microRNA profiling and target identification in a mouse model for allergic asthma

Nikola Schulz

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Nikola Schulz
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Erstgutachter:	Frau Prof. Dr. Elisabeth Weiß
Zweitgutachter:	Herr PD Dr. Daniel Krappmann
Mitgutachter:	Herr Prof. Dr. Thomas Lahaye
	Herr Prof. Dr. Michael Schleicher

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OUTLINE

1. SUMMARY

Allergic asthma has a high prevalence and is characterized by airway inflammation, tissue remodeling and a decline in respiratory function. Although the pathogenesis is well known, the underlying mechanisms are still poorly understood. It is believed that a fine interplay exists between the exposure to environmental stimuli and relatively small changes in expression of several genes with inter-individual variation. As microRNAs (miRNAs) are known to be responsive to environmental exposures and show dysregulated levels in diseased states, their function as regulators of gene expression might be a missing link for the changes seen in asthma.
In this project, changes in miRNA expression in a mouse model for allergic asthma were investigated and the interaction with possible target genes was analyzed.

Female Balb/c mice were i. p. sensitized with ovalbumin (OVA) followed by aerosol challenge on two consecutive days. An asthmatic phenotype was confirmed by elevated total cell numbers due to a rise in inflammatory cells, as well as increased CCL17 levels in broncho-alveolar lavage (BAL). High titres of OVA-specific serum IgE were measured and lung histopathology revealed infiltration of inflammatory cells with eosinophilia.

To study changes in miRNA expression, whole lung RNA was subjected to miRNA-microarray analysis (Exiqon). From 580 screened miRNAs, 319 were found to be expressed, of which 36 were differentially regulated in the allergic asthma group compared to healthy control mice. A second, TaqMan® chemistry based array was performed for validation. Based on the overlap between the two arrays in addition to fold changes and *p*-values (Exiqon), eight miRNAs were selected for single RT-qPCR measurement. Dysregulated expression of six miRNAs could be confirmed (miRNA-21, -142-3p, -144, -205, -208, -451).

Due to relatively low fold changes and in order to monitor possible co-regulation, the top 100 differentially regulated miRNAs from the Exiqon array were included in an *in situ* target prediction. Applying a “full consensus” approach of five prediction algorithms, 961 putative target genes were identified. Based on the assumption, that target genes harboring multiple miRNA sites might be more relevant, 11 targets containing more than four miRNA binding sites were selected. From these, the transcription factor cAMP-responsive element-binding protein 1 (CREB1) was chosen for further analysis because of its previous association with asthmatic disease. Moreover, four miRNAs (miRNA-17, -22, -144, -181a) were predicted to

bind at eight different sites, one of them being miRNA-144, a significantly up-regulated miRNA identified in the initial asthma profile.
To experimentally test the functional interaction between *CREB1* and the predicted miRNAs, a *CREB1* 3´-untranslated region (UTR) containing luciferase based reporter plasmid vector was constructed and co-transfected with precursor (pre-) miRNAs into human bronchial epithelial cells. Binding of all four miRNAs could be confirmed by measuring luciferase expression. Furthermore, three of four miRNAs, when transfected alone, were able to down-regulate endogenous *CREB1* expression *in vitro*. In the lung tissue of asthma mice, *CREB1* mRNA levels were significantly reduced compared to healthy controls, in contrast to two miRNAs, miRNA-17 and -144, which showed up-regulation.

To gain further insight into expression patterns during sensitization and after challenge, expression of *CREB1*, the two validated binding partners miRNA-17, and -144, as well as the two miRNAs (miRNA-21, -451) with most significant *p*-values and high fold changes from the Exiqon array were analyzed. Clear expression changes happened after OVA aerosol challenge with *CREB1* levels being steadily decreased, whereas all tested miRNAs showed elevated levels at 24 h post challenge, which further intensified after 120 h. This increase resembles measurements of inflammatory cell counts in BAL pointing at a possible origin within this population.

In order to test whether findings can be translated into the human situation, miRNA changes in whole blood samples of mice were compared to miRNA patterns in peripheral blood of asthmatic children. In contrast to measurements in lung tissue, all four miRNAs showed markedly decreased expression in murine blood. In human samples this reduction was mirrored and significant for miRNA-144 and -451.

2. INTRODUCTION

2.1. Asthma

2.1.1. Clinical symptoms, prevalence and therapy

Asthma bronchiale belongs to the chronic lung diseases. The WHO (world health organization) defines asthma as “chronic disease of the bronchi with recurrent attacks of breathlessness and wheezing, varying in severity and frequency from person to person” (WHO 2011). Patients suffering from asthma experience airway obstruction due to swelling and subsequent narrowing of the airways. Although the fatality rate is low, asthma is a burden for every day life with reduced activity, sleeplessness, work loss and frequent hospitalizations making it a public health problem. Currently, asthma affects 235 million people worldwide. In Germany, 9.2 % of the population is asthmatic, prevalence in the United States reaches 12 %, and is highest in Australia with 20 % (RKI 2009; AAFA 2011). Asthma onset often happens during childhood and thus is the most common chronic disease of this age group (Sly et al. 2008). There exists a gender bias with males being more frequently affected in younger age and females accounting for the majority of adult asthmatics (Postma 2007). In more than half of all disease cases, patients show an additional allergic disease, also referred to as allergic asthma (Locksley 2010).

Asthma is described to be under-diagnosed and under-treated and this is partly due to the observed variety of disease phenotypes with differences in pathology, clinical symptoms or response to treatment (Anderson et al. 2007; Haldar et al. 2008). Severe asthma is known to affect 5 % of patients making up a big part of total asthma health care costs. (Wenzel and Busse 2007).

In general, half of all asthmatics experience poor disease control, nonetheless the other 50 % are treated very effectively (Barnes 2002; Partridge et al. 2006). Overall compliance to medication is low, mainly due to fear of long term side-effects and the intermittent nature of disease with symptom free periods.

Asthma therapy comprises treatment against bronchoconstriction using dilatory agents, like long-acting β_2-adrenoreceptor agonists, as well as treatment against inflammation by inhaled corticosteroids (Holgate et al. 2010). Besides these two classical medications, novel therapies include antibodies against IgE as well as blocking agents against lipid mediators

(leukotriene modifiers) (Holgate 2009). Other selective therapies, including the inhibition of T cells or blockade of single cytokines, have not improved asthma control so far.

2.1.2. Pathogenesis of allergic asthma

Three common hallmark features of asthmatic disease are: airway inflammation, tissue remodeling and decline in respiratory function. The airway inflammation comprises activation and infiltration of several types of immune cells, like mast cells, eosinophils, T helper 2 (Th2) cells and others, which again release multiple signaling molecules, like cytokines, in order to modulate an immune response. Persistence of inflammation causes structural changes in the airways including thickening of the smooth muscle layer, collagen deposition, neovascularization, fibrosis as well as mucus hyperplasia with elevated numbers of goblet cells (Barnes 2008; Holgate et al. 2010; Locksley 2010). These structural changes are subsumed as airway remodeling which in turn leads to a persistent and poorly reversible airflow limitation and airway hyperresponsiveness (AHR). Although asymptomatic periods are very common, the pulmonary changes in chronic asthma patients are persistent.

In allergic asthma, inhalation of an allergen, like pollen, grass, animal dander, mold or excreta from insects as dust mites or cockroaches, drive an immune response in the airways. First, allergens penetrate the mucus and epithelial barrier in the lung and come into contact with innate pattern recognition receptors on epithelia and resident myeloid cells, e. g. macrophages, dendritic cells or mast cells (see Figure 1). The epithelial cell releases several stimuli, e. g. IL-33 and stem cell factor (SCF) which recruits mast cells to the airway surface (Reber et al. 2006), thymic stromal lymphopoietin (TSLP) which activates and mobilizes dendritic cells, or CCL11 chemokine, attracting eosinophils to the site of inflammation. Mast cells release bronchoconstrictors, like histamine or lipid mediators such as leukotriene C_4, D_4, E_4 and prostaglandin D_2 mediating smooth muscle contraction. Next to epithelia and mast cells, dendritic cells are also involved in the first contact with allergens. Besides presenting processed antigen peptides they also release chemokines (CCL17 and CCL22) leading to activation of CD4+ T cells and differentiation into Th2 type cells (Ying et al. 2005; Hammad and Lambrecht 2006). The Th2 cell has a central role in promoting the inflammatory response in allergic asthma. Elevated numbers of these cells are a typical feature together with the secretion of several associated cytokines (Meyer et al. 2008). IL-9 stimulates mast cell proliferation, IL-5 drives eosinophil differentiation in the bone marrow and IL-4 and -13 are known to act on B-cells promoting immunoglobulin class switch to produce IgE (Kay 2006; Barnes 2008). Released IgE can bind to receptors present on mast cells and basophils as well as other inflammatory cells, like B cells or macrophages (Gould et al. 2000). The later

are found in high numbers in the lung after chemoattraction via CCL2 and CXCL1 and are involved in matrix remodeling and the retention of tissue inflammation. Besides, eosinophilia is another distinct feature of asthmatic disease and has been found to contribute to tissue remodeling and fibrosis (Leckie et al. 2000; Locksley 2010).

Regulatory T (Treg) cells have been reported to be comprised in asthma and thus missing their suppressive effect on the CD4+ T cells (Larche 2007; Meyer et al. 2008). However, their overall role is not clear yet.

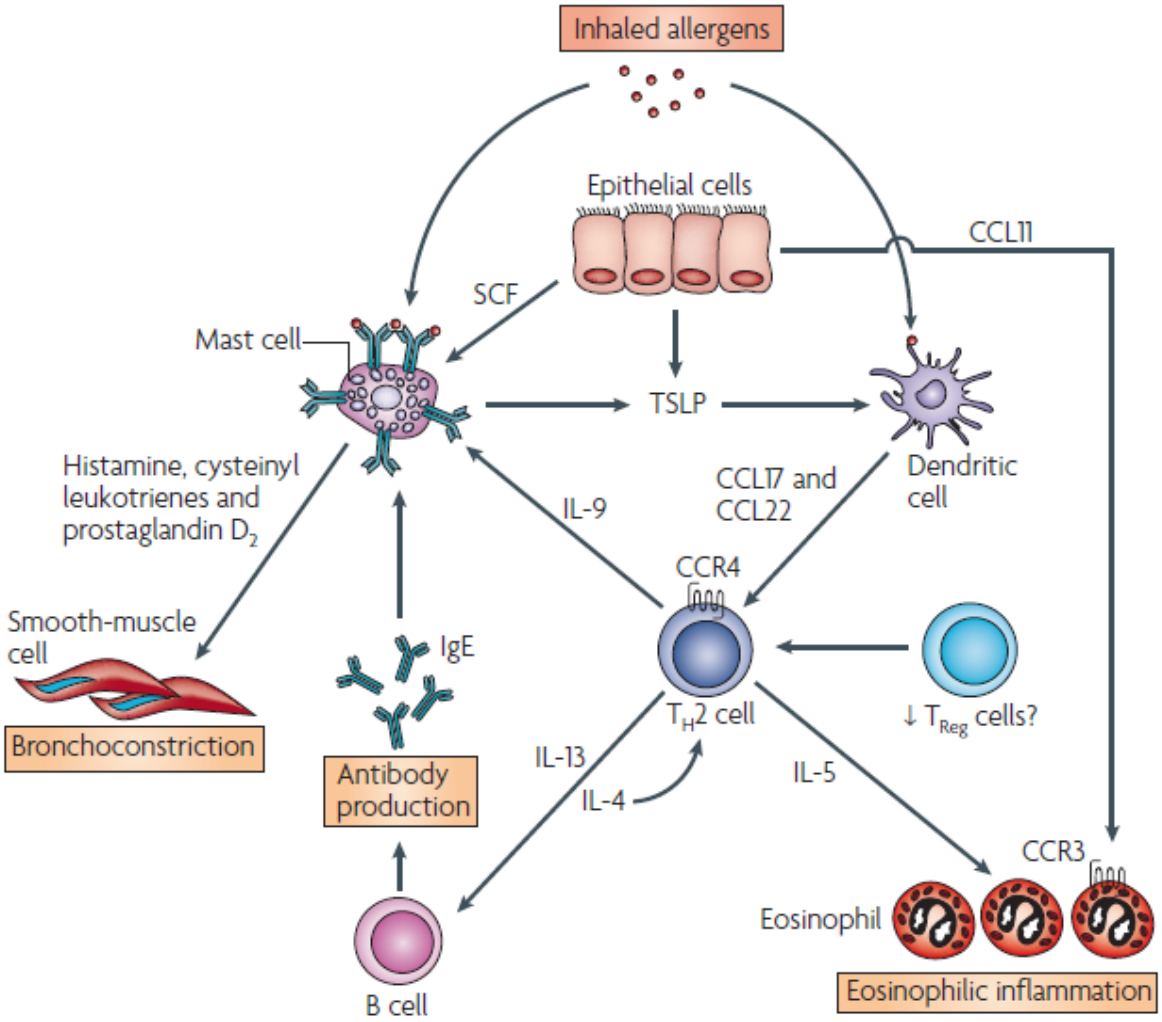


Figure 1: Inflammatory and immune cells in allergic asthma. Inhaled allergens come in contact with airway epithelia cells, which secrete signaling molecules to influence several other cell types: SCF stimulates mast cells, TSLP activates dendritic cells and CCL11 attracts eosinophils. Sensitized mast cells are also directly activated by allergens via surface-bound IgE molecules causing the release of bronchoconstrictors. Dendritic cells are capable to process antigen peptides and present them to other immune cells. Chemokine secretion (CCL17 and CCL22) attracts Th2 cells which themselves release a number of cytokines: IL-9 stimulates mast cell proliferation, IL-5 drives eosinophilic inflammation and IL-4 and IL-13 act on B-cells to produce IgE. In addition, possible defects in Treg cell function may hinder suppression of Th2 cells (Barnes 2008).

2.1.3. Risk factors and underlying mechanisms

Although symptoms and pathogenesis of allergic asthma are well understood, the underlying mechanisms and causes of the disease remain largely unresolved.
The WHO claims the strongest risk factors to be a combination of genetic predisposition and environmental exposure to inhaled substances and particles (WHO 2011). These include indoor allergens, such as house dust mite, pet dander or pollution, as well as outdoor allergens like pollen or molds. Other substances include tobacco smoke, chemical irritants, ozone or air pollution.
Notably, asthma prevalence in the western world has been rising during the second half of the 20th century, often referred to as “asthma epidemic” (Burr et al. 1989; Aberg et al. 1995) and has lately reached a plateau (Anderson et al. 2007; Lotvall et al. 2009). These epidemiological findings have led to the conclusion that this increase in allergic asthma over a relatively short period of time might be well attributed to environmental factors as overwhelming risk factors (Locksley 2010). With regard to the factors themselves, studies are ongoing, like increased risk for asthma due to urbanization of life style or after frequent lower respiratory infections in early childhood (Jackson et al. 2008; Wu, P. et al. 2008). After all, genetic predisposition seems clear, with family and twin studies estimating 60 % heritability (Duffy et al. 1990). Still, asthma is a genetically heterogeneous disease and more than 200 asthma candidate genes have been identified in the last decades (Vercelli 2008). During the last years, genome wide studies became feasible and results suggest relatively small contribution by many different loci (Moffatt et al. 2010; Ricci et al. 2011).

New aspects are arising from studies about epigenetic and prenatal influences on asthma. Epigenetics is defined as heritable changes in gene expression without alteration of the genetic code. Thereby, key environmental exposures are thought to induce epigenetic changes in gene expression which hence alters disease risk. This concept has been proposed and evaluated for microbial factors (Conrad et al. 2009; von Mutius and Vercelli 2010), diet (Chatzi et al. 2008; Hollingsworth et al. 2008), tobacco exposure (Hylkema and Blacquiere 2009) and pollutants (Liu, J. et al. 2008; Perera et al. 2009).

The factors which are thought to play major roles in the development and persistence of asthma are thus genes, environment and epigenetics. All three can be brought together in context with a new class of posttranscriptional regulators, named miRNAs.
miRNAs regulate gene expression, are responsive to environmental stimuli and regulate components of the epigenetic machinery. A number of studies have reported dysregulated levels of miRNAs in diseased states (Calin and Croce 2006; Kloosterman and Plasterk 2006)

and loss-of function studies *in vivo* show their contribution to disease development (Rodriguez et al. 2007; Ventura et al. 2008).
In 2007, Tan and colleagues reported the first connection between miRNAs and asthma risk. They found that the identified single nucleotide polymorphism in the asthma susceptibility gene *human leukocyte antigen* (*HLA)-G* did not affect gene expression but binding of three miRNAs to the 3´-UTR of the gene. This influence on miRNA targeting was associated with increased risk of asthma (Tan, Z. et al. 2007).

2.2. miRNAs

2.2.1. Occurence and importance

In 1993 V. Ambros and coworkers discovered the first miRNA, lin-4, in the worm *Caenorhabditis (C.) elegans* (Lee, R. C. et al. 1993). Additionally they reported sequence complementarity between lin-4 and the 3´-UTR of *lin-14* mRNA, a sequence known to play a role in the development of the worm (Wightman et al. 1991). This was the first hint at a new mechanism of gene regulation. However, it took almost seven years until the second miRNA, let-7 was identified, again in *C. elegans* (Reinhart et al. 2000). Similar to lin-4, also let-7 was found to function through binding to regions in the 3´-UTR of a gene, namely *lin-41* (Vella and Slack 2005). The fact that both let-7 and *lin-41* were already known to be evolutionarily conserved throughout metazoans, with homologues detected in several species, including mice and humans, indicated a more general role of these small RNAs (Pasquinelli et al. 2000). In 2001, three parallel reports were published, describing a large class of small RNAs with regulatory roles. They were named “miRNAs” (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee, R. C. and Ambros 2001). Today, the 19- to 21-nucleotide long miRNAs are integrated in the class of small non-coding RNAs, next to siRNAs or other small RNAs like tRNA or rRNA (Storz 2002).

Until now, more than 1400 human and 720 murine miRNAs have been identified (miRbase version 17), showing high conservation between species. Generally, miRNAs have been discovered in animal branches of eukaryota as well as in plants (Reinhart et al. 2002; Carthew and Sontheimer 2009). The biological effects of miRNAs are generally inhibitory and thus subsumed as RNA silencing. It is estimated that miRNAs affect at least 30 % of genes in the human genome (Bentwich et al. 2005; Lewis et al. 2005) and new studies propose

their function as large gene regulatory networks rather than solely being inhibitors of single genes (O'Connell et al. 2010).

The role of miRNAs includes many aspects of development and physiology, such as differentiation, proliferation and growth, mobility or apoptosis (Ambros 2004; Bartel 2004; Hwang and Mendell 2006). As a consequence, dysregulation of miRNA levels can lead or contribute to diseases and has been observed in cardiovascular and liver disease, situations of immune dysfunction or metabolic disorders (Poy et al. 2004; Lindsay 2008; Bostjancic et al. 2009; Shu et al. 2011). Implication in cancer was one of the first explorations with Calin et al. (2002) showing reduced miRNA-15 and -16 levels in most chronic lymphocytic leukemias.

Giving the fact of dysregulation in disease, therapeutic intervention could be either restoring decreased levels of miRNAs or inhibiting over-expression. miRNA silencing *in vivo* was shown to be successful when chemically engineered oligonucleotides, termed “antagomirs” were introduced in mice (Krutzfeldt et al. 2005).

The first therapeutic approach in the miRNA field aimed at inhibiting miRNA-122, a miRNA important for Hepatitis C viral replication (Santaris Pharma A/S). After initiation of clinical trials in may 2008, phase IIa assessements are ongoing since September 2010. Other fields of therapeutic investigation include cardiovascular diseases (miRagen Therapeutics), other viral and inflammatory diseases (GlaxoSmithKline and Regulus Therapeutics) or cancer (Enzon Pharmazceuticals) as well as miRNA-based diagnostic tests (i. e. by Rosetta Genomics, AsuraGen).

2.2.2. Biogenesis

miRNAs are encoded by genomic DNA with mostly intronic but also intergenic locations (Bartel 2004; Kim, V. N. 2005). Transcription is commonly performed by RNA polymerase II or to a lesser extent polymerase III. The primary transcripts (pri-miRNAs), which are capped and poly-adenylated can produce a single miRNA or, as many miRNAs are clustered, give rise to several miRNAs (Bartel 2004; Baskerville and Bartel 2005). Characteristics of pri-miRNAs are an imperfectly paired stem of around 33 bp with a terminal loop and flanking segments. The pri-miRNA undergoes two sequential processing reactions (see Figure 2) which have been described to happen co-transcriptionally (Ballarino et al. 2009).

The first processing step is performed by Drosha, also called ribonuclease 3, leading to the excision of the stem loop (Lee, Y. et al. 2003; Kim, V. N. 2005). For efficient and precise work, Drosha needs the cofactor DiGeorge syndrome critical region gene 8 (DGCR8), which interacts with the pri-miRNA stem and flanks the single stranded segments (Denli et al. 2004; Han et al. 2006). This interaction causes proper positioning of Drosha´s catalytic site and is

therefore critical for precise cleavage. The result of this first processing step is the so called precursor miRNA (pre-miRNA).
Until this point, reactions happen in the nucleus, whereas exportin 5 now transports the pre-miRNA into the cytoplasm. Here, a second excision step by Dicer leads to a duplex formation with around 22 bp length (Hutvagner et al. 2001; Bartel 2004). Due to the double stranded nature of the pre-miRNA, Dicer needs RNAse III enzymatic function. Under normal conditions Dicer is present as heterodimeric complex together with proteins, which contain additional double stranded RNA binding domains (TRBP and R2D2). In mammals, a single form of Dicer exists (Tomari and Zamore 2005). After the second processing reaction by Dicer, the mature miRNA has now reached its final length but still exists as double strand.

Regulation of expression and function of miRNAs may happen at three levels. First, transcription can be controlled by transcription factors which are able to influence the production of miRNA transcripts. A second point is post-transcriptional regulation of miRNA processing. One example is the tumour suppressor protein p53, which forms a complex with Drosha and induces increased processing in DNA damaging situations (Suzuki et al. 2009). Moreover, interferons and cellular stress have been reported to inhibit Dicer expression, thus limiting processing of pre-miRNAs (Wiesen and Tomasi 2009). A third regulatory mechanism is subcellular localization, for example by association of miRNAs with stress granules (Leung et al. 2006). Beyond that, some reports hint towards regulation of miRNAs by negative feedback loops, like let-7, which is repressed by its own target gene *lin-48* in *C. elegans* (Seggerson et al. 2002). Although yet not completely understood, tight regulation of miRNAs seems to be crucial for miRNA function and adaption.

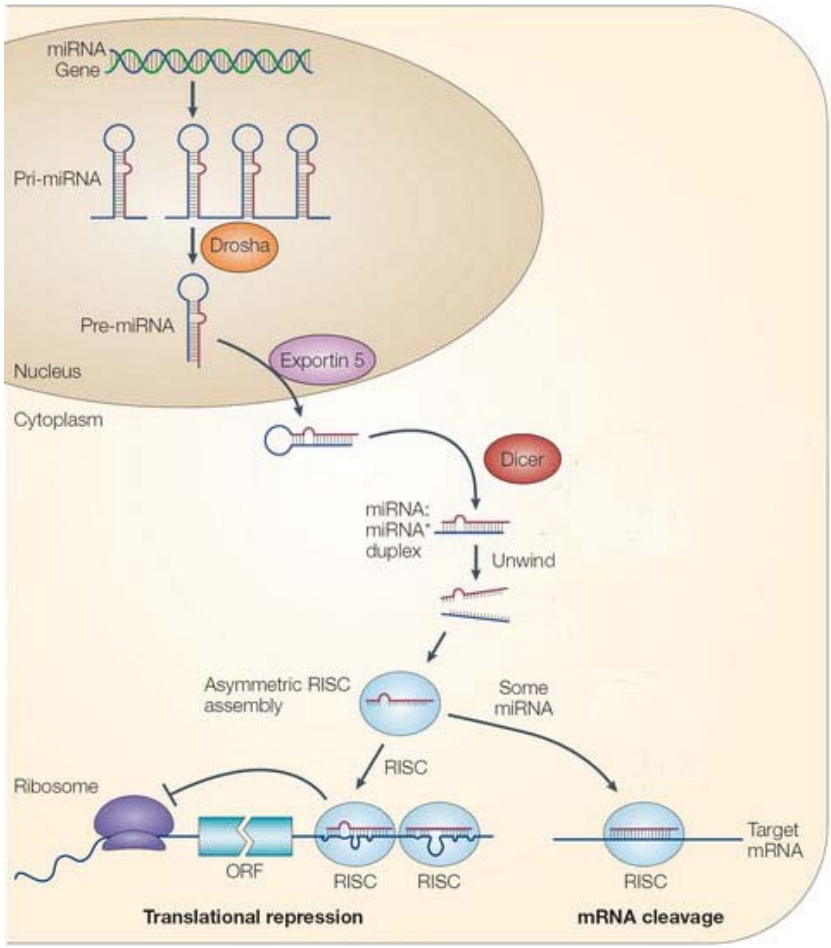


Figure 2: Biogenesis and suppressive function of miRNAs in animal cells. Pri-miRNA molecules are transcribed by RNA polymerase II or III and subsequently processed by Drosha and its cofactor DGCR8 into pre-miRNAs (~70 nt). After transport from the nucleus to the cytoplasm by exportin 5, a second processing step by Dicer (together with TRBP) leads to a duplex formation of ~22 nt length. Incorporation into the RISC complex includes association of one strand with the effector protein Ago whereas the other strand is degraded. The RISC bound miRNA strand binds to target mRNAs leading to mRNA cleavage or translational repression depending on the level of complementarity. ORF, open reading frame. (adapted from He and Hannon 2004).

2.2.3. Function

After processing, the mature miRNA duplex is incorporated into the RNA induced silencing complex (RISC) in order to be functionally active. Within the complex, the duplex is unwound and one strand is stably packaged in association with an effector protein, named Argonaute (Ago) (Meister and Tuschl 2004; Meister et al. 2005; Tomari and Zamore 2005). Belonging to the Ago superfamily of RNA binding proteins, four of eight human gene paralogs are known to function in association with miRNAs or siRNAs. When associated with an Ago, the double stranded miRNA is rapidly unwound and one strand has to get retained. This strand selection is based on relative thermodynamic stability. The 5´ terminus of the chosen strand is the one

with less stable base-pairing (Kim, V. N. 2005). The process of strand selection is still not fully understood considering the fact that also minor strands, which are supposed to be less probably associated in the RISC, have been found expressed as well (Okamura et al. 2008).
The rapid unwinding is due to the presence of Ago in complex with Dicer and TRBP, the very factors involved in the generation of the mature duplex from the precursor form. In consequence, transfer of the selected single strand to Ago happens in close proximity (Gregory, R. I. et al. 2005; Tang et al. 2008).
Moreover, the Dicer/Ago/miRNA complex is associated with further proteins, of which GW182 (also called TNRC6A) was found to be necessary for Ago to exhibit silencing (Bartel 2004; Liu, J. et al. 2005; Meister et al. 2005; Eulalio et al. 2008).
After association to the RISC complex, the miRNA strand guides the way to a target mRNA and binding sites have mostly been found in the 3´-UTR of genes. In contrast to siRNAs, miRNAs have the ability to bind to their target mRNAs with mismatches and bulges. Nevertheless, accurate base pairing seems to be necessary within the so called “seed region” of the miRNA, which comprises nucleotides 2-8 (Carthew and Sontheimer 2009).
Although not fully necessary for the binding, complementarity is described as determinant of the mechanism of inhibition. Perfect complementarity thereby leads to Ago catalyzed cleavage of the target mRNA whereas central mismatches lead to repression of translation. The two mechanisms are subject to ongoing debate. One question is whether degradation might be a consequence of a primary effect on translation as observed in some experimental settings (Mathonnet et al. 2007; Meister 2007). On the other hand, there exist several reports concluding that destabilization of target mRNA is the predominant reason for reduced protein output (Bagga et al. 2005; Lim et al. 2005; Guo et al. 2010; Wu, D. et al. 2010). Degradation of mRNA is based on de-adenylation, de-capping and exonucleolytic digestion (Behm-Ansmant et al. 2006).

3. AIMS and OBJECTIVES

miRNAs are small, non-coding RNAs regulating gene expression on a posttranscriptional level. Lately, several miRNAs have been implicated to play important roles in the immune system and dysregulated levels have been found in complex diseases. Their mode of action allows a small number of miRNAs to regulate whole signaling pathways, making them key players in disease biology.

