Effects of Toll-like receptor agonists on the pathogenesis of atopic asthma in mice

Dissertation

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Ich versichere, dass die vorliegende Arbeit nur unter Verwendung der angegebenen

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Diese Dissertation wurde weder in gleicher noch in ähnlicher Form in einem anderen

Prüfungsverfahren vorgelegt.

Neben dem akademischen Grad „Diplom-Biologe Univ." habe ich keine weiteren

akademischen Grade erworben.

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I. AIM OF STUDY

The aim of this study was to investigate if the immune stimulatory properties of Toll-like receptor (TLR) agonists can be utilized to develop novel therapeutic intervention strategies for the treatment of asthma. For this purpose five different TLR agonists were tested in preclinical murine models of acute and chronic asthma, both in preventive and therapeutic settings. Efficacy and safety of treatment were analyzed by assessing clinical relevant parameters including allergic inflammation, cytokine production, airway hyperreactivity, mucus production, smooth muscle hypertrophy, and fibrosis in the airways. Anti-asthmatic effects and pro-inflammatory capacities of the following five TLR agonists were directly compared, TLR2 agonist-lipoteichonic acid (LTA), TLR3 agonist-double stranded RNA (poly(I:C)), TLR4 agonist-lipopolysaccharide (LPS), TLR7 agonist-resiquimod (R848) and TLR9 agonist-DNA bearing unmethylated CpG base pairs (CpG).

II. TABLE OF CONTENT

III. ABBREVIATIONS

ANOVA = Analysis of variance

APC = Antigen presenting cell

BAL = Bronchoalveolar lavage

BSA = Bovine serum albumin

CCL = Chemokine C-C motif ligand

CD = Cluster of differentiation

CRA = Cockroach extract

DC = Dendritic cell

DNA = Deoxyribonucleic acid

EDTA = Ethylendiaminetetraacetic acid

e.g. = "exempli gratia" = for example

ELISA = Enzyme-linked immunosorbent assay

EtOH = Ethanol

FACS = Fluorescence-activated cell sorting

FOXP3 = Forkhead box P3

GATA-3 = Trans-acting T cell-specific transcription factor 3

GINA = Global Initiative for Asthma

GM-CSF = Granulocyte macrophage colony stimulating factor

HDM = House dust mite extract

HE = Hematoxylin and eosin staining

HRP = Horse reddish peroxidase

IFN = Interferon

Ig = Immunoglobulin

IL = Interleukin

ISS = Immunostimulatory sequences

MCh = Methacholine chloride

MDC = Human macrophage-derived chemokine

MHC = Major histocompatibility complex

MLP = Monophosphoryl lipid A

MMP-9 = Matrix metalloproteinase 9

NKT cell = Natural killer T cell

ODN = Oligodeoxynucleotides

OPD = O-phenylenediamine

OVA = Ovalbumin

PAMPs = Pathogen associated molecular patterns

PAS = Periodic acid-Schiff stain

PBS = Phosphate buffered saline

PenH = Enhanced pause

Poly(I:C) = Polyinosinic-polycytidylic acid

RNA = Ribonucleic acid

RSV = Respiratory syncytial virus

SEM = Standard error of the mean

SIT = Specific immunotherapy

TARC = Thymus and activation-regulated chemokine

TCR = T cell receptor

TGF = Transforming growth factor

T_H cell = T helper cell

TLR = Toll-like receptor

TNF-α = Tumor necrosis factor α

T_r cell = Regulatory T cell

STAT-6: Signal transducer and activator of transcription 6

VCAM-1 = Vascular cell adhesion molecule 1

INTRODUCTION

1. Asthma

The term "asthma" derives from the Greek verb ἀσθμαίνω, meaning to pant, to exhale with an open mouth, or to breathe sharply and the term άσθμα [aazein] meaning dyspnoea and shortness of breath. The first mention of "asthma" can be found in one of the oldest works of Western literature; Homer´s "Iliad". As a medical term asthma was first used in the " Corpus Hippocraticum" written ca. 460-360 BC by the Greek physician Hippocrates [1].

To date the "Global Initiative for Asthma" (GINA) 2004 describes asthma as a chronic inflammatory disorder of the airways with the involvement of different cell types and cellular elements. According to GINA, the chronic inflammation causes airway hyperresponsiveness leading to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly occurring at night or early in the morning. These episodes are usually associated with airflow obstruction, which in most of the cases abates either spontaneously or due to medical intervention [2]. In general, asthmatics suffer from an ongoing inflammation located in the lung tissue, show airway hyperresponsiveness, and variable obstructions of the airways.

