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mRNA mediated gene transfer of Toll-like receptors as treatment strategy for asthma *in vivo*

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Abbreviations

%	percent
µ	mikro
A549 cells	Human alveolar type 2 epithelial cells
AAV	Adeno-associated viral vectors
AHR	Airway hyperresponsiveness
AP-1	Activated protein 1
APC	Allophycocyanin
ApE	a plasmid editor
ARCA	Anti reverse cap analog
ATP	adenosine triphosphate
BAL(F)	Bronchoalveolar lavage (fluid)
bp	base pair
BSA	Bovine serum albumine
CTP	cytosine triphosphate
DC	Dendritic cell
DEPC	Diethylpyrocarbonate water
DMEM	Dulbecco’s modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothyocyanate

FoxP3	Forkhead box protein 3
FSC	Forward scatter
g	gravitation force or gram
GOI	Gene of interest
GTP	guanosine triphosphate
HDM	House Dust Mite
i.t.	intratracheal
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPL	Isolated, perfused and ventilated lung
kb	kilo base
LB	Luria Broth
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAL	MyD88 adaptor-like
m5C(TP)	5-methylcytidine (triphosphate)
MCh	Methacholine
MCS	Multiple cloning site
min	minute
MLE12 cells	Mouse transformed epithelial cells
mRNA	messenger RNA
MyD88	Myeloid differentiation factor 88
NK cell	Natural killer cell
NSAID	Non-steroidal anti-inflammatory drugs
OD	Optical density

ORMDL3	Orosomucoid like 3
PAS	Periodic acid-Schiff
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
RFP	Red fluorescent protein
RNA	Ribonucleic acid
rpm	rounds per minute
sec	seconds
SNP	Single nucleotide polymorphism
SSC	Side scatter
ssRNA	single strand ribonucleic acid
s2U(TP)	2-thiouridine (triphosphate)
TAE	tris-acetate EDTA
Th1 (2)	T helper cell type 1 (2)
TIR	Toll-IL-1 receptor domain
TLR	Toll-like receptor
TRAM	TRIF-related adaptor molecule
Tregs	Regulatory T cells
TRIF	TIR domain-containing adaptor-inducing IFNβ
UTP	uridine triphosphate
V	Volt
WHO	World Health Organisation

1 Introduction

1.1 Asthma Immunology

1.1.1 Epidemiology

Most recent studies estimate a number of 334 million people worldwide suffering from asthma [1]. The World Health Organization (WHO) forecasts that the number of asthma patients will even increase by more than 100 million by 2025 [2]. After decades of regarding asthma as a disease of Western or high-income countries, this historical view is no longer kept up. In fact, today most people with asthma are affected in low- and middle-income countries [1]. Whereas the prevalence of asthma is still increasing in these low- and middle-income countries, it might now be plateauing in some high-income countries [3, 4].

Although asthma is not a deadly disease, it can become very dangerous and even life-threatening if not treated and managed properly. According to the WHO 250,000 deaths are annually attributed to asthma [2]. Though fatal asthma is relatively rare among children, the disease represents a substantial burden especially to these young patients. In fact, asthma is the most common chronic disease in childhood. In Germany an estimated 10% of all children and 14% of all children worldwide suffer from asthma [5]. The illness often restricts their daily activities and interferes with family life. Reports published by the American Lung Association emphasize that asthma is one of the leading causes of hospitalization and emergency department visits among children and furthermore one of the most important reasons for missed school days [6]. Considering the impact and consequences of asthma it does not surprise that the disease is ranked 14th in the most important disorders of the world in terms of extent and duration of disability [1].

1.1.2 Classification of atopic and non-atopic asthma

1.1.2.1 Atopy

The term “atopy” describes the genetic predisposition to develop immunoglobulin (Ig) E antibodies against low doses of common environmental allergens (e.g. pollen, dander or food) [7]. IgE mediated allergic reactions trigger an exaggerated histamine release, causing the symptoms of the classical atopic disorders as allergic rhinitis, atopic dermatitis and allergic asthma. The classification of “extrinsic” (or atopic) and “intrinsic” (non-atopic) asthma was first introduced by Rackemann in 1947 [8]. He emphasized the triggering role of allergens in extrinsic asthma and in contrast considered intrinsic asthma to be a disease of non-allergic, unknown origin. The present study addresses the pathogenic principles of childhood atopic asthma.

1.1.2.2 Atopic asthma

Atopic (or extrinsic, allergic) asthma is characterized by an atopic, hence IgE mediated, pathogenesis of airway inflammation with an imbalance in T cell differentiation towards an exaggerated T helper cell type (Th) 2 immunity to allergens in the bronchial mucosa. Besides increased levels of total and allergen-specific serum IgE, a positive skin prick test to common allergens serves as a diagnostic test. Allergic asthma usually develops in childhood and is often accompanied by hay fever and seasonal exacerbations [9].

1.1.2.3 Non-atopic asthma

Most asthma patients are atopic, but a subgroup also suffers from non-atopic (or intrinsic) asthma, comprising

- asthma due to respiratory infections
- asthma through chemical or toxic irritants
- exercise-induced asthma
- analgesic-induced asthma and
- asthma complaints as a consequence of gastroesophageal reflux [10].

Here, as sensitization is absent, IgE plays a less important role in the pathogenesis of the disease [11] and symptoms are not associated with allergic reactions. Further immunological mechanisms are less characterized, particularly in children. Female predominance, a higher degree of severity, more frequent association to nasosinusal polyposis and a later onset in lifetime is observed in non-atopic asthma when compared to the atopic form of the disease [8, 9].

1.1.3 Clinical picture and etiology

Asthma is a chronic immune-inflammatory disease of the respiratory system. Main characteristics are reversible airway obstruction and airway hyperresponsiveness (AHR). Although asthma can develop at any stage of life, it most commonly develops in early childhood [1]. The character of symptoms and the severity of the disease may vary from patient to patient and can range from mild, occasional symptoms to severe, persistent complaints. Symptom-free periods often alternate with acute exacerbations. Common clinical features include wheezing, shortness of breath, chronic coughing, chest tightness or pain and increasing production of mucus in the airways (Fig. 1) [12].

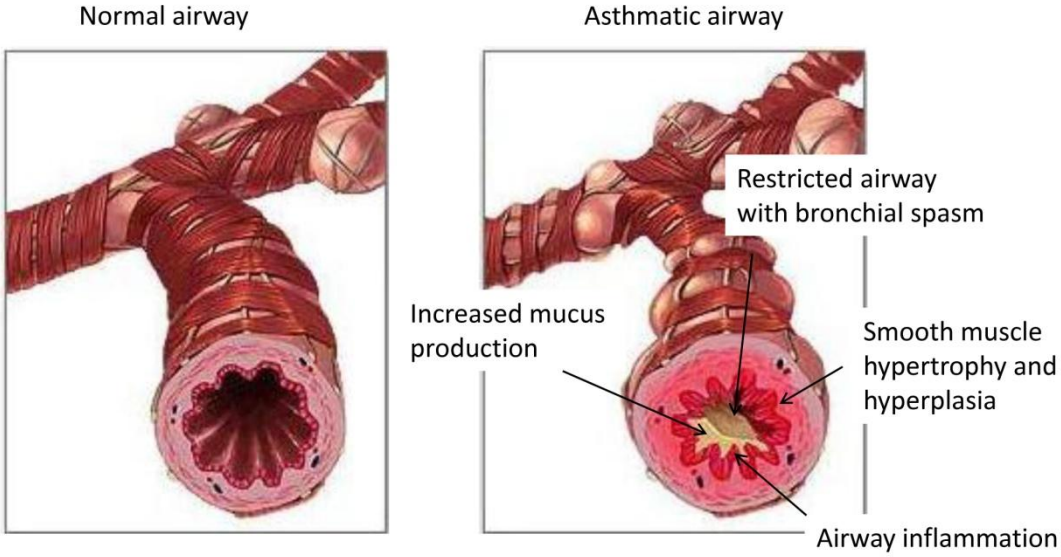

Fig. 1 Normal airway in comparison to an asthmatic bronchiole

Enlarged smooth muscles, bronchial spasm and excessive mucus secretion lead to an increase in breathing difficulty.
(Adapted and modified from *http://www.nlm.nih.gov/medlineplus/ency/imagepages/19346.htm*)

1.1.3.1 Pathogenesis and etiology

It is yet unclear what initiates the inflammatory process in asthma disease in the first place and which immunological mechanisms make some persons more susceptible to asthma than others. Nonetheless, during the past decades much has been learned about factors contributing to the development of asthma. Host factors, such as genetic predisposition seem to play an important role, as well as prenatal factors and environmental exposure. The influence of viral respiratory tract infections is controversially discussed [13-18]. However, the precise interactions of complex host, environmental and further site-specific factors are still subjects of active investigations.

Host factors

It is well recognized that asthma has strong inheritable components accounting for its pathogenesis. Children of atopic parents are more likely to develop atopy or asthma than children of parents not affected by atopy. In fact, if both parents have a history of asthma, the offspring's risk is estimated 60-80% to fall ill [10, 19]. Notably, maternal asthma increases the children's risk to develop the disease to a greater extent than paternal asthma [20, 21].

The development of asthma is dependent on multiple genetic variants and the environment. The genetics involved and their interaction are complex and still not fully understood [22, 23]. Some research efforts were able to identify genes or gene variants that may contribute to or even protect from the development of asthma. The gene *ORMDL3*, encoding transmembrane proteins in the endoplasmatic reticulum, has been identified as a susceptibility gene for childhood asthma in a genome-wide association study (GWAS) [24, 25]. Since its neighboring gene *GSDML* is co-regulated with *ORMDL3* [24, 26], making it difficult to clearly distinguish the effects of these two genes, it has been suggested to refer to the term "17q21 asthma locus". Several investigations were able to confirm this association [27-29]. Besides the 17q21 locus, meta-analysis of asthma GWAS suggest three more gene loci to be considered asthma susceptibility genes: *IL1RL1/IL18R1*, *TSLP* and *IL33* [30-32]. Asthma-associated genetic variants were also found in and close to *TLR1*, *TLR6* and *TLR10* [33, 34]. Furthermore, mutant alleles in the *filaggrin* gene were primarily

found to be associated with atopic dermatitis [35] and asthma, but only with concomitant atopic dermatitis [36, 37]. Interleukin-13 (IL-13) is a mediator of allergy produced by Th2 cells and several reports indicate that polymorphisms in the *IL-13* gene are associated with asthma and/or atopy [38-40].

More than 100 candidate genes are currently discussed, but most of them require further analysis and systematic studies to determine whether they really can be considered as susceptibility genes.

Sex and sex hormones might also contribute to the pathogenesis of asthma, but their relation remains still to be determined. Among school children and at puberty the prevalence of asthma is higher in girls, whereas early in life the disease is recognized to occur predominantly in boys [41].

Environmental factors

Depending on the agent, environmental influence on the maturing immune and organ system of children can both be protective or harmful.

The noxious effect of tobacco smoke, especially in early childhood, could be demonstrated by numerous studies and represents a crucial risk factor for the onset of many diseases, including wheezing and asthma [42-44]. Environmental tobacco smoke not only increases the prevalence of asthma but also the severity of the disease [45]. Furthermore, active smoking is associated with asthma in adults and adolescents [46, 47]. Also industrial and traffic air pollution during infancy contributes to the development of childhood asthma [48, 49].

Protective effects however, seem to emanate from the exposure to antigens or microbial products, childhood infections and bacterial endotoxin – in short, agents that are considered “unhygienic” in today’s industrialized lifestyle. While rising standards of household cleanliness, semi sterile diets, improving health care and declining family size are part of today’s Western lifestyle, this trend leads to reduced contact to antigens and microbes in early childhood. Simultaneously, the prevalence of asthma increased constantly during the past decades in developed industrialized countries [50, 51]. With rising standards of living and improved hygienic conditions, the prevalence of this disease also seems to increase in developing countries [50, 52]. The contributing factors and

underlying immunological mechanisms of this observation shall be argued in the next chapter (1.1.3.2).

1.1.3.2 The hygiene hypothesis

Microbes and other environmental antigens contribute materially to the onset of asthma in childhood. In fact, less exposure to these pathogens in early childhood is linked to a rise of atopy and allergic diseases during the past decades. This observation is referred to as the hygiene hypothesis and was first introduced by D. Strachan in 1989. In his study, Strachan followed a large sample of British children from birth until the age of 23 and was able to show that their risk to develop hay fever was inversely related to the number of children in the household [13]. He reasoned that infections in early childhood, possibly transmitted by older siblings, might have a preventive effect on later allergic diseases. Empirical findings of several studies supported this observation and confirmed that a higher number of siblings might have protective effect not only against hay fever but also against allergic eczema and asthma [14, 53, 54].

The role of recurrent infections in the development of childhood asthma is still not definitely determined. There is widespread evidence that certain infections, including measles, and exposure to infectious agents early in life protect against asthma [15-17]. Severe infections with respiratory syncytial virus in early childhood, however, are associated with an increased risk for the onset of asthma and allergic sensitization [14, 18].

Growing up in the environment of traditional farms, where exposure to a large range of microbes is typical, was also found to protect from childhood asthma and atopy [55-57], supporting the principles of the hygiene hypothesis. However, the term “farming” can cover a variety of dissimilar surroundings and one farm is not just like another: Children exposed to highly modernized concentrated animal feeding operations, mostly common in the USA, have an increased risk to develop asthma (but not atopy or allergies in general) [58, 59]. In fact, a greater diversity of microbial agents afforded on farms is associated with the protection from asthma [60].

Soon, immunological mechanisms were sought to explain and combine the findings of the hygiene hypothesis with the development of atopic asthma. In this regard, the maturation process of T helper cell responses in early life was found to play a major role. In the immune system, T cells are, beside B cells, one of the main types of lymphocytes and hold a key part in cell-mediated immunity. Another type are natural killer (NK) cells which have the same progenitors as T and B cells and are able to kill malignant or virus infected cells by secreting cytotoxic granules. T cells originate from the bone marrow but have to migrate to the thymus where naïve $CD4^+$ T cells differentiate into different T lymphocyte subsets such as Th1, Th2, Th17 or regulatory T cells (Tregs). The direction of the complex maturation process takes place according to the tissue cytokine context. It furthermore depends on the activation of specific translation factors and is influenced by surrounding partner cells secreting certain cytokines. The various subgroups of $CD4^+$ T cells show distinct cytokine expression profiles and carry out very different functions. Cytokines occupy a central position as they are both produced by the T lymphocytes to mediate many of their specific functions and also in turn influence the polarization and maturation of these T cell subsets. In brief, Th1 cells mainly produce IL-2 and IFNγ and play a pivotal role in cell-mediated immunity by stimulating phagocytosis. A Th1 predominated immune profile is associated with autoimmune diseases. Th2 cells initiate the humoral immunity via B cell activation and can promote allergic responses. In order to mediate allergic reactions these cells secrete IL-4, -5, -6, -9, -10 and -13. Accordingly, a predominant Th2 profile is associated with allergic asthma. Th17 cells secrete IL-17 and IL-22, play a crucial role in acute inflammation and are implicated in autoimmune reactions. Tregs however, are responsible to suppress T cell activity (once the pathogen has been eliminated) rather than inducing it. Thereby they maintain immunotolerance and prevent autoimmunity. Tregs produce TGF-β and IL-10. Of course a well-functioning immune system implies T cells and cytokines work together in a complex network with constant interaction.

In the newborn period the innate immune system begins to shift from a fetal Th2-dominated phenotype towards an adult, more balanced immune profile with increased Th1 reactivity [61, 62]. During this vulnerable time frame and already during pregnancy, various stimuli such as exposure to microbes, pathogens and viral infections are pivotal to induce this gradual maturation of the Th1 response [63, 64].

Absence of exposure to these agents is subsequently associated with a delay of T cell maturation and a tendency for the predominant Th2 profile to persist. This imbalance in favor of Th2 cells has been shown to play a determining role in the onset of atopy and allergic inflammation later in life.

Investigations in mouse models showed that cell transfer and overexpression of Th2 cytokines in murine airway epithelial cells lead to asthma symptoms such as increased secretion of mucus, AHR and eosinophilia [65, 66]. In fact, Th2 but not Th1 cells could be directly linked to the onset of AHR [67]. In humans, Th2 lymphocytes and Th2-like cytokines, mainly IL-4 and -5, were found to be increased in the bronchial mucosa of asthma patients and contribute to the development of the disease [68, 69]. Elevated levels of allergen induced IL-4 and IL-9 were detected in children with atopic asthma [70]. A primarily Th2-orientated immune system lays groundwork for the production of IgE antibodies to environmental antigens and subsequent development of atopic sensitization. The mechanism is initiated by Th2 cells secreting IL-4, thereby inducing an isotype switch and IgE production in B cells. T cell cytokines IL-13 and IL-9 further enhance IgE synthesis [71-73]. Th1 cells however are able to downregulate IgE synthesis by producing IFNγ. As mentioned previously, Tregs function as opponents as they secrete IL-10 to inhibit both Th1 and Th2 cell signaling and IgE production [72, 74]. As IgE activates its receptor, e.g. on mast cells, a wide range of mediators are released which promote the secretion of pro-inflammatory cytokines and initiate typical asthma symptoms such as bronchial spasm [75-77].

In summary, the hygiene hypothesis postulates that exposure to an environment rich in microbes in early childhood influences the maturation of the

immune system, promoting a “non-allergic” pathway, resulting in a decreased risk of asthma and other allergic diseases. An imbalance in T cell responses with a predominant Th2 profile is associated with the onset of asthma. However, many aspects of this theory are still fiercely debated. Although there is widespread evidence for this hypothesis being a crucial underlying cause for the rising of atopy in developed countries, the hygiene hypothesis should not be regarded as the sole explanation for the increasing and constantly high prevalence of asthma.

1.1.3.3 Pathophysiology

The interaction of genetic predisposition and further predisposing factors in the first years of life, eventually leading to the development of airway inflammation and asthma, is heterogeneous and still not fully understood. It is, however, generally agreed that inflammation plays a central role in the pathogenesis and pathophysiology of asthma. Trigger factors (Table 1 in chapter 1.1.3.4) are relevant when encountering hyperresponsive airways, initiating complex immunological and physiological mechanisms [78]. In brief, inhaled antigens activate mast cells in the airway. The activated cells secrete mediators such as histamine and leukotrienes, inducing not only bronchoconstriction but also promoting inflammation [79, 80]. Antigens further activate Th2 cells, which thereupon release cytokines such as IL-4, -5 and -13. As already described, some Th2 cytokines initiate IgE synthesis in B cells. IL-5 induces differentiation of eosinophils in the bone marrow. Eosinophils then enter the inflamed airway, release yet more pro-inflammatory cytokines and are kept alive by IL-4 contact. In addition, macrophages, smooth muscle cells, fibroblasts and also airway epithelial cells produce inflammatory mediators that amplify the inflammatory response [78, 81].

As in every inflammatory reaction, increased blood flow occurs, causing edema and swelling of the mucosa. Smooth muscle cells surrounding the bronchioles tighten and goblet epithelial cells produce more viscous thick mucus. Some patients can develop persistent changes in airway structure in later years, referred to as airway remodeling. These permanent alterations may include

smooth muscle hyperthrophy, thickening of the sub-basement membrane, sub-epithelial fibrosis and angiogenesis [78].

All these mechanisms cause airflow limitation by reducing the diameter of the airway where even a small decrease has a huge impact on airflow resistance and causes difficulty of breathing. Mainly expiration of breath is becoming difficult for asthma patients and the typical “wheezing” occurs. The less air the patient is able to breathe out, the less fresh air and oxygen he is able to inhale. This increasing breathlessness can lead to fear of asphyxiation, resulting in increasing stress, panic and even more severe airway obstruction. To early stop or preferentially to prevent this vicious circle, asthma needs to be treated and managed carefully.

Asthma medications include short- and long-term controller medications as well as rescue drugs. Inhaled corticosteroids dampen inflammation, are anti-allergic and immunosuppressive and therefore represent the foundation to provide good asthma control. In order to counteract the worsening of bronchial spasm, bronchodilators stimulate β2-receptors (therefore also referred to as β2-agonists) who are responsible for relaxing the tightened smooth muscles around the airways. Short-acting β2-agonists are taken as needed, quickly relieve or prevent asthma complaints and serve as rescue inhalers. Long-acting β2-agonists can be used in combination with inhaled corticosteroids in advanced stages of the disease to control chronic symptoms and prevent asthma attacks [10]. Leukotriene modifiers can serve as an alternative for some patients. These receptor antagonists block pro-inflammatory leukotrienes and prevent asthma symptoms [82]. Further alternative medications for moderate to severe persistent asthma are theophylline (anti-inflammatory, bronchodilatory) and omalizumab (a monoclonal IgE-antibody) [10].

Various therapeutic options are available to treat asthma, but most drugs have significant side effects or are not suitable for younger children. Although the knowledge about pathological mechanisms of the disease as well as research and development of new asthma drugs improved rapidly during the last years, severe or therapy-resistant asthma is still a major burden. In fact, some studies

estimate 5% of all asthma patients to suffer from difficult or drug-resistant asthma and new, targeted treatment approaches are necessary to mediacte these patients [83].

1.1.3.4 Trigger factors

Asthma exacerbations are caused by excessive reaction of the airways to various stimuli. Table 1 states common allergic triggers, leading to airway inflammation and smooth muscle contraction, and non-allergic triggers. These non-allergic triggers usually don't initiate inflammation but provoke irritable airways. Furthermore some drugs can trigger or induce asthma, such as NSAIDs (e.g. aspirin) or β-blockers [10]. Recurrent viral infections are the most common initial triggers in infants and toddlers [84, 85].

Table 1 Allergic and non-allergic factors that may trigger or worsen asthma symptoms

Allergic triggers	Non-allergic triggers
Pollen	Viral infections
House dust mite	Cold, dry air
Animal dander	Tobacco smoke
Molds	Exercise
Cockroach	Stress
Insect allergens	

1.2 The Toll-like receptor family

Toll-like receptors (TLRs) owe their name to the famous *Drosophila* receptor protein Toll, discovered by C. Nüsslein-Volhard in the early 1980s [86]. First identified to play a crucial role in the embryonic development of *Drosophila melanogaster*, years later Toll was furthermore found to be involved in several host-defense mechanisms. Soon, homologs of Toll, subsequently referred to as

Toll-like receptors, were also detected in mammalians. In 1997 the first human Toll protein and its capability to activate transcription factor NFκB was described by R. Medzhitov et al. [87]. Investigations in the field of TLRs during the following years resulted in the characterization and identification of a whole family of TLRs and many of their ligands. To date 10 functional TLRs in humans and 12 in mice have been discovered. Some of them are located on the cell-surface whereas others operate as intracellular receptors in endosomal membranes. Toll-like receptor proteins are expressed by many cell types, such as macrophages, dendritic cells, epithelial cells and other immune cells, where every TLR is dedicated to detect a distinct set of microbial ligands. Table 2 states ligands and cellular distribution of all 13 TLR proteins. In short, plasma membrane localized TLRs sense proteins and lipids, whereas those located in the membranes of endosomes are able to detect nucleic acids.

Table 2 Ligands and localization of TLRs

Adapted and modified from [88]

Toll-like receptor	Cellular distribution	Ligand
TLR1/2 heterodimer	Monocytes and mast cells, eosinophils, basophils, dendritic cells	Triacyl lipopeptides Lipomannans [89, 90]
TLR2/6 heterodimer	Monocytes and mast cells, eosinophils, basophils, dendritic cells	Diacyl lipopeptides Lipoteichoic acid Zymosan [91, 92]
TLR3 *intracellular*	NK cells	dsRNA [93]
TLR4	Macrophages and mast cells, eosinophils, dendritic cells	LPS [94]

Toll-like receptor	Cellular distribution	Ligand
TLR5	Intestinal epithelial cells	Flagellin [95]
TLR7 *intracellular*	NK cells, dendritic cells, eosinophils, B cells	ssRNA, bacterial RNA [96, 97]
TLR8 *intracellular*	NK cells, dendritic cells	ssRNA, bacterial RNA [96, 98]
TLR9 *intracellular*	Dendritic cells, eosinophils, basophils, B cells	unmethylated CpG DNA [99]
TLR10 (human only)	Dendritic cells, eosinophils, basophils, B cells	not determined
TLR11 (mouse only)	Macrophages, dendritic cells, epithelial cells	Profilin [100]
TLR12 (mouse only)	Macrophages, dendritic cells	Profilin [101]
TLR13 (mouse only)	not determined	rRNA [102, 103]

1.2.1 TLR structure and signaling

TLRs belong to the family of type 1 transmembrane proteins. They are characterized by an extracellular domain composed of leucine-rich repeats (LRRs) creating a horseshoe-shaped figure. The cytoplasmatic tail in mammalian TLRs consists of a Toll/IL-1 receptor (TIR) domain [104]. Whereas the LRRs represent a scaffold responsible for recognition and binding of ligands, the intracellular TIR domain interacts with adaptor proteins to induce a signaling cascade.