The aim of this project was to analyze dysregulation of miRNA expression in a murine OVA-model for allergic asthma and to investigate interaction with possible target genes. This should be achieved as outlined below:

- Characterization of the murine OVA model mimicking asthmatic disease
- Generation and analysis of miRNA profiles from asthma, sensitized and healthy control mice
- Identification of potential target genes and their experimental validation
- Following the kinetics of validated miRNA candidates during the sensitization and challenge phase of the protocol
- Investigation of the selected miRNAs as potential “biomarkers” in human and murine blood samples.

4. MATERIAL

4.1. Chemicals and reagents

Substance	Provider	
Acetic acid	Carl Roth	Karlsruhe (DE)
Acid Phenol:Chloroform	Ambion/ Applied Biosystems	Carlsbad (US)
Adenosine triphosphate (ATP)	pjk	Kleinblittersdorf (DE)
Agar-Agar	Carl Roth	Karlsruhe (DE)
Agarose	Invitrogen	Carlsbad (US)
Albumin from chicken egg white, grade V (OVA)	Sigma-Aldrich	Taufkirchen (DE)
Ampicillin	Carl Roth	Karlsruhe (DE)
Bio-Rad Protein Assay	BioRad	Hercules (US)
Bovine serum albumin (BSA)	New England BioLabs	Beverly (US)
Citric Acid Monohydrate	Sigma-Aldrich	Taufkirchen (DE)
Coelenterizine	SynChem	Illinois (US)
Coenzym A	pjk	Kleinblittersdorf (DE)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Taufkirchen (DE)
Dithiothreitol (DTT)	pjk	Kleinblittersdorf (DE)
D-Luciferin	SynChem	Illinois (US)
dNTP	Fermentas	Waltham (US)
ElectroMAX™ DH10B™ cells	Invitrogen	Carlsbad (US)
Entellan	Merck	Darmstadt (DE)
Ethidium bromide	Biomatik	Wilmington (US)
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich	Taufkirchen (DE)
Fetal calf serum (FCS)	Invitrogen	Carlsbad (US)
Filler DNA pUC21	Plasmid Factory	Bielefeld (DE)
Formaldehyde solution 32%	Science Services	München (DE)
Formamide	Carl Roth	Karlsruhe (DE)
Gene Ruler™ 1 kb DNA Ladder	Fermentas	Waltham (US)
Gene Ruler™ 50 bp DNA Ladder	Fermentas	Waltham (US)

Glycerol	Merck	Darmstadt (DE)
Hydrochloric acid (HCl), 2 M	Merck	Darmstadt (DE)
Hydrogen peroxide (H_2O_2)	Merck	Darmstadt (DE)
Imject® Alum	Thermo Fisher Scientific	Waltham (US)
Ketamin	CD-pharma	Québec (CAN)
LB (Luria Bertani) Fertigmedium	Carl Roth	Karlsruhe (DE)
Lipofectamine™ 2000	Invitrogen	Carlsbad (US)
Magnesium carbonate (pentahydrate) $(MgCO_3)_4Mg(OH)_2$ *5 H_2O	Carl Roth	Karlsruhe (DE)
Magnesium sulfate (heptahydrate) $MgSO_4$ *7 H_2O	Carl Roth	Karlsruhe (DE)
MEM (Minimal Essential Medium)	Invitrogen	Carlsbad (US)
Methanol	Carl Roth	Karlsruhe (DE)
Nuclease-free water	Ambion/ Applied Biosystems	Carlsbad (US)
Paraffin 52-54°	Carl Roth	Karlsruhe (DE)
Penicillin/Streptomycin	Invitrogen	Carlsbad (US)
PfuUltra® High-Fidelity DNA Polymerase	Stratagene/ Agilent Technologies	California (US)
Phosphate buffered saline (PBS)	Invitrogen	Carlsbad (US)
Power SYBR® Green Master Mix	Applied Biosystems	Carlsbad (US)
Protein standard	Sigma-Aldrich	Taufkirchen (DE)
psiCHECK™-2 Vector	Promega	Wisconsin (US)
QIAzol Lysis Reagent	Qiagen	Hilden (DE)
RNAlater®	Ambion/ Applied Biosystems	Carlsbad (US)
Rotiphorese® Gel 30	Carl Roth	Karlsruhe (DE)
Sodium bicarbonate ($NaHCO_3$)	Merck	Darmstadt (DE)
Sodium carbonate (Na_2CO_3)	Sigma-Aldrich	Taufkirchen (DE)
Sodium chloride (NaCl)	Merck	Darmstadt (DE)
Streptavidin-Peroxidase	Calbiochem/ Merck	Darmstadt (DE)
Sufluric acid (H_2SO_4)	Carl Roth	Karlsruhe (DE)
T4 DNA Ligase	Fermentas	Waltham (US)
TaqMan® 2x Universal PCR Master Mix, No AmpErase® UNG	Applied Biosystems	Carlsbad (US)
TaqMan® PreAmp Master Mix (2x)	Applied Biosystems	Carlsbad (US)
Tetramethylbenzidine (TMB)	Sigma-Aldrich	Taufkirchen (DE)

Tricine	Carl Roth	Karlsruhe (DE)
Tris	Carl Roth	Karlsruhe (DE)
Triton X-100	Sigma-Aldrich	Taufkirchen (DE)
Trizol	Invitrogen	Carlsbad (US)
Trypan blue	Sigma-Aldrich	Taufkirchen (DE)
Trypsin-EDTA	Invitrogen	Carlsbad (US)
Tween-20	Sigma-Aldrich	Taufkirchen (DE)
Xylazin	aniMedica	Senden-Bösensell (DE)
Xylene	Merck	Darmstadt (DE)

4.2. Buffers and solutions

Name	**Volume**	**Substance**
10x Tris/HCl (pH 7.4)	60.55 g	Tris
	ad 1 l	H_2O
Blocking Buffer/ Dilution Buffer	6 g	BSA
	ad 200 ml	1 x Tris
Coating Buffer (pH 9.5)	4.2 g	$NaHCO_3$
	1.78 g	Na_2CO_3
	ad 500 ml	H_2O
Coelenterizine stock	10 mg	Coelenterazine
	ad 1 ml	MeOH
Coelenterizine substrate buffer	69.32 µl	Coelenterizine stock
	32.93 ml	PBS
	protect from light	
	Use 1:5 diluted in PBS	

D-Luciferine substrate buffer	100 mg	D-Luciferin
	159 mg	Coenzym A
	3894 mg	DTT
	221 mg	ATP
	394 mg	$(MgCO_3)_4Mg(OH)_2$ *5 H_2O
	498 mg	$MgSO_4$ *7 H_2O
	2715 mg	Tricine
	28.2 mg	EDTA
	ad 757 ml	H_2O bidest
	30 min sonification	
Luria-Bertani (LB) medium	20 g	LB Fertigmedium
	ad 1000 ml	H_2O
Lysis buffer (pH 7.4)	15.14 g	Tris
	5 g	Triton X-100
	ad 500 ml	H_2O
Substrate Buffer (Gallati Buffer) (pH 3.9)	8.41 g	Citric acid monohydrate
	ad 200 ml	H_2O
TAE buffer (pH 8.5)	242 g	Tris
	57.1 ml	Acetic acid
	100 ml	EDTA, 0.5 M
	ad 1000 ml	H_2O
TMB solution	24 mg	TMB
	500 µl	EtOH absolute
	ad 1ml	DMSO
Wash Buffer (1x Tris/Tween)	100 ml	10 x Tris/HCl
	0.5 ml	Tween-20
	ad 1 l	H_2O

4.3. Antibodies

Step	Antibody/ Antigen	Provider	
Coating	Albumin from chicken egg white (OVA)	Sigma-Aldrich	Taufkirchen (DE)
OVA-specific IgE Detection	Biotin rat anti-mouse IgE (monoclonal)	BD Biosciences	Heidelberg (DE)
OVA-specific IgE Standard	Mouse anti-ovalbumin IgE (monoclonal)	Biozol	Eching (DE)
OVA-specific IgG1 Detection	Biotin rat-anti-mouse IgG1 (monoclonal)	BD Biosciences	Heidelberg (DE)
OVA-specific IgG1 Standard	Anti-chicken egg albumin clone OVA 14 (monoclonal)	Sigma-Aldrich	Taufkirchen (DE)

4.4. Oligonucleotides

Name	Sequence (5`- 3`)	Tm (°C)
CREB 1 fwd	CCCAGCAACCAAGTTGTTGTTC	62
CREB 1 rev	CTGCCTCCTGTTCTTCATTAGAC	65
CREB1 3´-UTR fwd	P-GTACTCGAGTTTGGGATTTAAGTTCTCTCCTGTTAC	73
CREB1 3´-UTR rev	GTAGCGGCCGCACACATTATGATTCCATTTATTTGAAATTTCTC	76
HPRT1 fwd	CTGGATTACATTAAAGCACTGAA	58
HPRT1 rev	TCAAGACATTCTTTCCAGTTAAAG	58

Oligonucleotides were synthesized by Metabion international AG, Martinsried (DE) unless noted otherwise. Melting temperature (Tm) was calculated by the Metabion using the formula 100.5 + (41* (yG+zC)/(wA+xT+yG+zC)) - (820/(wA+xT+yG+zC)) + 16.6*log10([Na+]).

miRNA probes by Ambion/Applied Biosystems:

TaqMan®-microRNA-Assay	miRNA sequence
hsa/mmu-miR-17	CAAAGUGCUUACAGUGCAGGUAG
hsa/mmu-miR-21	UAGCUUAUCAGACUGAUGUUGA
hsa/mmu-miR-22	AAGCUGCCAGUUGAAGAACUGU
hsa/mmu-miR-142-3p	UGUAGUGUUUCCUACUUUAUGGA

hsa/mmu-miR-144	UACAGUAUAGAUGAUGUACU
hsa/mmu-miR-181a	AACAUUCAACGCUGUCGGUGAGU
mmu-miR-193b	AACUGGCCCUCAAAGUCCCGCU
hsa/mmu-miR-205	UCCUUCAUUCCACCGGAGUCUG
hsa/mmu-miR-208	AUAAGACGAGCAAAAAGCUUGU
hsa/mmu-miR-451	AAACCGUUACCAUUACUGAGUU
mmu-miR-763	CCAGCUGGGAAGAACCAGUGGC
hsa RNU48	CTTTTGGACTGAATCTAAGTGATTTAAAATTCGTCACTACCACTGAGA
mammalian RNU6B	TCGCGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTTT
mmu snoRNA-202	GCTGTACTGACTTGATGAAAGTACTTTTGAACCCTTTTCCATCTGATG
mmu snoRNA-234	AGTGATGATGACCCCAGGTAACTCTGAGTGTGTCGCTGATGCCAT CACCGCAGCGCTCTG

Pre-miRNA precursor molecules by Ambion/Applied Biosystems:

Pre-miRNA	**Sequence (5´-3´)**
Pre-miR™ hsa-miR-17	CAAAGUGCUUACAGUGCAGGUAG
Pre-miR™ hsa-miR-22	AAGCUGCCAGUUGAAGAACUGU
Pre-miR™ hsa-miR-144	UACAGUAUAGAUGAUGUACU
Pre-miR™ hsa-miR-181a	AACAUUCAACGCUGUCGGUGAGU
Pre-miR™ negative control #1	Sequence is not provided

4.5. Restriction enzymes

Enzyme	**Buffer**	**Tempera-ture**	**Recognition site**	**Provider**	
Cla I	Buffer 4	37°C	AT/CGAT TAGC/TA	New England BioLabs	Beverly (US)
Not I	Buffer O	37°C	GC/GGCCGC CGCCGG/CG	Fermentas	Waltham (US)
Sma I	Buffer 4	25°C	CCC/GGG GGG/CCC	New England BioLabs	Beverly (US)
Xho I	Buffer R	37°C	C/TCGAG GAGCT/C	Fermentas	Waltham (US)

4.6. Commercial kits

Kit	Provider	
Artisan™ Congo Red Stain Kit	DakoCytomation	Glostrup (DK)
Agilent RNA 6000 Nano Kit	Agilent/ ThermoFisher Scientific	Waltham (US)
Diff-Quick Kit	Medion Diagnostics	Düdingen (CH)
DNA-free™ Kit	Ambion/ Applied Biosystems	Carlsbad (US)
DNeasy Blood & Tissue Kit	Qiagen	Hilden (DE)
High Capacity RNA-to-cDNA Kit	Applied Biosystems	Carlsbad (US)
Megaplex™ PreAmp Primers, Rodent Pool A and B	Applied Biosystems	Carlsbad (US)
Megaplex™ RT Primers, Rodent Pool A and B	Applied Biosystems	Carlsbad (US)
miRCURY LNA™ microRNA Array Kit	Exiqon	Vedbaek (DK)
miRCURY LNA™ microRNA Array Power Labeling Kit	Exiqon	Vedbaek (DK)
miRNeasy Mini Kit	Qiagen	Hilden (DE)
NucleoSpin® Extract II	Macherey-Nagel	Düren (DE)
QIAprep® Miniprep Kit (Plasmid DNA purification)	Qiagen	Hilden (DE)
Quantikine mouse CCL17/TARC	R&D Systems	Minneapolis (US)
TaqMan® microRNA Array A and B	Applied Biosystems	Carlsbad (US)
TaqMan® microRNA Reverse Transcription Kit	Applied Biosystems	Carlsbad (US)

4.7. Model systems

4.7.1. *In vitro* cell culture

Cells	Description/ Provider
BEAS-2B	Isolated from human bronchial epithelium from autopsy of non-cancerous individuals. Infected with adenovirus 12-SV40 virus hybrid (Ad12SV40). ATCC (CRL-9609)
16HBE14o-	Isolated from human bronchial epithelium. Transformed with SV-40 virus.
ElectroMAX™ DH10B™ cells	Electrocompetent *Escherichia coli* cells. Invitrogen, Carlsbad (US)

4.7.2. Mice

Strain	Provider	
Balb/c	Charles River	Sulzfled (DE)

4.7.3. Patient samples

Whole peripheral blood samples stored in Trizol reagent from a multicenter, double-blind, randomized intervention study (German Infant Nutrition Intervention study) were used.
Samples were selected based on the following criteria: current allergic asthma, lack of acute infections (in the last 4 weeks) and absence of environmental tobacco smoke exposure. Age matched controls from the same cohort were included in case they never had asthma and were non-atopic.

4.8. Miscellaneous Consumables

Miscellaneous consumable	Provider	
24-well plates Nunclon™	Nunc, ThermoFisher Scientific	Waltham (US)
Cell culture flasks, 75 and 175 cm^2	Greiner bio-one	Frickenhausen (DE)
Cryotubes	Sarstedt	Nümbrecht (DE)
Gene Pulser® Cuvettes (0.1 cm gap)	BioRad	Hercules (US)

Glass slides	Gerhard Menzel	Braunschweig (DE)
Histology cassettes	ThermoFisher Scientific	Waltham (US)
LumiNunc, F96	Nunc, ThermoFisher Scientific	Waltham (US)
MicroAmp™ Fast Optical 96-well Reaction Plate	Applied Biosystems	Carlsbad (US)
MicroAmp™ 96-well Optical Adhesive Film	Applied Biosystems	Carlsbad (US)
Micropipettes (10µl)	Marienfeld	Lauda-Königshofen (DE)
NUNC-MaxiSorp, round bottom	Nunc, ThermoFisher Scientific	Waltham (US)
Petri-dishes	Greiner bio-one	Frickenhausen (DE)
Shandon cytofunnels	ThermoFisher Scientific	Waltham (US)

4.9. Equipment and devices

Equipment/ device	**Name**	**Provider**	
Bioanalyzer	Agilent 2100 Bioanalyzer	Thermo Fisher Scientific	Waltham (US)
Array reader	GenePix 4000A Scanner	Axon Instruments	Foster City (US)
Centrifuge	Centrifuge 5810R	Eppendorf	Hamburg (DE)
	Heraeus Multifuge 3S+	Thermo Fisher Scientific	Waltham (US)
	Mikro 200R	Andreas Hettich	Tuttlingen (DE)
Cytospin centrifuge	Shandon Cytospin 3	Thermo Fisher Scientific	Waltham (US)
Lyophilizer	MICRO STP 420D	Thermo Fisher Scientific	Waltham (US)
Electroporation device	Capacitance Extender Plus	BioRad	Hercules (US)
	Gene Pulser II	BioRad	Hercules (US)
	Pulse Controller Plus	BioRad	Hercules (US)
ELISA reader	MRXII	Thermo Labsystems	Egelsbach (DE)
Embedding machine	MICROM EC 350-1	Thermo Fisher Scientific	Waltham (US)
Freezer	Heraeus Hera freeze -80°C	Thermo Fisher Scientific	Waltham (US)
Gel Imager	Intas	Science Imaging Instrument GmbH	Göttingen (DE)
Hemocytometer	Neubauer cell chamber	GLW	Würzburg (DE)

Histology Staining automat	MICROM HMS 740	Thermo Fisher Scientific	Waltham (US)
Homogenizer	Polytron TP2100	Kinematika	Luzern (CH)
Incubator	HeraCell 240 Incubator	Thermo Fisher Scientific	Waltham (US)
	Heraeus Function Line Incubator	Thermo Fisher Scientific	Waltham (US)
Liquid nitrogen tank	MVE 600 series	Chart Industries	Burnsville (US)
Luminometer	Wallac Victor2, 1420 Multilabel Counter	Perkin Elmer	Massachusetts (US)
Microscope	DMIL	Leica Microsystems	Wetzlar (DE)
Microtom	HYRAX M55	Zeiss	Oberkochen (DE)
Nebulizer	Pari boy	Pari	Starnberg (DE)
Real-time Thermocycler	ABI 7900HT Fast Real-Time PCR System	Applied Biosystems	Carlsbad (US)
	ABI StepOne™ Plus Real-Time PCR System	Applied Biosystems	Carlsbad (US)
Spectrophotometer	Nanodrop ND-1000	Thermo Fisher Scientific	Waltham (US)
Thermocycler	peqStar 96 universal gradient	peqLab Biotechnology	Erlangen (DE)
Water bath	1008	GFL	Burgwedel (DE)

4.10. Software and internet resources

Software and Internet resources

GenePix Pro 6.0	Axon Instruments, Foster City (US)
GraphPad Prism v5.0	GraphPad Prism software, San Diego (US)
miRanda	http://www.microrna.org//miranda.html
miRNA registry/ miRBase	http://microrna.sanger.ac.uk
PicTar	http://pictar.bio.nyu.edu
PITA	http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html
SDS v2.2	Applied Biosystems, Carlsbad (US)
Target spy	http://www.targetspy.org
TargetScanS	http://genes.mit.edu/targetscan

5. METHODS

5.1. Mice

5.1.1. Animal maintenance

Female Balb/c mice (Charles River, Sulzfled, Germany) were housed in individually ventilated cages and received a standard pellet diet and water *ad libitum*. The study was conducted under the federal guidelines for the use and care of laboratory animals and was approved by the government of the district of Upper Bavaria.

5.1.2. Treatment protocol

At an age of 6-7 weeks mice underwent treatment consisting of 6 intra-peritoneal (i. p.) injections of either PBS or OVA (1 µg in 200 µl of PBS) together with 2.5 mg of alum adjuvant (Imject® alum containing aluminum hydroxide and magnesium hydroxide) on days 0, 7, 15, 28, 42 and 56. On days 70 and 71 mice received an aerosol challenge (Pari) for 20 min consisting of either nebulized PBS or ovalbumin (1 % in PBS). All analyses were performed after a lethal i. p. anesthesia with 20 µl/g/body weight Ketamin (10 %) and Xylazin (2 %).

5.2. Serum analysis

Blood was taken from the retrobulbar veins. After coagulation for 1-2 h at room temperature serum was separated by centrifugation (10 000 rpm, 10 min at room temperature) and stored at -80°C.

5.2.1. OVA-specific Ig ELISA

OVA-specific IgE and IgG1 were measured in serum samples of mice by ELISA. For coating, a stock solution of OVA (1 mg/ml in PBS) was diluted 1:100 in coating buffer and 100 µl were added to each well of a 96-well plate (NUNC-MaxiSorp, round bottom). After overnight

incubation at 4°C plates were washed three times with washing buffer followed by blocking with 200 µl of blocking buffer for 2 h at room temperature and subsequent washing. Serum samples and serial dilutions of standard for IgE or IgG1 in blocking buffer were prepared. All samples were analyzed in duplicates using a volume of 50 µl per well and incubated at 4°C overnight. After washing, 50 µl of detection antibody (biotin anti-mouse IgE or IgG1, diluted 1:400 in blocking buffer) was added per well and incubated for 2 h at room temperature After washing, 50 µl of streptavidin-peroxidase (diluted 1:1000 in blocking buffer) was added for 30 min at room temperature before plates were washed five times. The substrate for the enzymatic reaction was mixed just before use (single preparation for each plate using 50 µl per well) consisting of 55 µl TMB solution and 2.55 µl of cold H_2O_2 (30 %) filled up to 5.5 ml with substrate buffer. After stopping the colorimetric reaction with 25 µl of 2 M sulfuric acid per well, plates were analyzed with a microplate reader at 450 nm.

5.3. BAL analysis

BAL fluid was obtained by intra-tracheal instillation of three times 0.8 ml of PBS. Centrifugation at 1200 rpm for 10 min at 4°C separated cells and fluid. The cellular fraction was promptly investigated, fluid was stored at -80°C until further use.

5.3.1. Total cell counts

The BAL cell pellet was resuspended in 160 µl of cold PBS (5 % FCS) and total cell numbers were counted using a hemocytometer. Briefly, 10 µl of a 1:2 dilution of cell suspension and trypan blue were added to a hemocytometer and four big squares were counted. The number of cells per ml was calculated considering dilutions and chamber volume
(counted cells in four squares / 4 x 2 (dilution) x 10^4/ml = cells per ml).

5.3.2. Differential cell counts

According to cell numbers, 10000 to 100000 cells in 150 µl of PBS (5 % FCS) were transferred to a sample slide using cytocentrifugation (400 rpm, 10 min at room temperature). Samples were left to dry overnight and cells stained using the Diff-Quick Kit. After drying, samples were embedded using Entellan.
Counting of different cell types was performed by light microscopy using 100 x magnification and counting a minimum of 500 cells per slide. Cells were morphologically distinguished into

macrophages, neutrophils, lymphocytes and eosinophils. The Diff-Quick Kit stains cells according to the method of May-Giemsa-Grünwald. The DNA-rich nuclei are stained by alkaline components of the dye (methylenblue and azur B) and appear dark blue or violet. The cytoplasma shows a lighter blue, with dark blue granula in basophils or acidophilic pink granula in eosinophils (stained by eosin). Neutrophils contain weak acidophilic granula in their cytoplasm, which are practically not visible. Epithelial cells were not included in the cell counts. Their pink staining and lack of visible intracellular components were used to distinguish them from other cell types. The epithelial cells present in BAL were squamous epithelium with a characteristic oval form and ciliated epithelium.

5.3.3. CCL17 ELISA

Cell-free BAL fluid was used for chemokine measurement. CCL17 (also named TARC) protein levels were analyzed by ELISA following instructions of the Quantikine® mouse CCL17/TARC kit (R&D Systems, MN).

5.4. Histopathology

For histopathological analysis, either the whole lung or the left lung lobe was fixed by intra-tracheal instillation and subsequent immersion with 4 % formaldehyde for 24 h at 4°C. Lungs were washed in PBS and trimmed according to histological standard analyzing left lobe and/or right cranial, middle, caudal and accessory lobe. Tissue slices were transferred to histology cassettes and immersed in 70 % ethanol for up to three days before being dehydrated applying washing steps with solutions of increasing ethanol concentrations (ethanol 70 %, 80 %, two times 95 %, two times 100 %). After clearance in xylene (2 washes for 5 min), samples were automatically embedded in paraffin (MICROM EC 350-1). The tissue blocks were cut into sections of 5-8 µm thickness on a microtom (HYRAX M55) and transferred to glass slides. After drying, slides were deparaffinized in xylene (2 washes for 5 min) and hydrated applying washing steps with solutions of decreasing ethanol concentrations (two times 2 min in 100 %, 1 min in 90 %, 1 min in 80 %, 1 min in 70 % and 30 sec in H_2O). Staining of lung tissue slides was done with Congo Red staining kit (Dako) using a histology staining automate (MICROM HMS 740). The Congo Red staining procedure includes three steps: an alcoholic Congo Red staining (8 % alcohol, 0.2 % Congo Red and NaCl in deionized H_2O), an alkaline alcohol step (80 % alcohol and NaCl in deionized H_2O) and a counterstain with Mayer´s Hematoxylin (0.1 % hematoxylin, 5 %

aluminum ammonium sulfate and additives in deionized H_2O). After staining, slides were washed in H_2O for 10 min before being dehydrated (ethanol each for 1 min in 70 %, 80 %, 90 %, 100 %). Clearance with two washes for 5 min in xylene was followed by addition of cover slips using Entellan mounting medium.
Analysis using light microscopy reveals pale pink colours for amyloid, collagen or fibrous material whereas nuclei have a blue appearance. Qualitative changes in lung tissue structure and cell composition were evaluated in stained sections.

5.5. RNA analysis

5.5.1. Isolation of total RNA including small RNAs

For depletion of blood from lung tissue, trans-cardial perfusion was undertaken. Approximately 8 ml of PBS were injected via the right ventricle and loss of blood was indicated by a change in colour of lung tissue.
Lung or spleen tissue was cut in pieces and stored in RNAlater® until further processing. Tissue was transferred to Qiazol reagent and underwent homogenization (Polytron TP2100). Approximately 70 mg of lung or 10 mg of spleen tissue were used for a volume of 700 µl Qiazol. For blood miRNA analysis, whole blood was directly transferred to Qiazol solution (approximate ratio 1: 6). Cell culture samples were directly dissolved by addition of Qiazol. All samples were processed following instructions of the miRNeasy Mini Kit (Qiagen).

5.5.2. Quality testing

Concentration and quality was monitored by absorbance measurement (Nanodrop). Acceptable values were defined as 260/280 nm ratios above 1.8 and 260/230 nm ratios between 1.8 and 2.2. In addition, RNA integrity was analyzed by native agarose gel electrophoresis inspecting 28S and 18S rRNA bands. Therefore, 1 µg of RNA was added to 5 µl formamide (total volume 10 µl) and treated with 65°C for 15 min. After cooling on ice and addition of 2 µl loading dye, samples were loaded on a 0.8 % agarose gel in TAE buffer with 0.5 µg/ml ethidium bromide added to the gel and run at ~65 V.
For further testing the Bioanalyzer system (Agilent) was applied following manufacturer´s guidelines for the Agilent RNA 6000 Nano Kit. RNA integrity numbers (RIN) above 7 indicate sufficient RNA quality.

5.5.3. miRNA analysis

5.5.3.1. miRNA profiling by microarray (Exiqon)

The Exiqon miRNA microarray experiment includes 2 steps: labeling of RNA molecules (miRCURY^{TM} LNA microRNA Power Labeling Kit) and second, hybridization of the labeled RNA to pre-spotted microarrays (miRCURY^{TM} LNA microRNA Array Kit).
For normalization, 10 different synthetic miRNAs in various concentrations are spiked into a RNA sample. The spike-in miRNAs bind to corresponding capture probes on the array. Additionally, positive and negative miRNA probes are available. The Exiqon array is based on locked nucleic acid (LNA)-modified capture probes which have been tested to achieve high detection sensitivity (Castoldi et al. 2006).

All steps were performed according to manufacturer´s guidelines. Briefly, 1 µg total RNA was combined with spike-in miRNAs and calf intestinal alkaline phosphatase plus buffer, incubated for 30 min at 37°C followed by heating for 5 min at 95°C. After cooling on ice for 2 min, labeling enzyme catalyzed the attachment of fluorescent labels (Hy3^{TM} or Hy5^{TM} respectively) in presence of labeling buffer and DMSO during 1 h at 16°C followed by a stop reaction for 15 min at 65°C. Next, the two samples aimed to be compared were mixed (e.g. healthy control RNA/Hy3^{TM}-labeled plus asthma RNA/Hy5^{TM}-labeled) and denatured with hybridization buffer at 95°C for 2 min followed by 2 min on ice. Moreover, every comparison was done twice including a colour-swop (reversed dyes) in order to minimize differences originating from the labeling reaction. The RNA preparation was then pipetted into the prepared microarray slide and incubated for hybridization within a tightly closed slide chamber for 16-18 h in a water bath at 56°C. Afterwards, microarray slides underwent a series of stringency washes (with decreasing concentrations of salt buffer and detergent solution) before drying. Fluorophore emissions of 556 nm and 656 nm (Hy3^{TM} and Hy5^{TM}) were measured by scanning at 5 µM resolution using GenePix 4000A Scanner (Axon Instruments, Foster City, US).
Prior to data analysis, fluorescent signals were matched to species-specific GenePix® Array Lists (GAL) files consistent with the micorarray layout. The matching was verified manually for each slide using GenePix Pro 6.0 (Axon Instruments, Foster City, US).
Bioinformatic analyses were performed by PD Dr. Philip Pagel (Institute for Bioinformatics and Systems biology/MIPS, Helmholtz Zentrum München). Analyses were generated with the R statistical language (R Development Core Team 2008) using packages of the Bioconductor framework (Gentleman et al. 2004). Quality control comprised spot and background as well as raw colour intensities of all arrays. For data processing, background

correction and within-array normalization was considered. Repeatability was tested as correlation between replicate spots within arrays and between arrays with colour-swop (reversed dyes). Analysis of differentially expressed miRNAs (given as log fold change and average expression) was carried out with the limma package (Smyth, G.K. 2005) which fits a linear model for each gene and computes a moderated t-statistic and its *p*-value (Smyth, G. K. 2004). Adjustment for multiple testing (adjusted *p*-value) was done using the method by Benjamini and Hochberg (Benjamini and Hochberg 1995).