Apart from these unifying characteristics, asthma is a very heterogeneous disease that can vary strongly in the specific clinical phenotype [3]. Main differences seen in patients suffering from asthma are the involvement of allergic reactions, type of inflammation, stimuli specificity of disease and response to treatment [4-6]. Depending on the involvement of allergic reactions, asthma can be separated into atopic/allergic or intrinsic/non-atopic asthma. In atopic asthma, pulmonary inflammation is thought to be triggered by exposure to aero-allergens like pollens, house dust mite or animal dander, whereas in non-atopic asthma the inflammatory trigger is unknown. Inherited factors like atopy, which can be defined as a genetic predisposition toward the development of an allergic hypersensitivity, are risk factors for the emergence of asthma and genetic analysis found many correlations between disease and genetic variations. However, the exact clinical implication of most of these genetic differences remain undetermined and from the genetic background alone it can not be diagnosed if someone will develop asthma [7]. Therefore, it is suggested that unspecific environmental factors such as viral infections, air pollution or smoke contribute to a higher susceptibility to developing asthma [8;9]. However, the exact causes and how different factors impact on the development of asthma is still not fully understood.

Today, asthma affects approximately 300 million people worldwide, making it one of the most common chronic diseases. The prevalence of asthma is highest in Western Europe, Australia, New Zealand and North America, but the incidence of asthma is increasing globally [2]. In Western Europe, the incidence doubled over the last decade with currently estimated 30 million asthmatics [10]. Alarming is that the increase of prevalence is highest in children under the age of 12, making it the most common chronic condition among children in North America [11]. The high numbers of asthma sufferers is also an important factor with regard to health cost. In 1987, annual costs for asthma treatment was estimated at approximately $6 billion in North America, rising to estimated costs of $18 billion in 1998 [12;13]. Beside the economic cost, asthma is a considerable burden for the affected, leading to a loss of productivity, constrain of activity and lowered quality of life [14]. This is the case for the mild or moderate form of the disease which can normally be well controlled by therapeutic intervention. However, around 5-10% of asthmatics suffer from the severe form of asthma, which can become a life-threatening disease. Patients suffering from the severe form of asthma are prone to severe exacerbations and this group has the highest percentage of corticosteroid insensitive patients, which show a poor or no response to treatment [15;16]. Therefore, these asthmatics exert the highest medical need for novel therapies and effective therapeutics. The main pathological difference that separates mild from severe chronic asthma is the remodeling process in the lung, which unlike in mild asthma, is more pronounced and permanent. More precisely, lungs of patients suffering from the severe form of the disease exhibit strong fibrosis and a hyperplasia of goblet cells, which is accompanied with increased production of mucus. This mucus production eventually leads to mucus plugging of small airways, a severe symptom closely associated with acute lung failure. Another symptom predominantly seen in severe chronic asthma is the thickening of smooth muscle surrounding the airways [17-20]. These patients account for approximately 30-50% of the healthcare cost of all asthmatics. Furthermore, they are prone to severe exacerbations and exhibit the highest mortality [21]. Worldwide, asthma is estimated to account for one in 250 deaths and in 2005, it was estimated that 255,000 people died of the disease [22]. Taken together, the incidence, morbidity and mortality of allergic asthma is increasing, thus making asthma a major health issue that needs to be addressed.

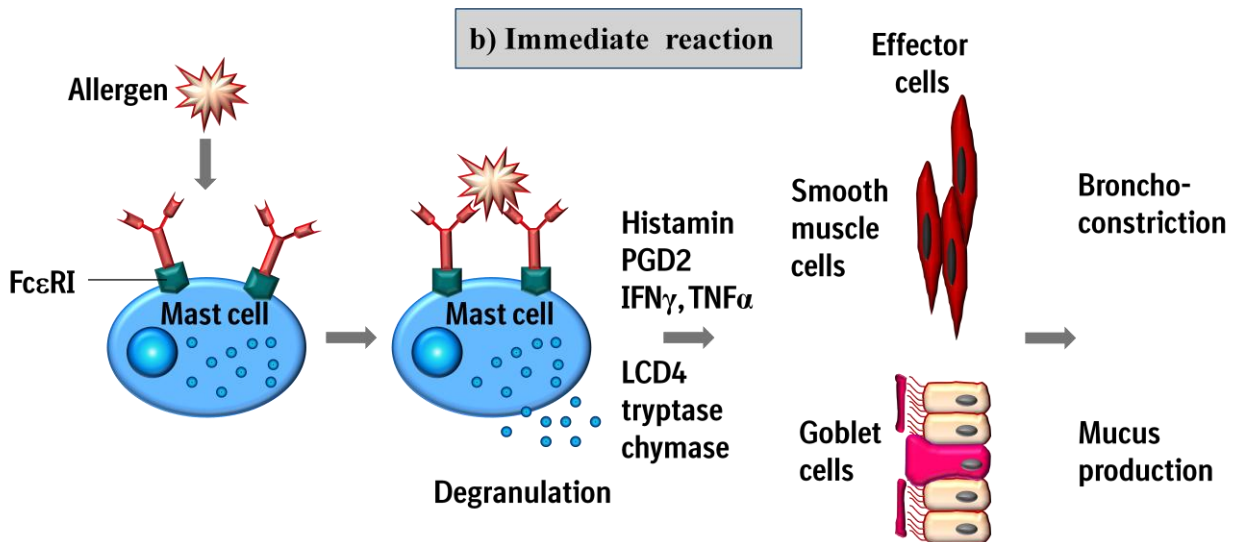## 2. Characteristics of atopic and non-atopic asthma

