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ALAR AAB

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Insights into molecular mechanisms of asthma and atopic dermatitis

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RNA Biology Research Group, Department of Biomedicine, Institute of Biomedicine and Translational Medicine, University of Tartu.

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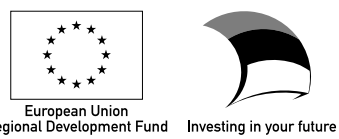

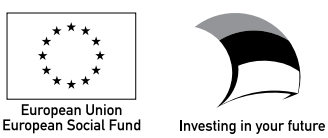

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II Jakiela B, Gielicz A, Plutecka H, Hubalewska-Mazgaj M, Mastalerz L, Bochenek G, Soja J, Januszek R, **Aab A**, Musial J, Akdis M, Akdis CA, Sanak M. Th2-type cytokine-induced mucus metaplasia decreases susceptibility of human bronchial epithelium to rhinovirus infection. Am J Respir Cell Mol Biol. 2014 Aug;51(2):229–41

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IV Rebane A, Zimmermann M, **Aab A**, Baurecht H, Koreck A, Karelson M, Abram K, Metsalu T, Pihlap M, Meyer N, Fölster-Holst R, Nagy N, Kemeny L, Kingo K, Vilo J, Illig T, Akdis M, Franke A, Novak N, Weidinger S, Akdis CA. Mechanisms of IFN-γ-induced apoptosis of human skin keratinocytes in patients with atopic dermatitis. J Allergy Clin Immunol. 2012 May;129(5):1297–306

Contribution of Alar Aab to original publications:

Study I: responsible for the study design, performing the experiments and analyzing the data, assessing the outcome and writing the manuscript.

Study II: designing the qPCR method for rhinovirus detection; involved in critical revision of the manuscript.

Study III: performing gene expression analyses, immunofluorescence, cryo-sectioning; involved in writing of the manuscript.

Study IV: performing gene expression analyses, immunofluorescence, cryo-sectioning; involved in critical revision of the manuscript.

ABBREVIATIONS

AD	atopic dermatitis
ALI	air–liquid interface
ANXA	annexin
APC	antibody presenting cell
BCR	B cell receptor
BSA	bovine serum albumin
CARD	caspase recruitment domain
CCDC	coiled Coil Domain Containing
CCL	CC chemokine ligand
CD	cluster of differentiation
CDHR3	cadherin-related family member 3
CFSE	carboxyfluorescein succinimidyl ester
CXCL	chemokine (C-X-C motif) ligand
DAPI	4’,6-diamidino-2-phenylindole
DC	dendritic cell
Drosha-DGCR8	DiGeorge syndrome critical region gene 8
EF	elongation factor
FAS	TNF receptor superfamily member 6
FcεRI	high-affinity IgE receptor
FLG	filaggrin
GINA	Global Initiative for Asthma
GM-CSF	granulocyte macrophage colony-stimulating factor
HBEC	human bronchial epithelial cell
HRV	human rhinovirus
ICAM	inter-cellular adhesion molecule
IF	immunofluorescence
IFI	interferon gamma inducible
IFITM	interferon induced transmembrane
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
IRAK	interleukin 1 receptor associated kinase
ISH	*in situ* hybridization
KC	keratinocyte
LDLR	low-density lipoprotein receptor family
LRT	lower respiratory tract
LYN	LYN proto-oncogene, Src family tyrosine kinase
mAb	monoclonal antibody
miRNA/miR-	microRNA
MOI	multiplicity of infection
mRNA	messenger RNA

MWCO	molecular weight cut off
MUC5AC	mucin 5AC
NK	natural killer
NOD	nucleotide binding oligomerization domain containing
PBMC	peripheral blood mononuclear cell
PFA	paraformaldehyde
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RAB	ras-related gtp-binding protein
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
TCID50	50% tissue culture infective dose
TD	T cell dependent
Th	T helper
TI	T cell independent
TNF	tumor necrosis factor
TNF-R	TNF receptor
TSLP	thymic stromal lymphopoietin
VP	viral protein
WT	wild type
URT	upper respiratory tract
UTR	untranslated region

1. INTRODUCTION

During last decades, asthma and atopic dermatitis (AD) have rapidly spread in many countries around the world, including Estonia [1, 2]. In majority of cases, these diseases are associated with recurring allergic hypersensitivity reactions to common environmental substances, i.e. people with asthma and AD are often allergic or atopic [3]. Although the prevalence of allergic diseases has increased dramatically during the last 50–60 years, the reasons for this are not fully understood. It seems that atopic diseases have mostly reached their plateau in western cultural area where approximately 20% of population is affected by the condition. However, the prevalence of allergic diseases is still rapidly increasing in many other parts of the world, notably in Eastern Europe where the „epidemic“ start was delayed as it has been clearly demonstrated by comparing the former West and East German populations.

Often, there occurs the sequential development of the atopic diseases known as the *atopic march* and characterized by the progression of AD to asthma and/or allergic rhinitis. However, despite apparent association between AD and asthma, there is no definitive proof that AD is causal for the subsequent development of asthma. Hygiene hypothesis, as proposed 1989 by Strachan, has been used to explain the spread of allergic diseases associated with urbanization. The hypothesis assumes that the T helper 1 (Th1) cell response is not induced early in life leaving the body more susceptible to Th2 induced disease. Testing the validity of the hygiene hypothesis has ended so far with contradicting research results showing that the real reasons behind allergic diseases might be much more complex.

Although the family history is a strong predicting factor, especially in case of AD [4], genetic susceptibility cannot explain a marked increase in prevalence of atopic diseases in such a short time period, suggesting that our modern environment is supporting the development of allergic diseases.

It is somewhat unexpected that in our immune system, where both innate and adaptive immune response mechanisms function with astonishing precision, a sub-program exists where large number of responses are misguided to usually non-harmful environmental substances. Understanding the immunological similarities and discrepancies in etiology and progression of AD and asthma may reveal early preventative and management strategies aimed towards reducing the global burden of these chronic inflammatory diseases.

We are only beginning to understand the full spectrum of factors that influence the development of allergic diseases. Probably the most intriguing question is why the rise of prevalence of AD and asthma among different cultural populations has happened almost at the same time. It strongly indicates that mechanisms of these two diseases are partially overlapping. In our work, we explored different aspects in the molecular mechanisms associated with AD and asthma. We decided to use less traditional approaches to describe new features of these two diseases, which eventually might lead to better under-

standing of common reasons of AD and asthma. In the first part of the study, we explored cellular responses and tested which cell types are infected by human rhinoviruses (HRVs) in *in vitro* reconstituted bronchial epithelium and human peripheral blood mononuclear cells (PBMCs). In the second part, we studied gene expression changes in the skin and keratinocytes from AD patients and explored a role of apoptosis of keratinocytes and functions of microRNAs (miRNAs) in AD. Our work describes different molecular aspects of AD and asthma and points out that these diseases are very complex and heterogenic conditions.

2. REVIEW OF LITERATURE

2.1. Atopic dermatitis and asthma: parallels and discrepancies

2.1.1. Epidemiology in the historical viewpoint

AD and asthma are becoming the most common inflammatory diseases affecting both children and adults with prevalence rate of up to 20–30% in some countries [4, 5]. The prevalence of these diseases has increased dramatically during last 60 years, starting first from „westernized“ culture area and shifting nowadays into east European and other countries [4–7]. In most cases, AD and asthma can be considered allergic or atopic, i.e. they are associated with recurrent hypersensitivity to common environmental substances: allergens. The increase in atopic diseases has been so intensive that we are in the midst of an allergy pandemic [8]. As an example, among immigrants who arrive from areas with low incidence of asthma, asthma prevalence increases to that of the general local population over a considerably short time [9, 10]. Apparently, atopic and other "modern" diseases, for example type II diabetes are increasingly associated with demographic variables uncommon in earlier human generations factors such as Caesarean birth, formula feeding, repeated exposure to antibiotics, consumption of processed foods and probably some other unknown factors [11, 12].

Before 1870, the cases of allergic diseases were almost not documented because of their minimal occurrence. This is still true for prehygiene societies in Africa nowadays. Asthma and AD prevalence started to increase around 1960s, first in industrialized western countries. Lately, as a new "epidemic", there has been dramatic increase in peanut allergy cases during last 20–30 years (figure 1) [5, 6]. It is alarming that sequential changes in our lifestyle seem to bring us next types of allergic "epidemics". So, to address this concern, we need to further deepen our understanding of the causes of allergic diseases and apparently non-allergic forms of AD and asthma, not only to be able to relieve the symptoms, but to treat the causes themselves.

The AD risk factors associated with increased prevalence of the disease are so-called western diet with high amounts of sugar and polyunsaturated fatty acids, small family size, high education level in the household, and living in urban settings and regions with low exposure to ultraviolet radiation and low humidity [8]. Among others, the strongest risk factor is a positive family history for atopic diseases, particularly for AD [9] for which twin studies suggests a heritability of more than 80% [4]. Children often develop AD during their first year of life (roughly 60% of cases), but for most of children, it disappears around age 3 years [10]. However, AD can start at any age [10, 11] and often, for adults, AD is a more serious condition than it is for children [12].

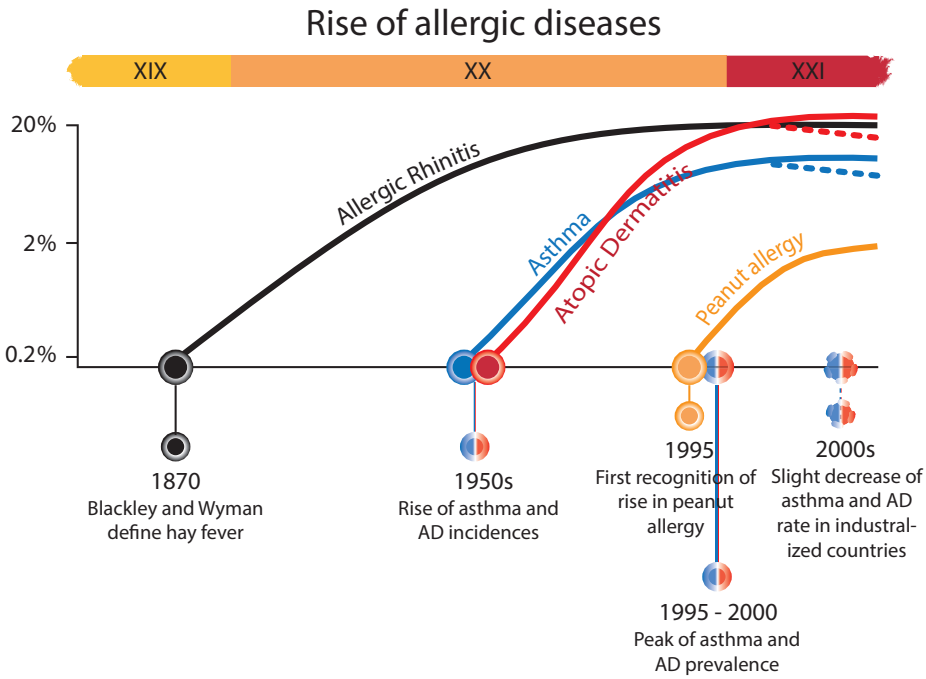

Figure 1. Sequential rises in allergic rhinitis, pediatric asthma, atopic dermatitis and peanut allergy among population of Western societies during last two centuries. Prepared based on information from [7].

The incidence of asthma is the highest in childhood with a gradual decrease after adolescence. Boys have a greater risk of asthma in early childhood, whereas girls are more frequently affected after puberty [13]. Adult females have more severe asthma than males, with more hospital admissions [14–16], slower improvement [17], longer hospital stays [14] and higher rates of readmission [14–16].

2.1.2. Asthma definition

"Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation" [18]. According to the Global Initiative for Asthma report (GINA 2017), past definitions of asthma have been long and descriptive – mostly describing the types of inflammatory cells, hyperresponsiveness, symptoms, and the assumed relationship between these features [18]. On one hand, the need to simplify asthma definition was to simplify the process of diagnosing asthma in the clinical practice. On the other hand, it has been recognized now that asthma phenotype of a specific patient is not stable. The symptom pattern may shift, so diagnosis

and treatment should be reviewed regularly. For this reason, the term “asthma” is now deliberately used as a general umbrella term like “anemia”, “arthritis” and “cancer” to overcome the limitation caused by asthma heterogeneity and phenotype instability [19].

The other problem with asthma classification lies in the fact that the clinical phenotypes are often mixed up with the inflammatory, immune or molecular endotypes. The former are aimed to characterize clinical outcomes of the treatment and the latter are aimed to describe pathological side of the asthma condition. In both cases, the terminology has not been yet standardized and GINA initiatives are aimed to globally solve the problem.

In the current thesis, we use the simplified (umbrella) definition of asthma.

2.1.3. Pathophysiology of atopic dermatitis and asthma

The primary events and key elements leading to AD and asthma are still not precisely known and are under heavy debate.

One can characterize AD as an inflammatory, relapsing, noncontagious and itchy skin disorder. It is characterized by the presence of dry and scaly patches of the skin [20]. It has traditionally been regarded as a childhood disorder with imbalance of the immune system towards Th2 responses and exaggerated IgE responses to allergens, however, AD can occur as a lifelong clinical condition as well [21]. The AD course can be continuous for long periods or relapsing with repeated flare-ups [10, 11]. In about 80% of affected children, the disease is mild [22]. Patients who seem to be outgrown from the disease might continue to have sensitive hyper-reactive skin and might have recurrences after long symptom-free periods [11]. Usually, allergies to milk, egg and wheat resolve during childhood, but allergies to nuts and fish often persist [23]. When children become older, the sensitization is shifted towards inhalant allergens [24]. A subgroup of patients are sensitive to microbial antigens, often originated from yeast or *Staphylococcus aureus* [25]. Most of the children get relief from inflammatory skin diseases when they get older. For adults, AD might be much more serious condition, characterized by chronic, thick, red, lichenified plaques. Patients can experience acute, vesicular or oozing flare-ups. Pruritus is intensive, strongly affecting daily life, sleep and activity of adult patients. In adults, AD affects different body parts as in infants. Commonly, hands, face and especially the eyelids are affected. The skin all over the body can be covered by inflammatory flare-ups – erythroderma. It is much more common for adult group of patients to develop infections and metabolic disorders which necessitate hospitalization [12].

Asthma pathology involves abnormal structural changes in the airway epithelium and submucosa. In the epithelium, we see the goblet cell metaplasia, hyperplasia and increase in epithelial mucin stores. In the submucosa, the subepithelial fibrosis [26–28], alterations in submucosal gland cells (increased gland volume), smooth muscle cells (hypertrophy and hyperplasia) [29–31] and

blood vessel cells (increased number of blood vessels) occur (figure 2) [32]. These changes in the airway may to lead to exacerbations when baseline airway caliber is narrowed and structural elements are altered in a manner that leads to exaggerated responses to inhaled exacerbating factors [33]. Concomitant sensitization to aeroallergens is very characteristic to the childhood-onset asthma, whereas adult one is less related to atopic sensitization [13].

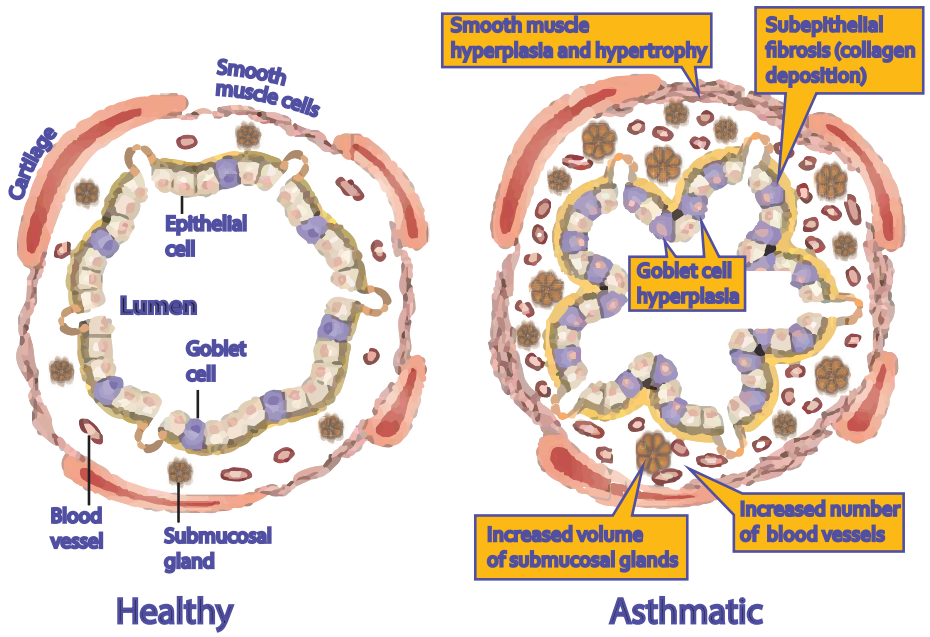

Figure 2. Asthmatic airways are characterized by significant structural changes. The mucous hyperplasia and hypersecretion, significant basement membrane thickening, subepithelial fibrosis, increased number of blood vessels and enlarged submucosal glands and smooth muscle are characteristic for asthmatic airways and epithelium. Prepared according to the information from [34].

2.1.4. Allergic march

Allergic march is the central hypothesis what refers to the phenomena of temporal progression from the childhood AD to allergic asthma and to allergic rhinitis (figure 3, [35–37]). The observation that AD tends to be the first manifestation of these inflammatory processes is strongly supported by some epidemiological data [38]. The allergic march typically appears first in the skin, which partially might be due the weaker skin barrier of very young children [39], or suggesting that this site might have either a particular role in initiating allergic sensitization or the lowest threshold to reveal symptoms. Controversially, wheezing might coincide or even precede development of skin symptoms [22, 40]. But, the evidences are still debated. Furthermore, even if

there is a tendency of developing asthma after AD, only one third of such children develop asthma later in life [41]. Thus, the risk seems to be lower than widely assumed. Nevertheless, there exist several possible mechanisms linking AD and allergic asthma. These include:

- A systemic immune disorders with enhanced Th2 responses to allergens in exposed epithelium [42];
- Barrier defects in the skin or airway epithelium that may lead to over-stimulation of the immune cells by allergens [43];
- a systemic defect causing immune cells to induce an allergic inflammation at any allergen-exposed epithelial surface [43].