5.5.3.2. miRNA profiling by TaqMan® MicroRNA Array

The TaqMan® array is based on TaqMan® RT-PCR technology. The reaction comprises a high-throughput system with a multiplex RT and qPCR reaction on pre-configured micro fluidic cards (Lao et al. 2006; Mestdagh et al. 2008). An optional pre-amplification step between RT and PCR is supposed to increase sensitivity. Each array of the two-array set contains endogenous control assays for species-specific normalization.

All experimental steps were performed according to manufacturer´s guidelines. Briefly, 350 ng of isolated RNA including small RNAs (see 5.5.1) was subjected to RT including Megaplex™ RT primers and TaqMan® MicroRNA Reverse Transcription Kit (including MulitScribe™ Reverse Transriptase, RT buffer, dNTPs, RNase inhibitor and nuclease-free water). Thermal cycling conditions were set to 40 cycles including 2 min at 16°C, 1 min at 42°C, 1 sec at 50°C followed by a terminal heating to 85°C for 5 min and cool down to 4°C. The achieved RT product was then combined with Megaplex™ PreAmp Primers and TaqMan® PreAmp Master Mix under the following thermal cycling conditions: 10 min at 95°C, 2 min at 55°C, 2 min at 72°C followed by 12 cycles including 15 sec at 95°C and 4 min at 60°C, ending with a cool down to 4°C. Next, the diluted pre-amplified RT product was mixed with TaqMan® Universal PCR Master Mix (No AmpErase® UNG) and the 384-well microfluidic card of the TaqMan® Array was loaded, centrifuged and sealed. The SDS v2.2 software was applied to import the plate layout and perform the experiment on the Applied Biosystems 7900HT Fast Real-Time PCR System before analyzing relative quantification (ΔΔCt method).

5.5.3.3. miRNA expression quantification by RT-qPCR

Expression of single miRNAs was tested using TaqMan® MicroRNA Assays in a two-step reaction setting.

In the RT reaction, a miRNA-specific stem-loop primer is extended to bind to the miRNA before a cDNA strand is synthesized (Chen, C. et al. 2005). Therefore, 50 ng total RNA was

combined with TaqMan® MicroRNA RT primer and given amounts of the TaqMan® MicroRNA Reverse Transcription Kit following manufacturer´s guidelines. Thermal cycling conditions were 30 min at 16°C, 30 min at 42°C followed by 5 min at 85°C.
In the second step, PCR products were amplified from cDNA samples using the TaqMan® MicroRNA Assay (specific forward and reverse PCR primers and a specific TaqMan® probe) together with the TaqMan® Universal PCR Master Mix, No AmpErase® UNG. Thermal cycling conditions for the PCR reaction included 10 min at 95°C followed by 40 cycles of 15 sec at 95°C (denaturation) and 60 sec at 60°C (annealing/extension). The PCR reaction was carried out in 96-well format using Applied Biosystems StepOnePlus™ Real-Time PCR system and analyzed using SDS software. All reactions were run in triplicate.
Data analysis included setting of baseline and threshold values and calculation of differential expression using the ΔΔCt method. Only samples run in the same RT reaction were considered for direct comparison.

5.5.4. mRNA analysis

Total RNA was isolated using the miRNeasy Mini Kit (Qiagen) and quality testing was performed as described in section 5.5.2. Prior to real-time PCR, DNase treatment was done following instructions of the DNA-free™ Kit (Ambion, Applied Biosystems).

5.5.4.1. mRNA Expression quantification by RT-qPCR

The High Capacity RNA-to-cDNA Kit (Applied Biosystems) was used for reverse transcription. In every experiment, “no amplification” controls were included for each sample. According to manufacturer´s instructions, RT buffer (containing dNTPs) and enzyme mix (containing MuLV and RNase inhibitor protein) were combined with 900 ng RNA per reaction for 60 min at 37°C followed by 5 min at 95°C.
For real-time qPCR the Power SYBR® Green PCR Master Mix (Applied Biosystems) was used containing SYBR® Green I Dye, AmpliTaq Gold® DNA Polymerase, dNTPs, passive reference (ROX) and optimized buffer components. Reactions were run in triplicates and “no amplification” as well as “no template” controls were included. Applied Biosystems StepOnePlus™ Real-Time PCR System was used and a melting curve was processed after each run.

Optimized reaction set up:

		Concentration for	
Reagent	**Volume [µl]**	*CREB1*	*HPRT1*
Power SYBR Green Mix	10		
cDNA	1		
Primer forward	1	375 nM	1 µM
Primer reverse	1	375 nM	1 µM
nuclease-free water	7		
Total	20		

Thermal cycling conditions:

	Step	**Temperature**	**Time**	
1	Denaturation/Enzyme activation	95°C	10 min	
2	Denaturation	95°C	15 sec	40 cycles
3	Annealing/Extension	60°C	60 sec	
4	Melting Curve	95°C	15 sec	
5		60°C	60 sec	with 0.3°C
6		95°C	15 sec	increments

Correct size of PCR products was tested by agarose gel electrophoresis using 2.5 % agarose in TAE buffer with 0.5 µg/ml ethidiumbromid run at 100 V for ~90 min. For size discrimination a 50 bp ladder was applied in a concentration of 0.5 µg/well. Data analysis comprised baseline and threshold settings using the SDS software (Applied Biosystems) and calculation of differential expression using the ΔΔCt method.

5.5.5. Normalization

Microarray and real-time qPCR based data for miRNA expression was normalized using control genes. These were either provided in the assay, namely RNU6B (Exiqon), determined to be most stable (snoRNA-202 for TaqMan® array), or experimentally verified to fulfill the role of control genes in different tissues and organs analyzed in the present study. For example, levels of snoRNA-234 were found to be particularly stable in murine lungs and spleen whereas snoRNA-202 was the candidate of choice for murine blood. For human blood samples RNU48 was selected. For the analysis of human cell lines RNU6B was applied as control gene.

Expression of *CREB1* gene was normalized using *Hypoxanthine guanine phosphoribosyl transferase 1* (*HPRT1*) which was employed for both human cell lines and mouse tissue samples.

5.6. Cell culture

5.6.1. Culture of cell lines

Adherent cell lines (BEAS-2B, 16HBE14o-) were cultured in MEM medium containing 10 % FCS and 1 % Penicillin/Streptomycin at 37°C and 5 % CO_2 under standard conditions. Detachment of cells was achieved by addition of Trypsin-EDTA (0.05 % (w/v) Trypsin and 0.5 mM EDTA, Invitrogen) when cells were needed for experiments or reached high confluency. After addition of 2 ml of Trypsin-EDTA (175 cm^2 cell culture flasks), cells were incubated for 4 min at 37°C. As soon as detachment was visible, cell culture medium was added to stop the enzymatic reaction.

5.6.2. Cell counts

Cell numbers were examined with a Hemocytometer as described in section 5.3.1.

5.6.3. Cryopreservation

For preservation, cells were spun for 5 min at 300 g and the pellet was resuspended in cell freezing medium consisting of MEM, 20 % FCS and 10 % DMSO. A number of 2- 5 $x10^6$ cells/ cryotube were slowly frozen at -80°C before transfer to cryopreservation in nitric oxygen at -196°C.
For defrosting of cryopreserved cells, tubes were directly set into a waterbath at 37°C until defrozen, transferred to pre-warmed medium and the pellet resuspended and cultivated in fresh medium after centrifugation at 400 g for 5 min.

5.6.4. Transfection via lipofection

For transfection experiments, 80 000 cells/ well (BEAS-2B and 16HBE14o-) were seeded in a 24-well format and visually monitored for viability before and after every treatment. After 24 h cells reached ~95 % confluency and transfection was performed with Lipofectamine 2000

(Invitrogen) following manufacturer´s instructions. Either 20 pmol of precursor miRNA (Ambion) alone, or precursor miRNA plus 50 ng of 3´-UTR-plasmid-vector construct were transfected. In case of the co-transfection experiment, a non-specific filler DNA (pUC21, Plasmid factory; kindly provided by PD Dr. Carsten Rudolph) was added to the plasmid vector DNA to achieve a total amount of 400 ng and an according volume of 1 µl of Lipofectamine. miRNAs of interest were tested and compared to a negative control. In every experiment, untransfected cells and/ or cells transfected with *CREB1* 3´-UTR-plasmid only, were also monitored. Analysis was done 72 h after transfection.

5.7. Bacterial culture

5.7.1. Culture of bacteria

Transformed *E.coli* cells (ElectroMAX™ DH10B™ cells, Invitrogen) were cultured on agar plates (15 g Agar-Agar in 1 l LB medium) including ampicillin (75 µg/ml) for selection. Bacterial colonies were grown in shaking suspension of ~5 ml LB medium containing 100 µg/ml ampicillin. Both culturing conditions were performed overnight (12-16 h) at 37°C.

5.7.2. Glycerol stocks

To prepare frozen stocks of desired clones, 700 µl of an overnight culture grown in medium was mixed with 150 µl of glycerol (~17 %) and stored at -80°C. For inoculation, some crystals of frozen glycerol stock were transferred to ampicillin containing LB medium and cultured as shaking suspension at 37°C overnight

5.7.3. Transformation using electroporation

Electrocompetent *E.coli* cells underwent transformation by electroporation in order to take up and amplify plasmid vector constructs. Therefore, 2 µl of electrocompetent *E.coli* were combined with 50-100 ng plasmid vector filled up to 50 µl with nuclease-free water. Transferred to a pre-cooled electroporation cuvette (GenePulser® cuvettes, 0.1 cm gap, BioRad), the following conditions were applied: 1.8 kV, 100 Ω, 25 µF using a Gene Pulser II (BioRad). Transformed solution was pipetted into 2 ml of LB medium and cultivated for 1 h at 37°C before centrifugation at 4000 rpm for 4 min. Supernatant was decanted and the pellet resuspended with the remaining liquid. Two agar plates with ampicillin (75 µg/ml) were plated

with 50 µl and 100 µl of resuspension and incubated at 37°C overnight. On the next day, formed colonies were picked and transferred to ampicillin-LB medium as described in section 5.7.1.

5.7.4. Plasmid purification

To purify the plasmid vector DNA after amplification in *E.coli*, the QIAprep® Miniprep Kit (Qiagen) was applied following manufacturer´s instructions. Before starting the procedure, bacterial cultures were spun at 4000 rpm for 5-10 min and supernatant was decanted.

5.8. miRNA expression reporter gene assay

5.8.1. 3´-UTR amplification

For amplification of the *CREB1* 3´-UTR, genomic DNA was isolated from murine heart tissue using the DNeasy Blood & Tissue Kit (Qiagen). To obtain the complete 3´-UTR of *CREB1*, PCR primers were designed to target the 7.2 kb long segment and amplification was performed using *Pfu* Ultra High-Fidelity DNA Polymerase (Agilent). Suggested extension time of 2 min per kb was extended to ~2.6 min per kb (total extension time 19 min).

Optimized reaction set up:

Reagent	Volume [µl]	Concentration
10x Pfu Ultra HF buffer	3.75	
dNTPs	1.25	500 µM
DNA	2	250 ng
CREB1 3´-UTR Primer forward	1	500 nM
CREB1 3´-UTR Primer reverse	1	500 nM
Pfu Ultra HF DNA Polymerase	1	2.5 U
BSA	1	0.4 µg
DMSO	1.25	5 %
Total	25	

Thermal cycling conditions:

	Step	Temperature	Duration	
1	Denaturation	95°C	5 min	
2	Denaturation	95°C	30 sec	10 cycles
	Annealing	55°C	30 sec	
	Extension	68°C	19 min	
3	Denaturation	95°C	30 sec	20 cycles
	Annealing	55°C	30 sec	
	Extension	68°C	19 min plus 10 sec/cycle	

Successful reaction was ascertained by agarose gel electrophoresis followed by gel extraction and PCR clean-up of the 3´-UTR (NucleoSpin® Extract II, Macherey-Nagel).

5.8.2. Restriction enzyme digestion

Restriction enzyme digestions were performed with *Not* I and *Xho* I (Fermentas) as well as with *Cla* I and *Sma* I (New England Biolabs) according to manufacturer´s guidelines. Reaction volume was 20 µl including 2 µl of specific 10x buffer, 0.2 µl of enzyme (1 U/µl) and 0.5-1 µg DNA. Digestions were run at 37°C for 3 h unless stated otherwise. Size distribution of digested DNA was tested on a 0.8 % agarose gel (see section 5.5.4.1) using a 1 kb ladder.

5.8.3. Ligation

The complete *Creb1* 3´-UTR was ligated into psiCHECK^{TM}-2 reporter plasmid (see Figure 3; 100 ng DNA per reaction) using T4 DNA ligase (5 U/µl, Fermentas) following manufacturer´s instructions. In order to verify the identity of the cloned insert, a restriction map was prepared. Three different restriction enzymes were used (*Cla* I, *Sma* I and *Xho* I) and size distribution was determined by agarose gel electrophoresis.

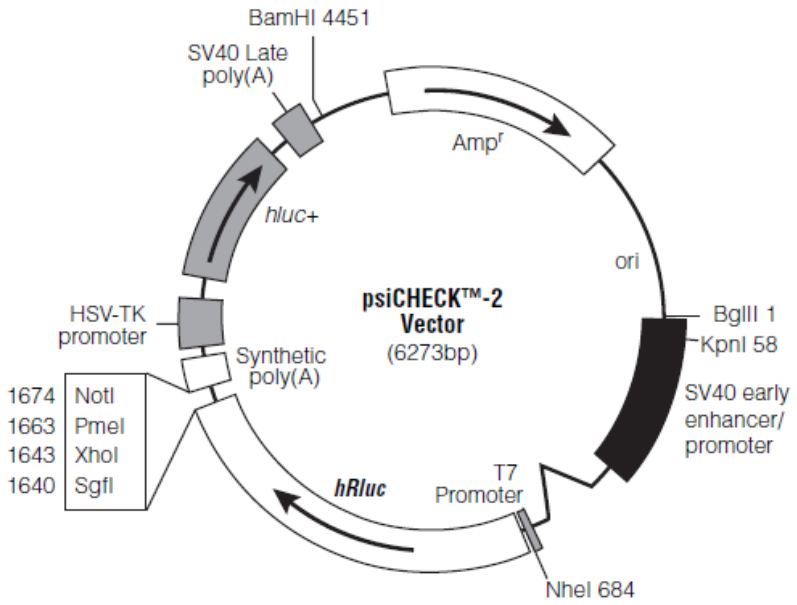


Figure 3: Plasmid map of psiCHECK^{TM}-2 vector Structure of reporter plasmid with a multiple cloning region downstream of a primary reporter gene (*Renilla* luciferase). The target 3´-UTR of interest is ligated to the reporter gene, which, after effective silencing by precursor miRNAs, leads to a reduction in *Renilla* luciferase activity. A secondary *Firefly* luciferase expression cassette can be used for normalization of the luciferase signals (Promega).

5.8.4. Measurement of reporter gene expression

Direct interaction between the target 3´-UTR of *CREB1* gene and the four predicted miRNAs was examined using a reporter gene assay. Activity of the cloned 3´-UTR could be indirectly measured by quantifying *Renilla* luciferase using coelenterizine as substrate. *Firefly* luciferase, encoded on the same plasmid, was used as a control for transfection efficiency and was quantified by addition of its substrate luciferin. Binding of a miRNA to target sites in the 3´-UTR hinders *Renilla* luciferase expression and thus lowers *Renilla/Firefly* ratio.

72 h after transfection, cells were lysed by addition of 120 µl of lysis buffer per well and incubated for 10 min at room temperature with constant agitation. Using a white 96-well plate (Nunc), 50 µl of lysed sample was pipetted per well. The according substrate buffers (luciferine or coelenterizine) were added automatically using a volume of 100 µl per well. Luminescence was measured with a Luminometer (Wallac Victor2).

5.8.5. Bradford protein measurement

After measurement of luminescence, 5 µl of each lysed sample was transferred to a 96-well microtiter plate and protein content was measured using Bio-Rad Protein Assay following manufacturer´s guidelines (Bradford method). 200 µl of dye reagent (diluted 1:5 in PBS) was

added per well and measured at 595 nm (Wallac Victor2). For calculation, a standard curve was prepared using BSA as standard protein in a concentration range of 0.2 –0.7 mg/ml.

5.9. Statistical analysis

Statistical analyses were performed with GraphPad Prism software version 5.0 (San Diego, US). The level of significance was accepted for p values ≤ 0.05.

For comparison of different treatment groups (means of two unmatched groups), the unpaired Student´s t-test was applied. In order to test association between two variables, Spearman rank correlation analysis was used. This non-parametric test gives a correlation coefficient “rho” that varies between -1 and +1, with ±1 describing perfect degree of correlation. Values near 0 point towards a weaker relationship of the two variables.

6. RESULTS

6.1. Mouse model characterization

The present study aimed at investigating miRNA dysregulation in allergic airway disease using an ovalbumin based mouse model. Sensitization and challenge scheme followed in this study is presented as Figure 4. Mice were analysed on day 72 post treatment, i. e. 24 h post final challenge, and none of the treatment groups showed any visible symptoms of physical distress. Before sacrificing the mice, body weight was measured and a small but significant decrease in body weight was observed for the asthma group (mean 19.69 g ± 1.87) compared to healthy control animals (mean 20.91 g ± 0.55). No significant difference could be observed between healthy control and sensitized groups (Figure 5).

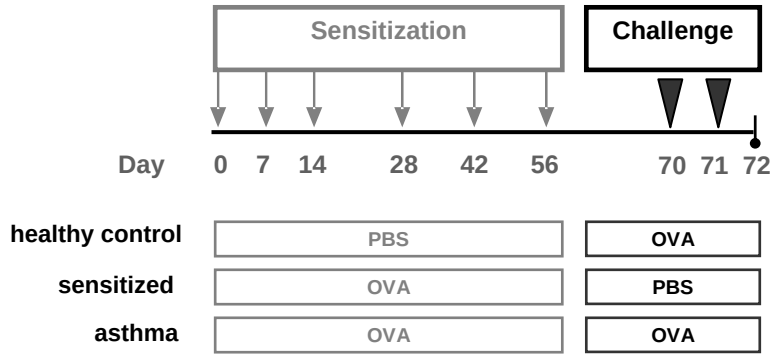


Figure 4: Treatment protocol. Six to seven week old female mice were i.p. sensitized on days 0, 7, 14, 28, 42 and 56 with either 1 µg OVA or PBS in 200 µl together with 2.5 mg alum, followed by aerosol challenge on two consecutive days (70 and 71) with 1 % OVA or PBS for 20 min. Three treatment groups were studied: healthy control, sensitized and asthma mice. Mice were sacrificed on day 72.

For characterization of the mouse model, the following analyses were performed: OVA-specific IgE and IgG1 titres in serum, total and differential cell counts as well as CCL17 chemokine levels in BAL, and lung histopathology.

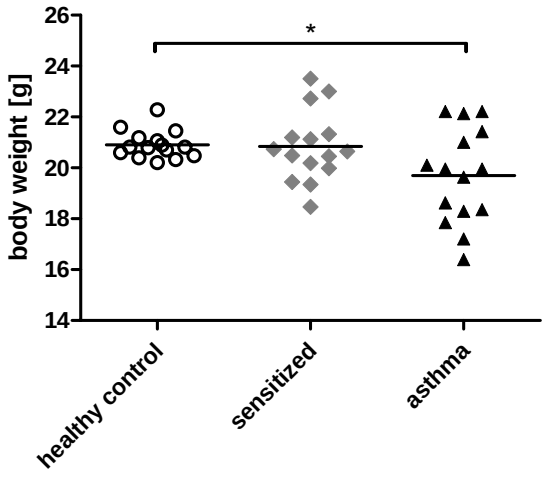


Figure 5: Body weight of mice in different treatment groups. Body weight was measured on day 72. 15 mice per group are shown with each point reflecting one animal (data from 3 independent experiments with n = 5); unpaired t-test with *p ≤ 0.05

6.1.1. OVA-specific Ig levels in serum

Sensitization with an antigen leads to a systemic immune reaction and raised Ig against the administered OVA can be detected in serum samples using ELISA technique. Mice that received i. p. OVA treatment (sensitized and asthma group) showed a significant increase in both IgE and IgG1 levels compared to healthy control mice (Figure 6A and B). Briefly, sensitized (305.40 ± 134.60 ng/ml) as well as asthma mice (301.00 ± 66.83 ng/ml) showed ~30-fold higher IgE levels compared to controls (10.04 ± 10.0 ng/ml). For IgG1, sensitized and asthma mice had serum levels of 4.69 (± 1.89) x 10^6 ng/ml and 4.75 (± 1.97) x 10^6 ng/ml whereas in control animals very little OVA-specific IgG1 could be detected (1.97 ± 4.21 x 10^{-5} ng/ml).

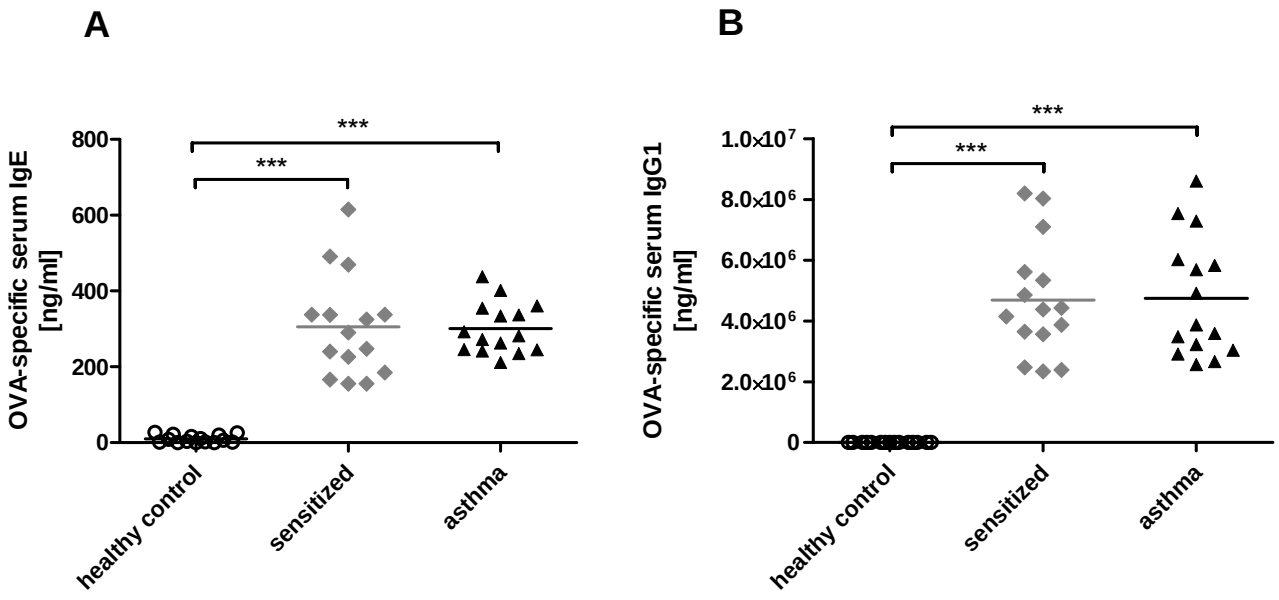


Figure 6: OVA-specific serum Ig levels in different treatment groups. OVA-specific IgE (A) and IgG1 (B) levels were detected in serum samples of healthy control, sensitized and asthma mice. Each point reflects one animal; n = 15 per group (data from 3 independent experiments with n = 5); unpaired t-test with ***p ≤ 0.0005.

6.1.2. BAL analysis

Performing BAL enables cells and signaling molecules to be retrieved from the airways which provide information about inflammatory changes. BAL was analysed with respect to total cell numbers and differential cell counts. As additional maker for inflammation CCL17 chemokine levels were investigated, which have previously been shown to be increased in asthmatic patients (Hartl et al. 2005).

6.1.2.1. Total cell counts

Total cell numbers, as presented in Figure 7, were significantly increased in BAL from asthma mice (16.11 x 10^4 cells/ml) compared to healthy control (3.11 x 10^4 cells/ml) and sensitized mice (2.41 x 10^4 cells/ml).

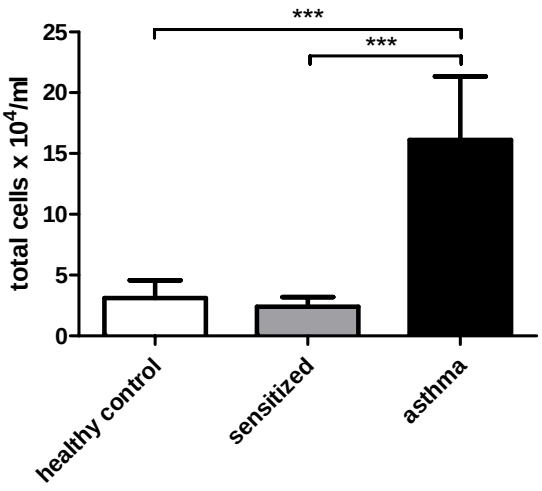


Figure 7: Total cell counts in BAL of different treatment groups of mice. Lungs were lavaged three times with 0.8 ml PBS and the cellular fraction was analyzed. Total cell counts were measured in healthy control, sensitized and asthma animals. Values represent mean x 10^4 cells/ ml ± SD with n = 7 per group (data from 2 independent experiments with n = 3 and n = 4); unpaired t-test with ***p ≤ 0.0005.

6.1.2.2. Differential cell counts

Differential cell counts in BAL were explored by cytocentrifugation and subsequent staining (Diff-Quik Kit, Figure 8). The different cell types were distinguished as described in 5.3.2. In both healthy control and sensitized mice, macrophages constituted the major cell type whereas all other cell types constituted less than 1 % (Fig. 9A). In asthma samples the proportion of macrophages was ~33 % due to a rise in neutrophils (~30 %), eosinophils (~34 %) and to a lower extent also lymphocytes (~3 %). Cell numbers per ml are depicted in Figure 9B. Next to the appearance of inflammatory cells in the asthma samples, morphology of macrophages changed due to activation and increased phagocytosis during inflammation. This was visible as increase in size as well as foam-like appearance of cytoplasma.

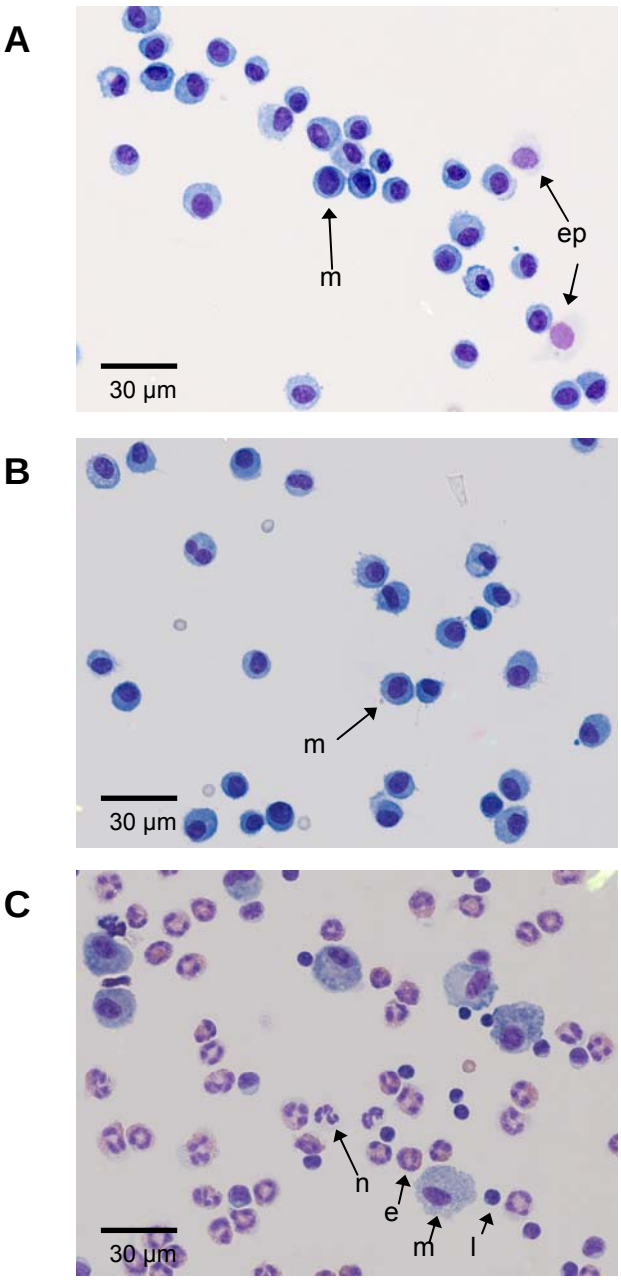


Figure 8: BAL cytospin pictures of different treatment groups. BAL was retrieved by intratracheal instillation of three times 0.8 ml PBS into the lungs. The cellular fraction was counted and cells were transferred to glass slides by cytocentrifugation. After staining with Diff-Quick, a minimum of 500 cells per slide were distinguished into macrophages (m), neutrophils (n), eosinophils (e), lymphocytes (l) or epithelial cells (ep); magnification 40x. Pictures are representative examples of (A) healthy control animals (B) sensitized animals and (C) asthma animals.