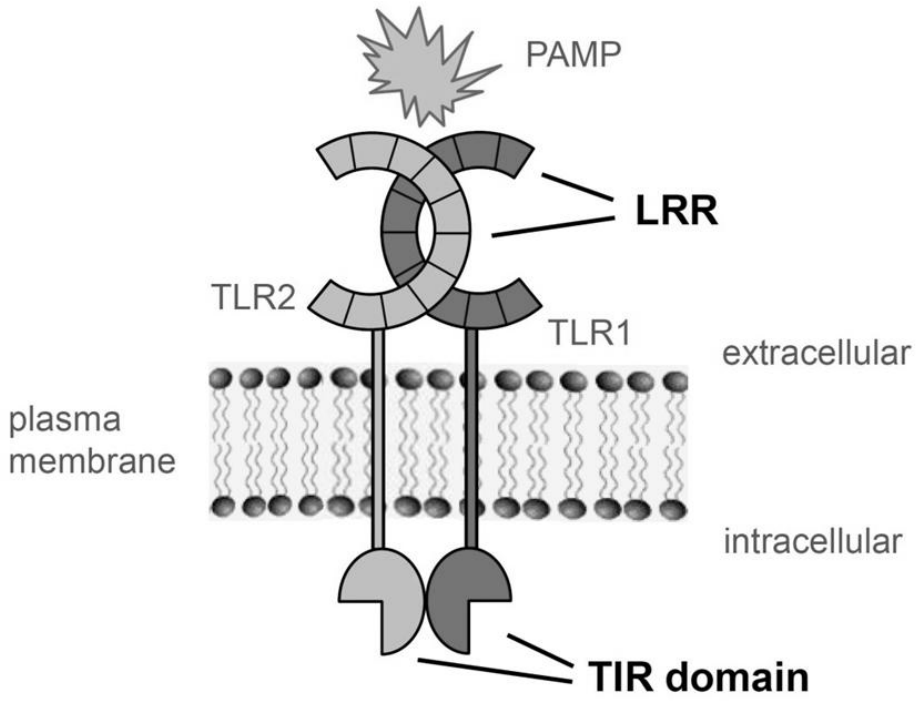

Fig. 2 Structure of the TLR protein

The structure of TLR proteins is shown, using the example of TLR1 and TLR2 forming a heterodimer. Binding of specific ligands induces dimerization of two TLRs, bringing their TIR domains close together to initiate the intracellular signaling pathway. LRR = leucine-rich repeat, TIR = Toll/IL-1 receptor

TLRs function as primary sensors of the immune system and are responsible for recognizing and responding to particular molecular pattern so called pathogen-associated molecular patterns (PAMPs) that are not naturally appearing in cells of healthy vertebrates. In order to identify and bind their specific ligands, TLRs form dimers, resulting in convergence of both the LRR and TIR region. Most TLRs act as homodimers to initiate the intracellular signaling pathway, however, TLR1 and TLR2 as well as TLR2 and TLR6 form heterodimers. Furthermore TLR heterodimerization of TLR4/6 with CD36 as a co-receptor has been described, promoting the activation of proinflammatory mediators [105]. Recent studies suggest heterodimerization of TLR2/4, which is thought to play a crucial role in inflammatory intracerebral hemorrhage [106].

Murine TLR11 and TLR12, both able to detect *T. gondii* protein profilin, are newly also assumed to form heterodimers [101].

Binding of the ligand to the extracellular TLR site initiates dimerization of two single receptors. Dimerization is a prerequisite to activate the signaling cascade (Fig. 3) as it allows close interaction of the two intracellular TIR domains. However, in the case of TLR4, the accessory proteins MD-2 and CD-14 are necessary for the recognition of its ligand LPS (Table 2) and activation of TLR4. Once activated, the receptors' TIR domains interact with both each other as well as with TIR domains of cytoplasmatic adaptor molecules. Mammalian TLRs use combinations of the following four different adaptor molecules to trigger the signaling pathway: TRIF (TIR domain-containing adaptor-inducing IFNβ), TRAF (TRIF related adaptor molecule), MyD88 (myeloid differentiation factor 88) and MAL (MyD88 adaptor-like). These molecules are able to initiate a cascade of events, including various enzymatic reactions, resulting in the activation of different transcription factors. With the exception of TLR3, MyD88 is a common adaptor molecule and involved in all TLR signaling pathways.

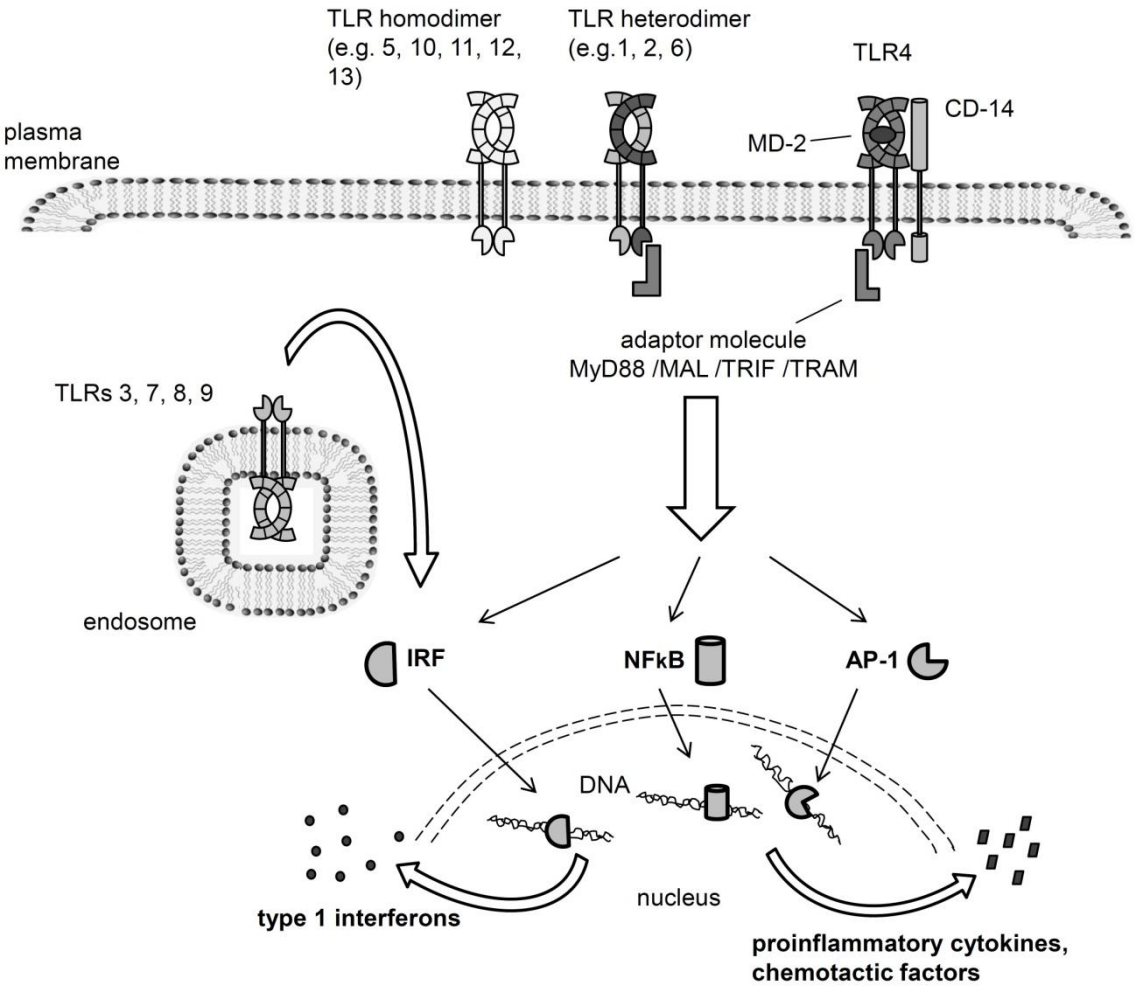

Fig. 3 Signaling pathway of TLRs

After ligand-induced dimerization, TLRs activate adaptor molecules (which are MyD88, MAL, TRIF and TRAM) in order to trigger gene transcription through transcription factors IRF, NFκB or AP-1. The cell consequently releases either antiviral type 1 interferons IFNα and IFNβ or proinflammatory cytokines.

Endosomal TLRs, mostly targeted and activated by viral components, aim at recruiting transcription factor IRF (interferon regulatory factor). IRF is responsible for inducing the production of antiviral type 1 interferons IFNα and IFNβ. All other TLRs are in addition capable of activating both NFκB as well as AP-1 (activated protein 1). These transcription factors induce the expression of cytokine and chemokine genes, leading to secretion of proinflammatory cytokines and chemotactic factors [107].

The intracellular signaling pathway of TLRs shows their remarkable capability to modify gene expression and immune responses, thereby being able to respond both to viral and bacterial threats as needed.

1.2.2 TLRs and asthma pathogenesis

Since PAMPS are thought to play a crucial role in the development of asthma, the role of TLRs as goal keepers of the innate immune system becomes an essential one. Consequently, research in the past decades has focused on the role of TLRs in the pathogenesis of asthma, atopy and other diseases.

In order to find out, why some people are more likely to develop atopic diseases than others, genetic variations in TLR genes have been studied elaborately. Single nucleotide polymorphisms (SNPs) are a common type of such genetic variations within one population. A typical and frequent SNP, for example, is the replacement of the base cytosine with thymine in a genetic sequence. In fact, both protective and disease promoting modulations in TLR expression and genetic polymorphisms have been identified.

In 2008, various genetic alterations in TLR genes and their association with childhood asthma were analyzed in a large German case-control study [34]. Interestingly, protective effects on atopic asthma could be demonstrated for SNPs in *TLR1*, *TLR6* and *TLR10*, all capable of forming heterodimers with TLR2. These findings included increased levels of *TLR* mRNA as well as protein and Th1 cytokines. Secretion of IL-4, which is Th2 associated, was decreased [34], indicating a Th2 to Th1 shift. The protective role of TLR6 activation in asthma and the resulting immunoregulatory beneficial effects were later also demonstrated in a HDM-induced mouse model of asthma by Moreira and colleagues [108].

A genetic variant in *TLR2* was found to be associated with asthma susceptibility in farm children. Here, children carrying a certain allele in *TLR2* were less likely to show asthma or atopic symptoms [109]. Another group investigating genetic polymorphisms in *TLR2* and *TLR4* demonstrated their association with disease severity, but not with the onset of allergic asthma [110].

Genetic alterations in *TLRs* can also have adverse effects on asthma disease. A polymorphism in *TLR4* was shown to be associated with a 4-fold higher risk to develop asthma in Swedish school children [111]. Increased expression of TLR2, 3 and 4 in airways was witnessed in victims of fatal asthma when compared to deceased control subjects [112]. An adverse effect on the development of atopic sensitization was demonstrated for a mutation in TLR2, relevantly impairing its receptor activity [113]. Evidence indicates that TLR5 seems to play a major role in the onset of allergic asthma. Interaction with its ligand bacterial flagellin lays groundwork for allergic sensitization [114].

As mediators of inflammation, TLRs have great impact on development and severity of a broad variety of other diseases aside from asthma. TLR function and TLR polymorphisms influence sarcoidosis, tuberculosis and COPD but also non-pulmonary diseases such as type 1 diabetes, atherosclerosis, leprosy and intestinal disorders [115-121].

The complex involvement of different TLRs in the pathogenesis of asthma and other diseases is still not fully understood. Still, these research results allow optimistic conclusions regarding the development of potential drugs and therapies targeting TLRs.

1.3 mRNA transcript therapy

The potential and applications of gene therapy in the field of immunology have been of great interest in recent years. One strategy consists of utilizing replication-deficient viruses as vectors for therapeutic genes [122, 123]. Despite high transfection efficiency many concerns are associated with this method since genomic integration as well as unwanted immunostimulation and even induction of oncogenesis remain problematic [124]. Although nonviral-vector mediated gene transfer is thought to be less immunostimulative, insufficient gene transfer efficiency often leads to failure in clinical trials [125].

As a nonviral alternative, the delivery of mRNA has become a promising therapeutic approach during the past decade. Since mRNA doesn’t enter the nucleus, the risk of genomic integration and the role of the nuclear envelope as

a major obstacle are not relevant anymore. The possibility to administer mRNA repeatedly and its effectiveness even in non-dividing cells are two more central advantages.

Still, also *in vitro* transcribed mRNA can trigger severe, undesirable immune responses. Besides its immunogenicity, mRNA in a conventional form is instable and labile, leading to early decay of the administered mRNA product in the target cell. However, with the discovery of the tool to chemically modify *in vitro* transcribed mRNA, both of these threats can be circumvented. With the incorporation of modified nucleotides such as pseudouridine [126] or 2-thiouridine (s2U) and 5-methyl-cytidine (m5C) [127], mRNAs have a higher translational capacity and are less immunogenic. In fact, replacement of 25% of uridine and cytidine with s2U and m5C was able to hinder binding of mRNA to pattern recognition receptors such as TLRs 3, 7, and 8, therefore reduced activation of immune responses *in vivo* [127, 128].

The therapeutic potential and efficacy of modified mRNA has been successfully shown in the case of the lethal congenital disease of surfactant protein B (SP-B) deficiency. Here, administration of chemically modified SP-B mRNA showed life prolonging effect *in vivo* as it was able to restore therapeutic relevant levels of SP-B [127]. Delivery of mRNA-encoded T cell transcription factor Foxp3 represents another successful example as a potential treatment strategy for pulmonary diseases. Administration of *Foxp3* mRNA rebalanced T helper cell responses and significantly improved the asthmatic phenotype in two distinct murine models of allergic asthma [129].

mRNA-based gene transfer represents a promising therapeutic tool for diseases and genetic disorders with limited treatment options. As a relatively new drug class, *in vitro* transcribed mRNA is already investigated in a broad application area. Beyond replacement and substitution of proteins *in vivo*, chemically modified mRNA encoding nucleases is recently even opening new perspectives in the field of genomic engineering [130].

1.4 Aim of this study

The aim of this study is to investigate whether preventative treatment of mice with chemically modified *Tlr1, 2* or *6* mRNA in an asthma model improves lung function and airway inflammation when compared to untreated controls. Consequently, it was examined how intrapulmonary application of combinations of chemically modified *Tlr* mRNA *in vivo* modulates immune response, lung function and inflammation in an HDM driven murine asthma model.

2 Materials and Methods

2.1 Cloning Strategy

Molecular cloning describes the process of moving any piece of DNA from one plasmid vector to another. Since restriction enzymes are playing a key role not only in isolating the DNA fragment but also in opening the plasmid vector, this method is also called “Plasmid Cloning by Restriction Enzyme Digest” or “Subcloning”. Once the specific piece of DNA, also referred to as “insert” or “Gene of Interest (GOI)”, has been successfully moved to the target vector, it can be easily amplified, stored, sequenced or used for expression studies. In the present study, the GOI *Tlr1, 2* or *6*, was cloned into the plasmid vector downstream of a T7 promoter and followed by a 120 bp polyA site. The T7 promoter sequence represents the starting point for the T7 polymerase to start with RNA synthesis. The polyA tail consists of multiple adenosine monophosphates and protects the mRNA from enzymatic degradation. Furthermore the polyA tail facilitates transcription termination and translation [131]. In this study *Tlr1, 2* and *6* genes initially were encoded on plasmid vectors with a pcDNA3.1 vector backbone (Table 3). Fig. 4 illustrates the steps of the cloning strategy to isolate *Tlr* inserts, move them to a standardized T7 promoter-containing pVAX vector to finally produce mRNA via *in vitro* transcription (chapter 2.2). *Tlr* sequences are stated as supplemental information.

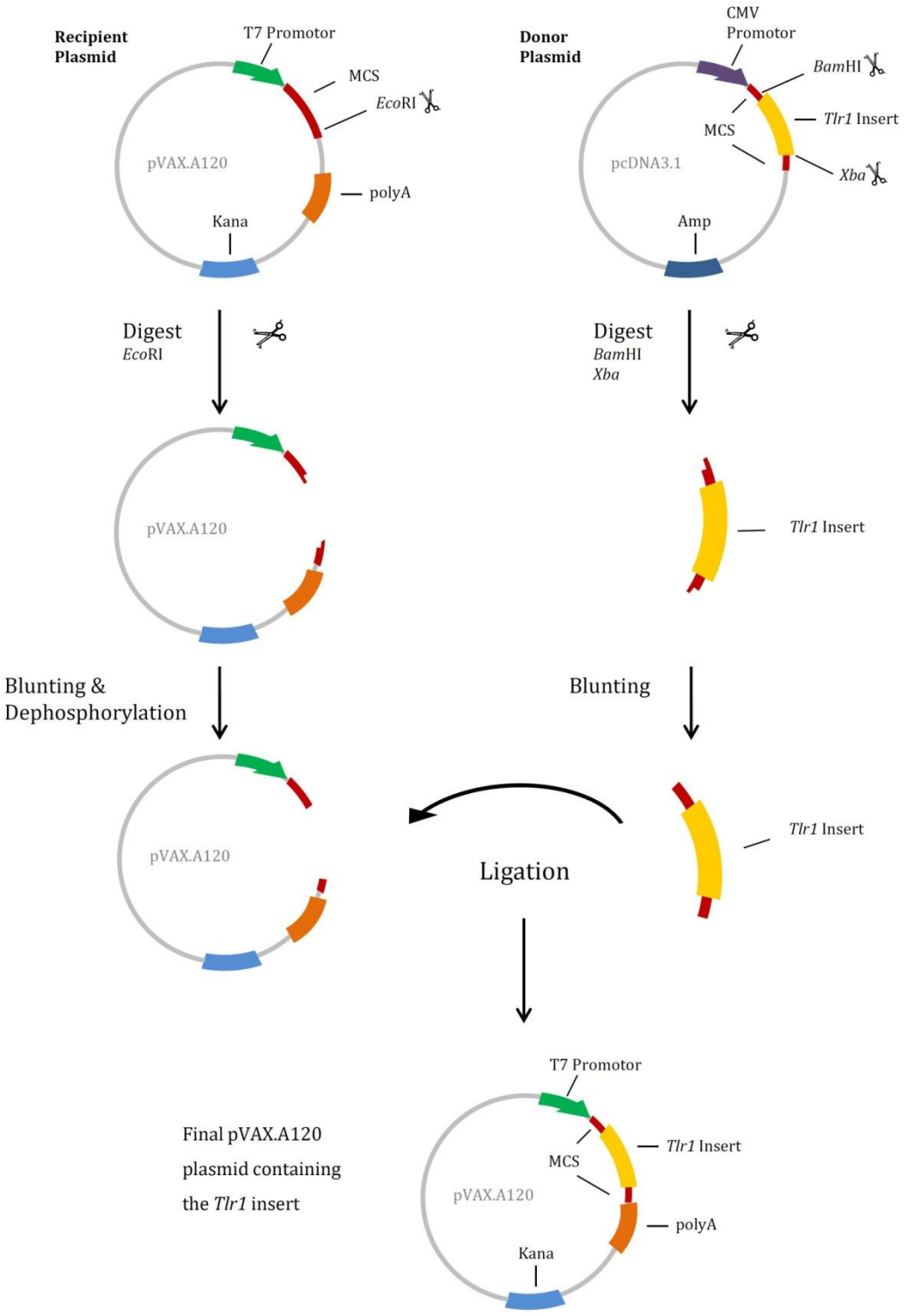

Fig. 4 Plasmid Cloning by Restriction Enzyme Digest

The graph illustrates the cloning strategy to move a *Tlr* insert from a pcDNA3.1 plasmid vector to the target vector pVAX.A120 using the example of the *Tlr1* insert. Labeled on the plasmid vectors are the promoter, the multiple cloning site (MCS) including various restriction sites (relevant restriction sites are indicated with scissors), the polyA tail (polyA), the *Tlr* insert and the resistance gene for ampicillin (Amp) or kanamycin (Kana) respectively.

Table 3 Original plasmids encoding mouse *Tlr1, 2* and *6*

Plasmid	Gene	Insert size	Backbone resistance	Supplier
pcDNA3.1_Tlr1	*Tlr1*	2388 bp	Ampicillin	Stefan Bauer, Marburg
pcDNA3.1_Tlr2	*Tlr2*	2300 bp	Ampicillin	Addgene, PI: Ruslan Medzhitov
pcDNA3.1_Tlr6	*Tlr6*	2421 bp	Ampicillin	Stefan Bauer, Marburg

2.1.1 Generation and transformation of chemically competent *E. coli*

Generation of chemically competent *E. coli*

Some bacteria are naturally capable of absorbing foreign DNA. It is, however, possible for other bacteria to gain this ability, called competence, via a special treatment. In this case the rubidium chloride method was used, which is a variant of the calcium chloride method yielding even higher competence [132].

In order to generate chemically competent bacteria, 500 ml LB Medium were inoculated with 3 ml of the respective *E. coli* strain. Bacteria were then grown up to an optical density OD_{600}=0.6-0.7 and afterwards centrifuged at 300 rpm for 8 min at 4°C. After resuspending the cell pellet in prechilled Buffer RF1 (Table 4), bacteria were incubated on ice for 15 min before centrifugation at 3000 rpm for 8 min at 4°C. The cell pellet was resuspended in Buffer RF2 (Table 5) and incubated on ice for another 15 min. The suspension was then resuspended again, aliquoted (100 µl) and immediately frozen in liquid nitrogen for storage at -80°C.

Heat shock transformation of chemically competent *E. coli*

Chemically competent *E. coli* were transformed with a plasmid (e.g. the initial plasmids, Table 3, or later the ligation product, 2.1.6) using the heat shock

method. 10 µl chemically competent *E. coli* were thawed on ice, gently mixed with ~10 ng of plasmid DNA and incubated on ice for 30 min. The suspension was then exposed to a heat shock at 42°C in water bath for 2 min, resulting in the bacteria membrane to release lipids and become porous. Furthermore the depolarization of bacterial cell membranes induced by heat shock leads to lower negative intracellular potential and facilitates therefore the uptake of negatively charged DNA into the competent cell. Following heat shock, bacteria were gently resuspended in 900 µl of SOC Medium and incubated on ice for 5 min. The suspension was then incubated for 2 hours at 37°C and 100 rpm vigorous shaking before centrifugation at 6000 rpm for 3 min. The supernatant was decanted and the pellet resuspended in the remaining fluid before being plated on agar plates containing the suitable antibiotic to select for the transformed bacteria. Agar plates were then incubated over night at 37°C to allow for growth of bacterial colonies.

Table 4 Ingredients of Buffer RF1

Buffer RF1	
Rubidium chloride	12 g
Manganese chloride tetrahydrate	9.9 g
Potassium acetate 1M	30 ml
Calcium chloride dihydrate	1.5 g
Glycerin	150 g
Dist H_2O	*ad* 1000 ml
Acetic acid	*ad* pH 5.8

Table 5 Ingredients of Buffer RF2

Buffer RF2	
Morpholino propanesulfonic acid	20 ml
Rubidium chloride	1.2 g
Calcium chloride dihydrate	11 g
Glycerin	150 g
Dist H_2O	*ad* 1000 ml
Sodium hydroxide	*ad* pH 5.8

2.1.2 Isolation of plasmid DNA

Plasmid DNA was isolated from recombinant *E. coli* using either the Metabion Plasmid Miniprep Kit for small amounts or the QIAGEN Plasmid Mega Kit to receive large amounts of plasmid DNA. These methods were performed according to the manufacturer's instructions as described in Table 3.

In principle, both Kits are based on the method of bacterial lysis under alkaline conditions in the presence of SDS, first published by Birnboim and Doly [133]. In this method, plasmid DNA as well as chromosomal DNA is denatured in the presence of an alkaline buffer. Due to subsequent neutralization, plasmid DNA becomes selectively renatured. However, chromosomal DNA remains single stranded, thereby forming an insoluble precipitate. Soluble plasmid DNA then undergoes several purifications steps before being eluted and redissolved in TE Buffer.

Table 6 Protocol to isolate plasmid DNA using a Mini or Mega Kit

	Plasmid Mini Kit	Plasmid Mega Kit
Supplied by	Metabion international AG, Martinsried, Germany	Qiagen GmbH, Hilden, Germany
Bacteria culture	3-5 ml	500 ml
Harvest of bacteria	The overnight culture was centrifuged at 5000 x g for 10 min and the supernatant was decanted.	The overnight culture was centrifuged at 6000 x g for 15 min and the supernatant was decanted.
Alkaline lysis	The bacterial pellet was resuspended in 200 µl Buffer MX1, 250 µl of Buffer MX2 were added and inverted 4-6 times. The solution was incubated at room temperature for 1-5 min before addition of 350 µl MX3 Buffer. Centrifugation at 10,000 x g for 5-10 min.	The bacterial pellet was resuspended in 50 ml Buffer P1, 50 ml Buffer P2 were added, inverted 4-6 times and incubated at room temperature for 5 min before addition of 50 ml prechilled Buffer P3. The solution was inverted 4-6 times and incubated on ice for 30 min before centrifugation at 20,000 x g for 15 min.
Equilibrate tips	-	35 ml Buffer QT were applied on the tip.
Binding mechanism	After centrifugation the supernatant was carefully transferred into the column and centrifuged at 7000 x g for 1 min. The flow-through was decanted.	After centrifugation the supernatant was carefully transferred on the tip.

	Plasmid Mini Kit	**Plasmid Mega Kit**
Washing step (1)	500 µl Buffer WN were applied on the tip, centrifuged at 7000 x g for 1 min before application of 700 µl Buffer WS. The solution was again centrifuged at 7000 x g for 1 min. Additional centrifugation at 10,000 x g for another 3 min ensures removal of residual ethanol.	200 ml of Buffer QC were applied on the tip. The flow-through was discarded.
Elution of DNA	DNA was eluted with 30 µl elution buffer, incubated at room temperature for 3 min and centrifuged at 10,000 x g for 3 min. This step was repeated with another 30 µl elution buffer.	DNA was eluted with 30 ml of prewarmed (50°C) elution buffer.
Precipitation	-	20 ml isopropanol were added before centrifugation at 15,000 x g for 30 min at 4°C, the supernatant was decanted.
Washing step (2)	-	The pellet was washed with 7 ml of 70% ethanol and centrifuged at 15,000 x g for 30 min at 4°C, the supernatant was again decanted carefully.

	Plasmid Mini Kit	Plasmid Mega Kit
Drying step	-	The pellet was allowed to air dry and subsequently redissolved in 100 µl TE-Buffer.

