRKS00004635; https://www.drks.de/drks_web/) and approved by the human ethics committee of the Bavarian Ethical Board, LMU Munich, Germany (Nr. 379-08).

2.2.2.1.1 Enrolment of participants for the present study

For this project, healthy control subjects (HC) and patients with mild-to-moderate allergic asthma (AA) were recruited between January 2015 and March 2016 [a]. Healthy controls were recruited at the Edith-Stein Gymnasium grammar school, Munich, as well as at the Dr. von Hauner Children´s Hospital, Munich, and as part of the 10.5 year follow-up of the PASTURE/EFRAIM study[296] cohort in Bad Tölz, Germany. More than 60 children (aged 10 to 14 years) at the Edith Stein grammar school were screened by questionnaires according to the inclusion criteria for HC (as will be described in detail below) prior to blood withdrawal. Patients with allergic asthma were recruited at the Dr. von Hauner Children´s Hospital, Munich, at the paediatrician's offices of Dr. med. Christina v. Bredow & Dr. med. Beate Kusser and of Dr. med. Christian Schröter, both located in Munich, Germany.
Upon enrolment, parents completed a detailed questionnaire assessing data on their children's health as well as environmental and socioeconomic factors. Core questions regarding wheeze/asthma, hay fever and atopic dermatitis were based on the systematic, standardised questionnaire from the International Study of Asthma and Allergies in Childhood (ISAAC)[34,297]. Questions have been internationally validated and show a high specificity for the diagnosis of asthma[298-300].
The 60-item questionnaire of the CLARA/CLAUS study further contains detailed questions on pregnancy, early childhood, parental health, among others (see original questionnaire provided in the **appendix (10.2)**). Furthermore, data on common potential confounding variables such as family size, breast-feeding, parental education, family history of atopy or passive smoking were obtained from the questionnaires. Plausibility checks and checks for the completeness of questionnaire data were performed. Oral and written informed consent was obtained from the children's parents for participation in the study and blood sample collection.

2.2.2.1.2 Blood sampling and clinical assessment

Upon enrolment, 28 ml of peripheral blood was collected from all study participants (n = 48). Complete blood count (CBC), C-reactive protein (CRP) values and total immunoglobulin (Ig) E levels were measured in the children's blood samples. Furthermore, sensitisation to allergens was assessed by specific IgE in serum samples via radio-allergo-sorbent test (RAST). Positive sensitisation was defined as at least one positive test result for specific IgE [≥ 0.35 IU/ml], i.e. specific IgE class (SCI) 1 or greater, to a panel of 20 common allergens (Mediwiss Analytic, Moers, Germany), including *dermatophagoides pteronyssinus*, *dermatophagoides farinae*, birch pollen, grass pollen mix, hen's egg, cow´s milk, soybean, hazelnut, peanut, carrot, wheat flour, latex, hazel, cat epithelium, horse epithelium, dog epithelium, *Alternaria alternata*, mugwort, timothy grass, plantain[294].

[a] For enrolment of participants including clinical assessment and blood sampling, the author was partly supported by the study doctor or a study nurse in charge.

All asthmatic children underwent full clinical examination and pulmonary function tests. Lung function parameters were assessed by body plethysmography using the MasterScreen™ PFT System (CareFusion, Höchberg, Germany), following international standards (American Thoracic Society (ATS)/ European Respiratory Society (ERS) guidelines)[301]. Children were instructed in detail before the measurement. The children's height and weight without shoes were determined. Pulmonary function parameters, including the forced expiratory volume at the timed interval of one second (FEV_1), forced vital capacity (FVC), mid-expiratory flow rate at 75%, 50% and 25% of FVC (MEF_{75}, MEF_{50} and MEF_{25}, respectively), and measures of airway resistance and residual volume were evaluated at baseline ('*pre*') and after ('*post*') inhalation of the rapid-acting bronchodilator (i.e. β_2-adrenergic receptor agonist) salbutamol. Individual z-scores of FEV_1 ($zFEV_1$), and of FEV_1/FVC ratio ($zFEV_1$/FVC) were calculated, thus adjusting measured patient's values for sex, age, height and ethnicity[302,303]. According to the Global Initiative for Asthma (GINA) guidelines[304], reversibility of airflow limitation was defined as a significant relative increase in FEV_1 of >12% predicted upon bronchodilator administration (bronchodilator (BD) response [($FEV_{1\ post}$ - $FEV_{1\ pre}$)/ $FEV_{1\ pre}$] x 100).

2.2.2.1.3 Inclusion and exclusion criteria – definition of cases (AA) and controls (HC)

Inclusion criteria for asthmatic children comprised typical asthma symptoms of wheezing, whistling in the chest, cough or shortness of breath triggered by allergens, exercise or infection at several time points (at least 3 episodes) during the last year AND/OR a doctor's diagnosis of asthma AND/OR a history of asthma medication (such as inhaled β_2-adrenergic receptor agonists, inhaled glucocorticoids or leukotriene receptor antagonists) AND/OR abnormal lung function tests, indicating significant variable airflow limitation (as described above)[301]. A clinical diagnosis of asthma was made according to the international GINA guidelines[304] and the German „Nationale Versorgungs Leitlinie (NVL) Asthma"[305].

Children in this study were defined as *allergic asthmatics* (AA) based on the asthma criteria mentioned above, with pulmonary symptoms primarily triggered by allergens AND specific IgE levels ≥ 0.35 IU/ml to any of the assessed allergens. Patients with symptoms of wheeze induced by physical exercise, respiratory tract infections, the common cold, or by cold air, but not upon allergen-exposure, and without objective measures of atopic sensitisation or allergy, were not included ('non-allergic asthma', n = 1; see **Figure 10** in the **results**-section).

Hay fever and atopic dermatitis were defined as having ever had a doctor's diagnosis. Food allergy was defined as having distinct clinical symptoms of food allergy.

Healthy children without any clinical symptoms of wheeze/asthma and allergic disease (e.g. sneezing, a runny or blocked nose, or itchy-watery eyes during pollen season) were included as *healthy controls* (HC). Within the group of HCs, those were classified as healthy *farm-children* (F-HC) that met the farming-criteria from the PASTURE/EFRAIM study (see below, **2.2.2.2.2**).

Exclusion criteria for both AA and HC comprised any other diagnosis of acute infection, pulmonary, chronic, autoimmune or immunodeficiency disease. Current infections (i.e. within the last 14 days) were assessed by clinical examination and CRP levels. Children with a history of steroid-intake (including oral and inhaled glucocorticoids), or antibiotic or probiotic agents during the 14 days before blood withdrawal, were not included in the study. Likewise, children born preterm (defined as delivery at gestational age (GA) < 37 weeks) or with a birthweight below 2500 g were excluded.

A final number of 43 samples was analysed, comprising 24 healthy children (HC) including non-farm (NF-HC, n = 19) and farm-children (F-HC, n = 5), and 19 patients with allergic asthma (AA). The number for single analyses, however, may differ slightly in some cases, due to technical issues and experimental specifics.

2.2.2.2 PASTURE/EFRAIM study

The Protection Against Allergy: Study in Rural Environments (PASTURE)/Mechanisms of Early Protective Exposures on Allergy Development (EFRAIM) study is a multinational prospective birth cohort study funded by the European Commission, which includes 1,133 farm-exposed and non-farm-exposed children from rural environments in Germany, Austria, Switzerland, Finland and France[296]. Of these, one subgroup from the 4.5-year follow-up (n = 37 comprising German (n = 24) and Finnish (n = 13) children) and another from the 6-year follow-up (n = 63, with German (n = 24), Finnish (n = 18) and French (n = 21) children) were selected for assessment in this work. The study was approved by the local research ethics committee of each country (Germany ethics number 02046, amendment 2014; Austria E Nr. 401; Switzerland EKSG 021056; Finland 10/2008; France 07/448).

2.2.2.2.1 Study design and follow-up[*]

Pregnant women either from family-run livestock farms (these were assigned to the farm-group) or from the same rural areas but not living on a farm (the non-farming reference group)[187] had been recruited between August 2002 and March 2005. Follow-up was performed from the third trimenon of pregnancy until the children's age of 10.5 years, with a 16-year follow-up currently ongoing (10/2019). Comprehensive questionnaires were administered to the child's mother at certain intervals (i.e. at the end of pregnancy, at birth and at the child's age of 2 months, and 1, 1.5, 2, 3, 4, 5, 6 and 10.5 years), assessing details on farm characteristics, farm-related exposures, and the prevalence of allergic or asthma symptoms and diagnoses. Questionnaires further contained information on lifestyle factors such as nutrition, breastfeeding and maternal smoking, as well as family characteristics, socioeconomic status and family history of atopy. Items were largely derived from the earlier ALlergy and EndotoXin study (ALEX)[109] and the Prevention of Allergy: Risk Factors for Sensitization In Children Related to

[*] Not performed by the author. See explanations above (2.2.1)

Farming and Anthroposophic Lifestyle (PARSIFAL) study[111] as well as from the Asthma Multicenter Infants Cohort Study (AMICS)[306] and the American Thoracic Society questionnaire[307]. Written informed consent was obtained from all parents.

Blood sampling and clinical examination was performed during clinical visits at birth, and at 1, 4.5 and 6 years of age[187]. A total of 628 children participated in blood sampling at 4.5 and 6 years[308]. Specific IgE for common inhalant and food allergens was measured in serum samples (Allergy Screen test panel for atopy; Mediwiss Analytic, Moers, Germany). The 13 inhalant allergens comprised *dermatophagoides pteronyssinus*, *dermatophagoides farinae*, cat, horse and dog epithelium, *Alternaria* species, mugwort, plantain, alder, birch, hazel, rye pollen and grass pollen mix. The 6 food allergens included hen's egg, cow's milk, peanut, hazelnut, carrot, and wheat flour[187]. Positive atopic sensitisation against seasonal, perennial or food allergens was defined by using the cut-off for specific IgE of 0.7 IU/ml or greater (i.e. specific IgE class (SCI) 2).

2.2.2.2.2 Definition of farm exposures and allergic disease outcomes

Children born to women living on a farm run by the family were defined as *farm* children (F), while children with mothers from the same rural areas but not living on a farm were assigned to the *non-farm* (NF) reference group[296]. Farming was characterised by traditional farming practices such as milking, feeding animals, removing dung, cleaning animal sheds and placing litter, among others. Detailed information on timing, frequency, and intensity of exposure to hay barns, stables, farm and pet animals and consumption of farm milk were assessed annually by means of questionnaires in both groups.

Data on farm exposures presented in this thesis (**Table 12**) refer to the children's first, second, third, fourth, fifth, and sixth years of life, with 'regular' exposure defined as at least once per week. An exposure of 'farm milk consumption' was indicated if children drank cow's milk directly from a farm[187]. Contact with hay refers to any exposure to hay, such as by playing in the hay[187]. Contact with cows was defined as contact to (dairy) cattle or their calves during the fourth, fifth or sixth year of life (comparable variable not available for years 1-3). Exposures to animal stables and barns were defined as spending time in a stable or barn, respectively. Exposure time was assessed continuously in days per week and hours per day for farm-children, or hours per month for non-farm children, respectively, and respective exposure variables were dichotomised (yes/no) subsequently[175]. A single contact to a stable or barn was therefore sufficient to assign a child to the exposure category 'stay in stable/barn'[187].

Asthma was defined as at least one parent-reported physician's diagnosis of asthma OR recurrent diagnoses of spastic, obstructive, or asthmatic bronchitis[187], assessed at the age of 6, when the clinical phenotype of asthma is rather stable[10]. The great majority of asthmatic children selected for the present study were allergic asthmatics, with a specific IgE level ≥ 0.7 IU/ml in RAST.

Hay fever was defined as having ever had nasal symptoms such as sneezing or a runny nose, independent of upper respiratory tract infections, together with itchy or watery eyes OR a doctor's diagnosis of hay fever reported by the parents at the child's age of 4 and 6 years, respectively. Atopic

dermatitis and food allergy 'ever' were defined as a doctor's diagnosis reported in year 1 or annually till year 4 or 6, respectively.

The two populations selected for this work were enriched for asthmatic children among available samples of the whole PASTURE/EFRAIM cohort at ages 4.5 and 6 years and balanced for farming status (18 F vs. 19 NF at age 4.5 years and 32 F vs. 31 NF at age 6, respectively). F and NF children were equally distributed in terms of gender as well as asthma and allergic disease status.

2.2.3 Farm dust samples

2.2.3.1 Dust collection and extraction*

Environmental dust samples that were used for PBMC stimulation in this project had been acquired from distinct rural environments in Germany (G), Finland (Fi), and China (Ch) within the TRILATERAL[b] multi-centre project. Dust samples were also collected from barns of the Amish people (Am), a traditional farming population living in the U.S.[309]. Amish dust samples were collected in Middlebury, Indiana, USA by scraping from the walls in a cowshed. Settled airborne dust from G, Fi and Ch was collected with 'Electrostatic Dustfall Collectors' (EDCs), an effective and reliable method that has been validated in different farming environments[310,311]. EDCs are passive dust sampling devices consisting of a plastic folder containing electrostatic cloths which are exposed to the air. In 2013 and 2014, EDCs had been deployed on traditional farms in Icking, Bavaria, Germany (G; ID PB838), in Kitee, Finland (Fi; ID TN01), and in rural Conghua, China (Ch; ID CH_5109), respectively, for a 4-week period[312]. In Conghua, China, EDCs were placed in living rooms, whereas both German and Finnish dust was collected in cowsheds from dairy farms. Date and time of EDC placement, as well as position of the folders in the room were documented in detail. Cloths from EDCs were immediately stored at -80°C after sampling and shipped to Munich for further processing.
Preparation of aqueous dust extracts used in PBMC stimulation experiments was performed by Dr. P. C. Schröder, a former member of the research group. Dust was extracted from the cloths according to the method of cold sodium chloride extraction, established by Prof. Dr. Otto Holst (research centre Borstel, Germany)[313]. In brief, dust from cloths was dissolved in NaCl, 9% conc., in a sterile, pyrogen-free glass container and stirred for 6 hours. The solution was centrifuged for 30 minutes at 10,000 rpm, 4°C, and supernatant was desalinated by dialysis technique. Then, vacuum sterile filtration was performed, and dry extracts were obtained from dust-filtrates, using Freeze Dryer Lyovac® GT (GEA). Dried and processed dust from four cloths was pooled for each of the samples (G, Fi, Ch), stored at room temperature and protected from light and moisture. For cell culture experiments, dust extracts

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* Not performed by the author. See explanations above (2.2.1)

[b] TRILATERAL is a multi-centre project between collaborating research groups from Kuopio (Finland), Hong Kong, Guangzhou (China) and from Munich (Germany). Funded by the German Research Foundation (DFG), SCHA-997/3-1.

were resuspended in sterile PBS to a stock solution of 1mg/ml weight per volume (w/v) concentration and mixed 5 minutes before use for sample suspension and homogenisation.

2.2.3.2 Assessment of endotoxin levels in farm dust samples

Endotoxin concentration was measured in the German, Finnish and Chinese farm dust extracts using the endpoint chromogenic Limulus Amoebocyte Lysate (LAL) assay (QCL-1000™, Lonza), as described in previous studies[314]. Due to logistic reasons, the Amish dust sample was not measured. The assay was performed according to the manufacturer's instructions. Only pyrogen-free borosilicate glass tubes (Lonza) and accessory materials that have been qualified for endotoxin testing were used in the assay. ß-1,3-Glucan-blocker (Lonza) was applied to reduce false positive assay results caused by yeast or cellulose-containing residues in the dust extracts. In order to evaluate product inhibition, an aliquot of every dust sample was spiked with endotoxin of a known concentration (1.0 EU/ml). The spiked solution was assayed along with the unspiked samples, and the difference between their determined endotoxin concentrations was supposed to equal the known concentration of the spike ± 25%. A standard curve was generated using dilutions of endotoxin standards (Escherichia coli serotype O111:B4, Lonza) from 1.0 to 0.1 EU/ml. All dust samples and standards were run in duplicates. The assay was performed in a 96-well microplate format and kept at a constant temperature of 37°C during all steps. Standards and samples were analysed at a wavelength of 405 nm using the microplate reader Synergy™ running Gen5™ software (BioTek). Endotoxin concentrations of the samples were determined from their absorbance values based on the standard curve. Results are expressed as endotoxin units per millilitre of aqueous dust extract (EU/ml).

2.2.4 PBMC isolation and stimulation

Whole blood from sodium heparin collection tubes (Vacutainer®, BD) was used for cell isolation and culture experiments. Blood samples of poor quality (i.e. haemolytic blood) or with insufficient cell count (< 15 x 10^6 cells) due to low total blood volume were excluded from final analyses ('technical exclusion' (n = 4), see **Figure 10** in the **results-**section).

Blood was processed within a 6- to maximum 24-hour-interval after blood withdrawal. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation. Whole blood was diluted 2:1 with phosphate-buffered saline (PBS, Thermo Fisher Scientific, Gibco™), and equal parts (5-8 ml) were aliquoted in LeucoSep™ tubes (Greiner Bio-One) filled with 3 ml of PBMC-separation medium Ficoll-Paque™ PLUS (GE Healthcare). Tubes were centrifuged at 1,400 x g for 10 minutes at 20°C, acceleration 9, deceleration 0 (no brake). PBMC layer was carefully transferred to 15 ml conical tubes using a sterile Pasteur pipette. RPMI 1640 cell culture medium (Thermo Fisher Scientific, Gibco™) was added to the cells up to a total volume of 14 ml, and the cell-medium suspension was centrifuged again at 1,100 x g for 10 minutes, at 20°C, accel 9, decel 9. Supernatant was decanted and cell pellet resuspended and homogenised softly in a final volume of 10 ml of RPMI 1640.

Red blood cell (RBC) lysis was performed in a 50 µl aliquot used for cell counting. Therefore, 50 µl of ACK Lysing Buffer (Lonza) was added to the 50 µl sample aliquot, and cells were incubated on ice for 7 minutes. 50 µl of trypan blue solution 0.4% (Sigma-Aldrich) was added, and the sample was mixed well. 10 µl of cell suspension was transferred into a Neubauer counting chamber (Karl Hecht, Assistent®), and PBMC count was assessed.

Total cells in RPMI medium suspension were pelleted by centrifugation at 1,100 x g for 10 minutes at 20°C, and supernatant was discarded. PBMCs were diluted to a concentration of 5 x 10^6 cells/ml by adding the appropriate volume of X-VIVO™ 15 cell medium (Lonza).

A number of 2.5 x 10^6 cells per well were cultured in a 24-well plate format (Corning) in X-VIVO™ for 24 hours at 37°C, 5% CO_2 either without stimulation (unstimulated U) or following stimulation with lipopolysaccharide (LPS, from E.coli-O111:B4) at a final concentration (f.c.) of 0.1 µg/ml, or with farm dust extracts (f.c. 40 µg/ml) from Germany (G), Finland (Fi), China (Ch) and from the Amish population (Am). LPS concentration had been selected based on previous findings from farming studies[175,187,308]. Concentration for farm dust stimulation was derived from preliminary experiments in which ideal conditions had been tested for the experimental setup of this project.

Harvested cells were split in two parts. One fifth was used for protein extraction for subsequent Western Blot analyses (0.5 x 10^6 cells at least), the remaining cells were used to isolate mRNA for gene expression studies. Supernatants were collected, and 110 µl aliquots were immediately frozen at -80°C for use in cytokine studies.

Blood samples from the 4.5- and 6-year-old PASTURE/EFRAIM children were processed as described previously[187], using a standardised protocol at the local study centres. In brief, heparinised whole blood was diluted with RPMI medium supplemented with 10% human serum (Sigma-Aldrich) to a final concentration of 5 x 10^6 cells/ml and incubated for 24 hours at 37°C, 5% CO_2 unstimulated (U) or stimulated with 0.1 µg/ml LPS. For the present study, frozen samples were analysed.

2.2.5 Cell lysis for RNA and protein extraction

A total number of 2.0 x 10^6 cultured cells were harvested after 24 hours incubation and centrifuged at 1,100 x g for 10 minutes at 20°C. Supernatants were collected and stored, and remaining cell pellet was resuspended in 1 ml PBS. Cells were pelleted by centrifugation at 1,100 x g for 10 minutes at 20°C, and supernatant was aspirated and discarded. Cells in pellet were disrupted properly and homogenised in 350 µl of buffer RLT (Qiagen) plus 1% (v/v) 2-mercaptoethanol (β-ME). Lysates were stored at -80°C until use.

Cells for subsequent protein expression studies in Western Blot were pelleted by spinning at 3,700 x g for 10 minutes at 4°C. Supernatant was aspirated and discarded, and cell pellets were lysed in 100 µl of ice cold radioimmunoprecipitation assay (RIPA) lysis buffer plus freshly added protease inhibitor cocktail (cOmplete™ Mini, Roche). Samples were incubated on ice for 30 minutes and mixed by

repeated flicking of the tubes. To pellet the cell debris, lysates were centrifuged at 20,200 x g for 15 minutes at 4°C. Cell lysate supernatant was quickly collected, aliquoted and stored at -80°C. Pellet was discarded. Cells and lysates were kept on ice during all steps.

2.2.6 RNA extraction and cDNA synthesis

Total RNA of PBMCs was purified using the RNeasy® Mini Kit (Qiagen) according to the manufacturer's instructions. Frozen cell lysates were thawed, 1 volume (350 µl) of 70% ethanol was added, and suspension was mixed well by vigorous pipetting. Lysates were transferred onto silica-membrane coated RNeasy® spin columns that had been placed into a 2 ml collection tube. Tubes were centrifuged at 8,000 x g for 15 seconds at 20°C, and flow-through was decanted. In order to eliminate all contaminants effectively from the spin column membrane, a number of centrifugation steps (8,000 x g, 15 seconds, at 20°C) were performed after addition of washing buffers RW1 and RPE (Qiagen), and flow through was decanted after each centrifugation. Steps were performed very carefully in order to avoid carryover of ethanol. Spin columns were placed in a new 2 ml collection tube and centrifuged at full speed for 1 minute, then transferred to a fresh 1.5 ml elution tube. Pure, concentrated sample RNA was eluted in 30 µl of RNase-free water during a last centrifugation step at 8,000 x g for 1 minute.

RNA of whole blood samples from the PASTURE/EFRAIM cohort was extracted using QIAzol and chloroform.

Sample RNA concentration was assayed with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). The absorbance of sample RNA was read at 260 nm. To rule out the presence of potential contaminants in the sample, the ratios of absorbance at 260 nm and 280 nm (A260/A280) as well as A260/A230 were determined. RNA purity was assumed for a A260/A280 value in the range of 2.0 to 2.1 and a A260/A230 value range of 2.0 to 2.2.

1 µg of total sample RNA underwent cDNA synthesis, using QuantiTect® Reverse Transcription Kit (Qiagen). RT reaction was run on PCR Thermocycler PeqSTAR 96 universal (Peqlab Biotechnologies). All reactions were set on ice to minimise the risk of RNA degradation. Genomic DNA (gDNA) wipe-out buffer, RNase-free water and RNA sample were combined in 0.2 ml tubes. Samples were incubated at 42°C for 2 minutes to eliminate gDNA residues. A master mix containing the reverse transcriptase enzyme, RT buffer, and a RT primer mix was prepared and distributed to the individual tubes, resulting in a total reaction volume of 20 µl. All components were mixed, and the tubes were centrifuged briefly. RT reaction was run at 42°C for 15 minutes, followed by an enzyme inactivation step at 95°C for 3 minutes. cDNA samples of a final concentration of 50 ng/µl were obtained and stored immediately upon receipt at -20°C for subsequent gene expression studies.

2.2.7 Gene expression analysis

2.2.7.1 Primer design

mRNA-specific oligonucleotide primers (forward/reverse) that were used for gene expression analysis in quantitative real-time PCR (qPCR) were designed using Vector NTI 10 Advance 11.5 program (Thermo Fisher Scientific, invitrogen™), and Primer-BLAST designing tool, provided by the National Center for Biotechnology Information (NCBI), Bethesda MD, USA[315]. PCR templates were based on DNA sequences listed in the genome database *Ensembl*[316].
Quality criteria regarding forward (fw) and reverse (rv) primer sequences for use in qPCR were as follows: Primer length between 18 and 27 base pairs for good specifity and binding abilities, obligatory primer location behind the gene's ATG sequence, at least one guanine or cytosine at the 3' end, 40-60% of guanine and cytosine with no more than 10% difference between fw and rv primer, primer melting temperature (T_m) ranging between 54°C and 65°C with compatible T_m values of fw and rv primer (maximum of 0.5°C difference), primer-dimer- or hairpin-building energy lower than ± 2 kcal/mol for good annealing efficiency, generated PCR product (amplicon) size of a maximum of 200 base pairs, and – if possible – at least one primer to span an exon-exon junction site, and primer pair to be separated by at least one intron of great size to avoid amplification of gDNA residues.

Primers were ordered from Thermo Fisher Scientific, invitrogen™, and were diluted in nuclease-free water (Qiagen) to a stock of 1 mM concentration. A 1 µM dilution containing both fw and rv primers was obtained after an intermediate step of a 0.1 mM dilution. 0.1 mM primer stock was stored at -20°C, 1 µM primers were kept at +4°C for use in qPCR. Diluted primers underwent quality and specifity testing to ensure efficiency of reactions and avoid mispriming events. Optimal concentrations for primers in the qPCR setup were determined. Thorough melting curve analysis was performed to exclude primers with amplification of unintended mRNA, gDNA or RNA targets (see also **2.2.7.3**). Primer sequences for the targets investigated in this work are listed in **2.1.7**.

2.2.7.2 Quantitative real-time PCR

mRNA expression of the selected target genes (*TLR4, MyD88, TRAF6, TNFAIP3, TNIP2, TAX1BP1, MALT1, CD80, CD86, CD274, PDCD1*) was assessed in the samples by quantitative real-time PCR (qPCR). 18S-ribosomal-RNA (rRNA) was used as an endogenous reference gene in all experiments. In a 96-well format (skirted PCR plates, low-profile, Hard-Shell®, Bio-Rad), all reaction components were combined to a final volume of 10 µl per well. The qPCR reaction setup used for the assays in this project is shown in **Table 1**. All reagents and samples were processed on ice and briefly mixed and centrifuged just before assembling the reactions. Mastermix (SsoAdvanced™ Universal SYBR® Green Supermix, Bio-Rad) containing a thermostable Sso7d fusion polymerase, dNTPs, $MgCl_2$, and SYBR® Green I dye was mixed thoroughly and equal aliquots of 5 µl dispensed into the wells of the

PCR plate. 1.8 µl of cDNA mix containing cDNA template and nuclease-free water was added, followed by 3.2 µl of 1 µM sequence-specific primers (fw and rv). For 'no-template-controls' (NTC), cDNA-template was replaced by an equal amount of nuclease-free water. Thorough mixing of the reaction components was ensured. After the pipetting process, the PCR plate was sealed with optically transparent film (Microseal®, Bio-Rad) and centrifuged at 500 x g for 15 seconds to spin down droplets and eliminate any air bubbles in the wells. qPCR was started, and reactions were run on the CFX96 Touch™ Real-time PCR Detection System (Bio-Rad) according to the thermal cycling protocol depicted in **Table 2**. Data was processed and analysed by Bio-Rad CFX Manager™ software version 2.1.

Table 1. *qPCR reaction setup.*

Component	**Volume per 10 µl reaction**	**Final concentration**
SsoAdvanced™ Universal SYBR® Green Supermix (2x)	5 µl	1x
Forward and reverse primers	3.2 µl	160 nM each
cDNA template (50 ng/µl)	0.12 µl	0.6 ng/µl
Nuclease-free H_2O	1.68 µl	--
Total reaction mix volume	**10 µl**	--

Table 2. *qPCR thermal cycling protocol.*

Step	**Times repeated**	**Temperature**	**Time**
Cycle 1	1x		2 min
Initial denaturation		95.0°C	
Cycle 2	40x		
Denaturation		95.0°C	20 sec
Annealing + Elongation		62.5°C	30 sec
Cycle 3	1x		2 min
Elongation		72.0°C	
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

Amplification of gene-specific PCR products was monitored during 40 cycles by measuring the increase in fluorescence signal caused by binding of SYBR® Green to double-stranded DNA (dsDNA). Baseline fluorescence signal for SYBR® Green was automatically determined by the software and used for threshold setting. The cycle threshold (C_t) value describes the number of cycles in a given

amplification reaction, at which the fluorescence signal significantly exceeds the background fluorescence. It is assessed at the early exponential phase of amplification (**Figure 8**). During this phase, the reaction is most effective and precise, with the amount of PCR product doubling at each cycle. Since fewer PCR cycles are required for high amounts of starting template to be amplified and detected over the background signal, a lower C_t value is associated with a higher initial amount of template in the reaction. Individual C_t values in this work were normalised to 18S, which was used as reference gene. Data of the presented target genes (T) are reported as ΔC_t values; $\Delta C_{t\,(T)} = C_{t\,(T)} - C_{t\,(18S)}$ (see also **2.2.9**).

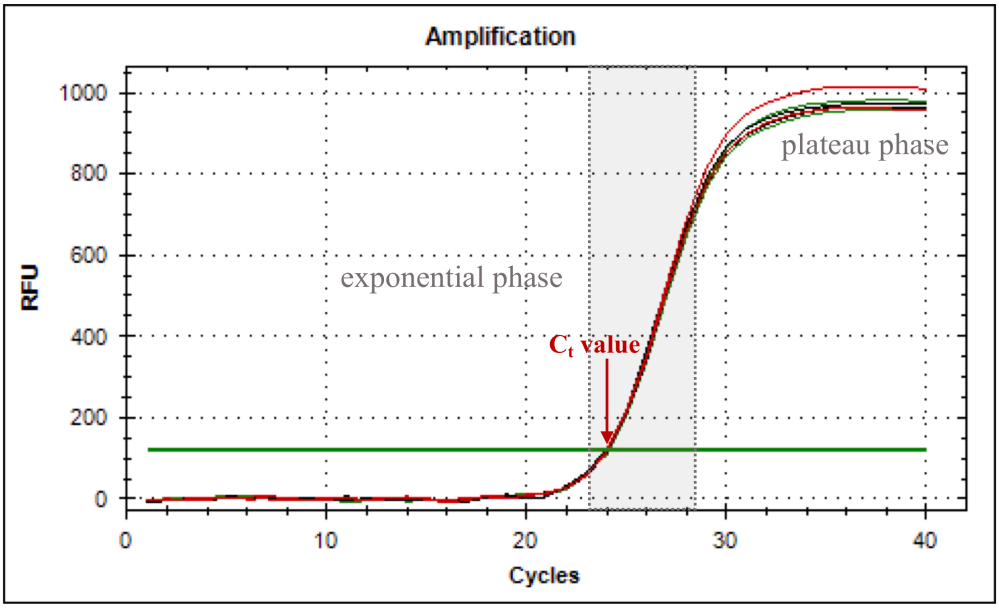

Figure 8. Graphical representation of qPCR data. Exemplary reaction amplification plot. Relative fluorescence units (RFU) are plotted against qPCR cycle number. Threshold line is set automatically above the baseline fluorescence signal (green horizontal line) in the early exponential phase of the plot. C_t (threshold cycle) value is determined at the intersection between the amplification curve and the threshold line. The different amplification curves in this chart represent data from different samples and their duplicates.

2.2.7.3 qPCR assay optimisation and validation

In accordance with the MIQE guidelines for qPCR experiments[317], all relevant experimental conditions and assay characteristics were optimised prior to starting the project and evaluated throughout the whole process in order to generate reliable qPCR data allowing accurate and precise quantification of target gene expression.

Sample quality control was ensured during RNA extraction and reverse transcription steps, as described above (**2.2.6**). Primer characteristics and concentrations were optimised (see **2.2.7.1**) and ideal cDNA template concentrations were assessed. To ensure reproducibility, all reactions were processed under

equal conditions including assay reagents, equipment and accessory items, and thermal-cycling protocol settings.

Good pipetting practice was employed in advance to achieve maximum assay accuracy. All samples and controls were run in duplicates and standard deviation measures were determined. A C_t difference between duplicates of greater than 0.4 was set as cut-off to rule out values and repeat the sample. Samples with a C_t value below the detection limit (i.e. 40 PCR cycles) were excluded. No-template-controls (NTC) were included in every reaction setup to identify potential contamination or primer-dimer formation. Specificity of the reactions and reliability of the obtained signals was further evaluated by melting curve analysis assessing the presence of nonspecific products. A melting curve (**Figure 9**) charts the sudden change in fluorescence signal observed when double-stranded DNA (dsDNA) bound with SYBR® Green dye dissociates into single-stranded DNA (ssDNA) at a certain melting temperature (T_m). Since different properties of nucleic acids (such as length, GC content among others) result in modified melting characteristics, different PCR products can be distinguished by their specific melting temperature.

For reliable quantitative gene expression analysis, 18S rRNA was validated as a reference control gene. It has shown to maintain stability of expression across samples regardless of treatment or clinical phenotype in our study group's previous validation experiments.

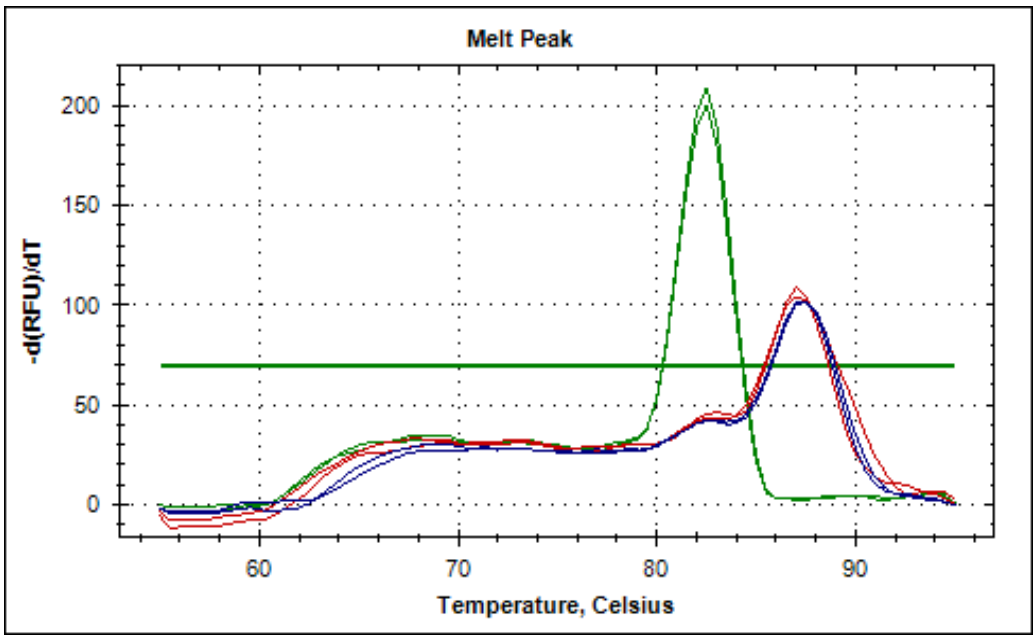

Figure 9. Exemplary representation of melting curve analysis showing off-target amplicons. The negative first derivative of the change in relative fluorescence (-d(RFU)/dT) is plotted as a function of temperature to obtain a clear view of the melting dynamics. In this case, additional peaks (red and blue plots) are detected to the right of the target product-specific melting peaks at 82.5°C (green plots), indicating the presence of off-target products.