The knowledge of Filaggrin (FLG), a skin cornified envelope protein absent from the lung epithelium, supports the idea of "atopic march" phenomena [44, 45]. Although controversial, AD patients with *FLG* loss-of-function mutations have increased incidence of asthma, indicating that the loss of an epidermal-specific barrier protein can induce systemic atopy in humans [46–48]. It is speculated that epicutaneous sensitization with allergens might be behind the development of airway hyper-reactivity [46, 47, 49].

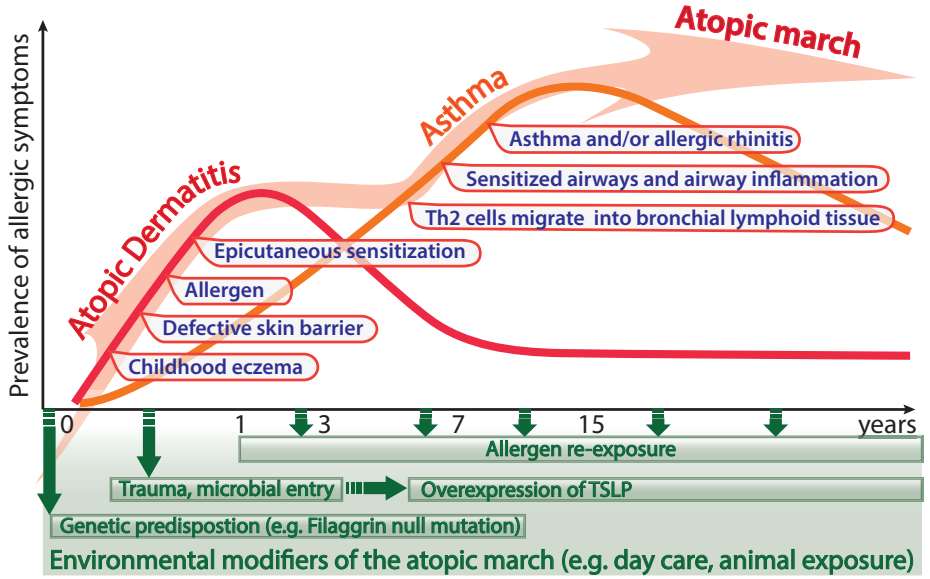

Figure 3. A proposed pathway of atopic march from childhood atopic dermatitis (AD) to the development of asthma. Common genetic and environmental risk factors sum up sequentially along an atopic pathway from AD to asthma. TSLP – thymic stromal lymphopoietin. Prepared according to information from [35, 50]

Still, to be sure that there exists a causal relationship from AD to allergic airway diseases, we need clear evidence that an effective intervention for AD leads to reduced asthma incidence. In other words, we need to have a therapy targeting AD in infants that prevents the subsequent development of asthma [51].

2.1.5. The microbiome and its role in inflammatory diseases

The hypothesis of the atopic march as an explanation of the causal link between AD and respiratory allergic diseases may be erroneous. One might say that if AD would be a causal "entry point" for subsequent asthma and allergic rhinitis, then effective management of AD could possibly deter the progression of the atopic march, but this concept has not been proved yet.

Recently, it has been proposed that our microbiota or more precisely its imbalance might be a casual factor for AD and asthma. The microbiota is a subgroup of all microbes (bacteria, fungi, protozoa, and viruses) that live in or on our body. Microbes outnumber human cells approximately 10 times. Most commensal microbes live in the gut, particularly in large intestine. The composition of intestinal bacterial communities is plastic and can change rapidly in response to changing environmental factors, including invasion by pathogenic microorganisms, antibiotic treatment and diet [52], and this tends to affect immune homeostasis as well.

Beside bacteria, the microbiota includes viruses – so called "virobiota" [53]. It has been shown that virobiota actively shapes the composition of commensal bacterial population [54], thereby influencing the way the host immune system responds to harmful substances and organisms. For example, the microbiome plays important role shaping the responses of the intestinal T lymphocytes [55, 56], directing them towards either being more pro- inflammatory or anti-inflammatory type. In context of our work, it would be interesting to know if there are other factors besides direct infection how HRV might influence immune response of the host.

The other way how the virobiota could impact the host immunity is by triggering immune responses that protect against other pathogenic viral and/or bacterial infections. For example, most people are infected by the herpes viruses during their childhood. Latent herpes infection produces the viral particles throughout our life. Generally, herpes virus infection, being either latent or active, is considered pathogenic. However, it apparently helps to protect the host from other viral and bacterial infections in a long run [57]. Protection is especially characteristic to the chronic infection, which increases the basal interferon γ (IFN-γ) expression and causes the activation of macrophages, which limits the spread of other infecting bacteria and viruses [57].

The nature of microbial exposure early in life appears to be important for the development of robust immune regulation. Disruption of either the microbiota or the host response can trigger different chronic inflammatory diseases and make individual hypersensitive to the allergens [58, 59].

However, similarly to the "atopic march" concept, the association between disruption of the skin or lung microbiota and inflammatory skin and lung diseases is still a mystery, and further studies focusing on the effects of the microbiota are needed.

2.1.6. Genetic predisposition

Epidemiologic observations suggest that a western lifestyle and urbanization have important roles in the increasing prevalence of AD and asthma [60]. It is also difficult to find a genetic explanation for the observation that AD is more common in wealthier and more educated families [61, 62] or in the ethnic groups recently migrated from a country of low prevalence to a country of high prevalence [63]. However, despite exposure to similar environmental factors, only some people develop allergies and therefore, a family history is considered a strong predisposing factor for AD and to some extent for asthma [60].

Common chromosomal linkages for AD and asthma have been identified on chromosomes 5q31–33, 11q13, 13q, 6p12.3 and 12q21.3 [64, 65]. Among these, Th2 type cluster of cytokines (encoding IL-3, IL-4, IL-5, IL-9, IL-13) and granulocyte macrophage colony–stimulating factor (GM-CSF) encoding genes are located on 5q31–33 [66]. Genome-wide association studies (GWASs) of asthma have added many more susceptibility factors. Most prominent are the *IL1RL1*/*IL18R1*, *IL33*, *protocadherin 1* (*PCDH1*), *cadherin-related family member 3* (*CDHR3*), *HLA-DQ*, *serine protease inhibitor Kazal-type 5* (*SPINK5*), *G protein–coupled receptor A* (*GPRA*), and *orosomucoid-like 3* (*ORMDL3*) at the 17q12-21 locus. However, it should be noted that GWAS method has its known limitations. So, these asthma-associated alleles have only small effect sizes. Presumably, the influence of these genes is strongly affected by other factors like the presence of allergens, pollution, viral infections, etc. [67]. As for AD, a number of factors like *TLR2*, *TLR9*, *CD14*, *TOLLIP*, *MYD88*, *MAL*, *NOD1*, *NOD2* and *NALP12*, have been reported to be associated with the condition in GWAS studies. However, one must understand that the GWAS studies give us only the list of candidate genes which might be associated with these diseases. It is still critical to validate the functionality of these candidate genes with the other methods.

Thus far, loss-of-function mutations in a key epidermal structural protein *filaggrin* (*FLG*) are thought to be as a major genetic factor for the development of AD and progression to AD-associated asthma [68–70]. However, most patients with AD do not have any *FLG* mutation, and up to 60% of carriers do not develop atopic diseases [68], indicating that *FLG* mutations are neither necessary nor sufficient to cause AD. It has been suggested that in case of mutated *FLG,* allergens reach more easily through the *startum corneum* and therefore immune cells and keratinocytes get activated [71]. During the development of AD and asthma, accumulation of multiple promoting genetic and environmental factors take place. Thus, although the rapid increases in disease prevalence is hard to explain by genetics, there seem to be certain set of genetic factors, which increase susceptibility to AD and asthma.

2.1.7. Immune responses in asthma and atopic dermatitis

AD and asthma both are chronic and relapsing in their nature. Traditionally, the pathogenesis of allergic asthma and AD has been interpreted as immune dysregulation with predominant Th2 cytokines, such as IL-4, IL-5 and IL-13. Th2 cytokines IL-4 and IL-13 support immunoglobulin switching in B cells, inducing IgE synthesis and the expression of adhesion molecules and recruiting various immune cells into skin or lung subepithelial layer. IL-4 and IL-13 have a permissive effect on microbial invasion and epidermal barrier disruption [72], and induce spongiosis [73]. IL-3, IL-5 and GM-CSF promote bone marrow differentiation and maturation of eosinophils [74, 75]. IL-3 and GM-CSF induce basophil recruitment [76]. Lesions of AD are mostly caused by Th2 and Th22 responses [77], to lesser extend by Th17 [78, 79] in Asian cohorts and Th1 response in the chronic skin lesions [80].

Lately, it has been shown that innate lymphoid cell 2 (ILC2) and ILC3 are capable of mediating the development of inflammation in asthma or AD without allergen specific activation of T lymphocytes [81]. Furthermore, the increased numbers of ILC2 are found in AD lesions [82, 83]. ILCs are recently identified immune cells that are similar to T helper cells, but lack an antigen receptor. Similar to Th1, Th2 and Th17 cells, ILCs can be grouped based on cytokines they produce to ILC1, ILC2 and ILC3 cells. ILCs can be activated by cytokines and danger signals produced by other cells and thereby contribute to the development of Th2 type immunity in allergic AD and asthma. As ILCs have shown to be capable of initiating of chronic tissue inflammation without functional adaptive immunity, they have been associated with the development of non-allergic forms of asthma and AD [81, 84].

Still, the reasons why Th2 type cytokines and immune responses are prevalent in asthma and AD are not well understood. It may be that immune programs become fixed because they are established in early life when the immune system is particularly plastic [85]. Type 2 inflammatory responses are often initiates in the airways in childhood, when viral respiratory tract infections and/or exposures to oxidants (such as cigarette smoke or other airborne pollutants) tend to activate airway epithelial cells to produce IL-25, IL-33 or TSLP. This initiates a pathogenic cascade, which leads to the development of asthma in children who are susceptible because they have pre-existing atopy, specific genetic risk factors in regulators of type 2 inflammation or other less well-understood vulnerabilities [86].

We do not know exactly why allergic diseases, including the most common forms of AD and asthma, exist in the first place. The prevalent hypothesis suggests that Th2 type cytokines and immunity, including Th2 cells, eosinophils and ILC2s are aimed at the elimination of worms and parasites and that the allergy represents a misfiring of this response to otherwise harmful substances [81, 87–89]. Parasitic infections trigger similar inflammatory responses, suggesting that allergy might be as a manifestation to ‘frustrated’ parasite elimination.

However, the majority of allergens are not helminths or their products. Most of the allergens are a diverse group of molecules with no apparent similarity in their structure or biological activity. Most often, we see in this group proteins associated with pollens, shellfish, nuts, animal dander, venoms and penicillin [90]. IgE-mediated sensitization is not the major shared mechanism driving the excess comorbidity of asthma and AD as it has been shown by a cross-sectional analysis in 12 European birth cohorts [91].

Even Th2 type responses of the immune system are considered triggering in AD and asthma, the chronic inflammation in the tissues is associated with activation of multiple cytokines and recruitment of different types of immune cells, which thereby all contribute to the severity of the disease. For example, it has been shown that Th1 type responses are dysregulated and can be the main cause of the apoptosis of keratinocytes in cases of AD [92, 93]. Th1 cell-mediated apoptosis via the Fas ligand resulted in spongiosis, which is a very characteristic feature of AD [93]. The keratinocyte apoptosis itself initiates increased expression of the factors that increase T-cell infiltration into epidermis, which then leads to the enhanced expression of other key elements for apoptosis, such as interferons and TNF receptor superfamily member 6 (FAS) [94]. In addition, Th1 cells in peripheral blood high in IFN-γ selectively undergo activation-induced cell death and skew the immune response toward Th2 cells in patients with AD [95], suggesting that there exist multiple pathways to suppress Th1 type immune responses in allergic diseases.

2.1.8. Treatment

AD and asthma cannot be cured at present; thus, the aims of the current therapies are to relieve symptoms and achieve long-term disease control.

Present prevention and treatment of AD focus first on restoration of epidermal barrier function, which is best achieved through the use of emollients, and use anti-inflammatory medicines like topical corticosteroids or calcineurin inhibitors. Also, it is important to avoid AD triggering factors [96]. After stabilization of the AD condition, it is strongly suggested to continue with emollient treatment [97]. When topical treatment fails, systemic immunosuppressive therapy is sometimes the option. The most widely used agents are cyclosporin, azathioprine, methotrexate and mycophenolate mofetil. The usefulness of these general suppressors of immune responses for AD is still unclear [98]. During recent years, multiple specific biological treatments have been tested. Dupilumab is the newest very promising immunotherapeutic to improve the signs and symptoms of severe AD, including pruritus, symptoms of anxiety and depression. Dupilumab blocks the alpha subunit of the interleukin-4 receptor (IL-4Rα), and thereby inhibits signaling of both the IL-4 and IL-13 pathway [99].

Similarly to the therapy of AD, current asthma treatments can in most cases effectively control symptoms and the inflammation, but do not affect the

underlying inadequate immune response. The disease-modifying drugs are almost missing. The current therapies mostly rely on bronchodilators, corticosteroids and β2-adrenergic receptor agonists that are used to treat the episodes of shortness of breath (dyspnea). Although a large subgroup of asthma patients has overactive Th2 type responses and a strong eosinophilia, the targeting of allergic asthma with selective therapies has not been as successful as hoped. However, recently among biological therapeutic agents, a monoclonal antibody (mAb) to IL-5 has been demonstrated to be effective in a subgroup of asthma patients with severe eosinophilic form of the disease [100]. The somehow disappointing slowness in the drug development reflects the complexity of the underlying pathogenic mechanisms of AD and asthma.

2.2. miRNA in asthma and in atopic dermatitis

Asthma and AD are characterized by tissue inflammation and changes in the immune system, which along with other factors is regulated by miRNAs. miRNAs are small non-coding RNA molecules that inhibit the translation and/or induce degradation of their target mRNAs. To date, more than 2000 unique miRNAs are found to be encoded by the human genome [101, 102] and are thought to modulate expression of >60% of genes [103]. MiRNAs are encoded either within the introns of protein-coding genes or by independent genes and transcribed by RNA polymerase II [104]. After transcription, mature miRNAs are produced through a multi-step process [105]. First, the primary transcript (pri-miRNA) is processed by the nuclear RNase III Drosha – DiGeorge syndrome critical region gene 8 (DGCR8) complex, into an approximately 65 nucleotide hairpin precursor miRNA [106]. Pre-miRNAs are exported from the nucleus into the cytoplasm by exportin 5 and cleaved by the RNase III enzyme Dicer into a double-stranded miRNA-miRNA* duplex approximately 22 nucleotides in length [107, 108]. This miRNA duplex is unwound by helicases and a mature single-stranded miRNA is incorporated into the RNA-induced silencing complex (RISC) [109]. The RISC complex binds to target mRNA through partially complementary sequence of incorporated miRNA [109, 110] (figure 4).

miRNAs typically change target mRNA levels by binding to the 3′ untranslated region (UTR) of mRNA transcripts [112]. This happens through complementary binding of specific seed sequence at the 5′ end of a miRNA to the target mRNA [113]. Sequence complementarity between the rest of the miRNA and the target mRNA is often quite low, allowing individual miRNAs to target multiple mRNA sequences and making the prediction of miRNA targets difficult [113–116]. MiRNA:mRNA interactions decrease protein translation and/or induce target mRNA degradation, resulting in reduced amount of mRNA and protein.

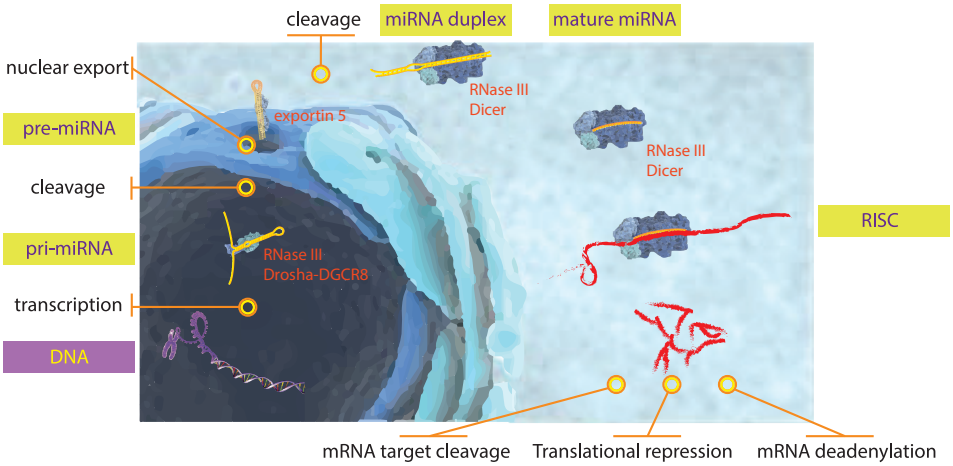

Figure 4. miRNA biogenesis and function. Primary miRNA transcript (pri-miRNA) is transcribed and cleaved to hairpin-shaped pre-miRNA, which is exported to the cytoplasm and further processed to miRNA duplex by Dicer. The duplex is unwound by helicases and a mature single-stranded miRNA is assembled into the RISC complex. The RISC complex typically modulates target mRNA stability or translation by complementary binding of miRNA to the 3′ UTR of mRNA. In case of full complementarity to the target mRNA, miRNAs also can mediate mRNA cleavage. Prepared according to information from [111].