2.1.3 Determining the concentration of nucleic acids

The concentration of DNA or RNA in aqueous solution was determined by measuring the absorbance at λ = 260 nm using a nanophotometer. Further measurements at λ = 280 nm allow for detection of protein. For pure DNA samples with low protein contamination the ratio OD_{260}/OD_{280} should yield results between 1.8 and 2.0 whereas pure RNA samples are required to yield an A260/230 ratio of not less than 2.0.

2.1.4 Restriction enzyme digestion and DNA purification

Restriction endonucleases are enzymes that cut DNA in a site-specific manner by catalyzing the hydrolysis of the DNA phosphodiester bond. Specific DNA sequences where restriction enzymes bind are usually four to eight bp long and often palindromic. Restriction enzymes produce either sticky ends by cutting double strand DNA in a staggered manner within the recognition site (resulting in single-stranded DNA ends) or blunt ends by cutting symmetrically with no overhangs being generated.

In molecular biology, restriction enzymes are frequently used to analyze plasmid DNA, to cut out a certain sequence from a plasmid vector or to prepare DNA fragments for ligation.

As soon as the plasmid vectors used for this study (pcDNA3.1_Tlr1, pcDNA3.1_Tlr2 and pcDNA3.1_Tlr6) were isolated and purified, control digestions and subsequent sequencing were performed to ensure that all plasmids contain the correct gene of interest. During control digestion procedure, the plasmid was digested with two restriction enzymes while the

expected length of digestion products was first calculated *in silico* using ApE software (ApE – A plasmid Editor vs2.0.45). In case of the gene of interest's correct sequence and orientation the calculated bands should correspond to the bands appearing during gel electrophoresis (chapter 2.1.5). Control digestions were also performed after ligation procedure to verify correct orientation of the gene of interest.

Further restriction enzyme digestions in this study were performed in order to:

- cut out the *Tlr* inserts from the pcDNA3.1 vector,
- linearize the pVAX.A120 vector in order to ligate the *Tlr* gene into the target vector and
- finally linearize the pVAX.A120_Tlr1, 2 or 6 vectors for subsequent mRNA production.

In our study the protocol described in Table 7 was used to perform restriction enzyme digestions. Table 8 states the specific restriction enzymes used in this study.

Table 7 Standard protocol for restriction enzyme digestion

Reagent	Volume	Supplier
Plasmid DNA	equal to ~500ng	Self generated
Buffer CutSmart	5 µl	New England Biolabs Inc., Ipswich, UK
Restriction Enzyme	1 µl per enzyme	New England Biolabs Inc., Ipswich, UK
H_2O	*ad* 50 µl	
Incubation	1-2 hours at 37°C	

Table 8 Application of different restriction enzymes for cloning procedure

Intention	Restriction Enzyme	Supplier
Control digestion of all pcDNA3.1 plasmids containing a *Tlr* sequence	*Nhe*I, *Not*I	New England Biolabs Inc., Ipswich, UK
Cutting out *Tlr* sequences		New England Biolabs Inc., Ipswich, UK
pcDNA3.1_Tlr1	*Bam*HI, *Xba*I	
pcDNA3.1_Tlr2	*Spe*I HF, *Xho*I	
pcDNA3.1_Tlr6	*Hind*III HF, *Xho*I	
Opening of pVAX.A120 vector in order to insert a *Tlr* sequence	*Eco*RI HF	New England Biolabs Inc., Ipswich, UK
Control digestion after ligation		New England Biolabs Inc., Ipswich, UK
pVAX.A120_Tlr1	*Hind*III HF	
pVAX.A120_Tlr2	*Eco*RI HF	
pVAX.A120_Tlr6	*Xba*I	
Linearization of pVAX.A120 containing the respective *Tlr* sequence in order to produce mRNA	*Xho*I	New England Biolabs Inc., Ipswich, UK

After incubation at 37°C for one to two hours the digestion reactions were either heat inactivated, if the restriction enzyme allows doing so, or purified by using the NucleoSpin Gel and PCR clean-up kit (Machery-Nagel GmbH, Düren, Germany). This method purifies DNA fragments from enzymatic reactions by means of silica based membranes. DNA is first mixed with a chaotropic salt containing Buffer to allow the DNA to bind to the silica membrane of a spin column. Chaotropic salt furthermore helps to dissolve agarose in case of purifying DNA fragments out of agarose gels. Washing steps with ethanolic Buffer ensure removal of contaminations while subsequent DNA elution is

carried out under low salt conditions with alkaline Buffer. Purification of enzymatic reaction products was assayed according to the manufacturer's instructions as described in Table 9.

Table 9 Standard protocol to purify enzymatic reaction products

Bind DNA	One volume reaction solution was mixed with two volumes of Buffer NTI and applied on the spin column
Centrifugation at 11,000 x g for 30 sec, flow-through discarded	
Washing step (performed twice)	700 µl Buffer NT3 was applied on the column
Centrifugation at 11,000 x g for 30 sec, flow-through discarded	
Drying step	Centrifugation at 11,000 x g for 1 min
Incubation at 70°C for 3 min to remove residual ethanol	
Elution Step (performed twice)	15 µl of up to 70°C preheated Buffer NE was applied to the column and incubated at room temperature for 1 min before centrifugation for 1min at 11,000 x g.

Correct digestion was subsequently verified by performing gel electrophoresis to analyze length of DNA fragments as described in 2.1.5.

2.1.5 Gel electrophoresis and isolation of DNA fragments from agarose gels

Mixtures of linearized DNA or RNA strands can be sorted and measured by agarose gel electrophoresis according to their size and charge.

Agarose was dissolved in TAE buffer at the appropriate concentration (1% agarose for DNA and 1.5% for mRNA samples) in a microwave oven. Subsequently 5 µl of 1x GelRed (GelRed Nucleic Acid Stain, 10,000x in water) were added, a fluorescent dye to detect nucleic acids. The solution was then allowed to cool slightly and poured into a cast stuffed with a comb to create wells. Once the gel solid, the cast was filled with TAE buffer as the running buffer. Samples were mixed 6:1 with 6x loading dye to increase the density of

the samples and make them sink to the bottom of the well. DNA or mRNA samples were then loaded into the wells and electrophoresed at 70-100 V and 400 milliampere. Furthermore a 1 kb DNA marker (GeneRuler; Thermo Fisher Scientific Inc., Waltham, USA) or ssRNA marker (New England Biolabs Inc., Ipswich, UK) was loaded into one additional well and co-electrophoresed with DNA samples. These markers contain fragments of standardized length. Comparison with these markers facilitates to determine the size of the samples on the gel.

Bands on the gel were detected using UV light under a dark hood and documented by taking a picture using the Biostep Argus X1 software.

For further use of DNA fragments the desired band was cut from the gel, weighed and purified using the High Pure PCR Product Purification Kit from Roche Diagnostics GmbH, Germany. Therefore the gel slice was dissolved in 200 µl Binding Buffer for every 100 mg of agarose gel by vortexing briefly and incubating the solution for 10 min at 56°C. Further purification steps were performed as described in Table 9.

2.1.6 Dephosphorylation and ligation

Restriction enzymes used for this study (as stated in Table 8) all produce different sticky ends. However, in order to ensure standardized ligation conditions as described in Table 12, all DNA inserts and plasmid vectors require blunt ends.

Therefore plasmid vectors and DNA inserts were treated with a $Vent_R$ DNA Polymerase (New England Biolabs Inc.) as described in Table 10 prior to ligation. This polymerase fills in 5’ overhangs and removes 3’ extensions.

Linearized plasmid vector pVAX.A120 was subsequently dephosphorylated at the 5’ end by treatment with an alkaline phosphatase according to the protocol described in Table 11 to prevent religation of the vector with itself.

Table 10 Protocol to generate blunt DNA fragments and vectors

DNA	According to rate of yield after purification	
dNTPs	1.0 µl	PeqLab
$Vent_R$ Polymerase	0.5 µl	New England Biolabs Inc.
ThermoPol Buffer (10% of final volume)	6.0 µl	New England Biolabs Inc.
H_2O	*ad* 60 µl	
Incubation	15 min at 72°C	

Table 11 Protocol for dephosphorylation of a DNA vector

DNA vector	Volume according to rate of yield after purification	
Antarctic Phosphatase Reaction Buffer (10% of final volume)	4.0 µl	New England Biolabs Inc.
Antarctic Phosphatase	1.5 µl	New England Biolabs Inc.
H_2O	*ad* 40 µl	
Incubation	15 min at 37°C	
Heat inactivation	5 min at 65°C	

DNA inserts were then included into the pVAX.A120 vector by means of an enzymatic reaction with a T4 DNA ligase. The enzyme ligase catalyzes the formation of phosphodiester bonds and therefore allows the joining of DNA double-strands.

In the presented work the ligation calculator from *www.insilico.uni-duesseldorf.de/Lig_Input.html* was used to calculate the amount of insert while several vector to insert ratios were tested, such as 1:5, 1:10 and 1:20.

Table 12 Protocol for ligation

Plasmid vector pVAX.A120	10 ng	
DNA insert	depending on vector to insert ratio and size of insert	
T4 DNA Reaction Buffer (10% of final volume)	1 µl	New England Biolabs Inc.
T4 DNA Ligase	1 µl	New England Biolabs Inc.
H2O	*ad* 10 µl	
Incubation	Overnight at 16°C	

After ligation the samples were finally inserted in bacteria using the heat shock transformation method. Plasmids were again isolated and checked for insertion of the correct plasmid: only inserts that were included into the plasmid vector in correct strand orientation during ligation can result in correct triplets in subsequent translation in 3'-5' direction. For this purpose, plasmids from several bacterial clones were isolated and checked for correct sequence via restriction enzyme digestion (2.1.4) and additional sequencing (2.1.7) for final validation.

2.1.7 Sequencing

In order to verify DNA sequences of plasmid vectors or inserts, DNA fragments with correspondent primers (Table 13) were send to GATC Biotech, Konstanz, Germany, where all sequencing analysis for this study was carried out. Primers were designed *in silico* using the software *http://primer3.ut.ee/* and purchased from Metabion GmbH, Martinsried, Germany.

With ApE software, sequence data in .seq or .ab1 format was afterwards analyzed and compared to original sequences of plasmid vectors and *Tlr* gene sequences as indicated at *www.ncbi.nlm.nih.gov.*

Table 13 Primers used for sequencing in this study

Sequencing of original plasmid vectors was performed using primers stated in (A). The primers to verify the sequences of final pVAX.A120 plasmid vectors containing *Tlr* sequences are stated in (B). All primers are stated in 5'-3' direction.

A **Plasmid**	**Gene**	**Forward**	**Reverse**
pcDNA3.1_Tlr1	*Tlr1*	CMV fwd	BGH rev
pcDNA3.1_Tlr2	*Tlr2*	CMV fwd	BGH rev
pcDNA3.1_Tlr6	*Tlr6*	CMV fwd	BGH rev

B **Plasmid**	**Gene**	**Primer 1**	**Primer 2**	**Primer 3**
pVAX.A120_Tlr1	*Tlr1*	CCACTGCTTACTGGCTTATCG	CCTCCAGATAGTTTGGCATACG	CATTCCAATGCACATGTGAGC
pVAX.A120_Tlr2	*Tlr2*	CCACTGCTTACTGGCTTATCG	CCAGGTTCCAGTTTTCACCA	TTTTCTTTGTTCTTGCCTCGG
pVAX.A120_Tlr6	*Tlr6*	CCACTGCTTACTGGCTTATCG	CTGAGAGAATCGACAGGGAAGA	ACAGGTGTGACTACCCAGAA

2.2 *In vitro* transcription of chemically modified mRNA

T7 promoter-containing pVAX.A120 vectors encoding for *Tlr1, 2* and *6* were linearized via restriction enzyme digestion as described in chapter 2.1.4 and transcribed *in vitro* into chemically modified mRNA, incorporating 25% 2-Thio-UTP and 25% 5-Methyl-CTP using the T7 MEGAscript kit (Ambion).

The T7 polymerase is a highly promoter-specific RNA polymerase originating in *E. coli* bacteriophages, that transcribes DNA only downstream a T7 promoter [134]. Double stranded DNA is separated and unwinded to allow for complementary base pairing on the codogenic strand.

Because high amounts of mRNA were required for *in vitro* and *in vivo* studies, all mRNA transcriptions were performed as 5x applications using 5 µg linearized vector DNA and 10 µl enzyme mix (Table 14) for each reaction. Since precision and cleanliness is crucial when working with mRNA, it has been taken care of an RNase free environment and lab space at all times.

In order to achieve low immunogenicity *in vivo* while at the same time achieving high gene transfer efficiency and stability an Anti Reverse Cap Analog (ARCA, TriLink Bio Technologies) and modified nucleotides were incorporated in terms of replacing 25% of UTP with 2-Thio-UTP and 25% of CTP with 5-Methyl-CTP [127]. The exact GTP to ARCA ratio is not defined, leading to overall higher amounts of guanine nucleotides in the reaction volume [126].

Before use, nucleotides were thawed on ice and reaction buffer at room temperature. Nuclease-free DEPC water was used for dilutions. All ingredients were pipetted carefully in a nuclease free reaction tube and incubated at 37°C for 2-3 hours to allow for mRNA transcription of the T7 polymerase. Subsequently 5 µl Turbo-DNase (MEGAscript T7, Ambion) was added and incubated at 37°C for 15 min to ensure degradation of residual DNA.

Table 14 Protocol for mRNA *in vitro* transcription of a T7 promoter containing linearized plasmid DNA using the MEGAscript kit

Reagent	Volume (µl)	Molarity (mM)	Final Concentration (mM)	Percentage (%)	Supplier
ATP	10	75	7.5	100	MEGAscript T7, Ambion
CTP	7.5	75	5.625	75	MEGAscript T7, Ambion
m5CTP	1.9	100	1.9	25	TriLink BioTechnologies
UTP	7.5	75	5.625	75	MEGAscript T7, Ambion
s2UTP	1.9	100	1.9	25	TriLink BioTechnologies
GTP	5	75	3.75	not defined	MEGAscript T7, Ambion
ARCA	6	100	6	not defined	TriLink BioTechnologies
Reaction Buffer (10x)	10				MEGAscript T7, Ambion
Plasmid DNA	equal to 5 µg				
Enzyme Mix	10				MEGAscript T7, Ambion
DEPC water	*ad* 100				

The MEGAclear kit (Ambion) was used for clean-up and purification of mRNA samples based on the principle of silicate based membranes [135]. To each reaction tube 350 µl binding solution concentrate and 250 µl 100% ethanol were added, the sample was supplied into a filter cartridge placed into a collection tube and centrifuged at 13,000 rpm for 30 sec. This was followed by a twofold performed washing step with 500 µl wash solution and centrifugation at 13,000 rpm for 30 sec each to remove residual nucleotides that haven't been incorporated during transcription. An additional centrifugation at 13,000 rpm for 30 sec ensured complete removal of remnants of wash solution and ethanol. New RNase-free tubes were now used for elution of mRNA using 42 µl elution solution, incubation at 70°C for 10 min and centrifugation at 13,000 rpm for 1 min. The elution step was repeated with another 42 µl elution solution to maximize the yield of mRNA. The reaction was then placed on ice, concentration of mRNA was measured and the samples were stored at -80°C. To check the mRNA for correct length gel electrophoresis was performed as described above (chapter 2.1.5) with the following changes in procedure: for mRNA samples, 1-3 µg mRNA were mixed with 2 µl 2x RNA Loading Dye (New England Biolabs Inc., Ipswich, UK) and filled up with DEPC water *ad* 12 µl. The mixture for 10 µl mRNA ladder was prepared using 2 µl ssRNA ladder (New England Biolabs Inc., Ipswich, UK), 3 µl DEPC water and 5 µl 2x Loading Dye. Samples were loaded onto a 1.5% agarose gel and electrophoresis was performed at 80 Volt and 400 milliampere until a sharp band was detectable.

2.3 Cultivation and transfection of A549 and MLE12 cells

Mouse transformed epithelial cells (MLE 12; deposited by ATCC American Type Culture Collection, Manassas, USA) and Human alveolar type 2 epithelial cells (A549; deposited by CLS Cell Line Service, Eppelheim, Germany) were grown in cell culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were cultured in a 175 cm^2 tissue culture flask under standardized conditions at 37°C and 5% CO_2.

Both cell types grow adherent and were splitted when reached 90% confluency. For this purpose the old medium was decanted, cells were washed with 10 ml PBS before being incubated with EDTA solution containing 0.25% trypsin at 37°C to allow for adherent cells to detach. Preheated DMEM stopped trypsin activity and cells were either used for experiments or seeded at different dilutions in order to grow again.

Transfection with Lipofectamine 2000

Lipofection is a transfection procedure using liposomes as vehicles to inject genetic material into eukaryotic cells. Cationic lipids interact spontaneously with anionic genetic material forming lipid-DNA or –RNA complexes, able to enter cells by fusion with their plasma membrane [136]. In doing so, lipofection is greater than 5- fold more effective than other transfection techniques, *e.g.* the calcium-phosphate or the DEAE-dextran transfection technique [137].

In the presented work Lipofectamine 2000 (Invitrogen Life Technologies Corporation) was used as a transfection reagent, containing 1 mg/ml neutral and kationic lipids.

One day prior to lipofection procedure, cells were seeded in a 24-well plate with a concentration of 100,000 cells and 500 µl medium per well. At the time of transfection cells should be 80-90% confluent. Each well was transfected with an amount of 0.5 µg mRNA (modified after Kormann, Depner et al. 2008). The transfection rate depends not only on cell lines, cell density and amount of nucleic acids [136] but also on incubation times [138]. Before transfection, cells received 400 µl fresh medium without antibiotics. Two reaction tubes for each well were prepared. In tube 1) 0.5 µg mRNA was filled up with Opti-MEM medium *ad* 50 µl, whereas tube 2) contained 2 µl Lipofectamine and 48 µl Opti-MEM. Both tubes were incubated at room temperature for 5 min. Carefully each solution from tube 1) (mRNA) was added to respective tube 2) (Lipofectamine) and incubated at room temperature for 20 min to allow for generation of lipid-mRNA complexes. This solution was then slowly pipetted into cell culture wells. Cells were again incubated at 37°C and 5% CO2. New medium was supplied

after 12 hours. Cells were stained and subjected to flow cytometry after 24 hours or 48 hours respectively.

2.4 Flow cytometry

Flow cytometry offers a method for quantitative analysis of cells in suspension [139]. The fundamental concept is based on scattered and fluorescent light. Cells or other microscopic particles in suspension pass by a laser beam and interact with the light. Light scatter as well as fluorescent scatter can be measured and are then detected separately. Whereas light scatter allows to determine not only size (via forward scatter, FSC) but also granularity (via side scatter, SSC) of the cell, fluorescent scatter can tell about cell specific components in case they have been stained with a fluorescent dye prior to flow cytometry. Various cell types and also dead cells can be distinguished according to their different physical properties. The use of different wavelengths of the laser stream enables the detection of various fluorescent signals. Usually flow cytometry data are shown as a density plot or on a histogram. On a density plot, each dot represents an individual cell while green and yellow areas indicate large number of events. On histograms, cell types can be analyzed separately and plotted as cell count on the y-axis versus fluorescence intensity on the x-axis.

In this study, flow cytometry was used to measure surface expression of TLR1, 2 and 6 on MLE12 and A549 cells *in vitro* as well as on lung and spleen tissue cells *in vivo*. This method was further used to quantify and differentiate different immune cells isolated from lung and spleen tissue after *in vivo* treatment with *Tlr* mRNA. Antibodies used for *in vitro* and *in vivo* experiments of this study are stated in Table 15.

Table 15 Antibodies to stain cells from whole lung and spleen tissues for T cells, T regs, B cells, eosinophils and dendritic cells

Antibody	Supplier
Anti-mouse TLR1-PE goat IgG	R&D Systems
Anti-mouse TLR2-FITC mouse IgG1	BioLegend
Anti-mouse TLR6-APC rat IgG2A	R&D Systems
Anti-mouse F4/80-Pacific Blue rat IgG2A	BioLegend
Anti-mouse Siglec F-PE rat IgG2A	BD Pharmingen
Anti-mouse CD19-PerCP/Cy5.5 rat IgG2A	BioLegend
Anti-mouse CD11b-PE.Cy7	BD Pharmingen
Anti-mouse CD11c-APC/Cy7 hamster IgG1	BD Pharmingen
Anti-mouse Ly6G-APC rat IgG2b	eBioscience
Anti-mouse CD3e-Brilliant Violet rat IgG2b	BioLegend
Anti-mouse CD4-APC rat IgG2b	eBioscience
Anti-mouse CD25-PE/Cy7 rat IgG1	eBioscience
Anti-mouse Foxp3-PE rat IgG2a	eBioscience
Rat IgG2a-PE	eBioscience
Foxp3 Fix/Perm Buffer Set	BioLegend

For *in vitro* experiments, A549 and MLE12 cells were prepared for flow cytometry analysis either 24 hours or 48 hours after transfection procedure. Cells were washed, detached from culture flasks as described above (chapter 2.3), washed again, centrifuged at 400 x g for 10 min, supernatant was decanted and cells were resuspended in 100 µl FACS buffer (1 x PBS with 0.1% BSA and 0.02% NaN_3) and stored on ice. Per 1 x 10^6 cells and 100 µl

volume, cells were stained with either 2 µl Anti-mouse TLR2, 8 µl Anti-mouse TLR1 or 8 µl Anti-mouse TLR6 per tube depending on their previous mRNA treatment. Non-transfected cells served as controls and were therefore pooled and stained with antibodies as mentioned above. An additional sample remained unstained as a control. All cells were incubated at 4°C for 30 min, washed and resuspended in 100-200 µl FACS buffer before being subjected to flow cytometry.

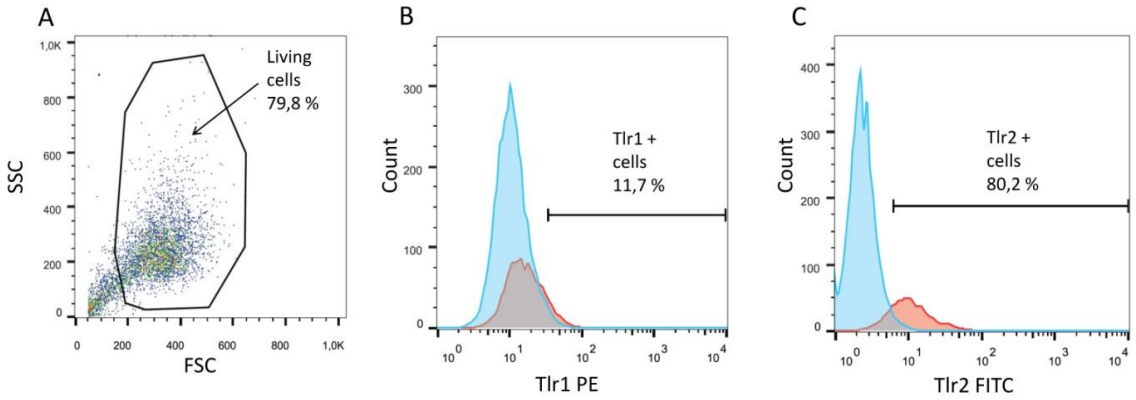

Fig. 5 Gating strategy for *in vitro* experiments to detect TLR expression in MLE12 and A549 cells

In the dot plot (A) cells were first gated for living cells by forward scatter (FSC) and side scatter (SSC). Histograms (B and C) only include living cells. Blue peaks indicate TLR expression on untransfected controls whereas red peaks show the positive dataset of increased TLR expression after transfection with chemically modified *Tlr* mRNA. Representative graphs of A549 cells after transfection with *Tlr1* (B) and *Tlr2* (C) mRNA are shown.

For *in vivo* experiments lung and spleen cells were isolated and stained as described in chapter 2.6. Cells from each mouse were separated whereas the first part (a) was stained for TLR1, 2 and 6 expression, the second part (b) for differential cell analysis and the third part (c) for Foxp3 as a marker for $CD4^+$ regulatory T cells. Each tube contained 500,000 cells in 100 µl FACS buffer and was incubated for 30 min at 4°C after the respective antibody mix was added. Cells were subsequently washed, centrifuged, resuspended in 100 µl FACS buffer and subjected to flow cytometry.

In order to investigate the expression of TLR1, 2 and 6 in lung and spleen tissue, cells were stained with a mixture of 10 µl Anti-mouse TLR1, 2 µl Anti-mouse TLR2 and 10 µl Anti-mouse TLR6 per tube.