2.2.8 SDS-PAGE and Western Blot analysis

TNFAIP3 protein expression was assessed by Western Blot analysis in a subsample of children. Western blotting was performed with kind collaboration of Professor Christoph Klein's lab and members, providing technical support and facilities at the Comprehensive Childhood Research Centre, Dr. von Hauner Children's Hospital, Munich.

The final protocol for this project has been established by the author herself after various preliminary experiments in accordance with recognised standardisation procedures[318]. All assay components and steps were extensively evaluated and optimised in advance, involving tests for ideal protein sample amount, gel type, appropriate primary and secondary antibody dilutions, incubation times, washing buffers for dilution and antibody incubation, and validation of the reference protein β-Actin against GAPDH.

2.2.8.1 Gel casting

15-well SDS 10%-gels of 1.5 mm thickness were hand-casted directly before use (**Table 3**), employing gel casting systems from Bio-Rad. For the separating monomer solution, diH_2O, SDS 20% (Carl Roth), acrylamide/bis-solution 30% (Rotiphorese®, Carl Roth) and 1.5 M Tris pH 8.8 were combined. Ammoniumperoxiddisulphate (APS, Carl Roth) and Tetramethylethylenediamine (TEMED, Carl Roth) were added for polymerisation, accurate mixing was ensured, and the separating solution was smoothly poured into the gel cassette. The solution was immediately overlaid with 1 ml of 2-propanol in order to prevent shrinkage at the interface with air. 2-propanol was decanted after 30 minutes, and the top of the solidified separating gel was dried with filter paper. Stacking monomer solution (diH_2O, SDS 20%, acrylamide, 0.5 M Tris pH 6.8, and APS and TEMED) was prepared and poured on top of the separating gel. Gel comb was inserted, and SDS polyacrylamide gels were ready for electrophoresis (SDS-PAGE) after solidification.

Table 3. *Pipetting volumes for 4 gels.*

separating gel 10%		**stacking gel**	
	40 ml		
diH_2O	16.0 ml		8.0 ml
1.5 M Tris (pH 8.8)	10.0 ml	0.5 M Tris (pH 6.8)	1440.0 µl
20% SDS	200.0 µl		57.5 µl
30% Acrylamide mix	13.3 ml		1950.0 µl
10% APS	400.0 µl		150.0 µl
TEMED	16.0 µl		11.5 µl

2.2.8.2 Protein quantification

Total protein content of sample cell lysates (**2.2.5**) was determined by Bradford protein quantification assay. Bradford staining solution with Coomassie Brilliant Blue Dye G250 (Roti®-Quant. 5X, Carl Roth) was used. Bovine serum albumin (BSA, heat shock fraction, Sigma-Aldrich) was used as calibrating standard protein, and serial dilutions were performed. In a 96-well microplate format, 200 µl of sterile filtered Bradford solution (diluted 1:2.75) was added to 50 µl of BSA-standards and diluted samples (1:40 in diH_2O). Plate was incubated for 5 minutes at room temperature. Standards, samples and blanks (negative controls) were analysed for absorbance at 595 nm wavelength, using Synergy H1 microplate reader running Gen5™ software (BioTek). Sample protein concentrations were determined by interpolation from a 10-point BSA-standard-curve (**2.2.9**). Quantification accuracy was ensured by a coefficient of determination $R^2 > 0.98$ for all calibrating standard curves. All standards, samples and blanks were run in duplicates, and values with a coefficient of variation (C.V.) absorbance of greater than 10% were excluded.

2.2.8.3 Sample preparation and SDS-PAGE gel electrophoresis

Protein concentration was normalised across samples in order to obtain equal amounts of protein samples to be loaded on one gel (40 µg to 55 µg). Determined volume of protein sample was combined with 7 µl of 5X Laemmli sample buffer (including bromphenolblue as loading dye and 3% 2-mercaptoethanol). Appropriate volume of 1X running buffer was added up to a total volume of 35 µl. Samples were boiled at 95°C for 10 minutes, centrifuged, and cooled on ice. Electrophoresis chamber (Bio-Rad) was assembled, casted gels were inserted, and chamber filled with 1X running buffer. 6 µl of PageRuler™ Prestained Protein Ladder (10 to 180 kDa, Thermo Fisher Scientific) and 35 µl samples were loaded onto the wells of the SDS-polyacrylamide gel. Gel electrophoresis was run at 80V for 10 minutes until protein was condensed to a flat front, then 120V was applied for 110 minutes.

2.2.8.4 Protein transfer

Separated proteins were transferred to a PVDF membrane (Roti®, 0.45 µm pore size, Carl Roth). Therefore, the gel was removed from the electrophoresis rack and incubated in 1X transfer buffer for 5 minutes. For improved transfer and protein binding, PVDF membrane was soaked in methanol for 2 minutes. Blotting sandwich comprising foam pads, Whatman filter papers, gel and transfer membrane was assembled in 1X transfer buffer, and the sandwich holder cassette was placed into the transfer chamber. Cooling unit and 1X transfer buffer were added. Protein transfer was performed at 400 mA for 90 minutes, 4°C using the wet tank blotting system Mini Trans-Blot® Cell (Bio-Rad).

2.2.8.5 Membrane blocking and antibody staining

After transfer, the blotted membrane was blocked in low-fat dry milk 5% (w/v) in 1X tris buffered saline (TBS) supplemented with 0.1% (v/v) Tween-20 (1X TBS-T), with gentle agitation for one hour at room temperature. Membranes were probed with primary antibody against A20/TNFAIP3 (#4625, Cell Signaling Technology), at 1:300 dilution in BSA 5% (w/v), 1X TBS-T, at 4°C with gentle agitation overnight. After subsequent washing steps (three times for 7 minutes each in 1X TBS-T), the membrane was incubated with Horseradish-peroxidase-conjugated goat anti-rabbit IgG (#7074, Cell Signaling Technology) at 1:1000 dilution in milk 5%, 1X TBS-T with gentle agitation for one hour at room temperature.
ß-actin was used as loading control. The membrane was incubated with HRP-linked antibody against ß-actin (sc-47778 HRP, Santa Cruz Biotechnologies), diluted 1:3000 in milk 5%, 1X TBS-T, with gentle agitation for one hour at room temperature.

2.2.8.6 Detection and imaging

Antibodies were discarded and membrane was washed another three times for 7 minutes each in 1X TBS-T. Enhanced chemiluminescence (ECL)-HRP substrate SuperSignal™ West Dura (Thermo Fisher Scientific) was used for protein detection. Equal volumes of Luminol solution and buffered hydrogen peroxide solution were combined to a substrate working solution. The membrane was incubated with the substrate solution for 5 minutes, while ensuring constant wetting of the membrane and limiting exposure to light. Chemiluminescence was visualised by CCD imaging system ChemiDoc™ MP (Bio-Rad) using Image Lab™ analytical software. Optimal exposure time was acquired for imaging to avoid band signal saturation and allow observation of minor variations in density. Detected protein bands were quantified using ImageJ Software (National Institutes of Health, Bethesda, USA).

2.2.9 Statistical analyses [c]

Entry of clinical data was done with SPSS for Windows Version 25. Statistical analyses were performed with GraphPad Prism 8 (GraphPad Software Inc.) and *R* software version 3.3.1. Results were considered statistically significant at an unadjusted p-value less than 0.05. Visualisation was performed using GraphPad Prism 8 software.

Differences in population and clinical characteristics were assessed by chi-square (χ^2) test for categorial data or by Mann-Whitney *U* test for continuous variables. Median was reported with interquartile range (IQR) for continuous data.

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[c] For statistical analyses, the author was partly supported by the biostatistician of the research group.

Gene expression data derived from qPCR were analysed using comparative quantitation algorithms. Mean C_t values were calculated for all technical replicates in qPCR. For comparative analysis, ΔC_t was calculated as the difference of mean C_t values between the target gene (T) and the reference control gene 18S for each individual sample (i.e. ID) and stimulating condition (mean $\Delta C_{t\,(T)}$ = mean $C_{t\,(T)}$ − mean $C_{t\,(18S)}$). Differences in target gene expression (ΔC_t comparison) between phenotypes (AA vs. HC) or exposure groups (F vs. NF) were assessed by parametric Welch's t test, not assuming equal standard deviations in the groups. For graphical representation, mRNA data were expressed as $2^{-\Delta Ct}$ values and plotted in bar charts with means and 95% confidence interval (CI). Gene expression changes upon stimulation were reported as fold change (fc) of expression compared to the unstimulated condition, expressed as $2^{-\Delta\Delta Ct\,(T)}$. Using the $\Delta\Delta C_t$ method, mRNA expression data from stimulated samples (s) were compared with both the unstimulated condition (u) and the reference gene 18S ($\Delta\Delta C_{t\,(T)} = \Delta C_{t\,(T,\,s)} - \Delta C_{t\,(T,\,u)}$). $\Delta\Delta C_t$ scale ($\log_2$ fold change) was used for statistical calculations as well as plotting purposes. Statistical significance of stimulation effects was assessed by paired t test.

Gene correlation analysis was performed using nonparametric spearman correlation.

Concentrations of endotoxin in farm dust samples as well as protein in sample cell lysates were quantified by interpolation from linear standard curves. Linear regression analysis was applied to define the function of the standard curves based on absorbance measures of standard samples at given concentrations.

Protein expression assessed in Western Blot was quantified by densitometric measurement using ImageJ Software (National Institutes of Health). Absolute density values from both target (TNFAIP3) and reference control (β-actin) protein bands were normalised within an individual sample (i.e. across stimulating conditions of a single sample ID). Therefore, each band's density value was divided by that of the band with the highest value in the respective sample. Relative TNFAIP3 expression was defined as the ratio of normalised TNFAIP3 protein expression related to the normalised expression of β-actin, expressed as arbitrary units (A.U.) and displayed as mean values with 95% CI. Differences in protein expression between phenotypes were assessed by Welch's t test, and significance of stimulating effects was determined using paired t test.

Due to the exploratory nature of the study, with the principal objective of pattern detection, no adjustment for multiple testing was applied, and results should therefore be interpreted in a descriptive manner.

3 Results

3.1 Immune regulation in school-aged children with allergic asthma

3.1.1 CLARA/CLAUS study population

3.1.1.1 Enrolment of allergic asthmatic (AA) and healthy control (HC) children

In order to study immune regulation of steroid-naive children with allergic asthma (hereinafter 'AA') as compared to their healthy peers ('healthy controls', HC), a new study population was recruited for this work. A total of 48 children, aged 4 to 14 years, were enrolled within the CLARA/CLAUS[294] project. All study participants underwent clinical examination, blood was taken from a peripheral vein, and parents were asked to complete a detailed questionnaire. A doctor's diagnosis, as well as questionnaire-data, clinical assessment, blood parameters and lung function testing were employed for the definition of cases (AA) and controls (HC). A number of 4 samples were excluded from the analyses for technical reasons. One child with the phenotype of non-allergic asthma (n = 1) was excluded, resulting in a final number of 43 samples with complete epidemiologic and immunologic data to be investigated (**Figure 10)**. Findings that were reported in this work comprise data from 19 cases with AA and 24 HC.

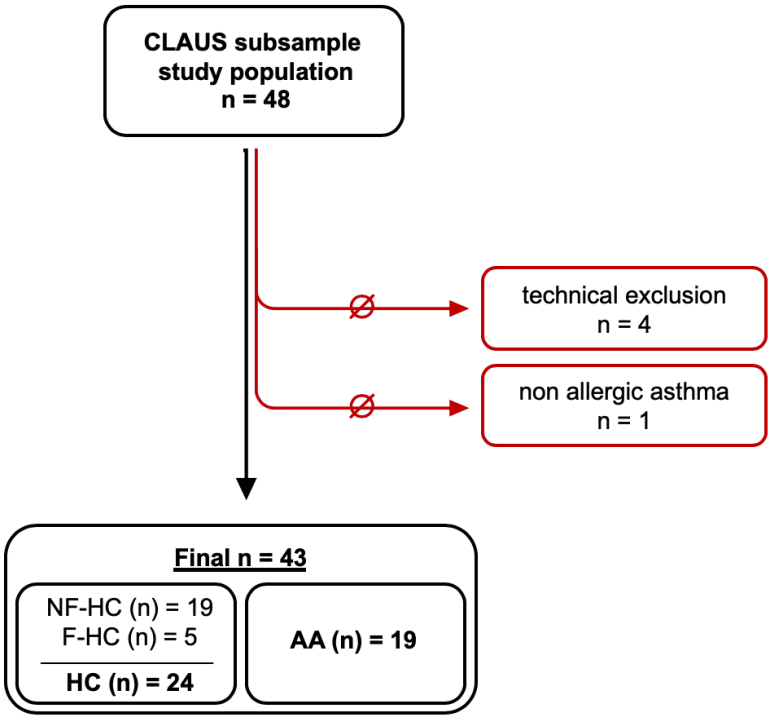

Figure 10. Enrolment flow chart CLARA/CLAUS study subsample.
After exclusion of samples, complete epidemiologic and immunologic data were available for a final number of 43 4-14-year-old children. *n,* number of cases*; HC*, healthy controls; *NF-HC*, healthy non-farm children*; F-HC*, healthy farm children; *AA*, steroid-naive patients with allergic asthma.

It is noteworthy that for additional immunological studies in farm children (see **3.3**), a subsample of 5 healthy children born and raised in a farming environment (hereinafter farm children, 'F-HC' - as opposed to healthy non-farm children, 'NF-HC', see **Figure 10**) was recruited.
The enrolment process as well as inclusion and exclusion criteria for participants were described in detail in the methods section **2.2.2.1** of this thesis.

3.1.1.2 Epidemiological and clinical characteristics of the study population

Based on the data derived from questionnaires, medical examination and from blood and lung function tests, the two study groups (HC and AA) were further characterised (**Tables 4-7**).

Demographic data and family characteristics of the study population were described in **Table 4**. Healthy controls (n = 24) and allergic asthma patients (n = 19) showed no significant differences in distribution of age and body-mass-index. Gender-distribution, however, was slightly unbalanced between the groups, with lower female proportions in the group of children with allergic asthma (36.8% vs. 58.3% female children). As differences in childhood asthma prevalence are known to be gender-related, analyses stratified for gender were conducted, but did not reveal different findings.
Analysed questionnaire-data provided information about the children's atopic conditions, namely hay fever, atopic dermatitis and food allergy as well as about parental history of atopy and asthma, socioeconomic status, number of siblings, and parental indoor smoking habits, among others. AA and HC differed significantly in all three atopic diseases with considerably higher proportions in patients with allergic asthma ($p \leq 0.002$; **Table 4**). The two groups did not differ significantly regarding the number of older siblings and indoor smoking at home.
It is noteworthy that maternal asthma and hay fever in particular were reported more often in the AA compared to the HC group ($p = 0.04$ and $p = 0.0007$, respectively). This was not seen for paternal atopy. Likewise, higher parental educational qualification was observed among AA children, with significant differences for maternal education ($p = 0.0007$). Despite potential confounding effects of maternal atopy[319,320] and high educational level, the limited sample size in the study groups did not allow for valid confounding analyses. Thus, only unadjusted data were presented within this work.

Neither AA nor HC displayed positive markers of ongoing or recent infection, as indicated by low levels of C-reactive protein (CRP) and normal white blood cell count (WBC) (**Table 5**). Neutrophils, lymphocyte and monocyte proportions of WBC did not differ between the groups and were within normal range.

Children with allergic asthma demonstrated typical clinical characteristics of allergic disease. Eosinophil count (**Table 5**) and serum levels of both total and specific IgE (**Table 6**) were found to be elevated in AA and significantly increased compared to the healthy reference group.

Table 4. *Demographic data and socioeconomic characteristics of the study population.*

Parameter	HC	AA	*P* value
n	24	19	
Female sex, n (%)	14 (58.3)	7 (36.8)	0.16
Age (y), median (IQR)	10.7 (10.4 – 12.6)	10.5 (9.3 – 11.4)	0.35†
BMI (kg/m^2), median (IQR)	17.4 (15.9 – 21.5)	17.3 (15.6 – 20.0)	0.54†
Doctor's diagnosis of hay fever (ever), n (%)	0 (0.0)	12 (63.2)	**< 0.0001***
Doctor's diagnosis of atopic dermatitis (ever), n (%)	0 (0.0)	11 (57.9)	**< 0.0001***
Food allergy, n (%)	0 (0.0)	6 (31.6)	**0.002***
Older siblings, n (%)	13 (54.2)	6 (31.6)	0.14
Smoking at home, n (%)	2 (8.3)	2 (10.5)	0.81
Maternal diagnosis of asthma (ever), n (%)	0 (0.0)	3 (15.8)	**0.04***
Maternal diagnosis of hay fever (ever), n (%)	1 (4.2)	9 (47.4)	**0.0007***
Maternal diagnosis of atopic dermatitis (ever), n (%)	2 (8.3)	1 (5.3)	0.67
Paternal diagnosis of asthma (ever), n (%)	4 (16.7)	3 (15.8)	0.95
Paternal diagnosis of hay fever (ever), n (%)	8 (33.3)	3 (15.8)	0.19
Paternal diagnosis of atopic dermatitis (ever), n (%)	0 (0.0)	2 (10.5)	0.09
Maternal education			**0.0007***
Elementary or middle school, n (%)	13 (54.2)	1 (5.3)	
High school or university, n (%)	11 (45.8)	18 (94.7)	
Paternal education			0.06
Elementary or middle school, n (%)	11 (45.8)	4 (21.1)	
High school or university, n (%)	11 (45.8)	15 (78.9)	
Missing/Other, n (%)	2 (8.3)	0 (0.0)	

HC, healthy controls; *AA*, patients with allergic asthma; *n,* number of cases; %, percentage of group total; *y*, years; *BMI*, body-mass-index. Values for continuous variables represent *median* and interquartile range (*IQR*). *P* values reflect χ^2 test for proportions, or the Mann-Whitney *U* test (†) for continuous variables, respectively. Tests were applied for non-missing data. *$p < 0.05$ (boldface). Total number of subjects might differ slightly for some parameters due to data availability.

Table 5. *Laboratory parameters of HC and AA.*

Parameter	HC (n = 24) *median*	AA (n = 19) *median*	*P* value
CRP (mg/L) (IQR)	0.1 (0.1 – 0.1)	0.1 (0.1 – 0.1)	--
WBC (cells x 10^9/L) (IQR)	5.95 (4.93 – 7.75)	6.80 (5.50 – 7.90)	0.32
Neutrophils (%) (IQR)	43.5 (37.5 – 52.0)	41.0 (36.0 – 48.0)	0.35
Lymphocytes (%) (IQR)	43.8 (35.5 – 48.8)	40.0 (37.0 – 47.0)	0.52
Monocytes (%) (IQR)	8.0 (7.0 – 8.3)	8.0 (7.0 – 10.0)	0.71
Eosinophils (%) (IQR)	3.0 (1.8 – 5.0)	9.0 (6.0 – 10.0)	**0.0003***

n, number of cases; *CRP*, C-reactive Protein; *WBC,* white blood cell count; *Neutrophils, Lymphocytes, Monocytes, Eosinophils*: percentage (%) of WBC. Values represent *median* and interquartile range (*IQR*). *P* values reflect the Mann-Whitney *U* test. Tests were applied for non-missing data. *$p < 0.05$ (boldface). Total number of subjects might differ slightly for some parameters due to data availability.

Specific IgE levels were dichotomised at the detection limit of 0.35 IU/ml (i.e. SCI 1), and three categories of sensitisation were defined: Positive specific IgE to *perennial* allergens (*dermatophagoides pteronyssinus, dermatophagoides farinae*, cat epithelium, horse epithelium, dog epithelium, *Alternaria alternata*); positive IgE to *seasonal* allergens (birch pollen, hazel, grass pollen mix and mugwort, timothy grass, plantain); and positive IgE to *food* allergens (hazelnut, peanut, hen's egg, cow's milk, carrot, wheat flour, soybean). Most of the AA within this study (73.7% of total) were sensitised to perennial allergens. Four healthy subjects showed a positive specific IgE to a single allergen (**Table 6**) without any clinical symptoms of allergic disease.

Table 6. *Sensitisation to allergens in HC and AA.*

Parameter	HC (n = 24)	AA (n = 19)	*P* value
Total serum IgE (IU/ml), median (IQR)	100.0 (45.5 – 206.0)	345.0 (234.3 – 817.0)	**0.0003*†**
Specific IgE (≥ 0.35 IU/ml)			
to perennial allergens, n (%)	2 (8.3)	14 (73.7)	**< 0.0001***
to seasonal allergens, n (%)	1 (4.2)	11 (57.9)	**< 0.0001***
to food allergens, n (%)	1 (4.2)	5 (26.3)	**0.04***

IU, international units; *n,* number of cases; %, percentage of group total. Total IgE values represent *median* and interquartile range (*IQR*). Specific IgE levels were dichotomised at the detection limit of 0.35 IU/ml. *P* values from Mann-Whitney *U* test (†) for continuous variables, or χ^2 test for proportions. *$p < 0.05$ (boldface).

Children who met the criteria of asthma according to the GINA guidelines[304], and had not taken steroids within the 14 days prior to blood withdrawal, were enrolled in the study. Lung function data of asthmatic children were depicted in **Table 7**. In the CLARA study population lung function parameters were shown to be within normal range in healthy control subjects[294]. In the specific subgroup presented in this work, lung function testing was not feasible due to logistic reasons.

Asthmatic patients in this study presented features of expiratory airflow obstruction, as demonstrated by reduced forced expiratory volume in one second (FEV_1) together with a reduced mean FEV_1/FVC ratio as compared to predicted reference population values (adjusted for age, sex, height and ethnicity using the 2012 Global Lungs Initiative (GLI) equations[303]). Decreased values for mid-expiratory flow at 50% (MEF_{50}, 75.6% predicted; **Table 7**) and especially at 25% of FVC (MEF_{25}, 69.5% predicted) particularly demonstrate affection of the small airways in the asthmatic children. Negative z-score values for FEV_1 before bronchodilation ($zFEV_{1\ pre}$, -0.88) and for FEV_1/FVC ($zFEV_1$/FVC, -1.11) in particular indicate asthmatic children's impaired lung function performance as compared to the reference population. Z-scores define how many standard deviations the individual measured value is from the predicted mean. A positive z-score indicates an individual's value above the population mean, while a negative z-score indicates a value below the mean. The majority of AA patients demonstrated positive reversibility testing, as illustrated by a significant mean increase in FEV_1 of 18.5% after inhalation of rapid-acting bronchodilator (BD) salbutamol[304].

Table 7. *Descriptive measures of lung function in AA.*

Parameter	***mean***	***95% CI***
FEV_1 (% predicted)	89.5	(81.5 to 97.5)
FEV_1/FVC (% predicted)	90.4	(84.6 to 96.1)
MEF_{50} (% predicted)	75.6	(61.3 to 90.0)
MEF_{25} (% predicted)	69.5	(48.6 to 90.3)
$zFEV_1/FVC_{\ pre}$	-1.11	(-1.73 to -0.50)
$zFEV_{1\ pre}$	-0.88	(-1.56 to -0.20)
$zFEV_{1\ post}$	-0.12	(-0.91 to 0.66)
Bronchodilator response ($\Delta\ FEV_1$/ $FEV_{1\ pre}$) x 100 (%)	18.5	(2.0 to 34.9)

Lung function parameters from study patients with allergic asthma (n = 19), presented as mean values with 95% CI. Total number of subjects might differ slightly for some parameters due to data availability.

Abbreviations: FEV_1, forced expiratory volume in one second; *FVC,* forced vital capacity; MEF_{50} / MEF_{25}, mid-expiratory flow rate at 50 % (or 25%, respectively) of FVC; *z[...],* z-score for specific parameter, dimensionless quantity; $FEV_{1\ pre}$, FEV_1 before bronchodilation; $FEV_{1\ post}$, FEV_1 after bronchodilation; $\Delta\ FEV_1$, $FEV_{1\ post}$ - $FEV_{1\ pre}$

The subgroup presented in this thesis was comparable to the CLARA/CLAUS cohort[294] regarding distribution of sex, age, the number of older siblings, indoor smoking, breastfeeding, and parental atopy and asthma. Leukocyte proportions including eosinophils were found to be similar between the two cohorts. HC in the present study population had higher IgE-levels compared to HC of the whole cohort (data not shown).

3.1.1.3 Differential baseline expression levels of target genes in PBMCs of study participants

Baseline mRNA expression levels of *TLR4, MyD88, TRAF6, TNFAIP3, TNIP2, TAX1BP1, MALT1, CD80, CD86, CD274*, and *PDCD1* were measured in both AA and HC children's PBMCs (total n=43). Optimal qPCR performance was achieved throughout all experiments and uniform sampling conditions were ensured for valid comparisons (see **2.2.7.3**). Target gene ΔC_t values from qPCR analysis express gene expression values, relative to the reference control gene *18S*. In order to demonstrate dispersion of data for each gene in the human immune cell samples, ΔC_t values were displayed in scatter dot plots in **Figure 11**, representing data points with mean ΔC_t values and their range from minimum to maximum. Since a lower ΔC_t value corresponds to a higher expression level, the y-axis was displayed in reverse direction, which may facilitate graphical interpretation of the data.

As can be seen in **Figure 11**, all genes were expressed and detected in qPCR in children's PBMC samples. *TNFAIP3* was the gene at highest, *PDCD1* at lowest expression among all genes. Individual values scattered widely in some genes (e.g. *TLR4, MyD88, CD274* and *PDCD1*), while other genes, particularly those involving *TNFAIP3* and its regulatory molecules (*TNIP2*, *TAX1BP1*, *MALT1*), were confined within a narrow expression range.

PDCD1 was the only gene not detectable in a limited number of samples (n = 2). A C_t value below the detection limit (i.e. 40 PCR cycles) did not occur in any of the other target genes. Since a considerable amount of data was censored for *PDCD1,* and overall expression was very low-level in all children, *PDCD1* was excluded from further statistical analyses, and data for this gene were not reported in this work. Detailed information on sample exclusion is provided in **Table E1,** which can be found in **the appendix** of this thesis.

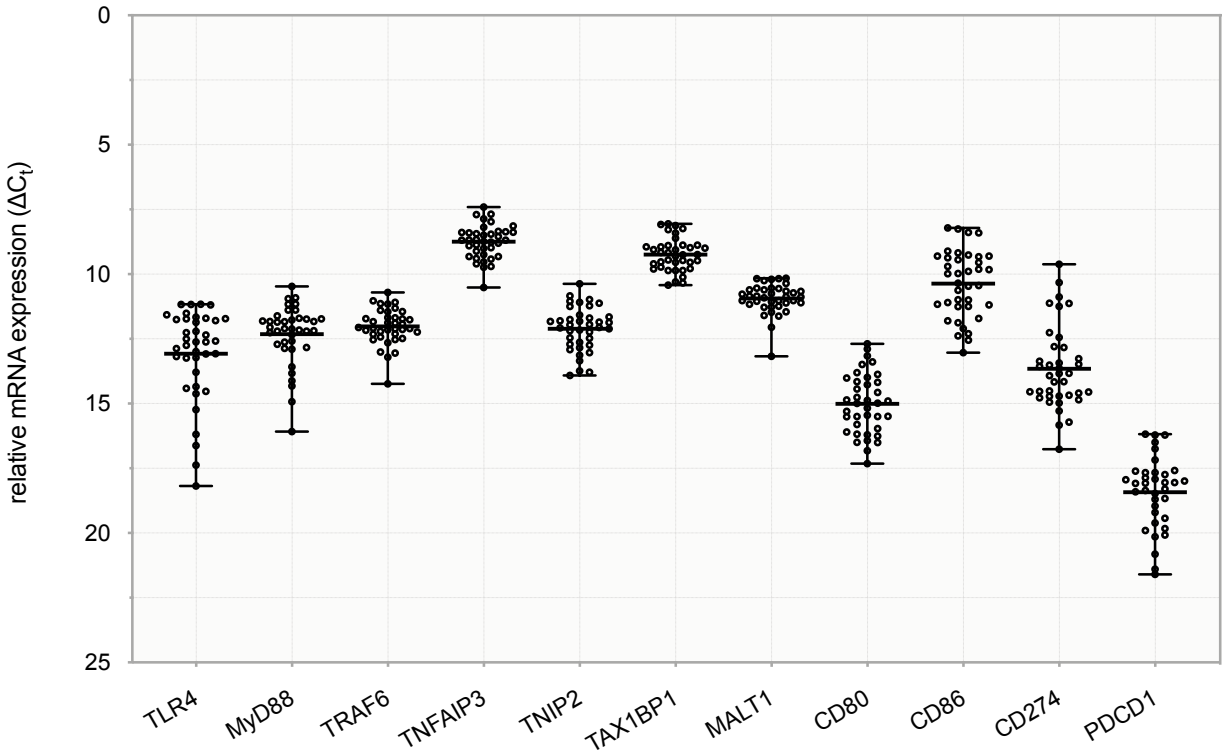

Figure 11. Overview of baseline mRNA expression levels of all target genes in the CLAUS subsample. Comparative expression levels, relative to 18S (ΔC_t values), in unstimulated peripheral blood mononuclear cells (PBMCs) of all study patients, aged 4 to 14 years (AA and HC, n=43 for TNFAIP3; n=38 for all other genes except PDCD1 (n=36)), as assessed by quantitative real time PCR (qPCR). Scatter dot plots show single sample data points and mean ΔC_t values with minimum and maximum range. Higher values reflect lower expression (y-axis in reverse direction).

3.1.2 Inflammatory innate immune phenotype in AA children

The first aim of this work was to investigate whether TNFAIP3 gene and protein expression and the expression of genes related to NF-κB signalling and dendritic cell maturation was dysregulated in PBMCs of children with allergic asthma (AA) compared to healthy control subjects (HC).

mRNA expression of the selected target genes was assessed at baseline (i.e. in cultured, unstimulated PBMCs) and data was analysed for differences between the two phenotypes. Statistical significance was determined using Welch's t test. The following graphs show relative mRNA expression data ($2^{-\Delta Ct}$ values), displayed in bar charts indicating mean values ± 95% confidence interval (CI).

3.1.2.1 Increased *TLR4* and *MyD88* expression in AA

In unstimulated cells, asthmatic children displayed significantly increased *TLR4* expression compared to healthy controls ($p < 0.001$; **Figure 12, A**). Likewise, expression of its adaptor molecule *MyD88* was significantly elevated ($p = 0.03$; **Figure 12, B**), whereas no difference between AA and HC was detected for *TRAF6* expression **(Figure 12, C)**.

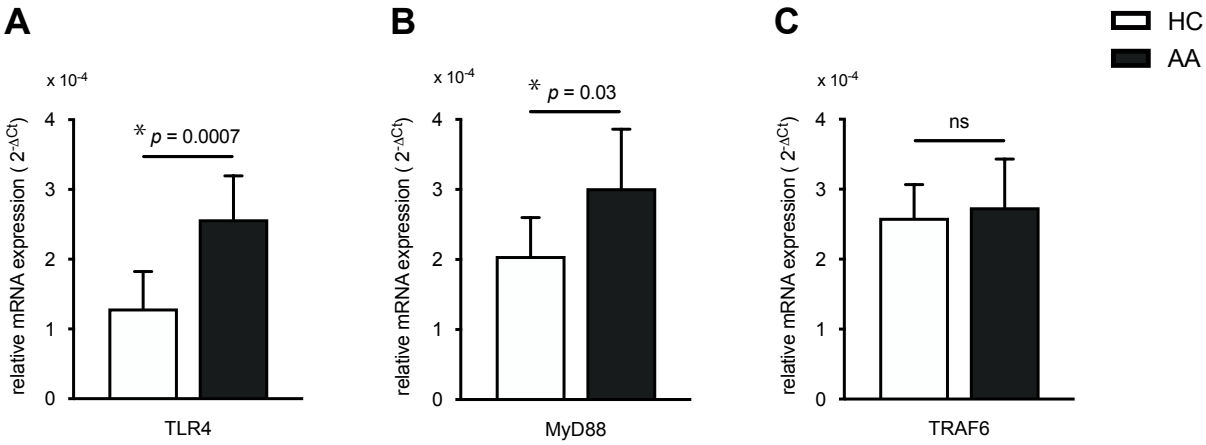

Figure 12. Expression of TLR4-pathway genes in HC and AA.
(A) TLR4, **(B)** MyD88 and **(C)** TRAF6 baseline mRNA expression levels, as measured by qPCR in unstimulated PBMCs of HC (n=22) and AA (n=16). Expression relative to 18S, displayed as $2^{-\Delta Ct}$ mean values, error bars indicate 95% CI. *P* values reflect Welch's t test for ΔCt comparison between HC and AA. *$p < 0.05$; *ns*, not significant.

3.1.2.2 Decreased TNFAIP3 gene and protein expression in AA

Under unstimulated conditions, AA children displayed significantly decreased anti-inflammatory *TNFAIP3* gene expression compared to their healthy peers ($p = 0.02$; **Figure 13, A**). Of note, stratified analysis for comorbid allergic diseases (i.e. hay fever, atopic dermatitis and food allergy) revealed significantly ($p = 0.035$) decreased *TNFAIP3* gene expression levels in children with asthma and additional hay fever (n = 12) compared to children suffering from allergic asthma only (n = 7) (ΔC_t 9.41 (+/- 0.76) vs. ΔC_t 8.74 (+/- 0.50)). However, no similar effect was found for asthmatic children with combined food allergy or atopic dermatitis, respectively[321].