Among immune system-related miRNAs, miRNA-155, miRNA-21 and miRNA-146a are very intensively studied and have been also shown to affect tissue inflammation and immune responses in allergic diseases. In addition to these three, multiple other miRNAs have been demonstrated to be dysregulated and affect the development of asthma and AD. At present, there have been performed many miRNA expression profiling studies on samples from patients with asthma and AD [117, 118]. Profiling has identified tens of differentially expressed miRNAs in bronchial epithelial cells [119, 120], exosomes from bronchial alveolar lavage fluids [121] and peripheral blood samples from patients with asthma [122–124]. The roles of particular miRNAs in AD and asthma are studied less, however, in some cases the functions have been elucidated. For example, miRNA-19a expression has been shown to be upregulated in T cells from asthmatic airways, where it was shown to promote Th2 type cytokine production [125].

Patients with AD have been shown to have increased expression of miRNA-155 in T lymphocytes and in the skin where it is induced by allergens and superantigens [126]. Numerous studies have demonstrated that miRNA-155 functions as an activating factor of the innate and acquired immune responses, immune cell development and as a suppressor carcinogenesis [127]. As the presence of miRNA-155 is needed for the development and functions of Th2 cells, it accordingly has been shown to contribute to allergic inflammation in animal models of airway inflammation and helminth infection [128, 129]. In the

immune system, miRNA-146a appears to be more important for controlling of Th17/Th1 responses and the function of Treg cells, but not Th2 cells [128]. miRNA-21 has been found to have a critical role in polarization and activation of Th2 cells, and it contributes to the development of allergic airway inflammation in mice [130, 131]. miRNA-21 and miRNA-223 regulate development of eosinophils, and thereby also contribute to the development of type 2 immunity [130, 132]. miRNA-375 is downregulated and plays important role in triggering the IL-13-driven epithelial responses [133].

In addition to the regulation of immune responses, miRNAs can influence the course and severity of allergic diseases through the modulation of inflammatory responses of the epithelial cells, such as skin keratinocytes and bronchial epithelial cells [134].

2.3. Human rhinoviruses

HRVs are large group of viral pathogens in humans. They were discovered 1950s and for today, we know three genetically distinct HRV species – HRV-A, HRV-B and HRV-C (figure 5). HRV infections have typically two seasonal peaks in the spring and autumn all around the world. HRVs usually infect the upper respiratory tract (URT) with characteristics of rhinitis or nasal congestion, although up to 15% of patients may be clinically asymptomatic [135].

In the early 90s, HRV-A and HRV-B group were distinguished from each other serologically [136] and later, this distinction was more deeply characterized using molecular analysis. HRV-C as a new rhinovirus subtype has only been recognized since 2009 [137]. For the moment, there are 74 HRV-A, 26 HRV-B and at least 50 HRV-C known subtypes [138]. HRV strains are also classified based on the receptor they use to enter to cells. Eleven members of the HRV-A gain cellular entry through interaction with members of the low-density lipoprotein receptor family (LDLR). The remaining strains of HRV-A and all members of the HRV-B bind to intercellular adhesion molecule 1 (ICAM-1) [139]. The asthma susceptibility gene product cadherin-related family member 3 (CDHR3) has been shown to mediate HRV-C binding and entry [140].

HRV is a single-stranded, positive-sense RNA virus of the *Picornaviridae* family with an approximately 7.2-kb genome enclosed in a protein capsid of roughly 27 nm in diameter. HRVs are non-enveloped viruses, which capsid consist of four viral proteins, VP1, VP2, VP3 and VP4. HRVs are assigned to subtypes according to the sequence of VP4/VP2 or VP1 proteins [141].

The airway epithelium is the primary site of HRV infections in both the upper and the lower airways [142, 143]. Because HRV infection does not cause strong epithelial cytotoxicity, alterations of epithelial biology are believed to be the initiating events in the pathogenesis of HRV infections. In healthy individuals, HRV infection of the upper airways is the major cause of the common cold and spread of HRV infection to the lower airway epithelium generally is

very unlikely. In patients with asthma, the increased asthma symptoms can occur several days after peak of nasal symptoms, indicating that subsequent spread of HRV to lower airways might trigger acute exacerbations of asthma.

Upon attachment to the cell, the virion is taken up by the endosomal pathway. Low pH in the endosomes causes uncoating of the virion and release of positive-sense strand RNA, consequent translation of viral proteins, replication of viral RNA through formation of negative-sense complementary strands that then allow transcription of further positive strands, and formation of new viral particles. Newly assembled viruses are released through epithelial cell lysis and distributed onto neighboring cells [138].

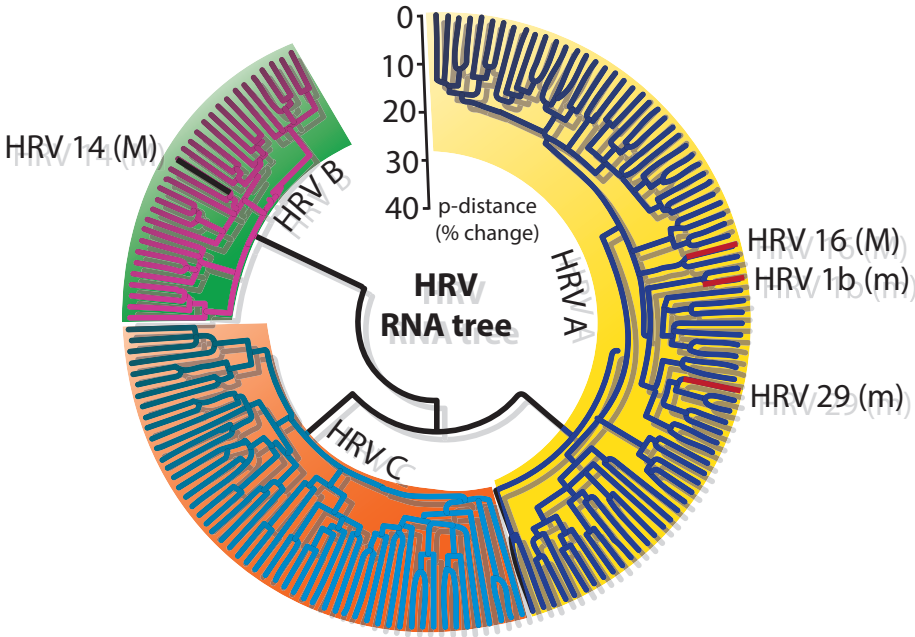

Figure 5. Circle phylogram of HRV-A, HRV-B and HRV-C according the viral RNA (vRNA) composition. The relative position of minor group HRV1b, HRV29 ('m', LDLR) and major group HRV14, HRV16 ('M', ICAM-1) is shown with red/black bar. Prepared according to information from [144].

2.4. Human rhinovirus infection

The airway epithelium is the first line of defense against HRV infections. Even so, most of the HRV infections appear to directly affect a small subset of cells and cell lysis is generally not extensive. Studies to map infected cells in the airways have generally found evidence of infected patches, and this corresponds to 1–5% infection rate of epithelial cells *in vitro* even after inoculation with high titers of virus [143, 145]. Well-differentiated epithelial cells are relatively

resistant to HRV infection [146]. HRV replication is enhanced when apical cells of well-differentiated epithelial cell cultures are either damaged or stripped away [147]. The epithelial barrier in asthma may be compromised and allergic inflammation and exposure to pollutants may exacerbate this condition. It is therefore possible that a reduced barrier function in asthma could promote more severe HRV infections. HRV infection itself can also disrupt epithelial barrier function, and perhaps, this effect contributes to secondary bacterial infection [148].

When exposed to HRV, dendritic cells (DC), which serve as primary antigen-presenting cells that bring signals of infection to the adaptive immune system, migrate more slowly to the lymph nodes and provide less inflammatory signals than without viral exposure [149]. Following HRV infection, T cells move from the circulation to the lung [150]. It has been shown that major group of rhinoviruses - HRV-16 (HRV-A) or HRV-14 (HRV-B) – significantly inhibit T cell proliferation [151, 152]. Inhibition of T cell proliferation is dependent on HRV binding to ICAM1 on monocytes, indicating that the virus interferes with lymphocyte activation indirectly through effects on antibody presenting cell (APC). This reduction is greater when more DCs are exposed to the major group HRVs [152]. In addition to proliferation, HRV inhibits T-cell cytotoxic responses, but not NK cell activity [151].

2.5. Human rhinoviruses and asthma exacerbation

Asthma exacerbation is a complex clinical phenomenon involving a loss of asthma control that leads to a set of symptoms, including shortness of breath, wheeze, cough and sputum production. Airway narrowing during asthma exacerbations results not only from concentric smooth muscle contraction but also from mucosal edema and the formation of pathological intraluminal mucus. Exacerbations can range from mild to severe and can result in near-fatal or fatal episodes of respiratory failure. The most common cause of asthma exacerbations is viral upper respiratory tract infections [153]. Common causative viruses include respiratory syncytial virus in children and HRV in adults [33]. It has been shown that recurring severe HRV infections with wheezing in early life is the strongest predictor of asthma development later in life [154].

The effects of HRV on asthma exacerbations are greatest in allergic individuals [155] and may be amplified by exposure to allergens [156] and possibly by exposure to greater levels of air pollutants [157]. In non-asthmatic subjects, virus infection is completely cleared by the host immune system causing only minimal cell death and often not inducing long-term immune memory. Interestingly, HRV infection of subjects with asthma does not usually provoke acute asthma symptoms [158, 159] and furthermore, most HRV infections in patients with asthma cause only upper respiratory symptoms similar to responses of non-asthmatic people.

Recent studies utilizing prospective monitoring of nasal secretions of school-aged children indicate that HRV infections are nearly ubiquitous in children

regardless of asthma [160]. Many of these infections are either asymptomatic or mild, even in children with asthma. This suggests that viral infections, most often due to HRV, are necessary but often not sufficient to cause acute exacerbations of asthma. Probably there are cofactors that either increase the severity of HRV infections, or else have additive effects on airway physiology to promote airway obstruction and acute symptoms of asthma [161].

Many findings suggest that reduced IFN responses could contribute to HRV-induced exacerbations of asthma, but the nature and mechanisms of deficient IFN responses and asthma is still an area of controversy. According to some studies, IFN responses of PBMC [162, 163] and pDCs [164] are reduced in asthma. It has also been reported that HRV-induced production of IFN-β and IFN-λ is impaired in *ex vivo* cultured epithelial cells of asthma patients [165, 166], raising the possibility that asthma is associated with a global defect in IFN production. However, other studies have reported that asthma does not have significant effects on IFN responses in cultured epithelial cells [167–169]. Moreover, an observational study of naturally acquired colds found similar viral shedding in children with vs. without asthma [169], while studies of experimentally inoculated volunteers have shown both – no difference or there is a difference in the extent of immune responses to HRV between these two groups [158, 169–171].

Even being the major viral finding in respiratory virus-associated asthma exacerbation, the evidence supporting a causal relationship of HRV infection and asthma exacerbation is weak and mechanisms are poorly understood.

2.6. B cell responses in atopic dermatitis and asthma

Immunologically, atopy is recognized as an increase in the Th2 and IgE-expressing memory/effector B cell numbers and appearance of plasma cells producing IgE antibodies specific to common environmental allergens. Although T cells are key players in the pathogenesis of AD and asthma, B cells are also found in the dermis of AD lesions [172] and in the airways in allergic inflammation [173]. B cell derived IgE contributes to the infiltration of eosinophils, as they express low-affinity IgE receptor FcεRII, into the epithelial layer of asthma patients [174]. Undoubtedly, B cell responses to common allergens play significant role in the development of these chronic inflammatory diseases.

B cells can present antigens to $CD4^+$ T cells and activate T cells. B cells interact with T cells via MHC class II molecule/T cell receptor and co-stimulatory molecules, as well as their ligands. Co-stimulatory molecules include CD40 and CD80/CD86 on B cells and their ligands, CD40L and CD28 on T cells. The expression of costimulatory molecule CD86 is increased on B cells in AD [175]. Th2 cytokine IL-4 induces immunoglobulin switching in B cells, resulting in IgE synthesis, which then promotes the expression of adhesion molecules and recruits various immune cells. B cells also produce chemokines

CCL17, CCL22 and IL-16, attracting T cells into the inflammatory tissue. IgE also contributes to IgE-mediated inflammation by stimulating FcεRI-expressing cells, such as mast cells and basophils. However, the role of IgE in pathogenesis of AD and asthma might be different: while attempts to inhibit IgE with omalizumab have shown heterogeneous therapeutic efficacy in AD [176], they are promising in reducing inflammatory symptoms in allergic asthma in case of patients with increased IgE [177].

How HRV enhances inflammatory processes in the airways, is most probably different from classical Th2 pathway. HRV infection of primary epithelial cells and epithelial cell lines is accompanied by a release of inflammatory mediators *in vivo* and *in vitro*, such as pro-inflammatory cytokines IL-1β, TNF-α, IL-8, IL-6 and IL-11, and the vasoactive peptides, bradykinin and lysyl bradykinin. It appears that a small number of HRV-infected epithelial cells release a variety of inflammatory factors. These soluble mediators are thought to orchestrate proliferation, chemotaxis, and activation of inflammatory cells, resulting in an amplification of the inflammatory process [149].

We need to take into consideration that B cell activation might be initiated following engagement of the B cell receptor (BCR) by a specific antigen in either a T-cell-dependent (TD) or T-cell-independent (TI) manner [178]. It has also been suggested that B-lymphocytes may become increasingly relevant as antigen presenting cells when antigen load is low [179] and play important role in airway hyper-reactivity and airway inflammation, even without the presence of T-lymphocytes [180]. So, it is possible that B-lymphocytes induce asthmatic response without the help of T-lymphocytes [180].

2.7. Summary of the literature

Despite much progress, interpretation of research findings for AD and asthma has been difficult. Our understanding of the history of these diseases and the factors determining the remission and persistence of these conditions is still incomplete, as is the knowledge on epidemiology and molecular mechanisms behind clinical features. Epidermal dysfunction in AD and abnormal structural changes in the airway epithelium in asthma are undoubtedly among the major pathogenic mechanisms. However, whether these are the primary drivers, how exactly they affect the individual patients, and what might be the most optimal strategies for treatment are not yet satisfactory understood.

The research included in the current thesis was aimed from the gaps in our knowledge and state-of-the-art discoveries in the field in the period from 2007 to 2010. At that time, studies on HRV infection and asthma exacerbation were initiated in multiple labs to find a way to reduce the number of asthma exacerbations. At the same time, a few large scale array analysis studies about mRNA expression in the skin of AD patients had been published, however, because of differences in the array platforms and patient selection criteria, the results from those studies were not always well overlapping. Similarly, the roles

of some particular miRNAs in the immune system had been described, however, functions of particular miRNAs in allergic inflammation was almost fully undescribed.

"Allergic march" is one of the intriguing hypotheses that might connect AD and asthma and give us an extra edge for understanding molecular mechanisms behind these two conditions. Another link between these two diseases is that they both are characterized by chronic inflammation of the epithelium and multiple similarities can be found in the inflammatory responses of bronchial epithelial cells and keratinocytes. However, in most of the cases, these two diseases are still studied separately. Taken together, the research performed within the current thesis was planned with the aim to elucidate particular molecular mechanisms contributing to the development of AD and asthma.

3. AIMS OF THE STUDY

The general aim of the current thesis was to explore particular molecular mechanisms involved in progression of two chronic inflammatory diseases – asthma and atopic dermatitis.

More specific hypotheses and aims were:

Study I: HRVs are one of the main causes of virus induced asthma exacerbations. It was known that HRVs affect epithelial cells as well as immune cells, and that lymphocytes infiltrate into the subepithelial tissue of the airways during HRV infection in allergic individuals. However, the mechanisms through which HRVs infect and modulate the immune responses of lymphocytes were not yet well described. The aim of this study was to visualize the dynamics of HRV uptake, to describe HRV impact on molecular responses of monocytes and lymphocytes, and to clarify whether these cell types can be infected by HRVs.

Study II: Goblet cell metaplasia and mucus hypersecretion are characteristic features of asthmatic epithelium. We hypothesized that HRV infection induces changes in mRNA expression of asthma candidate genes, and that different types of terminally differentiated epithelial cells might have variable susceptibility to HRV infection. Accordingly, we aimed to explore mRNA expression in bronchial epithelial cells from asthma patients cultured in air–liquid interface (ALI). In addition, we aimed to elucidate how Th2-type cytokines affect the differentiation of bronchial epithelial cells and HRV infection in ALI cultures.

Study III: Skin inflammation in AD was known to be associated with altered expression of pro-inflammatory genes and activation of innate immune responses in keratinocytes. We hypothesized that these processes are also affected by miRNAs. We aimed to describe miRNA expression profiles of keratinocytes from AD patients, and to elucidate the role of specific miRNAs in keratinocytes and skin inflammation in AD using relevant cell culture and mouse models.

Study IV: Enhanced apoptosis of keratinocytes was known to be the main cause of eczema and spongiosis in patients with the AD. IFN-γ was suggested to affect keratinocytes during chronic skin inflammation in AD. The aim of this study was to explore whether keratinocytes from patients with AD might exhibit differences in IFN-γ–induced apoptosis compared with keratinocytes from healthy subjects, and to describe alterations in mRNA expression in keratinocytes and skin from AD patients using mRNA array analysis.

4. METHODOLOGICAL CONSIDERATIONS

The material and methods are described in detail in the method and supplementary method sections of published papers I–IV [181–184]. The most important methods and materials are also given here.

4.1. Patient samples and cell cultures

The patient materials, cell-lines, human primary cells and related materials are described in table 1. For all collected patient and control samples, we obtained approvals from relevant institutional ethical committees and written informed consents from each participant (papers I–IV) [181–184].

PBMCs from voluntary healthy donors were used to estimate proliferation rates upon HRV stimulation using ^{3}H-thymidine or carboxyfluorescein succinimidyl ester (CFSE) staining assay, to be infected with different HRV serotypes, to analyze the infection kinetics by cell imaging flow cytometry and to detect HRV infection centers inside B lymphocytes by *in situ* hybridization (ISH) (paper I) [181].

Cytological airway brushings and microbiopsies of bronchial mucosa were used to obtain bronchial epithelial cells and to develop air-liquid interphase (ALI) cultures to study HRV16 replication and virus-induced changes in mRNA expression. Cytological brushings and microbiopsies of bronchial mucosa were obtained by bronchoscopy from 13 patients with a median duration of asthma of 16 years (38% with early onset) and mostly with partially controlled (38%) or uncontrolled disease (30%). We also collected bronchial epithelium samples from seven non-asthmatic donors (aged 27–62 years; three female subjects) who underwent diagnostic bronchoscopy, but chronic airway disease was ruled out during further investigation. All donors were current nonsmokers (paper II) [183].