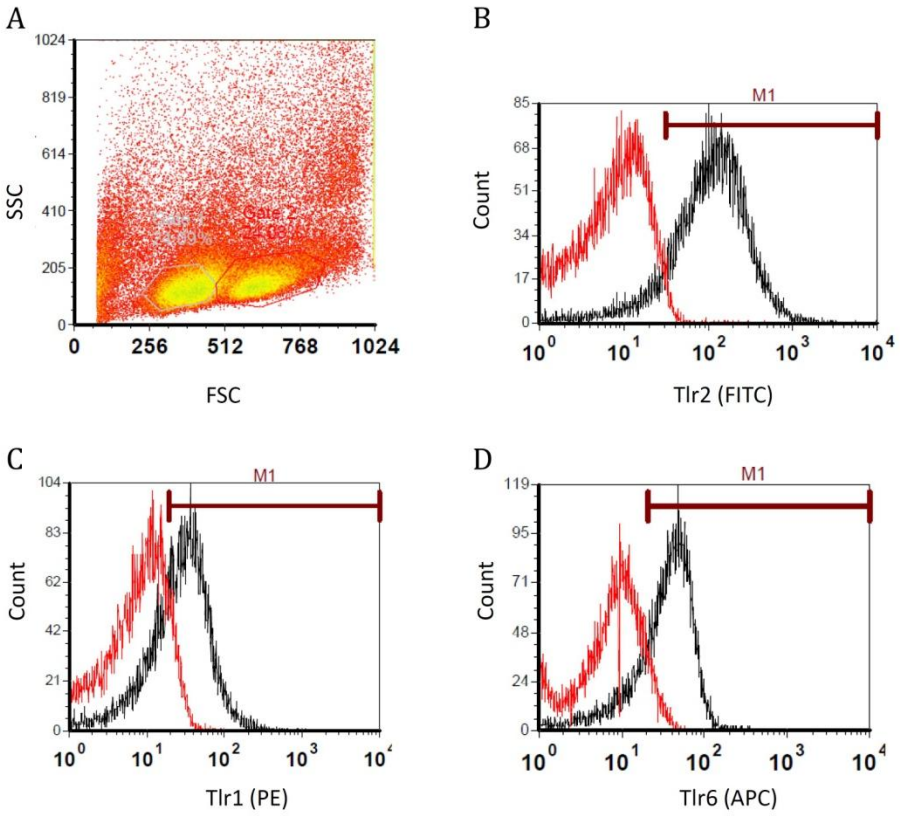

Fig. 6 Gating strategy for *in vivo* experiments to analyze TLR expression on lymphocytes, monocytes, PBMCs and neutrophils

Cells from whole lung and spleen tissue were isolated and stained for TLR1, 2 and 6. On the density plot. (A) cell types were analyzed separately and then plotted on histograms as cell count on the y-axis versus TLR fluorescence (PE for TLR1 (C), FITC for TLR2 (B) and APC for TLR6 (D)) on the x-axis. The red peak indicates the data of an unstained control, the black peak shows the data of stained samples. According to unstained samples, Marker (M1) was set as a threshold to define positive events.

Cell infiltration in lung and spleen tissue was differentially analyzed by adding the antibody mixture stated in Table 16 to each tube.

Table 16 Antibodies for cell differentiation by flow cytometry

Antibody	Volume
Anti-mouse F4/80	0.5 µl
Anti-mouse Siglec F	0.5 µl
Anti-mouse CD19	0.25 µl
Anti-mouse CD11b	0.25 µl
Anti-mouse Ly6G	6 µl of a 1:100 dilution
Anti-mouse CD3	2.5 µl
Anti-mouse CD11c	1 µl of a 1:10 dilution
FACS buffer	*ad* 50 µl

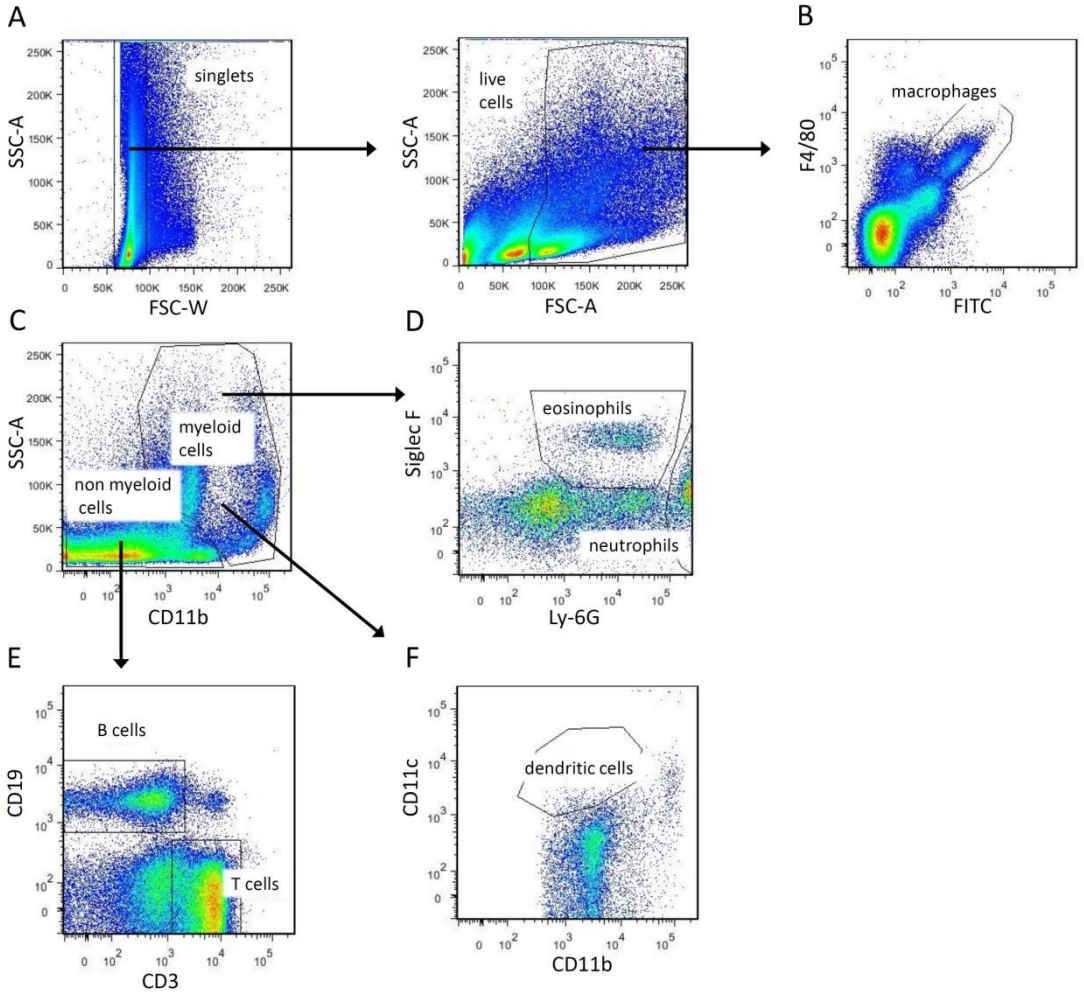

Fig. 7 Flow cytometry gating strategy for *in vivo* experiments to detect T cells, B cells, eosinophils and dendritic cells from lung and spleen tissue

A: Single cells and live cells were first identified by forward scatter (FSC) and side scatter (SSC). B: Macrophages were then identified as F4/80^{+} and FITC^{+} cells. C: Cells other than macrophages were fragmented into myeloid (SSC and CD11b^{+}) and non-myeloid (SSC^{low} and CD11b^{low}) cells. Among myeloid cells, eosinophils were defined as Siglec F^{+} and Ly-6G^{mid}, whereas neutrophils showed Ly-6G^{high+}. Dendritic cells (DCs) were identified within a CD11c$^{high/mid}$ and CD11b^{+} gate. Non myeloid cells were then identified as B cells (CD19^{+} CD3$^{low/neg}$) and T cells (CD3^{+} CD19^{low}).

In order to indentify regulatory T cells (Tregs), the fraction of CD4^{+} CD25^{+} Foxp3^{+} positive cells was measured via intra- and extracellular staining. Prepared cells were first stained for extracellular CD4 and CD25 (1 µl Anti-mouse CD4 and 3 µl Anti-mouse CD25 per tube). After incubation (30 min, 4°C) cells were washed, resuspended in 150 µl FACS buffer and 200 µl 1% Foxp3-Fix/Perm solution to fix cells prior to permeabilization treatment. After incubation at room temperature for 20 min in the dark, the solution was centrifuged and the

supernatant was decanted. Fixed cells were resuspended in 200 µl 1% Foxp3Perm Buffer to permeabilize the cells to allow for the fluorochrome reaching the interior of the cell. This solution was again incubated 15 min at room temperature in the dark, washed and resuspended in 50 µl Foxp3Perm Buffer. Cells were then stained with either 5 µl Anti-mouse Foxp3 or 5 µl Rat IgG2a as an isotype control. After another 30 min of incubation cells were washed and resuspended in 100 µl FACS Puffer for subsequent analysis via flow cytometry.

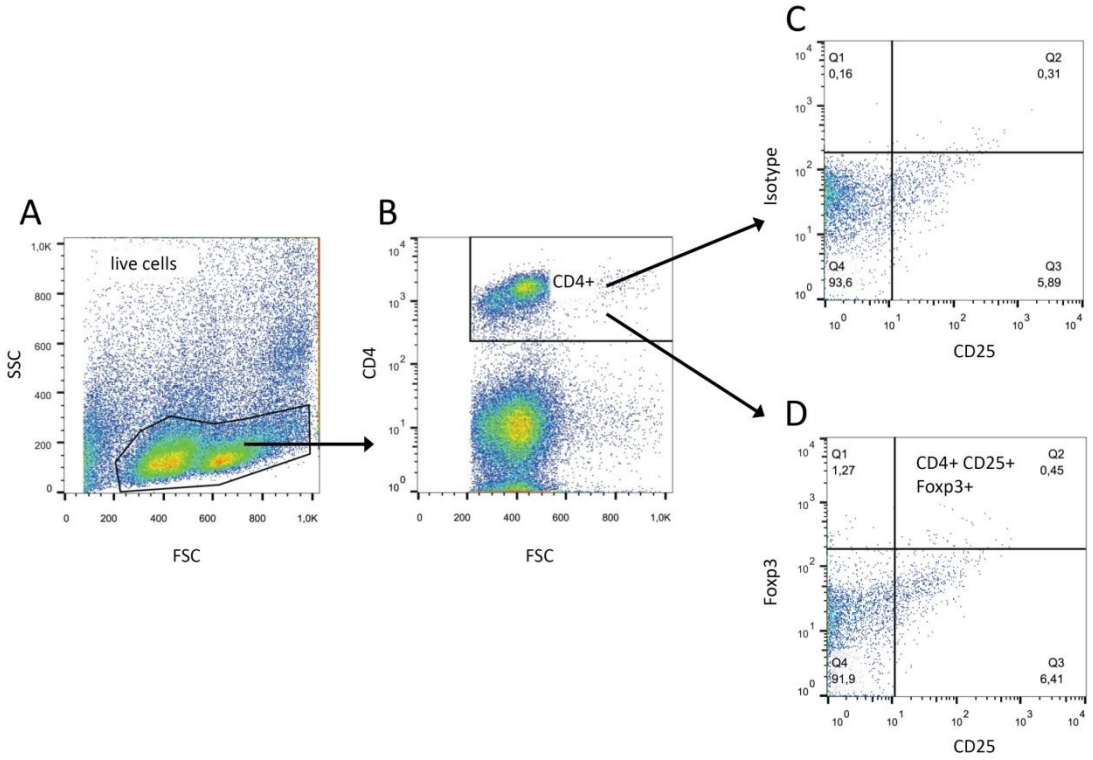

Fig. 8 Gating strategy for *in vivo* experiments to detect $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs

In a density plot for SSC versus FSC cells were gated for living cells (A). Living cells were then gated into $CD4^+$ cells (B). These were further gated for CD25 and Foxp3 fluorescence. Isotype controls were used to differentiate between $CD4^+$ $CD25^+$ but $Foxp3^-$ cells (C) and $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs (D), shown in the upper right quadrant of Foxp3 gate.

Cells were analyzed on FACS Calibur using CellQuest software. Data was further interpretated and analyzed with FACSDiva (vs 6.1.3) software for *in vitro* experiments. FCS Express and FlowJo software were used to analyze *in vivo* data.

2.5 Animal experiments

Six- to eight-week-old female BALB/c mice were purchased from Charles River Laboratories. Mice were kept under standardized specific pathogen-free conditions and maintained on a 12-h light-dark cycle. Food, water as well as nesting material were provided *ad libitum*. All animal experiments were approved by the local ethics committee and carried out according to the guidelines of the German Law for the Protection of Animals.

Intratracheal (i.t.) procedures were carried out under antagonizable anesthesia with a mixture of medetomidine (0.5 mg/kg), midazolam (5 mg/kg) and fentanyl (50 µg/kg). After treatment antidot was injected subcutaneously consisting of atipamezol (50 µg/kg), flumazenil (10 µg/kg) and naloxon (24 µg/kg).

At the age of 10-12 weeks, mice received their first i.t. treatment of the injection schedule (Fig. 18). This schedule was adapted from Mays et al. [129] and modified to enable a preventative treatment with *Tlr* mRNA prior to HDM exposure. Mice received either four instillations of *Tlr* mRNA combinations followed by three instillations of HDM extract (*Dermatophagoides pteronyssinus,* GREER laboratories, Lenoir, NC, USA), PBS instead of mRNA followed by HDM exposure as positive controls or PBS only as negative controls. Furthermore, mice were treated with *Tlr* mRNA followed by PBS administration instead of HDM, to observe whether mRNA application on its own may modify the asthmatic outcome *in vivo* (Table 17).

Table 17 Groups of mice and their respective treatment following the injection schedule as stated in Fig. 9

Group	n =	Treatment		Endpoint
		day -17, -14, -10, -7	day 0, 7, 14	
1	9	*Tlr1/2* mRNA	HDM	day 15
1.1	3	*Tlr1/2* mRNA	PBS	
2	9	*Tlr2* mRNA	HDM	
2.1	3	*Tlr2* mRNA	PBS	
3	9	*Tlr2/6* mRNA	HDM	
3.1	3	*Tlr2/6* mRNA	PBS	
4	9	PBS	HDM	
4.1	9	PBS	PBS	

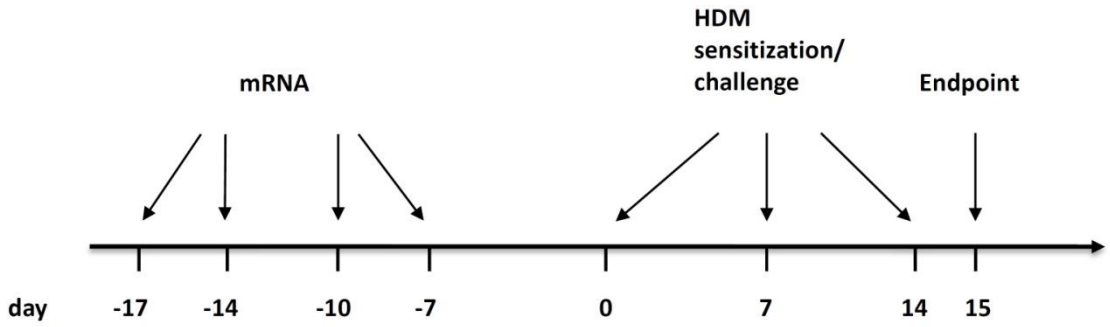

Fig. 9 Timeline illustrating the injection schedule of the HDM induced mouse model of asthma

Mice were treated intratracheally and received either 100 µg HDM, 20 µg mRNA per *Tlr* or PBS as a control.

2.6 Lung and spleen cell isolation

At experimental endpoints, mice were euthanized using 120 mg/kg pentobarbital sodium. Spleens were removed and squeezed through a 70 µm cell strainer while rinsing the sieve with 50 ml cold PBS. Lungs were removed and each left lobe was fixed in phosphate buffered formaldehyde and further

treated as described in chapter 2.11. Right lungs were shredded, incubated in 1 ml digestion solution (Table 18) for 1-2 hours at 37°C and subsequently squeezed through a 70 µm cell strainer rinsed with 50 ml cold PBS. Spleen and lung cells were centrifuged at 400 x g for 10 min before cell pellets were resuspended in 5 ml lysis buffer and incubated for 5 min at room temperature. 50 ml PBS per sample were added and cells were centrifuged again at 400 x g for 10 min. The cell pellet was finally resuspended in 3 ml PBS and live cells were counted.

Table 18 Solution to digest lung tissue prior to lung cell staining for flow cytometry analysis

Digestion solution	Final concentration	Supplier
Collagenase Type 1	1 mg	Life technologies
Dispase	1 mg	Corning
DNase	1% (500 U)	EPICENTRE Biotechnologies

2.7 Lung function test

At the predetermined endpoint of the study, airway resistance in response to methacholine (MCh, acetyl-β-methylcholine chloride; Sigma-Aldrich) was determined from n=6 mice per group using the model of the isolated, perfused and ventilated lung (IPL) [129].

Mice were deeply anaesthetized and toe reflex tests were performed to measure the depth of anaesthesia. The animal was fixed in a thorax chamber in supine position. The Trachea was cannulated for mechanical ventilation with 90 breaths per minute by negative pressure ventilation between -2.8 cm H_2O (end-expiratory) and -8.5 cm H_2O (end-inspiratory). Furthermore hyperinflation (-25 cm H_2O) was performed every 5 minutes to prevent atelectases of the lungs. After a laparatomy the diaphragm was dissected and heparin 500 IE/100g was injected into the right ventricle of the heart. The renal artery was then cut resulting in the mouse bleeding to death. The lower half of the body was

discarded and the thorax opened to cannulate the pulmonary artery and vein. Subsequently the chamber was closed and the pulmonary vessels were perfused at a constant flow rate (1 ml/min) in a nonrecirculating manner with a 4% hydroxyethyl starch containing perfusion buffer (Fig. 10). After a 20-minute equilibration period, the lungs were perfused with increasing concentrations of MCh (0.1 µM, 1 µM, 10 µM, and 100 µM) for 10 min each, separated by a 20-minute washout period with buffer.

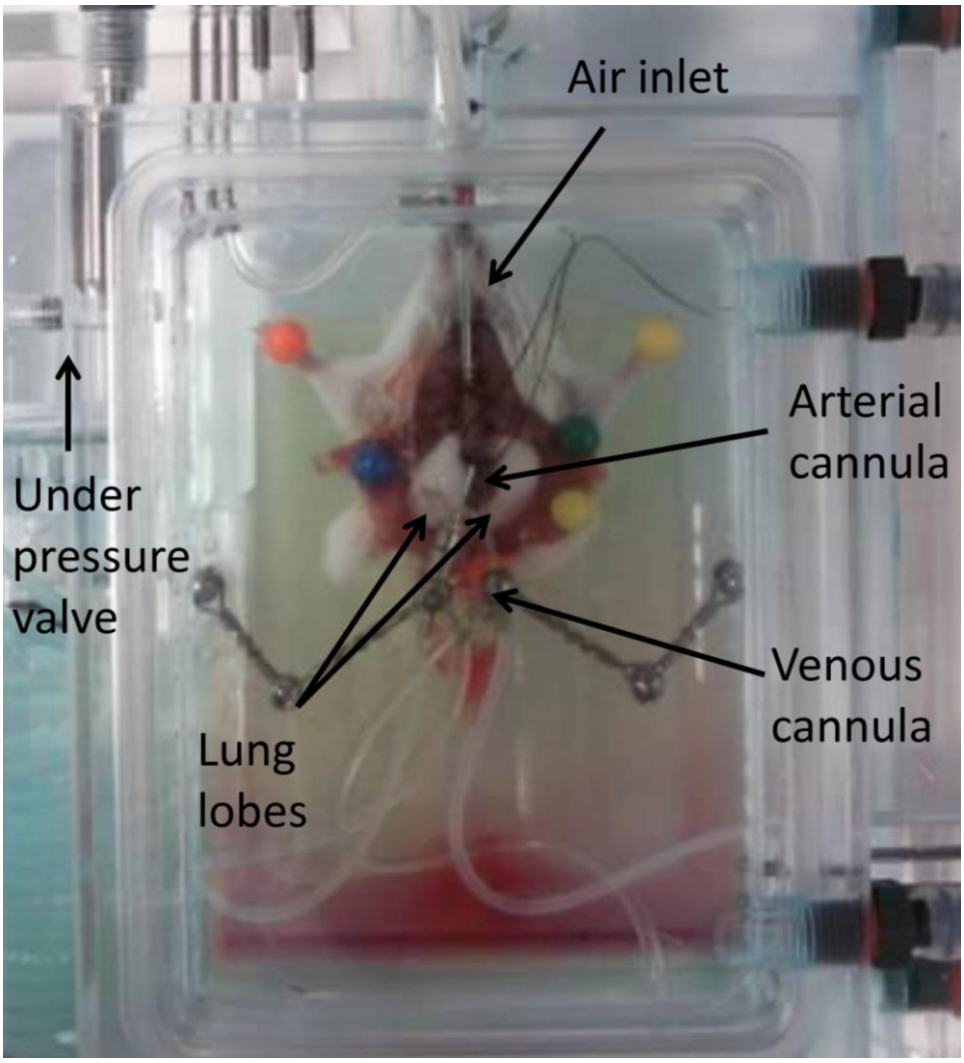

Fig. 10 Lung function and airway resistance was measured using the *ex vivo* IPL method

The trachea was mechanically ventilated through an air inlet in a vacuum chamber. The pulmonary artery and vein were cannulated and perfused with buffer.

The mean resistance values were calculated from the last 30 seconds of each 10-minute MCh exposure for statistical analysis. During this whole process,

Lung function parameters were analyzed automatically by HSE-HA Pulmodyn W Software (Harvard Apparatus).

2.8 Enzyme linked immunosorbent assay (ELISA)

Cytokines in BALF supernatant collected from mice at the predetermined endpoint of the study were assayed in triplicates by ELISA for IL-5, IL-13, IFNγ and CCL17 (Table 19) according to the manufacturer's instructions. Briefly, 96-well plates were precoated with antibody solution and then loaded with undiluted samples. After overnight incubation at 4°C chemokines were detected by horseradish peroxidase-linked antibodies and optical density values were measured in a microplate reader. Concentrations of chemokines were then calculated from standard curves.

Table 19 Chemokines investigated in this study via ELISA and suppliers of respective kits

Chemokine	Detection limit (minimum-maximum)	Supplier
IL-5	4 – 500 pg/ml	eBioscience
IL-13	4 – 500 pg/ml	eBioscience
IFNγ	15 – 2000 pg/ml	eBioscience
CCL17	7.8 – 500 pg/ml	R&D Systems

2.9 Cytokine multiplex panel

Cytokines in BALF supernatant were additionally analysed for IL-2, IL-4, IL-5, IL-10, IL-12p70, IL-17A, IFNγ and TNFα using a mouse cytokine multiplex panel according to the manufacturer's instructions (Bio-Plex Cytokine Assay, Bio-Rad Laboratories, USA). The Bio-Plex suspension array allows the detection of multiple cytokines in a single well while only a relatively small sample volume is

required. This technology combines the principles of flow cytometry and a sandwich immunoassay, using colored beads instead of a coated well [140].

In brief, antibodies directed to the cytokines of interest are covalently coupled to fluorescent beads. These antibody-coupled beads are allowed to react with an unknown amount of cytokines in any sample dilution. An additional analysis of samples containing a known amount of cytokines allows to establish a standard curve. After several washing steps, a specific detection antibody and finally streptavidin-PE is added to the reaction mixture. Lasers in the flow-based array system excite the fluorochromes and thereby detect and quantify the specific cytokine reaction in each well. Cytokine concentrations are automatically calculated by Bio-Plex Manager Software.

2.10 Differential cell count of murine bronchoalveolar lavage (BAL)

Bronchoalveolar lavage (BAL) fluid was obtained at the time of sacrifice. Gently, the trachea was dissected and carefully cannulated with a needle. Lungs were lavaged twice with 1 ml prechilled PBS. Each BALF sample was then centrifuged for 10 min. at 500 x g. The supernatants were collected and stored at -20°C for cytokine analysis. Cell pellets were subsequently resuspended in PBS and diluted with PBS 1:5. From this dilution a volume of 300 µl was used to prepare monolayer smears by cytocentrifugation at 800 rpm for 10 min at 4°C. The smears were then stained with May-Grünwald-Giemsa. Differential cell counts of BALF were performed from at least 100 cells per smear and analyzed using a Zeiss Axio Imager.M2 with the AxioCam MRc camera. Standard hemocytologic criteria were used to classify the cells as neutrophils, eosinophils, lymphocytes, macrophages and monocytes (Fig. 11).

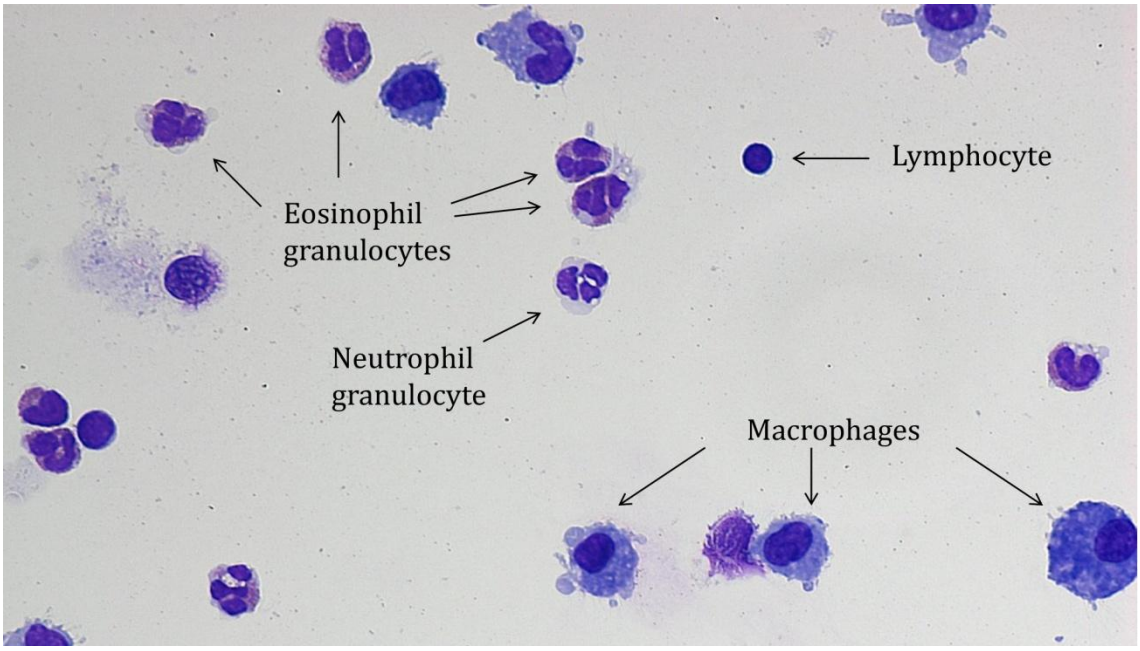

Fig. 11 Representative smear of BAL cells after cytospin procedure

Black arrows indicate different cell types of a BAL fluid sample.