To confirm lower TNFAIP3 levels of AA on protein level, Western Blot (WB) was performed in a subsample of 11 HC and 14 AA children. The two WB subsample groups did not differ significantly in age distribution (HC 11.2 years (IQR 10.3 – 12.6) vs. AA 10.4 years (IQR 7.2 – 11.7), $p = 0.30$) nor in terms of the potential confounders mentioned above. Clinical and population characteristics were

comparable with the whole groups (not shown). Consistent with the findings for *TNFAIP3* gene expression, significantly decreased levels of TNFAIP3 protein (82 kDa) were detected in AA patients when compared to the HC group *(p* = 0.03; **Figure 13, B)**.

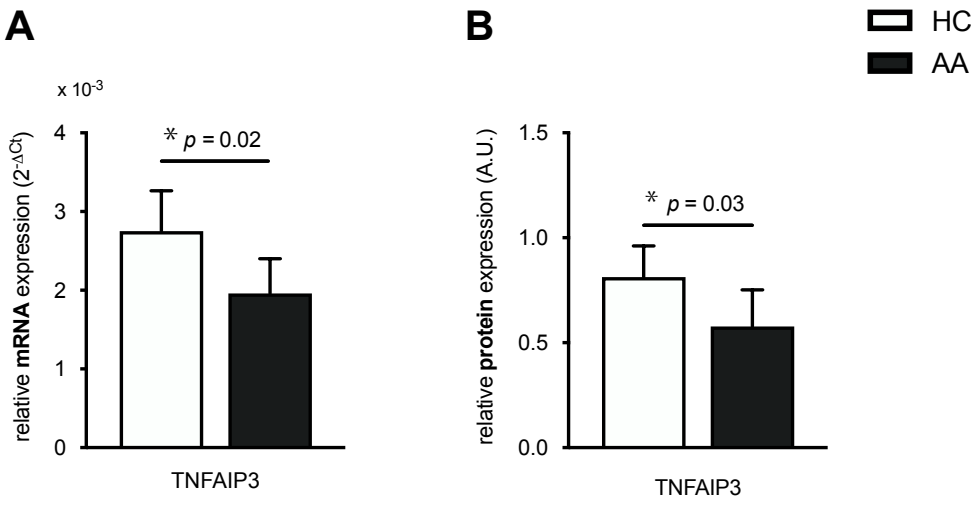

Figure 13. TNFAIP3 mRNA and protein expression in HC and AA.
(A) TNFAIP3 baseline gene expression levels, as measured by qPCR in unstimulated PBMCs of HC (n=24) and AA (n=19). Expression relative to 18S, displayed as $2^{-\Delta Ct}$-values. **(B)** Protein levels in HC (n=11) and AA (n=14), as analysed by Western Blot. Expression relative to β-Actin. *A.U.*, arbitrary units. Mean values with 95% CI. *P* values reflect Welch's t test. **p* < 0.05.

TNFAIP3 protein expression values scattered widely within the group of asthmatics, indicating great heterogeneity (**Figure 14**). While some patients presented with manifestly lacking protein, there were also a few with TNFAIP3 levels that were comparable to those seen in healthy subjects.
Remarkably, TNFAIP3 protein bands of two different sizes were visible on the immunoblots. A broad and stronger band was detected at the predicted molecular weight of 82 kDa. Additionally, a TNFAIP3 band that migrated more slowly in the gel (~ 100 kDa size) was observed in all measured PBMC samples (not shown here, see **Figure E1, appendix**). All analyses in this work refer to TNFAIP3 protein of 82 kDa size (as shown in **Figures 13, B and 14**).

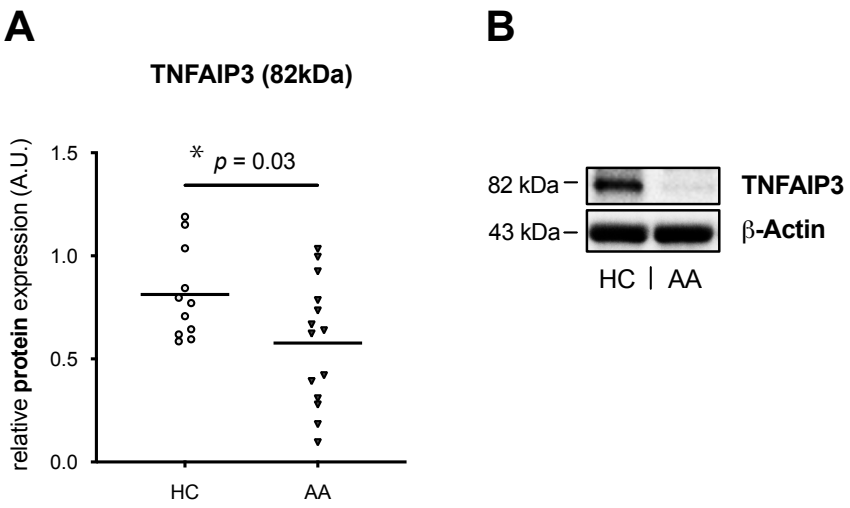

Figure 14. Scatter plot of TNFAIP3 protein expression.
Protein levels, as analysed by Western Blot in unstimulated PBMCs of HC (n=11) and AA (n=14). **(A)** Scatter plot with individual sample values and means of TNFAIP3 protein expression of predicted 82 kDa size. Results from densitometric analysis of protein bands. Normalized protein expression, relative to β-Actin. *A.U.*, arbitrary units. **(B)** Representative immunoblot membrane-cut-out. Molecular weight in kDa. β-Actin was used as loading-control. *P* values reflect Welch's t test. *$p < 0.05$.

To further investigate TNFAIP3-related pathways, gene expression of TNFAIP3 regulatory molecules, namely *TNIP2*, *TAX1BP1* and *MALT1* was analysed.

A trend for higher *TNIP2* expression was observed in AA compared to HC ($p = 0.06$; **Figure 15, A**). Neither the gene *TAX1BP1* nor *MALT1* appeared to be differentially expressed in AA and HC under unstimulated conditions (**Figure 15, B and C**).

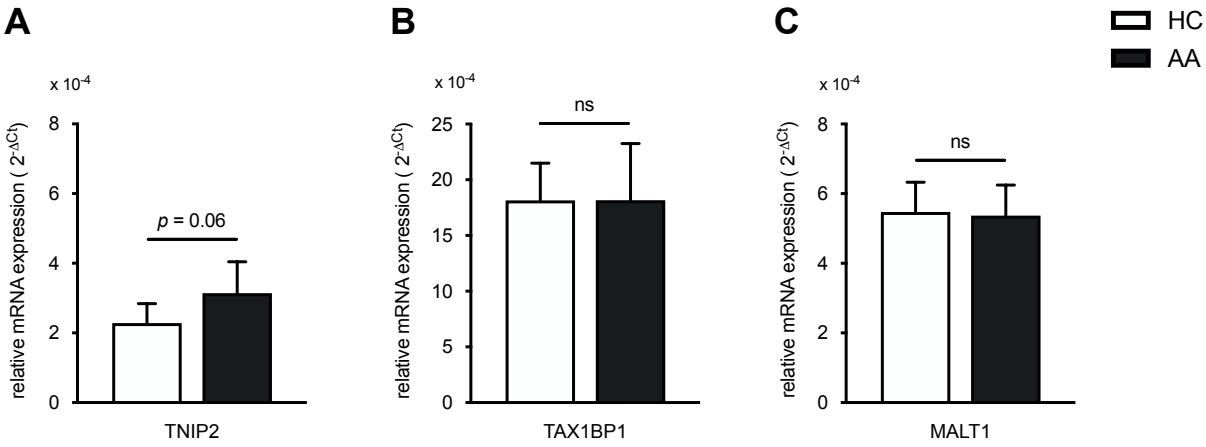

Figure 15. Expression of TNFAIP3 regulatory genes in HC and AA.
(A) TNIP2, **(B)** TAX1BP1 and **(C)** MALT1 baseline mRNA expression levels, as measured by qPCR in unstimulated PBMCs of HC (n=22) and AA (n=16). Expression relative to 18S, displayed as $2^{-\Delta Ct}$ mean values with 95% CI. *P* values reflect Welch's t test. *$p < 0.05$; *ns*, not significant.

3.1.2.3 Alterations in markers of dendritic cell activation in AA

In this study population, immune cells of AA expressed significantly lower levels of *CD80* ($p = 0.002$) than those of HC (**Figure 16, A**). In contrast, no significant effect was observed for the co-stimulatory molecule *CD86* **(Figure 16, B**). Levels of *CD274* were trend-wise lower ($p = 0.15$) in AA compared to the HC group (**Figure 16, C**). Since the function of DCs may depend on the net co-regulatory signal provided by co-stimulatory and co-inhibitory molecules, their expression was also determined relative to one another. In this context, the expression of *CD86* relative to *CD80*, i.e. *CD86/CD80* ratio, was significantly higher in AA compared to HC ($p = 0.02$; **Figure 16, D**).

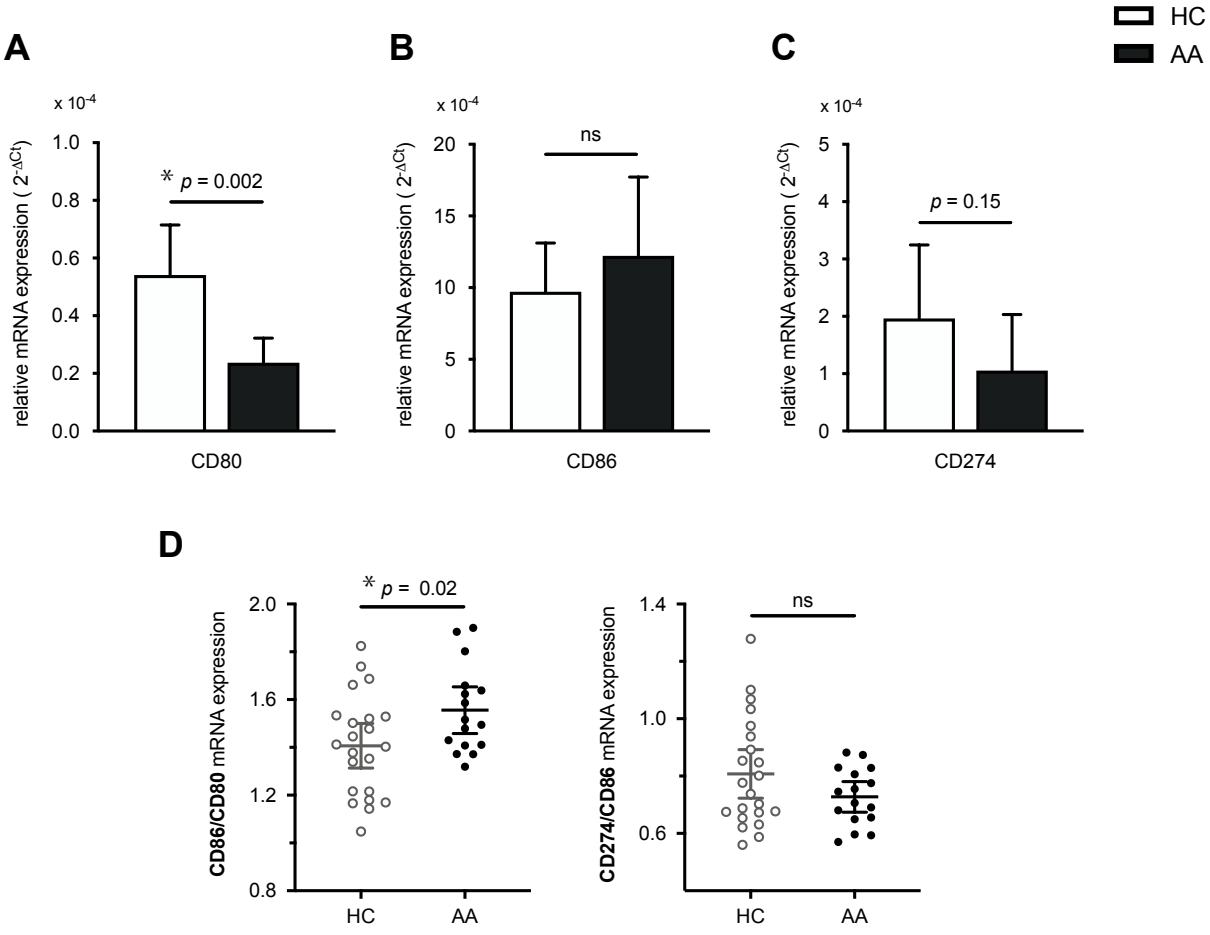

Figure 16. Expression of dendritic cell associated genes in HC and AA.
(A) CD80, **(B)** CD86, **(C)** CD274 baseline mRNA expression levels and **(D)** respective expression ratios, as measured by qPCR in unstimulated PBMCs of HC (n=22) and AA (n=16). Expression relative to 18S, displayed as $2^{-\Delta Ct}$ mean values with 95% CI. CD86/CD80 and CD274/CD86 expression ratios were built by relating ΔC_t values of CD80 to CD86 and of CD86 to CD274, respectively. *P* values reflect Welch's t test. *$p < 0.05$; *ns*, not significant.

3.1.2.4 Overview TNFAIP3, NF-κB pathway and DC- related gene expression in AA vs. HC

Table 8 may outline the findings of chapter **3.1.2**, reviewing baseline gene expression patterns of AA compared to HC children.

In summary, allergic asthmatic children showed significantly increased expression of *TLR4*-related genes, while *TNFAIP3* as a negative regulator of NF-κB mediated inflammation was significantly decreased in these children when compared to healthy subjects. Likewise, *CD80* as marker of dendritic cell activation was decreased in asthmatic children.

Table 8. *Differential baseline expression of all target genes among AA and HC children.*

Gene	Stimulus	mRNA expression level	
		AA vs. **HC**	***P* value**
TLR4	unstim.	↑ ***	0.0007
MyD88	unstim.	↑*	0.027
TRAF6	unstim.	↔	0.688
TNFAIP3	unstim.	↓*	0.015
TNIP2	unstim.	↑	0.061
TAX1BP1	unstim.	↔	0.784
MALT1	unstim.	↔	0.939
CD80	unstim.	↓ **	0.002
CD86	unstim.	↔	0.428
CD274	unstim.	↔	0.153

mRNA expression levels of 10 genes were measured by qPCR in unstimulated PBMCs. Comparison of gene expression between HC (n=22 and n=24 for TNFAIP3) and AA (n=16 and n=19 for TNFAIP3). ↑ up-arrow (blue) represents elevated mRNA expression in AA vs. HC, ↓ down-arrow (red) indicates decreased mRNA expression in AA vs. HC. Coloured arrows without asterisks indicate trend-wise effects. ↔ indicates no difference. Statistical significance of differences is indicated by the number of asterisks. *P* values from Welch's t test. *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

3.1.2.5 Strong correlation of TNFAIP3 with expression of the other innate genes in AA

As TNFAIP3 is considered to be one of the key players within this concept of 'immunological imbalance' in childhood allergic asthma, its correlation with the other genes that were determined in the children's PBMCs was assessed both in HC and AA.

As depicted in **Figure 17**, the expression of all target genes was highly correlated with *TNFAIP3* expression in AA, whereas only a weak or no linear relationship was visible in HC. TNFAIP3's regulatory molecules *TNIP2*, *TAX1BP1* and *MALT1*, and also the DC markers *CD80, CD86, CD274*,

and *TRAF6* in particular ($p = 0.0003$) were all positively correlated at a significant level with *TNFAIP3* expression in the AA group. Also, *TLR4* and *MyD88* levels showed borderline significance in AA ($p = 0.06$, and $p = 0.09$, respectively; data from statistical tests not shown).

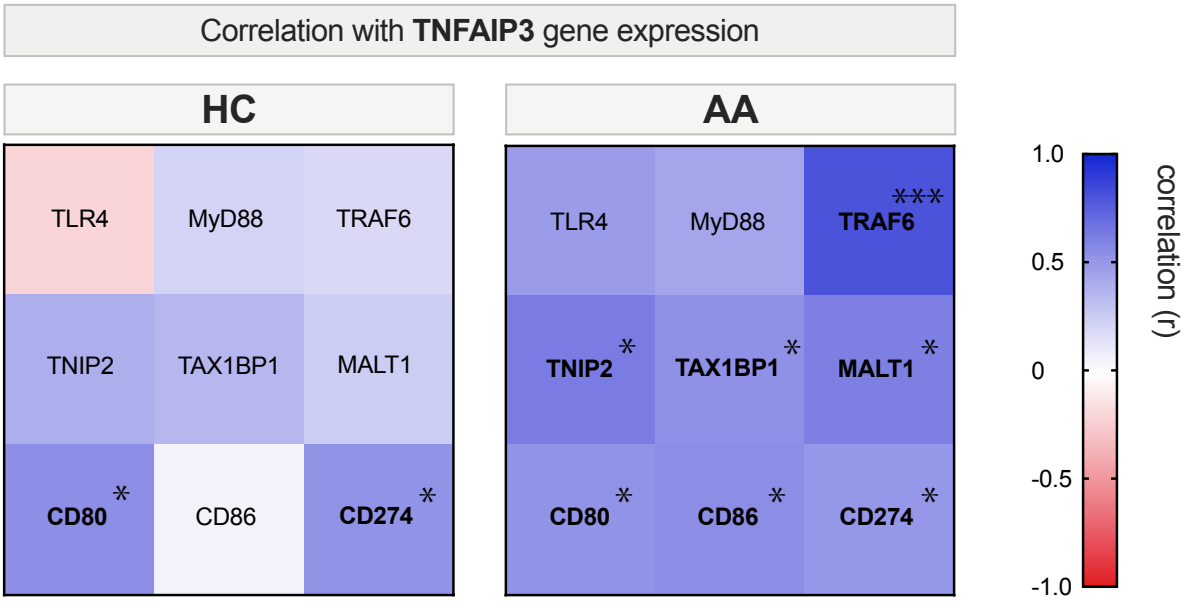

Figure 17. Correlation of all target genes with TNFAIP3 expression, stratified by clinical phenotype. Correlation of mRNA expression of TLR4, MyD88, TRAF6, TNIP2, TAX1BP1, MALT1, CD80, CD86 and CD274 with TNFAIP3 mRNA expression in healthy controls (HC, n=20) and allergic asthmatic patients (AA, n=16). Colour code of gene boxes reflects spearman correlation coefficient values (r). Blue colour indicates a positive correlation, red colour a negative correlation. Colour saturation reflects the strength of correlation. Statistical significance of correlation is indicated by the number of asterisks. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (boldface).

3.2 Studying asthma-protective farm exposure *in vitro* – immunomodulatory effects of farm dust

In mouse models, TNFAIP3 deficiency was reported to be a risk factor for disease, whereas normal or elevated levels were ascribed to have protective effects. Against this background, the present study **aimed to assess whether *in vitro* exposure to dust extracts from 'asthma-protective' farming environments as well as lipopolysaccharide (LPS) could upregulate reduced TNFAIP3 levels in asthmatic children's PBMCs and whether it could counterbalance increased pro-inflammatory gene expression in these patients.**

3.2.1 Characterisation of farm dust extracts

Dust extracts that had been collected from 'asthma-protective' farming environments in Germany (G), Finland (Fi), China (Ch) and from the Amish population (Am) were used for stimulation of study patients' PBMCs. The concentration of endotoxin (LPS), known as a major compound of farm dust, was determined in dust extracts (G, Fi, Ch) by Limulus Amoebocyte Lysate (LAL) assay prior to the start of the experiments. Due to logistical reasons, LPS concentration of the Amish dust sample could not be measured.

Endotoxin (LPS) concentrations measured in German and Finnish dust samples exceeded the greatest value of the test standards by far and were significantly higher than those of the Chinese samples (mean values 1082 EU/ml for the G sample, and 887 EU/ml for Fi, vs. 0.17 EU/ml for Ch, respectively; see **Figure 18**).

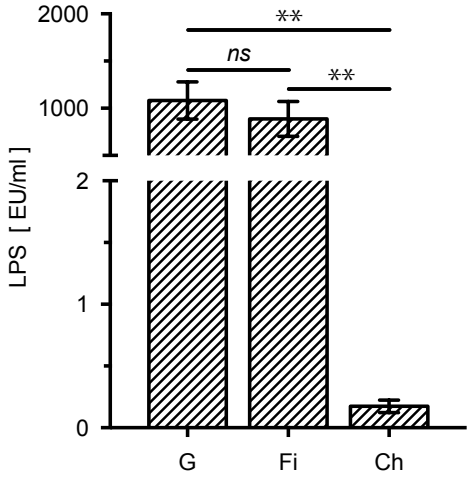

Figure 18. Endotoxin (LPS) concentration in farm dust extracts.
German (G), Finnish (Fi) and Chinese (Ch) farm dust. Different aliquots were used for each dust sample and assay. Concentration was measured by endpoint chromogenic Limulus Amoebocyte Lysate (LAL) assay. Mean values. Error bars indicate SEM. *P* values reflect Welch's t test. $*p < 0.05$, $**p < 0.01$; *ns*, not significant.

To further characterise the bacterial composition of the farm dust extracts, sequencing of bacterial 16S rRNA was performed in German and Finnish dust samples[d]. This revealed a high percentage of gram negative bacteria from the Proteobacteria phylum (90.1%), but also sequences from gram-positive Firmicutes (8.6%) and Actinobacteria (1%), respectively[321].

3.2.2 Anti-inflammatory immune response in AA and HC upon *in vitro* LPS and farm dust treatment

Changes in expression of the NF-κB signalling pathway genes and dendritic cell associated genes upon 24 hour-stimulation with LPS and the farm dust extracts were analysed.

3.2.2.1 Downregulated *TLR4* and *MyD88* expression

For *TLR4* and for its downstream molecules *MyD88* and *TRAF6* as well, a distinct pattern of mRNA-downregulation was observed upon treatment with all conditions except for Ch (**Figure 19, A-C**).
TLR4 level was significantly decreased, both in HC and in AA upon LPS and dust stimulation when compared to mRNA levels of the unstimulated (U) condition ($p < 0.0001$ for all stimulating conditions except Ch; **Figure 19, A**). Chinese dust treatment reduced *TLR4* expression only in the AA children, and showed overall the weakest effects. This pattern consistently continued in TLR4-adaptor *MyD88*, although effects in HC were not comparably strong to those in the AA children. Treatment with LPS and all dust samples significantly reduced *MyD88* expression in AA ($p < 0.001$ for all stimulating conditions except Ch; **Figure 19, B)**. Similar, yet weaker effects were visible for *TRAF6* in AA children, with significant downregulation upon G and Am stimulation ($p = 0.004$ and $p = 0.01$, respectively) and trend-wise effects for LPS and Fi ($p = 0.10$ and $p = 0.09$, respectively; **Figure 19**, **C**).

Overall, AA presented stronger responses to treatment than did HC. LPS and the dust extracts downregulated mRNA expression of both *TLR4* and *MyD88*, which was significantly, or at least trend-wise ($p \leq 0.10$), stronger in asthmatic patients compared to the healthy reference group (**Figure 20, A and B**). While *TLR4* expression changed impressively, *TRAF6* in particular was regulated within tighter limits, however significantly different between AA and HC (**Figure 20, C**).

In summary, asthmatics showed increased *TLR4* and adaptor *MyD88* gene expression compared to healthy controls in unstimulated cells, but expression was significantly downregulated upon *in vitro* stimulation with LPS and the farm dust extracts (G, Fi, Am).

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[d] 16S rRNA sequencing in the farm dust samples was not performed by the author herself, but by a member of the research group.

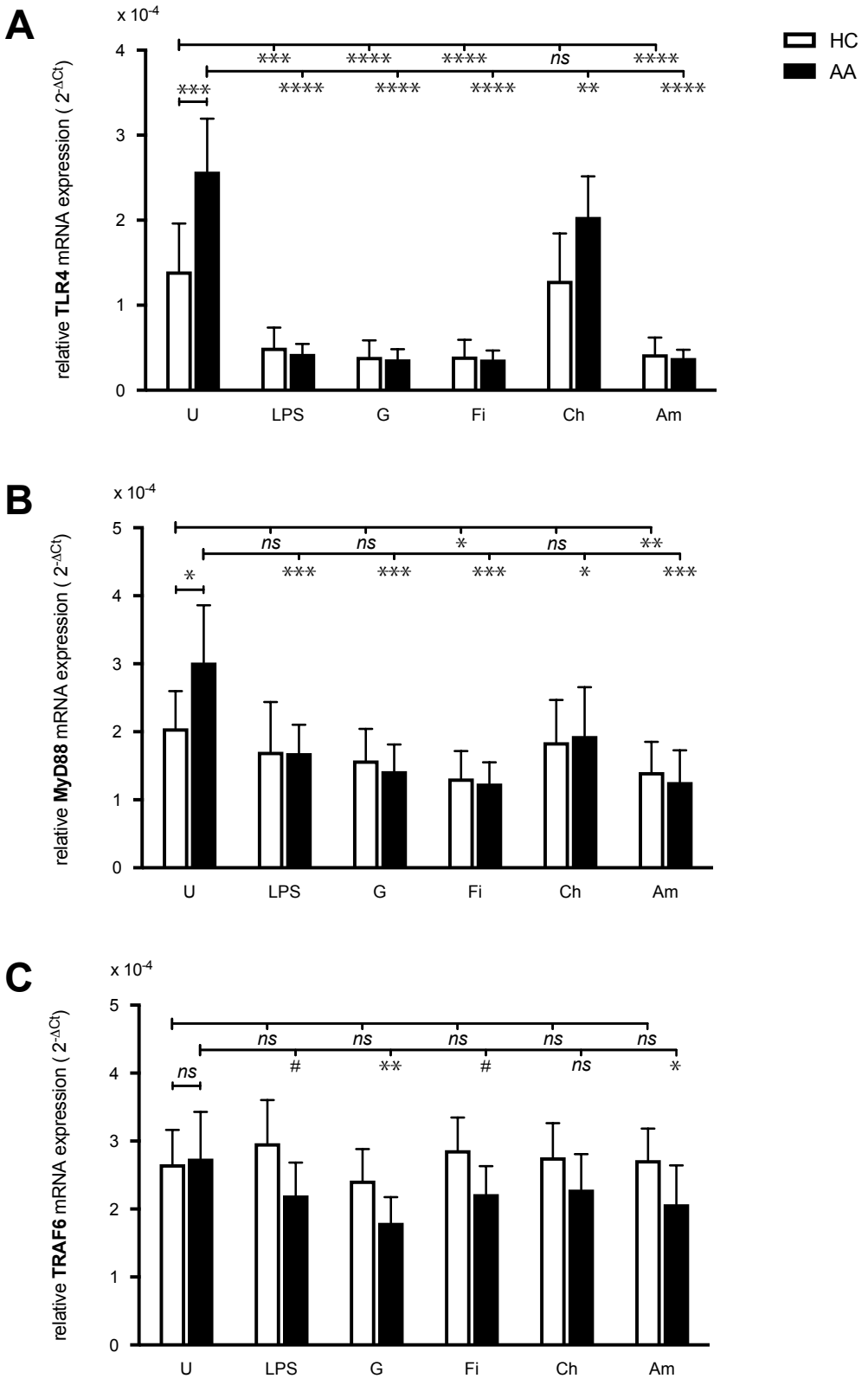

Figure 19. Effects of LPS and farm dust stimulation on expression of TLR4-pathway genes.
PBMCs of HC (n=22) and of patients with AA (n=16) were stimulated 24 hrs with either LPS [0.1µg/ml] or farm dust [40µg/ml] from Germany (G), Finland (Fi), China (Ch) and from the Amish people (Am). **(A)** TLR4, **(B)** MyD88 and **(C)** TRAF6 mRNA expression displayed as mean values ($2^{-\Delta Ct}$), error bars indicate 95% CI. *P* values from paired t test are indicated for comparisons of stimulated (LPS, G, Fi, Ch, Am) vs. unstimulated (U) condition in both AA and HC, or from Welch's t test for phenotype comparison (AA vs. HC) in unstimulated cells, respectively. # indicates trend-wise effects ($p \leq 0.10$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; *ns*, not significant.

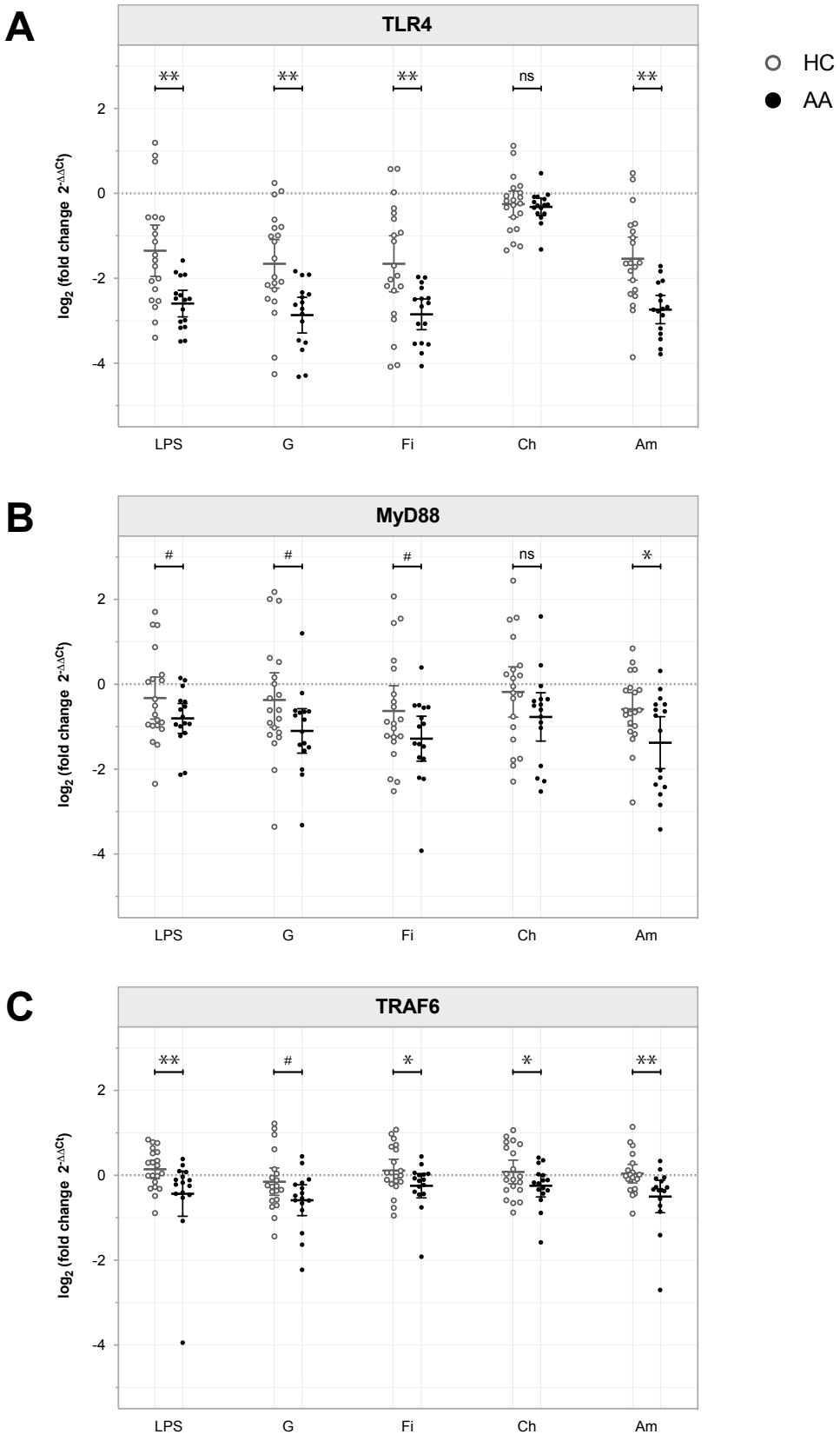

Figure 20. Fold change of TLR4-pathway gene expression upon LPS and farm dust stimulation.
(A) TLR4, **(B)** MyD88 and **(C)** TRAF6 gene expression fold change, compared to unstimulated conditions ($2^{-\Delta\Delta Ct}$, $\log_2$ scale), as assessed by qPCR in PBMCs of healthy control children (HC; n=22) and children with allergic asthma (AA; n=16) after 24 hrs stimulation with LPS or farm dust extracts from Germany (G), Finland (Fi), China (Ch) and from the Amish people (Am). Scatter dot plots represent single sample values with means and 95% CI. *P* values reflect Welch's t test for fold change comparison between HC and AA. # indicates trend-wise effects ($p \leq 0.10$). *$p < 0.05$, **$p < 0.01$; *ns*, not significant.

3.2.2.2 Upregulated TNFAIP3 gene and protein expression

Upon LPS stimulation, *TNFAIP3* mRNA expression was significantly increased in both HC and AA ($p < 0.001$; **Figure 21, A**). Although effects of the dust samples were not as strong as observed for LPS, treatment with the Finnish and the Amish sample significantly elevated *TNFAIP3* mRNA in AA children ($p = 0.03$, and $p = 0.005$, respectively), but not HC. In contrast, stimulation with farm dust samples from China significantly decreased *TNFAIP3* levels in PBMCs of both groups ($p = 0.004$ for HC and $p = 0.04$ for AA, respectively). No difference to the unstimulated condition was seen upon treatment with German dust extracts.

Western Blot analysis of TNFAIP3 confirmed findings on protein level (**Figure 21, B**), again with TNFAIP3 upregulation only seen in AA patients and again, with significant effects only for Fi and Am farm dust stimulation ($p = 0.02$ and $p = 0.04$, respectively). G and Ch stimulation demonstrated similar trend-wise effects in AA ($p = 0.09$ and $p = 0.08$, respectively). Of note, 24-hour stimulation with LPS did not affect TNFAIP3 protein expression in HC or in AA. Overall, no significant effects of any of the treatments were seen on TNFAIP3 protein levels in healthy children.

It is remarkable that, through stimulation with LPS and with the Finnish and Amish farm dust extracts, asthmatics reached TNFAIP3 levels comparable to those of healthy children under unstimulated conditions (**Figure 21, C**). This was true for both gene and protein expression. German farm dust stimulation also yielded a significant increase of TNFAIP3 protein to healthy levels (**Figure 21, C, right panel**).

A representative immunoblot membrane showing a single HC and AA sample with the different treatment conditions and TNFAIP3 and β-Actin protein bands is depicted in **Figure 21, D**.