HeLa cells were used to propagate HRV serotypes 1b, 14, 29 and 16 and to estimate HRV titer according to 50% tissue culture infective dose (TCID50)/ml of produced viruses and to reinfect with viruses originated from infected PBMCs (papers I, II)[181, 183].

Human primary keratinocytes were used to detect apoptotic cells, to perform miRNA and mRNA profiling using miRNA and mRNA Illumina arrays, to stimulate with different cytokines, to transfect with miR-146a precursors and inhibitors. Generation and maintenance of primary keratinocytes from 5 healthy subjects, 5 patients with AD, and 5 patients with psoriasis who went under cosmetic surgery are described previously [185, 186]. All included subjects were older than 18 years and did not receive systemic treatment and topical corticosteroids during at least 1 week before the study. Other functional experiments were perfomed using commercially available pooled human primary keratinocytes from adult donors (Promocell) (papers III, IV) [182, 184].

Table 1. Patient samples, primary cells, cell lines, viruses and mice.

Materials	Methods	Paper
PBMCs	to estimate proliferation rates upon HRV stimulation using ^{3}H-thymidine	Paper I
	to estimate proliferation rates upon HRV stimulation using CFSE staining assay	Paper I
	Infection with different HRV serotypes (HRV1b, HRV14,HRV16, HRV29)	Paper I
	to analyze the infection kinetics of different cell subtypes by cell imaging flow cytometry	Paper I
	in situ hybridization (ISH) of HRV	Paper I
cytological airway brushings, biopsies of bronchial mucosa and bronchial epithelium samples in ALI culture	HRV infection and replication	Paper II
	to analyze HRV impact on mRNA expression	Paper II
	immunofluorescence	Paper II
HeLa cells (Ohio)	to propagate HRV serotypes 14, 16, 1b, and 29	Paper I
	to estimate HRV titer	Paper I
	to reinfect with viruses originated from infected PBMCs	Paper I
primary keratinocytes from AD patients and controls (from L. Kemeny, University of Szeged) and commercial human primary keratinocytes (Promocell)	apoptosis analysis	Paper IV
	miRNA and mRNA expression analysis using Illumina arrays and RT-qPCR	Papers III, IV
	to stimulate with different cytokines	Papers III, IV
	to study the effect of miR-146a after transfection with miR-146a precursors or inhibitors	Paper III
skin biopsy specimens from AD patients and controls	mRNA and miRNA expression analysis, Western blot, laser microdissection, immunohistochemistry and immunofluorescence	Papers III, IV
DyLight650-labelled HRV1b, HRV16; from S.L. (Johnston lab, Imperial College London)	to measure the kinetics of HRV attachment to different PBMC cell types	Paper I
ultraviolet inactivated HRV1b and HRV16	negative control in HRV experiments	Paper I
HRV1b	to detect HRV replication centers in B lymphocytes by ISH	Paper I
HRV16	to infect ALI cultured HBECs	Paper II
miR-146a–/– mice in C57BL/6J background and C57BL/6J (B6) wild type mice (The Jacksons Laboratory)	to study the effect of miRNA–146a in MC903-dependent mouse model of AD	Paper III

Skin biopsies (diameter: 4 mm) were collected from 10 patients with chronic AD (5 females, 4 males, age 18–42) and 9 healthy subjects (4 males, 5 females, age 19–43). All patients had experienced 6- to 14-day-long severe exacerbation of the disease. None of the patients had been treated with systemic antihistamines or topical corticosteroids for at least 1 week before inclusion in the study.

4.2. Apoptosis detection

Generation and maintenance of primary keratinocytes for apoptosis assays was performed as described previously [185, 186]. In brief, keratinocyte viability was measured by means of flow cytometry after staining with 7-amino-actinomycin D (7-AAD) and annexin V (Beckman Coulter) according to the manufacturer's protocol. Viability represents the percentage of annexin V- and 7-AAD-negative cells. Cells that were early apoptotic were annexin V-positive, late apoptotic and necrotic cells were annexin V and 7-AAD-positive].

4.3. Generation of HRV stocks

HRV serotypes 1b, 14, 29 and 16 were propagated in Ohio HeLa cells using standard protocol [160]. Virus stocks were titrated by infecting HeLa monolayers with serially diluted HRV and assessing cytopathic effect to estimate their 50% tissue culture infective dose TCID50/ml by Kremser method. The identities of all HRVs were confirmed by neutralization using serotype-specific antibodies (ATCC). TCID50/ml was expressed as multiplicity of infection (MOI) based on TCID50; 0.1 ml of a TCID50 virus of 1e7 per ml on 1e6 cells would be an MOI of 1.

4.4. Ultraviolet inactivation of virus and 'Mock' solution

UV-inactivated virus was prepared by irradiating the virus suspensions in a 24-well tissue culture dish on ice for 10 min with a 75 W UV source (254 nm) at a distance of 5 cm. Treatment resulted in the complete loss of infectious titer as estimated by titration assay. To produce 'mock' solution, Ohio HeLa cells were cultured as for virus production, but without viruses to the culture.

4.5. Labeling of HRV with DyLight650

We developed a new method to visualize HRVs and measure the kinetics of infection based on labeling of viruses with DyLight650 (Thermo Scientific). First, media component in the virus stock (both active and UV inactivated) solutions was replaced with PBS by dialyzing it 2 times for 4 h at 4°C with cold

PBS (pH 7.0). For dialysis, membrane MWCO of 6–8 kDa Spectra/Por Dry Standard RC Dialysis Tubing (Spectrum Labs) was used. For the labelling, 1 mg of DyLight650 NHS Ester was added to 15 ml of virus samples, mixed, and stained at RT for 1 h protected from light. Labeled and starting nonlabeled virus solutions were titrated by endpoint titration assay in parallel and stored at –80°C. Labeling caused the drop in virus infectivity 5 times when compared with the starting nonlabeled virus (paper I, [181]).

4.6. Isolation and culturing of PBMCs

PBMCs were obtained from heparinized whole blood by Biocoll (Biochrom KG) density gradient centrifugation. The cells were washed three times with PBS (0.02% EDTA added) and suspended in RPMI 1640 medium supplemented with L-glutamine (2 mmol/l), MEM vitamin, penicillin (100 U/ml), streptomycin (100 lg/ml), kanamycin, nonessential amino acids, sodium pyruvate (Life Technologies), and 10% heat-inactivated fetal calf serum (FCS; Invitrogen, Cat.No. 10082147).

4.7. Proliferation assay with ^{3}H-thymidine

After 5 days of stimulation with unlabeled active or UV-inactivated or without viruses (mock) in triplicate, PBMCs were pulsed for 8 h with 3H-thymidine, harvested with a Tomtec plate washer, and counted on a Betaplate counter. The results were expressed as a stimulation index (SI).

4.8. CFSE labeling of PBMCs

PBMCs were washed in PBS twice and suspended in 10 ml PBS containing 5% FBS. CSFE (from 10 mM stock and at the final concentration of 5 μM) was added to the tube and immediately shaken vigorously. Labeling of PBMCs was done promptly 5 min at room temperature in the dark. The cells were washed three times in PBS supplemented with 5% FCS. Thereafter, complete RPMI 1640 (cRPMI) media were added and the cells were transferred to a cell culture incubator (at 37°C under 5% CO2) for further experiments.

4.9. Flow cytometry and cell sorting

For the expression of surface markers, the cells were stained with the following antibodies: CD3-FITC, CD16-FITC, CD19-ECD, CD19-PC5 (all from Beckman Coulter), CD4-PE/Cy7, CD14-APC/Cy7, CD19-Brilliant Violet 510, CD19-APC/Cy7 (all from BioLegend), TRAIL-R2-FITC (R&D Systems) and FN14-FITC (eBioscience). Matching isotype controls were used as negative controls.

Samples were measured with a FACSAria II instrument (Becton Dickinson) or Gallios flow cytometer and analyzed using Kaluza software (Beckman Coulter). Dead cells were excluded based on staining with the eFluor 450 dye. Before imaging flow cytometry, CD14+ cells were sorted out from the rest of the cells according to the CD14-APC/Cy7 signal. To obtain purified CD14+ cells, we excluded from this cell population eFluor 450-positive cells as dead cell population.

4.10. Imaging flow cytometry of PBMCs infected by HRV

For tracking HRV infection capacity, DyLight650 labeled viruses were used to infect PBMCs followed by imaging flow cytometry (Amnis). To avoid overlapping of the APC/Cy7 signal with the side scatter signal on the 'ImageStream', the monocytes were sorted out with the cell sorter FACSAria (Becton Dickinson) before taking images on the 'ImageStream'. Signal strength was compensated between different channels according to the manufacturer's protocols. Flow speed was stabilized before collecting 30 000 events and 40x magnification was used for taking images. Data were analyzed using 'Ideas' software (Amnis) (paper I, [181]).

4.11. QuantiGene ViewRNA *in situ* hybridization for vRNA

A very specific and sensitive method for HRV viral RNA *in situ* hybridization was developed in cooperation with Affymetrix (Cat.No. VF1-12634). Shortly, PBMCs were added on top of the polylysine-coated microscopic slides and infected with HRV or controls for 5 days. Before cell fixation, media was carefully replaced with PBS. Thereafter, the cells were fixed with 4% paraformaldehyde (PFA) and washed with PBS and dehydrated with ethanol at increasing concentrations (50%, 70%, and 100%). Slides were submerged in 100% ethanol at 20°C for storage. Before further analysis, the cells were rehydrated using decreasing concentrations of ethanol (70% and 50%) and washed with PBS. ISH was performed using QuantiGene ViewRNA protocols designed to detect specifically 20-nucleotide-long sequences in HRV vRNA of which15 sites were used for amplifying the signal and 5 sites were used for blocking the nonspecific binding. The cells on the slides were permeabilized with working detergent solution (Affymetrix) and hybridized for 3 h at 40°C with custom designed QuantiGene ViewRNA probes against positive strand HRV1b and human actin-beta. Unbound probes were flushed out with wash buffer (Affymetrix). The bound probes were amplified through PreAmp (Affymetrix) hybridization for 1 h at 40°C, followed by Amp (Affymetrix) hybridization for 1 h at 40°C. Labeled probes (Affymetrix) targeting the individual probe types were added for 1 h at 40°C. The slides were covered in 4',6-diamidino-2-phenylindole (DAPI)-containing mounting medium and examined using an LSM 510 confocal microscope (Zeiss) (paper I, [181]).

4.12. Detection of infectious HRV virions in PBMCs by qPCR

PBMCs were infected with unlabeled-HRV16 at MOI 10 and incubated for 24 h at 37°C in tissue culture incubator using regular cell culture conditions. Then, the PBMCs were washed three times with prewarmed PBS and added at a number of 800 000 cells per well (500 µl volume) in a 48-well plate seeded with HeLa cells. Uninfected HeLa cells were seeded in these wells 24 h before and were growing in log phase. In the ICAM-1-blocking condition, anti-ICAM1-Ab was added to HeLa cells at the concentration of 1 µg/ml 2 h before infected PBMCs were added. Three days after the addition of PBMCs, HeLa cells were washed thoroughly to remove nonadherent PBMCs and RNA was isolated from HeLa cells using the RNeasy Micro Plus kit (Qiagen). The expression of negative and positive strands of viral RNA was analyzed by RT-qPCR.

4.13. Isolation of Human Bronchial Epithelial Cells and Air–Liquid Interface Cultures

Human bronchial epithelial cells (HBECs) were isolated by enzymatic digestion of biopsy material with pronase and DNase (Sigma-Aldrich) and cultured in flasks coated with type IV collagen (Sigma-Aldrich) in supplemented bronchial epithelial growth medium (Lonza). Second-passage cells were seeded onto collagen-coated inserts (Costar) at a density of 1.0×10^5 cells/cm^2 and cultured in air–liquid interface (ALI) system using a 1:1 mixture of bronchial epithelial growth medium and Dulbecco's modified Eagle's medium (Lonza) enriched with 75 nM all-trans-retinoic acid (Sigma-Aldrich) as previously described [147]. To induce mucus metaplasia, cells were treated with IL-13 (5 ng/ml) (Sigma, France) for 8 days, with the addition of IL-4 (5 ng/ml) (R&D Systems Europe Ltd., UK) in the last 4 days. Aliquots of the bottom well culture medium were collected, spun, and stored at –80°C for eicosanoid measurements. Cells were harvested and lysed with TRI-reagent (Sigma-Aldrich) for RNA extraction or fixed in 4% PFA for immunofluorescence staining.

4.14. mRNA expression analysis of HRV16 infected human bronchial epithelial cells

Cytological brushings and microbiopsies of bronchial mucosa were obtained by bronchoscopy from 13 donors with asthma and from seven non-asthmatic donors. All donors were current nonsmokers. Bronchial epithelial cells were isolated, cultured in ALI system and mucus metaplasia was induced before infection with HRV16 (see 4.13). We washed epithelial cells surfaces to ensure optimal attachment of the virus. After 2 hours of early incubation with HRV16, cells were extensively washed to remove the unbound viruses. After 48h, epithelial cells were collected for transcriptome analyses.

Total RNA was isolated using Total RNA Kit (A&A Biotechnology) and reverse transcribed (HC Reverse Transcription KIT; Applied Biosystems). Relative mRNA expression was quantified using a predesigned Low-Density Array (Applied Biosystems) and 7900HT Fast Real Time PCR System (Applied Biosystems). Expression of selected genes (e.g., cilia transcriptome genes *EZR* or *SYK*) was assessed by SYBR green-real time (iCycler) using target specific primers (TIB Molbiol). A full list of analyzed genes is presented in Table E1 in the online supplement (paper II [183]). Data were normalized to *18S rRNA*, and relative quantities (RQ) of individual transcripts were calculated using a $2^{-\Delta\Delta}$CT method [187]. In volcano graphs, log2 RQ values (biological significance) were plotted against –log10 P values (statistical significance) estimated using a t test. A 2-fold change in the expression was considered biologically significant (log2 RQ < –1 or > 1).

4.15. miRNA and mRNA expression profiling, pathway analysis and miRNA target prediction (keratinocytes and skin samples)

A total RNA from keratinocytes or skin from AD patients and control subjects (3 donors in each group) were used for miRNA and mRNA profiling with Illumina miRNA Universal-16 and HumanHT-12 Expression BeadChips. miRNA and mRNA Illumina arrays were carried out at the Core Facility at the Department of Biotechnology, University of Tartu, using 500 ng of total RNA per each sample. The data were analyzed with GenomeStudio 2011.1 Gene Expression Module using average normalization for miRNA data and Illumina's custom rank invariant method for mRNA arrays.

Genes were considered to be expressed at detection $P < 0.05$ and differentially expressed at differential $P < 0.05$. Further analyses and visualizations were performed using Microsoft Excel and Multi Experiment Viewer 4.6.1 or 4.6.2. Unsupervised hierarchical clustering was done using Euclidean distance and average linkage analysis. Heatmaps were generated using Multi Experiment Viewer 4.6.1. Log2 values of expression levels that were mean-centered across all of the samples for each gene are presented. Color scale from green/blue (lower expression) to red (higher expression) represents deviation from the mean (black).

Pathway analysis was performed with g:Profiler (http://biit.cs.ut.ee/gprofiler), which retrieves the most significant gene ontology groups and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and enables one to estimate the significance of the search results by calculating enrichment P value using Fisher 1-tailed test. miRNA targets were predicted using Targetscan 6.2 [103, 188].

4.16. Isolation of RNA, cDNA synthesis, and qRT-PCR (human keratinocytes, human and mouse skin samples)

In papers III and IV, a total RNA was extracted using miRNAeasy Mini Kit (Qiagen). Homogenization of the human and mouse skin, epidermis, and dermis samples was done either by Ultra-Turrax T8 (IKA Labortechnik) or Precellys 24 (Precellys) homogenizer or gentleMACS diccociator (Miltenyi Biotec). cDNAwas synthesized from 200 to 900 ng of total RNA using oligo-dT and reagents from Thermo Scientific SuperScript III Reverse Transcriptase (Invitrogen) or reverse transcription reagents with random hexamers (Fermentas) according to manufacturers' protocols. RNA concentration and quality were assessed with NanoDrop ND-1000 and Agilent 2100 Bioanalyzer. TAQMAN (Applied Biosystems) or SYBR/Rox master mix (Bio-Rad Laboratories or Solis BioDyne) and ABI Prism 7900 were used for qRT-PCR. The relative gene expression levels were normalized to mouse or human EF1A levels and calculated using the comparative Ct ($^{\Delta\Delta}$Ct) method (Life Technologies). The mean level of control experiments or control group was equalized to 1. miRNA qPCR was carried out by using TaqMan micro-RNA Assays (Life Technologies) and were normalized to *Let-7a*. All PCR primers were designed with the assistance of Primer 3 software, and were ordered from Microsynth (Balgach).

4.17. RT-qPCR primers

Primers used in papers III and IV were designed with the assistance of Primer 3 and ordered from Microsynth. [182, 184].