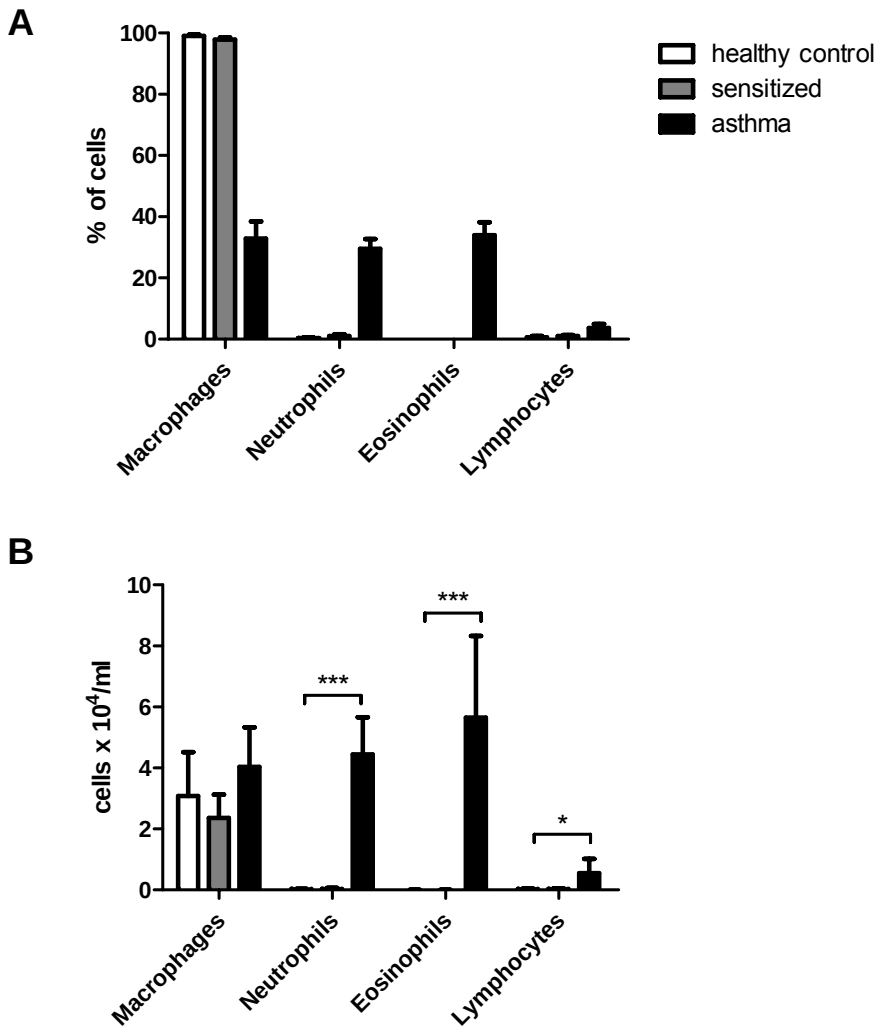


Figure 9: Differential cell counts of healthy control, sensitized and asthma mice. The cellular fraction of retrieved BAL was analyzed by cytospin technique using Diff-Quick staining. A minimum of 500 cells per slide were distinguished into macrophages, neutrophils, eosinophils or lymphocytes. (A) The proportion of each cell type is illustrated as percent of total cells or as (B) cells x 10^4/ml. Bars represent mean ± SD with n = 7 per group (data from 2 independent experiments with n = 3 and n = 4); unpaired t-test with *p ≤ 0.05 and ***p ≤ 0.0005.

6.1.2.3. CCL17 protein levels

Cell free BAL was used for chemokine measurement via ELISA. CCL17 is secreted by immune cells (dendritic cells, monocytes, $CD4^+$ T cells) and acts as a chemo-attractant recruiting mainly T cells of the Th2 type. Significantly higher CCL17 levels were observed in BAL from asthma mice (mean 101.20 ± 82.51 pg/ml) compared to healthy control and sensitized groups where CCL17 was undetectable (≤ 6 pg/ml; Figure 10).

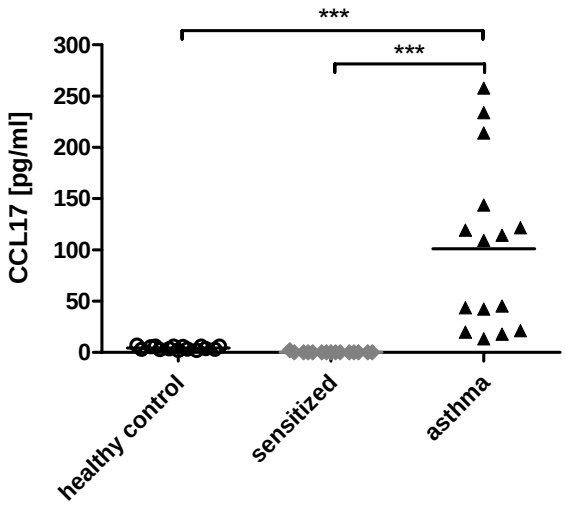


Figure 10: CCL17 protein levels in cell-free BAL in healthy control, sensitized and asthma mice. Cell-free fluid gained after BAL was assayed for CCL17 expression by ELISA. CCL17 levels of control, sensitized and asthma mice with n = 15 per group and each point representing one mouse (data from 3 independent experiments with n = 5). CCL17 levels of the asthma group were significantly higher with substantial variation within the asthma group when compared to controls and sensitized animals; unpaired t-test with ***$p \leq 0.0005$.

6.1.3. Histopathology

In order to gain insight into tissue changes after sensitization and challenge of mice with different treatments, histological analysis was undertaken. Samples were stained with Congo Red, a dye that intensively stains the cytoplasma of eosinophils. In Figure 8, microscopic pictures of lung tissue slices are shown with images 11A and 11B presenting healthy bronchial and vascular structures. In Figures 11C peri-vascular infiltration of cells is demonstrable. Higher magnification (Figure 11D) reveals enrichment of eosinophils with typically pink stained cytoplasm.

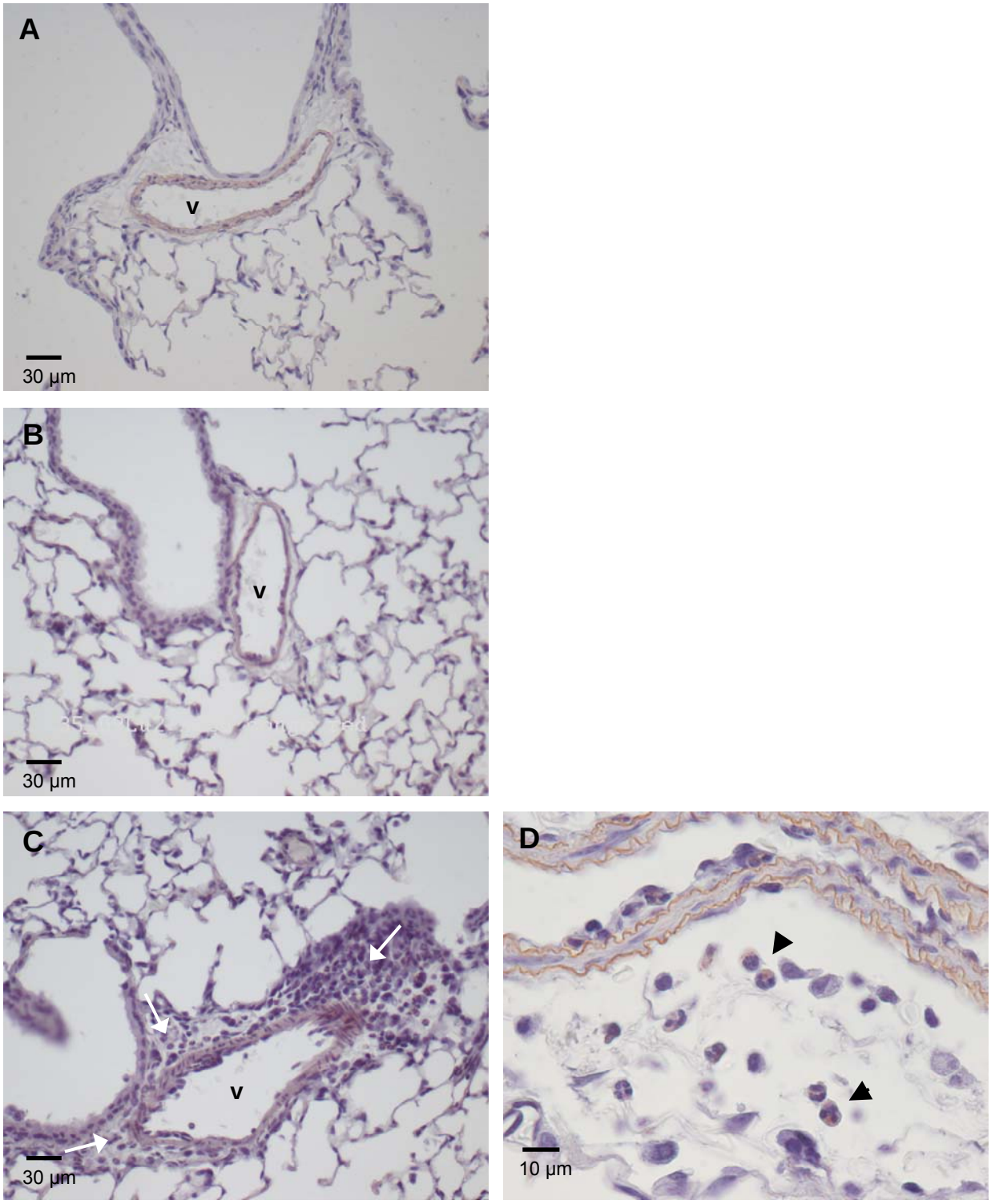


Figure 11: Histological evaluation of healthy control, sensitized and asthma lung tissue. After fixation lung lobes were prepared for morphological analysis from paraffin embedded samples. Staining was performed using Congo Red and pictures are taken in 20x magnification as representatives from groups of five mice per treatment. (A) healthy control (B) sensitized and (C) asthma lung tissue. In (D) a higher magnification of an asthma sample is shown (63x); white arrows show inflammatory infiltrate around vessels (v); black arrowheads point at eosinophils.

6.2. miRNA profiling of lung tissue

After confirming the successful development of an asthma-like allergic inflammation in the chosen mouse model, miRNA profiles were generated. Isolated lung RNA from three animals per group was pooled for analysis by microarray (Exiqon) and TaqMan® array. Afterwards, single samples were examined by miRNA specific RT-qPCR reactions. Differences between the groups were compared to identify candidate miRNAs dysregulated in asthmatic disease.

6.2.1. miRNA microarray (Exiqon)

To identify dysregulated miRNAs in asthma compared to control, an array based expression profile was performed. For comparison, miRNAs that are changed in sensitized versus control animals were also examined. Expression of 580 miRNAs (miRBase release 10.0) in whole lung tissue was investigated with a dual colour hybridization array (miRCury LNA^{TM} microRNA Array, Exiqon). Differential expression of miRNAs was identified using a linear model approach (limma, Bioconductor analysis suite). Bioinformatical data analysis included background correction as well as within-, and between-array normalization. Every miRNA is spotted in quadruplicates on an array and additionally each sample was probed on two arrays including a colour-swop (array 1: control sample Hy3^{TM}/asthma sample Hy5^{TM}; array 2: control sample Hy5^{TM}/asthma sample Hy3^{TM}) which in total leads to eight repeated measurements per miRNA. Data was further normalized to a constitutively expressed small nuclear RNA (RNU6B) and fold changes relative to the control samples were calculated. An adjusted *p*-value was built after correction for multiple testing. Significance was analyzed after Bonferroni correction. From 580 miRNAs included on the array, expression of 55 % was detected in lung tissue. To confine the resulting miRNA profile, only miRNAs with adjusted *p*-values under 10^{-4} were taken into account.

The comparison of asthma versus control animals revealed a signature of 36 statistically significant miRNAs of which five showed a fold change above 1.5 (Table 1). Additionally, two miRNAs with higher adjusted *p*-values but high fold changes were included (miRNA-193b and miRNA-205). In the sensitized versus control situation, a number of 11 miRNAs showed significant differences (Table 2) and of these, seven showed fold changes above 1.5.
When examining miRNAs of the two data sets one can find four miRNAs that are exclusively changed in the sensitized (atopic) state (miRNA-1, -133a, -133b and miRNA-665), whereas 15 miRNAs show dysregulation in asthma airways only (see Table 1, underlined). The overlap between the two data sets for sensitized and asthma revealed five miRNAs that

characterize changes seen in both conditions. These are miRNAs-126-5p, -144, -193a-5p, -208 and miRNA-451 (see Table 1 and 2 in grey). Notably, the coding regions of miRNA-144 and -451 are overlapping (clustered) and thus similar expression patterns can possibly be attributed to shared transcription.

Table 1: Differentially regulated miRNAs in lung tissue of asthma versus control mice evaluated by Exiqon microarray, TaqMan® miRNA array and single RT-qPCR. Dysregulated miRNAs in asthma compared to healthy control mice are depicted. Exiqon microarray gives statistical significance expressed by adjusted *p*-value (n = 3 per group; two experiments with reversed dyes; normalized to RNU6B). Verification was performed by TaqMan® microarray and fold changes (FC) are displayed (n = 3 per group; one experiment; normalized to snoRNA-202). In the right column, results of single RT-qPCR measurements of selected candidates are noted in FC (n = 3 per group; means of two independent experiments; normalized to snoRNA-234). FC above 1.5 are highlighted bold. Underlined miRNAs were found deregulated in asthma samples only (compared to healthy control); deregulated miRNAs in sensitized and asthma samples are highlighted in grey. Negative regulation is marked with a minus; n.d. not detected; / stands for miRNA not available on the array; n. a. stands for not analyzed; * stands for minor strand of miRNA

	Exiqon miRNA microarray		**TaqMan® miRNA array**	**TaqMan® RT-qPCR**
miRNA	**adj. p-value**	**FC**	**FC**	**FC**
hsa/mmu/rno-miRNA-451	1.47×10^{-14}	**1.97**	**1.90**	**1.98**
hsa/mmu/rno-miRNA-21	6.18×10^{-13}	**1.88**	1.40	**1.61**
hsa/mmu-miRNA-208/rno-miRNA-208	8.89×10^{-10}	**-1.57**	**-2.1**	-1.34
hsa/mmu/rno-miRNA-144	3.93×10^{-9}	**1.63**	/	**1.89**
hsa/rno-miRNA-126*/mmu-miRNA-126-5p	2.07×10^{-8}	-1.29	-1.16	n. a.
hsa142-5p/mmu/rno-miRNA-142-5p	2.84×10^{-7}	1.41	1.37	n. a.
hsa-miRNA-371-5p	4.98×10^{-7}	1.36	/	n. a.
hsa/mmu/rno-miRNA-30e*	1.12×10^{-6}	-1.31	-1.25	n. a.
hsa/mmu/rno-miRNA-125b-5p	5.31×10^{-6}	-1.20	-1.25	n. a.
hsa/mmu/rno-miRNA-365	5.31×10^{-6}	-1.37	-1.45	n. a.
hsa/mmu/rno-miRNA-125a-5p	1.0×10^{-5}	-1.23	**-1.82**	n. a.
hsa/mmu/rno-miRNA-142-3p	1.25×10^{-5}	1.40	1.46	1.36
hsa-miRNA-193a-5p	1.31×10^{-5}	1.35	/	n. a.
hsa/mmu/rno-miRNA-181a	1.34×10^{-5}	-1.37	-1.25	n. a.
hsa/mmu/rno-miRNA-100	1.73×10^{-5}	-1.27	-1.10	n. a.
Mmu-miRNA-199b*	2.73×10^{-5}	-1.32	**-1.69**	n. a.
hsa/mmu/rno-let-7b	4.58×10^{-5}	-1.26	-1.44	n. a.
hsa/mmu/rno-miRNA-145	4.73×10^{-5}	-1.18	-1.27	n. a.
hsa/mmu/rno-miRNA-101a	4.73×10^{-5}	-1.23	1.05	n. a.
mmu/rno-miRNA-21*	5.65×10^{-5}	1.37	**3.40**	n. a.

rno-miRNA-135a*	6.06 x10^{-5}	1.23	1.15	n. a.
hsa-miRNA-933	1.09 x10^{-4}	1.22	/	n. a.
hsa/mmu/rno-miRNA-24-2*	1.35 x10^{-4}	-1.28	-1.12	n. a.
mmu-miRNA-875-3p	1.35 x10^{-4}	1.16	n.d.	n. a.
hsa/mmu/rno-miRNA-26a	1.74 x10^{-4}	-1.24	-1.16	n. a.
hsa/mmu/rno-miRNA-193	2.01 x10^{-4}	-1.23	-1.18	n. a.
hsa/mmu/rno-miRNA-23b	3.21 x10^{-4}	-1.20	-1.20	n. a.
hsa/mmu-miRNA-668	3.47 x10^{-4}	1.22	n.d.	n. a.
mmu-miRNA-763	3.82 x10^{-4}	**1.70**	n.d.	1.12
hsa/mmu/rno-miRNA-30e	3.84 x10^{-4}	-1.18	-1.25	n. a.
mmu-miRNA-720	4.64 x10^{-4}	1.21	-1.35	n. a.
hsa-miRNA-519c-5p/-519b-5p/ -523*/ -518e*/ -522*/ -519a*	6.67 x10^{-4}	1.18	/	n. a.
mmu/rno-miRNA-290	6.73 x10^{-4}	1.22	**-2.60**	n. a.
hsa-miRNA-768-5p	6.80 x10^{-4}	-1.23	/	n. a.
hsa/mmu/rno-miRNA-152	7.21 x10^{-4}	-1.22	1	n. a.
hsa/mmu/rno-miRNA-29c	7.21 x10^{-4}	-1.18	1.1	n. a.
hsa/mmu/rno-miRNA-205	1.13 x10^{-3}	**1.61**	1.34	**2.43**
hsa/mmu-miRNA-193b	1.02 x10^{-2}	**2.73**	**-1.60**	-1.20

Table 2: Differentially expressed miRNAs in lung tissue of sensitized versus healthy control mice examined by Exiqon microarray and single TaqMan® RT-qPCR. Lung tissue RNA samples were profiled for dysregulated miRNAs in sensitized versus control mice using Exiqon microarray. miRNAs with statistical significance according to adjusted *p*-value are displayed (n = 3 per group; two experiments with reversed dyes; normalized to RNU6B) and FC are given. FC above 1.5 is highlighted bold. Underlined miRNAs were found in sensitized samples (compared to healthy control) only; deregulated miRNAs in sensitized and asthma samples are highlighted in grey. Validation of selected candidates by single TaqMan® RT-qPCR is shown in the right column (n = 3 per group; means of two independent experiments; normalized to snoRNA-234). Negative regulation is marked with a minus; n. a. not analyzed; * stands for minor strand of miRNA

	Exiqon miRNA microarray		**TaqMan® RT-qPCR**
miRNA	**adj. p-value**	**FC**	**FC**
hsa/mmu/rno-miRNA-451	1.68 x10^{-17}	**2.48**	**2,86**
hsa/mmu/rno-miRNA-144	7.51 x10^{-14}	**2.29**	**3,27**
hsa/mmu/rno-miRNA-208	9.94 x10^{-12}	**-1.75**	-1,36
mmu-miRNA-665	1.48 x10^{-11}	**2.22**	n. a.
hsa/mmu/rno-miRNA-133b	1.90 x10^{-11}	**-1.73**	n. a.
hsa/mmu-miRNA-1	4.31 x10^{-10}	**-1.58**	n. a.
hsa/mmu/rno-miRNA-133a	6.41 x10^{-9}	**-1.76**	n. a.
hsa-miRNA-193a-5p	1.34 x10^{-5}	1.36	n. a.
hsa/mmu/rno-miRNA-378	1.45 x10^{-5}	-1.46	n. a.

hsa/rno-miRNA-126*/mmu-miRNA-126-5p	1.54 x10^{-5}	-1.19	n. a.
mmu-miRNA-467e*	1.25 x10^{-4}	1.19	n. a.

6.2.2. Verification by miRNA TaqMan® array

In order to validate data from the Exiqon miRNA-microarray, a TaqMan® chemistry based array (TaqMan® microRNA Array, v2.0 miRNABase v.10.0) was performed using the same RNA samples as in the Exiqon array. On two 384-well plates, a number of 518 murine miRNAs can be profiled. After normalization to a small nucleolar RNA (snoRNA-202), differential expression of miRNAs in asthma versus control mice was calculated. Mean detection efficiency was 75 %.

In Table 1 (middle column), results from the TaqMan® array are matched to the Exiqon microarray. Fold change values of the 36 top miRNAs resulting from the Exiqon array correlate significantly (Spearman r = 0.62, ***p ≤ 0.0005) although six out of 36 miRNAs were not present on the TaqMan® array mainly because of strict limitation to murine sequences.

From the five miRNAs characterizing sensitization and asthma, three were validated with similar or higher fold changes (-126-5p, -208, -451) whereas the remaining two miRNAs were not available on the array (miRNA-144, -193a-5p). The signature for specific asthma-related miRNAs that comprised a number of 15 in the Exiqon array was validated for eight miRNAs with three showing augmented, and three having slightly lowered fold changes in the TaqMan® array (Table 1). Three miRNAs were not available on the array and four were not detected or revealed contradictory expression patterns.

6.2.3. Verification by miRNA TaqMan® RT-qPCR

As a third experiment for validating miRNA expression in asthma lung tissue compared to healthy control animals, specific miRNA RT-qPCR was undertaken using TaqMan® assays for single miRNAs selected from the achieved profile. Eight candidates were investigated and normalized to a small nucleolar RNA (snoRNA-234). The selection was based on the fold changes measured in the Exiqon array experiment which were also validated in the TaqMan® array. The three selected miRNAs demonstrating highest expression changes were miRNA-144, -208 and -451 for sensitization plus asthma and miRNA-21, -142-3p and -205 for asthma only. Two additional miRNAs, -193b and -763 were tested, that appeared clearly up-regulated in asthma versus control after Exiqon array with fold changes of 2.7 and 1.7 respectively, but which showed contradictory results in the TaqMan® array. Further analysis was done to examine the reliability of the two arrays. Expression levels of miRNA-193b showed down-regulation in both the TaqMan® array and the single PCR (-1.6 and -1.2-

fold change). This miRNA was included in the Exiqon array signature exhibiting the highest fold change measured (2.73-fold) but irrespective of a high *p*-value (above $x10^{-4}$). Due to these contradictory results, miRNA-193b was not investigated further. miRNA-763, selected based on *p*-value and high fold change (1.70-fold) in the Exiqon array was not detected in the TaqMan® array but could be analyzed by single RT-qPCR and asserted a 1.12-fold change which let to exclusion for further analysis.

As an overview, Figure 12 illustrates the expression of miRNAs investigated by the three methods. Six miRNAs could be validated with similar or intensified expression levels in asthma samples (miRNA-21, -142, -144, -205, -208 and miRNA-451). The three miRNAs that were similarly expressed in asthma and sensitized groups in the Exiqon microarray were tested, with miRNA-144 and -451 showing enhanced expression also in single RT-qPCR analysis (see table 3). Down-regulated expression of miRNA-208 can be seen in asthma and sensitized treatment although single RT-qPCR values were lower (-1.36 in sensitized and -1.34 in asthma respectively) than in the array analysis. Table 3 lists the expression of the eight selected and six validated miRNA candidates in asthma and sensitized lung tissue compared to the healthy control group.

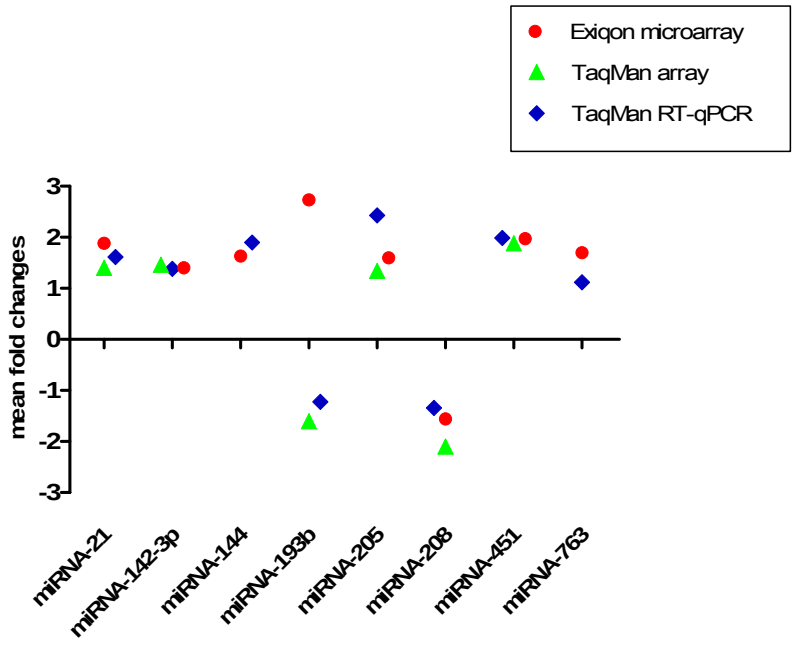


Figure 12: Differences in expression of eight miRNA candidates in lungs of asthma versus healthy control animals evaluated by three different methods. Isolated RNA samples from lungs of asthma and control mice underwent profiling for miRNA expression using Exiqon microarray (n = 3 per group; mean of two experiments with reversed dyes; normalized to RNU6B), TaqMan® array (n = 3 per group; one experiment; normalized to snoRNA-202) and single RT-qPCR (n = 3 per group; values describe mean of two experiments; normalized to snoRNA-234). Values are FC of asthma compared to healthy control samples with normalization to endogenously expressed small RNAs validated for each experiment, respectively.

Table 3: Overview of expression of eight selected miRNA candidates in asthma and sensitized lung tissue compared to healthy control. Results of the Exiqon microarray (n = 3 per group; mean of two experiments with reversed dyes; normalized to RNU6B) and validation by TaqMan® array (n = 3 per group; one experiment; normalized to snoRNA-202) and single RT-qPCR (n = 3 per group; values describe mean of two experiments; normalized to snoRNA-234). Values are FC. Negative regulation is marked with a minus; FC ≥ 1.5 is marked bold; n.a. not analyzed; n.s. not significantly changed; / stands for miRNA not available on the array; Chr stands for chromosomal location in mouse genome

Accession	miRNA	Asthma (Exiqon)	Asthma (TaqMan® array)	Asthma (RT-qPCR)	Sensitized (Exiqon)	Sensitized (RT-qPCR)	Chr
MI0001729	miRNA-451	**1.97**	**1.90**	**1.98**	**2.48**	**2.86**	11
MIMAT0000076	miRNA-21	**1.88**	1.40	**1.61**	n.s.	n.a.	11
MIMAT0000241	miRNA-208	**-1.57**	**-2.1**	-1.34	**-1.75**	-1.36	14
MIMAT0000436	miRNA-144	**1.63**	/	**1.89**	**2.29**	**3.27**	11
MIMAT0000434	miRNA-142-3p	1.40	1.46	1.36	n.s.	n.a.	11
MIMAT0003896	miRNA-763	**1.70**	n.d.	1.12	1.38	n.a.	10
MIMAT0000266	miRNA-205	**1.61**	1.34	**2.43**	n.s.	n.a.	1
MIMAT0002819	miRNA-193b	**2.73**	**-1.60**	-1.20	n.s.	n.a.	16

6.3. Target gene search

The profiling experiment revealed a number of miRNA candidates that were dysregulated in asthma mice compared to control animals. Although expression changes were reproducible for most miRNAs tested, fold changes stayed in a low range (highest expression 3.27-fold). As a consequence it was decided to include the top 100 miRNAs profiled by Exiqon microarray in “asthma versus healthy control mice” for a bioinformatical target gene search.