The three characteristic pathological hallmarks of asthma are airway obstruction, bronchial hyperresponsiveness and persistent airway inflammation [23]. Lately, the persistent inflammation of the lung has come into focus, not only as a mere symptom, but as the underlying cause for the development of asthma. As described above, depending on the origin of the inflammation, asthmatics are separated into two groups: non-atopic/intrinsic or atopic/allergic. People suffering from non-atopic or intrinsic asthma have no allergies and the cause of their airway inflammation is unclear. It is suggested that intrinsic asthma develops from chronic or recurrent infections of the bronchi, sinuses, tonsils and adenoids. In contrast, the pulmonary inflammation in atopic/allergic asthma is caused by exposure to an allergen.

In the 1920s, Arthur Fernández Coca and Robert Anderson Cooke coined the term "atopy" meaning "strange disease" for indications they found to be antigen-specific but for which they were not able to measure the precipitating antibodies [24]. Later on, the immunoglobulin isotype E (IgE) was identified to be responsible for the induction of humoral and cellular immune responses to allergens, and a direct correlation between serum IgE levels and asthma was found [25]. Today, atopy is defined as a predisposition to develop excessive IgE-mediated hypersensitivity reactions to otherwise innocuous molecules. Epidemiological studies suggest that in children and young adults more than half of the asthmatics suffer from the atopic type. As external stimuli are involved in this type of asthma it is also defined as extrinsic asthma.

In westernized countries, allergies against dust mites, pollens, pet dander and specific food proteins are very common. The Third National Health and Nutrition Examination Survey in the USA found that 54.3% of the tested population had positive test responses to one or more allergens [26]. In this study, the highest prevalence was measured for the following allergens: house dust mite, perennial rye, short ragweed and German cockroach. Atopic asthma is considered to be a hypersensitivity reaction of the immune response caused by an inherited tendency toward the development of allergies. Patients suffering from the atopic form of asthma exhibit elevated numbers of $CD4^+$ T helper type 2 (T_H2) cells in BALF and in blood [27]. Therefore, inflammation in asthma is considered to be predominantly driven by the aberrant expansion and activation of T_H2 cells [28]. These cells are able to initiate and enhance immune responses by attraction and activation of other immune cells. Figure 1 gives

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a general overview of the development of allergic responses and how T_H2 cells are believed to orchestrate the immune reaction.

The first step in the development of allergy is the sensitization reaction against the allergen. This reaction starts in the mucosa with antigen-presenting cells such as dendritic cells (DCs) which incorporate allergens and migrate to the T cell area of mediastinal lymph nodes where they can, supported by co-stimulatory signals, prime antigen-specific naïve T cells [29]. This essential role of DCs in asthma is supported by studies in which *in vivo* depletion of DCs abrogated characteristic features of experimental asthma [30]. During migration, DCs maturate, internalize and process the allergen and present small allergen fragments (antigens) on their major histocompatibility complex class II (MHC class II) [31]. Once in the lymph nodes, DCs are able to bind to naïve T cells provided that the T cells are able to fit to the antigen structure with their T cell receptors (TCR). This interaction was demonstrated by studies in mouse models in which lung DCs, but not B cells, stimulated a strong allergen-specific T cell response [32]. DC activation of naïve CD4+ cells induce differentiation to T_H2 cells, but can also induce the subtypes T_H1 or T_H17. The decision of which subtype is generated is influenced by many factors. Amongst others, the maturation state and the cell lineages of the interacting DC affect which T cell subtype is induced. Mature DCs of peripheral lymphoid organs and spleen preferentially induce T_H1, whereas immature mucosal DCs tend to induce differentiation into T_H2. T cell differentiation is tightly controlled and despite providing an essential signal, the cell-cell contact alone is not sufficient, as TCR signal alone induces anergy and/or apoptosis of the antigen-specific T cell [33]. Decisive factors for differentiation are the strength of the activating signal, which is decided by the TCR-antigen binding strength, the co-stimulatory binding of APC and T cell transmembran proteins such as CD28/B7, CD40/CD40L, inducing an intracellular signaling cascade, the presence of cytokines like IL-4, activating transcription factors like STAT6, and the genetic background of the T cell. Beside the essential antigen recognition, the most important factor for the T_H2 differentiation seems to be the composition of the surrounding cytokine milieu as it determines the activation of lineage-specific transcription factors to induce the T_H2 subtype [34]. Here, IL-4 is the most important cytokine inducing the T_H2 subtype and determining the lymphocyte´s production potential [35]. IL-4 elicits its function by activation of signal transducer and activator of transcription 6 (STAT6) which in turn leads to the upregulation of GATA-binding protein 3 (GATA3) expression, which is the master regulator for T_H2 differentiation and cytokine production [36].