2.11 Histopathology of murine lung tissue

In order to analyze inflammation and mucus production in lung tissues, each left lung was removed at the predetermined endpoint of the study. Lungs were fixed in Histofix (4.5%, Carl Roth) overnight and embedded in paraffin. Slices (4 µm) were stained with either H&E or periodic acid-Schiff (PAS). The samples were examined using a Zeiss Axio Imager.M2 with the AxioCam MRc camera. Multiple photographs were taken of the primary airway, perivascular and interstitial areas. Inflammation and infiltration of lung tissue was evaluated on H&E stained sections. Mucus-producing PAS-positive goblet cells were quantified in percent of counted cells (determined by visible nuclei).

2.12 Statistical analysis

Statistical significance of differences was defined as $P<0.05$ and denoted with asterisks: *0.05, **0.01 and ***0.001. Statistical analysis and generation of figures presented in this thesis were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, California) and SPSS Statistics Version 22

(IBM). Unless stated otherwise, data were statistically analyzed using Kruskal-Wallis oneway-analysis and Mann-Whitney U tests.

3 Results

3.1 Cloning of murine *Tlrs* for mRNA transcript therapy

3.1.1 Plasmidisolation and cloning of *Tlr1, 2* and *6* into a pVAX.A120 plasmid vector

In order to receive high amounts of plasmid vectors containing the respective *Tlr* genes, pcDNA3.1 plasmids encoding for murine *Tlr1, 2* or *6* were inserted into *E.coli* via heat shock transformation. Subsequently plasmids were isolated and *Tlr* gene inserts were cut out via restriction enzyme digestion. To further isolate the *Tlr* insert and check it for correct length of sequence, a gel electrophoresis and gel isolation were performed. Correct isolation of the *Tlr2* insert is shown in Fig. 12 representatively for all *Tlr* inserts.

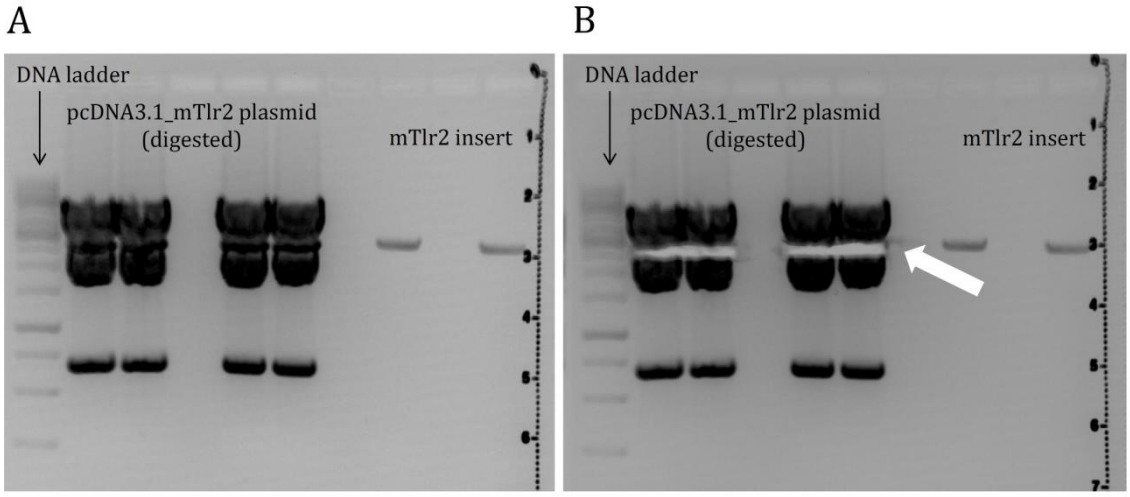

Fig. 12 Isolation of the *Tlr2* fragment from an agarose gel

(A) A pcDNA3.1 plasmid incorporating an *Tlr2* insert was digested with SpeI HF and XhoI and run on an agarose gel. The 2300 bp *Tlr2* fragment was identified by comparing the fragment of interest to the DNA ladder on the left and the previously extracted *Tlr2* fragment on the right. The desired band was removed with a razor blade, (B) the white arrow indicates the empty space after removal.

Following the isolation procedure, *Tlr1, 2* or *6* inserts were ligated into the pVAX.A120 plasmid vector.

3.1.2 Validation of the correct structure and sequence of pVAX.A120_Tlr plasmids

The final sequence of the pVAX.A120_Tlr plasmid construct after ligation was validated via control restriction enzyme digestion, gel electrophoresis (Fig. 13) and additional sequencing (Fig. 14).

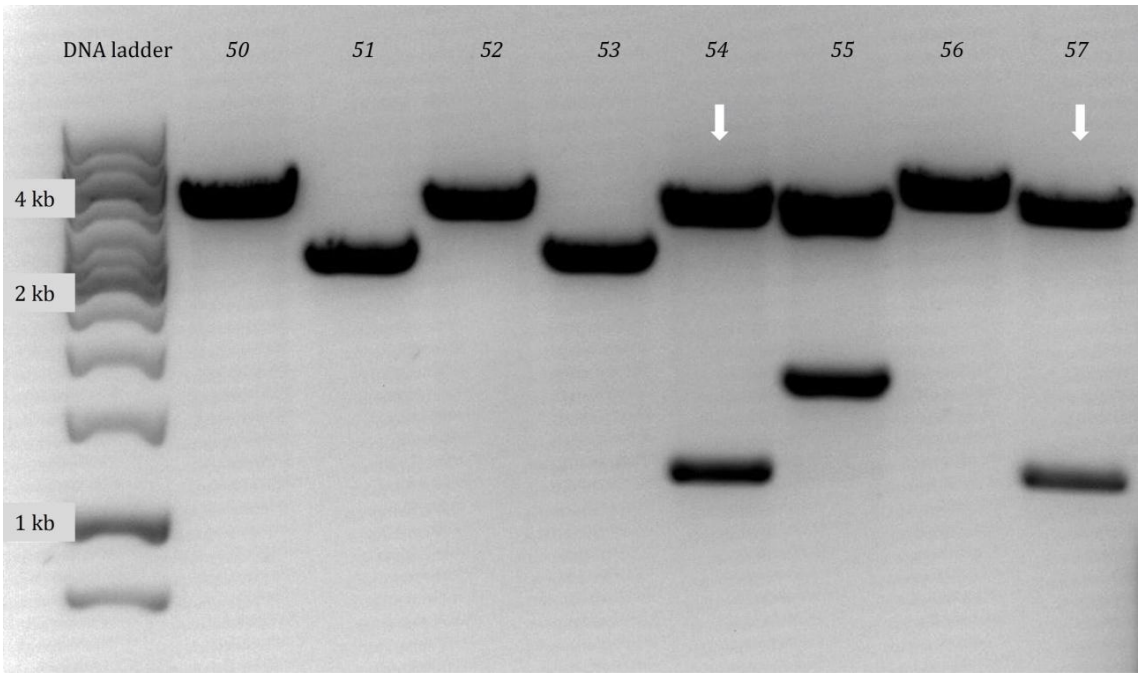

Fig. 13 Gel electrophoresis of pVAX.A120_Tlr2 plasmids from various bacterial clones after restriction enzyme digestion

In case of successful intake of the *Tlr* insert into pVAX.A120, restriction enzyme EcoRI HF produces fragments of 4191 bp and 1268 bp of length. In the picture above white arrows indicate plasmids from bacterial clones number 54 and 57 showing the desired pattern and thus correct intake and orientation of the fragment within the plasmid.

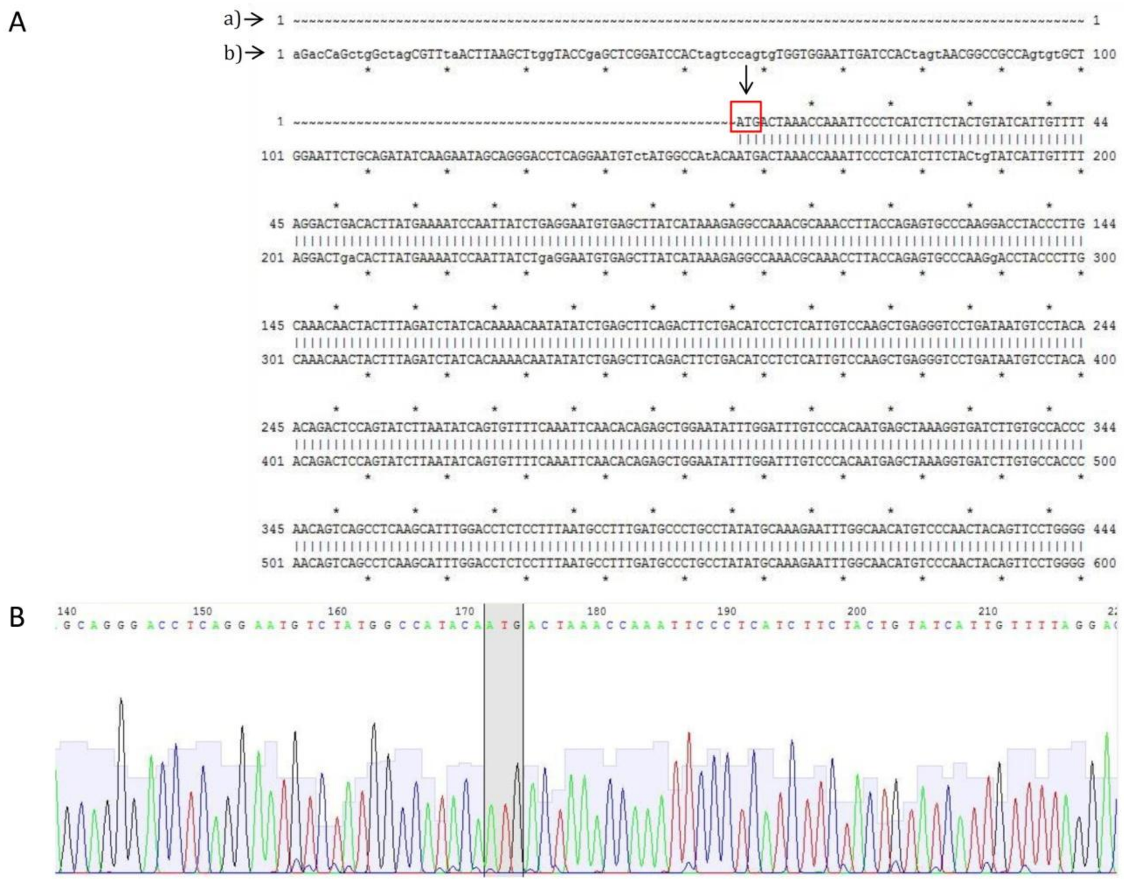

Fig. 14 Sequence data of plasmids was analyzed using ApE software

Representative data from *Tlr1* sequence is shown. (A) Alignment to compare the original *Tlr* sequence (a) as stated at www.ncbi.nlm.nih.gov to the sequencing results of *Tlr1* inserted into the pVAX.A120 plasmid vector (b). Here, the beginning of the coding sequence is shown, with the black arrow indicating the start codon. Mismatches or mutations did neither occur in this nor in the following part of the sequence (data not shown). (B) The electropherogram illustrates the origin of the same *Tlr1* coding sequence, showing distinct signal amplitudes without mutations or overlaps.

3.2 Generation of chemically modified *Tlr* mRNA

3.2.1 *In vitro* transcription of chemically modified *Tlr1, 2* and *6* mRNA

Chemically modified mRNA incorporating 25% 2-Thio-UTP and 25% 5-Methyl-CTP was produced via *in vitro* transcription. After the transcription process, a gel electrophoresis was performed with 1-3 µg mRNA of each sample to ensure the correct length of the mRNA strand. Fig. 15 presents *Tlr1, 2* and *6* mRNA samples of correct length after gel electrophoresis.

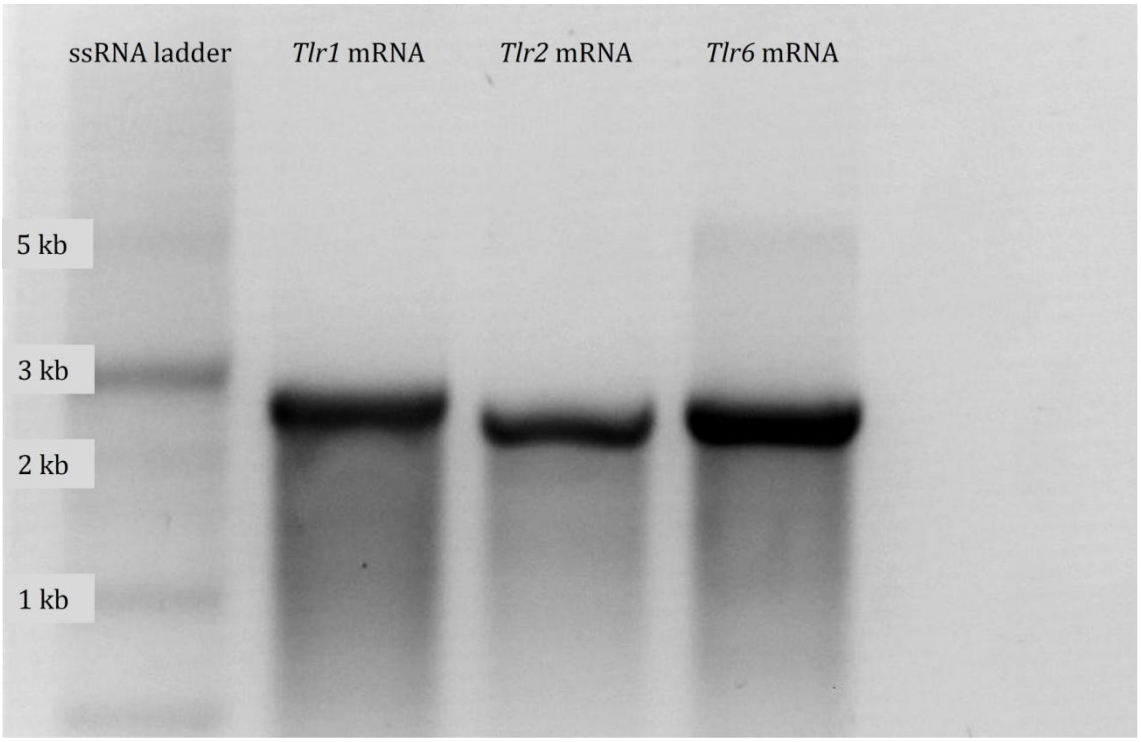

Fig. 15 Gel electrophoresis of *Tlr1, 2* and *6* mRNA after *in vitro* transcription

mRNA samples were run on an agarose gel. Bands appear as expected at approximately 2591bp for *Tlr1* mRNA, 2570bp for *Tlr2* mRNA and 2625bp for *Tlr6* mRNA.

3.2.2 Validation of TLR1, 2 and 6 expression *in vitro* after transfection of *Tlr* mRNA

Tlr1, 2 and *6* mRNA was transfected into A549 and MLE12 cells, in order to check the mRNA for correct function. Subsequently, cells underwent lipofection procedure and were subjected to flow cytometry to measure TLR expression after 24 hours or 48 hours respectively. TLR expression detected 48 hours post transfection (data not shown) was markedly reduced and cell numbers were decreased when compared to samples incubated only 24 hours after transfection procedure and have therefore not been analysed further. The steep increase in RFP positive cells after transfection of *RFP* mRNA is shown in Fig. 16A and served as a control to confirm successful lipofection procedure.

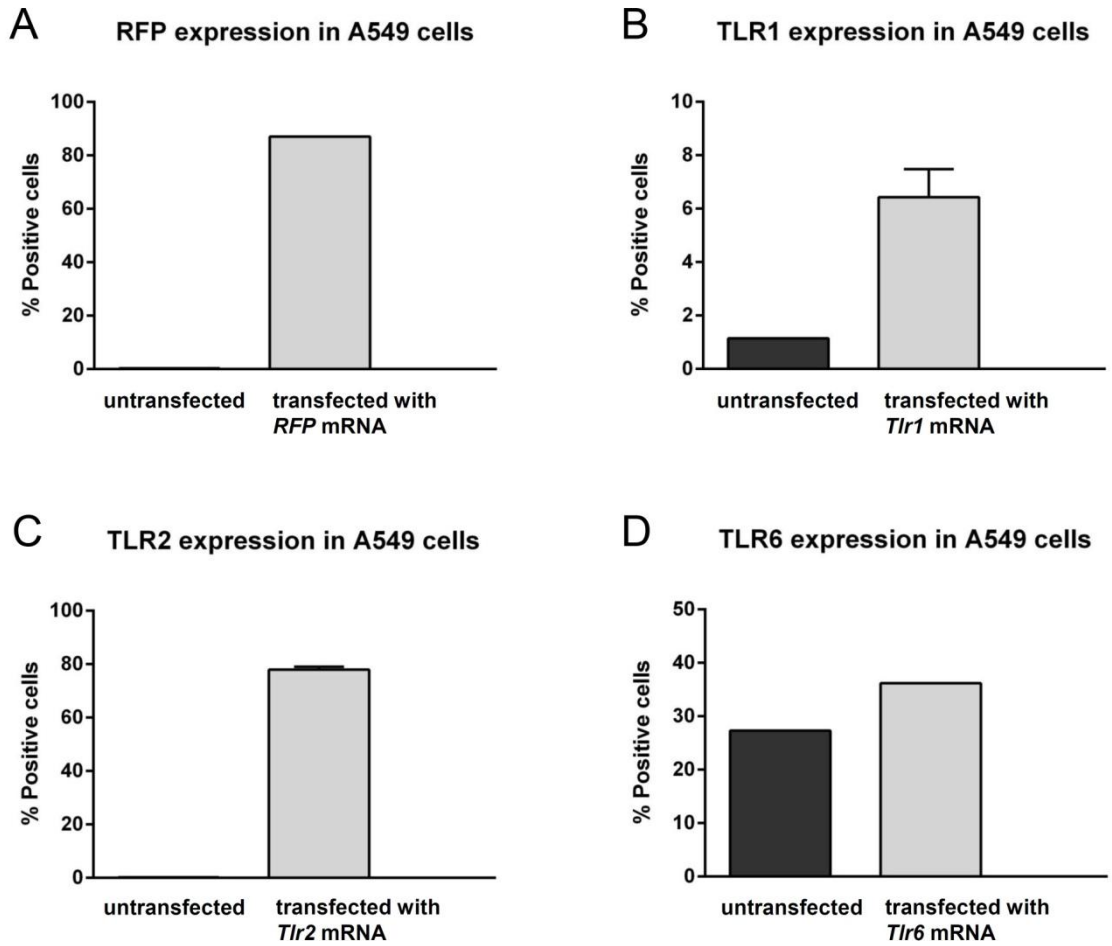

Fig. 16 TLR expression in A549 cells after transfection with *Tlr* mRNA

A549 cells were transfected with *Tlr1, 2* or *6* mRNA, harvested and stained after 24 hours and subjected to FACS analysis. (A) Transfection with *RFP* mRNA served as a control and confirmed successful lipofection procedure by a steep increase in RFP^+ cells. (B, D) $TLR1^+$ and $TLR6^+$ cells were elevated when compared to untransfected controls. (C) Expression of TLR2 increased markedly after transfection with respective mRNA. Data are represented as means ± SEM.

A549 cells showed higher levels of cells positive for TLR1 after transfection of *Tlr1* mRNA when compared to untreated control cells (Fig. 16B). Transfection of *Tlr2* mRNA resulted in highly elevated levels of $TLR1^+$ A549 cells compared to untransfected controls (Fig. 16C). A slight increase of cells positive for TLR6 could be detected after transfection of *Tlr6* mRNA (Fig. 16D).

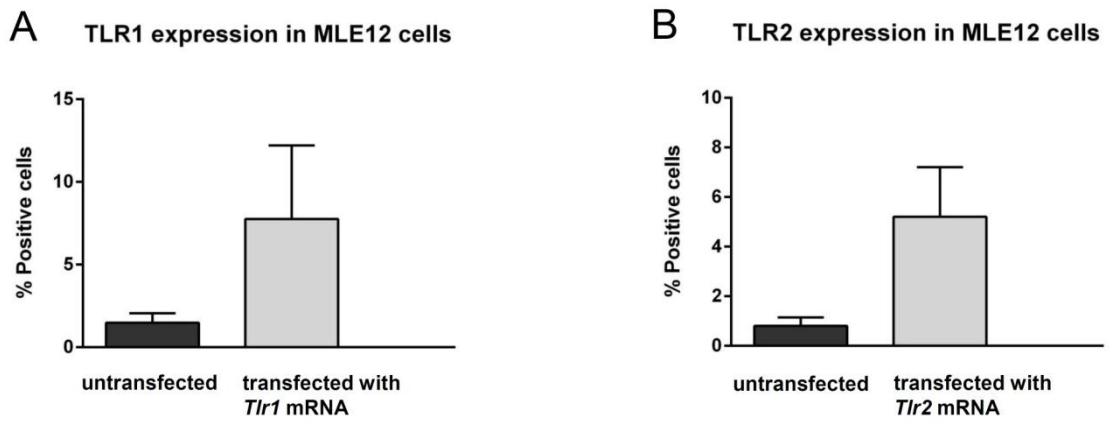

Fig. 17 TLR expression in MLE12 cells after transfection with *Tlr* mRNA

MLE12 cells were transfected with either *Tlr1* mRNA or *Tlr2* mRNA and subjected to FACS analysis 24 hour later. Transfection with *Tlr1* mRNA or *Tlr2* mRNA led to an increased expression of both TLR1 (A) and TLR2 respectively (B) in MLE12 cells. Data are represented as means ± SEM.

Tlr1 and *Tlr2* mRNA was furthermore tested in MLE12 cells. *Tlr* mRNA was administered to MLE12 cells via lipofection and incubated for 24 hours. The subsequent FACS analysis demonstrated upregulation of both TLR1 (Fig. 17A) as well as TLR2 (Fig. 17B) in MLE12 cells after transfection with respective mRNA, when compared to untransfected controls.

3.3 Effects of intratracheal *Tlr* mRNA delivery on systemic and local inflammation *in vivo*

3.3.1 Levels of inflammatory cells in BAL

Mice received combinations of *Tlr* mRNA at four determined time points before sensitization and challenge with HDM. Intratracheal applications were carried out under antagonizable anesthesia following the timeline as described in Fig. 18. At day 15, the predetermined endpoint of the study (32 days after the first injection), mice were sacrificed and different analysis were performed.

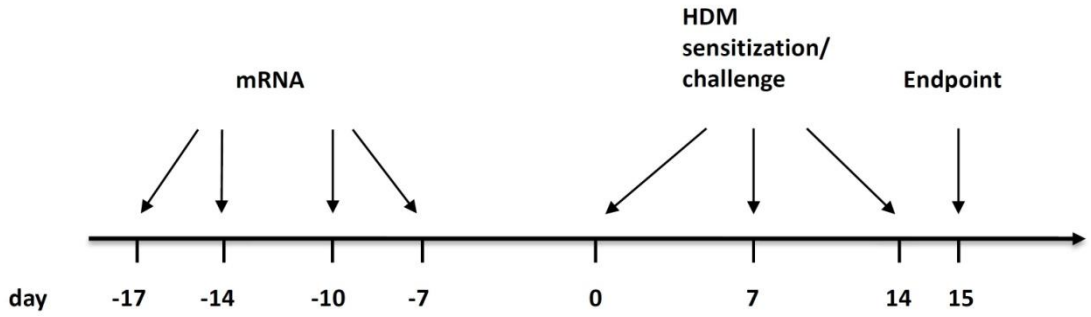

Fig. 18 Injection schedule for the HDM induced mouse model of asthma

Mice were treated intratracheally with combinations of *Tlr* mRNA at day -17, -14, -10 and -7 prior to the first sensitization with House dust mite extract (HDM), indicated as day 0. Further intratracheal injections with HDM followed on day 7 and 14. On day 15, the predetermined endpoint of the study, mice were sacrificed and several readouts were performed. (As published in [141])

Cells of BAL fluid were obtained at the time of sacrifice and differentially counted. Levels of inflammatory cells in BAL are presented in Fig. 19.