To elucidate the function of a miRNA, it is necessary to know the genes that are putative targets and thus can be regulated. Because of the imperfect complementarity that characterizes the binding between a miRNA and its target gene, different algorithms have been created that give a prediction for possible binding sites. Prediction criteria differ between algorithms, the most common ones are based on sequence complementarity

between miRNA seed region and 3´-UTR of the target mRNA, conservation of binding sites as well as number and thermodynamic stability of binding.

6.3.1. In silico prediction of target genes

Binding site prediction often leads to a high number of putative target genes for a miRNA. Figure 13 shows the number of predicted targets, depending on the algorithm chosen, for six validated miRNAs from the expression profiling in asthma lung tissue. To avoid high numbers of false positive target genes, a very strict, “full consensus approach”, based on five algorithms was applied. For bioinformatical analysis, four publically available algorithms (TargetScanS, miRNAanda, PITA and PicTar) as well as one newly created algorithm, “target spy”, were used (collaboration with PD Dr. Philip Pagel, Institute for Bioinformatics and Systems biology/MIPS, Helmholtz Zentrum München). The top 100 differentially expressed miRNAs from the Exiqon microarray (asthma versus healthy control) were included in the search and out of these, 33 families were built. miRNAs with identical seed regions (nucleotides 2-8 of mature sequence) were grouped into one family (mapping by TargetScan). By applying the strict “full consensus approach”, number of putative target genes could be diminished, for example for miRNA-144 from more than 490 or 2700 with single algorithms to 32 possible binding partners (Figure 13).

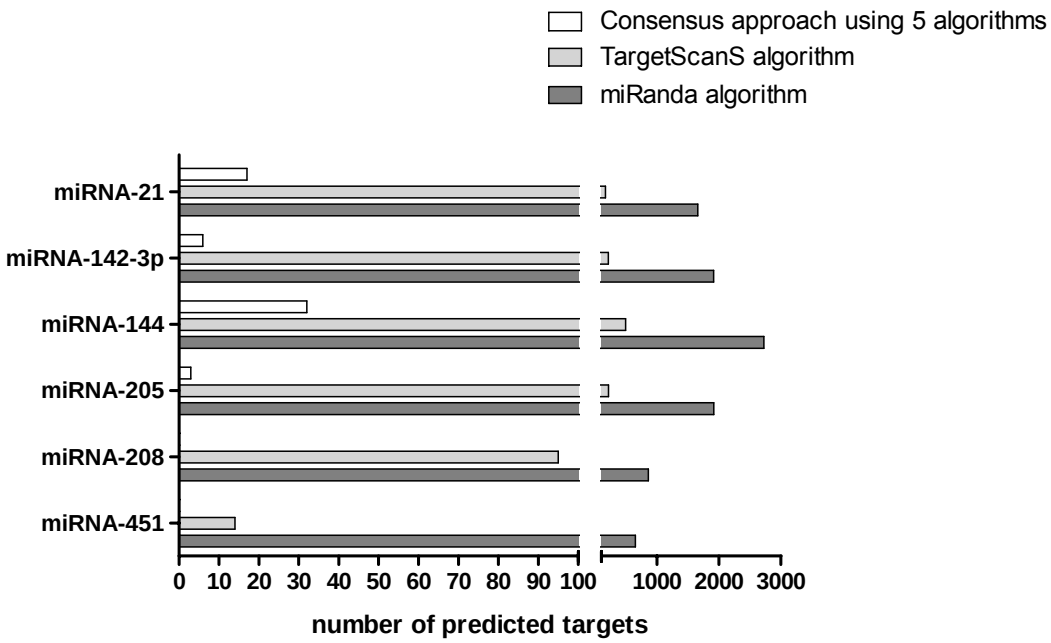


Figure 13: Differences in the number of predicted miRNA-targets according to used algorithms. For prediction of a putative binding between a miRNA and a target gene, different algorithms are available which consider aspects like complementarity, conservation or stability of the binding product. The number of target genes for the miRNAs-21, -142-3p, -144, -205, -208 and -451 (candidates from the profiling experiment) are shown either predicted by single algorithms (miRNAanda or TargetScanS) or by a full consensus approach considering five different algorithms (TargetScanS, miRNAanda, PITA, Target spy and pictar).

Target prediction analysis revealed 961 genes in total that are possible targets for miRNAs from the 33 families. Figure 14 depicts the number of target genes predicted for one miRNA or family of miRNAs. The miRNA-15/16/195/424/497 family shows the highest number of predicted genes with 149 hits whereas five miRNA families only have one or two hits.

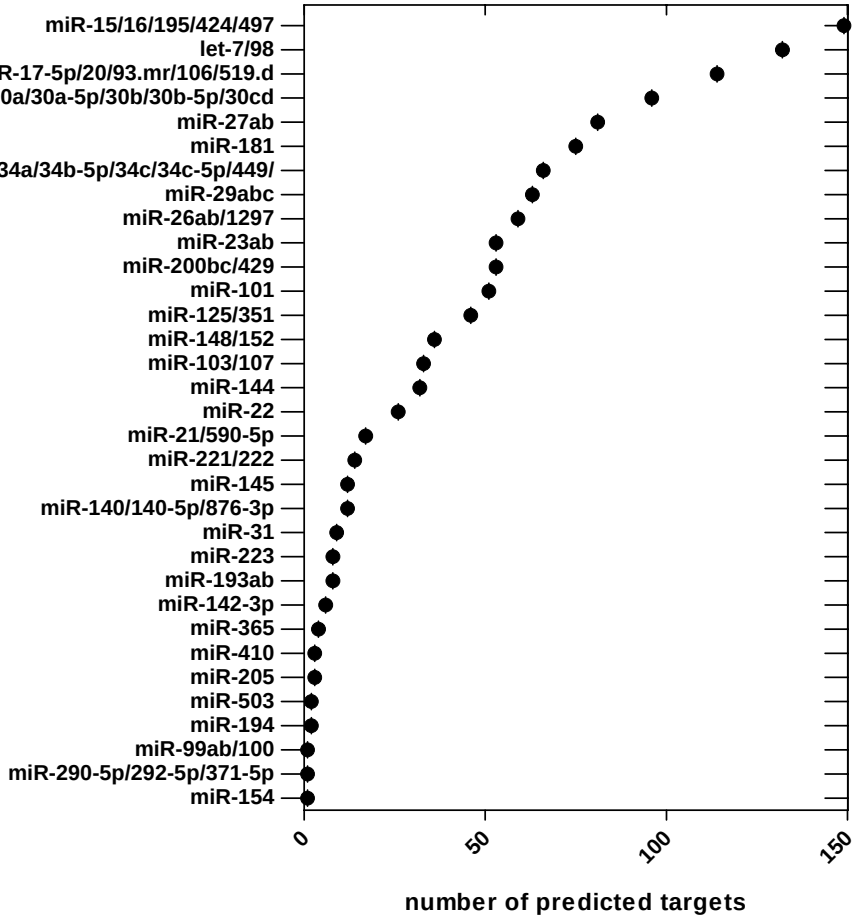


Figure 14: Number of predicted target genes for single miRNAs or miRNA families calculated by a full consensus approach of five algorithms. 100 miRNAs showing differential expression in asthma versus control lung samples analyzed by Exiqon microarray were grouped into 33 families according to identical seed regions (TargetScan). Putative gene binding sites were evaluated using five prediction algorithms (TargetScanS, miRNAanda, PITA, Target spy and pictar) and the resulting number of possible target genes per miRNA is depicted.

In most cases of the 961 targets identified, single genes offer binding sites for one or two miRNAs. Multiple binding of different miRNAs at the same gene might strengthen the regulatory effects. In Figure 15, the number of target genes that have either one or multiple binding sites, predicted by the “full consensus approach”, is indicated. Around 75 % of the identified target genes (723 of 961 in total) were predicted to have a single binding site for a miRNA, 20 % contain two sites, 4 % of target genes harbor three miRNA binding sites and a total number of 10 genes possess four sites. Only one target gene (*TNRC6B)* was predicted to have nine miRNA binding sites.

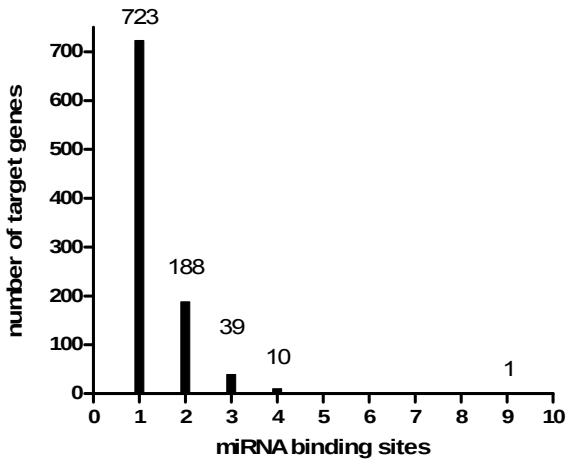


Figure 15: Number of target genes that harbor single or multiple binding sites for miRNAs. Applying the "full consensus approach" of five prediction algorithms led to 961 target genes for a total number of 100 miRNAs (summarized into 33 miRNA families). The number of miRNA binding sites on one gene are depicted with most target genes offering one or two binding sites for different miRNAs.

Of the predicted 961 target genes, 11 contain at least four miRNA binding sites. These targets were further investigated and are listed in Table 4 together with their binding miRNAs.

Table 4: Target genes with four or more binding sites and the corresponding miRNAs. Predicted miRNAs which harbor four or more binding sites for miRNAs are noted (abbreviation and name) as well as the miRNAs which are supposed to bind (by full consensus approach with five algorithms).

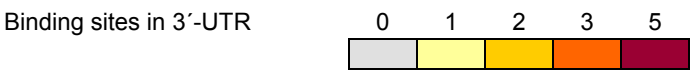
Target gene	Target gene name	Binding miRNAs
TNRC6B	Trinucleotide repeat-containing gene 6B protein	miRNA-15/16/195/424/497 miRNA-21/590-5p miRNA-22 miRNA-26ab/1297 miRNA-34a/34b-5p/34c/34c-5p/449/449abc/699 miRNA-101 miRNA-103/107 miRNA-148/152 miRNA-181
ACVR2A	Activin receptor type-2A	miRNA-15/16/195/424/497 miRNA-27ab miRNA-145 miRNA-223
BAZ2B	Bromodomain adjacent to zinc finger domain, 2B	miRNA-26ab/1297 miRNA-30a/30a-5p/30b/30b-5p/30cde/384-5p miRNA-181 miRNA-200bc/429

CREB1	cAMP responsive element binding protein 1	miRNA-17-5p/20/93/106/519d miRNA-22 miRNA-144 miRNA-181
ELAVL2	ELAV-like protein 2	miRNA-26ab/1297 miRNA-27ab miRNA-144 miRNA-200bc/429
HMGA2	High-mobility group AT-hook 2	let-7/98 miRNA-17-5p/20/93/106/519d miRNA-26ab/1297 miRNA-503
NFAT5	Nuclear factor of activated T-cells 5	miRNA-17-5p/20/93/106/519d miRNA-29abc miRNA-30a/30a-5p/30b/30b-5p/30cde/384-5p miRNA-181
SOCS6	Suppressor of cytokine signaling 6	miRNA-15/16/195/424/497 miRNA-17-5p/20/93/106/519d miRNA-23ab miRNA-27ab
SOX6	Transcription factor SOX-6	miRNA-15/16/195/424/497 miRNA-101 miRNA-181 miRNA-194
STYX	Serine/threonine/tyrosine-interacting protein	miRNA-15/16/195/424/497 miRNA-17-5p/20/93/106/519d miRNA-26ab/1297 miRNA-103/107
ZBTB39	Zinc finger and BTB domain-containing protein 39	miRNA-15/16/195/424/497 miRNA-27ab miRNA-30a/30a-5p/30b/30b-5p/30cde/384-5p miRNA-103/107

Besides different miRNAs being able to bind to one target gene at a time, single miRNAs can also bind at multiple locations within a given gene. The number of binding sites for a particular miRNA per target gene is presented in Table 5, concentrating on the genes with at least four binding sites for different miRNAs.

Table 5: Number of binding sites of one miRNA per gene. Genes which harbor four or more miRNA-binding sites are listed and the predicted miRNAs were searched for their binding frequency. Colours illustrate the number of binding sites for one miRNA in the 3´-UTR of a gene with grey = 0 sites, yellow = 1, orange = 2, dark orange = 3 and red = 5 sites. Binding site information is based on TargetScan and microRNA.org.

	TNRC6B	ACVR2A	BAZ2B	CREB1	ELAVL2	HMGA2	NFAT5	SOCS6	SOX6	STYX	ZBTB39
let-7/98											
miRNA-101											
miRNA-103/107											
miRNA-144											
miRNA-145											
miRNA-148/152											
miRNA-15/16/195/424/497											
miRNA-17-5p/20/93/106/519d											
miRNA-181											
miRNA-194											
miRNA-200bc/429											
miRNA-21/590-5p											
miRNA-22											
miRNA-223											
miRNA-23ab											
miRNA-26ab/1297											
miRNA-27ab											
miRNA-29abc											
miRNA-30a/30a-5p/30b/30b-											
miRNA-34a/34b-5p/34c/34c-											
miRNA-503											



In order to select which gene with its binding miRNAs should be investigated further, three aspects were taken into account. First, the total number of binding sites per gene, second, binding miRNAs that were found to be differentially regulated in allergic airway disease with a fold change above 1.5 (see Table 3 with selected candidates) and third, biological function of a gene with attention to asthmatic or inflammatory involvement.

As depicted in Table 5, *TNRC6B*, which is the gene with the highest number of different miRNAs predicted to attach, also contains the maximum number of binding sites in total (9 miRNAs binding at 14 sites). Of the remaining target genes, *HMGA2* has nine sites in total with the let-7/98 family adhering at five different locations within its 3´-UTR, miRNA-26ab/1297 family binding three times and miRNA-17-5p family as well as miRNA-503 binding at one site respectively. *CREB1* possesses eight binding sites for four different miRNAs in total, with the miRNA-17-5p family attaching three times, miRNAs-181 and -22 two times and miRNA-144 binding once. Of the remaining eight genes, half have four miRNAs binding at five sites, whereas the other half are supposed to bind four miRNAs at four sites.

The second criteria is the comparison of the significantly dysregulated miRNAs from the asthma profile (chapter 6.2) which showed fold changes above 1.5 with the miRNAs that are predicted to target the selected genes (Table 5). The profiling experiment revealed six miRNA candidates that were tested and validated with three different methods. After analysis with five target prediction algorithms, two miRNAs (miRNA-208 and -451) had no overlap and could thus not be included further. For miRNA-142-3p and miRNA-205 a number of six and three target genes were identified, respectively. However, the number of binding miRNAs per gene was 3 or less which was not compatible with the criteria of having at least four binding sites (see Table 4). miRNA-21 and -144 were both found to be differentially expressed in asthma and had a total number of 17 and 32 predicted target genes, including some with more than 4 binding sites. miRNA-21 was predicted to bind to *TNRC6B* at two different sites whereas miRNA-144 binds *CREB1* and *ELAVL2* at one site each (Table 5).

Looking at the third criteria, the biological function of genes, it should be mentioned that all 11 candidate genes are involved in regulatory processes, for example the transcription factor *SOX6*, the zinc finger protein *ZBTB39* or the transcriptional regulator *HMGA2*. The gene which inhabits most binding sites for miRNAs, *TNRC6B,* plays a role in miRNA-dependent translational repression by interacting with Ago complexes and thus is part of a negative feedback loop of the miRNA machinery (Meister et al. 2005; Lazzaretti et al. 2009). Focussing on an association with asthma or immune regulation, three genes can be named: the transcription factors *CREB1* and *NFAT5* and *SOCS6* protein. *SOCS6* is reported to play a role in the negative regulation of T cell activation (Choi et al. 2009) but has also been described to stimulate cytokine signaling through interaction with other SOCS family members (Piessevaux et al. 2006). *NFAT5* regulates the response of immune cells to osmotic stress by induction of gene expression and stimulation of cytokine expression. Such conditions are typically found in lymphoid organs and at inflammatory sites (Go et al. 2004; Berga-Bolanos et al. 2010). The third gene of interest, *CREB1* is known to regulate diverse cellular responses including many immune-related mechanisms. Examples are transcriptional regulation of IL-2, 6 or IL-10, inhibition of nuclear factor (NF) κB or regulation of macrophages and T and B cells (Wen et al. 2010). Moreover, the transcription factor is brought in context with lung diseases, like asthma or chronic obstructive pulmonary disease (COPD) (Chiappara et al. 2007; Mroz et al. 2007).

With regard to the aspects listed above, the target gene *CREB1* was chosen for further analysis. Taken together, *CREB1* offers a high number of binding sites in total (eight sites for four different miRNAs), miRNA-144 is both validated to be differentially regulated in allergic airway disease and predicted to bind *CREB1* and associations of *CREB1* with asthma have been previously reported.

6.3.2. Target gene *CREB1*

CREB1 is encoded on mouse chromosome 1 (human chromosome 2) and consists of nine exons. Alternate splicing results in several transcript variants encoding different isoforms (seven for mouse, two for human with identical 3´-UTR).

To study a possible interaction between the identified miRNAs in allergic lung inflammation and the 3´-UTR of *CREB1*, the next experiments were performed to test binding and interaction *in vitro* and besides, to evaluate expression levels in lung tissue of asthma and healthy mice. Based on the "full consensus approach", *CREB1* is predicted to contain binding sites for four miRNAs: miRNA-17, -22, -144 and miRNA-181a. The number, location and complementarity of binding sites for each miRNA is depicted in Figure 16.

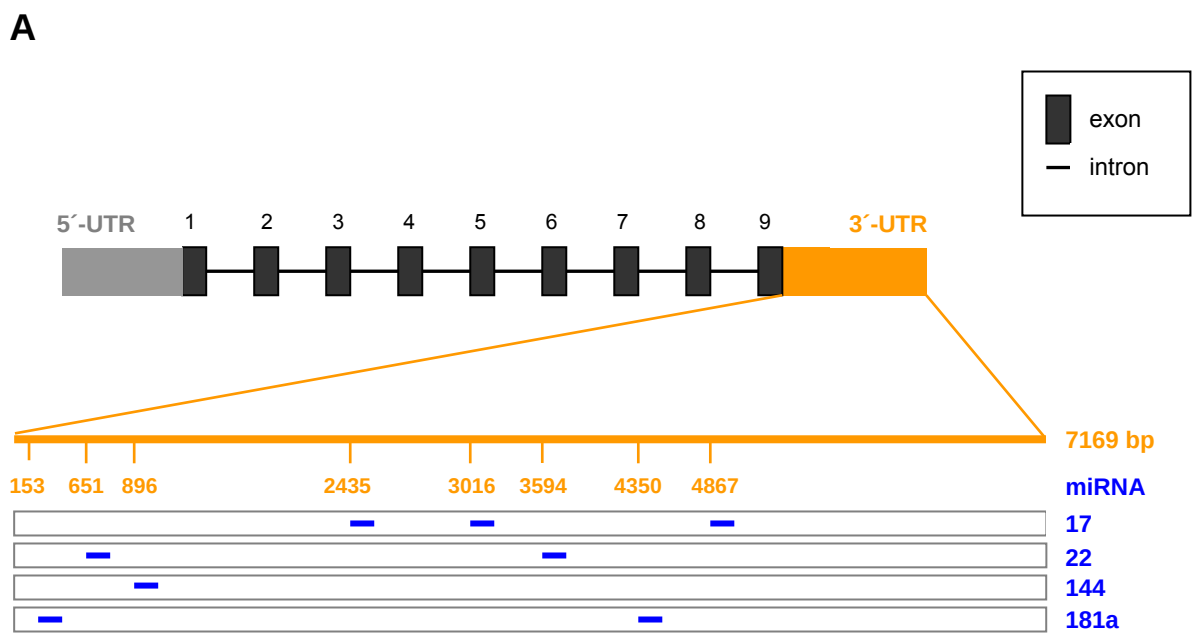


Figure 16: see next page

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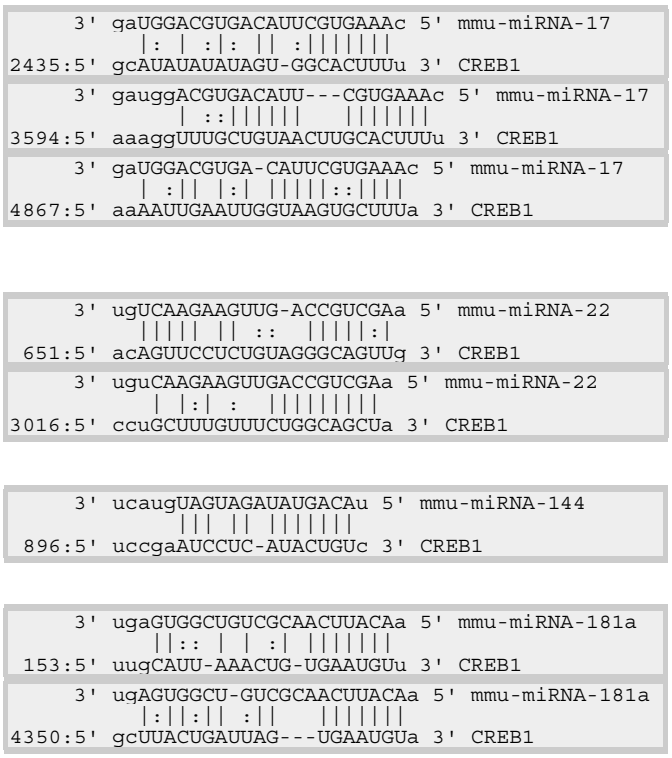

Figure 16: 3´-UTR of *CREB1* as target region for four miRNAs with their specific binding. *CREB1* offers eight binding sites for four different miRNAs (-17, -22, -144 and -181a). (A) Binding sites within the 3´-UTR of *CREB1* gene are depicted with starting points referring to the 3´-UTR sequence (in orange). In blue, the different miRNAs are shown. (B) Specific binding between the ~20 nt sequence of the miRNAs and *CREB1* 3´-UTR is presented with miRNA-144 having one, miRNA-22 and -181a having two and miRNA-17 showing three sites for potential binding.

6.4. miRNA-target gene interaction *in vitro*

To test whether the prediction of *CREB1* being a target gene for miRNA-17, -22, -144 and -181a is valid, in *vitro* experiments in human bronchial epithelial cells were undertaken. Firstly, a reporter gene assay was performed as indirect proof of binding, and secondly, the effect of increased miRNA levels on target gene expression was imitated by transfection of precursor miRNAs into cells followed by quantification of *CREB1* mRNA.

6.4.1. Co-transfection of *CREB1* 3´-UTR-vector construct and pre-miRNA-17, -22, -144 and -181a into human bronchial epithelial cells

Two human bronchial epithelial cell lines, BEAS-2B and 16 HBE-14o- were used for transfection experiments with reporter plasmid and miRNA precursors as described in section 5.6.

Figure 17 displays the ratio of *Renilla* to *Firefly* luciferase in both cell lines with the investigated pre-miRNAs. Significant reduction in the *Renilla/Firefly* ratio was observed after treatment with each of the four miRNAs in both cell lines. In BEAS-2B, this decrease was more than 40 % for miRNA-17 and -22 as well as 39 % for miRNA-181a and 25 % for miRNA-144 respectively (Figure 17A). In 16 HBE-14o- cells, luciferase signal was lowered by more than 55 % for miRNA-17 and -22 as well as 52 % for miRNA-181 and 46 % for miRNA-144 respectively (Figure 17B). In all experiments, total protein levels were measured by Bradford assay as supplementary control.

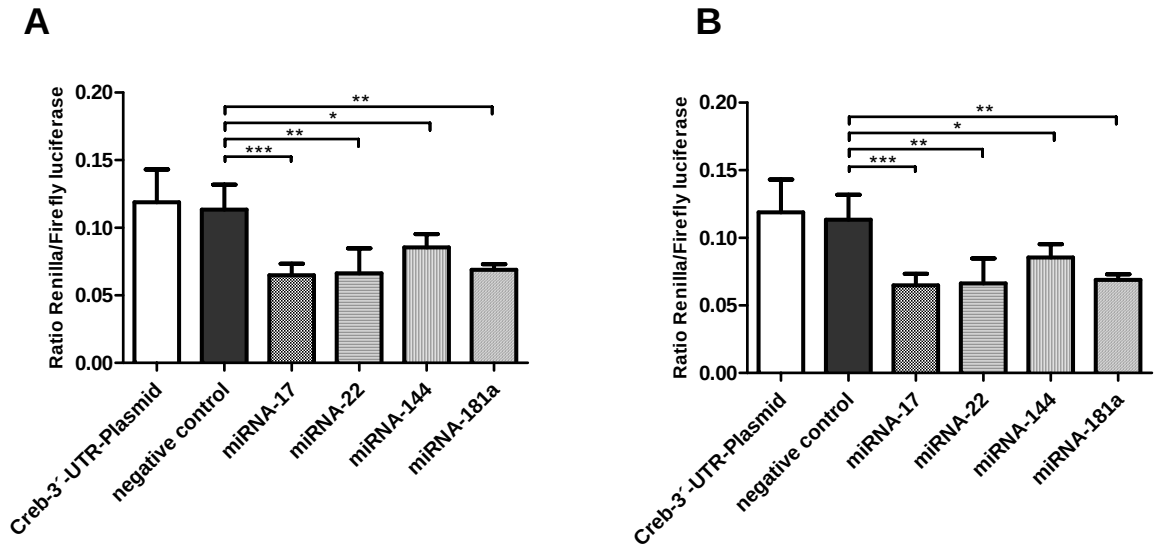

Figure 17: Reporter assay for testing the binding of four miRNAs to *CREB1*-3´-UTR in human bronchial epithelial cells. Pre-miRNAs-17, 22, 144 and 181a were co-transfected at a concentration of 20 pmol/well together with a plasmid-vector-construct containing the *CREB1*-3´-UTR binding site and two luciferase reporters. *Renilla* and *Firefly* luciferase signal was measured in cell lysates 72 h after transfection. Experiment in (A) BEAS-2B and (B) 16 HBE-14o- cell lines. Bars represent means ± SD of two independent transfections with n ≥ 4; unpaired t-test with ***p ≤ 0.0005, **p ≤ 0.005 and *p ≤ 0.05.

6.4.2. Analysis of *CREB1* expression after transfection of pre-miRNA-17, -22, -144 and -181a in human bronchial epithelial cells

The performed reporter assay proved interaction between the transfected miRNAs and the binding sites in the 3´-UTR of the gene of interest. However, this does not ascertain whether an increase in miRNA is sufficient to have an impact on physiological gene expression. Therefore, in the next experiment, the effect of increased miRNA levels on endogenous *CREB1* expression was tested by transfection of precursor miRNA-17, -22, -144 or -181a and subsequent quantification of target gene expression.

To quantify the corresponding miRNA levels post transfection, RT-qPCR (TaqMan®, ABI) was performed for miRNA-17, -22, -144 and -181a as well as a stably expressed small RNA (RNU6B, see figure 18). Cells transfected with a negative control were used for normalization. Though equal amounts of pre-miRNAs were transfected, varying amounts were observed for different miRNAs. Increase in values varied from more than 800-fold with miRNA-181a to more than 3000-fold increase after miRNA-144 transfection. Untreated BEAS-2B cells had higher endogenous expression of miRNA-17 and -22 and to a lower degree miRNA-181a when normalized to a negative control.