a) Sensitization

Allergen

DC

MHC II
1 antigen
TCR

OX40/OX40L
2 CD28/B7

Naive T cell

3 IL-4

IgM

B cell

TH2

IgE

B cell

IL-4
IL-13

IgE

1 = activation signal
2 = costimulation
3 = cytokine milieu

b) Immediate reaction

Allergen

FcεRI

Mast cell

Mast cell

Degranulation

Histamin
PGD2
IFNγ, TNFα

LCD4
tryptase
chymase

Effector cells

Smooth muscle cells

Goblet cells

Broncho-constriction

Mucus production

Fig. 1. Model of allergen-induced immune response in asthma. *(a) Allergen contact induces differentiation of naïve T cells into T_H2 cells. This is dependent on the activation signal, costimulatory binding to receptors such as OX40L or B7.1 (CD80) and presence of IL-4 in the surrounding milieu. When the T_H2 cell binds to the antigen presenting B-cell, Ig class switching is induced and the B-cell releases IgE. (b) Mast cells with allergen-specific IgE bound to high-affinity receptors for IgE (FcεRI) degranulate upon allergen encounter. The release of the mast cell factors histamine, prostaglandinD2 (PGD2), leukotriene C4(LCD4), tryptase, chymase and cytokines like IFN-γ and TNF-α trigger the immediate reaction characterized by smooth muscle contraction and mucus production. (c) In the late reaction, T_H2 cells that are activated by antigen presenting cells (APC) proliferate and release different cytokines such as IL-4, IL-5, IL-9, IL-13, IL-25, and IL-33. Activation of effector cells leads to chronic inflammation and airway remodeling.*

Another important factor is the presence of transmembrane proteins on the surface of the T cells. These molecules can support differentiation by the stabilization and narrowing of antigen mediated T cell and APC binding but can also, upon activation induce an intracellular signaling cascade [37]. In vitro experiments with human CD4+ cells showed that co-stimulation enhances early IL-4 production of the co-stimulated naïve T cells, promoting their own differentiation into high IL-4-producing effector cells [38]. The best characterized co-stimulatory molecules on the surface of T cells are CD27, CD28, OX40, CD30, CD40L (also referred as CD154) and ICOS with their respective binding partner CD70, B7-1 and B7-2 (also referred as CD80, CD86), OX40L, CD30L (also referred as CD153), CD40 and ICOS-L [39]. Some of the co-stimulatory molecules such as CD27 and CD28 are constitutively expressed whereas other molecules like ICOS, OX40 and CD30 are only present on stimulated T cells [37].

The differentiation of the naïve T cell to a T_H2 cell takes place in the lymph node, whereas for the effector phase the T_H2 cell has to migrate to the lung tissue. In allergic inflammation pulmonary allergen exposure leads to the increased production of a specific set of chemokines in alveolar tissue. The most important chemokines suggested to inducing positive chemotaxis of T_H2 cells are human macrophage-derived chemokine (MDC), Thymus and activation-regulated chemokine (TARC), and eotaxin [40;41]. These chemokines induce cellular migration and guide T cells to the site of allergen encounter. In the lung tissue the mature T_H2 cells enhance the T_H2 inflammation by the attraction and activation of additional immune cells. One very important aspect is the release of the cytokines IL-4 and IL-13 as these cytokines favor immunoglobulin-class switching of specific B cells from the IgD and IgM subtypes to IgE by activation of ε germline gene transcription [42;43]. B cells can produce immunoglobulins without T cell activation, for example by stimulation with bacterial lipopolysaccharides but most antibody responses require antigen-specific T cell – B cell interaction. In this T cell dependent response, B cells are activated by two different signals: the first signal is induced by the cross-linking of two allergen-specific membrane-bound immunoglobulins, whereas the second signal is provided by an activated T cell that is able to recognize the specific antigen presented by the B cell on the MHC class II with its TCR. The T cell is then able to co-stimulate the B cell for example by engaging the B cell receptor CD40 with CD40L and by the release of IL-4. These signals induce maturation of the B cell into antibody-releasing plasma and B memory cells. Furthermore, proliferation and a change in the expression of the heavy chain constant region of antibodies are induced. This change in the class of antibody synthesized, also called Ig class switch, results in the production of IgE instead of IgA, IgD, IgM or IgG [44]. After the initial sensitization, subsequent allergen contact boosts IgE$^+$ memory B cells, which leads to an increased production of allergen-specific IgE antibodies. These allergen-specific IgE molecules are loaded by means of specific receptors FcεRI and FcεRII onto mast cells, basophils, eosinophils, macrophages, dendritic cells, and B cells.