Fold change of *TNFAIP3* mRNA expression following LPS and dust treatment did not differ between AA and HC (**Figure 22, left panel**) and levels appeared to be confined at a defined range. Slight upregulation was seen in all treatment conditions except Ch. However, there were significant differences between AA and HC regarding TNFAIP3 regulation on protein level (**Figure 22, right panel**). AA patients displayed a stronger increase in TNFAIP3 protein expression than did HC. This was true for all the dust treatment conditions ($p = 0.03$ for G, $p = 0.009$ for Fi, $p = 0.01$ for Ch, and $p = 0.06$ for Am).

In summary, allergic asthmatic children exhibited decreased baseline TNFAIP3 expression (i.e. in unstimulated cells) on both transcriptional and translational levels compared to healthy control subjects, but it was possible to upregulate expression upon *in vitro* stimulation with LPS and farm dust extracts (Fi, Am).

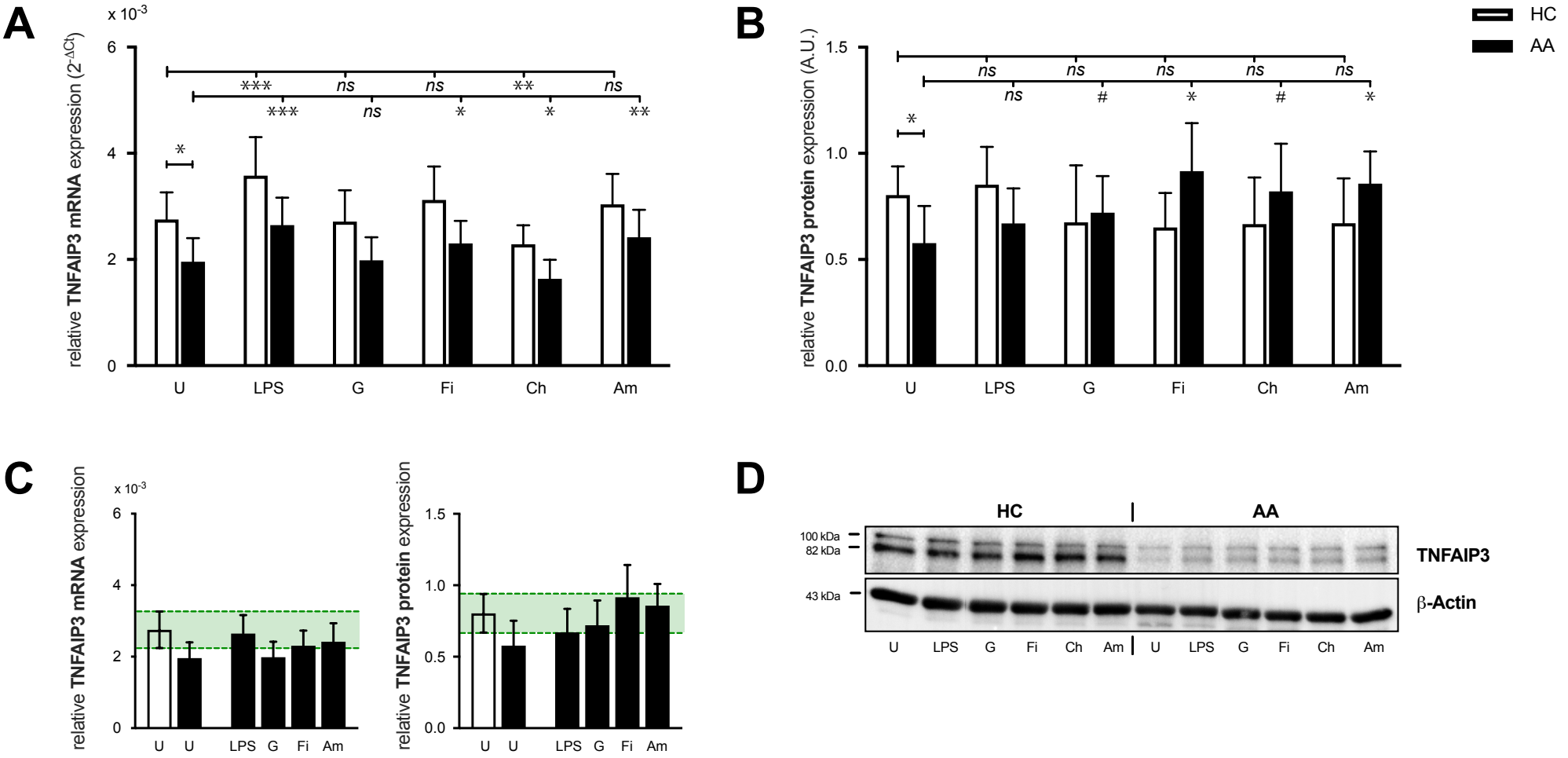

Figure 21. Effects of LPS and farm dust stimulation on TNFAIP3 mRNA and protein expression.
Mean values with 95% CI. **(A)** TNFAIP3 mRNA expression in PBMCs of HC (n=24) and AA (n=19) after 24 hrs stimulation with LPS or farm dust extracts (G, Fi, Ch, Am). **(B)** TNFAIP3 protein (82kDa) levels in HC (n=11) and AA (n=14), as assessed by Western Blot analysis after 24 hrs stimulation with LPS or farm dust extracts (G, Fi, Ch, Am). Results from densitometric analysis of protein bands. Normalized protein expression, relative to β-Actin. *A.U.*, arbitrary units. *P* values from paired t test are indicated for comparisons of stimulated (LPS, G, Fi, Ch, Am) vs. unstimulated (U) condition in both AA and HC, or from Welch's t test for phenotype comparison (AA vs. HC) in unstimulated cells, respectively. # indicates trend-wise effects ($p \leq 0.10$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; *ns*, not significant. **(C)** LPS- and farm dust-induced upregulation of TNFAIP3 mRNA and protein expression in AA compared to levels of HC under unstimulated conditions. Green-shaded area reflects 95% CI of TNFAIP3 expression, as measured in unstimulated samples of HC. **(D)** Representative image from TNFAIP3 immunoblot, showing detected protein bands of 1 HC (left) and 1 AA (right) sample in all stimulating conditions. β-Actin was used as loading-control. Molecular weight in kDa.

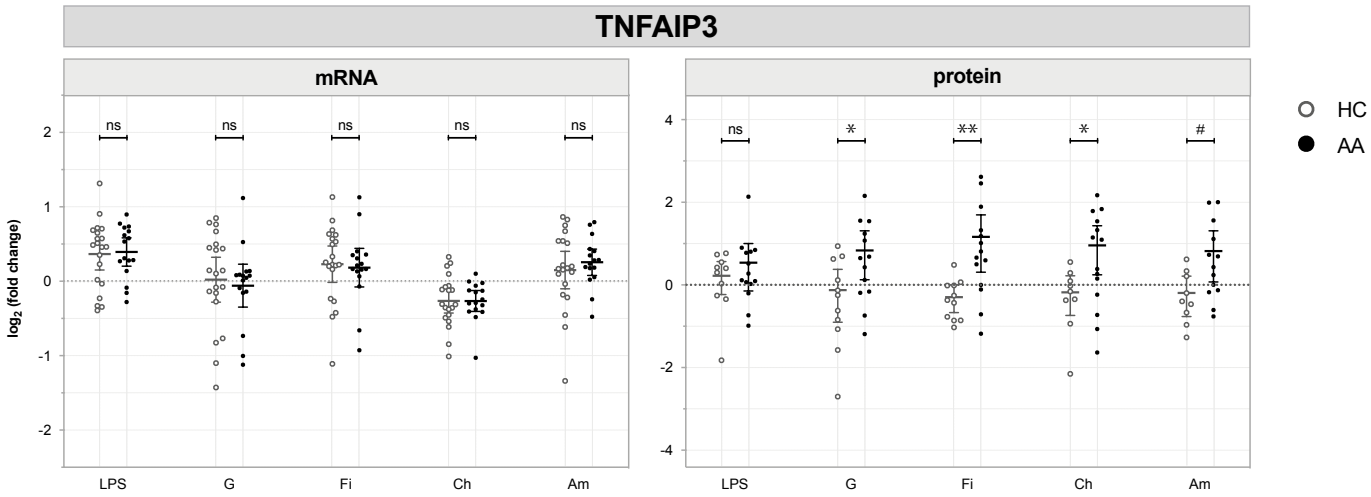

Figure 22. Fold change of TNFAIP3 mRNA and protein expression upon LPS and farm dust stimulation. TNFAIP3 gene **(left)** and protein **(right)** expression fold change, compared to unstimulated conditions (log_2 scale), as assessed in PBMCs of HC (n=24 for mRNA; n=11 for protein) and AA (n=19 for mRNA; n=14 for protein) after 24 hrs stimulation with LPS or farm dust extracts (G, Fi, Ch, Am). Scatter dot plots represent single sample values with means and 95% CI. *P* values reflect Welch's t test for fold change comparison between HC and AA. # indicates trend-wise effects ($p \leq 0.10$). *$p < 0.05$, **$p < 0.01$; *ns*, not significant.

TNIP2, TAX1BP1 and *MALT1* as regulatory factors of TNFAIP3's activity and function were also analysed for changes in expression levels after the treatment (**Figure 23, A-C**).

Neither in HC nor in AA was a clear pattern of up-or downregulation of the TNFAIP3 regulators visible upon LPS or dust treatment, respectively. In AA, mRNA levels of all three genes were decreased from baseline after treatment with the German dust. Yet, this was not the case in HC children. Chinese farm dust treatment significantly reduced *TNIP2* levels in both HC and AA ($p = 0.04$ and $p = 0.002$, respectively; **Figure 23, A**). Other treatment conditions (LPS, Fi and Am) did not exert any significant effects on *TNIP2, TAX1BP1* and *MALT1* expression in this experiment.

No difference between HC and AA regarding fold change of expression after stimulation was detected for the TNFAIP3 regulators (**Figure E2, appendix**).

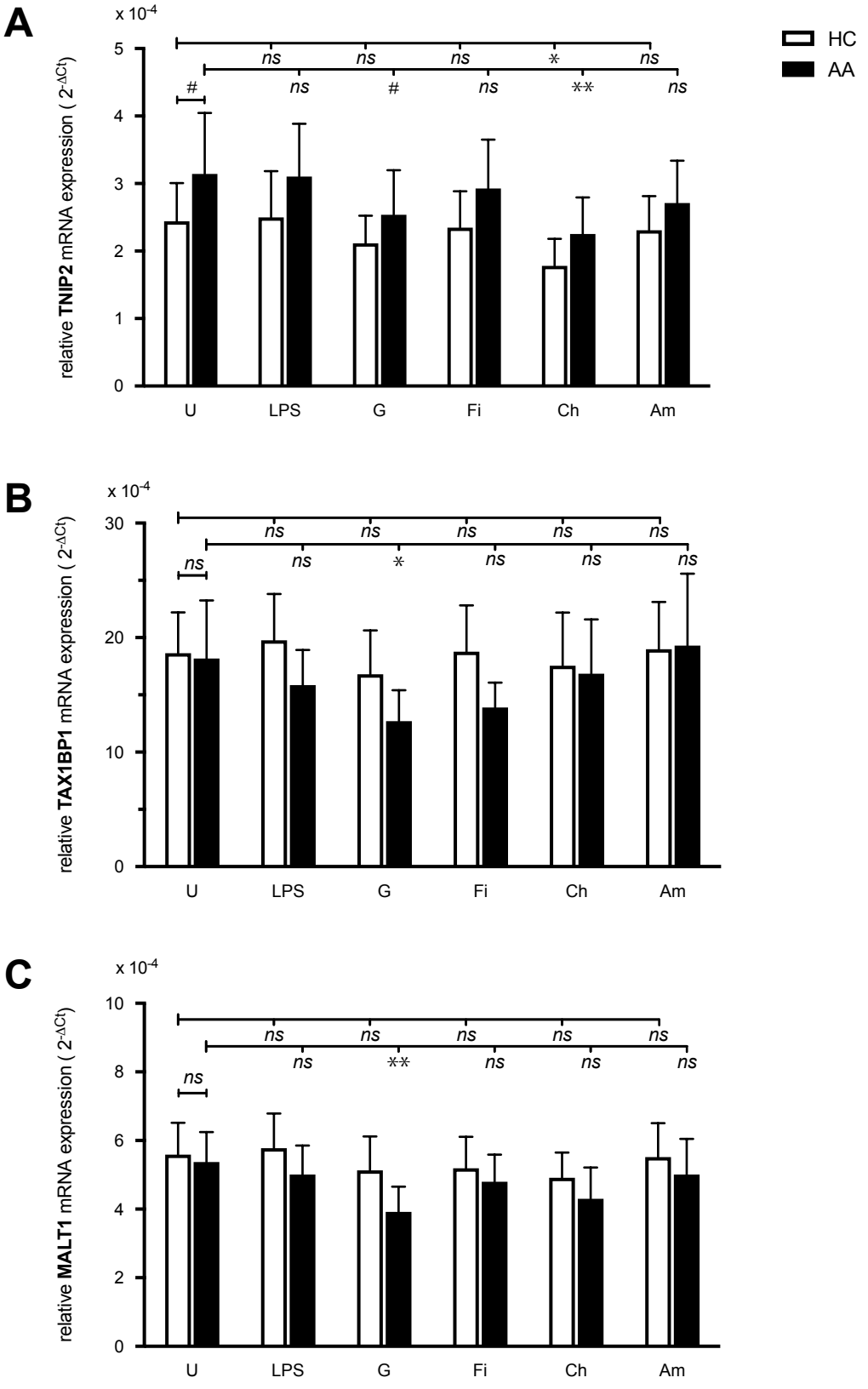

Figure 23. Effects of LPS and farm dust stimulation on expression of TNFAIP3 regulatory genes.
(A) TNIP2, **(B)** TAX1BP1 and **(C)** MALT1 mRNA expression in PBMCs of HC (n=22) and AA (n=16) after 24 hrs stimulation with LPS or farm dust extracts (G, Fi, Ch, Am). Expression displayed as mean values ($2^{-\Delta Ct}$) with 95% CI. *P* values from paired t test are indicated for comparisons of stimulated (LPS, G, Fi, Ch, Am) vs. unstimulated (U) condition in both AA and HC, or from Welch's t test for phenotype comparison (AA vs. HC) in unstimulated cells, respectively. # indicates trend-wise effects ($p \leq 0.10$). *$p < 0.05$, **$p < 0.01$; *ns*, not significant.

3.2.2.3 Opposing regulation of the co-stimulatory molecules *CD80* and *CD86*

To observe the influence of LPS and dust treatment on DC activation and maturation markers, *CD80*, *CD86* and *CD274* expression was analysed. Unlike TNFAIP3 and its regulators, DC markers showed very sensitive kinetics and a wide regulatory range of expression upon treatment with LPS and all dust samples except for Ch (**Figure 24, A-C**).
CD80 as a marker of DC activation was significantly increased from baseline expression in both healthy and asthmatic children's cells upon treatment with LPS and the Finnish and Amish farm dust ($p < 0.0001$ for AA and $p < 0.05$ for HC, respectively; **Figure 24, A**). In AA, *CD80* levels were also increased upon German dust treatment ($p = 0.002$).

In summary, *CD80* gene expression was significantly decreased in asthmatic children at baseline, but expression was upregulated upon LPS and farm dust (G, Fi, Am) stimulation. In contrast, the co-stimulatory molecule *CD86*, which was expressed at a high basal level in children's PBMCs, was significantly decreased in both groups after stimulation ($p < 0.001$ for treatment with LPS and G, Fi, Am dust in HC and AA, respectively; **Figure 24, B**). Again, only the Chinese dust did not induce such responses. **Like *CD80*, mRNA of *CD274*, the immune-suppressive marker on dendritic cells, was significantly upregulated after LPS and dust treatment in HC and AA** ($p < 0.0001$ for LPS and G, Fi and Am; **Figure 24, C**).

These findings were also expressed by a remarkable decrease in the *CD86/CD80* ratio, accompanied by an increased *CD274/CD86* ratio in response to LPS and all farm dust samples except Ch ($p < 0.0001$ for both HC and AA; **Figure 25, A and B**).

Overall, asthmatics showed a significantly stronger decrease in *CD86* mRNA expression after LPS treatment when compared to the healthy control group ($p = 0.009$; **Figure 26, B**). This was also observed for stimulation with the G, Fi, and Am farm dust samples *(*$p = 0.04$, $p = 0.06$ and $p = 0.09$, respectively). For *CD80* (**Figure 26, A**) and *CD274* (**Figure 26, C**), no differences between HC and AA regarding fold change of expression were seen.

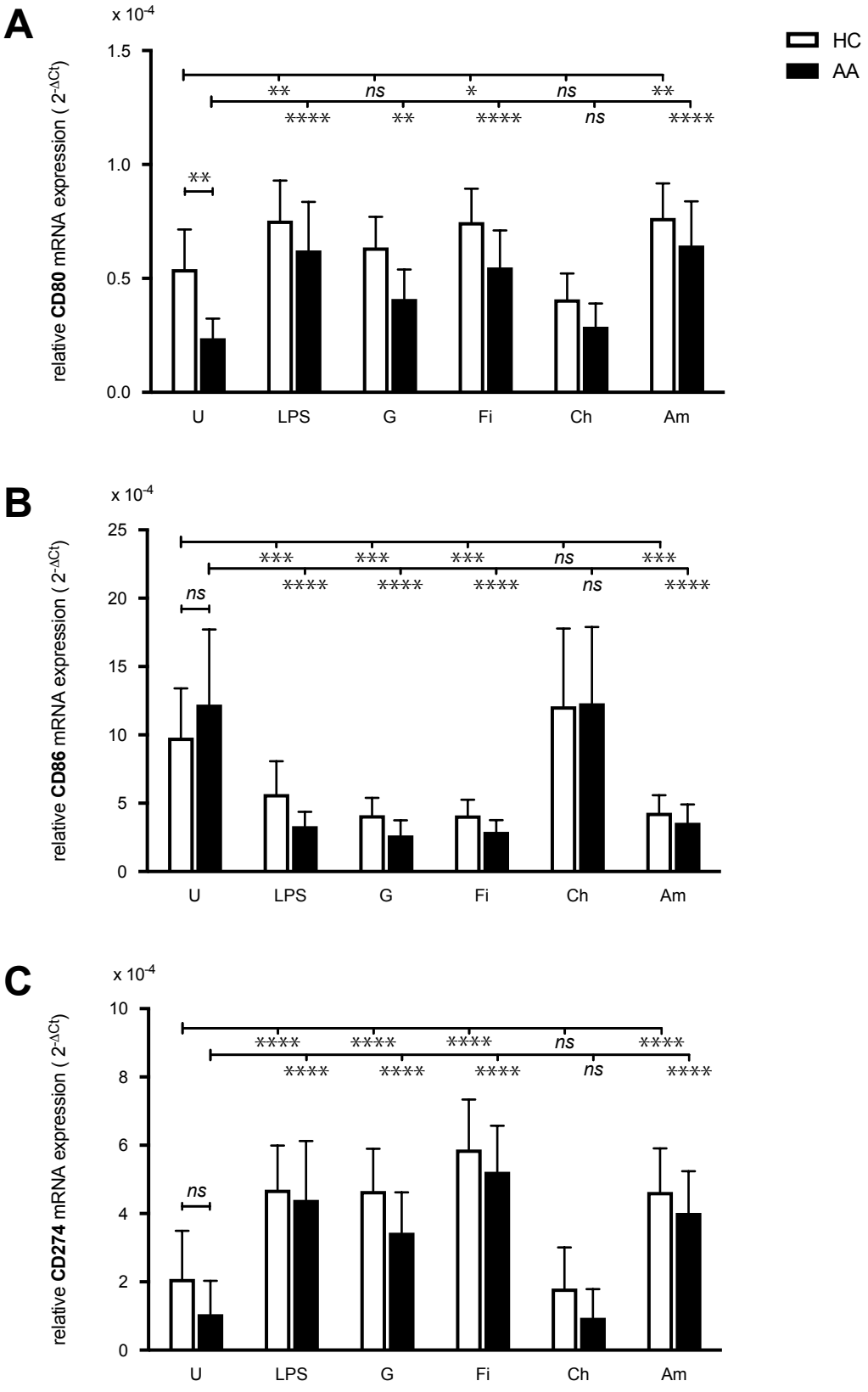

Figure 24. Effects of LPS and farm dust stimulation on expression of dendritic cell associated genes.
(A) CD80, **(B)** CD86 and **(C)** CD274 mRNA expression in PBMCs of HC (n=22) and AA (n=16) after 24 hrs stimulation with LPS or farm dust extracts (G, Fi, Ch, Am). Expression displayed as mean values ($2^{-\Delta Ct}$) with 95% CI. *P* values from paired t test are indicated for comparisons of stimulated (LPS, G, Fi, Ch, Am) vs. unstimulated (U) condition in both AA and HC, or from Welch's t test for phenotype comparison (AA vs. HC) in unstimulated cells, respectively. # indicates trend-wise effects ($p \leq 0.10$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; *ns*, not significant.

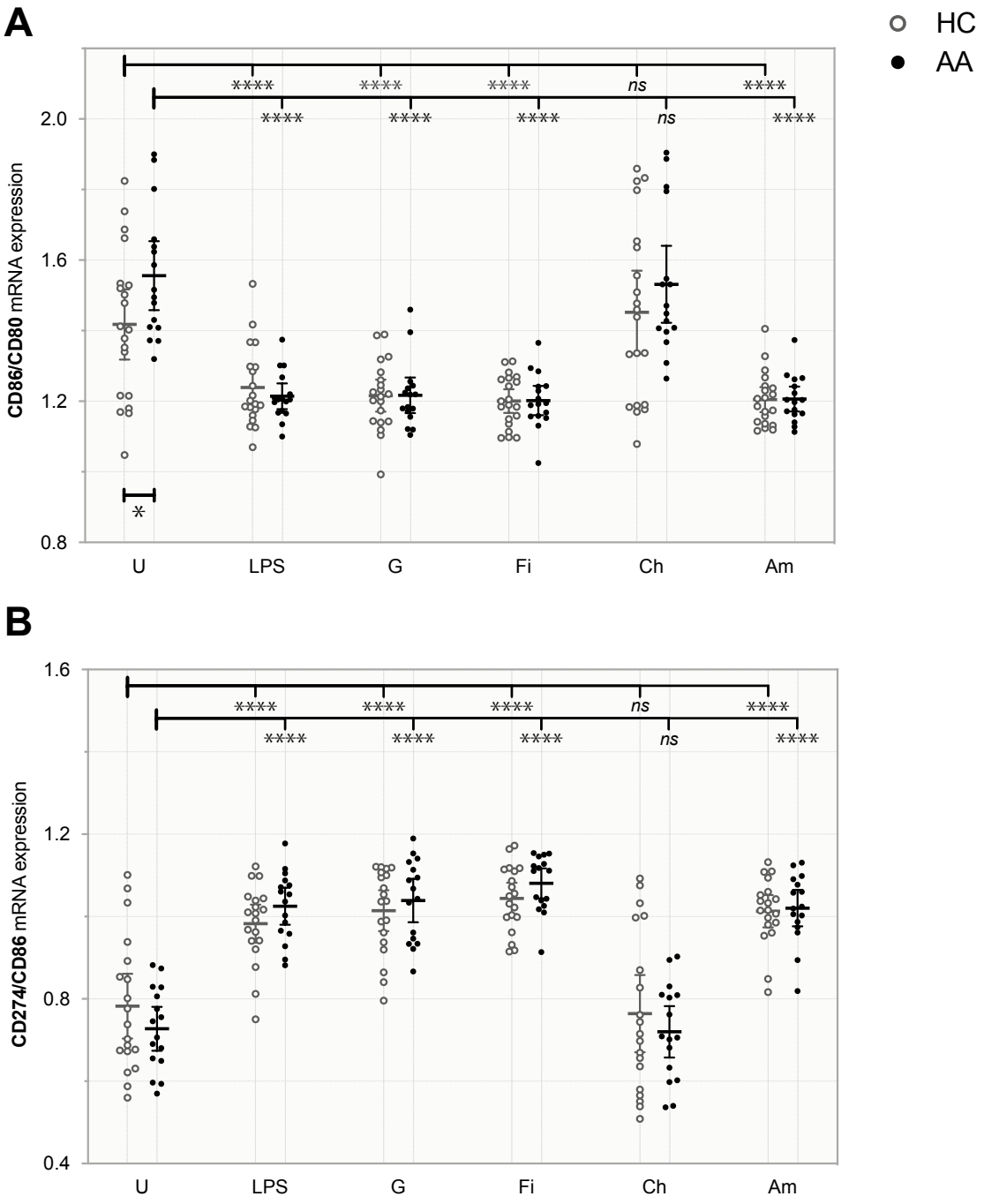

Figure 25. Effects of LPS and farm dust stimulation on CD86/CD80 and CD274/CD86 expression ratio. mRNA expression in PBMCs of HC (n=22) and AA (n=16) after 24 hrs stimulation with LPS or farm dust extracts (G, Fi, Ch, Am). **(A)** CD86/CD80 and **(B)** CD274/CD86 expression ratios were built by relating ΔC_t values of CD80 to CD86 and of CD86 to CD274, respectively. Scatter dot plots represent single sample values with means and 95% CI. *P* values from paired t test are indicated for comparisons of stimulated (LPS, G, Fi, Ch, Am) vs. unstimulated (U) condition in both AA and HC, or from Welch's t test for phenotype comparison (AA vs. HC) in unstimulated cells, respectively. ****$p < 0.0001$; *ns*, not significant.

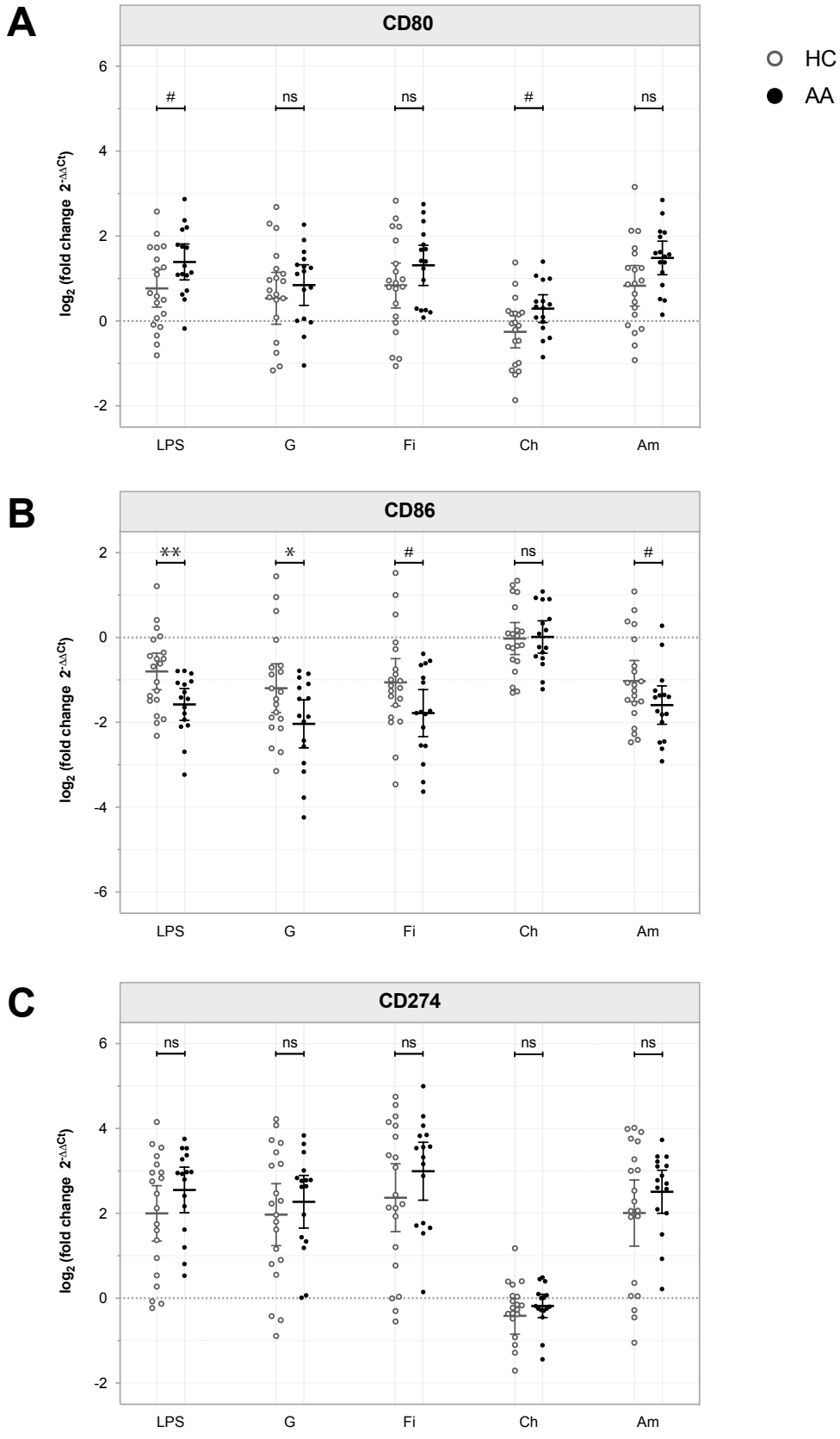

Figure 26. Fold change of dendritic cell associated gene expression upon LPS and farm dust stimulation. **(A)** CD80, **(B)** CD86 and **(C)** CD274 gene expression fold change, compared to unstimulated conditions ($2^{-\Delta\Delta Ct}$, $\log_2$ scale), as assessed by qPCR in PBMCs of HC (n=22) and AA (n=16) after 24 hrs stimulation with LPS or farm dust extracts (G, Fi, Ch, Am). Scatter dot plots represent single sample values with means and 95% CI. *P* values reflect Welch's t test for fold change comparison between HC and AA. # indicates trend-wise effects ($p \leq 0.10$). *$p < 0.05$, **$p < 0.01$; *ns*, not significant.

3.2.2.4 NF-κB signalling downstream effects – downregulation of *IL-18*

To further investigate TNFAIP3- and NF-κB related pathways in the context of allergic asthma, subsequent experiments were conducted by our research group in this specific cohort. Focussing on downstream effects of NF-κB signalling, gene expression of the pro-inflammatory cytokine interleukin 18 (*IL-18*) was assessed in allergic asthmatic and healthy children[e].

In line with the findings of increased *TLR4-/MyD88*-expression and decreased anti-inflammatory *TNFAIP3* in asthmatic children, significantly elevated mRNA levels of *IL-18* were found in unstimulated cells of AA compared to HC ($p = 0.002$; **Figure 27**).
However, upon stimulation with German, Finnish and Amish farm dust, *IL-18* was significantly downregulated in both HC and AA children ($p < 0.03$ in HC, $p < 0.0001$ in AA; **Figure 27**). As already observed for *TLR4, MyD88*, *TRAF6* and *CD86* expression, asthmatic children showed a significantly stronger decrease in *IL-18* expression upon LPS ($p = 0.002$) and German, Finnish and Amish farm dust stimulation when compared to healthy children ($p = 0.02$, $p = 0.04$, $p = 0.007$; data not shown).

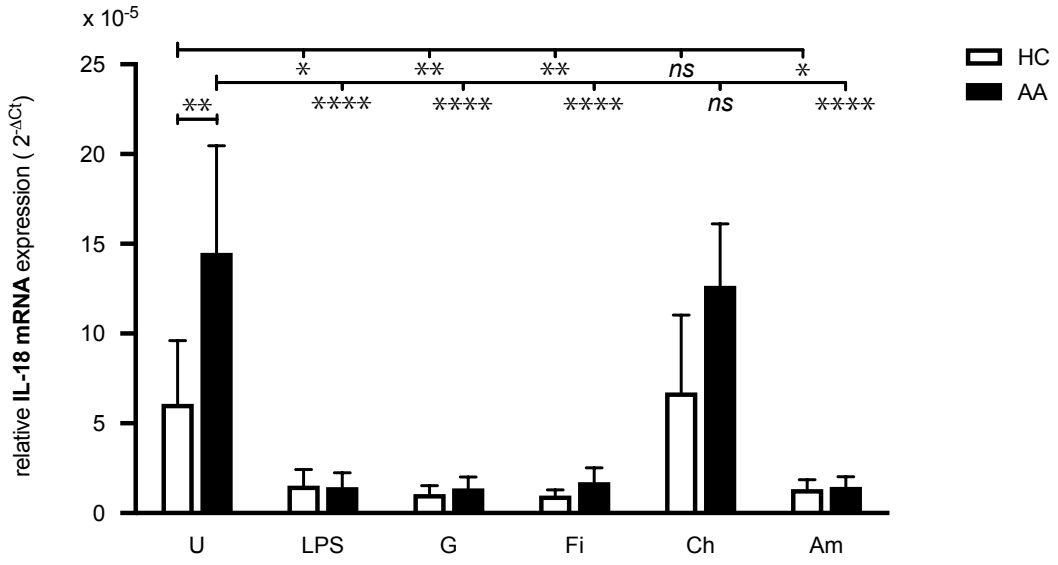

Figure 27. Effects of LPS and farm dust stimulation on IL-18 mRNA expression.
IL-18 gene expression in PBMCs of HC (n=17) and AA (n=17) after 24 hrs stimulation with LPS or farm dust extracts (G, Fi, Ch, Am). Expression displayed as mean values ($2^{-\Delta Ct}$) with 95% CI. P values from paired t test are indicated for comparisons of stimulated (LPS, G, Fi, Ch, Am) vs. unstimulated (U) condition in both AA and HC, or from Welch's t test for phenotype comparison (AA vs. HC) in unstimulated cells, respectively. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; ns, not significant.

[e] qPCR for IL-18 gene expression analysis was not performed by the author herself, but by a member of the research group.

3.2.2.5 Overview of *in vitro* LPS and farm dust stimulation effects in AA and HC

Heat maps presented in **Figure 28** summarise the findings of chapter **3.2.2**, providing colour-coded information about the mRNA response (i.e. up-/and downregulation) to the different treatment conditions for both phenotypes (AA and HC) and for each gene. The direction of regulation was represented as red-/blue-gradient, with blue indicating upregulation and red representing downregulation of gene expression. Colour saturation reflects the strength of the effect in terms of the fold change of expression ($2^{-\Delta\Delta Ct}$, $\log_2$ scale). Significant regulation compared to the unstimulated condition was indicated by asterisks (*$p < 0.05$, **$p < 0.01$. ***$p < 0.001$, ****$p < 0.0001$). Additionally, comparisons in fold changes of gene expression between the two phenotypes (allergic asthmatics (AA) vs. healthy controls (HC)) were included, and significant differences were marked with a black rectangle around the respective gene's cell.