Gene	Forward	Reverse
ANXA5	AGGCTTGGGCACAGATGAGGAGA	GCTGCAGAGATTTCCTGGCGCT
ADM	CTCTTAGCAGGGTCTGCGCTTCG	CCGACGCGACATCCAACCGA
CARD10	AGTGTGCCCAGCGGAAAGCC	GATGGCCCGGATCCTGCTGC
CCDC109B	CGCCCCAGGTTTTGCGTGTGA	TTTCATCAGGTGGCACCACGGTACT
CCL8	AGGGACTTGCTCAGCCAGATTCAGT	TCTTGTGTAGCTCTCCAGCCTCTGG
CCL5	AGTCGTCTTTGTCACCCGAAA	TCTCCATCCTAGCTCATCTCCAA
EF1Aex7-8	CCACCTTTGGGTCGCTTTGCTGT	TGCCAGCTCCAGCAGCCTTCTT
IRAK1	CACCTTCAGCTTTGGGGTGGTAGTG	CCAGCCTCCTCAGCCTCCTCT
IFI35	GGAACGGAGGTGGCGATGTGG	CAGACGCTGAGCCACTCCATCC
IFITM1	CAACACCCTCTTCTTGAACTGG	GCCGAATACCAGTAACAGGATG
IFITM2	GTTCAACACCCTCTTCATGAACA	GACGACCAACACTGGGATGAT
IL8	GCAGCTCTGTGTGAAGGTGCAGTT	TTCTGTGTTGGCGCAGTGTGGTC

LYN	AGCTCGTGAGGCTCTACGCTGT	CAGCAAACTGCCCTTGGCCATG
RAB39	GGGAGGTTCCCCTGAAGGATGCT	GGGTGGGATCTGGCGGCTGAT
PCSK9	ACGTCCTCACAGGCTGCAGC	TTGGGCTGACCTCGTGGCCT
NOD2	TGAGGTGGCTCAGCCTGGTGG	TGCCAGCATCAGTGCCAAGGC
hUBD	CAGAGATGGCTCCCAATGCT	CGCTGTCATATGGGTTGGCA
mCARD10	GCGAGGTCTACCCCATTGTC	CAACAGGCCCCTGATCTCAC
mCCL24	TCCCAAGGCAGGGGTCATCTTCA	TGCCTCTGAACCCACAGCAGC
mCCL5	GCCTCACCATATGGCTCGGACAC	TTGACGTGGGCACGAGGCAG
mCCL8	GGGCCAGATAAGGCTCCAGTCACC	TCAGCACCCGAAGGGGGATCT
mCXCL2	TGAACAAAGGCAAGGCTAACTG	CAGGTACGATCCAGGCTTCC
mEF1A	CGGCAGTCGCCTTGGACGTT	CGGTGGTTTTCACAACACCTGCGT
mIFNg	TGCCAAGTTTGAGGTCAACAACCCA	ACAGCTGGTGGACCACTCGGA
mIL1b	AGCTTCCTTGTGCAAGTGTCT	TGGGGTCCGTCAACTTCAAA
mIL4	CAGGAGAAGGGACGCCATGCAC	GCGAAGCACCTTGGAAGCCCT
mIL6	ACTTCACAAGTCGGAGGCTT	TGCCATTGCACAACTCTTTTCTC
mIRAK1	TGTGAGGACACAAGGTGCAA	TAGGCTGGGTGCTTTTCAGG
mTSLP	ATCGAGGACTGTGAGAGCAAGCCAG	GTGAAGGGCAGCCAGGGATAGGA
mUBD	TTCTGTCCGCACCTGTGTTG	GAGACCTTGGTTTGGGACCT
mLor	TCCCTGGTGCTTCAGGGTAAC	TCTTTCCACAACCCACAGGAG

4.18. Mouse AD model

miR-146a–/– mice in C57BL/6J background and C57BL/6J (B6) wild type (WT) mice were purchased from the Jackson Laboratory (Bar Harbor). The mice were maintained and bred in the animal facility at the Institute of Molecular and Cell Biology, University of Tartu, in accordance with the institute's regulations. Nine- to 10- week-old female mice were used for the experiments. Animal experiments were approved by the Animal Ethics Committee of the University of Tartu. MC903 (calcipotriol hydrate, C4369, Sigma-Aldrich) was dissolved in EtOH and topically applied on mouse ears (1 nmol per ear) on every other day 10 times starting at day 0. As vehicle control, the same amount of EtOH was applied. Analyses of the ear biopsies and lymph nodes were performed on day 19.

4.19. Immunofluorescence

For B lymphocytes, immunofluorecence (IF) was performed after ISH by incubating microscope slides with PBMCs for 30 min with anti-human CD20 (MS4A1 Rab-MAb, rabbit anti-human, Epitomics 1632-1, Epitomics) or with anti-rabbit polyclonal antibody (IC control). Then the slides were washed and further incubated for 30 min at RT in dark with secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG (H + L), A11034; Invitrogen). Then, slides were mounted with VectaShield mounting medium containing DAPI, dihydrochloride (Vector Laboratories) and analyzed with a Leica Fluorescence microscope (Leica Microsystems) (paper I, [181]).

Airway epithelial cells (paper II, [183]) were permeabilized with 0.3% Triton-X100, blocked (goat IgG and FCS), and stained with mAb for MUC5AC to detect goblet cells, β-Tubulin-IV to mark cilia (all from Sigma-Aldrich, USA), or HRV-capsid viral protein-2 (VP2) (mAb recognizing HRV capsid protein VP2 and its precursor) to detect HRV-infected cells. Cells were treated with secondary goat anti-mouse IgG antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 568 and Hoechst 33342 (all from Molecular Probes, USA).

4.20. Statistical analysis

Statistical tests were performed using GraphPad Prism 5.0 (GraphPad Software) and 'R' (R Core Team, 2017), specifically the ggplot2 package (H. Wickham – author). Statistical significance was determined using the 2-tailed Student's t-test or one-way ANOVA with Bonferroni's multiple comparison test (significance level is α/m, where α is the desired overall α level and m is the number of hypotheses) to pinpoint the difference between two groups in multiple selections (paper I, II, IV [181–183]).

For apoptosis, viability, and proliferation assays, statistical analysis between paired conditions (non-stimulated and stimulated keratinocytes from the same subject) was performed by using the Wilcoxon signed-rank test (paper IV [182]). The comparison between the groups in paper IV was performed with nonparametric Mann-Whitney U tests. Statistical analysis for paper III qRT-PCR and cytokine measurement results was performed by the unpaired Student *t* test (paper III [184]).

The results were considered significant at P value of less than 0.05 and highly significant at P values of less than 0.01 and 0.001 (papers I – IV [181–184]).

Statistical analysis of Illumina miRNA and mRNA array results and pathway analysis are described in subsection 4.15.

5. RESULTS AND DISCUSSION

5.1. Human rhinoviruses can attach or enter to monocytes, CD4+ and CD8+ T cells and B cells.

The principal site for HRV replication is respiratory epithelium. However, the capacity of HRVs to directly infect immune cells and to modulate immune responses has been studied less. Levandowski et al. [189] have demonstrated that the total lymphocyte count of infected individuals was significantly reduced on day 3 after the challenge with HRV. The reduced number of lymphocytes in the circulation can be at least partly explained by a massive lymphocytic and eosinophilic infiltration into the infected tissues. This cell infiltration to bronchial mucosa might be responsible for changes in airway hyper-responsiveness and asthma exacerbations [190]. Previously, it has been shown that under certain conditions (especially timing and choice of 'right' viral concentration are important), HRV is able to infect alveolar macrophages *in vivo* [191], as well as T cells [192] and Ramos cells (B-cell line) *in vitro* [193].

To study the dynamics of virus attachment and uptake by different PBMC populations, we used virus particles labeled with DyLight650 (paper I) [181]. We observed that monocytes internalize both HRV and UV-HRV (UV-inactivated) viruses (figure 6A, B), which indicates that the uptake of HRV particles by monocytes does not occur only via HRV-specific receptor-mediated infection. B and T lymphocytes had distinct attachment and/or internalization pattern in the case of functional virus and no signal with UV-treated viruses was detected. There was a lag time of around 8 h before HRV was detected in B, CD4+ and CD8+ T cells. In the case of CD4 T cells, the DyLight650-labeled virus signal overlapped CD4 signal that points to the attachment of virus to CD4 (figure 6B). Our results were concordant with the previously reported results about the attachment of HRV to different types of leukocytes [194]. This study looked in more detail at the T-cell part and showed that T-cells got activated by HRV induction with the maximum at HRV titer MOI = 1.0 according to a CD69 as T-cell activation marker. In study I, we observed activation of B-cells at MOI = 1–10.0. It might be that for the activation of lymphocytes, the concentration of HRV must be in a certain range, raising the interesting question as to why the exact viral concentration is important. It is possible that in allergic/asthmatic individuals, HRVs infect not only lower respiratory tract (LRT) epithelial cells, but also underlining infiltrating immune cells in the inflamed epithelium, which ultimately may lead to a strong nonspecific induction of B-cell proliferation in bronchial tissue. This might lead to a rapid increase in the total serum IgE, as observed previously [195] and therefore can provide a synergistic link between allergy, HRV infection and exacerbation of asthma [160].

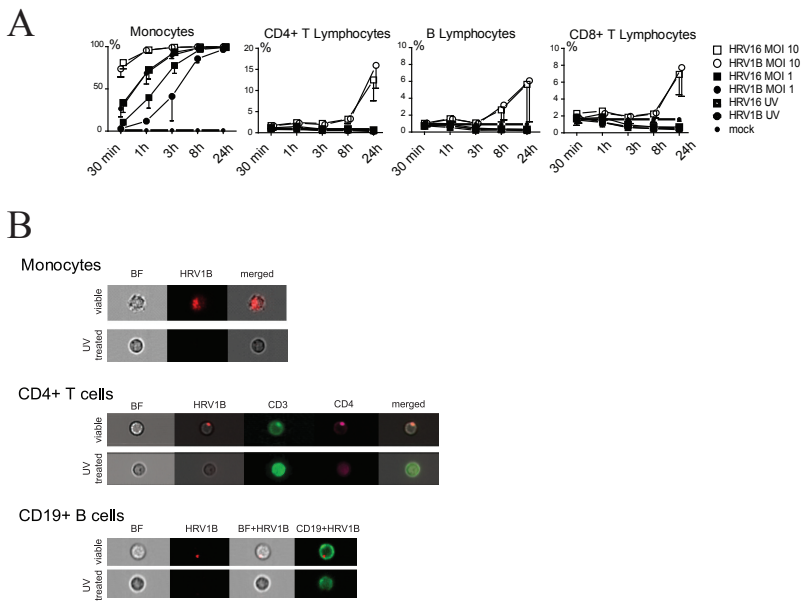

Figure 6. Analysis of DyLight650-labeled HRV1b uptake by PBMCs. PBMCs were cultured in the presence of DyLight650-labeled HRV1b or HRV16 at MOI = 1.0 and MOI = 10 or 'mock' and analyzed at the indicated time points by flow cytometry (A) or imaging flow cytometry (B). The UV-treated viruses were used at MOI = 10. (A) Data are mean with CI 95% of 6 donors. (B) Imaging of HRV1b interactions with monocytes, T cells, and B cells. Bright-field (BF) image shows the shape of cells. CD14+ monocytes were sorted out 1h after incubation with the virus by cell sorter and then analyzed for DyLight650-labeled HRV1b (red). T lymphocytes were analyzed for surface markers using anti-CD3 FITC (green) and anti-CD4 PE/Cy7 (purple) and for DyLight650-labeled HRV1b (red) 24 h after incubation with the virus. B lymphocytes were analyzed with anti-CD19 FITC (green) for DyLight650-labeled HRV1b (red) after 24h incubation with the virus (paper I) [181]. Reprinted with permission of the John Wiley and Sons. Copyright © 2017 John Wiley and Sons. Aab A, Wirz O, van de Veen W, Söllner S, Stanic B, Rückert B, Aniscenko J, Edwards MR, Johnston SL, Papadopoulos NG, Rebane A, Akdis CA, Akdis M. 2017. Human rhinovirus forms viral replication centers in B cells and induces the proliferation of B lymphocytes. Allergy. 2017 Feb;72(2):232–243. doi: 10.1111/all.12931

As HRV infects primarily URT and LRT epithelial cells, there are relatively few publications addressing the question that under certain circumstances HRV might infect other cell types. Recently, it has been shown that HRV can infect macrophages and T-cells [191, 192], so we focused our studies on another lymphocyte subgroup – B cells, with which we detected interaction of HRV1b and HRV16 (figure 6A).

To determine whether HRV can infect B cells, we used ISH for viral RNA (vRNA). Indeed, we were able to detect vRNA in B cells, whereas the size of the vRNA signal found in B cells indicated that these clusters were viral replication centers (figure 7).

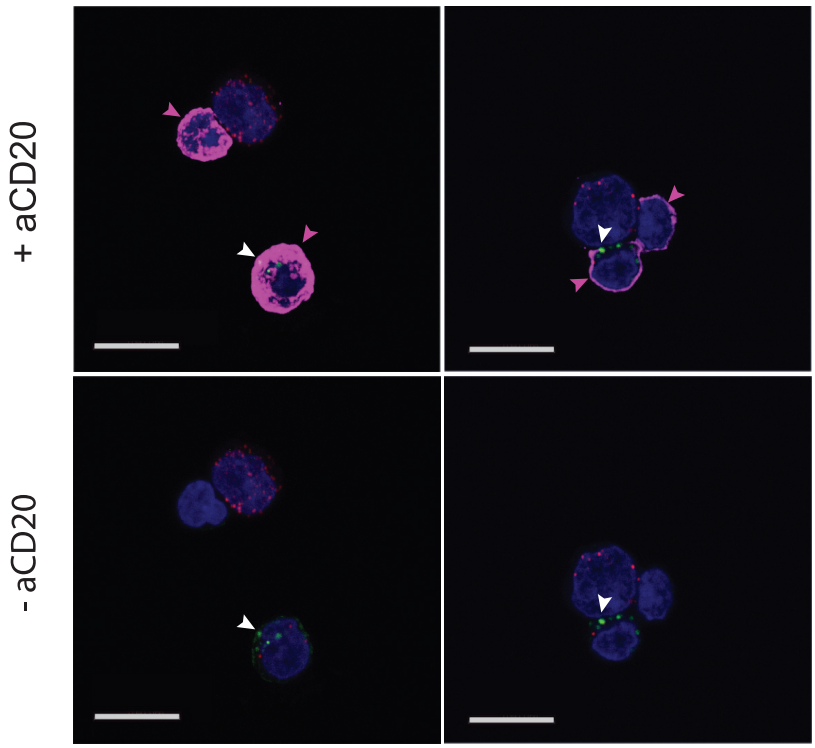

Figure 7. Visualization of HRV1b vRNA with *in situ* hybridization (ISH). PBMCs were cultured with HRV1b and subjected to ISH analysis on the 5th day. HRV1b vRNA is designated with green, human beta-actin mRNA with red, DAPI with blue, and anti-CD20 (purple arrow) as the marker for B cells with purple color. Representative images of 4 donors on 5th day are presented. White arrows indicate the vRNA signal. Bars indicate 10 μm (paper I) [181]. Reprinted with permission of the John Wiley and Sons. Copyright © 2017 John Wiley and Sons. Aab A, Wirz O, van de Veen W, Söllner S, Stanic B, Rückert B, Aniscenko J, Edwards MR, Johnston SL, Papadopoulos NG, Rebane A, Akdis CA, Akdis M. 2017. Human rhinovirus forms viral replication centers in B cells and induces the proliferation of B lymphocytes. Allergy. 2017 Feb;72(2):232–243. doi: 10.1111/all.12931

One of the main findings of us was the observation that HRVs were able to induce B-cell proliferation. In our experiments, the rate of proliferation was time-, HRV type-, and dose- dependent (figure 8, figure 1A–D (paper I) [181]). This ability of HRVs was not known before, but not surprisingly, other picornaviruses, such as poliovirus and Coxsackie virus, efficiently induce the proliferation of B cells even independently from T helper cells [196]. It is known that T-cell-independent antibody response results from extensive B-cell receptor cross-linking by the highly organized, repetitive picornavirus virion structure and is postulated to be generally characteristic of picornaviruses viruses [196], as is HRV. So, it is possible, that HRV's ability to infect B-cells *in vitro* is not the reason for the proliferation of these cells, but separate events,

such as crosslinking of B-cell receptors induces the B-cell proliferation. It would explain why we need the certain concentration of HRV particles to induce the cell proliferation. It has also been suggested that B lymphocytes may become increasingly relevant as antigen-presenting cells when antigen load is low, even without the help of CD4+ T lymphocytes [179]. It is possible that in allergic/asthmatic individuals, HRVs infect not only LRT epithelial cells but also underlining infiltrating immune cells in the inflamed epithelium, which ultimately may lead to a strong nonspecific induction of B-cell proliferation in bronchial tissue. This might lead to a rapid increase in the total serum IgE, as observed previously [195], and therefore can provide a synergistic link between allergy, HRV infection, and exacerbation of asthma [160]. Further, it would be interesting to examine whether infected B-cells become targets for other immune cells like natural killer or cytotoxic T-cells.

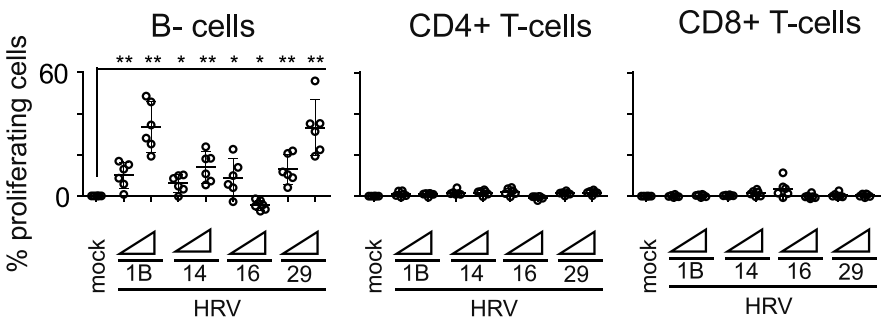

Figure 8. The capacity of HRV to induce the proliferation of B cells. PBMCs were incubated with mock, HRV1b, HRV16, HRV14 and HRV29 (MOI = 1.0 or 10.0). Proliferation rate of B cells and CD8+ and CD4+ cells was measured by CFSE labeling assay and anti-CD4, anti-CD8 and anti-CD19 antibodies were used as discrimination markers. Time – 5th day after activation with HRV. Data are mean with confidential interval 95%. Statistical significance was determined using one-way ANOVA with Bonferroni's multiple comparison test with $^{**}P < 0.01$, $^{*}P < 0.05$ from six different donors (paper I) [181]. Reprinted with permission of the John Wiley and Sons. Copyright © 2017 John Wiley and Sons. Aab A, Wirz O, van de Veen W, Söllner S, Stanic B, Rückert B, Aniscenko J, Edwards MR, Johnston SL, Papadopoulos NG, Rebane A, Akdis CA, Akdis M. 2017. Human rhinovirus forms viral replication centers in B cells and induces the proliferation of B lymphocytes. Allergy. 2017 Feb;72(2):232–243. doi: 10.1111/all.12931

5.2. Cultured primary airway epithelial cells associated with Th2-type cytokine-induced goblet cell metaplasia have decreased susceptibility to HRV-A16 infection

Although numerous studies have aimed to describe the role of inflammatory responses of bronchial epithelial cells in asthma [143, 145–148], these studies do not fully agree with each other and many questions still remain. To describe

the responses of the epithelium, we used ALI cultures of bronchial epithelial cells from patients with asthma and control subjects to compare HRV16-induced changes in mRNA expression of asthma candidate genes (paper II [183]). The experiments were performed in the presence or absence of IL-13, which induces mucus metaplasia.