After this initial sensitization, subsequent inhalation of allergen triggers the immediate reaction which leads to hypersensitivity and an acute inflammatory reaction. These reactions are mainly mediated by the release of mediators and cytokines deriving from activated mast cells. Mast cells are activated by the binding of allergen to allergen-specific IgE antibodies which are loaded on the Fcε receptors on the surface of these cells. The activated membrane spanning receptors induce an intracellular signaling cascade which leads to degranulation and

to the release of pro-inflammatory mediators such as histamine, TNF-α, prostaglandineD2, leukotriene B4 and C4 and proteases such as tryptase, chymase, and heparin [45]. These factors directly cause to the symptoms of the immediate reaction.

The late reaction in asthma is believed to be primarily induced by the presentation of antigen to specific T_H2 cells, leading to activation, proliferation and release of cytokines. After activation, T_H2 cells produce the T_H2 cell-associated cytokines IL-4, IL-5, IL-9, IL-13, IL-25 and IL-33 [46]. These cytokines play a crucial role amplifying the T_H2 response and directly promoting asthma symptoms (Table 1).

Table 1.　　　*Effects of T_H2 cytokines in asthma*

Cytokine	Effects on asthma
IL-4	Induction/maintenance of T_H2 response [47], IgE production [48], upregulation of low affinity IgE receptor [49], upregulation of endothelial adhesion molecules on eosinophils and basophils [50;51], alternative macrophage activation [52] , smooth muscle contraction [53], subepithelial fibrosis [54], airway hyperreactivity [55], goblet cell hyperplasia and mucus production [56].
IL-5	Enhancement of differentiation, survival, and growth of eosinophils [57], airway hyperreactivity [58], lung remodeling (potentially eosinophil-mediated) [59].
IL-9	Mastocytosis [60], upregulation of high affinity IgE receptor [61;62], development of eosinophils (only in synergy with IL-5) [61], T_H2 induction/maintenance [63].
IL-13	IgE production, eosinophilia, goblet cell hyperplasia, airway hyperreactivity [55;64], smooth muscle contraction [53], subepithelial fibrosis [54].
IL-25	Maintenance of T_H2 response [65].
IL-33	Amplification of T_H2 inflammation (but also T_H1), chemoattractant for T_H2 cells [66], mast cell activation [67;68], activation of basophils [69]
TSLP	Dendritic cell activation [70], CD4+ T cell proliferation [71], suppression of regulatory T cells [72], mast cell activation [73], early induction of T_H2 response

As stated earlier, IL-4 is an essential mediator activating T_H2 responses and the cytokine with arguably the most important and pleiotropic effects in allergic inflammation. Apart from the main source of IL-4, the T_H2 lymphocytes, this cytokine is also released by basophils, natural T killer cells (NKT), eosinophils, and mast cells [74;75]. IL-4 binds to type I- and type II IL-4 receptors which are present on T cells, B-cells, macrophages, mast cells and fibroblasts [76;77]. In epithelial cells, IL-4 induces the expression of vascular cell adhesion molecule 1

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(VCAM-1), which in turn increases the adhesiveness of the endothelia specifically for cells involved in allergic reactions such as eosinophils, basophils and monocytes [50]. In addition, IL-4 is important for maintaining immune responses by acting as a T cell survival factor preventing apoptosis of T lymphocytes [78]. Other important effects of IL-4 are activation of macrophages and mast cells, induction of goblet cell metaplasia, IgE class switch in B cells, sub epithelial fibrosis and smooth muscle hyperplasia. As mentioned before, IL-4 activates GATA-3 through STAT-6 activation of T_H2 cytokine production, while it simultaneously inhibits T_H1 differentiation and cytokine expression [36].