Overall, stimulation with LPS and dust extracts from German (G), Finnish (Fi) and Amish (Am) farms significantly decreased the expression of the pro-inflammatory genes *TLR4, MyD88* and *CD86* (red), while it increased anti-inflammatory *TNFAIP3, CD80* and *CD274* (blue) in both healthy and asthmatic children.

Regulation was significantly stronger for some of the genes (*TLR4, MyD88* (Am), *TRAF6, MALT1* (G), *CD86* (LPS, G)) in AA as compared to HC (**Figure 28,** black rectangle around the gene's cell for $p < 0.05$).

Similar effects were observed for T cell associated gene expression in the same cohort, with the pro-inflammatory T cell co-stimulatory molecule *CD28* being decreased and anti-inflammatory *CTLA-4* on T cells being significantly increased in asthmatic children after stimulation with LPS and farm dust extracts[f] (data not shown here, but included in the publication[321]). As mentioned before, stimulation with Chinese dust samples did not exert similar responses in healthy or in asthmatic children.

It is noteworthy that the key findings of *TLR4* downregulation and *TNFAIP3, CD80* and *CD274* upregulation respectively were replicated in a Chinese cohort of 49 children including AA (n = 20) and HC (n = 29) upon LPS stimulation (data not shown here[321]).

[f] qPCR for CD28 and CTLA-4 gene expression analysis was not performed by the author herself, but by a member of the research group.

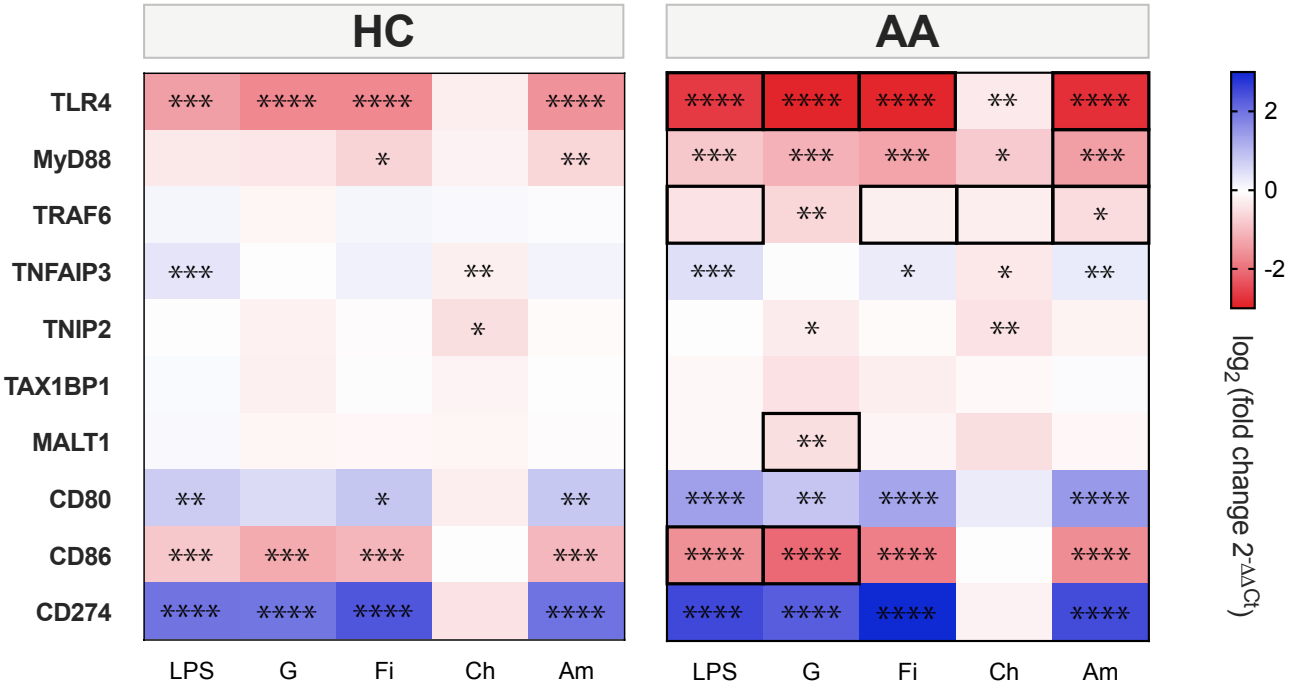

Figure 28. Overview of *in vitro* LPS and farm dust stimulation effects in HC and AA.
Colour-coded up-/and downregulation of mRNA expression of TLR4, MyD88, TRAF6, TNFAIP3, TNIP2, TAX1BP1, MALT1 and the dendritic cell associated genes CD80, CD86 and CD274. Fold change of gene expression compared to unstimulated conditions ($2^{-\Delta\Delta Ct}$, log_2 scale) in PBMCs of children with allergic asthma (AA; n=19 for TNFAIP3 and n=16 for all other genes) and healthy control children (HC; n=24 for TNFAIP3 and n=22 for all other genes respectively), measured by qPCR following 24 hrs treatment with either LPS [0.1µg/ml] or farm dust [40µg/ml] from Germany (G), Finland (Fi), China (Ch) and from the Amish people (Am). Blue colour of cells indicates upregulation, red colour indicates downregulation of gene expression. Saturation reflects the strength of the fold change effect. Statistical significance of differences of gene expression compared to the unstimulated condition is marked by asterisks based on paired t test analysis. *$p < 0.05$, **$p < 0.01$. ***$p < 0.001$, ****$p < 0.0001$. Significant differences of regulation between the two phenotypes (AA vs. HC), as assessed by Welch's t test are marked with a black rectangle around the respective gene's cell.

3.2.2.6 Overall stimulation effect – inflammation ratio

In order to further demonstrate the pooled effect of LPS and the environmental dust extracts on inflammatory immune responses, an 'inflammation ratio' was calculated by relating pro- to anti-inflammatory gene expression[321] (**Figure 29**). The average ΔC_t value of all investigated genes exhibiting anti-inflammatory properties (*TNFAIP3*, *TNIP2, TAX1BP1, CD274*) was divided by the average ΔC_t value of all pro-inflammatory genes (*TLR4, MyD88, TRAF6, MALT1, CD86)*.

Children with allergic asthma displayed a significantly increased inflammation ratio compared to healthy controls in unstimulated (U) cells ($p = 0.01$; **Figure 29**), indicating a higher pro-inflammatory basal immune state in asthmatic children.

Upon 24-hour stimulation with LPS and farm dust extracts (G, Fi, Am), this inflammation ratio was significantly reduced in both healthy and asthmatic children ($p < 0.001$; **Figure 29**), thus highlighting the anti-inflammatory properties of *in vitro* LPS and farm dust exposure on children's immune cells.

Significant differences could also be observed when the ratio was calculated by the two central genes of the pathway *TLR4* and *TNFAIP3* (data not shown).

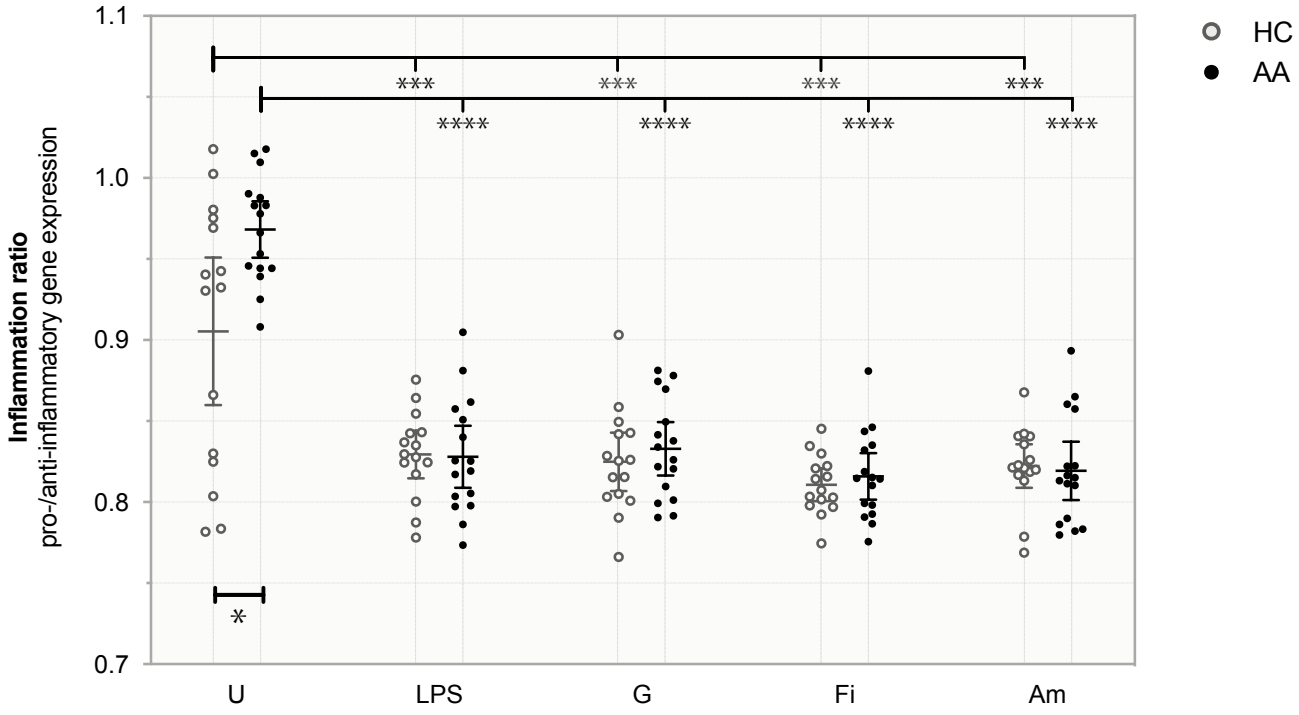

Figure 29. Inflammation ratio in HC and AA and the effect of LPS and farm dust stimulation.
Inflammation score defined as ratio of pro-inflammatory (TLR4, MyD88, TRAF6, MALT1, CD86) against anti-inflammatory gene expression (TNFAIP3, TNIP2, TAX1BP1, CD274) in unstimulated (U) and LPS or farm dust (G, Fi, Am) stimulated PBMCs of HC (n=15) and AA (n=16). Scatter dot plots represent single sample values (inflammation ratio) with means and 95% CI. *P* values from paired t test are indicated for comparisons of stimulation vs. unstimulated condition in both AA and HC, or from Welch's t test for phenotype comparison (AA vs. HC) in unstimulated cells, respectively. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

3.3 Gene expression changes upon chronic early life *in vivo* farm exposure

Children from farming environments were shown to be naturally protected from the development of atopic diseases and asthma. **Therefore, the third aim of this study was to investigate potential effects of sustained early life *in vivo* farm exposure on the expression of *TNFAIP3* and associated NF-κB pathway genes in immune cells of those children at the age of 4.5, 6 and 10 years.**

3.3.1 PASTURE/EFRAIM study cohort

Gene expression was assessed in a subset of the European farming birth cohort PASTURE/EFRAIM including both healthy and asthmatic children at three different time points. Whole blood samples from a 4.5-year-old group of 37 children, comprising a total of 19 non-farm (NF) and 18 farm children (F) from three different study centres (Germany, Finland, France), and samples from the follow-up 6-year-old group of 63 children, comprising 31 non-farm and 32 farm children were analysed (**Figure 30**). For study design and setup details, see **2.2.2.2** in the **methods** section of this thesis.
To analyse sustained immunomodulatory effects in older farm children as well, a small sample of 5 healthy German farm children (F-HC) was enrolled for this study within the PASTURE/EFRAIM 10-year follow-up (see **Figure 10**). Blood was drawn and processed as previously described (see **2.2.4**).

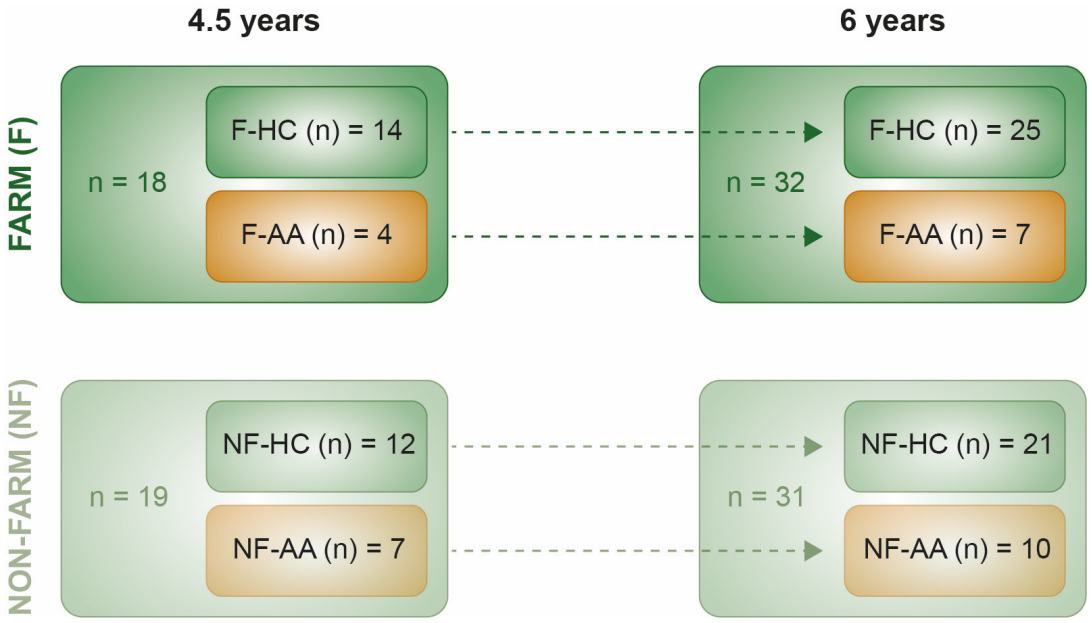

Figure 30. Enrolment flow chart PASTURE/EFRAIM study subsample.
n, number of cases*; HC*, healthy controls; *F-HC*, healthy farm children; *NF-HC*, healthy non-farm children; *AA*, patients with allergic asthma; *F-AA*, farm children with allergic asthma; *NF-AA*, non-farm children with allergic asthma.

3.3.1.1 Study population and clinical characteristics of farm (F) and non-farm (NF) children

Characteristics of the PASTURE/EFRAIM subpopulation at 4.5 and 6 years were given in **Tables 9-11**. Similar distribution of gender was ensured when selecting the two groups of farm (F) and non-farm (NF) children at both time points (**Table 9**). Likewise, both 4.5- and 6-year-old farm and non-farm groups were balanced in terms of potential confounding factors like comorbid allergic diseases (hay fever, atopic dermatitis, food allergy), parental atopy, indoor smoking at home and the number of older siblings. Significant differences were only observed for paternal education in the 6-year-old group, with a higher educational status of fathers from non-farm children when compared to the farming group. However, this was not seen in the 4.5-year-old children, and no differences were found for maternal education in both age groups.

Laboratory parameters of the 6-year-old F and NF children were outlined in **Table 10**. White blood cell count and lymphocyte proportions did not differ significantly between F and NF. Furthermore, F and NF were equally distributed regarding sensitisation to common perennial, seasonal or food allergens (**Table 11**).

In the 10-year-old group, F (n = 5) and NF children (n = 15) were unequally distributed in terms of sex (80% female children in NF-HC vs. 20% in F-HC; $p = 0.01$), mainly due to the small sample size of the farming subsample. Farm children were younger than their non-farming peers, with borderline statistical significance ($p = 0.07$; median age 10.4 (10.4 – 10.6) vs. 11.7 (10.4 – 13.3)). However, the two groups were not significantly different regarding blood cell counts, immune cell proportions and further clinical and epidemiological parameters (data not shown).

Table 9. *Study population characteristics of the PASTURE/EFRAIM subgroup, age 4.5 years and 6 years.*

	4.5 years			***6 years***		
Parameter	**NF**	**F**	***P* value**	**NF**	**F**	***P* value**
n	19	18		31	32	
Female sex, n (%)	10 (52.6)	7 (38.9)	0.40	15 (48.4)	15 (46.9)	0.90
BMI (kg/m^2), median (IQR)	15.8 (15.3 - 16.9)	15.4 (14.8 - 16.5)	0.37†	15.9 (14.9 - 17.1)	15.6 (14.4 - 16.5)	0.33†
Asthma (6y), n (%)	7 (36.8)	4 (22.2)	0.33	10 (32.3)	7 (21.9)	0.35
Hay fever (ever), n (%)	2 (10.5)	1 (5.6)	0.58	4 (12.9)	3 (9.4)	0.66
Doctor's diagnosis of atopic dermatitis (ever), n (%)	5 (26.3)	3 (16.7)	0.48	13 (41.9)	8 (25.0)	0.15
Doctor's diagnosis of food allergy (ever), n (%)	2 (10.5)	3 (16.7)	0.59	3 (9.7)	5 (15.6)	0.48
Maternal diagnosis of asthma (ever), n (%)	3 (15.8)	0 (0.0)	0.08	5 (16.1)	3 (9.4)	0.42
Maternal diagnosis of hay fever (ever), n (%)	8 (42.1)	3 (16.7)	0.09	9 (29.0)	7 (21.9)	0.51
Maternal diagnosis of atopic dermatitis (ever), n (%)	5 (26.3)	3 (16.7)	0.48	8 (25.8)	3 (9.4)	0.09
Paternal diagnosis of asthma (ever), n (%)	3 (15.8)	1 (5.6)	0.32	4 (12.9)	1 (3.1)	0.15
Paternal diagnosis of hay fever (ever), n (%)	6 (31.6)	3 (16.7)	0.29	8 (25.8)	3 (9.4)	0.09
Paternal diagnosis of atopic dermatitis (ever), n (%)	1 (5.3)	2 (11.1)	0.52	1 (3.2)	2 (6.2)	0.57
Smoking at home, n (%)	0 (0.0)	0 (0.0)	--	0 (0.0)	0 (0.0)	--
Maternal education			0.64			0.80
Elementary or middle school, n (%)	12 (63.2)	10 (55.6)		15 (48.4)	14 (43.8)	
High school or university, n (%)	7 (36.8)	8 (44.4)		16 (51.6)	17 (53.1)	
Other, n (%)	0 (0.0)	0 (0.0)		0 (0.0)	1 (3.1)	
Paternal education			0.58			**0.01***
Elementary or middle school, n (%)	10 (52.6)	11 (61.1)		13 (41.9)	24 (75.0)	
High school or university, n (%)	8 (42.1)	6 (33.3)		16 (51.6)	7 (21.9)	
Missing/Other, n (%)	1 (5.3)	1 (5.6)		2 (6.5)	1 (3.1)	
Older siblings, n (%)	11 (57.9)	11 (61.1)	0.84	21 (67.7)	22 (68.8)	0.93

NF, non-farm children; *F,* farm children. *n,* number of cases; %, percentage of group total*; BMI*, body-mass-index. Values for *BMI* represent *median* and interquartile range (*IQR*). *P* values reflect χ^2 test for proportions, or the Mann-Whitney *U* test (†) for continuous variables, respectively. Tests were applied for non-missing data. *$p < 0.05$ (boldface). Total number of subjects might differ slightly for some parameters due to data availability.

Table 10. *Laboratory parameters of NF and F children (6 years).*

Parameter	NF (n = 31) *median*	F (n = 32) *median*	*P* value
WBC (cells x 10^9/L) (IQR)	7.45 (5.58 – 9.10)	7.6 (6.70 – 9.10)	0.49
Lymphocytes (cells x 10^9/L) (IQR)	3.05 (2.58 – 3.75)	3.10 (2.30 – 3.80)	0.98

Healthy and asthmatic farm (*F*) and non-farm (*NF*) children. *n,* number of cases; *WBC,* white blood cell count. Values represent *median* and interquartile range (*IQR*). *P* values reflect the Mann-Whitney *U* test. **p* < 0.05 (boldface). Total number of subjects might differ slightly for some parameters due to data availability.

Table 11. *Sensitisation to allergens in NF and F children.*

Parameter	4.5 years			6 years		
	NF (n = 19)	F (n = 18)	*P* value	NF (n = 31)	F (n = 32)	*P* value
Specific IgE (≥ 0.70 IU/ml)						
to perennial allergens, n (%)	5 (26.3)	7 (38.9)	0.41	8 (25.8)	4 (12.5)	0.18
to seasonal allergens, n (%)	4 (21.1)	2 (11.1)	0.41	6 (19.4)	4 (12.5)	0.46
to food allergens, n (%)	3 (15.8)	5 (27.8)	0.38	4 (12.9)	8 (25.0)	0.22

Healthy and asthmatic farm (*F*) and non-farm (*NF*) children. *IU*, international units. *n,* number of cases. %, percentage of group total. Specific IgE levels were dichotomised at the detection limit of 0.70 IU/ml and categorised by perennial, seasonal and food allergens as described above. *P* values from χ^2 test. **p* < 0.05 (boldface).

3.3.1.2 Farming exposures of F and NF children

Data on children's early life farming activities were assessed annually by questionnaires among all farm and non-farm children. Specific farm exposures that have been shown relevant in the context of protection against asthma and allergic disease in different farming cohorts[112,122] were analysed in the 4.5- and 6-year-old PASTURE study subgroups (**Table 12**).

Farm children were documented to have higher regular consumption of farm-produced cow's milk (77.8 % in F vs. 5.3 % in NF; $p < 0.001$), more frequent exposure to stables and barns, and regular contact with hay and cows when compared to the non-farm group ($p < 0.001$ for all exposure variables; **Table 12**).

Table 12. *Specific farming exposures in NF and F children.*

Parameter	4.5 years			6 years		
	NF (n = 19)	**F (n = 18)**	***P* value**	**NF (n = 31)**	**F (n = 32)**	***P* value**
Regular# farm milk consumption, n (%)	1 (5.3)	14 (77.8)	**< 0.001***	5 (16.1)	26 (81.2)	**< 0.001***
Stay in stable, n (%)	3 (15.8)	17 (94.4)	**< 0.001***	8 (25.8)	30 (93.8)	**< 0.001***
Stay in barn, n (%)	0 (0.0)	12 (66.7)	**< 0.001***	5 (16.1)	25 (78.1)	**< 0.001***
Regular contact with hay, n (%)	4 (21.2)	15 (83.3)	**< 0.001***	8 (25.8)	28 (87.5)	**< 0.001***
Regular contact with cows, n (%)	0 (0.0)	13 (72.2)	**< 0.001***	1 (3.2)	25 (78.1)	**< 0.001***

Healthy and asthmatic farm (*F*) and non-farm (*NF*) children. n, number of cases. %, percentage of group total. # Regular is defined as at least weekly exposure. Exposures refer to the children's first, second, third, fourth, fifth or sixth year of life. *P* values reflect the χ^2 test. *$p < 0.05$ (boldface).

3.3.2 Decreased expression of *TNFAIP3* and dendritic cell marker genes in F children

Upon unstimulated conditions, differences regarding gene expression of *TNFAIP3* and *TLR4* as well as the dendritic cell associated genes *CD80* and *CD274* were analysed in the 4.5-, 6- and 10-year-old farm and non-farm children.

In the 4.5-year-old subjects, no significant difference in *TNFAIP3* expression between farm (n = 18) and non-farm (n = 19) children was detected (**Figure 31, A**). However, *TNFAIP3* was found significantly decreased in the 6-year-old group of 32 farm compared to 31 non-farm children ($p = 0.04$). Supporting these findings, baseline *TNFAIP3* levels in the small 10-year-old group was found trend-wise to be decreased in the farm (n = 5) compared to the non-farming (n = 17) cohort. No difference in *TLR4* expression levels was present between farm and non-farm children at ages 4.5, 6 and 10 years (**Figure 31, B**).

Gene expression of *CD80* and *CD274*, however, was lower in F compared to NF children among all ages, with significant effects at the age of 4.5 years, in particular ($p = 0.05$ for *CD80* and $p = 0.03$ for *CD274*, respectively; **Figure 32, A** and **B**). *CD274* expression was also significantly reduced in the 6-year-old farm children upon unstimulated conditions ($p = 0.03$; **Figure 32, B**).

It is noteworthy that the observed effects between F and NF remained significant in the 6-year-old children, and at least at a trend-wise level also in the smaller 4.5-year-old group, when the data for children with asthma were excluded from the analysis (see **Figures E3 and E4, appendix**).

All of the investigated genes were expressed at a significantly higher baseline level in the 10-year-old groups (PBMCs) compared to 4.5- and 6-year-old subjects (whole blood samples), regardless of the farming status.

mRNA expression was also assessed for the genes *MyD88, TRAF6, TNIP2, TAX1BP1, MALT1*, and *CD86*, but did not reveal differences in expression between farm and non-farm children (data not shown).

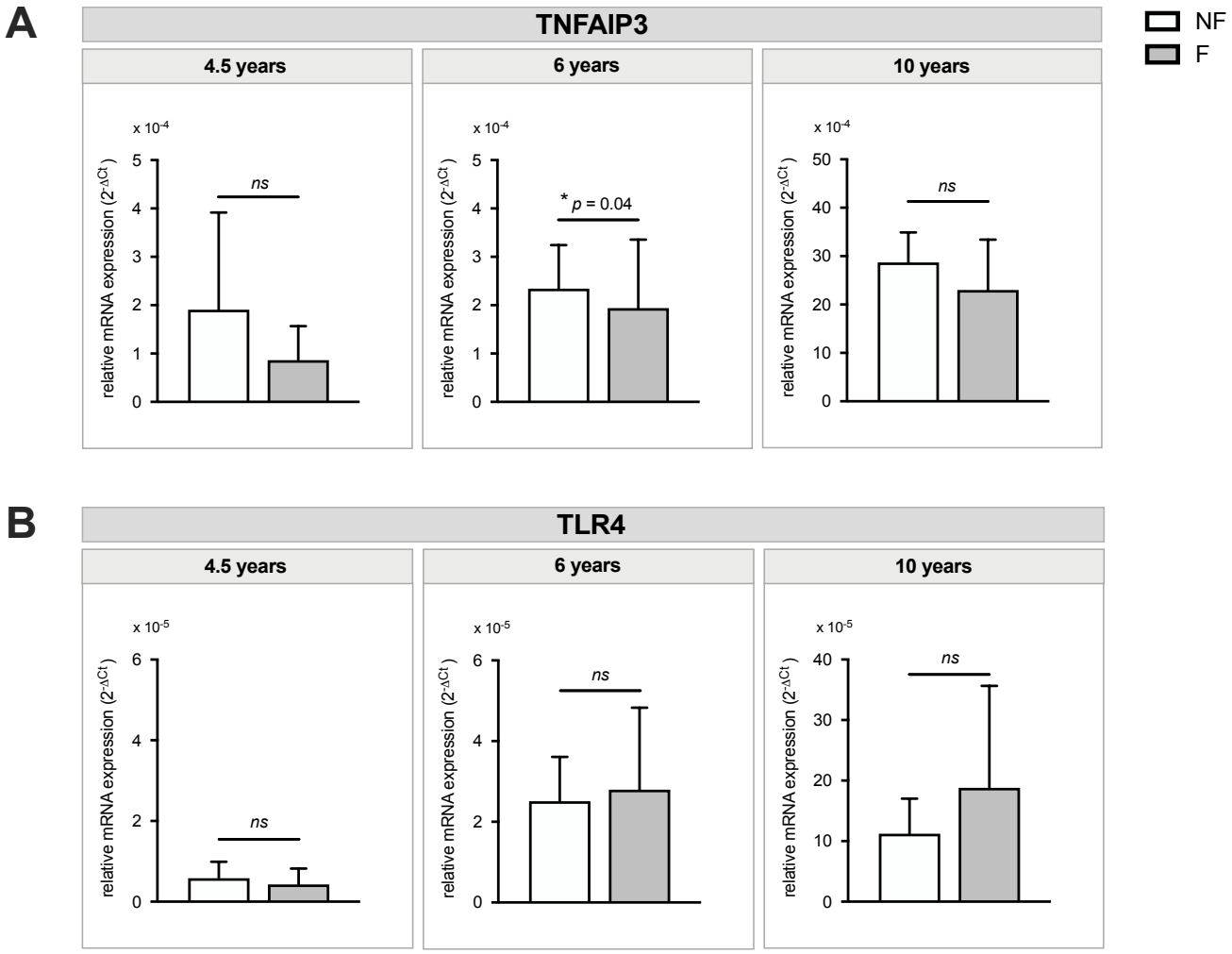

Figure 31. TNFAIP3 and TLR4 mRNA expression in NF and F children.
Baseline gene expression levels of TNFAIP3 **(A)** and TLR4 **(B)**, as measured by qPCR in unstimulated whole blood samples of healthy and asthmatic farm (F) and non-farm (NF) children, assessed at ages 4.5 years (n=37, 18 F/ 19 NF) and 6 years (n=63, 32 F/31 NF), and in unstimulated PBMCs of healthy 10-year-old children (n=22, 5 F/17 NF). Expression relative to 18S, displayed as $2^{-\Delta Ct}$ mean values with 95% CI. *P* values reflect Welch's t test for ΔCt comparison between NF and F. **p* < 0.05; *ns*, not significant.

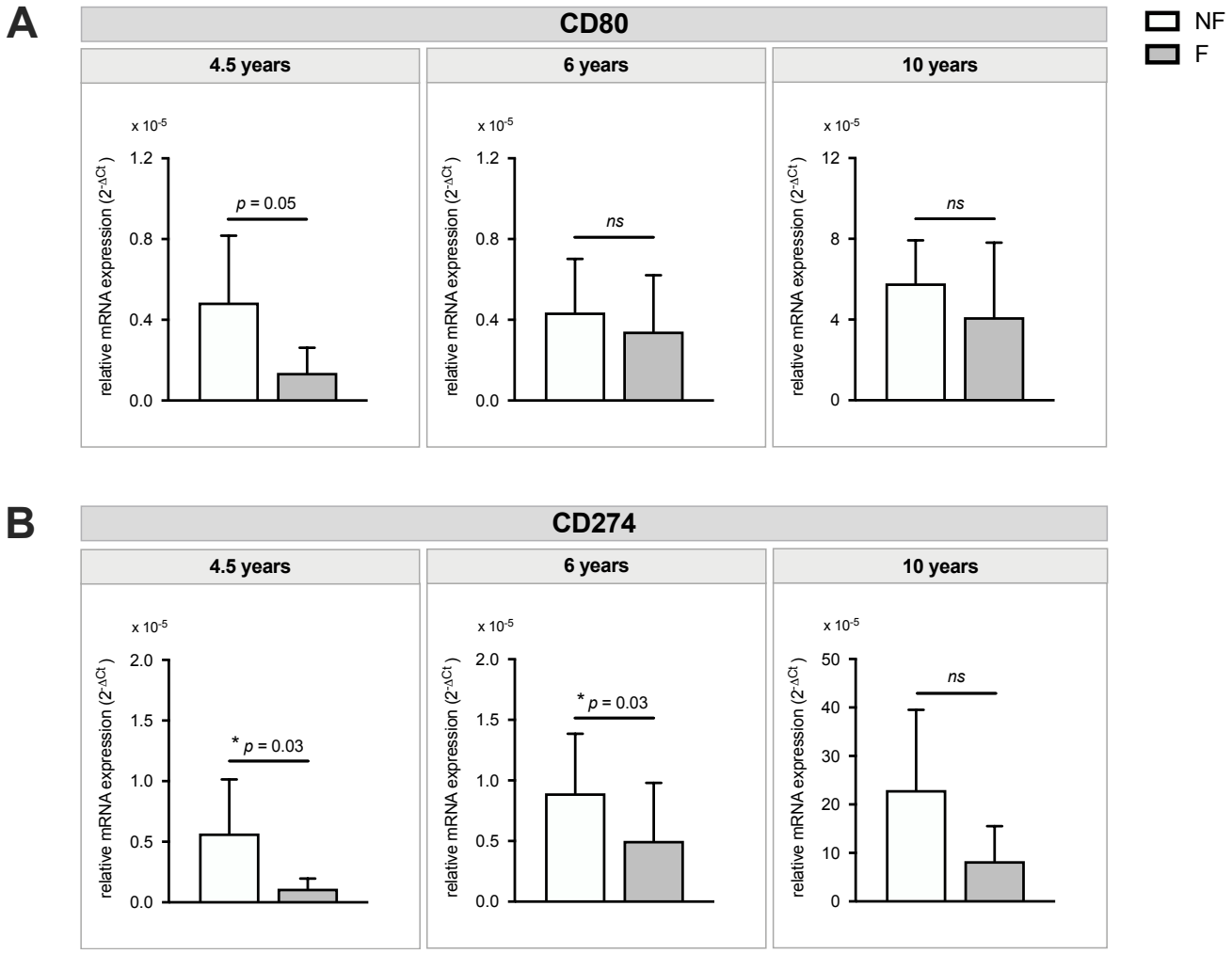

Figure 32. CD80 and CD274 mRNA expression in NF and F children.
Baseline gene expression levels of dendritic cell markers CD80 **(A)** and CD274 **(B)**, as measured by qPCR in unstimulated whole blood samples of healthy and asthmatic farm (F) and non-farm (NF) children, assessed at ages 4.5 years (n=37, 18 F/ 19 NF) and 6 years (n=63, 32 F/31 NF), and in unstimulated PBMCs of healthy 10-year-old children (n=22, 5 F/17 NF). Expression relative to 18S, displayed as $2^{-\Delta Ct}$ mean values with 95% CI. *P* values reflect Welch's t test for ΔCt comparison between NF and F. **p* < 0.05; *ns*, not significant.

Table 13 summarises the findings of chapter **3.3.2**, reviewing baseline gene expression patterns (i.e. in unstimulated cells) of farm (F) compared to non-farm (NF) children.

Altogether, farm children showed a significantly lower expression of *TNFAIP3* at the age of 6, while no difference in *TLR4* expression was seen when compared with non-farm children at all three time points of assessment (4.5, 6 and 10 years of age). Similarly, both *CD80* as a marker of dendritic cell activation and *CD274*, the T cell suppressive molecule on dendritic cells, were found decreased in farm children.

It is noteworthy that findings of significantly lower *TNFAIP3, CD80* and *CD274* gene expression in farm children have been replicated in another non-European cohort comprising a total of 112 7-year-old children from asthma-protective rural Conghua[108] (n = 58) and urban Hong Kong (n = 54) ($p < 0.001$; data not shown here, but integrated in the publication[321]).