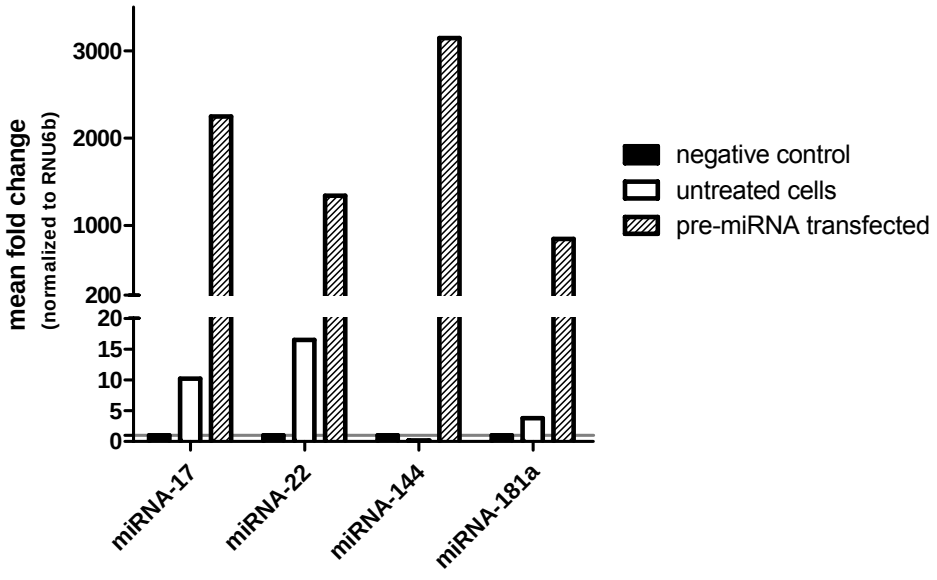

Figure 18: miRNA expression levels after pre-miRNA transfection into BEAS-2B cells. Cellular levels of miRNA-17, -22, -144 and -181a were increased using 20 pmol of pre-miRNA transfected with Lipofectamine 2000. RNA was isolated 72 h post transfection (miRNANeasy Mini Kit, Qiagen), single miRNAs evaluated by RT-qPCR normalized to RNU6B and compared to a negative control. miRNA levels of untreated cells were used as additional control. Bars represent mean fold change; grey line at 1.

To explore whether the miRNAs of interest are able to influence *CREB1* mRNA levels, gene expression was measured 72 h after transfection by real-time qPCR. Figure 19 illustrates significantly reduced *CREB1* mRNA levels after miRNA-17 and -22 transfections whereas no change was observed after transfection with miRNA-181. In cells transfected with miRNA-144, a trend of lowered *CREB1* levels is visible but is not significant.

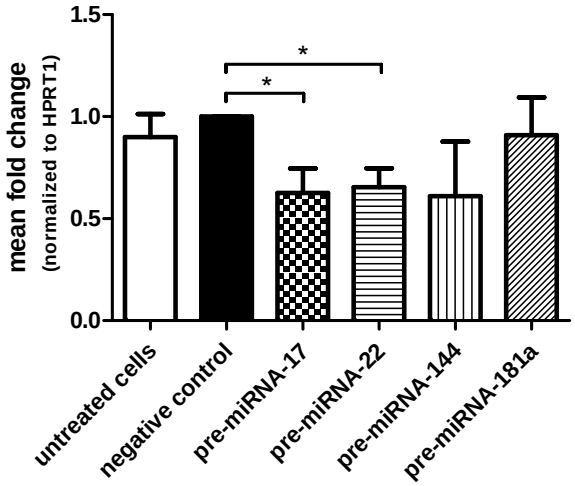

Figure 19: *CREB1* mRNA expression in BEAS-2B cells after transfection of miRNAs. Human bronchial epithelial BEAS-2B cells were transfected with 20 pmol of pre-miRNA-17, -22, -144 or -181a using Lipofectamine 2000. After 72 h post transfection, RNA was isolated and *CREB1* expression examined by RT-qPCR (normalized to *HPRT1*). Cells transfected with a negative control were used for comparison and *CREB1* gene expression in untreated cells was assayed as additional control. Bars represent mean ± SD of two independent experiments with each n = 4 wells/ transfection; unpaired t-test with ***p ≤ 0.0005, **p ≤ 0.005 and *p ≤ 0.05.

6.5. Quantification of *CREB1* and binding miRNAs in lung tissue

To further test relevance of *CREB1* and its four binding miRNAs in asthmatic conditions in this animal model, expression was quantified in lung tissues from healthy control, sensitized and asthma mice. The mice had been characterized with respect to the development of allergic asthma, OVA specific antibodies, BAL analysis and lung histology in section 6.1 (Figures 6-11). In accordance with bioinformatic prediction (Table 4 and 5) and *in vitro* transfection data presented above, highly significant down-regulation of *CREB1* in the asthma group (-1.36-fold) was observed. *CREB1* levels were unchanged in sensitized mice compared to their control counterparts (Figure 20).

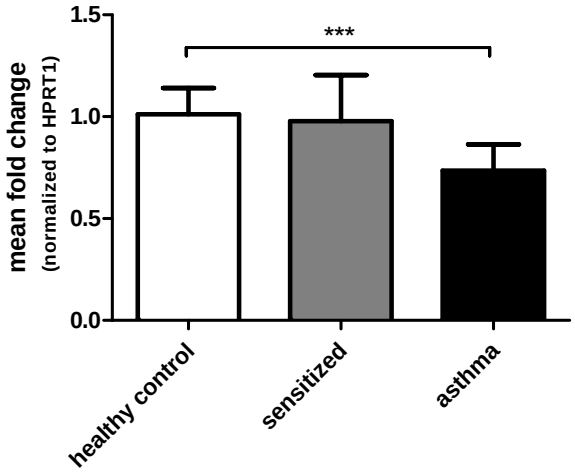

Figure 20: *CREB1* mRNA expression in lung tissue of healthy control, sensitized and asthma mice. RT-qPCR was performed to quantify expression levels of *CREB1* in lung tissue of different treatment groups. Mean expression of *CREB1* in healthy control, sensitized and asthma mice with n = 15 animals per group (data from 3 independent experiments with n = 5); bars represent mean ± SD normalized to *HPRT1* as endogenous control; unpaired t-test with ***p ≤ 0.0005.

As mRNA expression of *CREB1* was unchanged in the sensitized group, expression of miRNAs was further examined in the healthy control and asthma groups only. *CREB1* is predicted to contain binding sites for four miRNAs: miRNA-17, -22, -144 and miRNA-181a. Expression levels of these miRNAs are presented as Figure 21. In accordance with the Exiqon and TaqMan® array data, no differences were observed for miRNA-22 whereas significantly elevated levels of miRNA-144 could be measured in asthma-like murine lungs compared to controls (2.23-fold). miRNA-17 levels were also significantly elevated in asthma mice compared to controls (1.34-fold). Measured down-regulation of miRNA-181a in the two arrays was not reproduced by single RT-qPCR. As no significant difference could be observed for miRNA-22 and miRNA-181a, they were not investigated further.

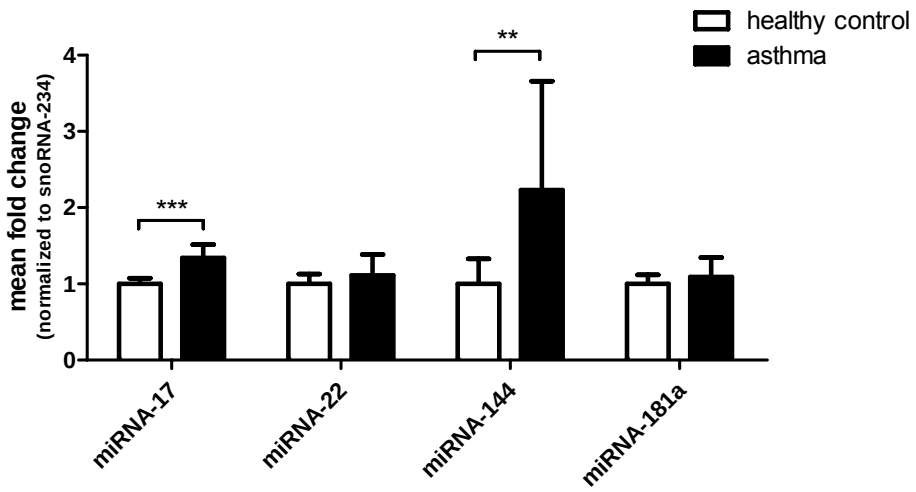

Figure 21: Relative expression of miRNA-17, -22, -144 and -181a in murine lung tissue of asthma and healthy control animals. Mean miRNA expression in RNA samples isolated from lung tissue was tested by RT-qPCR with n = 15 per group (data from 3 independent experiments with n = 5). Bars represent mean ± SD; unpaired t-test with **p ≤ 0.005 and ***p ≤ 0.0005

6.6. Kinetics of expression changes in murine lung during sensitization and after OVA challenge

In the interest of gaining more information about the dynamics of dysregulation in miRNA and target gene expression during the development of the asthma-like changes in the Balb/c OVA mouse model, different time points during the treatment protocol were evaluated. In the protocol depicted in Figure 22, a total of six different time points were selected for analysis. On four time points during the sensitization phase, mice were studied 24 h after i. p. treatment with OVA/alum or PBS/alum (days 15, 29, 43, 56) in order to evaluate whether miRNAs and/ or target gene respond to allergic treatment only. The two aerosol OVA challenges have been shown to lead to directed inflammatory changes in the lung of OVA sensitized animals (see 6.1). In addition to the acute time point analysed 24 h after the last challenge (d 72), a second time point, 120 h later (d 76), was investigated as well.

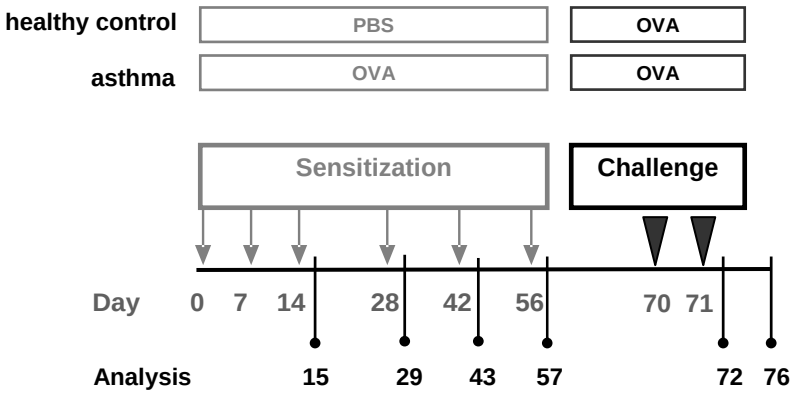

Figure 22: Treatment protocol of healthy control and asthma mice and analysis time points during sensitization and after challenge. Adult female Balb/c mice were treated with repeated i. p. sensitizations of PBS/alum or OVA/alum (1 µg in 200 µl total volume) followed by aerosol challenge with 1 % ovalbumin for 20 min on two consecutive days (day 70 and 71). Six analysis time points were studied, days 15, 29, 43 and 57 during sensitization and days 72 and 76 after challenge. The control and asthma treatment groups consisted of five mice per group for each time point.

6.6.1. BAL analysis

BAL total cell counts were uniform in all treatments on days 15, 29, 43 and 57 with a mean of 3.6 x 10^4 cells/ml BAL. After aerosol challenge with ovalbumin, control animals had consistent numbers of cells (2.8 x 10^4 cells/ml) whereas asthma animals showed markedly and significantly elevated numbers of cells in BAL with 15.1 x 10^4 cells/ml on day 72 and 112.2 x 10^4 cells/ml on day 76 (Figure 23).

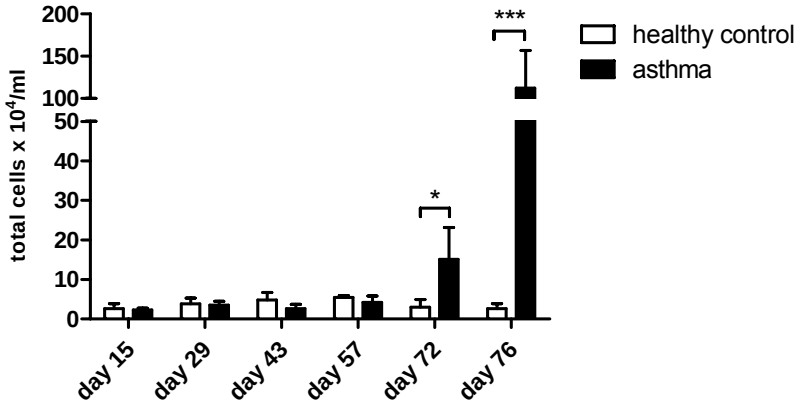

Figure 23: Kinetics of total cell counts in BAL of healthy control and asthma animals during sensitization and after challenge. BAL was performed by instillation of three times 0.8 ml of PBS into the lungs and subsequent analysis of the cellular fraction. Results for days 15, 29, 43 and 57 during sensitization and days 72 and 76 after challenge are depicted for control and asthma groups. Bars represent means ± SD; n=5 per group and time point; unpaired t-test with *p ≤ 0.05 and ***p ≤ 0.0005.

Due to high similarity with respect to total cell counts in BAL samples before ovalbumin challenge, differential cell counts are presented only for day 29 of the sensitization period (Figure 24A and B). Control values are shown as mean of control samples of day 29, 72 and 76 (macrophages 97.57 ± 0.40 %, neutrophils 0.97 ± 0.68 %, eosinophils 0 ± 0 %, lymphocytes 1.37 ± 0.29 %).

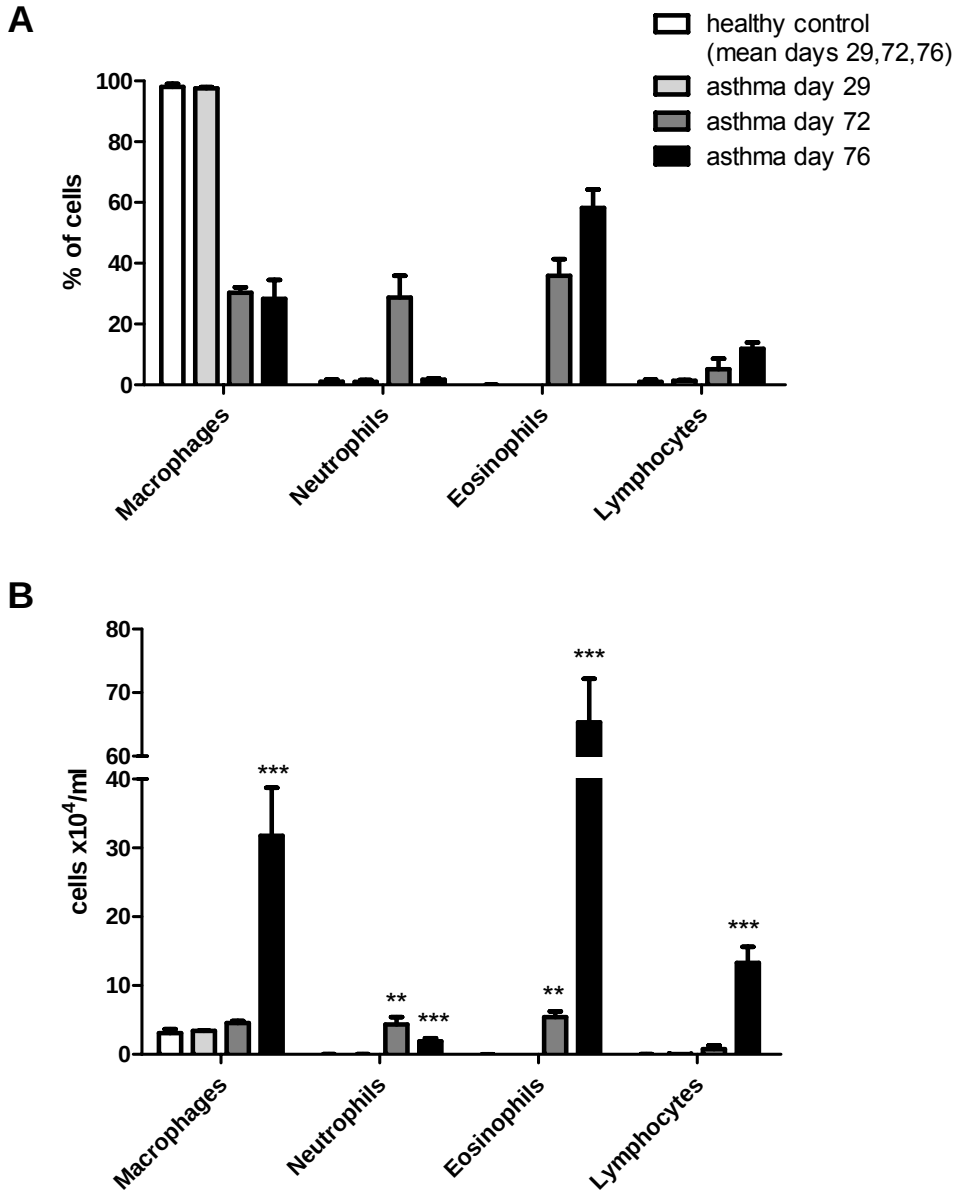

Figure 24: Differential cell counts in BAL of healthy control and asthma mice on day 29, 72 and 76. Cells from BAL fluid were analyzed after cytocentrifugation of a minimum of 10 000 cells per slide and staining with Diff-Quick. At least 500 cells per sample were distinguished into macrophages, neutrophils, eosinophils and lymphocytes. Values are presented as (A) percent of total cells and (B) cells x10^4/ml of control and asthma mice; mean ± SD of 5 animals per group and time point; unpaired t-test with **p ≤ 0.005 and ***p ≤ 0.0005; compared to the healthy group of each time point, respectively.

Cell type composition is shifted in asthma samples with an increase in eosinophils and lymphocytes. This change in cellular composition was clearly visible on day 72 and further intensified on day 76. Looking at Figure 24B, a strong increase in total numbers of macrophages, eosinophils and lymphocytes on day 76 is reflected.

6.6.2. miRNA-17, -21, -144 and -451 expression in lung tissue

The two significantly elevated miRNAs in lung tissue that bind to 3'-UTR of *CREB1*, miRNA-17 and -144, were investigated over time on four time points during sensitization and on two time points after aerosol challenge. In addition, the two miRNAs showing the highest changes in expression in asthma versus control animals from the profiling experiment, namely miRNA-21 and -451 were also included in the analysis (mean fold change with three different methods 1.63 and 1.95 respectively, see Table 3). Besides, miRNA-451 is encoded clustered with miRNA-144.

Levels of miRNA-17 remained unchanged during sensitization but increased 1.45-fold and 3.67-fold on day 72 and 76 respectively (Figure 25). miRNA-21 levels in ovalbumin sensitized animals resembled expression in controls but then increased 1.97-fold on day 72 and 4.86-fold on day 76. miRNA-144 levels were fluctuating before challenge, being elevated on day 15 (1.5-fold) but lowered on days 29, 43 and 57 (mean -1.5-fold) compared to control animals. On the contrary, expression was enhanced after challenge to 2.00-fold and 4.19-fold on days 72 and 76 respectively. Figure 25 shows the expression pattern of the clustered miRNA-451 with an increase at day 15 (1.96-fold) and a reduction on day 57 (-2.00-fold) compared to control mice. Challenge lead to 1.72-fold and 3.44-fold up-regulation.

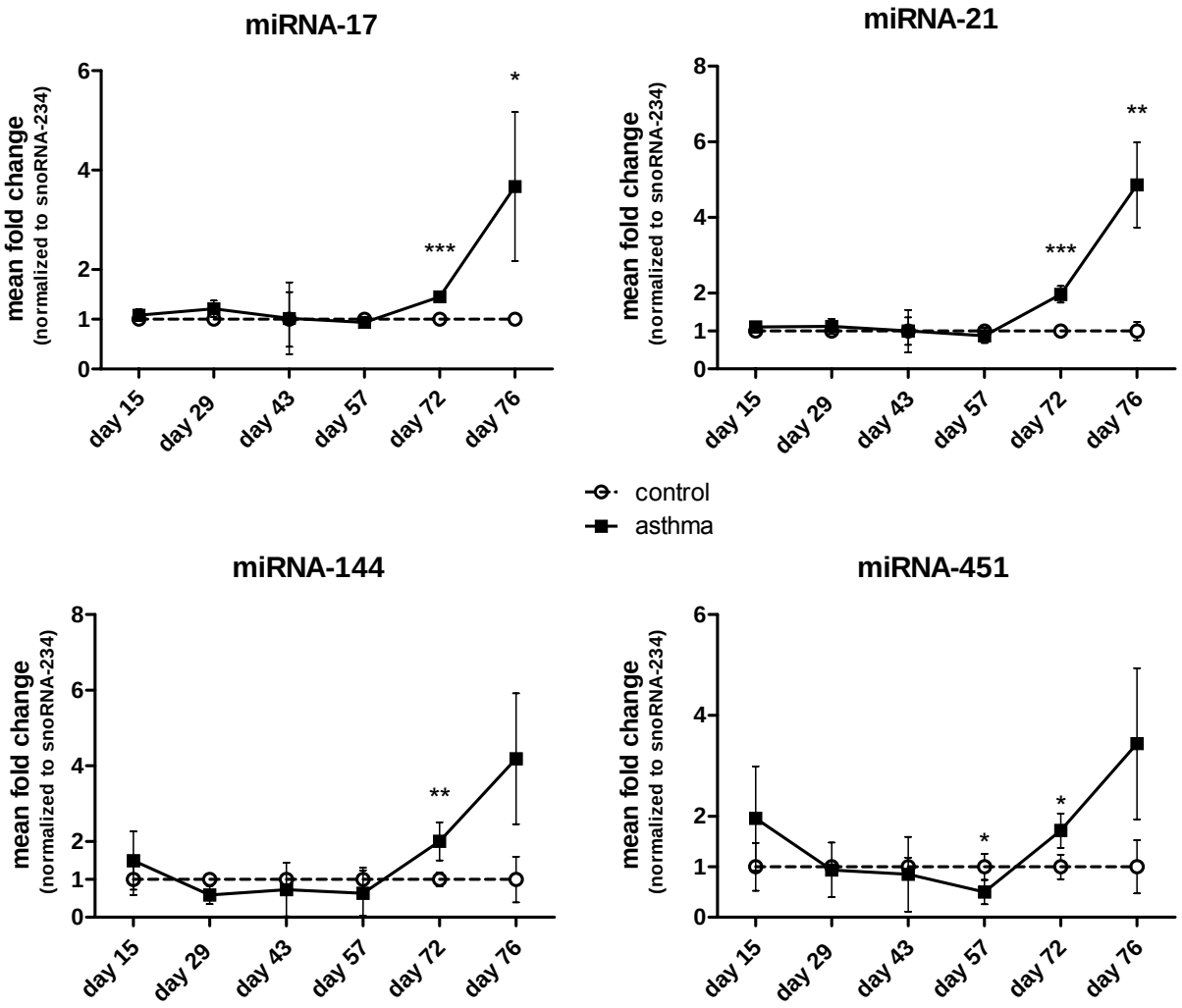

Figure 25: Expression pattern of miRNA-17, -21, -144 and -451 in lung tissue of healthy control and asthma mice during sensitization and after challenge. RNA including miRNAs was isolated from lung tissue on days 15, 29, 43, 57, 72 and 76 of the study protocol and RT-qPCR was performed. Expression of miRNA-17, -144, -21 and -451 was monitored, normalized to snoRNA-234 and the comparison of asthma versus control animals is shown. Points illustrate mean ± SD with n = 5 mice per group and time point; unpaired t-test with *p ≤ 0.05, **p ≤ 0.005 and ***p ≤ 0.0005.

6.6.3. *CREB1* mRNA expression

After exploring miRNA changes during the treatment protocol, *CREB1* mRNA levels in lung tissue were evaluated. Since expression of the binding miRNAs-17 and -144 was only slightly changed during sensitization phase, day 29 was chosen as a representative measurement for this period. mRNA analysis revealed no changes in asthma samples compared to controls after sensitization only (Figure 26). *CREB1* expression was significantly reduced after OVA challenge on days 72 (-1.30) and 76 (-1.35) in asthma mice.

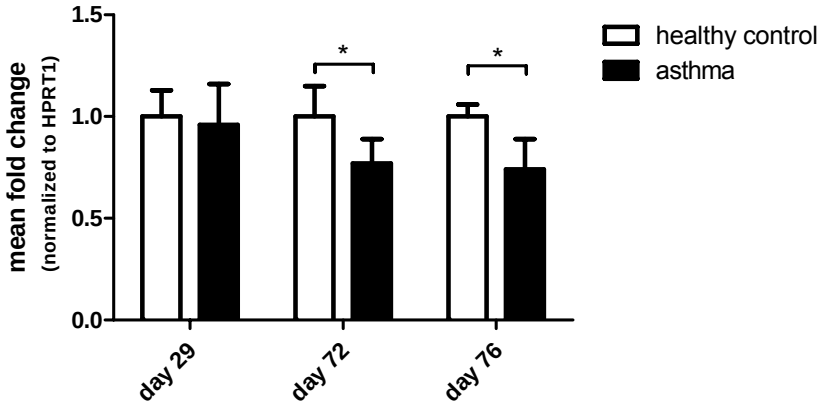

Figure 26: Expression of *CREB1* mRNA in lung tissue of healthy control and asthma mice on days 29, 72 and 76 of treatment. *CREB1* gene expression was analyzed at one time point before (day 29) and two time points (day 72 and day 76) after challenge with OVA. Mice of the asthma group are compared to control mice and data is normalized to *HPRT1* as control. Bars represent mean ± SD; n = 5 animals per group and time point.

6.7. miRNA expression in spleen and blood

Expression of miRNAs in lung tissue was followed through the sensitization phase and after OVA challenge in asthma and control mice. To gain information about possible changes in other immunologically important compartments, miRNA-17, -21, -144, and -451 expression was studied in spleen tissue and blood samples. For analysis, time points of day 29, 72 and 76 were tested.

6.7.1. Expression of selected miRNAs in murine spleen samples

miRNA expression in spleen remained mostly unchanged during sensitization, tested on day 29 (Figure 27). After aerosol challenge on days 70 and 71, a slight increase was noted on day 72 for miRNA-17 (1.30-fold), miRNA-144 (1.48-fold) and miRNA-451 (1.33-fold). Significant up-regulation was seen on day 76 for all four miRNAs with miRNA-144 being most prominent with 7.51-fold change, miRNA-451 being 3.0-fold increased and miRNAs-17 and -21 with 2.42, and 1.46-fold enhancement compared to controls.

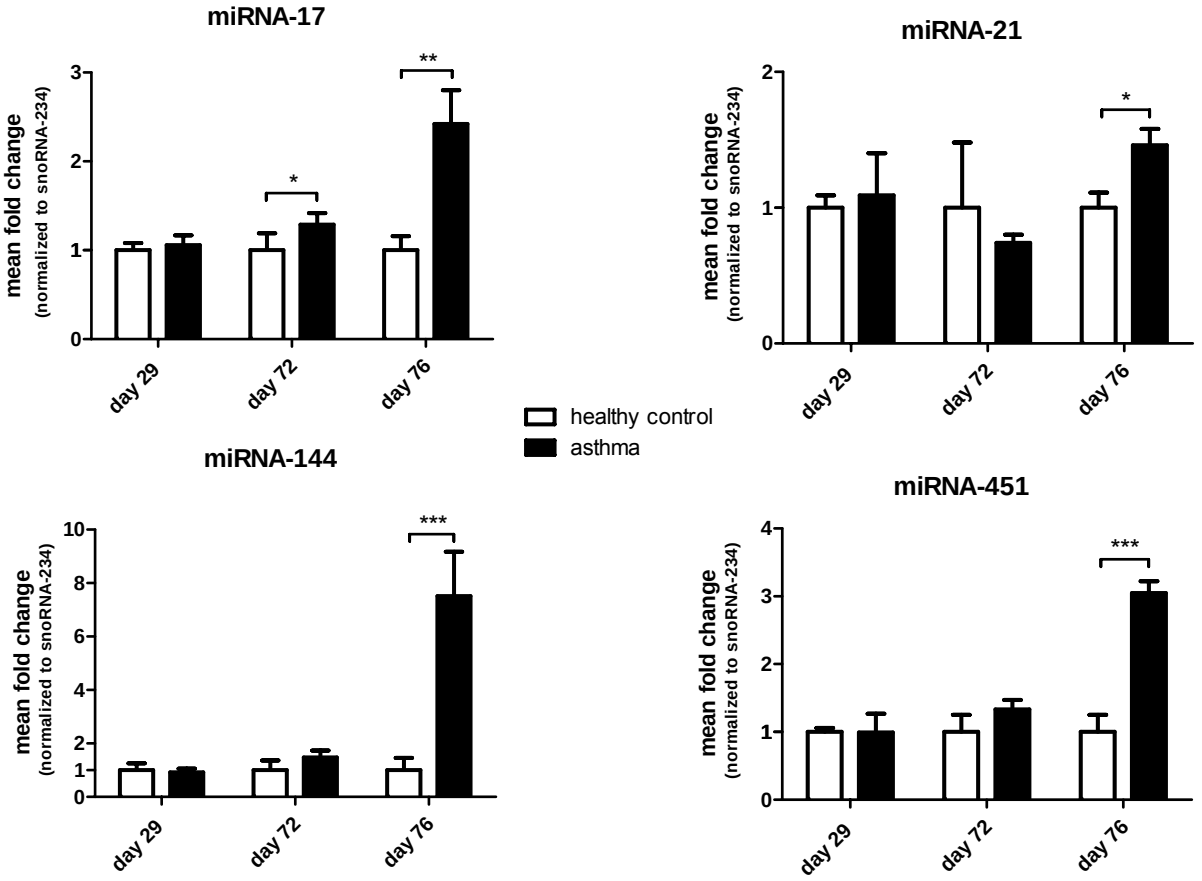

Figure 27: Expression of miRNA-17, -21, -144 and -451 in spleen tissue of healthy control and asthma mice. Spleen samples from days 29, 72 and 76 of control and asthma mice were investigated for miRNA expression after isolation of RNA (miRNANeasy Mini Kit, Qiagen). RT-qPCR was performed normalizing to snoRNA-234. (A) miRNA-17 (B) miRNA-22 (C) miRNA-144 and (D) miRNA-451. Bars represent mean ± SD with n = 5 mice per group and time point; unpaired t-test with ***p ≤ 0.0005, **p ≤ 0.005 and *p ≤ 0.05.