Interestingly, we identified ciliary cells as the main target for HRV16 by immunofluorescence imaging and demonstrated that the numbers of ciliary cells decreased in HRV16-infected epithelium also in the absence of IL-13 (figure 9). At the same time, mucus metaplastic epithelium was characterized by a 20-fold less replication of HRV16 (figure 2A in paper II [183]). This effect was seen to the same extent in patients with asthma and control subjects.

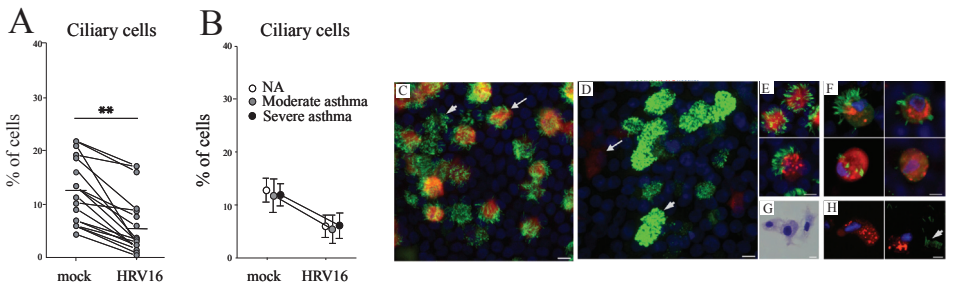

Figure 9. Ciliated cells are the target for HRV16. A) Decrease in the percentage of ciliated cells in mucociliary epithelium (30-d culture) 48h after infection. **P<0.01. B) Percentage of ciliary cells in HRV16-infected or mock treated epithelium 48h after infection in different asthma severity groups. C) A representative composite image of HRV16-infected (8h) mucociliary epithelium stained with anti-HRV-VP2 antibody (red), antitubulin-βIV (green) and Hoechst (blue). Examples of HRV16 infected ciliated cells (arrows) and uninfected ciliated cell (arrowheads) are shown. D) A representative image of HRV16 infected (8h) mucus metaplastic epithelium anti-HRV-VP2 antibody (red) and MUC5AC (green). An exemplary HRV infected cell is noted with an arrow, MUC5AC-bright goblet cell (mucus granules) is noted with and arrowhead. E) Typical images of HRV16 infected ciliary cells (8h) after attachment of virus. F) Typical images of HRV16 infected ciliated cells (red) at 16h. Majority of cells are round shaped, with distorted or reduced cilia (green). G) Detached cells (and debris) in cytospin preparation of apical secretions collected 48h after infection with HRV16 (stained with May-Grünwald-Giemsa). H) Example image showing HRV16 positive cells (red) and debris with cilia (arrowhead) in cytospin preparation of apical secretions (48h) (paper II) [183]. Reprinted with permission of the American Thoracic Society. Copyright © 2016 American Thoracic Society. Jakiela B1, Gielicz A, Plutecka H, Hubalewska-Mazgaj M, Mastalerz L, Bochenek G, Soja J, Januszek R, Aab A, Musial J, Akdis M, Akdis CA, Sanak M. 2014. Th2-type cytokine-induced mucus metaplasia decreases susceptibility of human bronchial epithelium to rhinovirus infection. The American Journal of Respiratory and Critical Care Medicine; 51(2):229–41

To study changes in gene expression, we analyzed three groups of genes: inflammation-associated genes, genes involved in bronchial remodeling and eicosanoids. As expected, the expression changes were detected for the genes in the IFN and NF-κB pathways, including *CXCL10*, *CXCL5* and *STAT1* both in mucociliary differentiation (figure 10A, B) and mucous metaplastic condition (figure 10C, D). Altered expression of particular eicosanoids (*PTGER2*, *PTGFR*, *LTC4S* and *ALOX15*) was also observed in both conditions (figure 10).

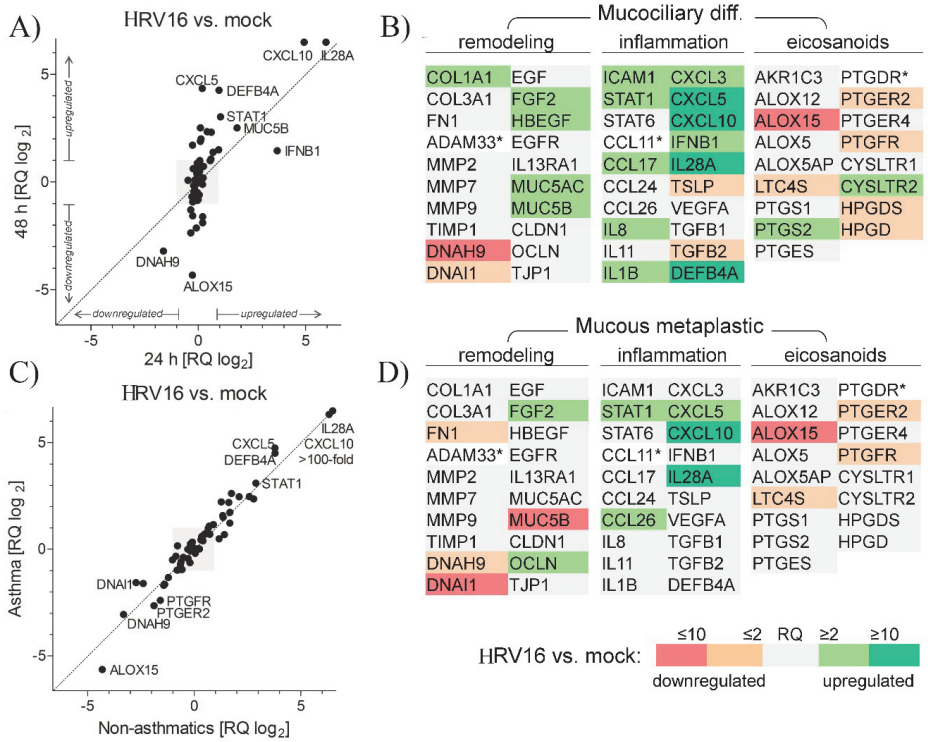

Figure 10. Gene expression in HRV16 infected ALI-differentiated airway epithelium. A) Scatter plot showing HRV-induced differences in gene expression determined at 24 or 48 hours after infection (gray square indicates the area of no biological significance. Only selected genes are labeled. B and D) Table summarizing the differences in gene expression upon RV16 infection (48h incubation) of mucociliary (B) or mucus metaplastic (D) epithelium. For mucociliary differentiating conditions, human bronchial epithelial cells (HBECs) were cultured in the presence of IL-13 (5 ng/ml, days 22–26) and a combination of IL-13 and IL-4 (5 ng/ml, days 26–30). C) Scatter plot showing HRV-related differences in gene expression (48h incubation) in 30-day ALI cultures from non-asthmatic subjects and from patients with asthma (paper II) [183]. Reprinted with permission of the American Thoracic Society. Copyright © 2016 American Thoracic Society. Jakiela B1, Gielicz A, Plutecka H, Hubalewska-Mazgaj M, Mastalerz L, Bochenek G, Soja J, Januszek R, Aab A, Musial J, Akdis M, Akdis CA, Sanak M. 2014. Th2-type cytokine-induced mucus metaplasia decreases susceptibility of human bronchial epithelium to rhinovirus infection. The American Journal of Respiratory and Critical Care Medicine; 51(2):229–41

When the expression levels of these genes in HRV16-infected mucociliary epithelium were compared with mucous methaplastic epithelium, the over-expression of genes associated with bronchial remodeling (e.g., *MUC5AC* and *FGF2*) and altered expression of eicosanoids was observed (figure 11).

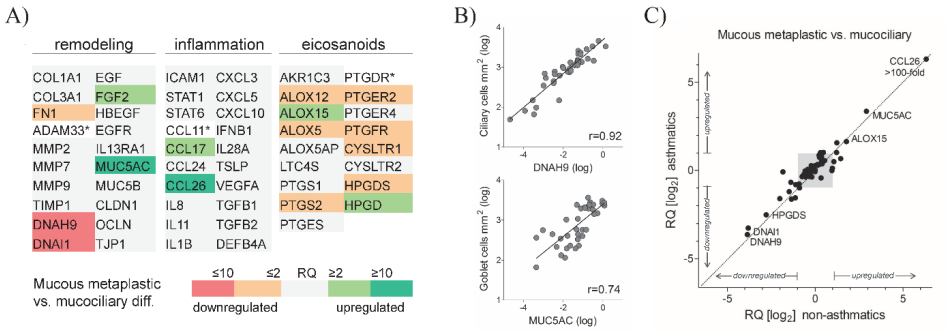

Figure 11. Comparison of HRV16-induced gene expression changes in HBEC cells cultured in mucus metaplasia or mucociliary differentiation conditions. A) Table summarizing the differences in gene expression in airway epithelium upon induction of mucus metaplasia as compared to mucociliary differentiation conditions. Only genes significantly (t test, P<0.05) up-regulated (with RQ > 2-fold, green) or down-regulated (with RQ < 2-fold, red) upon Th2-type cytokine treatment are marked (*not detected). B) Correlation of the number of ciliary cells per mm^2 with relative expression of *dynein chain* (*DNAH9*) and the number of goblet cells per mm^2 with *mucin-5AC* (*MUC5AC*). *DNAH9* and *MUC5AC* relative expressions are normalized to 18S rRNA (in gene expression analyses). C) Scatter plot showing changes in gene expression (RQ) in airway epithelium related to mucus metaplasia (gray square indicates the area of no biological significance) in asthmatic and non-asthmatic patients (paper II) [183]. Reprinted with permission of the American Thoracic Society. Copyright © 2016 American Thoracic Society. Jakiela B1, Gielicz A, Plutecka H, Hubalewska-Mazgaj M, Mastalerz L, Bochenek G, Soja J, Januszek R, Aab A, Musial J, Akdis M, Akdis CA, Sanak M. 2014. Th2-type cytokine-induced mucus metaplasia decreases susceptibility of human bronchial epithelium to rhinovirus infection. The American Journal of Respiratory and Critical Care Medicine; 51(2):229–41

In addition, arachidonic acid–derived eicosanoids were measured in basolateral supernatants from mock-treated or HRV16-infected (48h) airway epithelial cells cultured either in mucus metaplasia or mucociliary differentiation conditions (figure 12). In both conditions, the concentration of particular arachidonic acid–derived eicosanoids and prostaglandins was increased upon infection with HRV16.

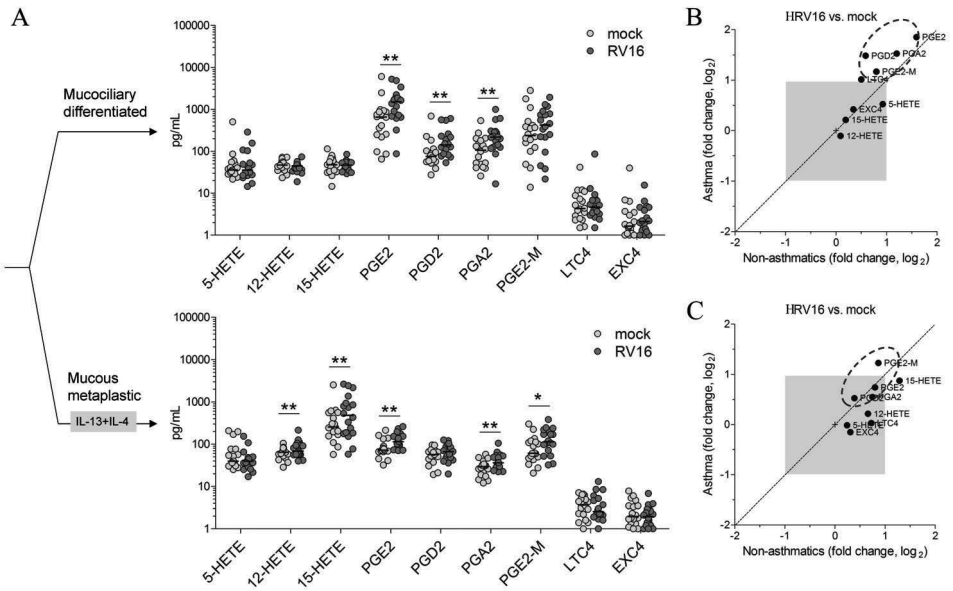

Figure 12. Changes in lipidomic profile after HRV16 infection of in vitro differentiated airway epithelium. A) Arachidonic acid–derived eicosanoids were measured in basolateral supernatants from mock-treated or HRV16-infected (48h) airway epithelial cells cultured for 30 days in ALI. Horizontal bars represent medians. *P<0.05; **P<0.01. B). Scatter plot showing fold-change in concentration of eicosanoids after HRV16 infection in mucociliary differentiated epithelium. Results from patients with asthma and non-asthmatic subjects are contrasted. Area of no biological significance is marked in gray. Prostaglandins are encircled with a dashed line. C) Scatter plot showing fold changes in eicosanoid concentration in HRV-infected mucus metaplastic epithelium. Results from patients with asthma and non-asthmatic subjects are contrasted. Area of no biological significance is marked in gray. Prostaglandins are encircled with a dashed line (paper II) [183]. Reprinted with permission of the American Thoracic Society. Copyright © 2016 American Thoracic Society. Jakiela B1, Gielicz A, Plutecka H, Hubalewska-Mazgaj M, Mastalerz L, Bochenek G, Soja J, Januszek R, Aab A, Musial J, Akdis M, Akdis CA, Sanak M. 2014. Th2-type cytokine-induced mucus metaplasia decreases susceptibility of human bronchial epithelium to rhinovirus infection. The American Journal of Respiratory and Critical Care Medicine; 51(2):229–41

According to these results, we speculate that increased release of prostaglandins associated with HRV infection may contribute to the overproduction of mucus and facilitate the development of mucus metaplastic phenotype of airway epithelium [147].

Although alterations in inflammatory responses of bronchial epithelial cells from asthmatic patients are described before [168], in this study, we could not detect significant differences in gene expression pattern and lipidomics of HRV16A infected epithelial cells from control subjects versus asthmatic patients. Similar trends in the gene expression changes in patients with asthma and non-asthmatic subjects were observed (figure 10C, 11C and 12B, C).

5.3. Possible mechanisms of virus induced asthma exacerbation

Asthma is a very diverse and individual condition where personal risk factors shape the severity of the disease. Environmental factors like pollutants, irritants, and allergens, might promote the exacerbation of the disease.

In addition, it seems that, there is an additive and/or synergistic effect between allergic sensitization and viral illnesses often leading to greatly increased severity of asthma symptoms [161]. Principal site for HRV infection is URT epithelial cell lining [197]. However, more than 50% of bronchial epithelial biopsies collected from patients during virus-induced asthma exacerbation have found to be HRV positive [198]. It is known that the HRV infections enhance allergic inflammation in airways [141, 199–201], suggesting that non-adequate responses of lymphocytes might also contribute to the exacerbated inflammatory condition. Especially among children, the most exacerbation-prone subgroup of patients is highly atopic, with high IgE and eosinophilia. Accordingly, a very recent study demonstrates that in this subgroup, seasonal treatment with omalizumab decreased the duration of HRV infections and the risk of HRV-induced illnesses [202]. These findings indicate that there is a clear link between the atopy and HRV-induced asthma exacerbation as blocking of IgE decreases susceptibility to HRV infections and illnesses.

Here we were interested whether HRV has particular properties that are shaping non-adequate immune responses. As a novel idea, we investigated the ability of HRVs to bind and activate lymphocytes using *in vitro* cultured PBMCs. We were able to show that indeed monocytes, CD4+ T-cells, CD8+ T-cells and B-cells interact and respond to HRV. More importantly, we found that the activation of B-cells by HRVs is time- and viral concentration- dependent, which raises a question about the nature of the interaction of HRV with B cells. Is HRV able to infect B-cells or/and is it possible that the shape and size of HRV virion can induce BCR crosslinking and this way activate the B-cells? Does the activation of the B-cells somehow cause a switch of B-cell type, for example to produce more IgE? Further studies are needed to answer to these questions.

It has been suggested that HRV might increase the severity of asthma by damaging bronchial epithelium, which is followed by non-adequate inflammatory responses and secondary dysfunction of the airways. However, as HRVs generally infect a small number of airway epithelial cells, it is very unlikely that the biggest contributor for asthma exacerbation is the HRV infection itself and rather it is the inflammation induced by the infection. Several studies have shown that HRV induces acute-phase cytokines, such as IL-1β and type I and III interferons [165, 166] and mediators (prostaglandins, kinins) that can damage bronchial epithelium or induce secretion of chemokines and mediators of inflammation [203, 204]. In the later stage of infection, HRV might also induce mucus secretion [205, 206].

An interesting point to note is that respiratory allergies seems to inhibit antiviral responses, as allergic asthma patients have been found to be more prone to HRV infection-related exacerbation of asthma [207, 208]. Some studies suggest that type 2 inflammation (e.g. ILC2 and Th2 cells controlled) could inhibit virus-induced interferon responses [163]. It is likely that for asthma outcome, the shape of virus-induced interferon responses is an important determinant. Too little interferon in the beginning of the viral infection probably allows more efficient viral replication, whereas excessive interferon secretion later might worsen the symptoms of the asthma [209–211]. At the time when, we started our experiments inducing PBMCs with HRVs, we were not aware of the fact that the activation of ILC2s by viruses can also facilitate Th2 type inflammation independent of adaptive immunity [212]. So, our finding that the HRV can induce B-cell proliferation without noticeable activation of CD4+ T cells might present another novel side of the story.