Table 13. *Differential baseline expression of TNFAIP3, TLR4, CD80, CD274 among F and NF.*

Gene	Stimulus	mRNA expression level – F vs. NF		
		4.5 years	6 years	10 years
TNFAIP3	unstim.	↔	↓*	↔
TLR4	unstim.	↔	↔	↔
CD80	unstim.	↓	↔	↔
CD274	unstim.	↓*	↓*	↔

mRNA expression levels of TNFAIP3, TLR4, CD80 and CD274, as measured by qPCR in unstimulated whole blood (for 4.5- and 6-year-old children) or PBMCs, respectively (10-year-old children). Comparison of gene expression between farm children (F) and non-farm children (NF) at ages 4.5 years (18 F vs. 19 NF), 6 years (32 F vs. 31 NF) and 10 years (5 F vs. 17 NF). ↓ down-arrow (red) represents decreased mRNA expression in F vs. NF. Coloured arrows without asterisks indicate trend-wise effects. ↔ indicates no significant difference. Statistical significance of differences is indicated by the number of asterisks. *P* values from Welch's t test for ΔCt comparison between NF and F. *$p < 0.05$.

3.3.3 Decreased *TNFAIP3* expression in farm children with allergic asthma

To study *TNFAIP3* expression at the very interface between protection and disease, stratified analysis for farm exposure and clinical phenotype was performed in a small subset of 6-year-old children within the German PASTURE/EFRAIM cohort (total n = 22). Immune cells of farm children with allergic asthma (F-AA; n = 4) were analysed for *TNFAIP3* expression and compared to their healthy farm (F-HC; n = 7) and healthy and asthmatic non-farming peers (NF-HC; n = 7 and NF-AA; n = 4). The limited number of cases is partly due to the very low natural prevalence of atopic conditions and asthma in farm children.

First, the previous finding of decreased baseline *TNFAIP3* levels in patients with allergic asthma (see **3.1.2.2**) was confirmed in this subsample cohort (14 HC vs. 8 AA, $p = 0.05$; **Figure 33, 'all'**). When stratifying for farming exposure, consistently lower *TNFAIP3* expression was found in F-AA compared to F-HC ($p = 0.047$; **Figure 33, 'F'-bars**), while phenotype differences were not significant among non-farm children under unstimulated conditions (**Figure 33, 'NF'-bars**).

Within the group of allergic asthmatics (n = 8), F-AA children exhibited even decreased *TNFAIP3* expression compared to NF-AA children ($p = 0.05$). **Overall, *TNFAIP3* expression levels were lowest in farm children suffering from allergic asthma.**

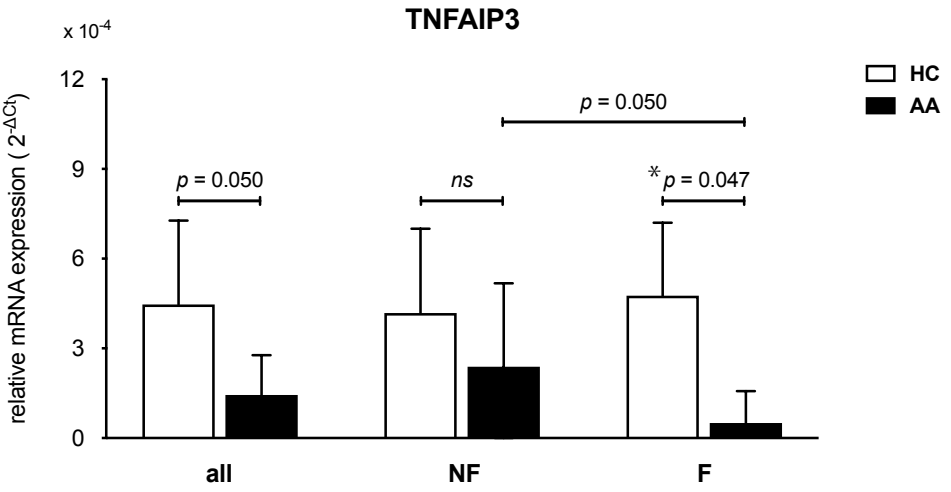

Figure 33. TNFAIP3 mRNA expression in healthy and asthmatic NF and F children.
Gene expression levels, as measured in unstimulated whole blood samples of 6-year-old healthy and asthmatic children from the German PASTURE subset ('all' n=22, 14 HC/8 AA). Healthy non-farm (NF-HC, n=7) and farm children (F-HC, n=7) and asthmatics (NF-AA, n =4, and F-AA, n=4, respectively). Mean values with 95% CI. *P* values reflect Welch's t test for phenotype comparisons (AA vs. HC). *$p < 0.05$; *ns*, not significant.

3.3.4 Anti-inflammatory immune response upon *in vitro* LPS and farm dust stimulation in F and NF

In order to assess specific immune responses of the farm children upon acute *in vitro* LPS and farm dust exposure, changes in expression of the selected genes *TNFAIP3, TLR4* and *CD80, CD274* were analysed upon 24 hour-stimulation with LPS and the farm dust extracts (G, Fi, Am).

In line with the findings observed in the urban CLARA/CLAUS cohort, *in vitro* LPS stimulation resulted in upregulation of anti-inflammatory *TNFAIP3* in farm and non-farm children's immune cells of all age groups (*$p < 0.05$, **$p < 0.01$; **Figure 34, A**). Significant effects were also achieved for stimulation with the Amish farm dust extract in the 10-year-old farm children ($p = 0.002$; **Figure 34, A, 10-year-panel**).

Furthermore, *TLR4* expression was significantly downregulated in both F and NF children at all ages and upon both LPS and farm dust (G, Fi, Am) stimulation (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; **Figure 34, B**), again confirming the results from stimulation experiments in the urban cohort that have been described in chapter **3.2.**

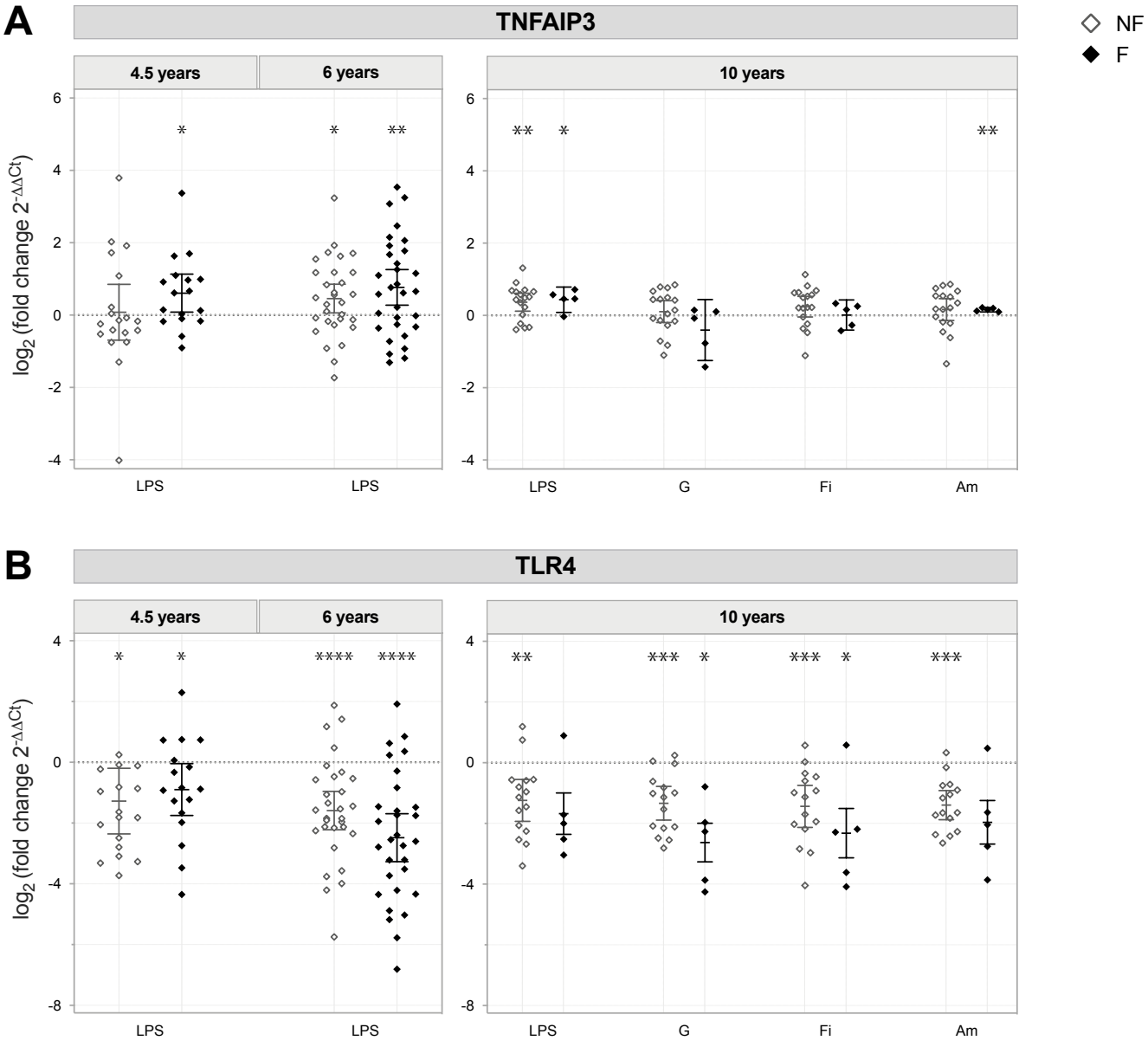

Figure 34. Fold change of TNFAIP3 and TLR4 mRNA expression upon stimulation in NF and F.
(A) TNFAIP3 and **(B)** TLR4 gene expression fold change, compared to unstimulated conditions ($2^{-\Delta\Delta Ct}$, $\log_2$ scale), as assessed by qPCR after 24 hrs stimulation with LPS and farm dust extracts (G, Fi, Am). LPS stimulated whole blood samples of 4.5 year-old farm (F) (n=17) and non-farm (NF) (n=19) and 6 year-old F (n=30) and NF (n=30) healthy and asthmatic children, and LPS and farm dust (G, Fi, Am) stimulated PBMCs of 10-year-old healthy F (n=5) and NF (n=17) children. Scatter dot plots represent single raw data fold change values with sample means and 95% CI. Statistical significance of differences of gene expression compared to the unstimulated condition is marked by asterisks based on paired t test analysis. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Consistent findings were also observed for gene expression of the dendritic cell molecules *CD80* and *CD274*. LPS and dust stimulation resulted in a strong upregulation of these genes both in F and NF children (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; **Figure 35, A and B**).

In summary, the capacity to increase the expression of the anti-inflammatory genes *TNFAIP3* and *CD274* and to decrease the expression of pro-inflammatory *TLR4* was preserved in farm children upon *in vitro* LPS and farm dust exposure.

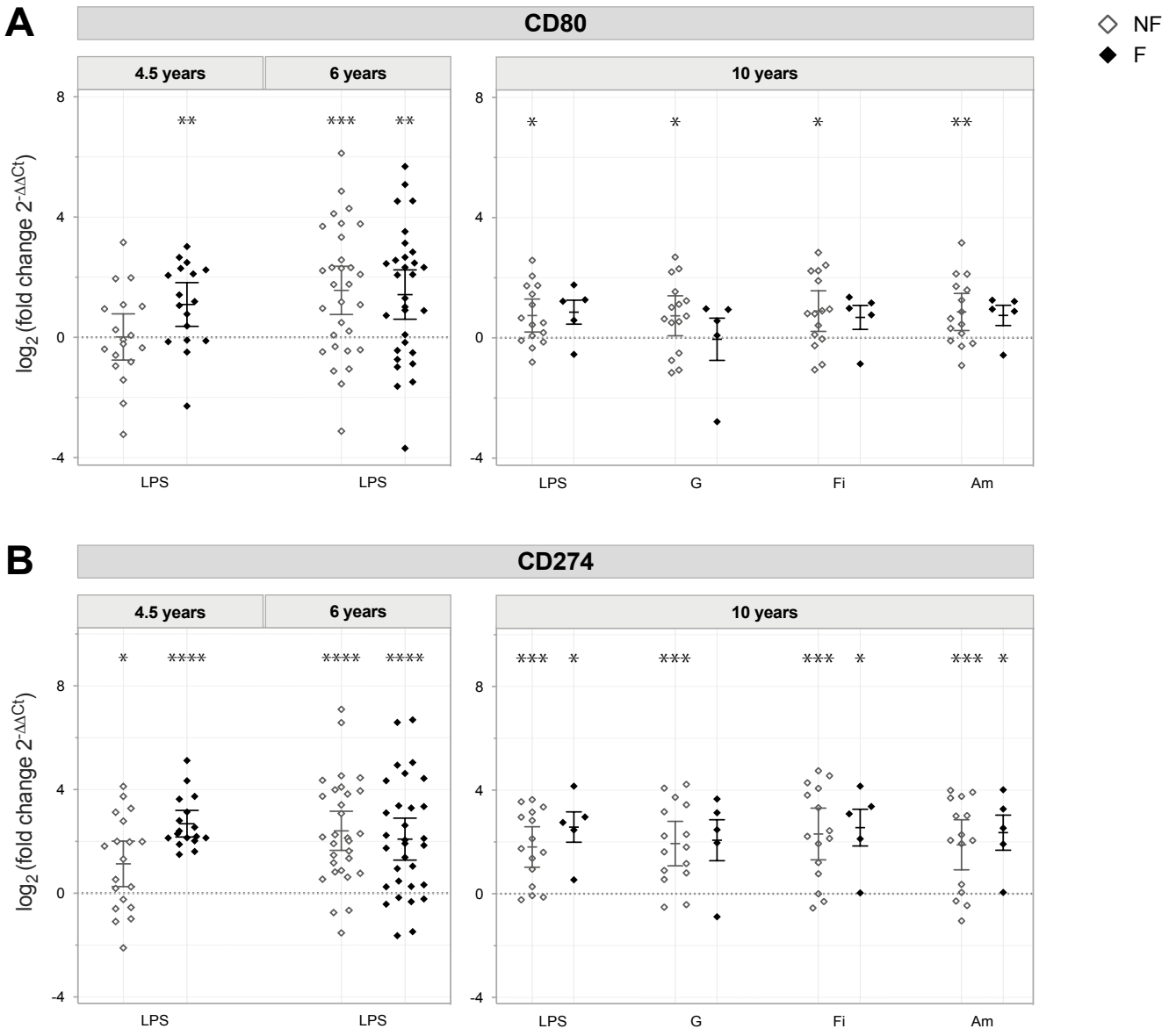

Figure 35. Fold change of CD80 and CD274 mRNA expression upon stimulation in NF and F.
(A) CD80 and **(B)** CD274 gene expression fold change, compared to unstimulated conditions ($2^{-\Delta\Delta Ct}$, $\log_2$ scale), as assessed by qPCR after 24 hrs stimulation with LPS and farm dust extracts (G, Fi, Am). LPS stimulated whole blood samples of 4.5 year-old farm (F) (n=17) and non-farm (NF) (n=19) and 6 year-old F (n=30) and NF (n=30) healthy and asthmatic children, and LPS and farm dust (G, Fi, Am) stimulated PBMCs of 10-year-old healthy F (n=5) and NF (n=17) children. Scatter dot plots represent single raw data fold change values with sample means and 95% CI. Statistical significance of differences of gene expression compared to the unstimulated condition is marked by asterisks based on paired t test analysis. *$p < 0.05$, **$p < 0.01$. ***$p < 0.001$, ****$p < 0.0001$.

Heat maps depicted in **Figure 36** summarise the findings of chapter **3.3.4**, providing colour-coded information about the mRNA response (i.e. up-/and downregulation) to the different treatment conditions for farm and non-farm children (F and NF) and for each of the genes *TNFAIP3, TLR4, CD80, CD274*. The direction of regulation is represented as red-/blue-gradient, with blue indicating upregulation and red representing downregulation of gene expression. Colour saturation reflects the strength of the effect in terms of the fold change of expression ($2^{-\Delta\Delta Ct}$, $\log_2$ scale). Significant regulation compared to the unstimulated condition is indicated by asterisks (*$p < 0.05$, **$p < 0.01$. ***$p < 0.001$, ****$p < 0.0001$).

Overall, stimulation with LPS and dust extracts from German (G), Finnish (Fi) and Amish (Am) farms significantly decreased gene expression of the pro-inflammatory *TLR4* (red), while it increased anti-inflammatory *TNFAIP3, CD80* and *CD274* (blue), with similar effects in F and NF children at all investigated time points (4.5, 6 and 10 years of age).

In the 10-year-old farm children, stimulation with German (G) dust samples yielded different responses regarding the strength and direction of effects when compared to Finnish (Fi) and Amish (Fi) dust stimulation (**Figure 36, panel F, 10y**).

Again, *TLR4* downregulation and *TNFAIP3, CD80* and *CD274* upregulation effects have been replicated in PBMCs of both rural (n = 55) and urban (n = 49) children from the above-mentioned Chinese TRILATERAL cohort upon LPS stimulation (data not shown here, see publication[321]).

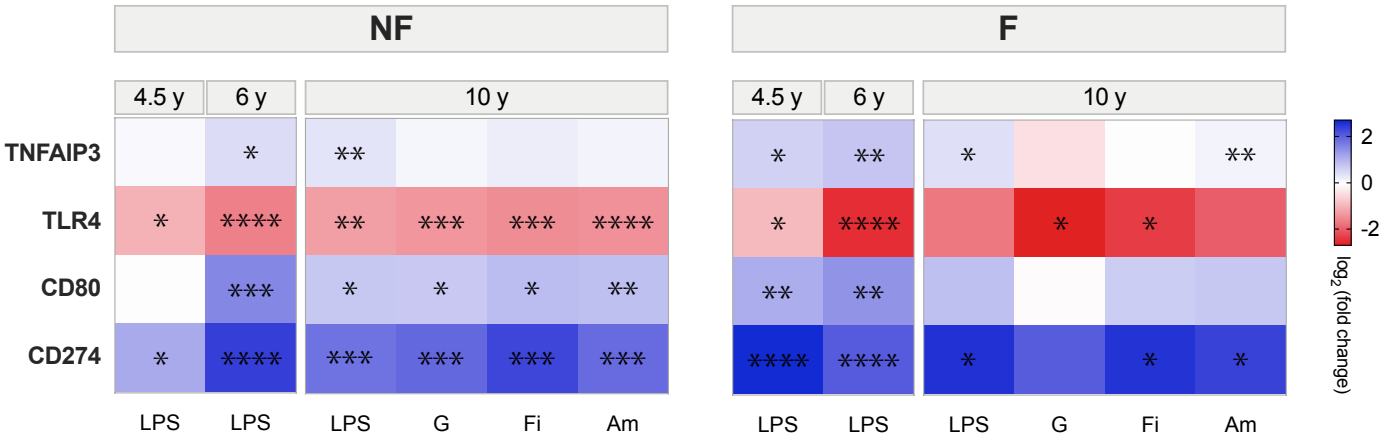

Figure 36. Overview of *in vitro* LPS and farm dust stimulation effects in NF and F.
Colour-coded up-/and downregulation of mRNA expression of TNFAIP3, TLR4, CD80 and CD274. Fold change of gene expression compared to unstimulated conditions ($2^{-\Delta\Delta Ct}$, $\log_2$ scale) in LPS stimulated whole blood samples of 4.5- and 6-year-old farm (F) (n=17 and n=30) and non-farm (NF) (n=19 and n=30) healthy and asthmatic children, and LPS and farm dust (G, Fi, Am) stimulated PBMCs of 10-year-old healthy F (n=5) and NF (n=17) children. Blue colour of cells indicates upregulation, red colour indicates downregulation of gene expression. Saturation reflects the strength of the fold change effect. Statistical significance of differences of gene expression compared to the unstimulated condition is marked by asterisks based on paired t test analysis. *$p < 0.05$, **$p < 0.01$. ***$p < 0.001$, ****$p < 0.0001$.

As outlined before (**Figure 36**)**,** in the 10-year-old group of German farm children, stimulation with the German dust samples resulted in gene expression responses that differed from those observed after stimulation with LPS and the Finnish or Amish dust samples (**Figure 37**). *TLR4* downregulation was significantly stronger upon German dust stimulation, while anti-inflammatory *TNFAIP3* as well as *CD80* and *CD274* were less upregulated compared to LPS, Fi and Am. Despite the very small sample size of this farming children subgroup (n = 5), effects partly yielded significant levels (for *TLR4* $p = 0.031$ for comparison G vs. LPS, $p = 0.037$ for G vs. Am; **Figure 37**). However, the majority was seen as trend-wise effects ($p \leq 0.10$).

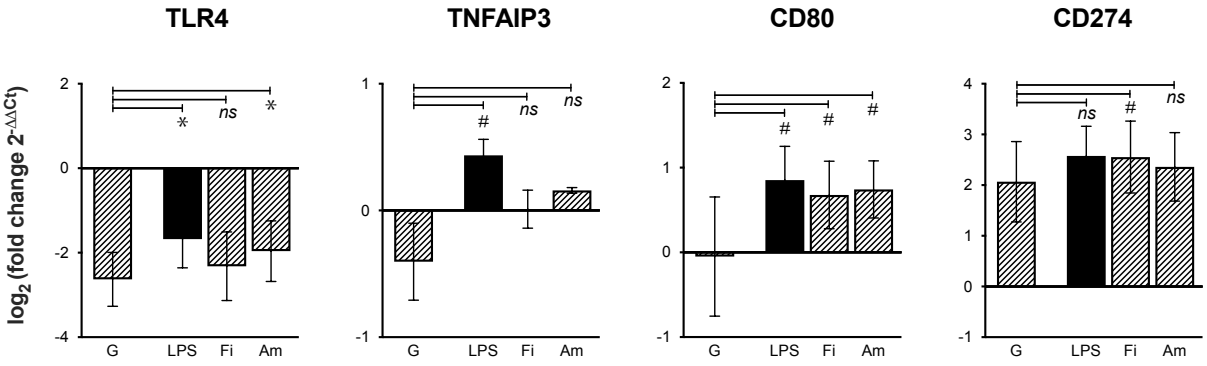

Figure 37. Fold change of TLR4, TNFAIP3, CD80 and CD274 mRNA expression upon stimulation in German F children.

Gene expression fold change, as measured in PBMCs of 10-year-old German farm children (n=5) after 24 hrs stimulation with German (G) farm dust extract as well as LPS, Finnish (Fi) and Amish (Am) farm dust. Mean $\log_2$ of fold change values ($2^{-\Delta\Delta Ct}$). Error bars indicate SEM. *P* values based on paired t test analysis for comparisons of G stimulation vs. LPS, Fi and Am, respectively. # indicates trend-wise effects ($p \leq 0.10$). *$p < 0.05$; *ns*, not significant.

4 Discussion

4.1 Summary of the main findings

The aim of this study was to investigate the role of TNFAIP3 and related signalling pathways in the development of allergic asthma and its environmentally mediated protection in children. Gene expression and protein levels were assessed in 148 peripheral blood immune cell samples of healthy and asthmatic children of different ages from two distinct paediatric study cohorts. Analyses in peripheral blood mononuclear cells revealed a reduced expression of anti-inflammatory TNFAIP3 gene and protein in allergic asthmatic children, while the expression of the pro-inflammatory pathway genes *TLR4* and *MyD88* was increased. *In vitro* stimulation with LPS as well as dust extracts from different asthma-protective farming environments restored TNFAIP3 expression of asthmatic patients to healthy levels and reduced the expression of the pro-inflammatory TLR4-pathway-related target genes (*TLR4, MyD88, TRAF6*). The observed anti-inflammatory effects were accompanied by significant downregulation of dendritic cell associated co-stimulatory molecule *CD86* and upregulation of the co-inhibitory molecule *CD274* upon stimulation. Whole blood samples of farm children at ages 4.5, 6 and 10 years exhibited decreased expression levels of both pro- and anti-inflammatory genes compared to their non-farming peers, but the capacity to induce *TNFAIP3* and *CD274* expression and to downregulate *TLR4* levels upon LPS and farm-dust stimulation was preserved at all ages.
In the following these findings will be discussed in the context of the present state of relevant studies, considering both methodological and content-related aspects.

4.2 Discussion of the study methods and experimental design

4.2.1 Survey data and conjoint analyses in the PASTURE/EFRAIM study population

Blood samples from the PASTURE/EFRAIM farming birth cohort provided a unique opportunity to examine (innate) immune regulation in farmer's children within the scope of this work. Experimental research studying the immunology of the protective 'farm effect' on asthma in humans is rarely found, thereby highlighting the significance of the current investigations. The prospective nature of the study avoided recall bias and supports reliability of questionnaire-based data on farming exposures and atopic outcomes. Since a random selection of farm and non-farm children was not possible due to limited sample availability and the aim of a balanced distribution regarding demographic and clinical aspects, the presence of sample selection bias cannot be excluded.

Samples from three different study centres (Germany, Finland, France) were assayed for immunological analyses. Both fieldwork and laboratory procedures that were conducted prior to the experiments demonstrated herein had been checked for coherence between the centres. Blood sampling

and processing steps in particular had been standardised and validated by a series of extensive quality control measures beforehand[296]. Therefore, conjoint analysis of the present data was possible. Please refer to corresponding earlier publications of the PASTURE study project for more detailed considerations regarding the study design.

4.2.2 Time points of clinical and immunological assessment

Blood samples of the PASTURE/EFRAIM farming cohort were screened for the selected target genes at three different time points, thus making it possible to address the question of long-lasting effects of early pre- and postnatal farming exposures[322]. However, valid longitudinal analysis was only possible to a limited extent, since blood samples of some children were not available at age 4.5 years. Moreover, blood samples from children younger than 4.5 years were not available for analysis in this particular study cohort. However, protective effects of microbial exposure are considered to be greatest during early infancy[109], and similar timeframes for concomitant immunological changes are proposed[323]. It must be conceded that, on the basis of the above mentioned, the crucial time window in which decisive immunological processes may develop might have been missed in the current immunological assessment. On the other hand, assessment at ages 4.5 to 10 years captures the time period when allergic asthma manifests itself in most children, thus making it possible to investigate immunoregulatory effects in the context of both farming exposures and children's health outcomes at the same time. This is a major strength of this study despite limited group sizes particularly of asthmatic farm children, which is largely due to the naturally low prevalence of atopic conditions in farm children.

Since a doctor's diagnosis of asthma is a criterion possibly to be biased in patients under 6 years of age, as children with transient wheeze may also be included[324,325], asthma cases in farm and non-farm children of the PASTURE/EFRAIM population were investigated only at the age of 6 years.
In the CLARA/CLAUS study sample, however, a small number of children under 6 years (n = 2) were also included as 'allergic asthmatics'. Since these two patients were seen by a paediatric allergology specialist, demonstrating typical asthma symptoms, with corresponding clinical history and impaired lung function results, inclusion in the present study was possible.

4.2.3 Investigated sample material – PBMCs and whole blood

Principal among the limitations of the study is the fact that immunological assessment focussed on systemic immunity by using peripheral blood samples as the primary material. It is conceded that any such model system may not adequately represent the *in vivo* local situation. Undoubtedly, decisive disease determining inflammatory changes do occur in the respiratory tract[209], and circulating peripheral immune cells may be unlikely to reflect these local immunological processes. Furthermore, a considerable proportion of PBMCs isolated from whole blood is likely to be phenotypically and functionally distinct from immune cells of the respiratory mucosa or pulmonary lymphatic tissue.

In vitro findings from children's PBMCs can therefore not be directly related to immune responses which occur at the critical site of action, i.e. the lungs.
The demonstrated experimental approach may yet be interpreted in the light of the inherent limitations of a clinically based study in young children, which requires an easy and ethically reasonable access to appropriate sample material. In fact, any immunological study involving the acquisition of bronchoalveolar lavage (BAL) or even bronchial biopsy samples in a paediatric cohort of non- or mildly to moderately disease-affected individuals would not undergo approval by the human ethics committee, nor by the children's parents for comprehensible reasons.
Notwithstanding this, pulmonary immune cells are also largely recruited from systemic circulation, and increasing evidence suggests that processes other than those happening locally in the airways may be involved in the pathogenesis of childhood asthma. The proposed causal relationship between adipose-related systemic chronic inflammation and asthma[326] may exemplify this conception. Not least because of the farming studies and recent microbiome research, it has become clear that different sites of the body are most likely involved in controlling innate and adaptive immune functions influencing asthma development[126,327,328]. In view of this, and because of the fact that the above-reported *in vitro* findings obtained from children's PBMCs replicated previous findings from animal *in vivo* model systems[118,261,329], relevant physiological significance of the observed effects may well be assumed.

Data derived from unfractionated cells (i.e. PBMCs, or whole blood samples in the PASTURE cohort, respectively) are likely to be confounded by even minor differences in cell proportions between groups. PBMCs constitute a heterogeneous group of peripheral immune cells, comprising the lymphocyte populations (T cells, B cells, and NK cells), as well as monocytes and a small proportion of dendritic cells[330], whereas whole blood samples additionally contain nucleus-free red blood cells and polymorphonuclear granulocytes (neutrophils, basophils and eosinophils). Since peripheral blood samples of cases (AA) and controls (HC), or farm and non-farm children respectively, exhibited similar proportions of leukocyte subsets, the observed gene expression profiles are largely unlikely to be due to differential composition of cell populations.
Mechanistic interpretation of the immunological findings gained from PBMCs is further complicated by the fact that an observed effect cannot be allocated to a distinct cellular source. This particularly applies to the dendritic cell population, which accounts for only about 1-2 percent of human PBMCs[331]. Cell-type specific gene expression effects might be overlaid by opposing effects or even be lost within the summed signal of the bulk sample.

Notwithstanding these considerations, the use of unfractionated cells constituted a reasonable experimental approach in the present project, whose objective was considered to be essentially exploratory. In fact, the current data provided the basis for experiments subsequent to this work, which were able to confirm the present findings in isolated cell populations[321]. Several follow-up projects on isolated dendritic cells as well as regulatory T cells are in progress, with data to be published by the research group in the near future. These more elaborate model systems are certainly required in order

to gain further mechanistic insights and ultimately be able to translate findings into the human *in vivo* system.

4.2.4 Immunological assessment methods

Immunological phenotype signatures as well as *in vitro* stimulation effects in children's PBMCs and whole blood leukocytes were assessed by analysing gene expression and protein levels. Quantitative real time PCR (qPCR) was considered an appropriate experimental readout method for the purposes of this work, as it allows objective and precise quantification of target genes. Given the fact that qPCR is still a technique very liable to interference, extensive quality control measures were performed (as outlined in the methods section), allowing high process standards throughout all experiments.
Gene expression profiling *per se*, however, provides only limited information about functional outcomes. For evaluating DC-T cell-dependent immune responses in particular, further experimental readouts such as cytokine studies are required. Furthermore, it remains subject to speculation whether detected mRNA is also translated into protein, and to what extent mRNA regulation is associated with changes in functional protein at all. To answer this question at least for TNFAIP3 as the key molecule in this project, Western Blot analysis was performed. Particular effort was made to optimise all process steps and components and establish an ideal standard operating procedure (see methods section), which provided the basic framework for further experimental projects of the study group.

4.2.5 *In vitro* LPS and farm dust stimulation model

The *in vitro* farm dust stimulation model performed in this work was a valuable experimental approach towards a clearer understanding of the mechanisms underlying the farm effect. Limitations and challenges inherent in this model-based approach will be reflected in the following.

Firstly, a single dose stimulation regimen was employed, although it must be considered that this may not ideally mirror the effects of chronic *in vivo* exposure in the natural farming environment. Since stimulation significantly affects the natural viability of human primary immune cells, however, repetitive stimulation and a long-term cell-culture setting would have biased immunological outcomes due to increased cytotoxic side effects. Moreover, experimental studies in animal models have shown that single high dose LPS as well as farm dust exposure generated similar beneficial effects as did chronic low-dose administration[261].
A robust protective effect of a certain exposure is characterised by preventing or suppressing the development of pathophysiological features defining the disease phenotype. Inflammatory responses in allergic asthmatic patients are mostly triggered when re-exposed to specific allergens, which highlights the importance of allergen challenge in any model-based approach. In fact, unlike previous *in vivo* and *in vitro* experimental studies investigating the protective effect of farming exposures[118,178,238,261,332], the current experiments did not employ assessment of immunological

outcomes upon allergen exposure. Thus, the observed treatment effects of LPS and the farm dust extracts may predict the *in vivo* outcome in allergic asthmatic children only to a limited extent, and care must be taken when drawing conclusions about their asthma-protective properties from the present model system.

To address the issue of dose-dependency of effects and gene expression kinetics, preliminary experiments were carried out in children's PBMCs prior to the start of the study. Comparative analyses of two time points of assessment (8 hrs and 24 hrs), and of three different doses of farm dust extracts respectively, revealed only minimal differences regarding target gene expression (data not shown), suggesting that the potency of farm dust treatment is widely guaranteed. Considering the observation of a dose-dependent dual effect of LPS *in vivo*[333], however, it is admitted that further experiments are necessary to address the question of a safe and 'protective' LPS and farm dust dose.

Finally, *in vitro* LPS and farm dust exposure was modelled in peripheral blood immune cells, most equivalent to a systemic treatment approach. Following the 'proof of concept' principle, this was a reasonable and straight-forward approach to further elucidate the characteristic immunomodulatory effects of farm dust exposure in the human system. Although asthma-protective effects have actually been demonstrated for systemic (i.e. intravenous or intraperitoneal respectively) LPS exposure in murine model systems[239,238], it is conceded that this constitutes an artificial route of exposure. *In vivo* farming exposures are most likely to occur through inhalation and oral ingestion[126], which strengthens the notion of the mucosal barriers as being the critical site of action in the natural system. There is increasing evidence to suggest that the complex interplay of incorporated farm-derived microbiota and allergens with the interacting network of immune cells and barrier epithelial cells is decisive for the protective *in vivo* farm-effect[33]. In the natural setting, direct engagement between immune cells and certain farm dust constituents might be limited, since macromolecules, for instance, may not cross the epithelial tight-junction barrier. Organotypic cultures of primary human cells are therefore required to illustrate processes at the mucosal interface in the best way possible. In this context, elaborate work establishing air-liquid interface (ALI) cultures of human adult airway epithelial cells (ECs) has already been done[261]. Findings from such models, together with a more detailed knowledge about chemical and physical properties of composed dust samples (e.g. molecular weight fractions (*work in progress*)), will greatly improve our understanding and provide the basis for subsequent experimental approaches in paediatric cohorts as well.

Only if there is absolute certainty about the impact of dosage, timing, composition and mode of exposure may the vision of a farm dust-derived treatment strategy for childhood asthma be further pursued.