Another cytokine produced after GATA-3 activation is IL- 5, the most important factor for eosinophilic development, attraction, differentiation, activation, and survival [79]. In mouse models, it was shown that IL-5 is a necessary factor for airway hyperreactivity (AHR) and lung damage [80]. IL-9, first described as a mast cell growth factor [81], is involved in lung eosinophilia, mucus hypersecretion, IgE production, and pulmonary mastocytosis [82-85]. Publications suggest that IL-9 alone is not sufficient to cause these effects and requires additional signals of IL-4, IL-5, and IL-13 [84]. Another factor associated with T_H2 responses is IL-25, which is produced by epithelial-, T_H2- and mast cells, and basophils. The main effect of IL-25 seems to be the maintenance of T_H2 response by initiation of cell expansion, enhanced maturation and cytokine production in T_H2 cells [65]. The IL-1-like cytokine IL-33 is yet another cytokine with important properties for the onset of T_H2 inflammation. IL-33 is expressed by epithelial cells, fibroblasts, and smooth muscle cells, affecting basophils, T_H2 cells, NK cells, and mast cells. In mast cells, IL-33 increases survival, adhesion, and cytokine production of IL-5, IL-13, granulocyte macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor-α (TNF-α) [86;87]. *In vivo* administration of IL-33 in naive mice induces the expression of IL-4, IL-5, IL-13 and leads to severe pathological changes in lungs [88]. Thymic stromal lymphopoietin (TSLP) is an epithelial, mast cell or basophil-derived cytokine that activates DCs and can directly induce T_H2 differentiation of naïve CD4$^+$ T cells [89]. TSLP is released by epithelial cells and through its potential to activate DCs and is important predominantly for the early onset of allergic reactions. The important role of TSLP in asthma was shown in mice models where TSLP was able to induce severe airway hyperreactivity [90]. In conclusion, various T_H2 cytokines contribute to the pathophysiology of asthma by orchestrating, perpetuating and amplifying the inflammatory response in asthma, making cytokines a major indicator in therapy, as well as potential target for treatment.

Table 2.   *Different cells types and their main function in asthma*

Cell type	Main function in asthma
Epithelial cells	Sensing of antigens, recruitment of DCs, induction of T_H2 response
Dentritic cells	Antigen processing and presentation, activation of T cells
Basophils	Induction of T_H2 differentiation and T_H2 maintenance, effector-cell recruitment
T_H2 cells	Induction and amplification of T_H2 response, activation of effector-cells
Eosinophils	Induction of airway remodeling, mucus secretion , broncho-constriction
Mast cells	Broncho-constriction, airway remodeling, mucus secretion, induction of inflammation, effector-cell recruitment
B-cells	IgE production
T_H17 cells	Potential y maintaining inflammation in severe disease by activation of effector-cells
Neutrophils	Airway remodeling, airway hyperreactivity (especially in non atopic or severe asthma)
Fibroblasts	Airway remodeling
Goblet cells	Effector cells for production and release of mucus, airway narrowing
Smooth muscle cells	Effector cells for broncho-constriction, airway narrowing

The lung consists of approximately 30 different cell types and has indigenous cell populations such as bronchiolar cells and alveolar macrophages. In an allergic reaction, many of these cells can interact and influence each other adding to the complexity of asthmatic inflammation which is still not fully understood. In the respiratory tract, the first cells coming into contact with allergens are epithelial cells and airway dendritic cells (DCs). Cells of the epithelium were initially considered to function solely as a physical barrier, but more recent results highlight their role as central players in the sensitization process. It has been reported that epithelial cells release the T_H2 cell-promoting cytokines TSLP, IL-25, and IL-33. In addition, epithelial cells can directly influence functions of DCs [91].

DCs are key players in the sensitization reaction to allergens as they are considered responsible for the initiation and the maintenance of T_H2 cell immune responses. DCs, and more recently, basophilic granulocytes (basophils) were identified as professional antigen-presenting cells with the capabilities of sampling allergens [92]. Once activated, DCs can induce both T_H2 and T_H1 inflammatory responses. In contrast, basophils are associated only with the induction of T_H2 responses [93;94]. Activated T_H2 cells can enhance the immune

response via cytokine release leading to direct activation of different immune cells such as eosinophilic granulocytes (eosinophils), mast cells, B cells or macrophages.

Eosinophils are considered the most prominent cellular infiltrate of atopic asthma occurring in the late asthmatic reaction. Increased numbers of eosinophils can be found in the peripheral blood, sputum, and bronchoalveolar lavage of asthmatics and are considered to reflect the severity of the disease [95;96]. Main effects in which eosinophils are involved are airway remodelling, airway hyperresponsiveness and mucus accumulation [97]. Because of their fundamental role in asthma, reduced levels of eosinophils and their granule proteins in the airways are one of the most reliable indicators of successful treatment of allergen-induced asthma exacerbations [98]. Most of the research in the field of asthma is focused on the eosinophilic-, T_H2 cell- and IgE-driven type of inflammation. In non-atopic asthma, these cells play a minor role and a different type of inflammation and inflammatory cells seem to elicit symptoms of the disease. Lung neutrophilia on the other hand is a frequently reported feature in non-atopic asthma, and the amount of neutrophilic granulocytes (neutrophils) appears to correlate with the severity in this form of the disease [99;100].

Activated neutrophils release increased amounts of TGF-ß [101] and proteases such as elastase and matrix metalloproteinase 9 (MMP-9) [102], which are factors suggested to contribute to airway remodeling. Besides being a feature of non-atopic asthma, neutrophils seem to play a crucial role in severe and fatal asthma [103;104]. One property of neutrophils is their resistance to glucocorticoid treatment [105], which may explain their increased numbers found in the more severe form of asthma. Glucocorticoid treatment can even boost neutrophilia by inhibiting apoptosis in neutrophils [106], which is supported by reports that treatment increases neutrophilic counts in airways of patients [107].