Several other features of HRV infection in lower airways could enhance the severity of asthma. HRV infection stimulates mucus secretion, edema and bronchospasms. These effects contribute to airway narrowing and thereby increase a risk of exacerbation. Well-differentiated epithelium is relatively resistant to HRV infection [146], but impaired epithelium allows increased HRV replication allowing more severe HRV infection [147].

Still, relationship between HRV infection and asthma exacerbation is poorly understood and has been mostly limited to epidemiological studies. As virus-induced exacerbations are still one the major causes of morbidity and mortality among asthma patients, and HRVs are the most common triggering factors of acute asthma exacerbations [141, 213], further studies are needed to explore the role of HRV in the modulation of immune responses during asthma exacerbation.

5.4. The level of miR-146a is increased in keratinocytes and skin from patients with atopic dermatitis

MiRNAs are energetically effective way to manipulate with high precision cellular and immunological responses. Therefore, it has been suggested that miRNAs are used a lot by our organism to fine-tune the molecular responses to different challenges. Understanding the role of miRNAs in the regulation of molecular pathways might lead to better understanding of atopic and chronic inflammatory diseases.

In the beginning of our studies, there was very little information available about the role of miRNAs in allergic diseases [214, 215]. We focused our studies on the role of miRNA-146a in the skin inflammation in AD as it was upregulated in cultured primary keratinocytes from patients with AD compared to cells from control subjects (figure 13A). We confirmed that miRNA-146a expression was increased both in nonlesional and lesional skin of patients with AD (figure 13B). In human keratinocytes form non-atopic donors, miRNA-146a was strongly upregulated by IL-1β, TNF-α, and IL-17A, with a synergistic

effect between the three cytokines (figure 13C) demonstrating that miRNA-146a is induced by proinflammatory cytokines and in the skin of patients with AD.

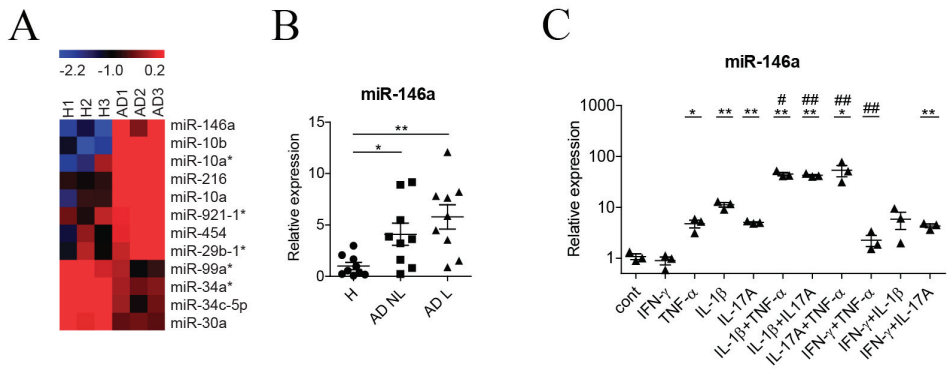

Figure 13. miRNA-146a expression in the skin and keratinocytes. A) Heatmap of miRNAs that are differentially expressed in keratinocytes from patients with AD (AD1-AD3) and healthy individuals (H1-H3). Log2 values of miRNA expression signals are mean-centered for each gene separately. Color scale from blue (lower) to red (higher) represents deviation from the mean (black). B) The relative miRNA-146a expression in lesional (L) and nonlesional (NL) skin of patients with AD compared with the mean of healthy (H) controls. C) miRNA-146a expression in keratinocytes from non-atopic donor stimulated or costimulated with indicated cytokines. Data on (C) are presented in log10 scale. Statistical analysis was performed relative to unstimulated control (cont) (*) or single treatments (#). Data are mean with SEM, **P < 0.01, *P = 0.01/0.05. (paper III) [184]. Reprinted with permission of the ELSEVIER. Copyright © 2016 ELSEVIER. Rebane A, Runnel T, Aab A, Maslovskaja J, Rückert B, Zimmermann M, Plaas M, Kärner J, Treis A, Pihlap M, Haljasorg U, Hermann H, Nagy N, Kemeny L, Erm T, Kingo K, Li M, Boldin MP, Akdis CA. 2014. MicroRNA-146a alleviates chronic skin inflammation in atopic dermatitis through suppression of innate immune responses in keratinocytes. J Allergy Clin Immunol. 2014 Oct;134(4):836–847.e11. doi: 10.1016/j.jaci.2014.05.022.

5.5. miRNA-146a controls keratinocyte responses to IFN-γ and TNF-α

To elucidate the role of miRNA-146a function in keratinocytes, we next overexpressed miRNA-146a in unstimulated and IFN-γ– or TNF-α–stimulated keratinocytes and performed mRNA array analysis (figure 14A). TNF-α was used as a strong stimulant of miRNA-146a and IFN-γ as a hallmark cytokine of chronic skin inflammation in AD known to cause global changes in gene expression in keratinocytes. The transfection of miR-146a resulted in down-regulation of 102, 37 and 410 genes in unstimulated, TNF-α-stimulated and IFN-γ-stimulated keratinocytes, respectively (paper III, online repository figure E1). According to Targetscan 6.2 [103, 188], 21 predicted targets were suppressed by miRNA-146a in both IFN-γ–stimulated and unstimulated conditions, including the previously identified miRNA-146a direct targets IRAK1 [216] and CARD10 [217]. miR-146a strongly inhibited chemokine signaling

pathway (figure 13A) and the NF-κB pathway (paper III, figure 2D, E). Among genes, suppressed by miR-146a, three genes: *CCL5*, *UBD* and *IL-8* were observed to be increased in the skin of AD patients (paper III, figure 3A). Suppression of miR-146a direct targets, *CARD10* and *IRAK1* as well as miR-146a influenced genes, *CCL5* and *IL-8*, was confirmed by RT-qPCR (figure 14B). Reduced amounts of *CCL5* and *CXCL10* in IFN-γ–stimulated, *IL-6* in TNF-α– and IFN-γ–stimulated, and *IL-8* in all conditions were detected in supernatants of keratinocytes on overexpression of miRNA-146a (figure 14C). Together, these results demonstrated that miR-146a inhibits the expression of numerous pro-inflammatory factors, including IFN-γ-inducible and AD-associated genes *CCL5*, *CCL8* and *UBD* in human primary keratinocytes.

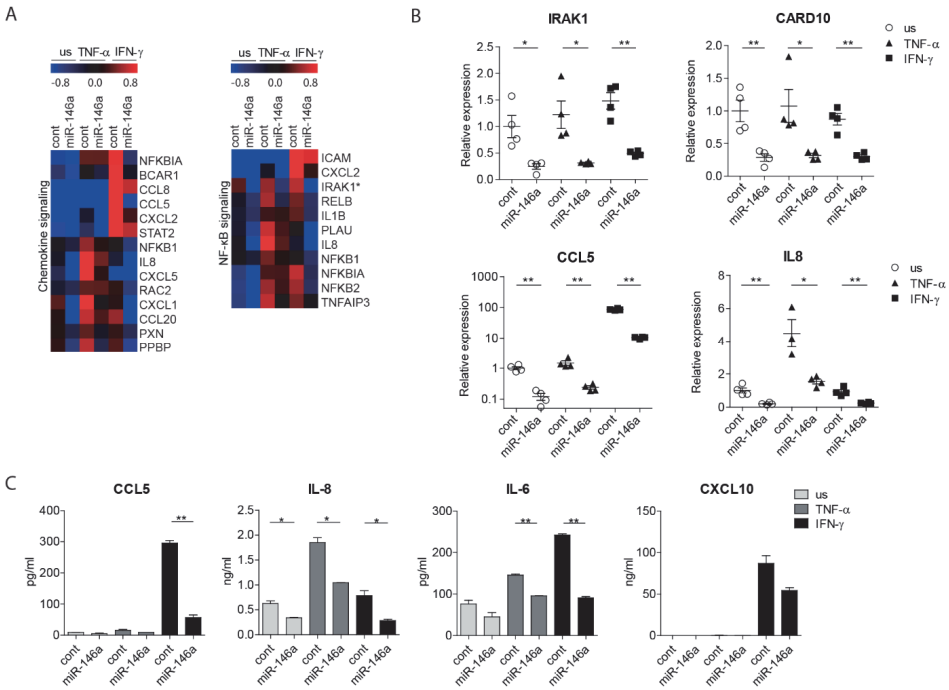

Figure 14. miRNA-146a inhibits the expression of NF-κB–dependent genes in primary keratinocytes (KCs). A–C) KCs were transfected either with control (cont) or pre-miRNA-146a (miRNA-146a) for 24 hours and then stimulated with IFN-γ or TNF-α for 48 hours or left unstimulated (us). Heatmap of the predicted direct targets. B) Relative mRNA expression for CCL5 is presented in log10 scale. C) Chemokine levels (pg/ml) in supernatants of keratinocyte cultures (n = 2). Data are represented as mean with SEM. Students t-test, * $P < 0.05$, ** $P < 0.01$ (paper III) [184]. Reprinted with permission of the ELSEVIER. Copyright © 2016 ELSEVIER. Rebane A, Runnel T, Aab A, Maslovskaja J, Rückert B, Zimmermann M, Plaas M, Kärner J, Treis A, Pihlap M, Haljasorg U, Hermann H, Nagy N, Kemeny L, Erm T, Kingo K, Li M, Boldin MP, Akdis CA. 2014. MicroRNA-146a alleviates chronic skin inflammation in atopic dermatitis through suppression of innate immune responses in keratinocytes. J Allergy Clin Immunol. 2014 Oct;134(4):836–847.e11. doi: 10.1016/j.jaci.2014.05.022.

To study how miRNA-146a affects AD-related inflammation *in vivo*, we used MC903-dependent mouse model of AD [218]. In this model, miR-146a was increased upon the treatment with MC903 in WT mice, whereas the direct targets of miR-146a, *CARD10* and *IRAK1* were downregulated in WT mice (figure 15A). In line with experiments in human keratinocytes, we observed increased mRNA expression of *IFN-γ*, *CCL5*, *CCL8*, and *UBD* in the skin of MC903-treated *miR-146a*$^{-/-}$ mice as compared to MC903-treated WT mice (figure 15B). These results demonstrate that in case of lack of miRNA-146a, there is increased inflammation in the skin in MC903-dependent mouse model of AD, which involves targeting of the NF-κB pathway-activating elements CARD10 and IRAK1 and suppression of the AD-related genes *CCL5*, *CCL8*, and *UBD* (figure 15B).

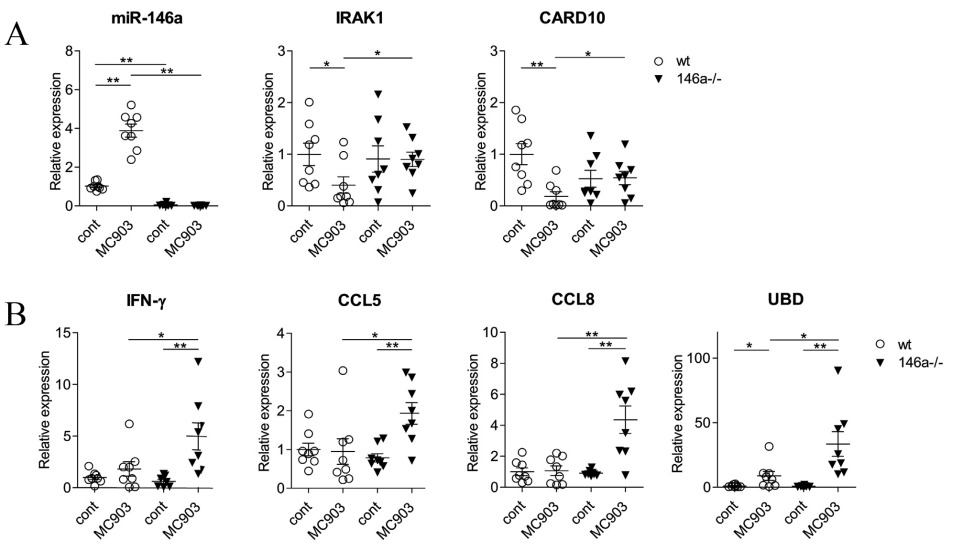

Figure 15. Increased expression of proinflammatory cytokines in inflamed skin of *miRNA-146a*–deficient mice. WT and *miRNA-146a*−/− (146a−/−) mice were topically treated with MC903 or ethanol as a control (cont). **A** and **B)** The relative expression of miRNA-146a and mRNA levels compared with the WT cont. Data are represented as mean with SEM. Students t-test, * $P < 0.05$, **$P < 0.01$. (paper III) [184] Reprinted with permission of the ELSEVIER. Copyright © 2016 ELSEVIER. Rebane A, Runnel T, Aab A, Maslovskaja J, Rückert B, Zimmermann M, Plaas M, Kärner J, Treis A, Pihlap M, Haljasorg U, Hermann H, Nagy N, Kemeny L, Erm T, Kingo K, Li M, Boldin MP, Akdis CA. 2014. MicroRNA-146a alleviates chronic skin inflammation in atopic dermatitis through suppression of innate immune responses in keratinocytes. J Allergy Clin Immunol. 2014 Oct;134(4):836–847.e11. doi: 10.1016/j.jaci.2014.05.022.

As we did not observe significant influence of miR-146a to Th2 type cytokines, such as TSLP and IL-4, in used mouse model of AD (paper III, online supplementary figure E4) ([181–184, 219], we propose that in this mouse model of AD, miRNA-146a mainly influences the chronic inflammation of AD.

Although shown previously in other cell types [220, 221], we could not see significant influence of miR-146a on JAK-STAT pathway as no reduction in phosphorylation of STAT1 was detected (paper III, figure 2E). As siRNA suppression of miR-146a direct targets from the NF-κB pathway (*CARD10* and *IRAK1*) also inhibited AD-associated target genes upon stimulation of IFN-γ (paper III, figure 4), we propose that in keratinocytes, miR-146a primarily inhibits the activation of the NF-κB pathway. However, it is not clear, how IFN-γ activates the NF-κB pathway in keratinocytes. As the siRNA suppression of *CARD10*, but not *IRAK1* affected *CCL5* level upon stimulation by IFN-γ (paper III, figure 4), our results indicate, that the activation of the NF-κB pathway by IFN-γ involves CARD10 in keratinocytes.

Still, multiple open questions remain. First, it is possible that miRNA-146a is involved in the suppression of Th2 responses because it has been show to down-regulate T cell receptor-driven NF-kB activation [222]. In addition, the effect of miR-146a on allergen-induced activation of dendritic cells and epithelial cells, as well as the impact on the immune responses caused by pathogens characteristic to AD, such as *Staphylococcus Aureus*, remain to be studied.

Interestingly, our results suggest that manipulation of the NF-κB pathway through miRNA-146a might give us a potential treatment of chronic inflammatory conditions in the skin or even in the airways in the case of asthma. Recently, a well-known allergen, the house dust mite was shown to directly activate bronchial epithelial cells and cause airway inflammation in mice through one of the main targets of miR-146a, CARD10 [223]. In addition, CARD10 has been shown to function as an activating factor of the NF-κB pathway during viral infection [224]. These studies indicate that miR-146a might actually also inhibit initiation of allergic inflammation in the bronchial epithelium and suppress immune responses to the HRVs through targeting of CARD10. In conclusion, our results demonstrate that miR-146a inhibits innate immune responses in keratinocytes and suggest that overexpression of miR-146a may be a potential treatment of chronic inflammatory conditions in the skin and perhaps also in other tissues.

5.6. Increased IFN-γ and IFN-γ-inducible genes in atopic dermatitis skin might mediate enhanced apoptosis of keratinocytes

Although Th2 type immune responses are considered more important in the triggering of AD, in the chronic phase of skin inflammation in AD, multiple types of immune cells and a mixture of pro-inflammatory cytokines strongly influence the disease pathogenesis [93, 225]. Among others, IFN-γ, IL-22 and TNF-α are widely known to be associated with chronic tissue inflammation. Besides other functions, IFN-γ is known to induce activation-induced apoptosis of keratinocytes in various skin diseases [225, 226]. Our aim was to investigate

whether intrinsic features of keratinocytes may be responsible for their increased apoptosis in AD. In addition, to find novel factors that might be responsible for keratinocyte apoptosis in the skin, we performed mRNA array analysis of skin biopsies and keratinocytes from AD patients.

First, keratinocytes from AD patients and control individuals were studied for the activation-induced apoptosis caused by IFN-γ. Our results show that IFN-γ induced significantly more apoptosis of keratinocytes from patients with AD (27.1±13.8 % Annexin V positive cells) than keratinocytes from healthy subjects (15.4±3.8 % Annexin V positive cells) (figure 16A), suggesting that keratinocytes from patients with AD are more susceptible to IFN-γ–induced apoptosis.

We next performed mRNA profiling of skin biopsies from AD patients with chronic AD lesions. Comparing the skin array results with a previously published dataset of IFN-γ–inducible genes from keratinocytes [227], we found 9 overlapping genes, which indicates that gene expression changes in the skin indeed reflect the influence of IFN-γ (figure 16B).