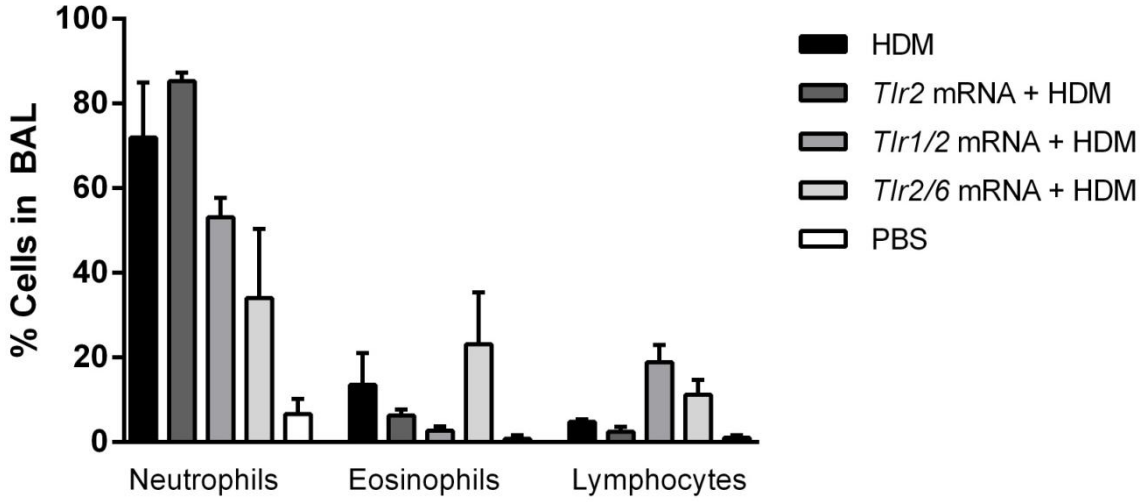

Fig. 19 Inflammatory cells in BALF

Intratracheal application of *Tlr1/2* mRNA resulted in decreased levels of neutrophils and eosinophils in bronchoalveolar lavage fluid (BALF). However treatment with *Tlr2/6* mRNA led to higher amounts of eosinophils compared to untreated controls but still dampened neutrophilic inflammation. Differences between mRNA treated groups and untreated controls were non-significant with $P > 0.05$. Data are represented as means ± SEM. (As published in [141])

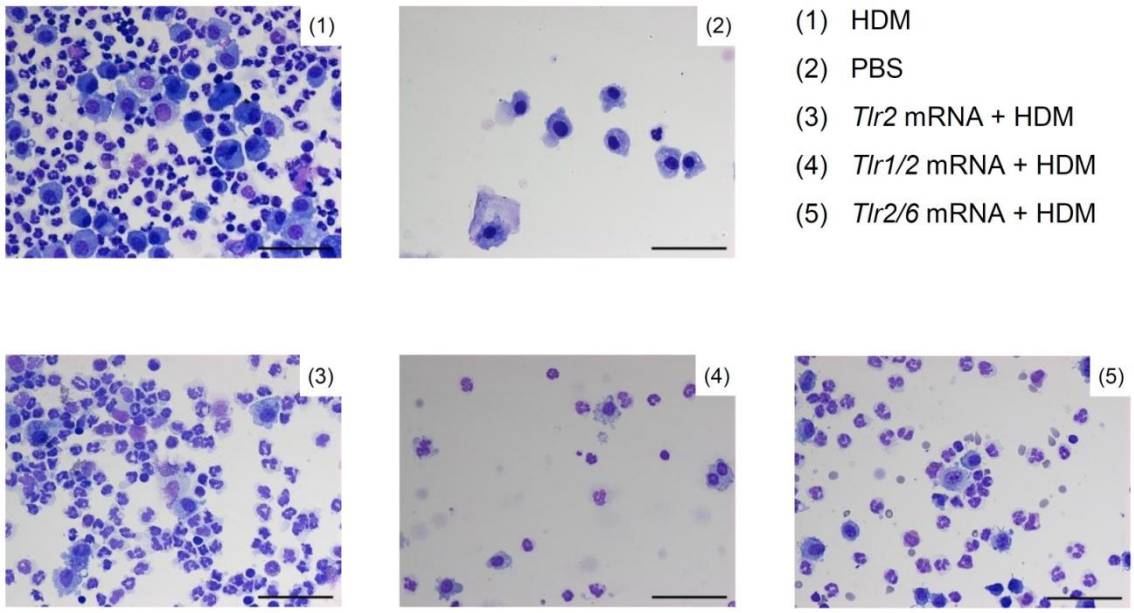

Fig. 20 Representative micrographs of BALF cellspin preparations

Lungs were lavaged with PBS and differential cell counts were performed microscopically. Scale 100 µm, magnification x200. (As published in [141])

Levels of both neutrophils and eosinophils in BAL were decreased after delivery of *Tlr1/2* mRNA when compared to untreated controls. Application of *Tlr2/6* mRNA led to higher amounts of eosinophils but still dampened neutrophilic inflammation. Treatment with *Tlr2* mRNA resulted in increased levels of neutrophils whereas eosinophils and lymphocytes were slightly reduced. However all differences were non-significant ($P > 0.05$) (Fig. 19). Representative micrographs stated in Fig. 20 additionally illustrate the distribution of macrophages and inflammatory cells in BALF of differently treated groups.

In order to directly compare the composition of BAL cells of treated and untreated mice, Fig. 21 states the data as parts of whole, indicating the considerable decline in neutrophils and eosinophils after treatment with *Tlr1/2* mRNA.

The results presented in this chapter 3.3.1 have been published in [141].

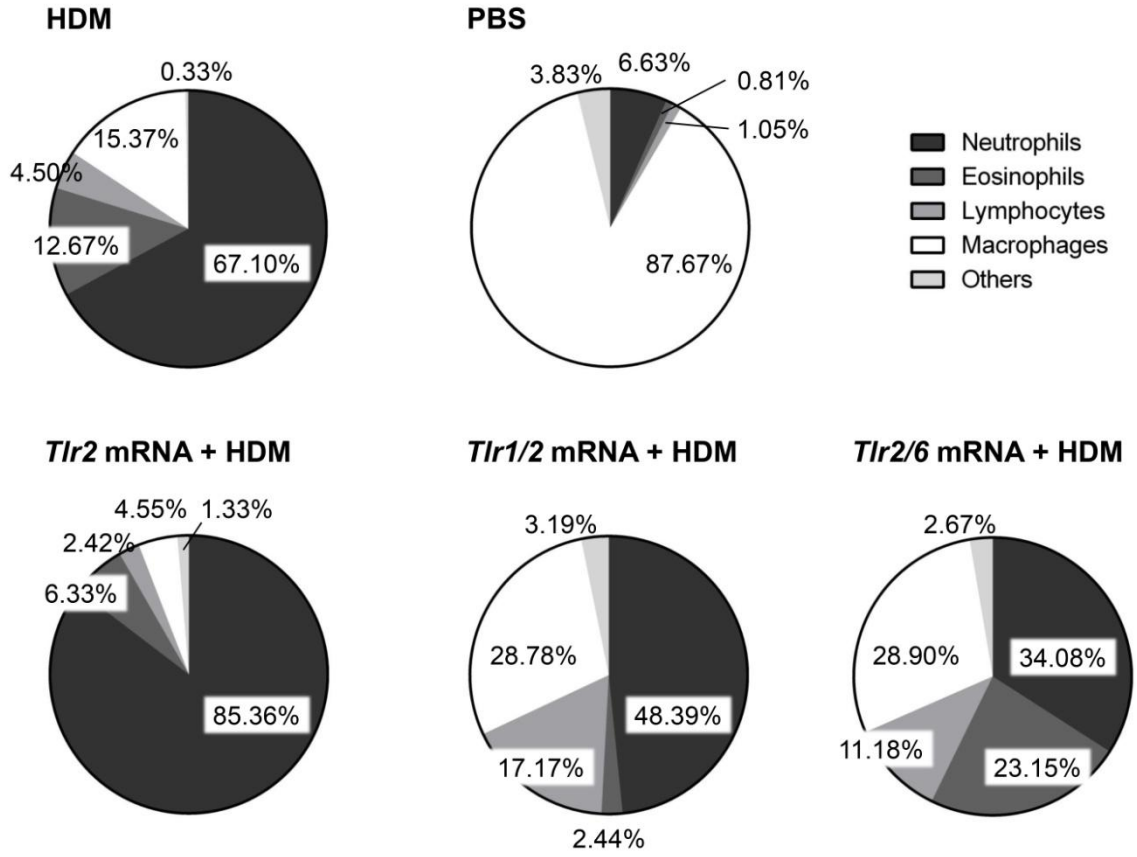

Fig. 21 Cells of BALF presented as parts of whole

The various BAL cells per group as stated in Fig. 19 are here presented as parts of whole. Data are represented as mean percentages.

3.3.2 Cytokine levels in BAL

The effect of *Tlr* mRNA treatment prior to HDM sensitization and challenge was further investigated on cytokine levels in BALF supernatant (Fig. 22). BAL fluid was collected at the time of sacrifice and centrifuged to obtain the supernatant. ELISA technique was used to subsequently determine cytokines in the BAL supernatant.

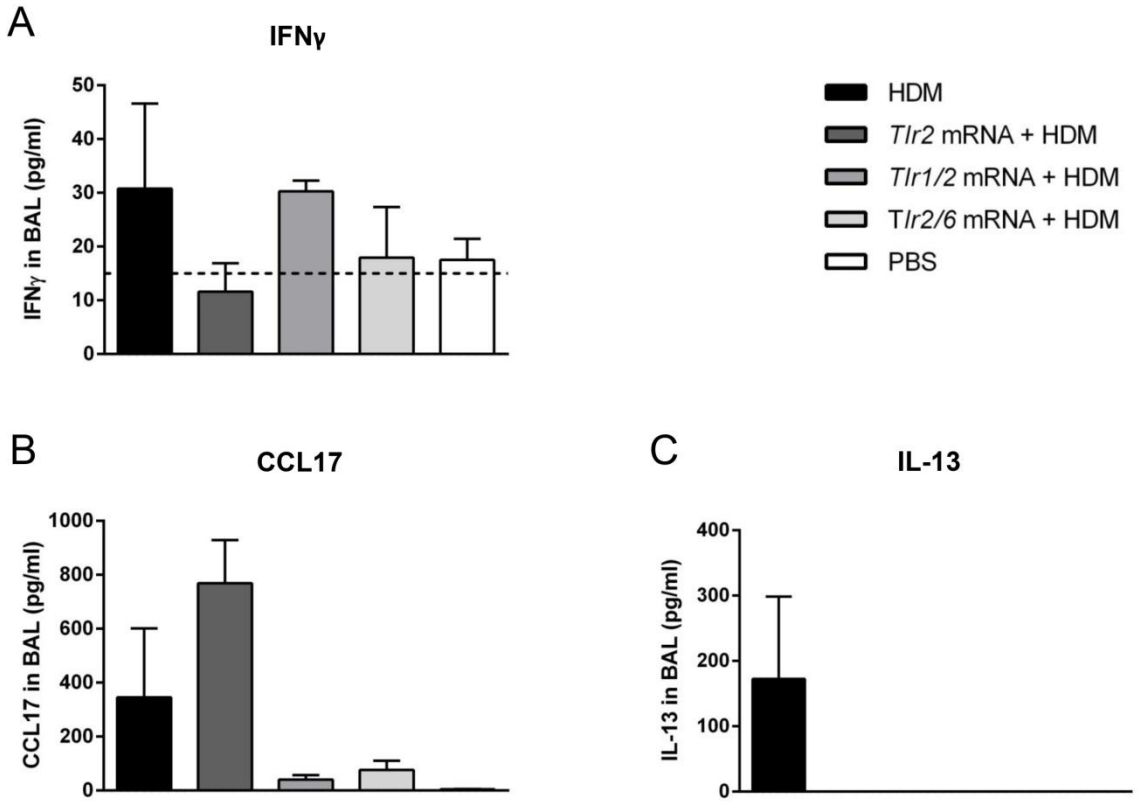

Fig. 22 Cytokine levels in BALF analysed using ELISA

Concentrations of cytokines in bronchoalveolar lavage (BAL) fluid of groups of differently treated mice are presented. (A) IFNγ levels were highest after delivery of *Tlr1/2* mRNA when compared to other mRNA treatments. The dashed line indicates the detection limit of 15 pg/ml. (B) Administration of *Tlr1/2* and *Tlr2/6* mRNA resulted in decreased, administration of *Tlr2* mRNA however in increased CCL17 concentrations. (C) IL-13 was not detectable in all groups except for the HDM control group. IL-5 was not detectable (n.d.) in all samples (data not shown). Differences were non-significant. Data are represented as means ± SEM.

Treatment with *Tlr2* and *Tlr2/6* mRNA resulted in a moderate decrease of IFNγ levels in BALF compared to the HDM control group. Administration of *Tlr1/2* mRNA resulted in the highest IFNγ concentrations compared to other mRNA treatments.

CCL17 levels were markedly reduced after administration of *Tlr2/6* and *Tlr1/2* mRNA. Mice treated with *Tlr2* mRNA though, showed levels of CCL17 almost twice as high as the HDM control group. Concentration of CCL17 in the PBS group remained below the assay's detection limit of 7.8 pg/ml.

Whereas IL-5 levels were measured but not detectable in all groups, levels of IL-13 were decreased after application of *Tlr1/2*, *Tlr2* and *Tlr2/6* mRNA when compared to mice without *Tlr* mRNA treatment prior to HDM administration.

In order to detect cytokines in BALF more precisely, a bead-based bio-plex cytokine assay was performed additionally to the conventional ELISA tests.

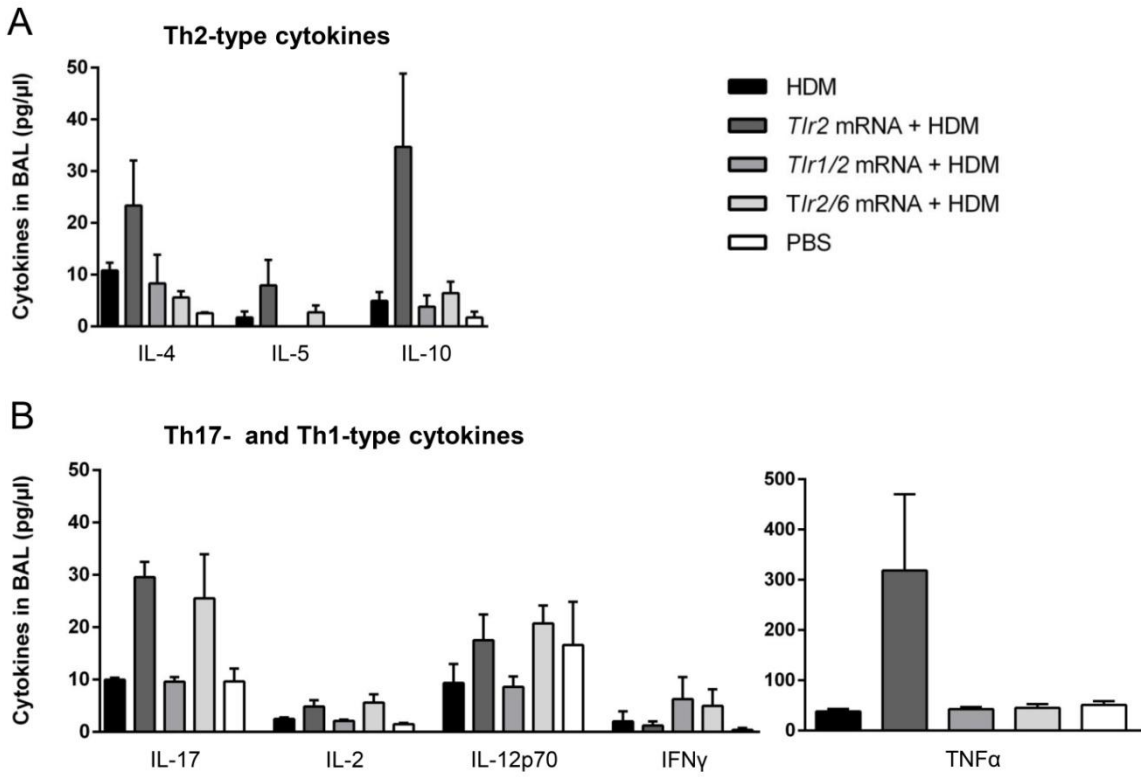

Fig. 23 Cytokine levels in BALF analysed using a Bio-Plex cytokine panel

T cell cytokines in BALF supernatants were quantified using a Th1/Th2 multiplex panel. (A) Th2-like cytokines were increased after administration of *Tlr2* mRNA and slightly decreased after treatment with *Tlr1/2* mRNA. (B) Levels of Th17-like cytokine IL-17 were elevated after *Tlr2* and *Tlr2/6* mRNA treatment, but not in response to *Tlr1/2* mRNA. Administration of *Tlr2* mRNA also led to increased levels of most Th1-type cytokines (except IFNγ), whereas *Tlr1/2* mRNA slightly reduced IFNγ concentrations in BALF. Differences remained non-significant. Data are represented as means ± SEM.

Cytokine levels of IL-4, IL-5 and IL-10, mainly secreted by Th2 cells, were increased after *Tlr2* mRNA treatment and remained unchanged or slightly reduced after *Tlr1/2* mRNA treatment. Administration of *Tlr2/6* mRNA resulted in marginally diminished IL-4 levels and barely influenced levels of IL-5 or IL-10 (Fig. 23 A).

Concentration of Th17-secreted cytokine IL-17 was increased in response to *Tlr2* mRNA and *Tlr2/6* mRNA treatment and remained unaltered after *Tlr1/2* mRNA treatment (Fig. 23 B).

Levels of Th1-type cytokines IL-2, IFNγ and IL-12 were elevated after *Tlr2/6* mRNA treatment. *Tlr2* mRNA treatment resulted in higher concentrations of IL-2 and markedly increased concentrations of TNFα, also mainly secreted by Th1 cells. Administration of *Tlr1/2* mRNA led to diminished levels of IL-2 and IL-12 and slightly increased concentrations of IFNγ (Fig. 23 B).

Parts of the results presented in this chapter 3.3.2 have been published in [141].

3.3.3 TLR Expression in lung and spleen tissue at the endpoint of the study

The expression of TLR1, 2 and 6 on day 15 (endpoint) was analyzed via Flow cytometry to determine whether the administration of *Tlr* mRNA may have long term effects on systemic and/or local TLR expression. Lung and spleen tissue was prepared and stained prior to FACS analysis. TLR expression detected in lymphocytes, monocytes, PBMCs and neutrophils in spleen tissue of mice treated with *Tlr* mRNA did not differ from TLR expression detected in these cells of mice treated with either HDM alone or neither mRNA nor HDM (data not shown). TLR expression in cells of lung tissue did also not differ significantly from treated to untreated mice. However, an increase in TLR1 and TLR2 expression on neutrophils was detected after administration of *Tlr1/2*, *Tlr2* as well as *Tlr2/6* mRNA (Supplement Fig. 1). Furthermore $Foxp3^+$ Tregs were analyzed in the same manner and again no differences could be discerned between differently treated groups (data not shown).

3.3.4 Immigration of inflammatory cells into lung and spleen tissue

In order to investigate both the local effect of *Tlr* mRNA application on immune cells in lung and the systemic effect on spleen tissue, lung and spleen cells were isolated, stained and subjected to FACS analysis.

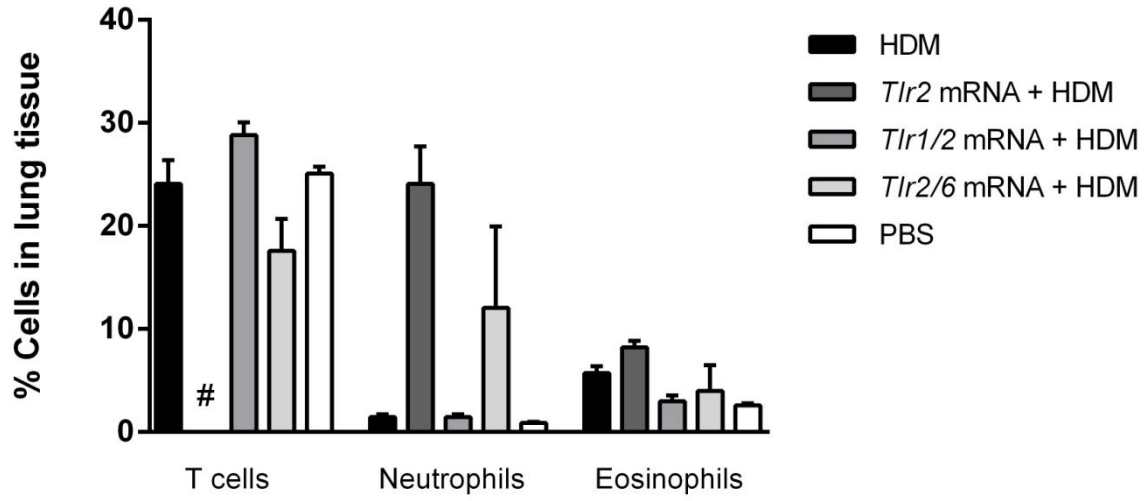

Fig. 24 Inflammatory cells in lung tissue

Levels of T cells, neutrophils and eosinophils were measured via flow cytometry. T cell levels in the *Tlr2* mRNA+HDM group could technically not be detected (#). Differences were non-significant. Data are represented as means ± SEM.

Numbers of T cells in lung tissue were reduced after administration of *Tlr2/*6 mRNA. Delivery of *Tlr1/*2 mRNA was able to dampen eosinophils but resulted in increased numbers of T cells. *Tlr2* mRNA resulted in augmented levels of eosinophils and considerably increased neutrophils in lung tissue (Fig. 24).

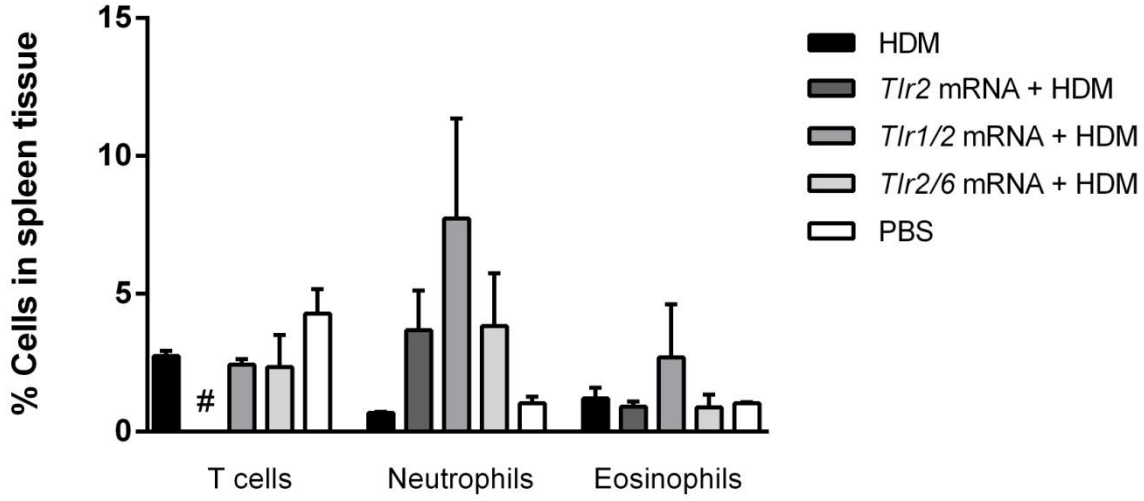

Fig. 25 Inflammatory cells in spleen tissue

T cells, neutrophils and eosinophils were analyzed using flow cytometry. T cell levels in *Tlr2* mRNA+HDM group could technically not be detected (#). Differences were non-significant. Data are represented as means ± SEM.

In order to further determine a possible systemic effect of *Tlr* mRNA treatment prior to HDM administration, immune cells of spleen tissue were analyzed (Fig. 25). Here, *Tlr2* mRNA led to increased numbers of neutrophils but slightly decreased numbers of eosinophils when compared to untreated controls. Levels of neutrophils and eosinophils but not T cells in spleen tissue cells were increased after delivery of *Tlr1/2* mRNA. All differences remained non-significant.

Parts of the results presented in this chapter 3.3.4 have been published in [141].

3.3.5 Inflammation and mucus production in lung tissue

At the time of sacrifice, whole lungs were obtained, embedded in paraffin and finally cut in sections. Lung tissue sections were then stained with either H&E to analyze tissue inflammation or PAS to evaluate mucus production.

Peribronchial, perivascular and interstitial tissue inflammation in lungs of mice treated with *Tlr1/2* mRNA was markedly reduced (Fig. 26). Administration of *Tlr1/2* mRNA resulted in significantly reduced ($P = 0.007$) numbers of mucus producing goblet cells in airways (Fig. 27). Treatment with *Tlr2* mRNA led to higher degrees of goblet cell metaplasia when compared to untreated controls (Fig. 27) and did not dampen lung inflammation. Inflammation of lung tissue was slightly diminished after delivery of *Tlr2/6* mRNA. No differences in goblet cell metaplasia were detected in this group.