Lately T_H1 cells have received increasing attention for their potential contribution to the inflammation in asthma. There are studies claiming that the pro-inflammatory effect of T_H1 cells potentiates the inflammatory response in asthma [108;109]. In a study of experimental asthma using an adoptive transfer system, T_H1 cells did not attenuate T_H2 cells but rather caused severe airway inflammation [110]. Publications suggest that despite being predominantly a T_H2 and eosinophilic disorder, asthma adopts T_H1-type characteristics as the disease becomes more severe and chronic [111]. It is hypothesized that in this severe form of asthma, a T_H1 component is necessary for sustained inflammation. A candidate key mediator

in this process is the pro-inflammatory T_H1 cytokine TNF-α. Supporting this, clinical data show that patients with severe corticosteroid-dependent asthma have higher concentrations of TNF-α in their bronchoalveolar lavage fluid which was not seen in patients with mild asthma or healthy control subjects [112]. In line with this, a first clinical trial using anti-TNF therapy resulted in a marked improvement of lung function and bronchial hyperreactivity [112]. These results could not be confirmed by a second study treating patients with mild asthma, in which anti-TNF therapy failed as a method reducing airway inflammation [113]. It was assumed that TNF-α may be a mediator important primarily in the severe form of asthma. However, reported safety concerns and lack of overall efficacy in recent anti-TNF-α studies do not favor this approach.

Because of the potential influence on neutrophils and macrophages during inflammation, the T_H17 subtype has recently come into focus as a factor contributing to severity in steroid resistant asthma. Activated T_H17 cells secrete IL-26, TNF-α, lymphotoxin-β, IL-22 [114] and are considered a main source of the different IL-17 subtypes which were in turn found to be elevated in asthmatics [115;116]. Known IL-17 subtypes are IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also defined as IL-25), and IL-17F. The levels of the first member of the IL-17 family, IL-17A, were found to correlate with severity and increased neutrophilia [116]. Interestingly, in patients and healthy subjects, a loss-of-function mutation in the gene locus of IL-17F was found to be inversely related to asthma risk [114] and knock-out experiments performed in mice showed that IL-17A is essential during antigen sensitization to establish allergic asthma [117]. On the other hand, animal studies by the same group reported an inhibitory effects of IL-17A on allergic airway inflammation with the researcher concluding that IL-17A has a dual role, also functioning as a negative regulator in established allergic asthma [117]. As these effects were accompanied by increased airway neutrophilia, the exact function of IL-17A and other IL-17 family members might not only depend on the bias of inflammation but also on its severity.

In summary, inflammation in atopic asthma is considered a predominantly T_H2, eosinophilic- and IgE-mediated disorder with T_H2 cytokines contributing to the development of different asthma symptoms, whereas the non-atopic form of asthma shows a different type of inflammation often characterized by influx of neutrophilis. In the more severe form of atopic asthma, recent data suggest an involvement of the T_H1 and the T_H17 subtypes in sustaining and exacerbating the pre-existing inflammation.

3. Hygiene Hypothesis

Epidemiological studies, as well as numerous animal experiments, have given rise to the "Hygiene Hypothesis" as a means to explain the cause for the increased prevalence in allergic diseases in high income societies [118]. This hypothesis proposes that a lifestyle with high hygiene standards and sanitation leads to a decreased exposure to microbes, which in turn results in an overall decline in early-childhood infections. This decline in infections affects the developing immune system and increases the risk for atopy and asthma [119]. One mechanism frequently associated with the hygiene hypothesis is the aberrance of T_H1/T_H2 balance, caused by the lack of stimuli, which leads to a reduction of T_H1 cells and an increase in allergy-promoting T_H2 cells [120]. On the other hand it is hypothesized that frequent early life viral and bacterial infections direct the maturing immune system toward T_H1 bias, counterbalancing pro-allergic responses of T_H2 cells. In consequence, the reduction in overall microbial burden results in a weak T_H1 imprinting and the unrestrained T_H2 responses are leading to an increase of atopic diseases.