4.3 Discussion of the main findings

4.3.1 Clinical characterisation of the allergic asthmatic phenotype

Allergic asthma is the most common asthma phenotype in children and is represented by a clear pattern of well-established clinical and biological features[11,29,334]. Allergic asthmatic children that were recruited for the current project demonstrated the distinctive biological characteristics of the phenotype, as defined by positive sensitisation to allergens, elevated levels of total IgE and peripheral blood eosinophilia[28]. Consistent with a mild to moderate disease severity profile which has been typically associated with this phenotype[11], prebronchodilator lung function parameters were only mildly affected (FEV_1 > 80% predicted in most AA patients) and were reversed to normal in the majority of children. The varying degree of clinical symptom severity among AA children, which was also reflected in the large variance of lung function measures, may be partly due to recruitment during different pollen seasons.

In accordance with the commonly defined risk factors for early-onset allergic asthma, questionnaire data revealed an increased rate of allergic comorbidities such as hay fever and atopic dermatitis among AA children[29,11], as well as an increased prevalence of a maternal history of asthma and allergic disease[335,336] and a higher level of parental education. Despite a strong association of atopy and asthma in school-age children, atopic sensitisation *per se* has not been finally proven to be an independent predictive factor for asthma susceptibility[337]. In fact, around 17% of healthy control children enrolled in the study exhibited positive atopic sensitisation to a single allergen without clinical symptoms of allergic disease, which is consistent with prevalence rates derived from large-scale epidemiological studies[338]. In contrast, poly-sensitisation (i.e. sensitisation to multiple allergens) and higher levels of allergen-specific IgE were observed in AA children, supporting the assumption that the intensity of sensitisation largely determines asthma risk in this age group[182]. Both allergen specific IgE and comorbid allergic diseases remained strong discriminating factors between AA and HC in the present study. Since valid confounding analyses were not feasible due to limited sample size, however, it cannot be excluded that the observed immunological effects in AA children were at least partly mediated by the atopy component rather than arising from the asthmatic condition *per se*.

Together, the clear characterisation and precise definition of AA cases and HC subjects underscores the validity of the reported findings with respect to the distinct clinical phenotype of allergic asthma in school-age children.

4.3.2 Inflammatory innate immune signature of school-age children with allergic asthma

The present study revealed a distinct and conclusive gene expression profile in asthmatic children's peripheral blood mononuclear cells, particularly highlighting the role of TNFAIP3 and NF-κB pathway-related target genes. Compared to healthy controls, school-aged urban children with allergic

asthma showed significantly increased expression of *TLR4* and its adaptor *MyD88*, while levels of the anti-inflammatory molecule *TNFAIP3* were significantly reduced on transcriptional and translational level under unstimulated conditions (i.e. at baseline). Similarly, baseline mRNA expression of the co-stimulatory dendritic cell surface molecule *CD80* was decreased.

It is now well known that TLRs as innate immune receptors do not only play a vital role in sensing and transmitting signals, but are also critically involved in modulating adaptive immune responses[232]. Direct TLR stimulation is required for effective DC activation and subsequent T cell priming[231]. The role of TLRs - especially TLR4 - and ensuing signalling in the development of allergic responses and asthma pathogenesis is complex, and its contribution to airway inflammatory responses remains incompletely understood. Genetic alterations in TLR4 were found to modify asthma risk, although results are conflicting (as reviewed by *Tesse et al.*[234]). In the present study population, significantly elevated *TLR4* expression was visible in asthmatic patients' PBMCs compared to healthy controls. So far, few other studies have assessed the role of TLR4 in patients with allergic asthma, and those studies conducted in adult patients exhibited major limitations and provided inconsistent results[339-341]. Aside from differences in the patients' age, a valid comparison with these studies is hampered by a number of differing factors concerning methodology and study design (i.e. clinical characteristics including atopy status, investigated sample material, and readout method, among others). Furthermore, the highly dynamic nature that *TLR4* expression *in vivo* and *in vitro* is known for makes it difficult to interpret and compare data between experimental settings, and it is certainly one reason for the discrepancy of findings on *TLR4* expression in human immune cells. However, recent evidence has supported the present data with respect to the atopy component of allergic asthma, linking elevated TLR4 expression to high levels of IgE in asthmatic patients[342]. In fact, TLR4-mediated signalling, particularly from airway epithelial cells[343,344], has been proven essential for the determination of Th2-dominated immune responses to allergens and airway inflammation in experimental animal models of allergic asthma[345,346]. Landmark studies have demonstrated that some common aeroallergens can elicit allergic responses through direct or indirect engagement with TLR4[233,347,348]. In light of these findings, increased *TLR4* expression in allergic asthmatic children in the present study may constitute a reduced baseline threshold within innate immunity for mounting Th2-mediated inflammatory responses to varying environmental stimuli. Given the fact that the expression of TLR4 signalling adaptor *MyD88* was equally increased in asthmatic subjects, relevant downstream effects of enhanced TLR4 signalling may indeed be assumed. Moreover, *MyD88* levels paralleling those of *TLR4* may indicate that the MyD88-dependent rather than the TRIF-dependent TLR4 signalling pathway is affected in children with allergic asthma. Since MyD88 is involved in multiple convergent innate receptor signalling cascades, however, the contribution of other non-TLR4-related pathways must still be considered.

Chronic inflammation in asthma may result from a skewed balance of activation and control mechanisms of immunological processes and signalling pathways. Thus, impaired regulatory mechanisms would lead to exaggerated activation of immune responses, causing the development of

inflammatory disease. TNFAIP3 is critically involved at this point, acting as a negative regulator of TLR4-induced NF-κB activity, which has been associated with the pathogenesis of allergic asthma[248]. Few animal studies have provided evidence for a key function of TNFAIP3 in suppressing airway inflammatory responses upon allergen exposure[349,261]. Subsequent human *in vitro* approaches have primarily focussed on local processes in the lung, investigating the role of TNFAIP3 in airway epithelial cells[350,261]. However, TNFAIP3 expression in myeloid cells and especially DCs has recently been proven to be equally required for suppressing allergen induced airway inflammation[260]. Against this background, the present finding of decreased TNFAIP3 gene and protein expression in peripheral blood immune cells of allergic asthmatic children is of particular relevance. The fact that *TNFAIP3* was highly correlated with all other NF-κB pathway-related target genes in these patients further underscores the conception of TNFAIP3 as a biological determinant of dysregulated innate immunity pathways in childhood asthma. Recent data from our study group have reinforced this assumption with particular regard to the early determination of the disease. Cord blood mononuclear cell (CMBC) samples of newborns with subsequent asthma development in childhood exhibited decreased *TNFAIP3* expression, suggesting that *TNFAIP3* levels may even help to predict the individual's asthma risk at birth (see publication[321]). Since a similar association was found for *TNFAIP3* expression and comorbid hay fever in the present AA group, it may be hypothesised that TNFAIP3-dependent regulatory processes are particularly relevant in the development of the early-onset allergic asthma phenotype rather than the asthmatic condition *per se*.

Overall, the present data strongly point towards a pivotal role of the anti-inflammatory molecule TNFAIP3 in the development of allergic asthma in children. The current observations of elevated levels of *TLR4* and *MyD88* along with decreased TNFAIP3 gene and protein expression in asthmatic subjects strongly suggest that inflammatory processes are insufficiently contained in these patients.

Decreased TNFAIP3 protein levels were found in asthmatic children at a statistically significant level, although a remarkably large variance for the individual values was observed within this group. *Schuijs et al.* have shown a relation between TNFAIP3 levels in airway epithelial cells and asthma severity in a small sample of adult patients[261]. Although some heterogeneity in disease severity was actually observed in the present paediatric cohort, the current sample size was not sufficient to detect a reliable effect on TNFAIP3 protein levels. Thus, it may only be speculated on a similar association at this point. On this score, recent findings suggest that TNFAIP3 expression in DCs is essential for controlling the development of Th17-mediated neutrophilic airway inflammation, associated with a more severe asthma phenotype[260]. These observations are most intriguing due to the fact that the functional role of TNFAIP3 in DCs is directly linked to the determination of a specific immunological phenotype.

Notwithstanding this, the observed heterogeneity of expression may also reflect a different genetic makeup in these patients. Several functional domains have been identified in the *TNFAIP3* gene, and two of these have been directly linked to its ubiquitin-modifying activity[351,352]. Animal studies have

impressively demonstrated that selective disruptions in these domains can result in varying phenotypic traits[257,277]. This is particularly intriguing considering the fact that a distinct gene polymorphism located in the functional deubiquitinase domain of TNFAIP3 has recently been associated with increased susceptibility for asthma in a large-scale GWAS[261]. The variety of polymorphisms in the TNFAIP3 gene locus which have been associated with many other inflammatory and autoimmune diseases[263,353-358] highlights the great potential for further attempts in genetic research in paediatric asthma.

Apart from genetic modifications, a vast number of mechanisms have been determined to regulate the functional activity of TNFAIP3[264]. Post-translational modifications such as phosphorylation were shown to critically affect its enzymatic activity[359]. The additional TNFAIP3 protein band, which had been detected at slightly higher molecular weight than expected in all children's samples in Western Blot, may be interpreted against this background, although its definite relevance certainly needs to be addressed in future mechanistic approaches.

The activity of TNFAIP3 is further regulated by direct interactions with specific proteins, such as TNIP2, TAX1BP1 and MALT1, which were also addressed in the present study. Unlike TNFAIP3, the genes encoding these proteins did not appear to be differentially expressed in healthy and asthmatic children. TNIP2 (ABIN-2) constitutes one of three known TNIP (ABIN) proteins which act as negative regulators of NF-κB by enhancing TNFAIP3's ubiquitin-modifying activities on target proteins[267,266]. The functional role of the TNFAIP3- and ubiquitin-binding protein TAX1BP1 has been characterised very similarly[265]. Both TNIP1 and TNIP2 have been independently shown to be critically involved in regulating allergic airway inflammation in mice[360,270], but a potential relevance for human asthma has only been demonstrated for TNIP1[361]. **The current findings might therefore illustrate the minor role TNIP2 plays in mediating TNFAIP3's anti-inflammatory function.** However, it may well be supposed that the complex regulation of TNFAIP3's activity provided by a variety of molecules and mechanisms is governed by additive and complementary rather than exclusive effects. This also applies to the negative regulation of TNFAIP3, where proteolytic processing by MALT1 is only one among many other mechanisms that suppress TNFAIP3's inhibitory function on NF-κB dependent inflammatory responses[272,362]. Therefore, redundancy of biological activities within the whole regulatory network may explain why no differences between phenotypes were observed for the individual genes.

By controlling NF-κB activation, TNFAIP3 critically regulates the biology and function of dendritic cells. Selective TNFAIP3 deletion in murine DCs results in spontaneous DC maturation, hyperresponsiveness to TLR-signals, and aberrant adaptive immune responses[363]. In the context of asthma, the functional role of TNFAIP3 in DCs has been further defined as particularly regulating T cell differentiation into the Th17 cell subset[260].

Acting as 'gatekeepers of the immune system'[364], DCs are the key checkpoints in governing both immunity and tolerance. Changes in their phenotypic features, involving the expression of co-regulatory molecules, usually coincide with changes in their functional activity. The outcome of DC-

T cell encounter, i.e. the resulting T cell-mediated immune response, however, is not determined by the co-regulatory molecule expression status *per se*, but by the intricate network of immunogenic or tolerogenic signals transmitted during interaction with the different receptors on T cells. Bearing this in mind, the present data demonstrating gene expression of co-regulatory molecules unrelated to their counterpart receptors are of little significance. Considering the distinct receptor binding affinities of the co-regulatory molecules, however, more valid assumptions can be made about the T cell response they might induce. The finding of significantly decreased *CD80* expression in asthmatic children's PBMCs shall be discussed against this background.

It was very early proposed that CD80 (B7-1) and CD86 (B7-2) differentially affect T cell development[365]. Like CD86, CD80 is known to interact with the stimulatory receptor CD28 on T cells, but engages the inhibitory molecules CTLA-4 (CD152) and CD274 (PD-L1) with higher affinity[366,367]. Interaction of CD80, yet not CD86 with CTLA-4, was shown to enhance the suppressive capacity of regulatory T (Treg) cells[368]. Likewise, signalling through the co-inhibitory molecule CD274, which can specifically bind CD80[287], promotes the development and functional maintenance of Treg cells[369,370]. Although there is still some disagreement regarding the role of CD274 in the development of allergic asthma[371,292,293] more recent studies have provided evidence of its inhibitory function, particularly in controlling severe, Th17-mediated disease[372]. In contrast, CD86 expression appears to be essential for DC-dependent Th17 differentiation[373] and has been particularly associated with allergen-induced Th2 cell immune responses in both animal[278,279] and human[282] experimental models. A higher CD86/CD80 expression ratio on human DCs was associated with an allergic phenotype and a Th2-dominated rather than Th1-preferential immune response upon *in vitro* allergen exposure[282]. Thus, increasing evidence suggests that CD80 and CD86 may have opposing roles in regulating Th cell differentiation and that the type of co-regulatory molecules expressed on DCs and their relative expression levels may substantially determine the outcome of the adaptive immune response in the development of allergic asthma. **The current finding of a significantly elevated *CD86/CD80*-ratio in asthmatic children may therefore indicate a shift in the balance of co-regulatory molecules on activated DCs favouring the induction of inflammatory Th2 and Th17 cells rather than suppressive Treg or counterregulatory Th1 cells under steady-state conditions.**

However, mechanistic interpretation is complicated by the fact that DCs are not the only source of *CD80* and *CD86* expression in PBMCs. T cells were also shown to constitutively and inducibly express both molecules on their cell surface, thereby modulating immune responses through mainly CTLA-4-mediated T-T cell interactions[374]. Therefore, co-regulatory molecule expression cannot be exclusively related to the function of DCs. Furthermore, it may well be questioned whether the assessment of the steady, inactive state of circulating peripheral blood DCs, i.e. their maturation state under unstimulated conditions, represents an adequate model to illustrate the true pathological immunophenotype, which may manifest only upon allergen challenge and particularly in the diseased tissue, i.e. the lungs of asthmatic subjects. However, the strength and consistency of effects observed under unstimulated conditions suggests that the immune phenotype of allergic asthmatic children is determined by major

disruptions in innate immune pathways, which are even clearly distinguishable during steady state in the peripheral blood.

Taken together, the present study revealed features of an inflammatory immune phenotype in school-aged children with allergic asthma, suggestive of dysregulated TLR4-induced NF-κB mediated signalling. It may be proposed that impaired negative regulation by TNFAIP3 in asthmatic children results in altered DC biology, potentially driving aberrant immune responses in allergic asthmatic subjects.

4.3.3 Anti-inflammatory effects of *in vitro* farm dust exposure

Growing up in a farming environment constitutes a strong protective factor against childhood asthma and allergic disease[375]. The protective *in vivo* 'farm effect' is supposed to be mediated by exposure not only to high concentrations, but also to a great diversity of microbial agents[135,143]. It has been shown that airborne dust samples collected from traditional farming sites can highly reflect this *in vivo* environmental exposure of farm children[128]. To disentangle innate immune mechanisms that may explain this 'farm effect' on asthma, *in vitro* stimulation experiments with different farm-derived dust extracts and LPS were performed in children's PBMCs of the CLARA/CLAUS study cohort.
The present data clearly demonstrate that *in vitro* exposure to LPS and German (G), Finnish (Fi) and Amish (Am) farm dust extracts induces anti-inflammatory processes by increasing the expression of NF-κB inhibitor TNFAIP3, while consistently suppressing pro-inflammatory *TLR4*-pathway signalling genes in both healthy and asthmatic children. In the presence of LPS or farm dust (G, Fi, and Am) respectively, the expression of DC maturation markers *CD80, CD86, CD274* changed significantly, which indicates a crucial role for DCs in mediating the anti-inflammatory effects of LPS and farm dust treatment.

Numerous animal studies have shown the beneficial effects of exposure to microbial products[238,376], single bacterial species[329,142,332], or farm-derived dust samples[178,261,118] in controlling allergic airway inflammation and reducing cardinal features of allergic asthma. Mechanistic approaches have demonstrated that innate immune signalling particularly via the TLR4[329,238]-MyD88[118]-dependent pathway plays a key role in mediating these asthma-protective properties. **The present results of farm dust induced downregulation of the LPS receptor *TLR4* and its associated signalling adaptors *MyD88* and *TRAF6* corroborate these findings, indicating an immediate counter-regulatory process upon high-dose microbial exposure.** It may well be supposed that persistent signalling through the same pathway results in a sustained tolerogenic state characterised by non- or hypo-responsiveness to different immunogenic environmental stimuli[377]. Maintenance of any such state may be ensured by regulatory mechanisms at multiple levels, including effective control of proximal signalling as well as downstream NF-κB dependent pathways[377,378]. TNFAIP3 is known to attenuate

TLR4-induced NF-κB activation by inhibiting the activation and functional activity of TRAF6, and possibly other signalling intermediates upstream of IKK. Recent findings from a murine model of allergic asthma have attributed a key role to TNFAIP3 in mediating the protective effect of *in vivo* LPS and farm dust exposure, and have allocated the effect particularly to the structural cells of the airway epithelium[261]. The current study was the first to replicate these findings in primary human cells *in vitro*. **Analyses revealed significant upregulation of TNFAIP3 mRNA and protein levels in asthmatic children's PBMCs in response to LPS and farm dust stimulation, providing evidence for its relevant functional role in microbial-induced immunoregulation in humans as well.** Given the fact that it was possible to detect these effects in peripheral immune cells, a ubiquitously relevant rather than cell-type specific molecular mechanism may be proposed as determining the protective effect of the diverse microbial environment. In fact, findings from animal models have also suggested a similar role of TNFAIP3 in intestinal epithelial cells[379]. This is particularly intriguing as it may provide an explanatory molecular approach to the extensively studied protective effect of farm milk consumption, which may hardly involve the airway epithelium but may certainly implicate immunological processes at the mucosal surface of the intestine.

Notably, the expression of both positive and negative regulators of TNFAIP3 was virtually unaffected, suggesting the existence of alternative molecular mechanisms to regulate TNFAIP3 activity upon farm dust exposure.

Upregulation of TNFAIP3 may reflect a crucial regulatory mechanism through which farm dust exposure can suppress TLR4-mediated NF-κB induced inflammatory responses. The significant decrease in *IL-18* levels in the presence of farm dust and LPS clearly supports this assumption, as it suggests that the NF-κB dependent induction of other pro-inflammatory target genes may be reduced as well. However, more functional readouts are certainly needed to assess further downstream effects associated with the inhibition of NF-κB activation.

Besides barrier epithelial cells in the lung, gut and skin, antigen-presenting DCs are decisive sensors and transducers of TLR-mediated microbial-derived signals encountered in the farming environment[33]. A large body of evidence from animal and human studies points to their crucial involvement in the asthma-protective effect of farming exposures[142,178,206-208,332]. In fact, the above-discussed findings have been consistently replicated in DCs isolated from PBMCs from a small subsample of the present cohort (see publication[321]). Farm dust and LPS exposure may modulate immune responses by substantially influencing the phenotype and activation state of DCs, as indicated by the striking changes in expression of the co-regulatory molecules *CD80, CD86* and *CD274*. Strong upregulation of *CD80,* accompanied by significant downregulation of *CD86,* was observed in response to LPS and G, Fi, and Am farm dust treatment. This also became evident in a significant decrease of *CD86/CD80* ratio. Such opposing dynamics of the two co-stimulatory molecules may well be considered as an intriguing finding, since a comparable dichotomy in *CD80/CD86* surface expression has not been reported in similar studies before. In view of the above-mentioned assumption of the differential roles of CD80

vs. CD86 in modulating T cell development, however, elevated *CD80* together with reduced *CD86* levels may point to a DC-induced Th1/Treg polarising programme, which may inhibit pathogenic Th2 cell development in asthmatic subjects. In support of this, additional measurements in the same study population revealed significant farm dust induced upregulation of *CTLA-4*, the inhibitory receptor for CD80 and CD86, while expression of the stimulatory receptor *CD28* was reduced[321]. In line with these findings, *Schuijs et al.* recently demonstrated in their landmark study that chronic low-dose LPS or farm dust treatment significantly decreased the allergen-induced production of GM-CSF[261], a cytokine which was shown to programme maturing DCs for the induction of Th2 cells[380] and to reduce the threshold for allergen responsiveness[381]. Likewise, two independent studies conducted in human DCs have shown that incubation with different bacterial species isolated from cowshed dust modulated the immune response by inducing DC-dependent Th1 polarisation[142,332].

The net co-regulatory signal delivered by DCs of healthy and asthmatic children is further represented by the relation of co-inhibitory *CD274* to co-stimulatory *CD86* molecule expression. The remarkable increase observed for *CD274* expression and concomitant *CD274/CD86* ratio following LPS and farm dust stimulation supported the hypothesis of a farm dust induced 'inhibitory' phenotype of DCs. The vital role of CD274 (PD-L1) in mediating regulatory T (Treg) cell induction has been characterised previously[369]. A high CD274/CD86 ratio on plasmacytoid DCs (pDCs) in particular has been associated with elevated numbers of Treg cells[382]. The ability of pulmonary pDCs to suppress features of allergic asthma and inhibit the generation of effector T cells[201] has been linked to their increased expression of CD274[202]. Therefore, it seems likely that microbial exposure delivered by LPS or farm dust stimulation may induce the development of Treg cells through a CD274-dependent mechanism, and there is recent evidence from animal models supporting this notion. *Gollwitzer et al.* have demonstrated that distinct changes in airway microbiota colonisation during the neonatal period sustainably prevented allergic airway inflammation by promoting the CD274-dependent induction of functional Treg cell subsets[383].

Taken together, the remarkable increase of the *CD274/CD86* ratio accompanied by a significantly reduced *CD86/CD80* ratio upon LPS and farm dust (G, Fi, Am) treatment may illustrate a switch in co-regulatory molecule expression on DCs promoting the development of Treg cells suppressing Th17- and/or Th2-mediated immune responses.

Regardless of potential limitations in methodology and experimental setup as discussed in section **4.2**, *in vitro* stimulation experiments yielded interesting results. The present data demonstrate strong anti-inflammatory effects and potential asthma-protective properties of LPS and farm dust treatment in allergic asthmatic children. They point to a multicomponent mode of action, most likely involving the NF-κB inhibitor TNFAIP3 as a major determinant through which farm dust develops its beneficial potential. **It may well be proposed that farming exposure leads to reduced proximal innate receptor signalling and, by activating TNFAIP3 and altering maturation processes and**

functional activities of DCs, may dampen the TLR4-driven NF-κB dependent innate and subsequent adaptive immune response to environmental allergens.

The anti-inflammatory effects of G, Fi and Am farm dust and LPS treatment were consistently observed in both healthy and asthmatic children, indicating that beneficial effects of farming exposure may be relevant regardless of the clinical phenotype. Yet, allergic asthmatic patients downregulated pro-inflammatory *TLR4, MyD88* and *TRAF6* as well as *CD86* to a significantly greater extent. Likewise, upregulation of TNFAIP3 protein was stronger in asthmatic compared to healthy children when stimulated with the German, Finnish and Amish farm dust extracts. In this way, stimulation was able to restore gene expression features of a healthy immunophenotype in asthmatic children, and in particular raised their comparatively low basal expression of NF-κB inhibitor TNFAIP3 to 'healthy levels'. This is a remarkable finding, as it strongly suggests that farming exposure may counterbalance, or rather overcome the manifest inflammatory immune phenotype of allergic asthmatic children at the early stage of symptomatic disease and may even be able to suppress an underlying predisposition in some children. Thus, the current data point to a significant potential for a farm dust derived treatment approach in asthmatic children.

The question of clinical applicability, however, implies the need of defining a distinct composition of farm-derived microbial agents and products which provides the 'most protective' effects. **The current study demonstrated very consistent anti-inflammatory effects for 24-hour stimulation with a 0.1μg/ml LPS dose and with the German, Finnish and Amish farm dust extracts, whereas greatly divergent effects were observed upon stimulation with the dust samples from Chinese rural Conghua.** These differences seemed to be largely due to the differential LPS content of the environmental dust extracts, which was by far the lowest in the Chinese samples. This is in line with previous epidemiological and immunological findings demonstrating a dose-dependent effect of endotoxin (LPS), with particularly high levels conferring asthma-protection[118,135,333]. In fact, the Chinese dust samples used in this work's experiments may not reflect the protective *in vivo* exposure that the other dust samples do, since they had been collected in the family's living rooms in China, where the amount of dust was rather low. Supporting this assumption, follow-up experiments of the study group using Chinese dust samples from hens' stables with higher LPS content again revealed comparable anti-inflammatory effects as observed for the German, Finnish and Amish samples (*personal communication*).

Remarkably, stimulation with 0.1μg/ml LPS yielded effects very similar to stimulation with the German, Finnish and Amish dust samples, suggesting no clear benefit of farm dust treatment in the present experimental approach. This was in line with findings from an animal model, which also showed comparable asthma-protective effects for LPS and farm dust[261]. However, since the different LPS concentrations between the farm dust extracts and LPS were not adjusted to a defined common stimulation dose, direct comparison of LPS and farm dust induced effects is not valid. The present data therefore cannot answer the widely discussed issue of defining a safe and 'protective' LPS dose, nor

do they help reveal potential 'add-on' signals in farm dust extracts, which might further contribute to the beneficial effects of *in vivo* farming exposure. In particular, it was not possible to identify distinctively protective microbial species, since unfractionated dust samples were used in the current model. Although high concentrations of environmental endotoxin may clearly represent the relative abundance of gram-negative bacteria, they cannot sufficiently reflect the diversity of microbial sources encountered in asthma-protective farming environments[143,384]. Indeed, 16S rRNA sequencing analyses performed in the German and Finnish dust samples also revealed a certain percentage of gram-positive non-LPS containing bacteria from the Firmicutes and Actinobacter phyla. According to the current state of knowledge, it remains unclear whether the relative contribution of these and other microbes found in farm-derived dust samples, including gram-positive bacterial species[141,144], parasites and fungi[385], or even plant-derived products[179], plays a role in mediating the protective effects. Most recent studies, however, have provided substantial evidence for a specific composition of bacterial communities rather than individual species being the key determinant for asthma-protection[386,146], and thereby support the present model-based approach.

4.3.4 Immune profile of farm children at ages 4.5, 6 and 10 years

The current *in vitro* model suggested profound effects of farming exposures on major signalling pathways within innate immunity. In order to investigate how sustained microbial stimulation *in vivo* might affect these regulatory innate immune genes, gene expression analyses were performed in immune cells of farm children at three different time points (4.5 years, 6 years, 10 years of age). Indeed, continuous farm exposure was reflected in the innate immune signature of farm children's peripheral blood cells when compared to non-farm children. **Analyses revealed a distinct pattern of consistently decreased pro- and anti-inflammatory genes particularly in the 4.5- and 6-year-old farm children.**

It is reasonable to assume that the immune homoeostasis of farm children, who face repeated stimulation by a wide range of microbial antigens and environmental allergens, depends largely on developing and maintaining a state of non-responsiveness to these stimuli. From a molecular perspective, this is considered to be the result of a multi-levelled counter-regulatory process, similar to the phenomenon described for chronic low-dose LPS (endotoxin) exposure, termed 'endotoxin tolerance' (ET)[387,388]. Since LPS is known as a major component within the microbial cocktail of the farming environment, a comparable mechanism may well be proposed for chronic farm exposure. Induced by repeated and prolonged (LPS) stimulation, TLRs, the first site to sense antigens, change into a refractory, tolerogenic state, and they become hypo-responsive or even insensitive to ligand re-stimulation[378]. During ET, profound gene reprogramming processes lead to transient silencing of pro-inflammatory genes such as TLRs, while genes encoding anti-inflammatory cytokines or regulatory effector molecules were shown to be over-expressed upon LPS re-exposure[389]. Molecular mechanisms

underlying the regulation of ET-associated genes are considered to involve TLR4-induced epigenetic changes such as chromatin remodelling[389].

The farming environment may serve as an *in vivo* model to put some of these *in vitro* findings into a practical context. Gene expression in farm children is likely to be governed by epigenetic modifications[390] as well as genetic variants, which may therefore also contribute to defining the individual 'protected' immune phenotype. Such genetic alterations have been observed for both the *TLR4*[235,391,392] and the *TNFAIP3*[261] gene in response to environmental microbial exposures and may also explain the partial discrepancy of the current data compared to previous findings from farming cohorts. Analyses in the present PASTURE/EFRAIM study subsample did not reveal significant differences in *TLR4* gene expression between farm and non-farm children at any age. In contrast, elevated levels of *TLR4* and several other innate immune receptor genes and related signalling cascade molecules were found in immune cells of children with early life or prenatal farm exposure in the ALEX[241], PARSIFAL[240,122,188] and also the PASTURE[393] study.

Consistent with the ET hypothesis, TNFAIP3 along with other negative regulators of TLR-induced signalling was shown to be induced in LPS tolerised macrophages[378]. Experimental studies in mice have further demonstrated that TNFAIP3 is critically involved in maintaining immune homoeostasis in the gut by regulating constitutive TLR stimulation derived from commensal microbiota[394]. Based on these findings, increased levels of *TNFAIP3*, which were found in children from the U.S. Amish farming population[118], indicate a molecular mechanism to control constant TLR4-dependent signalling induced by the variety of microbial cues in the farming environment. However, the phenotype of farm children's immune cells in the present PASTURE study cohort did not fully reflect the ET model and did not confirm the findings from the Amish farming cohort in particular. Data from this work showing significantly decreased tolerogenic markers *TNFAIP3* and *CD274* in the farm children compared to non-farm children at the age of 4.5 and 6 are somehow surprising. The findings were replicated at a highly significant level in the TRILATERAL cohort comprising 112 7-year-old children from rural Conghua and urban Hong Kong[321], which suggests a stable and true finding.

It is the current conception that the farming environment exerts most effective protective effects when exposure happens in the very early period of life and already prenatally[395]. Although it is known that major maturation processes and long-term antigen memory are also established during that phase, our common understanding of the exact kinetics and the speed of the principal immunological changes that occur postnatally and during infancy is still limited[115]. An experimental animal model of asthma has shown that the CD274-dependent development of Treg cells suppressing the development of aberrant inflammatory processes is induced by microbial stimulation immediately after birth[383]. Similarly, analyses in cord blood samples of infants with maternal farm exposure already exhibited increased numbers of Treg cells[186], and a higher percentage of Tregs was still found in farm-exposed children of the PASTURE cohort at the age of 4.5 years[187]. However, members of our research group have found a significant decrease in Treg cells between the age of 4.5 and 6 years in the same population[308], and likewise, school-aged Amish farm children did not show differential Treg numbers when compared to

the Hutterite children[118]. These findings suggest that distinct timeframes in which regulatory immune processes develop may exist, and that effects of long-term microbial exposure may manifest in a certain immune phenotype at different stages. On the basis of that notion, the present data may not reflect the immediate changes during the early establishment of immune homoeostasis in farm children. **Decreased expression of both regulatory and inflammatory innate immune genes in farm children at school age may therefore either be causally linked to their protection from allergic disease, or may be due to a secondary effect of downregulation, reflecting a suppressed steady state, a 'tolerant' immune phenotype maintained after a period of continuous *in vivo* microbial exposure.** Data from U.S. and European farming studies reinforce this conception, showing generally decreased levels of Th2- and Th17-, but also Th1- and Treg cell-associated cytokines in LPS-stimulated peripheral blood leucocytes of farm children at school age[118,135]. A recently published study by *Kirjavainen et al*. could demonstrate similar effects of suppressed cytokine responses to LPS even in PASTURE/LUKAS 6-year-old *non*-farm children when they were exposed to an indoor microbiota composition similar to farm homes[146].

Given the understanding that the generation of tolerance is a dynamic process, it is reasonable to suggest that key regulatory molecules such as TNFAIP3, CD274 and CD80 are upregulated in farm children's immune cells during the crucial phase of immune maturation in infancy, while downregulation may occur once immune homoeostasis is established in the healthy child. Such an educated, or 'tolerised' immune system of farm children may not be set at a defined state of constitutive unresponsiveness, but rather allow dynamic responses to different environmental cues. This was shown upon stimulation with LPS and the different farm dust extracts in the present farming cohort. Similar to their non-farming or urban peers respectively, all 4.5-, 6- and 10-year-old farm children consistently demonstrated a significant decrease in expression of *TLR4*, while increasing *TNFAIP3, CD274* and *CD80* in response to LPS and farm dust stimulation. **This indicates that the capacity to downregulate inflammatory molecules and to induce regulatory anti-inflammatory processes is preserved in farm children.**

Interestingly, the 10-year-old German farm children exhibited significantly altered immune responses upon stimulation with the German farm dust sample compared to those observed when treated with LPS or the Amish or Finnish dust samples, respectively. This is a striking finding, as it strongly argues that the immune response is specific to the environmental exposure that the system had been conditioned for. Although these preliminary findings definitely need confirmation on a larger scale, **they point to a mechanism of long-term immunological tolerance in farm children, with sustainably altered immune responses to a distinctive 'cocktail' of microbial stimuli 'known' from continuous early life *in vivo* exposure.**

Despite the protective effect of such environmental exposure, a small number of farm children still develop asthma, which illustrates clearly the contribution of non-environmental factors when

determining the manifestation of the disease. Indeed, the current data supported this notion in some part. When stratified by phenotype in the 6-year-old German farming group, peripheral blood samples of asthmatic farm children exhibited significantly decreased *TNFAIP3* expression when compared to their healthy farming peers. This finding provides further evidence for a key role of TNFAIP3 at the interface between disease-determining and disease-protecting factors and further reinforces the notion that - apart from environmental determinants - inherited factors may also play a decisive role in defining the allergic asthmatic phenotype in children[396]. **While strong genetic factors may primarily determine the development of asthma in farm children, non-inheritable environmental exposures may be more relevant for disease manifestation in non-farm children. Thus, protective environmental conditions may not be strong enough to overcome a certain genetic risk profile.** This further implies that the individual's effective protection from asthma and allergic disease also requires a genotype which is accessible to the protective environmental stimuli. These considerations, however, remain speculative at this point, since the genetic background of the investigated study cohort will still be unknown when this work is completed. However, genome-wide analysis data from the whole PASTURE study population are to be expected in the near future and may provide a clearer view on the heritability component of allergic asthma, particularly in the farming population. Notwithstanding this, previous large-scale GWAS studies in asthma[397,398] have already taught us that complementary explanations besides single heritable factors must exist to determine the phenotype of allergic asthma in children[399]. It is becoming clear that the impact of non-heritable environmental factors on the human immune system cannot be underestimated[400], and that this particularly applies to exposures in farming environments and the associated changes in innate immunity functions and asthma risk[118].