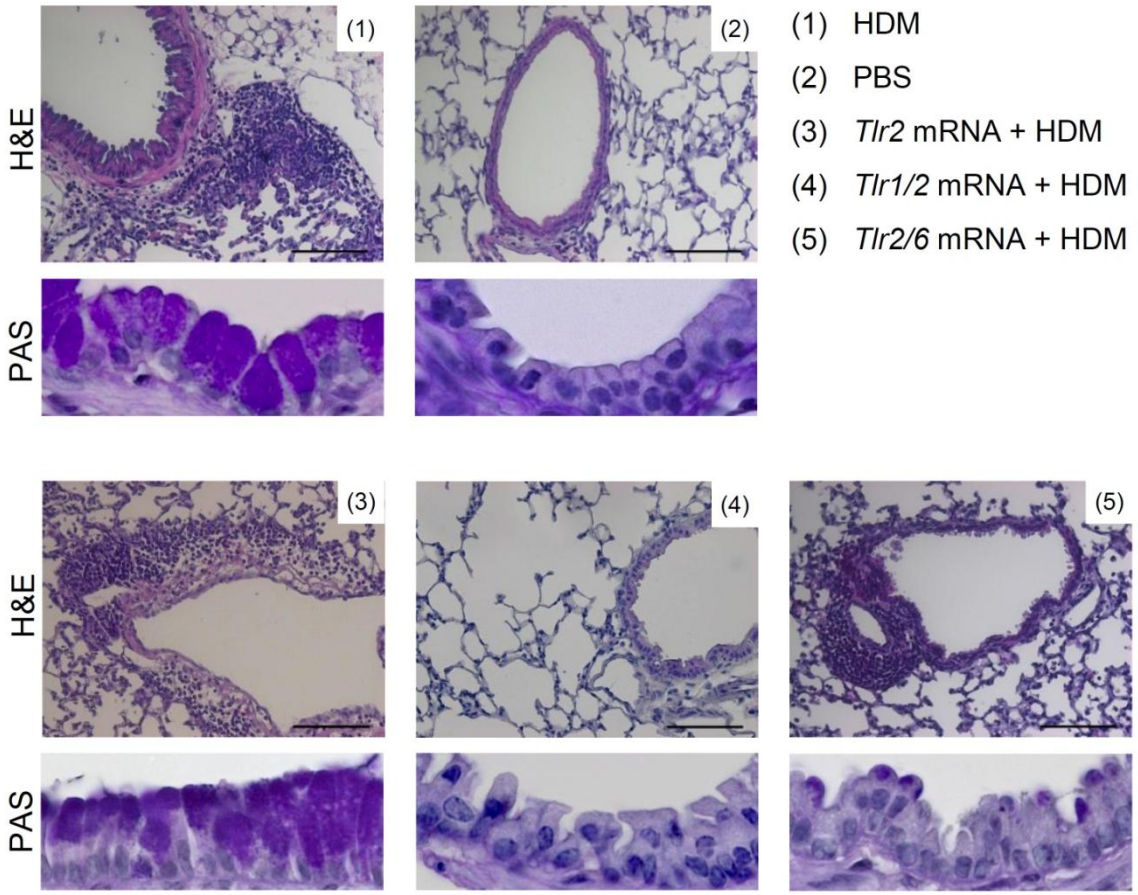

Fig. 26 Inflammation and mucus production in lung sections

Tissue inflammation and goblet cell metaplasia was analyzed on H&E- and PAS-stained lung sections. Representative micrographs are shown (scale 100 µm, magnification of H&E sections: x200, PAS sections: x400). (As published in [141])

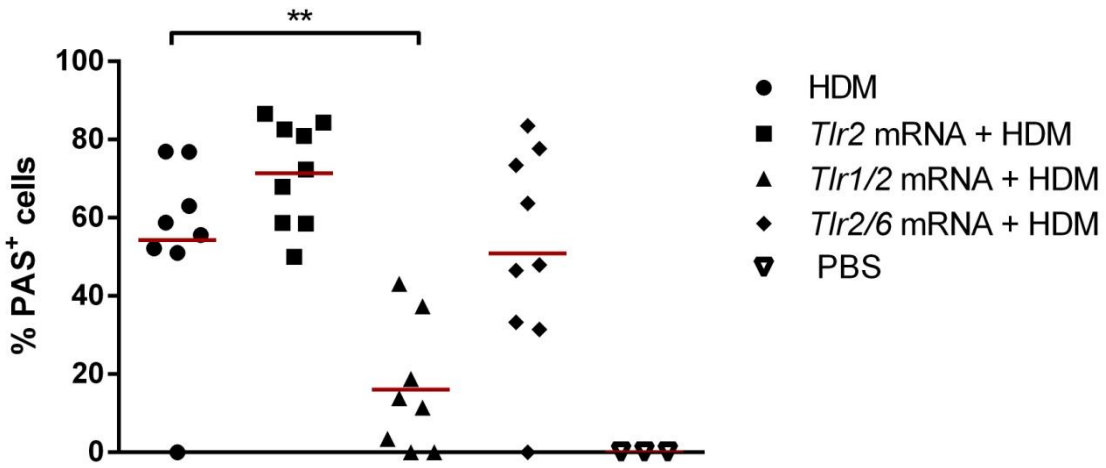

Fig. 27 Scoring for mucus producing PAS$^+$ goblet cells

PAS stained lung sections were analyzed to quantify PAS$^+$ goblet cells. Delivery of *Tlr2* mRNA resulted in increased levels of PAS$^+$ cells, whereas delivery of *Tlr1/2* mRNA led to significantly reduced goblet cell metaplasia (P = 0.007). Data are represented as individual mice, horizontal lines state means. (As published in [141])

The results presented in this chapter 3.3.5 have been published in [141].

3.4 Effects of *Tlr* mRNA treatment on lung function

Next, it was investigated whether the delivery of combinations of chemically modified *Tlr1, 2* and *6* mRNA modulates airway hyperresponsiveness of mice in an HDM induced model of asthma. For this purpose, lung function in terms of airway resistance was determined by using the model of the isolated, perfused and ventilated lung (IPL).

Here, markedly reduced airway resistance values following methacholine (MCh) challenge after administration of *Tlr1/2* mRNA prior to HDM challenge were observed (Fig. 28). Delivery of *Tlr2/6* mRNA was as well associated with decreased airway resistance values, hence lower airway hyperresponsiveness. However, treatment with *Tlr2* mRNA led to increased airway resistance and thus a decline in lung function. These mice showed significantly higher resistance values than PBS controls and *Tlr1/2* mRNA treated mice (Fig. 28).

The results presented in this chapter 3.4 have been published in [141].

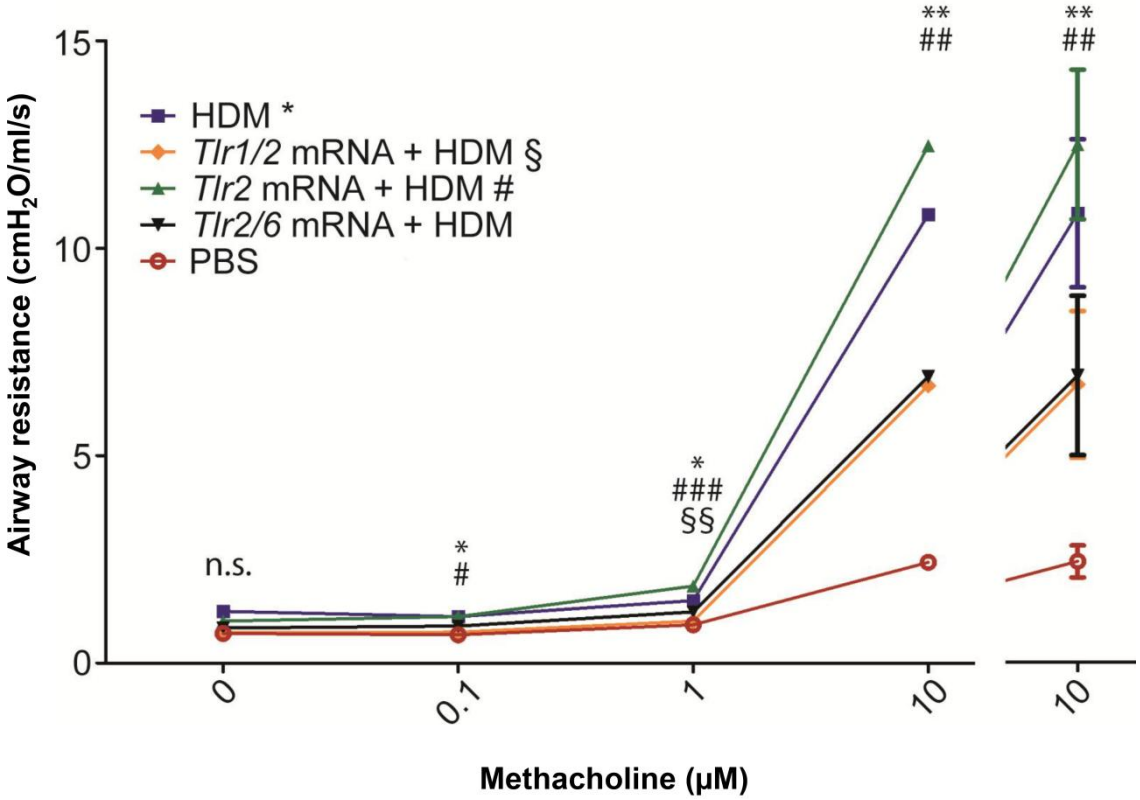

Fig. 28 Airway resistance values in response to MCh

Airway resistance was measured in response to rising concentrations of methacholine (MCh) using the isolated, perfused and ventilated lung (IPL). Treatment with *Tlr1/2* mRNA resulted in reduced airway resistance implying an increase of lung function. Statistical analysis was performed separately for each MCh concentration, * and # are PBS vs. the respective group, § is *Tlr1/2* vs. *Tlr2*. Data are represented as means, data at 10 µM MCh is additionally stated as means ± SEM. (As published in [141])

In conclusion, intratracheal administration of combinations of *Tlr1/2* and *Tlr2/6* mRNA resulted in improved lung function whereas intratracheal administration of *Tlr2* mRNA was associated with reduced lung function when compared to negative controls.

4 Discussion

4.1 General remarks

To elucidate the relationship between TLRs and asthma has been subject of ongoing research for some years. Various SNPs have been identified and new ligands were discovered. In fact, stimulation of TLRs with specific agonists is already subject of clinical studies. Though, *TLR* gene supplementation in terms of mRNA administration hasn't been investigated so far. This thesis provides first insights in the potential of this strategy and suggests *Tlr1/2* mRNA treatment as asthma-protective [141].

In the following sections, the present findings will be discussed in the context of previous and current study results, future perspectives and challenges.

4.2 The importance of mRNA modification and application route

Chemically modified mRNA has proven to hold great therapeutic potential in various fields of applications. Nevertheless, methodological improvements and the specific manner of nucleoside modification are still subjects of ongoing research. In the present study, mRNA incorporating 25% s2U and 25% m5C was used. This modification has proven/shown to yield efficient expression with high stability combined with low immunogenicity [127, 130]. In line with these findings, at the endpoint of our study, no significant differences in immune responses comparing mice treated with mRNA and PBS control groups were detected (Supplement Fig. 2). Here, treatment with *Tlr2*, *Tlr1/2* or *Tlr2/6* mRNA did not promote pulmonary inflammation in terms of increased immigration of T cells, neutrophils or eosinophils into lung tissue when compared to PBS control mice. However, other modifications, such as 10% s2U and m5C [129], pseudouridine-containing mRNA [126, 142, 143] or N(1)-methylpseurdouridine (also in combination with m5C) [144] also seem capable of ensuring low immunogenicity and high transfection efficiency. Beside biological stability and immunological safety, the application manner can be optimized as well. In

previous work of our group [127, 129] and also in the present study, mRNA (naked, dissolved in nuclease-free water) was delivered intratracheally by using a high pressure spraying device. Ambitious efforts to even further improve safety and efficacy of an mRNA delivery strategy resulted in introducing nanoparticles as vehicles for mRNA. This innovative tool enables various local and systemic routes of administration with very interesting results in recent publications [130, 145, 146]. Regarding the variety of possibilities, it becomes apparent that in terms of mRNA modification and delivery, there may not be one universal method or strategy. Rather it will be the challenge of future research to adapt chemical modifications and routes of administration precisely to the particular target organ or cell type and clinical implementation.

Continuative studies could also aim to vary the injection schedule of the murine asthma model. The injection schedule was designed based on the work of Mays et al. (2013) [129] and modified the application time points in order to create a preventative approach. Due to its relatively short half-time, mRNA was administered not only once but at four time points before the HDM challenge. Thereby, it was aimed to create a time frame of temporary TLR-upregulation. Interestingly, there is evidence to suggest that this sensitive time frame might already take place during pregnancy. In a large cross-sectional study, Ege et al. (2006) described that prenatal - thus maternal - exposure to farming environments has protective effects against asthma and atopy in children, probably via upregulation of innate immune receptors [147]. This observation was confirmed by Lundell and colleagues 2015, linking the farm exposure to increased levels of the B-cell activating factor. At the level of mouse models, maternal exposure to the cowshed-derived bacterium Acinetobacter Iwoffii protected the progeny against the onset of asthma through TLR signaling mechanisms [148]. The exposure of pregnant mice to combustion derived particles, however, interfered with the natural Th1/Th2 cell maturation of the offspring’s immune system and resulted in impaired airway hyperresponsiveness in the progeny [149].

The detailed understanding of prenatal immune processes and their influence on the offspring’s immune system remains still to be further investigated. A

modification of our injection schedule combined with a pregnant mouse model could, for example, enable maternal transient upregulation of certain TLRs. The subsequent analysis of both immunophenotype and atopic predisposition of their progenies could be an interesting subject of further research in this field.

4.3 The role of *Tlr1, 2* and *6* mRNA application in a mouse asthma model

4.3.1 *Tlr1/2* mRNA application improved asthma phenotype

In our study, preventative treatment with *Tlr1/2* mRNA resulted in decreased levels of both eosinophils and neutrophils in BALF. Whereas numbers of neutrophils remained constant, eosinophils were also reduced in FACS analysis of lung tissue cells. The reduction of these inflammatory cells in treated mice indicates a decline of the essential inflammatory component of asthma. Concordantly, markedly reduced interstitial and peribronchial tissue inflammation in histological lung sections were detected in this group.

Inflammatory cytokines CCL-17, IL-5 and IL-13, mostly secreted by Th2 cells, were slightly reduced after *Tlr1/2* mRNA treatment whereas IFNγ as a Th1 cytokine was increased. However, it might be too early to interpret these results as a switch in Th2/Th1 immune response since other Th1 and Th2 cytokines remained on the control group's level after *Tlr1/2* mRNA treatment. A general problem in our cytokine analysis (regarding *Tlr1/2* as well as *Tlr2* and *Tlr2/6* mRNA results) could be the overall low concentration of cytokines in all BALF samples. For future experiments, less volume of PBS to perform the BALF in order to receive higher concentrations of cytokines in each sample could help to facilitate cytokine analysis. To overcome this problem, a highly sensitive cytokine Multiplex-panel was used additionally to ELISA tests, which allows to detect the differences in IL-5 and IFNγ and to run more tests with only small sample volumes.

Besides inflammation, excessive mucus production is another central manifestation of asthma. This symptom was significantly decreased after *Tlr1/2* mRNA administration and measured in terms of quantifying peribronchial mucus-producing goblet cells. Airway hyperresponsiveness as another key

pathophysiological feature of asthma was investigated by means of airway resistance following *ex vivo* methacholine challenge. Here, markedly improved results for airway resistance (and hence lung function) were detected for mice treated with *Tlr1/2* mRNA.

The role of TLR1/2 positively modulating asthma and lung inflammation has also been suggested in some experimental and clinical reports. In a study from 2008, Koller and colleagues investigated TLR expression on airway neutrophils and lung function parameters of patients suffering from cystic fibrosis lung disease. Interestingly, improved lung function values correlated positively with increased TLR5 expression on airway neutrophils. This TLR5 upregulation turned out to be stimulated by bacterial Pam_3CSK_4 and mediated through TLR1/2 signaling [150]. Since TLR5 wasn't subject of investigation in the present study, the further exploration of TLR5 and its' role as a link between TLR1/2 signaling and improved lung function could serve as an interesting subject to investigate in an HDM induced mouse model of asthma. More recent studies report that stimulation with the TLR1/2 agonist Pam_3Cys was found to shape immune responses in human peripheral blood leucocytes [151] whereas stimulation with the novel lipopeptide LPGerD in a mouse model was able to induce an LPS tolerance state in antigen presenting cells, preventing mice from allergic sensitization [152].

Interestingly, the effect of *Tlr1/2* mRNA treatment resulting in improved lung function parameters and decreased bronchial inflammation could be repeated in a small-scale study performed subsequent to the thesis at hand. This study was conducted by Clara Will as her Bachelor thesis in Molecular Medicine at the University of Tuebingen. The experimental set-up of this study was designed and supervised by Prof. Kormann and me in order to validate the promising results of *Tlr1/2* mRNA administration obtained from this thesis. For this study, mice were treated intratracheally with either *Tlr1/2* mRNA or *RFP* mRNA as a mock control (both chemically modified with 25% s2U and 25% m5C). HDM and PBS groups served as controls. Mice were exposed to the same injection schedule described in Materials and Methods (Fig. 9) though HDM was administered intranasally. Concordantly with the findings at hand in this thesis,

preventative treatment with *Tlr1/2* mRNA again resulted in markedly improved lung function values (Supplement Fig. 3). In addition, both neutrophils and eosinophils in BAL fluid were significantly reduced after *Tlr1/2* mRNA treatment when compared to untreated HDM controls (Supplement Fig. 4).

4.3.2 Ambiguous effect of *Tlr2/6* mRNA on asthma outcome

In our study, treatment with *Tlr2/6* mRNA led to decreased levels of neutrophils in BALF, reduced pulmonary interstitial inflammation in histological sections and lower airway resistance, hence improved lung function. So far, these results seem to militate in favor of an asthma protecting effect of *Tlr2/6* mRNA treatment and in line with previous findings indicating a protective effect of TLR6 on asthma in humans and in a mouse model [34, 108, 153]. However some analysis yielded ambiguous results. In contrast to the results of the present study that were mentioned above, some of the findings also pointed rather towards enhanced inflammation, such as increased eosinophils in BALF and increased neutrophils in FACS analysis of lung tissue. Eosinophil levels in lung tissue remained unaltered when compared to untreated controls as were many BALF cytokine levels. In Multiplex analysis, IL-17A as a Th17 cytokine was distinctly elevated. Regarding the elevated levels of both eosinophils and IL-17A in BALF together, it is interesting to mention that eosinophils being elevated in sputum and BALF of asthmatic patients have been discussed to represent a source of IL-17A (additionally to Th17 cells), driving inflammatory responses through the release of proinflammatory mediators [154]. Schnyder-Candrian and colleagues reported about IL-17A in a mouse model to play a crucial role in both establishing asthma (through inflammatory mediators) but also being able to dampen allergic responses, emphasizing its dual role in immune responses [155]. When describing the protective role of TLR6 in both a fungal and an HDM driven mouse model of asthma, Moreira and colleagues demonstrated this effect to be IL-23 and IL17A dependent. In this context, TLR6 activation is responsible for Th17 activation through IL-23 and Th17 cells secrete IL17A to regulate and restrict allergic inflammatory responses [108]. In HDM and OVA induced mouse models of asthma, exogenous administered IL-17A vitiated the

protective and therapeutic effect of *Foxp3* mRNA treatment on allergic asthma [129]. Taken together, these findings indicate that the balanced signaling of Th17, Tregs and Th2 cells and their chemokine mediators are of crucial importance in allergic asthma immune responses and might explain, in parts, the diverse results of the present read-outs after *Tlr2/6* mRNA treatment.

4.3.3 Treatment with *Tlr2* mRNA alone worsened asthma symptoms

Additionally to combinations of heterodimer forming *Tlr1/2* and *Tlr2/6* mRNA, *Tlr2* mRNA was administered alone. Remarkably, treatment with *Tlr2* mRNA *in vivo* promoted a rather proinflammatory phenotype in our HDM induced mouse model of asthma. Percentages of neutrophils, but not eosinophils and lymphocytes, were increased in BALF samples at the endpoint of the study. In FACS analysis of lung tissue, neutrophils and eosinophils were markedly elevated when compared to HDM controls. Inflammation was also intensified in pulmonary interstitium and mucus producing goblet cells were augmented, analyzed on histological lung sections. Interestingly, also airway resistance of *Tlr2* mRNA treated mice was markedly increased. These mice had even worse lung function values than HDM control mice without mRNA treatment.

Contradictory results have been reported regarding the association of TLR2 and asthma. In view of the hygiene hypothesis stating that farmers' children are less affected by atopic asthma, Lauener and colleagues found increased TLR2 expression in farmers' compared to non-farmers' children. They suggested this finding to represent the link between (farming-) environment and decreased risk of the onset of allergic asthma [156]. Already previous reports demonstrated a therapeutic effect of TLR2 stimulation with lipopeptides such as LP40 [157] or Pam3CSK4 [158] in experimental asthma. Liu et al. demonstrated the inseparable co-stimulation of TLR2 and TLR4 by HDM, the close cross-talk between the two receptor pathways and the resulting Th2-promoting immune response [159]. This specific interaction of HDM components and TLR2 mustn't be disregarded in this context though cannot be fully distinguished since TLR4 was not part of the present study. Finally, in a clinical study following up Finnish children, no association between bronchiolitis severity or postbronchiolitis

wheezing (frequent pre-stages of childhood asthma) and polymorphisms in *TLR2* was found [160]. Also an additional follow-up study conducted by this group, investigating lung-function in association with *TLR* polymorphisms, did not deliver clearer results in this regard [161].

On the contrary and in line with the findings presented in this thesis, there is also widespread evidence for TLR2 to be a major contributor in asthma pathogenesis. TLR2 has been found to be overexpressed in both patients suffering from persistent allergic rhinitis [162] and in fatal asthma patients [112]. A mutation in TLR2 was found to relevantly increase not the risk of asthma specifically, but the risk of atopy in general in a German population [113]. Another polymorphism in TLR2 was significantly associated with asthma and also diabetes type 1 (an immune-mediated type of diabetes characterized by destruction of insulin-producing beta cells) in a large cohort of Norwegian children [117]. In a mouse model using ovalbumin instead of HDM to induce asthma, allergic responses in terms of AHR, airway inflammation, Th2 cytokine levels and mucus cell metaplasia were significantly reduced in TLR2 knockouts compared to TLR2 wildtype mice. The authors reasoned, TLR2 might function as a crucial mediator of the Th2-cytokine-driven inflammatory component of asthma [163]. The importance of the interrelation between TLR2 activation and a Th2 biased immune response was already proposed by Redecke et al., who observed how TLR2 activation aggravated experimental asthma [164].

When viewed in combination, the results of these reports suggest a complex role of TLR2 in the development of allergic asthma. Some of these controversial results might arise, in part, from imprecise differentiation into whether results are consequences of TLR2 protein expression levels, gene polymorphisms or heterodimerization with either TLR1 or TLR6. Particular single nucleotide polymorphism, specific TLR ligands, gene-gene interactions and the combination of these factors probably collude to orchestrate Th immune responses, in interaction leading or not leading to the onset of allergic asthma in children.

4.4 Future prospects

Recapitulating the immunological mechanisms that are contributing to atopy and allergy, it seems tempting to simply manipulate TLR pattern and Th1/Th2 balance in order to interfere with the pathogenesis of asthma. Treatment with tailored *TLR* mRNA transcripts could represent such a conceivable therapeutic approach and has been introduced in an animal model in the present study.

However, one of the main difficulties remains the dual role of TLRs. Depending on the disease, both excessive TLR signaling or insufficient TLR function can lead to pathological conditions. Hence, treating one condition might lead to the next, undesirable immune-mediated illness. Both TLR agonists (e.g. a TLR9 agonist [165, 166]) and antagonists (e.g. for TLR2 or 4, reviewed in [167]) for various applications are currently evaluated in clinical trials. However, TLR1/2 or TLR2/6 agonists are to date not yet part of these clinical studies. Systemically influenced TLR pattern may change TLR homeostasis entirely different in various organ systems (such as airways, urinary or gastrointestinal tract), thus therapeutic manipulation should be carefully considered. Downstream signaling mechanisms in terms of influencing Th1/Th2 responses can appear as a logical target for treating auto-inflammatory (mostly caused by exaggerated Th1 response) or atopic (driven by Th2 biased immune responses) diseases. Precise dosing and timing would be essential to ensure that treatment of one condition doesn’t result in the other.

Even though much has been learned about TLRs and their respective ligands, their interaction is still complex. Cross-reactions of TLR signaling should be taken into consideration and also the fact that agonists might act differently in natural occurring combinations and doses than purified ligands as tested under sterile conditions. Furthermore, the actual ligand dose is also likely to influence signaling, as shown for LPS-stimulation of TLR4 [168]. It becomes evident that deep and thorough understanding of the role of TLRs is a crucial precondition when composing TLR-based therapies.

The progress accomplished in the last years in designing highly effective chemically modified mRNA transcripts might represent the ideal tool for such

TLR-targeting therapies. Current efforts to introduce nanoparticles as vehicles for guiding mRNA to cellular destinations even increased the efficacy [130, 146]. Further studies should now optimize custom-designed chemical mRNA modifications and application routes. Overall, this novel method might be available for broader fields of application in the future.

However, the present data should be assessed considering some limitations this study comprises. The experimental design implies a rather small number of mice and matches the character of a pilot-study with implications for subsequent large-scale studies. Preliminary work [34] emphasizes the role of *TLR* SNPs in human asthma and their manifold impact on downstream signaling and onset of the disease. In the present study, conventional Balb/C mice and not humanized mice were used, with the consensus coding sequence (CCDS) murine *Tlr* genes as stated in the CCDS database at *www.ncbi.nlm.nih.gov.*

Despite various challenges, the need for new, innovative treatment options is obvious: Asthma is a major burden worldwide and available asthma medications are often accompanied by disturbing side effects. Current treatment approaches affect asthma symptoms but are unable to prevent the development of the disease. They furthermore do not account for patient specific immune backgrounds and are sometimes not even approved for application in children. Especially cases of severe asthma are still hard to control and can be life threatening [169-171]. Controlled, site-specific modulation of TLR expression via chemically modified mRNA might represent such a therapeutic tool. This novel approach may furthermore have preventative potential. The concept introduced in this thesis might serve as a starting point for further large-scale studies to both validate the present results and broaden the analysis to gain more detailed information. The concept of this study could also be applicable to other atopic and immune-mediated diseases with implications for additional investigations aiming beyond allergic asthma.

5 Summary

Asthma is the most common chronic disease in childhood. Environmental factors and genetic predisposition are key factors influencing the pathogenesis of the disease, operating in constant interaction.

Considering the environmental factors, exposure to an environment rich in microbes in early life (and already during pregnancy [148]) reduces the risk of atopic asthma [13, 55, 172]. Beside environmental aspects, the atopic genetic predisposition to produce IgE-antibodies in response to environmental allergens [7], plays a crucial role in the pathogenesis and facilitates the onset of the disease. At the interface between genes and environment, Toll-like receptors (TLRs) are viewed as gate-keepers of the immune system. They are capable of recognizing microbial components and influence immune responses as well as gene expression via intracellular signaling.

Studies investigating the role of TLRs in asthma pathogenesis suggest a protective role of TLR6 activation in mice [108]. Heterodimer variants of TLR1, 6 and 10 (all capable of forming heterodimers with TLR2) and increased protein expression of these TLRs were found to have protective effects on childhood asthma [34].