6.7.2. Expression of selected miRNAs in murine blood samples

Due to their great stability compared to other types of RNA, the study of miRNA levels in biofluids, like blood or serum, is profitable (Chen, X. et al. 2008). The perspective to use them as biomarkers for diagnosis or disease activity is attractive and has gained attention in the last years, mainly for malignant disease (Hu et al. 2010; Liu, X. G. et al. 2011). By analyzing expression of selected miRNAs in blood, the following information was supposed to be gained: first, are the observed miRNA changes in lung or spleen also visible in blood and, second, do differentially expressed miRNAs detected in blood of mice with an asthma phenotype resemble human blood samples of asthmatic children.

With regard to the most pronounced augmentation of miRNA expression in lung and spleen tissue on day 76, whole blood samples from this time point were evaluated. All four selected miRNAs (miRNA-17, -21, -144 and -451) showed reduced expression levels in asthma compared to control samples (Figure 28). Down-regulation was not significant for miRNA-17

showing a -1.79-fold change. Highest decreases could be observed for miRNAs-144 and -451 (each -2.70-fold), followed by miRNA-21 showing -2.63-fold reduction compared to control.

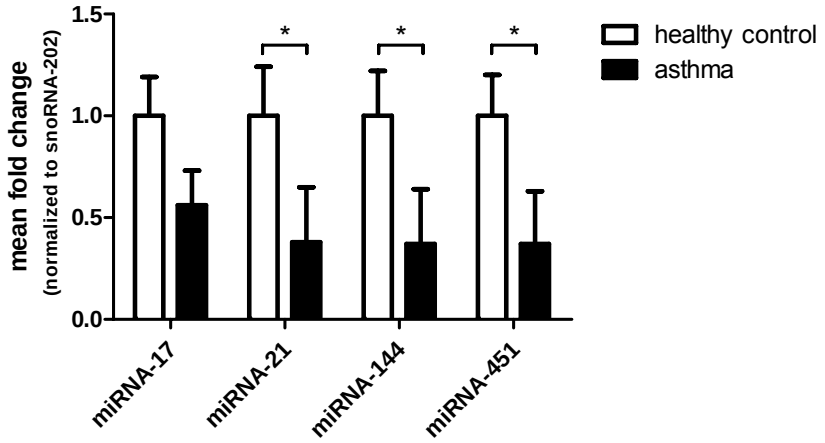

Figure 28: Expression of miRNA-17, -21, -144 and -451 in murine blood samples of healthy control and asthma groups. RNA including miRNAs was isolated (miRNANeasy Mini Kit, Qiagen) from whole blood of healthy control and asthma mice gained on day 76. For normalization snoRNA-202 was used and bars represent mean ± SD of asthma compared to control mice with n = 4 per group; unpaired t-test with *p ≤ 0.05.

6.7.3. Expression of selected miRNAs in human blood samples of asthmatic children

For direct comparison to the human situation, bio-banked blood samples of 10 year old children participating in a multicenter, double-blind, randomized intervention study (German Infant Nutrition Intervention study) were analyzed. Out of 8 children with diagnosed asthma, five had a record of atopic eczema at any time (four current) and four children were positive for rhinitis ever (Table 6).

Table 6: Patient characteristics. Peripheral blood samples of healthy and asthmatic children from a multicenter, double-blind, randomized intervention study (GINI) were analyzed. Characteristics of the selected groups are shown. f female; m male.

	Healthy children	Asthmatic children
N	8	8
Age (years)	10	10
Sex (f/m)	4/4	4/4
Asthma onset (mean age)	-	5.1 (1-8)
Atopy ever	0	8
Atopic eczema ever	0	5
Rhinitis ever	0	4

In Figure 29 miRNA expression in human blood samples from asthmatic and healthy children is depicted. Similar to observations made in murine blood, miRNAs-144 and -451 are significantly decreased in asthmatic samples with fold changes of -1.75 and -1.89 respectively. miRNA-17 exhibits only slight changes (-1.27-fold) and miRNA-21 shows no detectable differences.

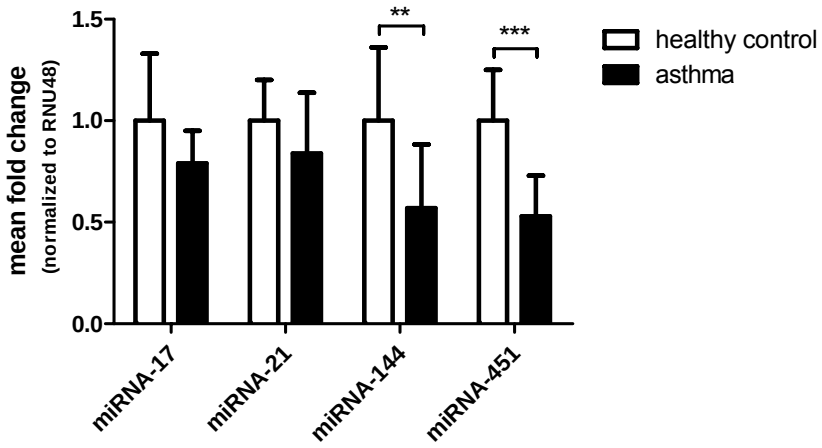

Figure 29: Expression of human miRNAs in blood of healthy and asthmatic children. RNA was isolated (miRNANeasy Mini Kit, Qiagen) from bio-banked blood samples of healthy and asthmatic children participating in the GINI study. The miRNAs-17, -21, -144 and -451 are conserved in human and mouse and were normalized to hsa RNU48. Bars represent mean ± SD with n = 8 children per group; unpaired t-test with ***p ≤ 0.0005 and **p ≤ 0.005.

7. DISCUSSION

7.1. The chosen Balb/c OVA model reflects a mild form of acute asthma

In the present project, mice of Balb/c background were subjected to treatment with the allergen OVA in order to mimic allergic asthma. The mouse strain has been widely used for the study of allergic immune reactions and is known for its susceptibility to mount prominent Th2 responses, high antigen-specific titres of IgE and elevated cytokine concentrations in BAL (Shinagawa and Kojima 2003; Zosky et al. 2009). Treatment protocol included a number of six i. p. injections of 1 µg OVA/alum, leading to allergen-specific immunity, followed by two pulmonary aerosol challenges. The applied i. p. dose of OVA is comparatively low as other studies applied doses between 10 and 50 µg. (Liu, X. et al. 2009; Swedin et al. 2010; Garbacki et al. 2011). The sensitization period of 70 days stands in contrast to other descriptions in literature, where protocols between 18 and 30 days were reported. However, a short, high dose allergen exposure only reflects very strong acute human asthma. The treatment used here, led to markedly increased allergen-specific titres of IgE and IgG1 and to an increase in total cells in BAL. The absolute numbers and percentages of cells, including macrophages, neutrophils, eosinophils and lymphocytes were in the lower range of comparable studies indicating a milder, yet evident form of acute airway disease (Kumar, R. K. et al. 2008; Liu, X. et al. 2009; Zosky et al. 2009; Swedin et al. 2010; Garbacki et al. 2011).

Several time points during sensitization and after challenge were investigated and inflammatory cell numbers in BAL were further increased at 120 h compared to 24 h after the last challenge. This rise is due to higher numbers of macrophages (7-fold increase), eosinophils (12-fold increase) and lymphocytes (18-fold increase). A study analyzing BAL cells 24 and 48 h after allergen challenge confirms augmentation in cell numbers at the later time point (Swedin et al. 2010).

Eosinophilic inflammation is one important hallmark of allergic asthmatic disease which was also evident in our histological analysis. Compared to BAL samples from asthmatic patients, levels of eosinophils are exaggerated in mice, representing up to 80% of the inflammatory cells in BAL (Hartl et al. 2005; Mattes et al. 2009; Garbacki et al. 2011). Moreover,

histopathology revealed more perivascular than peribronchial infiltrates of inflammatory cells in mice which is not the case in lungs of asthmatic patients (Cohn 2001).

The Balb/c OVA model used in our studies has been established previously by our collaborators who had already demonstrated increased AHR and mucus hyper-secretion as well as enhanced IL-5 and IL-13 levels in BAL (Alessandrini et al. 2009). Nonetheless, aspects of chronic disease, such as recurrent episodes of inflammation or airway remodeling are not mirrored in this model adding to the general limitations of any asthma mouse model such as the absence of spontaneous disease, differences in airway anatomy, the transient nature of AHR after induction of asthma as well as mechanistic differences in the generation of allergic asthma (Epstein 2004).

7.2. Pulmonary miRNA expression is changed in experimental asthma

In the last years, numerous miRNA profiling studies have been performed to enlarge the understanding of regulatory mechanisms in specific organs, tissues or cell types. In the first part of this project, a miRNA signature characteristic for changes in lung tissue of Balb/c mice treated according to our protocol was established. Most profiling approaches are undertaken using either microarray technology (Lu, Z. et al. 2008; Mattes et al. 2009; Garbacki et al. 2011) or, to a lesser extent, RT-qPCR based signatures (Williams, A. E. et al. 2007; Williams, A. E. et al. 2009). In the present study, three different methods were used, an Exiqon microarray for the initial profile, a TaqMan® array for a validating screen and third, single TaqMan® RT-qPCR for a smaller number of selected miRNAs. The second profiling experiment was aimed at broadly validating the identified signature, which was evidenced by a positive correlation.

The Exiqon array version used (v.10) offered 580 miRNAs for profiling of which ~55 %, a number of 319 miRNAs, were expressed in lung tissue. One study measured 256 miRNAs in whole lung tissue of healthy Balb/c mice (Williams, A. E. et al. 2007). Comparison of the miRNAs with our assessed profile of 36 significantly changed candidates from the Balb/c OVA model shows an overlap of 24 miRNAs. This indicates that the assessment of pulmonary miRNA profiles by different investigators yields comparable results. Furthermore, a relatively high number of miRNAs seem to be abundantly present, whereas only a smaller proportion of miRNAs are induced de novo after experimental induction of allergic asthma. In 2007, Landgraf et al. set up an expression atlas of miRNAs in different tissues and species

applying next generation sequencing technique. It became clear that only few miRNAs are found exclusively in a single tissue or cell type. This result was replicated in another study where the inquiry for specific lung miRNAs in rats revealed only two miRNAs that were not found to be expressed in any other organ tested (Wang, Y. et al. 2007).

The number of up- versus down-regulated miRNAs in our profiling experiment is almost balanced (16 up-regulated, 20 down-regulated). This is also reported in other asthma profiling studies (Polikepahad et al. 2010; Garbacki et al. 2011). In contrast, measurements in lung tissue after aerosolised lipopolisaccharide (LPS) (Moschos et al. 2007) or diesel exhaust particle inhalation (Jardim et al. 2009) showed overall up-regulation of miRNAs. On the other hand, studies of cigarette smoke exposure in lungs of rats and humans found miRNAs to be almost exclusively down-regulated (Izzotti et al. 2009; Schembri et al. 2009). Thus, direction of miRNA regulation in the lung may be dependent on the type of provocation. As a consequence, application of different allergens for the induction of airway disease in mice might lead to different results and needs confirmation in various models.

In the last two years, six reports published lung miRNA profiles in asthma mouse models (Lu, Z. et al. 2008; Mattes et al. 2009; Polikepahad et al. 2010; Collison et al. 2011a; Collison et al. 2011b; Garbacki et al. 2011). One further study analyzed airway biopsies from asthmatic patients (Williams, A. E. et al. 2009). The later did not find any differences in levels of miRNAs expressed in patients suffering from mild asthma compared to healthy donors. The authors suggest that this may be due to the mild form of disease. Moreover, variation in expression between individuals might have hindered the exploration of changed miRNAs. Nevertheless, they described expression of miRNAs in lung tissue with high abundance, like members of the let-7 family, miRNA-23b, -24, -26, -30 and -125. This confirmed findings from previous studies, which were performed mainly in murine samples (Landgraf, Pablo et al. 2007a; Williams, A. E. et al. 2007). Notably, the named miRNAs were also present in our OVA model with some showing expression changes in the asthma phenotype. When comparing our miRNA profile with the results gained in the six studies, a total number of 16 miRNAs are overlapping. From these, eight miRNAs are regulated in a similar manner: miRNAs-29c, 101a, 142-3p, 142-5p and 720 were each found in two other studies whereas miRNAs-152, 208 and 451 each appeared in one other study.
However, five miRNAs showed contradictory expression changes. Up-regulation of miRNA-145 was replicated in two OVA and one house-dust mite model for asthma (Collison et al. 2011a; Collison et al. 2011b; Garbacki et al. 2011) whereas it was found down-regulated in our signature. Notably, inhibition of miRNA-145 by Collison and coworkers led to diminished eosinophilic inflammation, mucus hypersecretion, Th2 cytokine production and AHR. These

effects were comparable to steroid treatment which makes this miRNA a new candidate for further research. In the very same study, let-7b, was up-regulated, yet again levels appeared reduced in our profile. These contradictory results might be due to the use of different allergens and treatment protocols, like house-dust mite versus ovalbumin treatment.
Two other recent studies concentrated on the role of let-7 in allergic asthma. In 2010, Polikepahad et al. stated a proinflammatory role of let-7 miRNAs in experimental asthma, whereas another group (Kumar, M. et al. 2011) recently published incompatible findings. They showed decreased levels of let-7 miRNAs in disease and detected reduction of asthmatic features after administration of let-7 miRNA mimics *in vivo* by repression of its target gene IL-13.
Looking at the performed array, several members of the let-7 family have been detected but did not reach significance according to *p*-values. All let-7 members show a moderate decrease in asthma compared to control samples. Members of the let-7 family have also been found down-regulated in other chronic lung diseases, like fibrosis, cancer or after cigarette smoke exposure (Takamizawa et al. 2004; Izzotti et al. 2009; Pandit et al. 2010).

One of the best-studied miRNAs up to date, miRNA-21, was up-regulated in our profiling experiment, which was confirmed in three other asthma studies (Lu, T. X. et al. 2009; Mattes et al. 2009), but expression decreased in one OVA-model (Garbacki et al. 2011). Just as miRNA-21, miRNA-126 appeared in four out of six other studies with two showing up-regulation (Mattes et al. 2009; Collison et al. 2011a) and two showing down-regulation as here (Lu, T. X. et al. 2009; Garbacki et al. 2011). This miRNA was extensively studied by Mattes et al. in a house-dust mite model where they could repress features of the asthmatic phenotype by selective inhibition of miRNA-126 using inhalative “antagomiRs”. In addition, blocking the miRNA led to a decrease in *GATA3* expression and Th2 cell function. The group followed miRNA-126 in a second, chronic OVA asthma model where they could confirm the increase of miRNA-126 predominantly in early phases of asthmatic lesions in the airways (Collison et al. 2011a). miRNA-126 has also been associated with various other lung diseases, such as idiopathic pulmonary fibrosis, cystic fibrosis or cancer, which points to a possible role in common mechanisms (Oglesby et al. 2010; Pandit et al. 2010; Miko et al. 2011).

Next to the 16 miRNAs that have been detected in other studies as well, 20 miRNAs have not been monitored in other murine asthma studies before. Notably, seven “minor sequence” miRNAs, marked with a star, were described (miR-21*, -24-2*, -30e*, -135a*, -199b*, -522* and -523*). The minor sequence is defined as the strand which is less likely incorporated in the RISC complex and thus is less abundant. Some arrays do not offer more rare sequences

which might be a reason why they were not detected so far. Thereby, some miRNAs are present with both strands, major and minor at the same time, like miRNA-21 and -21* and miRNA-30e and -30e*. This raises the question why and how these miRNA strands are expressed in parallel as it is believed that after selection of one strand, the other, in general the minor strand, gets degraded. However, one paper describes cooperative and dynamic cooperation of miRNA-155 and its minor strand partner in the regulation of type I interferon production in dendritic cells (Zhou, H. et al. 2010).

Of the 20 miRNAs which were not previously reported in asthma, five have been associated with pulmonary fibrosis (miRNA-26a, -30e, -125a, -144 and -205) and of these, miRNA-26a and -125a have been down-regulated upon cigarette smoke exposure. miRNA-125a has been found to be expressed in alveolar and bronchial epithelium, whereas miR-26a was located in hematopoietic cells and neutrophils. In addition, miRNA-26a expression increased after mechanical stretch in human airway smooth muscle cells leading to hypertrophy via binding to its target gene *glycogen synthase kinase-3β* (*GSK3*) (Mohamed et al. 2010). miRNA-26a is one of two miRNAs brought in context with smooth muscle cell physiology, which is markedly changed in asthma and a major cause of airway obstruction. The second, miRNA-133a, was not identified in our asthma signature. Reasons might be the analysis of whole lung tissue in contrast to Chiba et al. (2009), who evaluated a reduction in miRNA-133a and concordant increase of the target gene RhoA in bronchial smooth muscle in an OVA mouse model.

The asthma mouse models available for comparison had different experimental backgrounds: IL-13 transgenic mice, house-dust-mite or ovalbumin provocation.
The study with the highest overlap is from Lu and colleagues (Lu, T. X. et al. 2009), who analyzed an IL-13 transgenic mouse model and all eight miRNAs that are overlapping are also regulated in the same direction. In addition, Lu et al. reproduced their findings, at least for their top candidate miRNA-21, in three additional asthma models: two allergen-induced models with OVA and *Aspergillus fumigatus* and one IL-4 lung transgenic model. For miRNA-21 highest expression changes in airway inflammation versus control animals was measured in the IL-13 and IL-4 lung transgenic mice with 6-fold induction each. This was followed by a 4-fold elevation in the *Aspergillus fumigatus* model and a 2.6-fold increase in the OVA model. This finding suggests that different models of the same disease can cause variances in miRNA expression levels.
It is easily imagined that also within similar models, like the applied OVA model, differences in concentration and duration of allergen exposure are the cause of possible variation. It might be hypothesized that the 2.6-fold induction seen by Lu et al. is well comparable to the

measured 1.8-fold induction in our model, considering a concentration difference in OVA sensitization of 100 µg versus 1 µg.
Overall, the fold changes measured in the performed profile are comparatively low with 2.7-fold being the highest change. Looking at the other available profiling studies, highest fold changes are between 5- and 12-fold. Besides the differences in model or allergen concentration, also the number of challenges were more numerous (between 4 and 8 times) compared to two challenges in our milder model, which might add to the more pronounced changes in miRNA levels observed in other studies. Nonetheless, till date it is not clear whether and in which range a fold change of a miRNA can be defined as “biologically relevant”.

7.3. Selective analysis of miRNA-17, -21, -142-3p, -144, -205, -208, -451

After setting up the signature of 36 significantly changed miRNAs in the OVA model for allergic airway disease, six candidates were selected for further analysis. The criteria for selection were level of fold change and reproducibility with three methods.
Lately, miRNA-208 has been found to be expressed in embryonic stem cells and to play a role in cardiac differentiation (Wilson et al. 2010) with implications on the regulation of cardiac hypertrophy and stress-dependent cardiac growth (van Rooij et al. 2007; Callis et al. 2009). Notably, miRNA-208 is differentially expressed in myocardial infarction together with miRNA-133a (Bostjancic et al. 2009), a key regulatory miRNA of bronchial smooth muscle changes in a previously mentioned model for allergic AHR (Chiba et al. 2009). Besides, Lu et al. (Lu, T. X. et al. 2009) identified miRNA-208 to be equally down-regulated in their IL-13 transgenic asthma-model. Until now, there are no validated target genes for this miRNA.

The highest up-regulation was measured for miRNA-205 (2.4-fold). A number of studies have been conducted in order to elucidate miRNA-205 function, the majority pointing at a connection with carcinogenesis (Dar et al. 2011; Tellez et al. 2011). Moreover, studies exploring its utility as sputum biomarker for squamous cell lung cancer are ongoing (Lebanony et al. 2009; Xing et al. 2010). Although miRNA-205 has not been considered to be changed in allergic airway inflammation so far, it has been implicated in the regulation of epithelial to mesenchymal transition (Gregory, P. A. et al. 2008) and in idiopathic pulmonary fibrosis (Pandit et al. 2010).

miRNA-142-3p showed slight up-regulation in the asthma group and a similar change in expression has been examined in two other asthma models thus strengthening its possible role in the disease (Lu, T. X. et al. 2009; Polikepahad et al. 2010). Profiling studies have identified miRNA-142-3p to be specifically expressed in the hematopoietic lineage and being part of a distinct profile of miRNAs found in human bronchial stem cells (Brown, B. D. et al. 2006; Landgraf, P. et al. 2007b; Qian et al. 2008; Bissels et al. 2011). Furthermore, studies investigating single immune cell types, like macrophages or $CD8^+$ T cells could measure expression of this miRNA (Wu, H. et al. 2007; Williams, A. E. et al. 2009). *Adenylate cyclase* (*AC*) *9* is a validated target gene of miRNA-142-3p leading to a restriction of cAMP production in regulatory T cells and their subsequently suppressed function. At the same time, forkhead box protein (FOX) P3, a selective transcription factor of regulatory T cells, inhibits miRNA-142-3p, thus drawing a negative feedback loop (Huang et al. 2009). A decrease in *FOXP3* has been found in patients with asthma (Provoost et al. 2009) pointing towards a possible connection with increased miRNA-142-3p levels found in this and other models of asthmatic airway disease. In addition, examination of samples from asthmatic children revealed functional impairment of a subtype of pulmonary regulatory T cells (Hartl et al. 2007) which further underlines a possible involvement in functional aspects.
miRNA expression can be regulated dynamically in response to specific immune and inflammatory stimuli. Several studies have investigated the response of miRNAs to lipopolysacchride (LPS), the Toll-like receptor (TLR) 4 ligand (Taganov et al. 2006; Moschos et al. 2007) demonstrating up-regulation of miRNA-142-3p and other miRNAs.

Another candidate from our profiling experiment in allergic airway disease, miRNA-21, has also been found up-regulated upon LPS stimulation. Induction of TLR4 signaling leads to augmented expression of miRNA-21 and subsequent inhibition of the target gene *programmed cell death (PDCD) 4*, which is a signaling molecule contributing to apoptosis. As a consequence, miRNA-21 protects against the pro-inflammatory response by limiting LPS induced lethality (Sheedy and O'Neill 2008; Sheedy et al. 2009; Quinn and O'Neill 2011). miRNA-21 further has a role in *ras* induced tumor growth (Frezzetti et al. 2010) and was identified in multiple studies of various types of cancer (Lu, Z. et al. 2008; Liu, G. et al. 2010; Wei et al. 2011; Yu, L. et al. 2011). *PDCD4* seems to represent a general binding partner, as its inhibition was also confirmed to play a role in the regulation of transforming growth factor (TGF) β induced myofibroblast differentiation (Lu, Z. et al. 2008; Yao et al. 2010). Moreover, this miRNA-target gene couple has been observed in autoimmunity (Iliopoulos et al. 2011) and Lupus disease (Stagakis et al. 2011).
The analyzed up-regulation of miRNA-21 in our model of allergic airway inflammation might as well lead to inhibition of *PDCD4,* and as a consequence, might enable protection against

apoptosis in situations of strong inflammation. This interaction yet has to be proven experimentally.

Looking at other diseases, dysregulation of miRNA-21 expression has been described in fibroblasts of myocardial disease as well as idiopathic pulmonary fibrosis (Thum et al. 2008; Liu, G. et al. 2010; Xie et al. 2011). With regard to allergic diseases, expression changes were found in atopic eczema (Sonkoly et al. 2007) as well as in three studies for allergic airway disease (Lu, T. X. et al. 2009; Mattes et al. 2009; Collison et al. 2011a). Lu et al. (2009), as mentioned before, investigated miRNA-21 expression in four parallel models, two transgenic and two allergen based provocation models. Besides the common up-regulation of miRNA-21, the authors localized elevated miRNA-21 levels in inflammatory leukocytes and myeloid cells and identified *IL-12p35* as target gene of miRNA-21 in allergic airway disease. These findings are in line with an observed decrease in IL-12 and a polarization of Th1/Th2 immune responses in asthma (Trinchieri 2003).

Next to miRNA-21, two more miRNAs were selected from the initial signature of allergic airway inflammation to be further analyzed. miRNA-144 and -451 are encoded ~100 bp apart from each other on mouse chromosome 11, being transcribed as a bicistronic transcript that gives rise to the two mature forms which are highly conserved in evolution (Dore et al. 2008). As they are highly expressed in bone marrow, spleen and erythropoietic cells, the two miRNAs and their contribution to erythroid development and homeostasis has been investigated in zebrafish (Wienholds et al. 2005; Du et al. 2009), mouse (Patrick et al. 2010; Rasmussen et al. 2010) and humans (Masaki et al. 2007; Zhan et al. 2007). Both miRNA-144 and -451 are regulated by the erythroid-specific transcription factor *GATA1* (Dore et al. 2008), and both have been shown to functionally inhibit negative regulators of erythropoiesis (Zhan et al. 2007; Du et al. 2009; Fu et al. 2009; Pase et al. 2009). Notably, it seems that miRNA-451 is more relevant for erythropoiesis, giving the fact that a single knock-out of miRNA-451 but not 144 is sufficient to display the seen abnormalities in erythropoiesis (Patrick et al. 2010; Rasmussen et al. 2010).

Although some studies monitored miRNA-144 and -451 to be mainly expressed in erythropoietic cells (Merkerova et al. 2008), others found different sources like neurons or hematopoietic cells (Gentleman et al. 2004; Landgraf, P. et al. 2007b; Zhang, H. Y. et al. 2011). Expression in lung tissue and disease has been reported, such as miRNA-144 in fibrosis (Xie et al. 2011) and miRNA-451 in pulmonary hypertension (Caruso et al. 2010) as well as in asthmatic disease (Garbacki et al. 2011).

miRNA-451 has also been brought in context with cancerous disease, bearing tumor suppressive functions (Li, X. et al. 2011; Wang, R. et al. 2011). Several studies point to therapeutic relevance of miRNA-451, as restoring its levels improved cancer drug resistance

(Gal et al. 2008; Kovalchuk et al. 2008; Bandres et al. 2009). In glioma cells, miRNA-451 is able to regulate adaptation to metabolic stress by reacting to changes in glucose levels (Godlewski et al. 2010). A stress-protective role has also been stated in a miRNA-451 knock-out study where Patrick et al. detected ineffective erythropoiesis in response to oxidative stress. They identified the chaperone protein 14-3-3ζ as direct target which coordinates signal transduction downstream from hematopoietic growth factor receptors by interacting with granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as with IL-3, -5 and -9 receptors (Stomski et al. 1999; Sliva et al. 2000; Barry et al. 2009). Until now, there are no reports to whether miRNA-451 changes in asthma models might have effects on 14-3-3ζ levels and as a consequence influence signaling of GM-CSF, IL-3, -5 or -9, all of which are being associated to the pathogenesis of allergic asthma.
In parallel, another group attributed the protective effect against oxidant stress by repressed 14-3-3ζ to up-regulation of *FOXO3*, a regulator of the anti-oxidative response (Yu, D. et al. 2010). The miRNA-451-14-3-3ζ–*FOXO3* axis may be of interest in stress situations beyond erythropoiesis as *FOXO3* has been found to work also in other tissues, like the heart (Tan, W. Q. et al. 2008). It thus might be, that elevated miRNA-451 levels in allergic airway disease lead to anti-oxidative properties regulated via 14-3-3ζ and *FOXO3*.
FOXO3 is a predicted transcriptional activator of miRNA-21 (transmiR analysis) which was found to be up-regulated in this study as well and which would highlight a possible protective circuit including anti-oxidative pathways via *FOXO3* and, for example reduced *PDCD4* levels via the induced miRNA-21, thereby diminishing apoptosis due to inflammatory reactions. However, most of this information was gained very recently and detailed investigations into this direction still need to be undertaken.