Overall, the current results indicate that sustained early life *in vivo* farming exposure modulates the expression of genes encoding innate immune signalling molecules, although the immunological mechanisms underlying the observed effects remain undefined. Future large-scale studies may be able to particularly elucidate the role of TNFAIP3 and related innate immune pathways in the context of environmentally mediated childhood asthma protection.

4.4 Concluding remarks and outlook

In conclusion, the immunological studies conducted on children's peripheral blood immune cells within the scope of this work demonstrated a straightforward approach providing robust and consistent findings on the role of TNFAIP3 and related innate immune signalling pathways in the development of childhood allergic asthma and its environmentally mediated protection. The findings presented provide further insight into the potential mechanisms underlying the epidemiological 'farm-effect' on asthma by revealing significant immunomodulatory properties of *in vitro* and *in vivo* farming exposure. Corroborated by earlier studies in both mice and humans, the present data suggest that strong

immunostimulatory signals encountered in the farming environment may decrease asthma risk by activating regulatory TNFAIP3-dependent processes and sustainably altering innate immune pathways. Even if definite practical implications cannot yet be deduced, the results of this study point to a significant potential of a microbial-based treatment approach for the prevention of allergic asthma in children.

Considerable support for this concept comes from the emerging field of microbiome research, which continues to reinforce the major impact of the human microbial ecosystem on the development of immune-mediated disorders[401]. Growing evidence suggests that reduced exposure to a biodiverse microbial environment early in life may cause major disruptions in endogenous commensal microbiota composition, which in turn results in dysregulated, i.e. non-tolerant immune function promoting allergic inflammation[145,402,403]. The notion that 'appropriate' intestinal colonisation early in life may prevent allergic disease has fuelled the development of bacterial probiotics[404,405], but clinical effects of interventions have so far been disappointing[406]. From the clinical perspective, however, adjusting microbiota imbalances through targeted modification of the *external* microbial exposures and/or the *internal* lung and gut microenvironment remains a promising concept for future asthma treatment. Coupling findings from the farming studies with the novel possibilities in microbiome profiling and profound immunological research might help to overcome the challenges of designing such a novel interventional approach[405]. Based on most recent findings, it may now be possible to select specific compositions of beneficial farm-related microbes, to use them in well-structured interventional studies[146] and evaluate the immunological changes and related clinical outcomes they may provoke. Modifying the indoor microbial environment may thereby constitute a feasible, non-invasive approach, implying the natural routes of exposure via inhalation, oral intake, or skin contact.

Considering the vast number of children with severely uncontrolled or residual disease under current standard treatment, the key to reducing the global burden of asthma will essentially rely on establishing novel effective strategies for both disease prevention and therapy. Many years have passed since the 'hygiene hypothesis' was posed, and its theories have inspired researchers around the globe. Today, human research is on the verge of ultimately deciphering a causal relationship between early life microbial exposures and childhood asthma protection. Taking advantage of the valuable knowledge provided by epidemiological and immunological research over the last few decades may now allow for the development of translational interdisciplinary clinical trials and thus eventually transfer immunological findings from 'bench to bedside'.

5 Summary

Asthma has become one of the most common chronic diseases among children in developed Western countries, with its prevalence still rising in other parts of the world. Distinct microbial exposures encountered in traditional farming environments early in life have been associated with robust protection from developing the disease. The practical relevance of this protective 'farm-effect', however, remains limited as long as the causal mediators and molecular mechanisms have not been fully identified. In this context, recent findings in mouse models of asthma have suggested a key role for the enzyme TNFAIP3, which attenuates NF-κB dependent inflammation, but equivalent immunological studies in humans are missing.

The objective of the present study was therefore to unravel the role of the anti-inflammatory regulator TNFAIP3 and related innate immune signalling pathways in the development of allergic asthma and its environmentally mediated protection in children. The study aimed to investigate target gene and protein expression in peripheral blood mononuclear cells (PBMCs) of allergic asthmatic children during disease manifestation. It further aimed to examine the effect of *in vitro* stimulation with farm dust extracts and bacterial lipopolysaccharide (LPS) on the expression of these target molecules. Moreover, the study aimed to assess whether children with continuous early life *in vivo* farm exposure showed modified *TNFAIP3* pathway gene expression in immune cells at the age of 4.5, 6 and 10 years.

Therefore, peripheral blood samples of asthmatic and healthy children from urban and farm environments from two distinct study population cohorts were analysed. As part of the cross-sectional CLARA/CLAUS study, a representative nested study sample comprising allergic asthmatic (AA) and healthy control (HC) school-age children (n = 48) was recruited. PBMCs were isolated from whole blood samples and stimulated *in vitro* with four different farm-derived dust extracts (from Germany, Finland, China and from the Amish farming environment) as well as LPS for 24 hours. Furthermore, from the prospective birth cohort study PASTURE/EFRAIM, a nested sample of farm (F) and non-farm (NF) children (n = 100) was selected. Unstimulated and LPS-stimulated whole blood samples were analysed at the children's age of 4.5, 6 and 10 years. For both cohorts, gene expression and protein levels of TNFAIP3 and NF-κB signalling-associated candidate molecules (TLR4, MyD88, TRAF6, TNIP2, TAX1BP1, MALT1, CD80, CD86, CD274, PDCD1) were assessed by qPCR and Western Blot analysis respectively.

The following results were achieved and are presented in this thesis:

(i) Analyses in unstimulated PBMC samples revealed significantly reduced expression of the anti-inflammatory TNFAIP3 gene and protein in allergic asthmatic children compared to healthy controls, while expression of the pro-inflammatory pathway genes *TLR4* and *MyD88* was increased in allergic asthmatic subjects.

(ii) *In vitro* stimulation with LPS and all four farm dust extracts except the Chinese sample restored TNFAIP3 expression of asthmatic patients to healthy levels and significantly reduced the expression of the pro-inflammatory TLR4-pathway-related target genes (*TLR4, MyD88, TRAF6*). The observed anti-inflammatory effects were accompanied by significant downregulation of dendritic cell associated co-stimulatory molecule *CD86* and upregulation of the co-inhibitory molecule *CD274* upon stimulation.

(iii) Unstimulated whole blood samples of farm children at ages 4.5, 6 and 10 years exhibited decreased expression of both pro- and anti-inflammatory genes compared to their non-farming peers. However, the capacity to induce anti-inflammatory *TNFAIP3* and *CD274* expression and to downregulate pro-inflammatory *TLR4* levels upon LPS and farm dust stimulation was preserved at all ages.

The results of this study in children's peripheral immune cells indicate that farming exposures *in vitro* and *in vivo* may inhibit critical inflammatory processes in human asthma development by activating regulatory TNFAIP3-dependent processes and shaping innate immune pathways. These findings increase the potential for novel farm-derived microbe-based treatment and prevention strategies, and thus may ultimately pave the way for improving asthma management on a global scale.

6 Zusammenfassung

Asthma bronchiale zählt in den westlichen Industrienationen mittlerweile zu einer der häufigsten chronischen Erkrankungen im Kindesalter, wobei in anderen Teilen der Welt weiterhin ein Anstieg der Prävalenz verzeichnet wird. Es hat sich gezeigt, dass insbesondere der frühe Kontakt zu bestimmten mikrobiellen Bestandteilen in der Bauernhof-Umgebung einen wirksamen Schutz vor der Erkrankung vermittelt. Dieser sogenannte "Bauernhof-Effekt" ist jedoch von geringer praktischer Relevanz, solange die ursächlichen Mediatoren und zugrundeliegenden molekularen Mechanismen nicht vollständig entschlüsselt sind. In diesem Zusammenhang weisen nun jüngste Forschungsergebnisse an Asthma-Mausmodellen auf eine Schlüsselrolle des Enzyms TNFAIP3 hin, welches NF-κB-vermittelte Entzündungsprozesse hemmt. Dabei fehlen aktuell entsprechende immunologische Untersuchungen am Menschen.

Ziel der vorliegenden Studie war es daher, die Rolle des entzündungshemmenden Regulators TNFAIP3 und zugehöriger Signalwege der angeborenen Immunität bei der Entstehung von allergischem Asthma bronchiale im Kindesalter und seinem umweltvermittelten Schutz zu entschlüsseln. Vor diesem Hintergrund sollten Gen- und Proteinexpression von TNFAIP3 und entsprechenden Zielmolekülen in peripheren mononukleären Blutzellen (PBMCs) von Kindern mit allergischem Asthma bei Krankheitsmanifestation untersucht und anschließend die Auswirkung von *in-vitro*-Stimulation mit Bauernhofstaubextrakten und bakteriellem Lipopolysaccharid (LPS) auf die Expression dieser Zielmoleküle betrachtet werden. Darüber hinaus sollte untersucht werden, ob Immunzellen von Bauernhofkindern nach anhaltender *in-vivo*-Bauernhofexposition im Alter von 4,5, 6 und 10 Jahren veränderte Expressionsmuster ausgewählter Zielgene innerhalb des TNFAIP3-Signalweges aufweisen.

Mithilfe von zwei verschiedenen Studienkohorten wurden hierfür Blutproben von Kindern mit Asthma bronchiale und von gesunden Kindern aus städtischen sowie ländlichen Gebieten analysiert. Im Rahmen der CLARA/CLAUS-Querschnittsstudie wurde eine repräsentative Kohorte von Schulkindern mit allergischem Asthma (AA) sowie gesunden Kontrollen (HC) (n = 48) rekrutiert. Aus Vollblutproben wurden PBMCs isoliert und *in vitro* mit vier verschiedenen Bauernhofstaubextrakten (aus Deutschland, Finnland, China und von den Amischen aus Amerika) sowie mit LPS für 24 Stunden stimuliert. Des Weiteren wurde aus der prospektiven Geburtskohorte PASTURE/EFRAIM eine Stichprobe von Kindern ausgewählt, die am Bauernhof aufgewachsen sind (F) und solchen, die nicht am Bauernhof aufgewachsen sind (NF) (n = 100). Deren unstimulierte und LPS-stimulierte Blutproben wurden im Alter von 4,5, 6 und 10 Jahren untersucht. In beiden Studienpopulationen erfolgte die Auswertung der Gen- und Proteinexpression von TNFAIP3 und Zielmolekülen des NF-κB Signalwegs (TLR4, MyD88, TRAF6, TNIP2, TAX1BP1, MALT1, CD80, CD86, CD274, PDCD1) mittels qPCR und Western-Blot.

Folgende Ergebnisse wurden erzielt und sind in der vorliegenden Arbeit dargestellt:

(i) In unstimulierten PBMCs zeigte sich bei Kindern mit allergischem Asthma im Vergleich zu gesunden Kontrollen eine signifikant reduzierte Expression des entzündungshemmenden Regulators TNFAIP3 auf Gen- und Proteinebene bei zugleich erhöhter Expression der entzündungsfördernden Moleküle des Signalwegs (*TLR4, MyD88*).

(ii) *In-vitro*-Stimulation mit LPS und allen vier Bauernhofextrakten mit Ausnahme der chinesischen Staubprobe konnte die verminderte Expression von TNFAIP3 bei Asthmatikern auf das Niveau gesunder Kinder anheben und zugleich die Expression der entzündungsfördernden Zielgene des TLR4-Signalwegs (*TLR4, MyD88, TRAF6*) signifikant reduzieren. Der beobachtete entzündungshemmende Effekt der Stimulation zeigte sich außerdem in einer deutlichen Expressionsreduktion des mit dendritischen Zellen assoziierten kostimulatorischen Moleküls *CD86* bei gleichzeitiger Expressionserhöhung des koinhibitorischen Moleküls *CD274*.

(iii) Unstimulierte Vollblutproben von Bauernhofkindern im Alter von 4,5, 6 und 10 Jahren wiesen im Vergleich zu Proben von nicht-Bauernhofkindern eine verminderte Expression sowohl entzündungsfördernder als auch -hemmender Gene auf. Die Fähigkeit zur Expressionserhöhung der entzündungshemmenden Regulatoren *TNFAIP3* sowie *CD274* sowie Reduktion des entzündungsfördernden Gens *TLR4* bei LPS- und Bauernhofstaubstimulation blieb jedoch in jedem Alter erhalten.

Die Daten der vorliegenden Studie an peripheren Immunzellen von Kindern deuten darauf hin, dass Bauernhof-Expositionen *in vitro* und *in vivo* maßgebliche Entzündungsprozesse bei der Entstehung von Asthma bronchiale hemmen, indem möglicherweise regulatorische TNFAIP3-vermittelte Prozesse aktiviert und Signalwege der angeborenen Immunität modifiziert werden. Diese Ergebnisse eröffnen neue Möglichkeiten für die Entwicklung mikrobiell-basierter Präventions- und Therapiestrategien und können somit letztlich zu einem optimierten und umfassenderen Konzept zur Entstehung und Behandlung von Asthma bronchiale bei Kindern beitragen.

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8 Abbreviations

AA	allergic asthmatic
ABIN	A20 binding inhibitor of NF-κB
AHR	airway hyperresponsiveness
ALEX	ALlergy and EndotoXin study
Am	Amish
APC	antigen-presenting cell
APS	Ammoniumperoxiddisulphate
ATS	American Thoracic Society
A.U.	arbitrary units
BAL	bronchoalveolar lavage
BD	bronchodilator
BMI	body-mass-index
BSA	bovine serum albumin
CBMC	cord blood mononuclear cell
Ch	Chinese
CD	cluster of differentiation
cDC	conventional dendritic cell
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
CLARA	CLinical Asthma Research Association
CO_2	carbon dioxide
CRP	C-reactive protein
C_t	cycle threshold
CTLA-4	cytotoxic T lymphocyte-associated protein 4
DAMP	damage-associated molecular pattern
DC	dendritic cell
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
EC	epithelial cell
ECL	enhanced chemiluminescence
E. coli	Escherichia coli
EDC	Electrostatic Dustfall Collector
EDTA	ethylenediaminetetraacetic acid
ERS	European Respiratory Society
ET	endotoxin tolerance
EU	endotoxin units

F	farm
fc	fold change
f.c.	final concentration
FcεRI	high-affinity receptor for the Fc region of immunoglobulin E
FEV_1	forced expiratory volume in one second
Fi	Finnish
FVC	forced vital capacity
fw	forward
G	German
g	gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	genomic DNA
GINA	Global Initiative for Asthma
GLI	Global Lung Function Initiative
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	genome-wide association study
HC	healthy control
HRP	horseradish peroxidase
H_2O	water
IFN-γ	interferon gamma
IgE	immunoglobulin E
IgG	immunoglobulin G
IκB	inhibitor of kappa B
IKK	inhibitor of nuclear factor kappa-B kinase
IL	interleukin
ILC	innate lymphoid cell
IQR	interquartile range
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor
ISAAC	International Study of Asthma and Allergies in Childhood
IU	international units
kDa	kilodalton
kg	kilogram
L	litre
LAL	Limulus Amoebocyte Lysate
LPS	lipopolysaccharide
M	molar
MALT1	mucosa-associated lymphoid tissue lymphoma translocation protein 1

mDC	myeloid dendritic cell
MD-2	myeloid-differentiation protein 2
$MEF_{75/50/25}$	mid-expiratory flow rate at 75%, 50% and 25% of FVC
MHC	major histocompatibility complex
ml	millilitre
mM	millimolar
mRNA	messenger RNA
MyD88	Myeloid differentiation primary response 88
n	number
NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
NF	non-farm
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B cells
NK cell	natural killer cell
NKT cell	natural killer T cell
nM	nanomolar
nm	nanometre
ns	not significant
NTC	no-template-control
p	p-value
PAMP	pathogen-associated molecular pattern
PARSIFAL	Prevention of Allergy: Risk Factors for Sensitization In Children Related to Farming and Anthroposophic Lifestyle
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
pDC	plasmacytoid dendritic cell
PD-L1	Programmed Death Ligand 1
PD-1	Programmed Death receptor 1
PRR	pattern recognition receptor
PVDF	polyvinylidene difluoride
qPCR	quantitative real-time polymerase chain reaction
RAST	radioallergosorbent test
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
rRNA	ribosomal RNA
rpm	rotations per minute
RT	reverse transcription
rv	reverse
SCI	specific IgE class

SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SNP	single nucleotide polymorphism
ssDNA	single-stranded DNA
TAK1	TGF-β-activated kinase 1
TAX1BP1	Tax1-binding protein 1
TBS-T	Tris-buffered saline with Tween20
TCR	T cell receptor
TEMED	tetramethylethylenediamine
TGF-β	transforming growth factor beta
Th cell	helper T cell
TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain-containing adaptor protein
TLR4	Toll-like receptor 4
T_m	melting temperature
TNFα	tumor necrosis factor alpha
TNFAIP3	tumor necrosis factor alpha induced protein 3
TNIP2	TNFAIP3 interacting protein 2
TRAF6	tumor necrosis factor receptor associated factor 6
TRAM	TRIF related adaptor molecule
TRIF	TIR domain-containing adaptor protein inducing interferon beta
Treg	regulatory T cell
Tris	trishydroxymethylaminomethane
U	unstimulated
V	volt
v/v	volume per volume
WB	Western Blot
WBC	white blood cell count
w/v	weight per volume
x g	times gravity
χ^2	chi-squared
z	z-score

9 List of figures and tables

9.1 List of figures

9.2 List of tables

10 Appendix

10.1 Supplementary tables and figures

Table E1 provides an overview of the number of samples that had been excluded from further statistical analysis. These samples did not meet the criteria of quality control (i.e. technical replicates with a difference of C_t values > 0.4, as well as melting curve abnormalities), as they are described in detail in the methods section of this thesis.

Table E1. *Number and percentage of excluded samples.*

Gene	**Stimulus**					
	U n (%)	**LPS** n (%)	**G** n (%)	**Fi** n (%)	**Ch** n (%)	**Am** n (%)
TLR4	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
MyD88	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
TRAF6	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
TNFAIP3	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
TNIP2	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
TAX1BP1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
MALT1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
CD80	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
CD86	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
CD274	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
PDCD1	2 (5.4)	2 (5.4)	3 (8.1)	4 (10.8)	2 (5.4)	3 (8.1)

Stimulating conditions were lipopolysaccharide (*LPS*) and dust extracts from German (*G*), Finnish (*Fi*), and Chinese (*Ch*) farming environments, and from farms of the Amish population (*Am*). *n,* number of excluded samples. %, percentage of total.

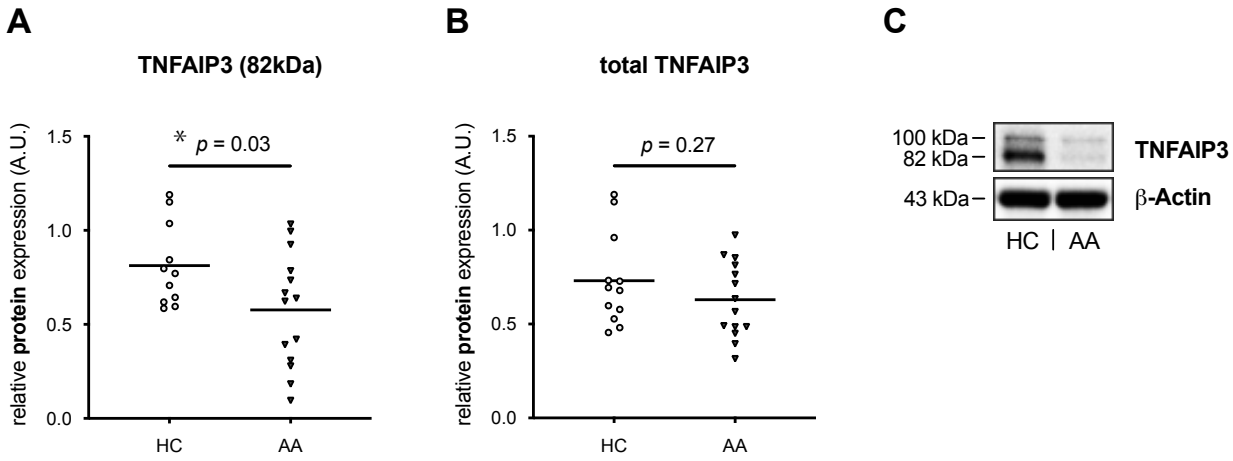

Figure E1. TNFAIP3 protein levels at different molecular weight.
TNFAIP3 protein levels, as assessed by Western Blot analysis in unstimulated PBMCs of HC (n=11) and AA (n=14). (**A**) TNFAIP3 protein, predicted 82kDa size. (**B**) 'total TNFAIP3' (~100 kDa + 82kDa). Results from densitometric analysis of protein bands. Normalized protein expression, relative to β-Actin. Lines at mean value. *A.U.*, arbitrary units. (**C**) representative immunoblot membrane-cut-out. Molecular weight in kDa. β-Actin was used as loading-control. *P* values reflect Welch's t test. *$p < 0.05$.

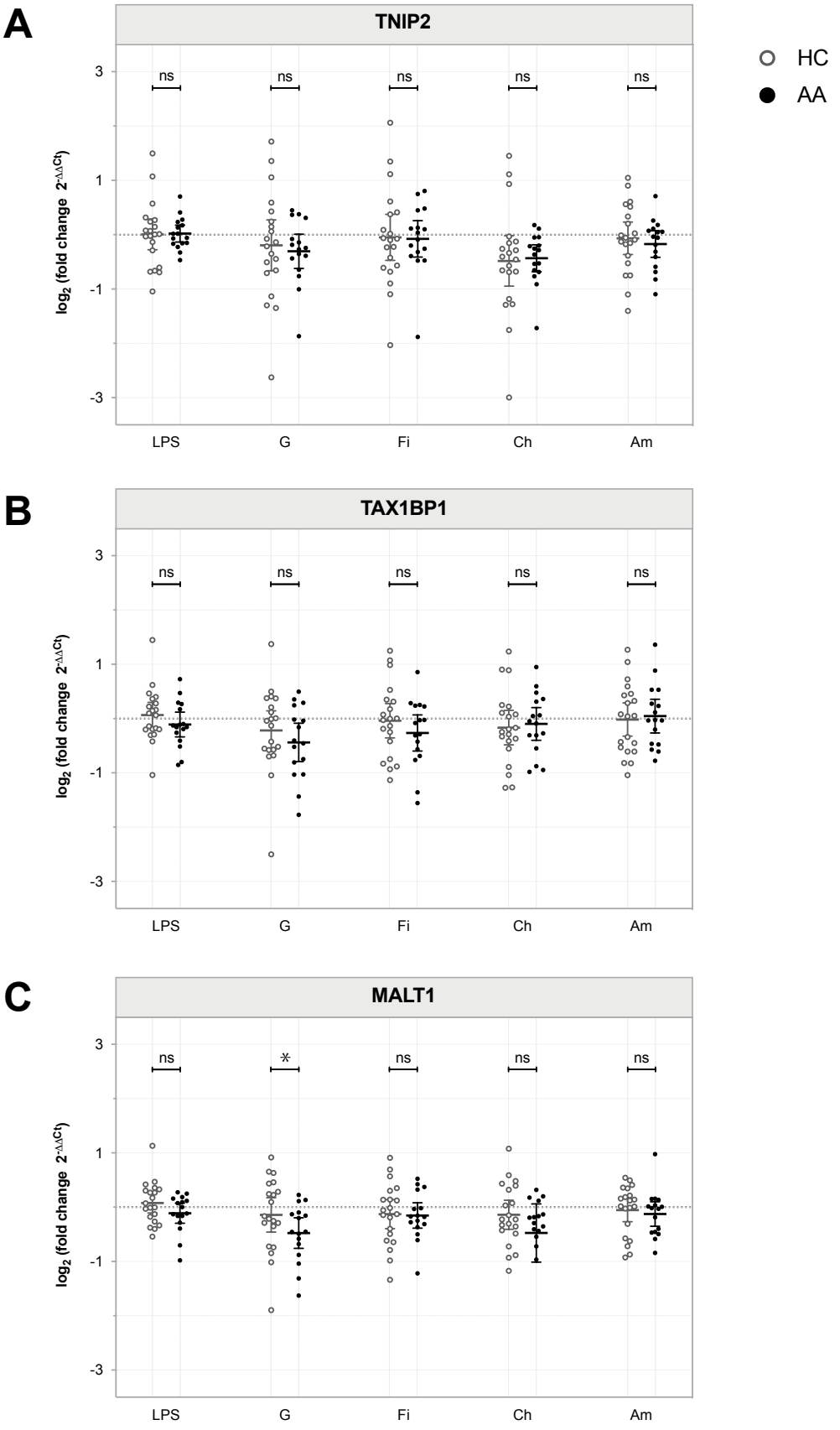

Figure E2. Fold change of TNFAIP3 regulatory gene expression upon LPS and farm dust stimulation. **(A)** TNIP2, **(B)** TAX1BP1 and **(C)** MALT1 gene expression fold change, compared to unstimulated conditions ($2^{-\Delta\Delta Ct}$, $\log_2$ scale), as assessed by qPCR in PBMCs of HC (n=22) and AA (n=16) after 24 hrs stimulation with LPS or farm dust extracts (G, Fi, Ch, Am). Scatter dot plots represent single sample values with means and 95% CI. *P* values reflect Welch's t test for fold change comparison between HC and AA. *$p < 0.05$; *ns*, not significant.

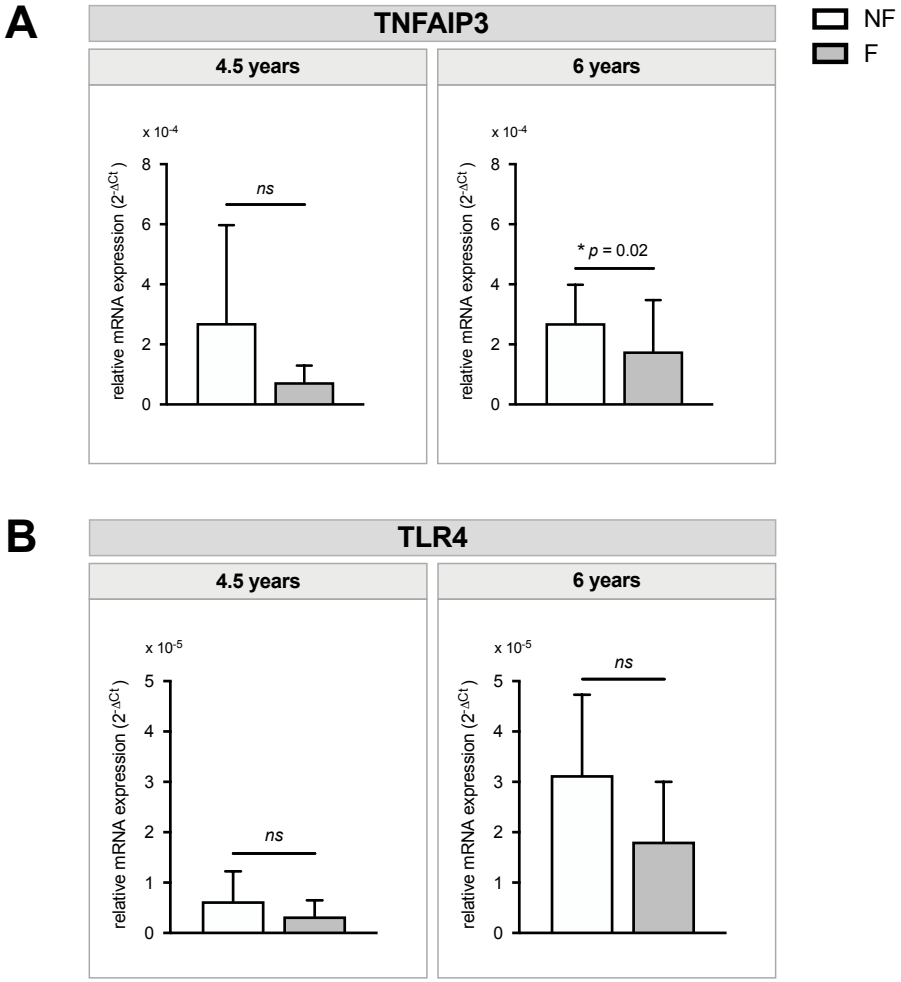

Figure E3. TNFAIP3 and TLR4 mRNA expression in NF and F children without asthma.
Baseline gene expression levels of TNFAIP3 **(A)** and TLR4 **(B)**, as measured by qPCR in unstimulated whole blood samples of healthy farm (F) and non-farm (NF) children, assessed at ages 4.5 years (n=25, 13 F/ 12 NF) and 6 years (n=43, 23 F/20 NF). Expression relative to 18S, displayed as $2^{-\Delta Ct}$ mean values with 95% CI. *P* values reflect Welch's t test for ΔCt comparison between NF and F. *$p < 0.05$; *ns*, not significant.

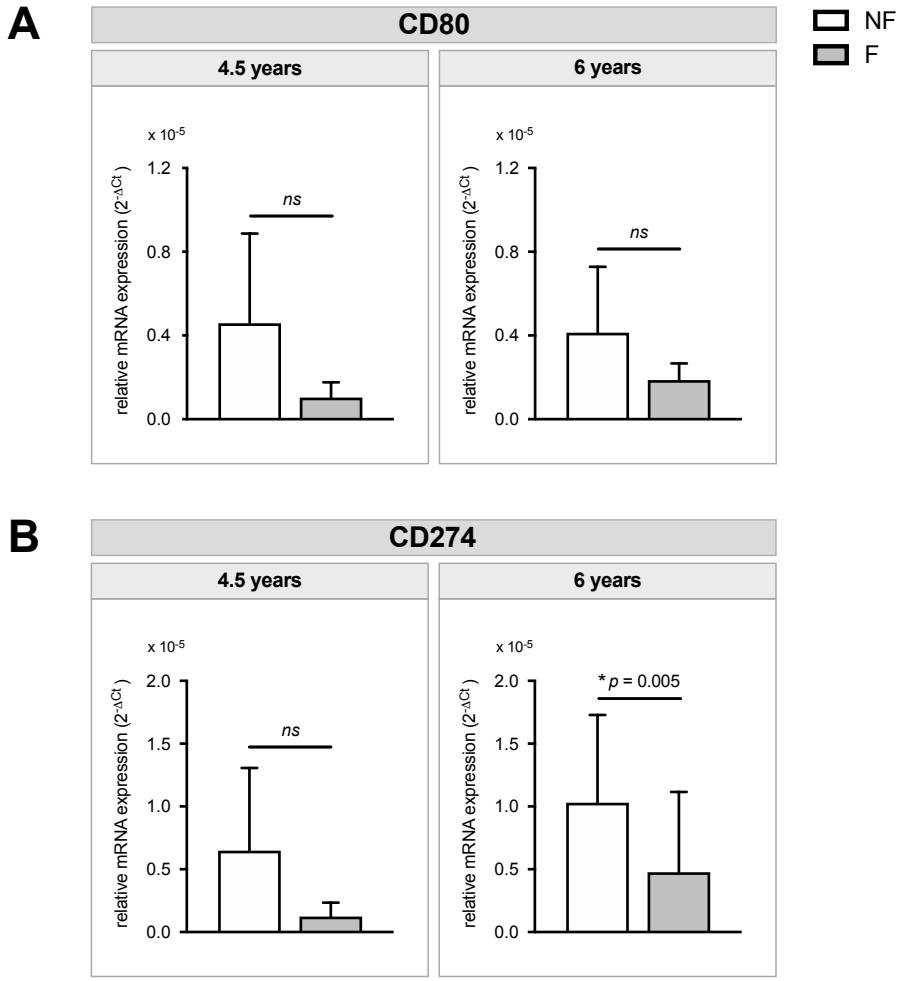

Figure E4. CD80 and CD274 mRNA expression in NF and F children without asthma.
Baseline gene expression levels of dendritic cell markers CD80 **(A)** and CD274 **(B)**, as measured by qPCR in unstimulated whole blood samples of healthy farm (F) and non-farm (NF) children, assessed at ages 4.5 years (n=25, 13 F/ 12 NF) and 6 years (n=43, 23 F/20 NF). Expression relative to 18S, displayed as $2^{-\Delta Ct}$ mean values with 95% CI. *P* values reflect Welch's t test for ΔCt comparison between NF and F. **p* < 0.05; *ns*, not significant.

10.2 CLARA/CLAUS questionnaire

Datum:_____ Studiennummer:_____

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

Asthmastudie CLARA

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 letzen 12 Monaten gleichzeitig mit diesen Nasenbeschwerden juckende oder tränende Augen?

Ja..... ☐

Nein ..... ☐

17. Wann in den letzen 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 nachts 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
Paracetamol.....	☐	☐

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 jemals 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 ohne 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 <u>bei dem Vater</u> 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 <u>.....</u>.geboren.
Es kam mit <u>.....</u>..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:

PD Dr. med. Bianca Schaub

Studienärztin Elisabeth Klucker, Tel: 089/ 44005-7878

11 Publication and presentations

11.1 Publication

Krusche J.*, Twardziok M.*, **Rehbach K.***, *et al*. (2019). TNF-alpha-induced protein 3 is a key player in childhood asthma development and environment-mediated protection. *The Journal of Allergy and Clinical Immunology*, *144*(6), 1684-1696 e1612. doi:10.1016/j.jaci.2019.07.029

* contributed equally to this work as first authors

11.2 Presentations

Work in Progress Report, Comprehensive Childhood Research Center, Dr. von Hauner Children's Hospital, Munich, Germany, January 2020. **Rehbach K.** The role of TNFAIP3 and NF-κB signalling in childhood asthma development and environment-mediated protection.

EAACI Congress, Munich, Germany, May 2018. Krusche J., Böck A., **Rehbach K.**, Twardziok M., Roponen M., Schaub B. Role of IL-18 activation in childhood asthma development and environment-mediated protection.

Final seminar of 'FöFoLe' doctoral study programme, Herrsching, Germany, May 2016. **Rehbach K.** Asthma-Protektion: Identifikation von neuen Immun-Regulations-Mechanismen vermittelt durch Umwelt-Exposition.

'Allergy on the Isle', doctoral students' workshop of the German Society of Allergology and Clinical Immunology (DGAKI), Frauenchiemsee, Germany, November 2015. **Rehbach K.** Childhood asthma protection through farm dust exposure?

Danksagung

Es ist mir ein großes Anliegen, mich an dieser Stelle bei all den Menschen zu bedanken, die auf vielfältige Weise zum Gelingen dieser Arbeit beigetragen haben.

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