The therapeutic administration of chemically modified mRNA represents a promising tool to interfere with these genetic processes therapeutically. Not only as a treatment strategy for severe lung diseases [129, 130] but also in other fields of application [143, 145] the therapeutical potential of mRNA has been demonstrated. In contrast to approaches in gene therapy utilizing AAV vectors, chemically modified mRNA circumvents the threat of genomic integration and yields high transfer efficiency combined with low immunogenicity.

Based upon these findings, the present study was designed to test the effect of a preventative treatment with combinations of *Tlr1*, *Tlr2* and *Tlr6* mRNA in a HDM driven mouse model of asthma. Murine *Tlrs* were cloned into a plasmid vector in order to subsequently produce *in vitro* transcribed chemically modified *Tlr1*, *Tlr2* and *Tlr6* mRNA. Balb/c mice received mRNA and HDM extract

intratracheally. FACS analysis of lung and spleen cells, differential cell count of BALF and histology of whole lung sections were performed. Additionally, BALF cytokines were detected and lung function as well as airway resistance *via* IPL was measured.

After treatment with *Tlr1/2* mRNA decreased portions of both neutrophils and eosinophils in BALF were detected. In addition, eosinophils were also diminished in lung tissue cells. Furthermore considerably reduced tissue inflammation and significantly decreased mucus producing goblet cells were observed in this group. Application of *Tlr1/2* mRNA resulted in markedly reduced airway resistance and hence improved lung function compared to untreated HDM controls. Mice treated with *Tlr2* mRNA presented aggravated asthma symptoms with an elevated numbers of neutrophils in BALF and lung cells. Further symptoms were increased tissue inflammation and mucus production in lung histology as well as declined lung function. Administration of *Tlr2/6* mRNA led to reduced portions of neutrophils in BALF yet elevated levels of these cells in lung tissue. Analysis of lung histology yielded a slight reduction of tissue inflammation but no decline of mucus production whereas lung function values were considerably improved in this group.

Summing up, a protective effect of the treatment with chemically modified *Tlr1/2* mRNA in an HDM induced mouse model of asthma was observed. Although application of *Tlr2/6* mRNA was also able to improve lung function, further studies will be necessary to prove a clear effect. Administration of *Tlr2* mRNA however, resulted in an aggravated asthma phenotype.

The concept of this study combines new insights into the role of TLRs in atopic asthma with novel therapeutic tools such as chemically modified mRNA transcripts. Early treatment with tailored mRNA transcripts could provide a new therapeutic and even preventative approach in asthma management. Modification of TLR expression via chemically modified mRNA might furthermore have protective effects on other atopic and inflammatory diseases, therefore representing a promising target of future research.

6 Zusammenfassung

Asthma ist die häufigste chronische Erkrankung im Kindesalter. Umweltfaktoren und genetische Veranlagung beeinflussen entscheidend die Entstehung der Erkrankung und stehen in enger Wechselwirkung.

Bezüglich der Umweltfaktoren verringert sich das Risiko, an atopischem Asthma zu erkranken, wenn der Organismus in früher Kindheit (oder schon während der Schwangerschaft [148]) einer Umgebung mit hoher mikrobieller Belastung ausgesetzt ist [13, 55, 172]. Neben Umweltfaktoren spielt die genetisch bedingte atopische Prädisposition eine entscheidende Rolle - eine überschießende IgE-Antikörperproduktion als Antwort auf Umweltallergene begünstigt hier die Entstehung der Erkrankung [7]. An der Schnittstelle zwischen Umwelt und Genetik fungieren Toll-like Rezeptoren (TLRs) als sogenannte „Torhüter“ des Immunsystems. Sie können mikrobielle Bestandteile aus der Umwelt erkennen und über intrazelluläre Signalwege sowohl die Immunantwort als auch die Genexpression modulieren. Studien zur Rolle der TLRs in der Asthma-Pathogenese deuten auf eine schützende Wirkung einer TLR6-Aktivierung im Mausmodell hin [108]. Heterodimer Varianten in TLR1, 6 und 10 sowie erhöhte Expression dieser TLRs erwiesen sich als protektiver Faktor bezüglich Asthma im Kindesalter [34].

Chemisch modifizierte mRNA bietet einen vielversprechenden Ansatz, in diese genetischen Abläufe therapeutisch einzugreifen. Nicht nur in der Behandlung schwerer Lungenerkrankungen [129, 130] sondern auch in anderen Anwendungsgebieten [143, 145] wurde das therapeutische Potenzial mRNA basierter Therapie bereits demonstriert. Im Gegensatz zum Ansatz einer Gentherapie mit AAV-Vektoren, umgeht chemisch modifizierte mRNA die Gefahr der genomischen Integration bei gleichzeitig niedriger Immunogenität.

Basierend auf diesen Erkenntnissen wurde die vorliegende Studie entwickelt, mit dem Ziel, den Effekt einer präventiven Behandlung mit Kombinationen von *Tlr1*, *Tlr2* und *Tlr6* mRNA in einem durch HDM induzierten Asthma-Mausmodell zu erforschen. Murine *Tlrs* wurden in einen Plasmidvektor geklont, um

anschließend *in vitro* transkribierte chemisch modifizierte *Tlr1*, *Tlr2*, und *Tlr6* mRNA herzustellen. Balb/c Mäuse erhielten mRNA und HDM Extrakt intratracheal. FACS Analysen von Lungen- und Milz-Zellen, sowie eine differentielle Zellzählung der BALF wurden durchgeführt. Außerdem wurden die Histologie von Lungenabschnitten untersucht, BALF-Zytokine detektiert und die Lungenfunktion und Atemwegswiderstände mittels IPL gemessen.

Nach der Behandlung mit *Tlr1/2* mRNA zeigte sich ein Rückgang sowohl von Neutrophilen als auch Eosinophilen in der BALF. Auch in den Lungengewebszellen ging die Zahl der Eosinophilen zurück. Zusätzlich konnte in dieser Gruppe in der Lungenhistologie ein deutlicher Rückgang der Gewebsentzündung sowie eine signifikant reduzierte Zahl Mukus-produzierender Becherzellen festgestellt werden. In der IPL zeigten sich erheblich reduzierte Atemwegswiderstände der *Tlr1/2* mRNA Gruppe, also eine verbesserte Lungenfunktion im Vergleich zu unbehandelten HDM Kontrolltieren. Die Behandlung mit *Tlr2* mRNA führte zu verschlechterten Asthmasymptomen mit einem Anstieg der Neutrophilen in BALF und Lungengewebe, gesteigerter Gewebsentzündung und Mukusproduktion sowie höheren Atemwegswiderständen als in der Asthmakontrollgruppe. Die Gabe von *Tlr2/6* mRNA führte zwar in der BALF zu erniedrigten Anteilen an Neutrophilen, welche jedoch im Lungengewebe erhöht waren. Die Analyse der Lungenhistologie zeigte einen leichten Rückgang der Gewebsentzündung mit etwa unveränderter Mukusproduktion. Die Lungenfunktion dieser Gruppe lieferte wiederum deutlich verbesserte Werte im Vergleich zur HDM Kontrollgruppe.

Zusammenfassend zeigte sich in dieser Studie ein protektiver Effekt der Behandlung mit chemisch modifizierter *Tlr1/2* mRNA auf Asthma in einem HDM-induzierten Mausmodell. Eine Behandlung mit *Tlr2/6* mRNA verbesserte zwar ebenfalls die Lungenfunktion, weiterführende Studien sind hier allerdings notwendig um einen eindeutigen Effekt nachzuweisen. Die Gabe von *Tlr2* mRNA hingegen hatte einen aggravierten Asthmaphänotyp zur Folge.

6 Zusammenfassung

Die vorliegende Studie liefert neue Einblicke in die Rolle von TLRs in atopischem Asthma kombiniert mit neuen therapeutischen Möglichkeiten, wie chemisch modifizierter mRNA. Eine frühe Therapie mit maßgeschneiderten mRNA Transkripten könnte einen neuen therapeutischen oder sogar präventiven Behandlungsansatz im Asthmamanagement liefern. Die Modifikation der TLR-Expression mittels modifizierter mRNA könnte desweiteren schützende Effekte auf weitere Erkrankungen des atopischen Formenkreises bieten und stellt folglich einen vielversprechenden Ansatzpunkt für weiterführende Studien dar.

7 Literature

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8 Figures

Supplement Figures

9 Tables

10 Erklärung zum Eigenanteil

Diese Arbeit wurde in der Universitätsklinik für Kinder- und Jugendmedizin, Abteilung 1, Sektion Pädiatrische Infektiologie & Immunologie, unter Betreuung von Prof. Michael Kormann durchgeführt.

Die Konzeption der Studie erfolgte durch Prof. Kormann und mich.

Die Methode „IPL“ zur Lungenfunktionsprüfung wurde von Dipl.-Biol. Benedikt Mothes, Institut für Pharmakologie und experimentelle Therapie der Universität Tübingen, durchgeführt und ausgewertet. Benedikt Mothes erstellte graphisch Figure 27, Figure 28 sowie Supplement Figure 3. Prof. Kormann führte die Tierversuche durch, wobei ich assistierte. Jennifer Rottenberger, MTA, führte die Paraffineinbettung und Färbung der Histologie durch. Sämtliche weiteren Versuche wurden nach Einarbeitung durch Prof. Kormann sowie verschiedenen Labormitgliedern von mir eigenständig durchgeführt. Die statistische Auswertung erfolgte nach Anleitung durch Prof. Kormann durch mich.

Ich versichere, das Manuskript selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Teile der vorliegenden Studie wurden in folgendem Publikationsorgan veröffentlicht: **Zeyer, F**., B. Mothes, C. Will, M. Carevic, J. Rottenberger, B. Nurnberg, D. Hartl, R. Handgretinger, S. Beer-Hammer, and M.S. Kormann (2016).”*mRNA-Mediated Gene Supplementation of Toll-Like Receptors as Treatment Strategy for Asthma In Vivo.”* PLoS One, **11**(4): p. e0154001. In dieser Veröffentlichung wurden Figure 2B, 2C und 2D von Benedikt Mothes erstellt. Alle weiteren Abbildung stammen von mir. Das Manuskript dieser Veröffentlichung wurde von mir verfasst und von Benedikt Mothes, Prof. Sandra Beer-Hammer und Prof. Kormann korrigiert.

Tübingen, den

11 Publications

Mahiny, A.J., A. Dewerth, L.E. Mays, M. Alkhaled, B. Mothes, E. Malaeksefat, B. Loretz, J. Rottenberger, D.M. Brosch, P. Reautschnig, P. Surapolchai, **F. Zeyer**, A. Schams, M. Carevic, M. Bakele, M. Griese, M. Schwab, B. Nurnberg, S. Beer-Hammer, R. Handgretinger, D. Hartl, C.M. Lehr, and M.S. Kormann (2015). "*In vivo genome editing using nuclease-encoding mRNA corrects SP-B deficiency."* Nat Biotechnol, **33**(6): p. 584-6.

Zeyer, F., B. Mothes, C. Will, M. Carevic, J. Rottenberger, B. Nurnberg, D. Hartl, R. Handgretinger, S. Beer-Hammer, and M.S. Kormann (2016)."*mRNA-Mediated Gene Supplementation of Toll-Like Receptors as Treatment Strategy for Asthma In Vivo."* PLoS One, **11**(4): p. e0154001

Supplement

Coding sequence of murine *Tlr1* insert

ATGACTAAACCAAATTCCCTCATCTTCTACTGTATCATTGTTTTAGGACTGA
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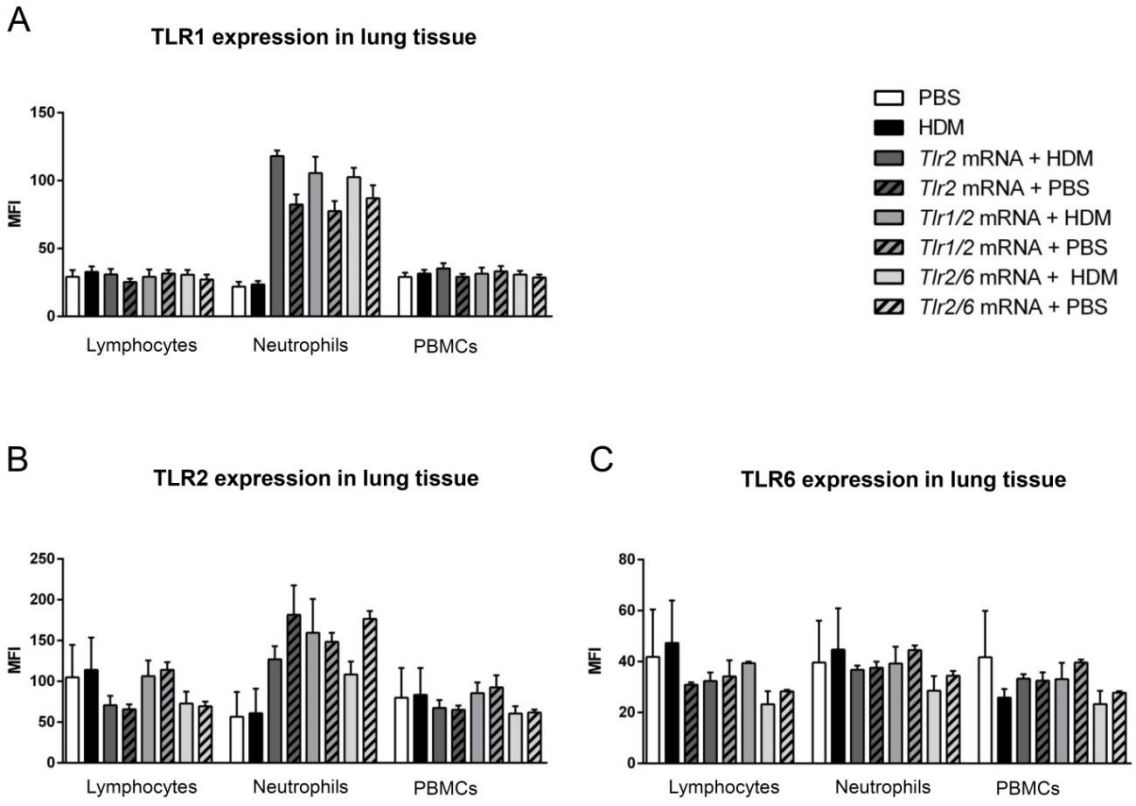
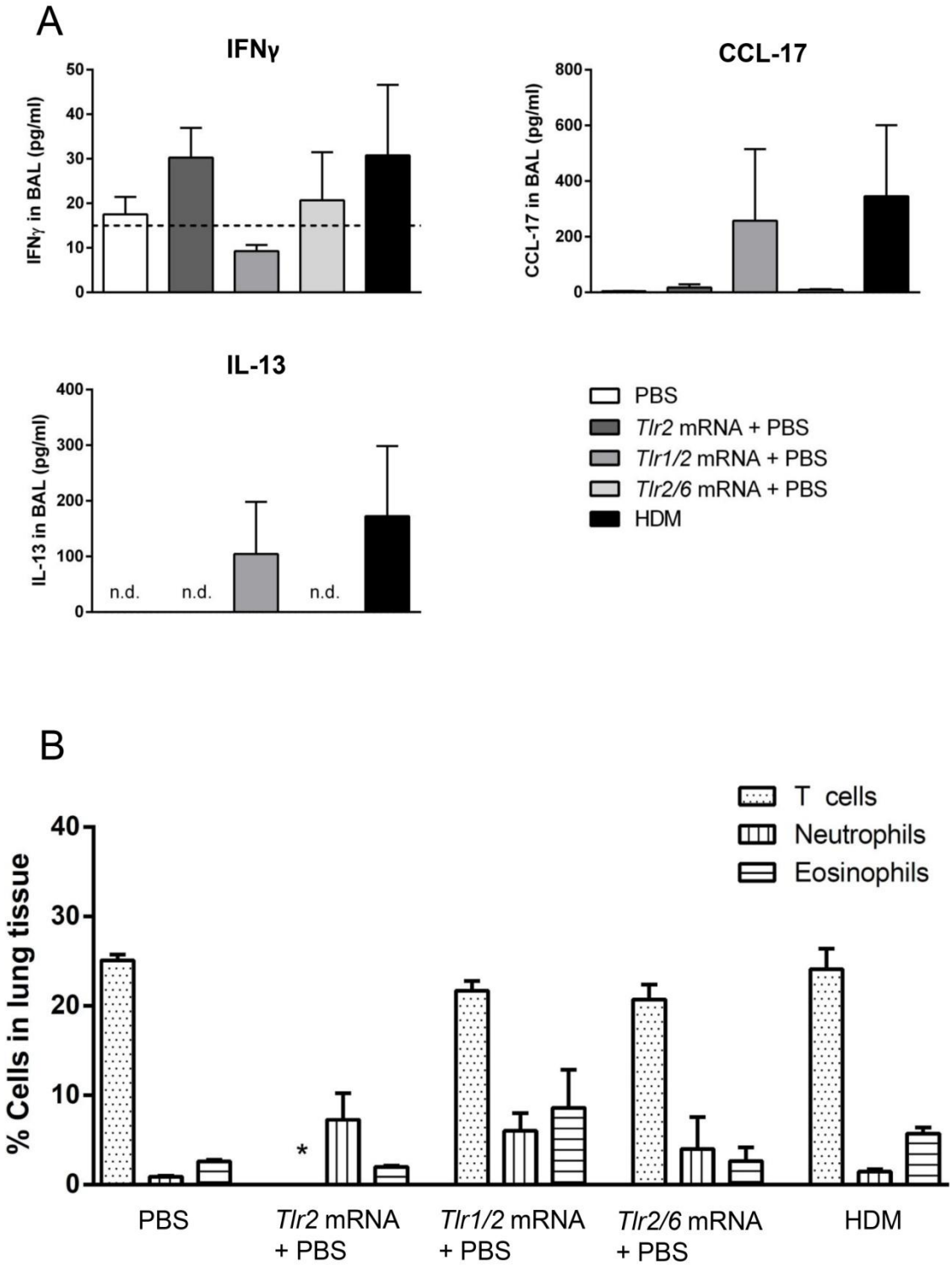


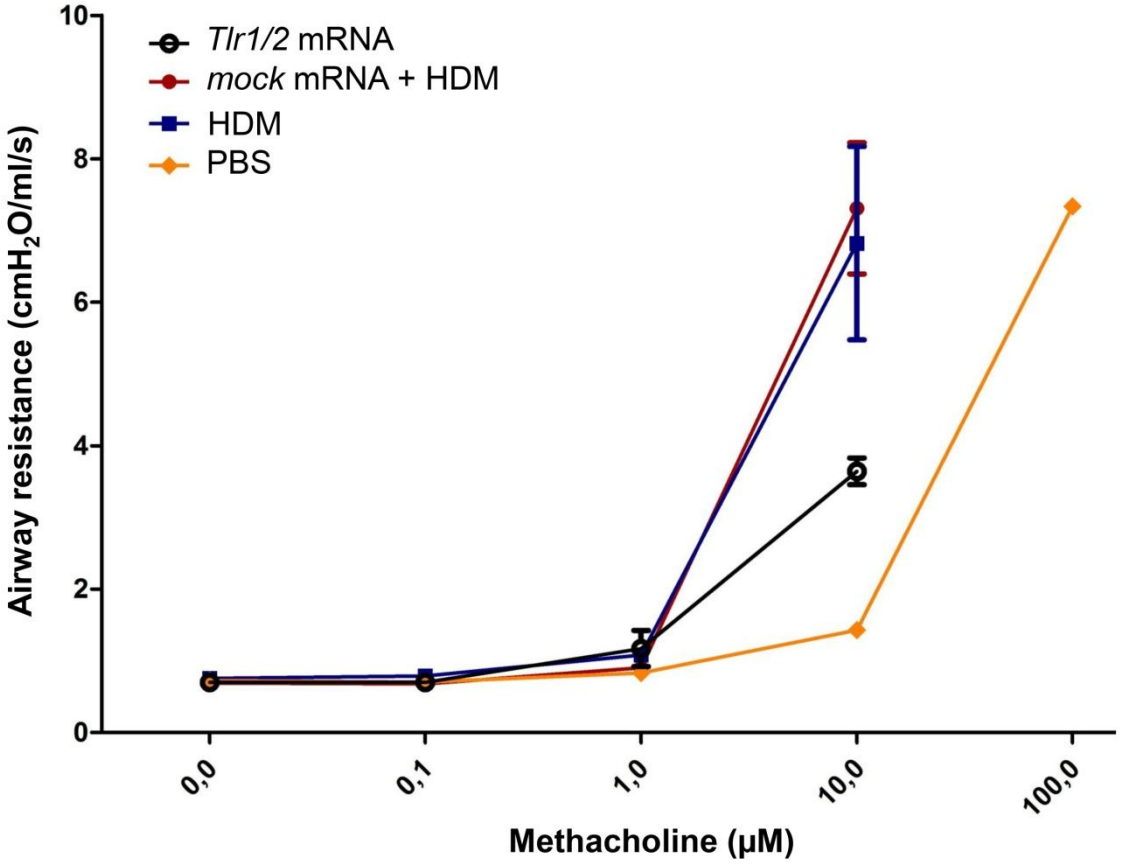
Supplement Fig. 1 TLR expression in lung tissue cells at the end of the study

Cells of lung tissue were harvested at the predetermined endpoint of the study, stained and subjected to flow cytometry analysis. Expression of TLR1, 2 and 6 were detected on lymphocytes, neutrophils and PBMCs. Data are presented as means ± SEM.



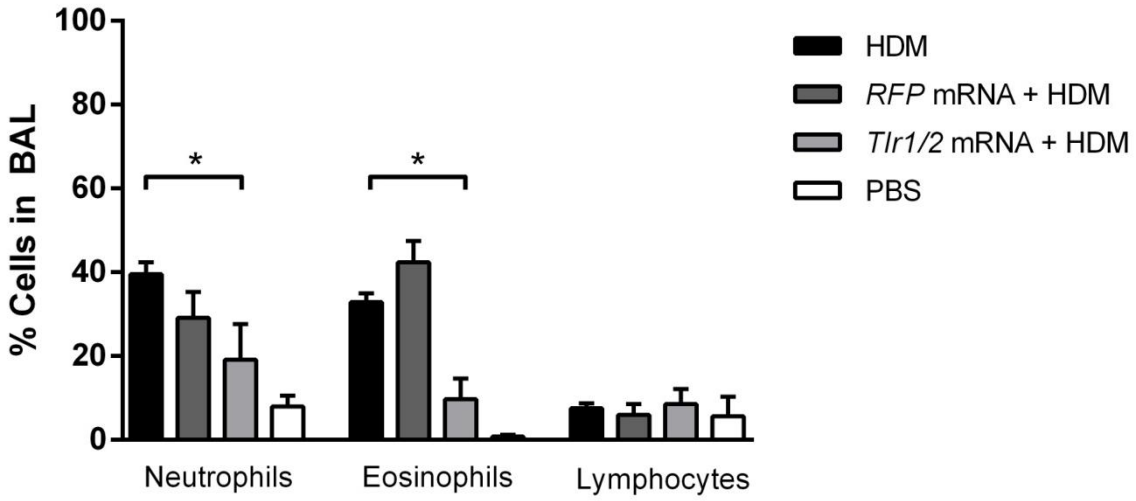
Supplement Fig. 2 Treatment with *Tlr* mRNA alone does not lead to increased lung inflammation

Mice were treated intratracheally with *Tlr1*, *2* and *6* mRNA according to the injection schedule. A) Cytokines in BAL were measured by ELISA, the dashed line indicates the detection limit of 15 pg/ml. IL-5 was measured but not detectable in all samples. n.d. = not detectable. B) Cell infiltration of T cells, neutrophils and eosinophils in lung tissue was analyzed via flow cytometry. * = no valid detection due to technical antibody issue. All data are stated as means ± SEM, n=3.



Supplement Fig. 3 Airway resistance values in response to MCh, Bachelor thesis C. Will

Airway resistance was measured in response to rising concentrations of methacholine (MCh) using the isolated, perfused and ventilated lung (IPL). Mice were treated with either *Tlr1/2* mRNA or mock *RFP* mRNA prior to HDM sensitization and challenge. Control groups received HDM without preventative mRNA treatment or only PBS. Differences were non-significant. Data are stated as means ± SEM. [Clara Will, Bachelor Thesis, Universität Tübingen, 2015]



Supplement Fig. 4 Inflammatory cells in BAL, Bachelor thesis C. Will

Differential cell count of bronchoalveolar lavage indicating percentages of inflammatory cells. Mice were treated with either *Tlr1/2* mRNA or mock *RFP* mRNA prior to HDM sensitization and challenge. Control groups received HDM without preventative mRNA treatment or only PBS. Intratracheal application of *Tlr1/2* mRNA led to significantly decreased levels of both neutrophils and eosinophils in BAL fluid ($P < 0.05$). Data are represented as means ± SEM. [Clara Will, Bachelor Thesis, Universität Tübingen, 2015]

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Curriculum Vitae

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Education

03|2013 – 06|2017	University Children's Hospital Tübingen, Pediatric Infectiology & Immunology **Doctoral thesis** under the supervision of Prof. Michael Kormann Title: *"mRNA mediated gene transfer of Toll-like receptors as treatment strategy for asthma in vivo"*
10|2009 – 11|2016	University of Tübingen **Study of Medicine** 17.11.2016: Second state examination ("good")
08|2014-01|2015	University of Istanbul, Turkey **Study of Medicine**
06|2008	Gymnasium Plochingen **Abitur**

Scholarships

04|2013- 03|2014	Scholarship "IZKF graduate programme", University of Tübingen
10|2010 - 11|2016	Scholarship of "Evangelisches Studienwerk Villigst e.V."

Language Skills	German (native), English (fluent), Swedish (very good), French (basic), Turkish (basic)