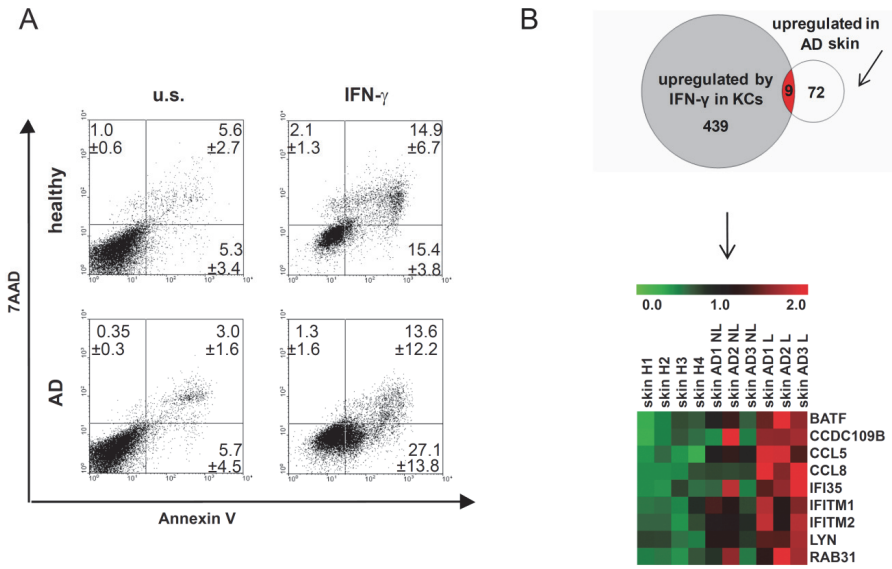

Figure 16. Increased IFN-γ stimulated apoptosis of AD keratinocytes and increased expression of IFN-γ–inducible genes in AD skin. (A) The viability of unstimulated (u.s.) or IFN-γ–stimulated keratinocytes was measured by staining with Annexin V and 7AAD four days after the stimulation. The results are presented as mean ±SD (n=2). (B) Increased expression of IFN-γ–regulated genes in lesional skin from patients with AD. Venn diagram of the selection process and heat map of log2 values of selected differentially expressed genes (paper IV [182]). Reprinted with permission of the ELSEVIER. Copyright © 2016 ELSEVIER. Rebane A, Zimmermann M, Aab A, Baurecht H, Koreck A, Karelson M, Abram K, Metsalu T, Pihlap M, Meyer N, Fölster-Holst R, Nagy N, Kemeny L, Kingo K, Vilo J, Illig T, Akdis M, Franke A, Novak N, Weidinger S, Akdis CA. 2012. Mechanisms of IFN-γ–induced apoptosis of human skin keratinocytes in patients with atopic dermatitis. J Allergy Clin Immunol. J Allergy Clin Immunol. 2012 May;129(5):1297–306. doi: 10.1016/j.jaci.2012.02.020

In addition, we performed mRNA array analyses of cultured keratinocytes from AD patients, which revealed differential expression of apoptosis-related genes in AD keratinocytes (paper IV, figure 3) [182]. Concordantly, three apoptosis-related (*NOD2*, *DUSP1* and *ADM*) and 8 genes upregulated in AD skin lesions (*CCDC109B*, *CCL5*, *CCL8*, *IFI35*, *LYN*, *RAB31*, *IFITM1* and *IFITM2*) were observed to be induced by IFN-γ in keratinocytes, while Th2 type cytokine IL-4 had no influence and TNF-α only moderately stimulated the expression of *CCL5* (paper IV, figure 6 [182]).

These results show that IFN-γ–inducible genes and apoptosis-related genes can be stimulated in AD skin, and thus might contribute to the disease related apoptosis, spongiosis and development of eczematous lesions in AD skin. Remarkably, although inflamed skin of AD patients has been shown to contain more Th2 type cells, and AD is known as Th2 type disease [228], we observed strong influence of IFN-γ on global gene expression pattern. It is possible that compared with other cytokines, IFN-γ is more powerful in the stimulation of inflammatory responses in keratinocytes, and, especially together with other cytokines, has a very strong effect even at relatively low concentrations produced by only a small fraction of IFN-γ–producing cells in the skin of AD patients.

Interestingly, it has been shown recently that in psoriasis, which is characterized with more intensive hyper-proliferation of keratinocytes and thickening of epidermis compared to AD, anti-apoptotic genes are overexpressed [229]. One probable explanation is that the presence of IFN-γ and apoptosis of keratinocytes might actually have protective role in the tissue, as apoptosis helps to clear damaged cells without increasing tissue inflammation and development of necrosis. A recent study using FAS-deficient mice as a model of AD demonstrated that in case of reduced apoptosis, the inflammation and changes in the epidermis are stronger, which indicates that apoptosis is beneficial for recovery [230].

It is also interesting that increased IFN-γ–induced apoptosis and differences in mRNA and miRNA (paper III [184]) expression profile of keratinocytes from AD patients can be detected even after several passages of keratinocytes in tissue culture. This hints at the underlying genetic differences or epigenetic changes, which take place in keratinocytes during the disease, and can persist over extended time scales sufficient to maintain chronic inflammatory state.

In conclusion, we propose that differentially-expressed apoptosis-related and IFN-γ–inducible genes, or both in keratinocytes identified herein might be involved in the pathogenesis of AD. However, it is not entirely clear, whether the increased expression of IFN-γ–inducible genes and related apoptosis of keratinocytes contribute to the long-lasting inflammation in AD, or if they are the signs of the recovery.

CONCLUDING REMARKS

Within the current thesis, four studies exploring different aspects in the molecular background of AD and asthma were carried out.

First, we found that beside the epithelial cells, HRV might actively interact with monocytes, CD4+ T cells, CD19+ B cells, and CD8+ T cells. More importantly, we show for the first time that HRVs enter and can induce the proliferation of CD19+ B cells. These results indicate that in allergic/asthmatic individuals, HRVs can infect not only LRT epithelial cells but also underlining infiltrating immune cells in the inflamed epithelium, which possibly may lead to a nonspecific induction of B-cell proliferation in bronchial tissue, a rapid increase in the total serum IgE, and therefore, can provide a synergistic link between allergy, HRV infection, and exacerbation of asthma. Thus, our results demonstrate that for understanding HRV-induced asthma exacerbation, we need to take a much broader approach to the problem than has been taken so far, and also give further consideration to the participation of immune system.

It is known that HRV infects a rather small number of respiratory epithelial cells. However, this phenomenon remains poorly-explained. In paper II, we studied more closely the ability of HRV to infect different types of respiratory epithelial cells and found that HRV preferentially infects ciliated epithelial cells. We observed that HRV16 infection led to a significant disappearance of ciliated cells from airway epithelium due to detachment of the cells. Because the epithelium in asthma is already compromised, HRV infection may aggravate a deficiency in mucociliary clearance due to preferential loss of ciliary cells and hypersecretion of mucus, both factors known for their detrimental impact on the function of the airways.

In paper III, we aimed to study the function miRNAs in AD. We found that miR-146a expression was increased in keratinocytes and lesional skin of patients with AD. In human primary keratinocytes, miRNA-146a was able to inhibit the expression of IFN-γ–inducible and AD associated genes *IL-8*, *CCL5*, *CCL8*, and *UBD*. In addition, miR-146a–deficient mice had elevated expression of the same factors in the skin as those of a MC903-dependent mouse model of AD. Considering that IFN-γ and the activation of the NF-κB pathway are both characteristic of chronic skin inflammation in AD, our results demonstrate that miR-146a has an anti-inflammatory function in the chronic AD skin. Our data also suggest that manipulation of the NF-κB pathway through miRNA-146a might give us a potential treatment for chronic inflammatory conditions in the skin (or even in airways in case of asthma), as the NF-κB pathway is also activated in tissues in other chronic inflammatory diseases and during viral infection.

Finally, in paper IV, we show the increased expression of IFN-γ–inducible genes in the skin and apoptosis-related genes in the keratinocytes from AD patients. Together with the result demonstrating the increased IFN-γ–induced apoptosis of keratinocytes from AD patients, our data suggest that besides Th2

type immune responses and cytokines, multiple other disease-related factors impact pathogenesis of AD. It was also interesting to observe that keratinocytes from AD patients showed differences in miRNA and mRNA expression compared to keratinocytes from control subjects even after several passages in tissue culture. This indicates that epigenetic changes might take place in cells affected by the diseases and these changes might affect the development of chronic conditions. Obviously, further studies are needed to explore epigenetic changes and their impact on chronic diseases, including AD and asthma.

In conclusion, our work describes multiple aspects at the molecular level in association with asthma and AD. Definitely, we have now a better understanding of the molecular mechanisms of asthma and AD. However, considering the striking heterogeneity of these diseases, which is exemplified by variations in clinical features, and individual risk factors and shown by the absence of reliable biomarkers, diagnostic tests, or specific treatments that would apply to all patients, many questions still remain to be answered.

CONCLUSIONS

1. Besides epithelial cells, HRVs have capacity to interact with monocytes, CD4+ T cells, CD19+ B cells, and CD8+ T cells. HRVs are able to infect and induce the proliferation of CD19+ B cells. These results indicate that HRVs may cause a nonspecific induction of B-cell proliferation in bronchial tissue, which might contribute to the exacerbation of asthma.
2. In *in vitro* differentiated bronchial epithelium, HRV16 preferentially infects and causes significant disappearance of ciliated cells. The infection with HRV16 causes changes in the expression of genes from the NF-κB pathway and upregulates interferon-inducible genes. HRV16 infection of mucociliary epithelium induced overexpression of genes associated with bronchial remodeling (*MUC5AC*, *FGF2*, and *HBEGF*), induction of *cyclooxygenase-2*, and increased secretion of prostaglandins.
3. miR-146a has increased expression in the skin of patients with AD and inhibits multiple factors from the NF-κB pathway, including IL-8, CCL5, CCL8, and UBD in keratinocytes and in a mouse model of AD. Therefore, we conclude that miR-146a has an anti-inflammatory function in the chronic inflammation in AD skin.
4. Increased expression of IFN-γ–inducible genes detected in the chronic skin lesions of AD patients and increased IFN-γ–induced apoptosis of cultured keratinocytes from AD patients may contribute to the long-lasting skin inflammation in AD.

FUTURE PROSPECTS

The data accumulated during recent years have led to a huge progress in our understanding of the environmental, genetic, tissue-specific and immunological factors mastering the inflammatory conditions in AD and asthma. Our own research results about capacity of HRVs to directly affect immune cells and about miR-146a as a suppressor of chronic inflammation in AD suggest new approaches for studying these diseased conditions, as well as hint at the possible future treatment approaches. In addition, recent studies by others suggest that we need to consider more about the surrounding environment. It certainly seems very important to take care of microbiome around us to maintain healthy and supportive environment. We also need to develop new ways for managing, treating, and, in the future, preventing these disorders. In addition to molecular studies similar to those described here, we also need to continue with the long-term longitudinal studies of phenotype, and integrate clinical and molecular data to effectively help patients with chronic inflammatory lung and skin diseases. In the near future, we are likely to be able to target more specifically the mediators of inflammatory diseases at the immune system level through the receptors, FcεRI for IgE triggering cytokines or effector cells in each particular case. This will be associated with the discovery of novel biomarkers, either proteins, mRNA or miRNAs that will help to better classify the patients. The new knowledge gained through all of our work would probably allow us also to prevent initial allergic sensitization, or induce safer forms of allergen-specific immunotherapy in the future, or perhaps even, for example, to use miRNAs as effective means to induce and maintain healthy immune responses. Time will tell us how successful we will be in managing these complex “disorders of advanced civilization”.

SUMMARY IN ESTONIAN

Ülevaade astma ja atoopilise dermatiidi molekulaarsetest mehhanismidest

Vaatamata edusammudele atoopilise dermatiidi (AD) ja astma tekkepõhjuste uurimisel, on saadud eksperimentaalsete tulemuste tõlgendamine ja rakendamine kliinilisse praktikasse osutunud oodatust raskemaks. Üheks põhilisteks patoloogiateks AD ja astma korral on vastavalt kas epidermise või epiteeli struktuursed muutused. Kuid kuidas need muutused on seotud neid esile kutsuvate molekulaarsete mehhanismidega, pole meile veel kahjuks piisavalt selge. See omakorda pole võimaldanud patsiente kõige optimaalsemal moel ravida. Ilmselt tuleks nende krooniliste põletikuliste haiguste molekulaarsetest tekkepõhjustest arusaamiseks kasutada senisest veelgi uudsemaid lähenemisteid ja uurimismeetodeid.

Üheks meile huvipakkuvaks teemaks oli uurida inimese rinoviiruse (HRV) nakkuse võimlikku osa astma ägenemise esilekutsumises. Kuigi HRV nakkuse põhiliseks märklauaks on hingamisteede epiteelirakud, siis oma töös me näitasime, et astma ägenemise põhjuste selgitamisel oleks oluline uurida ka HRV osa monotsüütide, CD4+ T, CD8+ T ja B-rakkude aktiviseerimisel. Samuti õnnestus meil esmakordselt näidata, et HRV viirus on võimeline nakatama B-rakke, seda küll *in vitro* tingimustes. Uus ja oluline avastus meie poolt on, et HRV võib esile kutsuda T-rakkudest sõltumatu mittespetsiifilise B-rakkude paljunemise. Kas selline protsess omab tegelikku rolli B-rakkude massilisel infiltreerumisel epiteelikoesse nagu see on täheldatav astma ägenemisega patsientide kopsubiopsia lõikudes, vajab veel edaspidist uurimist. Ülalmainitud protsessid võivad viia allergeen-spetsiifiliste B-rakkude paljunemisele ja seekaudu IgE oluliselt suurenenud kontsentratsioonile, mida on täheldatud HRV poolt esilekutsutud astma ägenemiste korral. See võib olla seletuseks, kuidas HRV nakkuse mõjul atoopilised põletikulised protsessid võivad kiiresti ägeneda.

On teada, et HRV nakatab ainult väga väheseid epiteelrakke (1 – 5%) ja meil õnnestus oma töös jõuda lähemale selle fenomeni selgitamiseni. Nakatades erinevaid tüüpi juhtehingamisteede epiteelrakke, õnnestus meil näidata, et HRV nakatab eelistatult ripsepiteeli rakke. Ripsepiteeli rakkude hävimine võib olla põhjuseks, miks HRV nakkuse korral mükotsiliaarne puhastumine on pärsitud. Lisades suurenenud limaerituse, saame olukorra, mis on iseloomulik astma ägenemisele, kus peale põletikuliste protsesside on halvenenud ka mehaaniline lima eemaldamine bronhidest.

Need avastused on viinud meie arusaamise oluliselt edasi viirusliku astma ägenemise protsessi mõistmisel ja andnud uued teaduslikud suunad nende protsesside uurimiseks.“Allergilise marsi” hüpotees seob omavahel astma ja AD. Epidemioloogistest andmetest on täheldatud, et AD on esmaseks põletikuliseks haiguseks, mis paljudel juhtudel võiks olla astma tekkimise eelduseks hilisemas vanuses. Nende mõlema kroonilise põletikulise haiguse kulus ja molekulaarsetest mehhanismides on palju sarnast. Selgusetu on veel, kas üks põhjustab

teist või kas inimestel kellel on soodumus üheks on soodumus ka teiseks allergiliseks haiguseks.

Seoses AD-ga uurisime oma töös kahte molekulaaarset aspekti: kirjeldasime miR-146a funktsioone keratinotsüütides ja mõju hiire AD mudelis ning kirjeldasime võimalikke IFN-γ–indutseeritud kertinotsüütide apoptoosi põhjuseid.

Oma uurimisobjektiks valisime miRNA-146a, kuna leidsime mikrokiibi tulemuste analüüsist, et miRNA-146a ekspressioon oli suurenenud AD patsientide keratinotsüütides ja nahas. Oma töös näitasime, et miRNA-146a inhibeerib IFN-γ–indutseeritavaid ja AD seonduvaid molekule – IL-8, CCL5, CCL8, ja UBD, millede ekspressioni aktivatsioon on sõltuv NF-κB signaalirajast. Lisaks rakendasime atoopilise dermatiidi mudelit metsikut tüüpi ja miR-146a–/– hiirtele, et välja selgitada, kas miR-146a võib omada mõju AD tekkele organismi tasemel. Meie katsed näitasid, et samad NF-κB signaalirajast sõltuvad proinflammatoorsed geenid, mis olid inhibeeritud miR-146a poolt inimese keratinotsüütides, olid mõjutatud ka hiire AD mudelis. Arvestades, et nii IFN-γ kui ka NF-κB raja aktiveerumine on iseloomulik krooonilisele AD-le, näitavad meie tulemused, et miRNA-146a võib omada AD haigete nahas põletikuvastast toimet haiguse krooonilises faasis. Oletame, et miR-146a võiks olla potentsiaalselt kasutatav ka krooniliste põletikuliste protsesside ravis.

Käesoleva doktoritöö neljandas artiklis leidsime, et IFN-γ–indutseeritud apoptoos on tugevamini avaldunud keratinotsüütides, mis pärinevad AD patsientidel võrreldes tervetest isikutest pärit keratinotsüütidega. Lisaks kirjeldasime geenieksрessiooni muutusi AD patsientide nahas ja keratinotsüütides. Nägime, et AD patsientidest pärinevatel keratinotsüütidel on mRNA ekspressioonis muutused toimunud apoptoosiga seotud geenides ning, et atoopilise dermatiidi haigete nahas on muutunud IFN-γ poolt mõjutatavad geenid. Nende tulemuste põhjal võib oletade, et lisaks Th2-tüüpi põletikule, mõjuavad AD patogeneesi ka teised bioloogilised protsessid koes.

Kokkuvõtteks võib öelda, et oma töös oleme käsitlenud mitmeid astma ja AD-ga seotud molekulaarseid aspekte. Kindlasti teame me nüüd rohkem faktoritest, mis on nende haigustega seotud, kuid on liiga vara öelda, et me mõistame täielikult ja piisavalt põhjusi, mis viivad iga konkreetse patsiendi puhul haiguse tekkeni. Samuti ei ole olemas ühtset raviskeemi, mis ühtviisi hästi mõjuks kõigile haigetele. Eelkõige on ebapiisavate teadmiste põhjuseks nende haiguste endi suur heterogeensus, mis väljendub näiteks ka juba kliinilise pildi mitmekesisuses. Neil haigustel on ilmselgelt olemas alamtüübid, mis on reguleeritud erinevate molekulaarsete mehhanismide poolt, mis mõjutavad erineval määral ja viisil immuunvastuste individuaalset kujunemist. Endiselt on palju tööd, et aru saada AD ja astma molekulaarsetest põhjustest, et leida biomarkereid, mis aitaksid grupeerida patsiente ning parandada ravi ja mõista keskkonna fakorite mõju, et vältida neid haigus.

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PUBLIKATSIOONID:

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