For miRNA-144, a recent study revealed elevated erythrocytic expression levels in a subgroup of patients with sickle cell disease characterized by more severe anemia (Sangokoya et al. 2010). The authors identified *nuclear factor-erythroid 2-related factor 2* (*NRF2)* as direct target, which is a central regulator of cellular response to oxidative stress. During oxidative stress, NRF2 binds to the antioxidant response element (ARE), which further activates key genes of the oxidative stress response, such as *superoxide dismutase*, *catalase* or *gluthathione synthetase H* connected enzymes. Notably, NRF2 not only plays a role in erythrocytes, but has repeatedly been identified as important protective factor in asthma (Rangasamy et al. 2005) with special involvement in the anti-oxidative pulmonary defense after provocation with diesel exhaust particles (Li, N. et al. 2004; Williams, M. A. et al. 2008; Li, Y. J. et al. 2010b).
Given the up-regulation of miRNA-144 in allergic airway disease, this would in theory lead to repression of NRF2 and thus hinder its anti-oxidative functions.

The possible pro-inflammatory role of miRNA-144 stands in contrast with another report where this miRNA was found to repress Caspase 3, a known factor of the tumor necrosis factor-related apoptosis inducing ligand (TRAIL) - induced apoptotic cascade (Ovcharenko et al. 2007). This is of special interest as TRAIL is known to be elevated in the airways of asthma patients (Williams, M. A. et al. 2008) and the fact that miRNA-144 is enhanced in this model of allergic airway disease might hint to a regulatory and anti-apoptotic function.

After all, it is surprising that the two miRNAs, -144 and -451, although being transcribed together seem to have distinct functions. Therefore, they most probably undergo additional posttranscriptional regulation. Zhang X. et al. (2010) found synergistic effects of miRNA-144 and -451, mediated by GATA4 in a study examining cardiomyocyte death after simulation of ischemic disease. Both miRNAs led to augmented survival which could be explained by direct inhibition of the RNA-binding protein (CUG triplet repeat-binding protein 2) which is known to repress translation of cyclooxygenase-2 (COX-2). This indicates increase of COX-2 as an indirect effect of miRNA-144/451 up-regulation. COX-2 has been found to be elevated in airway cells of asthmatic patients (Taha et al. 2000) although another study could not validate these findings (Demoly et al. 1997). In general, COX-2 has pro-inflammatory attributes and is activated exclusively after inflammation or induction by growth factors, cytokines and mitogens. COX-2 levels are decreased after corticosteroid treatment in airway epithelia (Aksoy et al. 1999) reflecting a common mechanism for treatment of asthmatic disease. The reported pro-survival effect in cardiomyocytes could thus be a pro-inflammatory effect in a setting of inflammation. There is not enough published information in order to decipher the possible consequences of miRNA up-regulation on COX-2 levels in asthma. However, a recent study claimed suppression of mast cells by bone marrow-derived stromal cells (BMSC) which was dependent on up-regulation of COX-2 in BMSCs (Brown, J. M. et al. 2011).

From the six selected miRNAs, four are encoded on mouse chromosome 11: miRNA-21, -142-3p, -144 and -451. Chromosome 11 encodes a total of 69 miRNA genes. Except for the clustered miRNAs-144 and 451, these coding regions are not in close proximity. *In silico* analysis of transcription factor binding sites (DIANA analysis) shows co-regulation of miRNA-144 and -451 by GATA1 and GATA4. A common transcription factor predicted to influence all four miRNAs is octamer-binding transcription factor (OCT-1). It has been described to act as a sensor for stress signals (Kang et al. 2009; Wang, P. and Jin 2010), which would be in line with the stress-associated target genes *NRF2*, *FOXO3* or *COX-2* which could be brought in context with the miRNAs-144 and -451. Additionally, OCT-1 was reported to play a role in asthma susceptibility (via IgE) and in polymorphic effects of the IL-4 promoter (Gervaziev et

al. 2009; Holt et al. 2011). However, real co-regulation would have to be proven experimentally.

As described in results section 6.3, a target prediction analysis was done and CREB1 was chosen to be studied in more detail. Two predicted miRNA binding partners could be confirmed to be differentially expressed in asthma lung tissue, one being miRNA-144, a candidate that had been selected because of its fold change beforehand and which was discussed above. The second predicted binding partner for *CREB1* is miRNA-17. Exiqon array analysis determined a 1.12-fold difference in asthma versus control mice, which led to initial exclusion in the miRNA signature. Being predicted to bind to *CREB1*, miRNA-17 expression was tested in a larger group of mice (n=15) where a 1.34-fold change was measured. So far, this miRNA has not been reported in allergic airway disease. However, one study measured up-regulation in atopic eczema (Sonkoly et al. 2007). miRNA-17 is expressed in most tissues (Landgraf, P. et al. 2007; Ventura et al. 2008) and belongs to the miRNA-17~92 cluster. Located on human chromosome 13, the cluster consists of six miRNAs (miRNA-17, 18a, 19a, 20a, 19b-1 and 92-1) which are transcribed as polycistronic primary transcript before being processed into individual mature miRNAs. As a typical trait for clustered miRNAs, they are highly conserved in vertebrates and their coordinated regulation and function is thought to reflect their important roles (Mendell 2008). There exist two cluster paralogs, the miRNA-106b~25 cluster on chromosome 7 (miRNA-25, 93 and 106b) and the miRNA-106a~363 cluster located on the X chromosome (miRNA-18b, 19b-2, 20b, 92-2, 106a and 363) which are explained by ancient gene duplications. Although all miRNAs from the 17~92 cluster and its two paralogs were available on the Exiqon microarray, only miRNA-17 and miRNA-18a (1.26-fold change) were measured to be differentially expressed in the asthma group.

There exists a large body of research about the 17~92 cluster and its members with most studies concentrating on the role in cancerous disease. miRNA-17, along with its clustered members, has been found up-regulated in several cancers with anti-apoptotic function (He et al. 2005; Matsubara et al. 2007; Chow et al. 2009; Li, H. et al. 2010a; Heegaard et al. 2011; Yu, L. et al. 2011). Mice with targeted deletion of the 17~92 cluster die shortly after birth with striking defects in lung and heart development and a defect in B cell development. Notably, deleting each of the two paralog clusters showed no phenotypic consequences whereas both triple and double knock-outs including the miRNA-17~92 cluster led to death even before birth (Ventura et al. 2008). miRNA-17~92 plays important roles in both B and T cell development (Lu, Y. et al. 2007; Xiao et al. 2008). As with the knock-out studies, a key function of miRNA-17~92 was identified to be inhibition of the pro-apoptotic protein BCL-2 interacting mediator of cell death (BIM), as well as the tumor suppressor PTEN. The

previously mentioned miRNA-21, which was found up-regulated in asthma compared to control samples, also targets PTEN which might point to a co-regulation of target genes by several miRNAs. miRNA-17 was additionally shown to play a role in monocyte differentiation and maturation by targeting *acute myeloid leukaemia-1* (*AML-1*, also called *RUNX1*), a factor known to induce expression of the receptor for M-CSF, as well as IL-3 and GM-CSF (Fontana et al. 2007). Another study identified miRNA-17 among others to regulate stress-induced immune responses (Stern-Ginossar et al. 2008) which further underlines this miRNA´s role in innate and adaptive immune responses.

Recently, Zhang M. et al. (2011) identified *signal transducer and activator of transcription* (*STAT) -3* to be a miRNA-17 target and its inhibition blocked the suppressive function of myeloid derived suppressor cells known to play a role in tumor immune tolerance. Blocking *STAT-3* signaling has been described beneficial in an asthma mouse model, where hallmarks of airway inflammation, like AHR and eosinophilia were markedly reduced. The authors explained the effects by up-regulation of counter-balancing immune factors like IL-10, IL-12 and enhanced *FOXP3* expression (Hausding et al. 2010). Regarding this, one might speculate whether elevated miRNA-17 levels in the asthma model might have the intention to regulate inflammatory changes via inhibition of *STAT-3*.

7.4. Temporal expression patterns of selected miRNAs in lung and spleen

Three miRNAs were chosen based on fold change (miRNA-144 and -451) as well as previous association to inflammation (miRNA-21). In addition, target prediction led to the inclusion of a fourth candidate, miRNA-17. With the exception of miRNA-21, which has lately been investigated in allergic asthma to target *IL-12p35* (Lu, T. X. et al. 2009), no other miRNA candidate of this selection has been investigated for its role in allergic asthma.

In a kinetic experiment all four miRNAs were examined in lung and spleen tissue at several time points during sensitization and after aerosol challenge.

miRNA-17 as well as miRNA-21 expression was increased only after allergen challenge in parallel with the inflammatory changes in lung tissue ascertained in the total and differential cell counts in BAL. The observed inflammatory changes on day 72 were intensified on day 76. This trend of increase was also visible in the expression levels of miRNA-144 and -451.

BAL samples on day 76 consisted of elevated numbers of macrophages, eosinophils and to a vast extent lymphocytes. As a consequence, the measured miRNAs might be located in these cells. For miRNA-21, macrophages as well as T cells have been found to be a source

before (Lu, T. X. et al. 2009; Salaun et al. 2011) whereas there is no report about expression in eosinophils so far.
Expression of miRNA-17 as well as 451 in spleen was unchanged prior to airway challenge and increased afterwards. Changes were again more prominent at the later time point.
The miRNA-144 experienced a boost in expression from a 1.5-fold change on day 72 to 7.5-fold elevation on day 76. This strong increase distinguishes miRNA-144 from its clustered miRNA-451. Except for this measurement in spleen, the two miRNAs were expressed very similarly in all other measurements.

7.5. Target gene search

The first part of this project aimed to set up an expression signature of allergic airway disease in our experimental model. After describing the changes in miRNA levels during disease, the question of function was addressed. miRNAs are post-transcriptional regulators and work through binding to a target mRNA which is subsequently repressed. There exist several possibilities to find miRNA-gene couples. The biological most meaningful approach might be to study a phenotypic change, as was done with the very first miRNA lin-4 and its target *lin-14*, which were discovered in a screen for larval defects in *C.elegans* (Lee, R. C. et al. 1993; Wightman et al. 1993). Other studies concentrated on genes that are known to be important in a biological context, such as IL-13 in asthma, and thus screen for putative regulatory miRNAs (Novershtern et al. 2008; Kumar, M. et al. 2011). Over-expression or knock-down experiments of single miRNAs have been undertaken and mostly followed by transcriptome and proteome analyses or immunoprecipitation studies in order to identify relevant gene targets (Ventura et al. 2008; Xiao et al. 2008; Thomas et al. 2010).

In this study, the miRNA profile did not project any single miRNA as a “hot candidate”, as fold changes were mostly less than 2-fold. Nevertheless, it is an emerging principle that co-regulation by several miRNAs can occur. Therefore, also small changes of single miRNAs might be relevant (Zhou, Y. et al. 2007; Xu and Wong 2008; Xiao and Rajewsky 2009).

We chose a target search that included the top 100 differentially expressed miRNAs identified in the Exiqon array experiment. Based on identical seed regions, the miRNAs were categorized into families, which led to the formation of 33 families. These were analyzed using five computational target prediction algorithms in a “full consensus” approach. Most prediction algorithms include criteria for seed region complementarity, conservation between

species and thermodynamic stability of the formed heteroduplex. Single algorithms take non-conserved sequences (Target Scan) or sequences outside the 3´-UTR of genes into account (PITA). Although seed based predictions still have the highest specificity and sensitivity, there are prominent examples of non-predictable, so called “seedless” targets (Rajewsky 2006; Lal et al. 2009).

In this study, application of 5 prediction algorithms led to a reduction in number of target genes and putative false positives results. The false positive rate of the most common algorithms is estimated to be between 22 and 30 % (Yoon 06). However, the disadvantage of a strict approach is a higher rate of false negatives, meaning the loss of possible target genes. As an example, the miRNAs-208 and -451 did not have a single target after the analysis. Applying only one prediction algorithm reveals 14 putative targets for miRNA-451 by TargetScan whereas PicTar does not give any target thus causing the complete loss of this miRNA in the full consensus approach which includes this prediction program.

Although some studies rely solely on one prediction algorithm, many studies are now combining two or three programs (Thum et al. 2008; Stagakis et al. 2011). The recently improved availability of bioinformatic programs that offer overlapping target predictions and combine several algorithms are gaining popularity (miRGator, miRecords).

Our strict prediction strategy still revealed 961 putative target genes. We reasoned that genes with multiple binding sites might have a more important biological role than less targeted genes. The majority of genes, around 95 %, offered one or two binding sites for miRNAs whereas 8 % of genes had three sites. Four or more miRNA binding sites were found with 11 genes (around 1 %) and these were further evaluated. Notably, all candidate genes were involved in regulatory mechanisms with most being involved in transcriptional regulation, like the transcription factors *CREB1*, *NFAT5, SOX6* or transcripts for regulatory proteins *ELAVL2, HMGA2, SOCS6, ZBTB39, STYX, BAZB2* and *TNRC6B*. Asirvatham et al. (2008) studied more than 600 genes involved in immune regulation and found that miRNAs preferentially target immune genes compared to the genome. They identified major gene targets to consist of transcription factors, cofactors and chromatin modifiers and to a much lesser extent ligands or receptors (Cui et al. 2007). This goes in line with the identified gene list in this study. In addition, miRNAs are frequently regulating themselves by repressing factors needed for their own generation or function, like in this case *TNRC6B*. Thereby, tight regulation could be a reflection of their importance and subsequent strict control (Carthew and Sontheimer 2009).

A comparison of the 11 identified genes that harbor multiple miRNA binding sites with the literature reveals that five genes have no reported and experimentally validated miRNA partners (*TNRC6B, BAZ2B, ELAVL2, STYX, ZBTB39*). Three genes (*NFAT5, SOCS6,*

SOX6) do have validated sites for miRNAs but these were not included in the profile of miRNAs in allergic airway disease. *CREB1* and *ACVR2A* are proven target genes of miRNA-34b (Pigazzi et al. 2009) and miRNA-29b (Li, Z. et al. 2009), respectively, and although these miRNAs can be found in the asthma profile to be differentially regulated, the applied prediction method does not suggest these interactions. Nevertheless, the let-7/miRNA-98 family has been predicted to target *HMGA2* and this validated couple was also predicted in the applied analysis (Pandit et al. 2010).
The target prediction has been done irrespective of miRNA fold changes. Looking at the miRNAs that had been selected based on their differential regulation and highest fold changes, two candidates also fulfill the criteria of having putative target genes that harbor four or more binding sites for different miRNAs and thus could include co-regulation. miRNA-21 binds *TNRC6B*, whereas miRNA-144 targets *CREB1* and *ELAVL2*.

7.6. Interaction of the target gene *CREB1* with the predicted miRNAs

After evaluating the list of predicted target genes, the transcription factor CREB1 was chosen for further analysis. This decision was based on 3 aspects: 1) its 3´-UTR can be targeted by several miRNAs at multiple sites, 2) miRNA-144 is a predicted binding partner and has been validated to be one of the highest up-regulated miRNAs in the performed asthma signature and 3) CREB1 has been previously associated with asthmatic disease (Couetil et al. 2006; Chiappara et al. 2007; Kim, C. H. et al. 2009).
To test functional interaction and ascertain the prediction, binding of the miRNAs-17, -22, -144 and -181a to the 3´-UTR of *CREB1* were tested. A reporter gene assay performed in two bronchial epithelial cell lines proved binding of all tested miRNAs. This finding confirms the prediction made by the bioinformatic analysis.

A second experiment showed significant down-regulation of endogenous *CREB1* gene levels by *in vitro* transfected miRNAs-17 and -22. Repression was also seen to a lesser extent with miRNA-144. The effect of miRNA-181a seen in the reporter gene assay could not be confirmed. It should be mentioned that levels of different miRNAs varied after transfection, but these differences had no visible effect on inhibition of *CREB1* mRNA. A speculative reason for this could be additional regulatory steps in the processing and incorporation of the single miRNAs into the RISC complex or differences in accessibility of the binding sites.

To elucidate biological relevance in allergic airway inflammation, expression levels of *CREB1* and the predicted miRNAs were measured in a large number of lung samples (n=15) of asthma and healthy mice. The reduction in *CREB1* level was highly significant, whereas only miRNA-17 and -144 showed a statistically relevant increase. The inverse relation of expression, together with data from the reporter assay point towards a role of miRNA-17 and -144 in repressing *CREB1* gene levels in this model of allergic asthma.
miRNA-mediated regulation of *CREB1* has been studied by one group in acute myeloid leukaemia (Pigazzi et al. 2009). They found disease-associated reduction of miRNA-34b and in parallel increased levels of *CREB1*. Restoring miRNA-34b levels not only let to a subsequent decrease in *CREB1* expression but also affected downstream targets like B cell lymphoma-2 (*BCL-2)*, *NFκB*, *STAT-3* and others, thus pointing towards broad effects following miRNA regulation.

Belonging to the CREB/ATF subfamily of cAMP responsive basic region-leucine zipper transcription factors, CREB1 influences expression of various genes with involvement in immune regulation, cell survival/ DNA repair, neuropeptides and others (Mayr and Montminy 2001). Concentrating on the function in immune regulation, CREB1 is involved in T and B cell activation and proliferation (Hsueh et al. 1997; MuthUSmy and Leiden 1998; Zhang, F. et al. 2000; Blois et al. 2004). Moreover, immune signaling via interleukins or chemokine receptors has been brought in context with CREB pathway activation (Kuipers et al. 2008; Kawaguchi et al. 2009). A reduction in *CREB1* levels determined in this model of allergic asthma could thus indicate a repression of immune response, perhaps in a counter-regulatory fashion.
Possible co-regulation of genes by miRNA-17 might be present in the repression of the apoptotic factor BIM. CREB1 is a known activator of *BIM* expression and both genes are validated target genes of miRNA-17. One could speculate that a reduction of *BIM* expression could lead to a pro-survival response, possibly protecting against apoptotic effects during strong inflammation.
On the other hand, CREB1 is known to promote anti-inflammatory immune responses through repression of NFκB signaling and induction of IL-10 and regulatory T cells (Ollivier et al. 1996; Parry and Mackman 1997; Kim, H. P. and Leonard 2007; Ananieva et al. 2008; Ruan et al. 2009). The observed decrease in *CREB1* would consequently support pro-inflammatory signaling. Heijink et al. (2005) investigated the impact of elevated CCL17 levels on T cells. They observed a shift from CREB1 signaling, which is associated with reduced T cell activity, to MAPK signaling leading to T cell mediated inflammation. A scenario like this would indicate a pro-inflammatory situation in the investigated model.

A study in asthma patients reported elevated CREB1 phosphorylation in severe disease and Kim et al could show involvement in mucus hypersecretion, which is a hallmark of asthmatic disease (Chiappara et al. 2007; Kim, C. H. et al. 2009). In contrast, a study in horses with airway obstruction saw negative correlation of CREB1 activation with disease (Couetil et al. 2006), eventually pointing at differences between humans and other species. Although phosphorylation of CREB1 rather than expression is mostly studied, one report in COPD patients showed overlapping patterns for *CREB1* expression and activation (Mroz et al. 2007) which might allow speculations towards similar regulation.
Nevertheless, the role of CREB1 in asthma pathology is not well understood, given the different implications of CREB1 function in immune regulation alone. In the applied asthma model, a reduction of *CREB1* gene expression was observed which goes in line with the increase of two miRNAs proven to bind to the 3´-UTR of *CREB1*.

7.7. miRNA expression in human and murine peripheral blood

Due to the high stability of miRNAs it is feasible to measure their expression in a multitude of body fluids, like urine, tear, ascetic or amniotic fluid as well as BAL fluid (Chen, X. et al. 2008; Gilad et al. 2008; Turchinovich et al. 2011).
In this study, it was possible to explore miRNA expression in stored peripheral blood samples of asthmatic children, originating from the GINI study (von Berg et al. 2008; Rzehak et al. 2011). The expression of candidate miRNAs, miRNA-17, -21, -144 and -451 were assessed and compared to age-matched, healthy children that served as control group.
Beyond the analysis of expression and possible changes in human asthmatic disease, interest was laid on the question whether murine blood miRNA expression resembles the human situation and how the tested miRNAs behave in blood versus lung tissue of one animal.

Analysis of human blood miRNAs revealed down-regulation of the measured miRNAs with miRNA-144 and -451 being reduced in asthma, miRNA-17 showing only slight reduction and miRNA-21 being unchanged. Comparison to murine blood samples mirrored these findings, with the exception of miRNA-21 which was 2.6-fold down-regulated. Fold changes were generally higher in mice. Interestingly, all miRNAs were found to be up-regulated in lung tissue of diseased mice giving an adverse picture of increase in lung versus decrease in blood samples. A study examining the miRNA response to LPS in human leukocytes found reduced miRNA-146b expression, a miRNA known to be typically up-regulated after endotoxin exposure which might hint towards an adverse link of expression in different

compartments (Taganov et al. 2006; Moschos et al. 2007; Schmidt et al. 2009). Although most miRNAs are found to be similarly expressed in serum and blood cells under normal conditions in literature, analysis of diseased states displayed some differences which indicates that further investigations should be done in restricted blood components, like serum, plasma or the different blood cells (Chen, X. et al. 2008).

Until now, origin and possible function of miRNAs in blood are not well understood. There exist reports proposing that extracellular miRNAs are of non-vesicular origin and stay associated to Ago or other stabilizing proteins (Wang, K. et al. 2010; Turchinovich et al. 2011). The authors propose that these miRNAs might be by-products of dead cells. The diagnostic specificity found in numerous biomarker studies yet does not support this idea. In contrast, specific loading of miRNAs into exosomes has been proposed as well as transfer between circulating cells, such as T cells and monocytes (Gibbings et al. 2009; Pegtel et al. 2010). A very recent study showed that plasma miRNAs are transported and delivered to recipient cells by high-density-lipoproteins and that these miRNAs do have functional targeting capabilities (Vickers et al. 2011). The hypothesis that circulating miRNAs can lead to signal transmission has been stated earlier (Valadi et al. 2007; Kosaka et al. 2010), for example during the initiation of immune responses in peripheral lymphoid tissues. Such a scenario could play a role in the situation of allergic asthma as well. Here, the lung might be a place of special interest, as it is a site of extensive contact between cellular tissue and blood.

7.8. Outlook

Beyond their role in the patholphysiology of disease, an increasing number of reports describe the potential of miRNAs as non-invasive biomarkers easily detectable by RT-qPCR methods. Application includes diagnosis, as well as prognosis, like survival prediction in non-small-cell lung cancer based on serum samples (Hu et al. 2010; Chen, X. et al. 2011; Liu, X. G. et al. 2011; Yaman Agaoglu et al. 2011; Yu, L. et al. 2011).

Due to difficulties in the diagnosis of asthmatic disease, identification of novel biomarkers is of great relevance. Especially in young children, wheezing or respiratory infections are hard to distinguish from developing asthma (Bacharier et al. 2008). Early diagnosis and therapy are important to avoid airway remodeling and to minimize allergen exposure.

The results gained in our experiments show translational potential of distinct miRNAs. In future experiments, miRNA expression profiles in human blood samples could be further analyzed with regard to their possible function as biomarkers for asthma.

Besides, the distinct roles of miRNA-17 and -144 and others, which have been found up-regulated in this mouse model for asthma, can be further explored. Validation in other asthma mouse models, as well as cell type specific localization of the identified miRNAs would be precious. Moreover, loss-of function studies, applying antisense-miRNAs (anti-miRNAs) can bring important information (Mattes et al. 2009; Pandit et al. 2010; Lu, T. X. et al. 2011). Overall consequences for disease outcome as well as the interaction with the *in vitro* validated target gene *CREB1* can thus be analyzed. Thereby, direct application of anti-miRNAs into the lungs has been proven to be effective, functional and attractive with regard to possible therapeutic use (Collison et al. 2011a; Collison et al. 2011b).

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9. APPENDIX

9.1. Abbreviations

Ago	Argonaute
alum	aluminium hydroxid/magnesium hydroxid
AML-1	Acute Myeloid Leukaemia-1
ARE	Antioxidant Response Element
ATP	Adenosine triphosphate
BAL	Broncho-Alveolar Lavage
BCL-2	B-cell lymphoma-2
BIM	BCL-2-Interacting Mediator of cell death
BMSC	Bone Marrow-derived Stromal Cells
bp	base pair
BSA	Bovine Serum Albumin
C. elegans	Caenorhabditis elegans
COX2	Cyclooxygenase 2
CREB	cAMP Responsive Element Binding protein
DGCR8	DiGeorge syndrome Critical Region gene 8
DMSO	Dimethyl Sulfoxide
DNA	Deoxy-Ribonucleic Acid
dNTP	deoxy-Nucleotide-Tri-Phosphate
DTT	Dithiothreitol
E. coli	Escherichia coli
ELISA	Enzyme Linked Immuno Sorbent Assay
FC	Fold change
FCS	Fetal calf serum
FOXP3	Forkhead box protein P3
GINI study	German Infant Nutrition Intervention study
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
h	hour
HLA-G	Human Leucocyte Antigen-G
HPRT1	Hypoxanthine guanine phosphoribosyl transferase 1
i. n.	intra-nasal
i. p.	intra-peritoneal
Ig	Immunoglobulin
IL	Interleukin
LB medium	Luria-Bertani medium
LNA	Locked Nucleic Acid
LPS	Lipopolysacchride
MEM	Minimum Essential Medium
min	minute
miRNA	microRNA
n. a.	not analyzed
n. d.	not detected
NFκB	Nuclear Factor-κB
NRF2	Nuclear factor-erythroid 2-Related Factor 2
OCT-1	octamer-binding transcription factor-1

OVA	Ovalbumin
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDCD4	Programmed Cell Death 4
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
qPCR	quantitative Polymerase Chain Reaction
RISC	RNA Induced Silencing Complex
RNA	Ribonucleic Acid
rpm	rounds per minute
RT	Reverse Transcription
SCF	Stem Cell Factor
sec	second
siRNA	small interfering RNA
snoRNA	small nuclear RNA
STAT-3	Signal Transducer and Activator of Transcription-3
TAE buffer	Tris Acetate EDTA buffer
TGFβ	Transforming Growth Factor beta
Th2	T helper 2
TLR4	Toll-Like Receptor 4
TMB	Tetramethylbenzidine
TRAIL	Tumor necrosis factor-Related Apoptosis Inducing Ligand
Treg	regulatory T cell
TSLP	Thymic Stromal Lymphopoietin
U	Units (enzyme activity)
UTR	Un-Translated Region
WHO	World Health Organisation

9.2. Tables

9.3. Figures

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Ich erkläre desweiteren, dass die Dissertation erstmalig einer Prüfungskommission vorgelegt worden ist und ich mich anderweitig einer Doktorprüfung ohne Erfolg nicht unterzogen habe.
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München, den 24. November 2011

…….
(Nikola Schulz)

NIKOLA SCHULZ

geboren am 12.08.1979 in München

STUDIUM UND AUSBILDUNG

12/07-05/11	**Promotion im Fach Biologie (Dr. rer. nat.) an der Ludwig-Maximilians-Universität und Helmholtz Zentrum München** (Comprehensive Pneumology Center) *Thema:* Analyse der microRNA Expression und Identifizierung von Zielgenen in einem Asthma-Mausmodell
07/05- 04/07	**Studium an der Technischen Universität München, Weihenstephan** Master of Science Ernährungswissenschaft (Gesamtnote 1,5) Studienrichtung Bio-Medizin Zusatzfächer Gastrointestinale Infektionen, Medizin *Thema Master´s thesis:* Einfluss einer eisen-armen Diät auf chronische Ileitis im $TNF^{\Delta ARE/wt}$ Mausmodell
09/02-07/05	**Studium an der Technische Universität München, Weihenstephan** Bachelor of Science Ernährungswissenschaft (Gesamtnote 1,9) Zusatzfächer Bio-Medizin, Biofunktionalität der Lebensmittel *Thema Bachelor´s thesis:* Gesundheitsfördernde Wirkung von Tomaten: Eine vergleichende Analyse zu Qualität und Quantität antioxidativer Inhaltsstoffe bei Tomaten
09/99-09/02	**Studium an der Ludwig-Maximilians-Universität München** Humanmedizin, 3 Semester
09/97-07/99	**Edith-Stein-Gymnasium** Allgemeine Hochschulreife (Gesamtnote 1,5) Haupfächer Sport, Französisch, Chemie
09/90-09/97	**Michaeli-Gymnasium München**

WEITERBILDUNGEN

03/10	Versuchstierkundlicher Kurs FELASA C (tiz-BIFO, München)
10/09	“Upward leadership and creativity in research” (Ludwig-Maximilians Universität München)
10/08	Seminar „Clear writing for science publications“ (Helmholtz Zentrum München)
06/08	Seminar zu Rethorik und Präsentationstechnik (Helmholtz Zentrum München)

München, den 24. November 2011 …….