As mentioned before, T_H2 cells produce IL-4, IL-5 IL-9, IL-13, IL-25, and IL-33. These cytokines mediate the allergen-specific immune response in atopic disorders. T_H1 cells on the other hand produce interferon γ (IFN-γ), IL-2, IL-12, IL-18, IL-23, and tumor necrosis factor β (TNF-β), and activate cell-mediated immunity and phagocyte-dependent inflammation. Both cell types release cytokines that enhance the differentiation of naïve T cells into the respective subtype. Because the surrounding cytokine milieu defines the T cell subtype, T_H1 and T_H2 cytokines have antagonistic effects in a direct competitive manner. Cytokines such as IL-12, IL-18 and IL-27 activate the transcription factors STAT-1 and STAT-4. STAT-4 in turn activates the transcription factor T-bet, which is the key regulator of T_H1-cell fate determination [121]. T-bet induces transcription of IFN-γ and the IL-12 receptor. IFN-γ itself increases T-bet transcription by STAT-1 activation and negatively regulates the T_H2 biased factor GATA-3 [122]. In line with this, it was shown that T-bet-deficient mice have impaired T_H1 immunity and develop spontaneous asthma [123].

Whilst there is a large body of evidence supporting the hygiene hypothesis, more recently some hypothesis conflicting observations have been made. Firstly, as well as atopic diseases, the prevalence of T_H1-autoimmune diseases is also increasing in high income societies [124]. Secondly, T_H2-inducing parasitic worms such as helminth infections are not associated with allergy. Instead, experiments even suggest a suppression of allergies mediated

by the helminth infection [125-127]. In line with this are observations in endemic areas in Africa, that parasitic infections are strongly inversely related to the development of atopic disease [128]. These findings suggest that a more complex picture must be drawn in order to explain the increase in atopic disease. Hence the "counter-regulation" theory was introduced. In this theory, the lack of infections does not lead to an immune deviation, rather to a less-well developed regulatory response (reviewed in [129]). In this regulatory response, the T cell subtype defined as suppressor or regulatory T cell (T_r) is believed to be the most important cell, as these cells have the ability to control T cell proliferation and can inhibit harmful immunopathological responses induced by both T_H1, T_H17 and T_H2 cells [130;131].
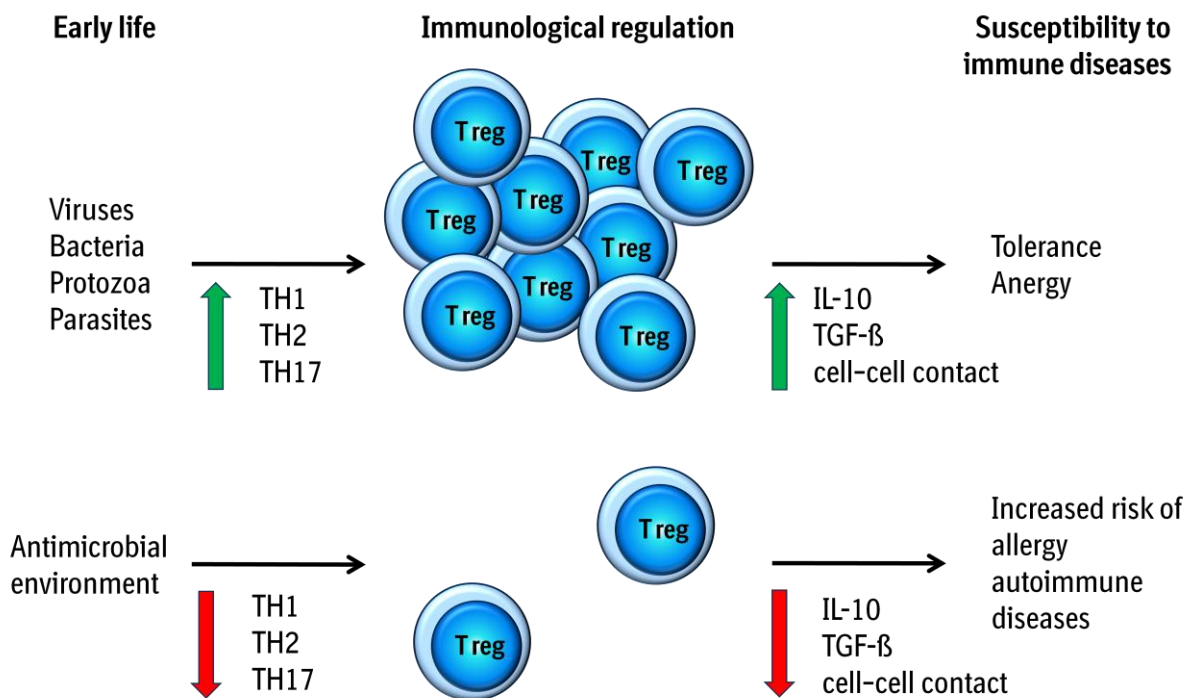
Fig. 2. Schematic view of the "counter-regulation" hypothesis. *In the counter regulation theory, the westernized lifestyle leads to a decrease in infections. This lack of activation leads to a rudimentary development of the counter-regulatory response of the developing immune system. One major process believed to be responsible for this is the impaired expansion of natural and allergen-specific T_r cells. T_r are able to secrete IL-10 and TGF-ß which are potent regulatory cytokines that can further suppress inflammatory processes by cell-cell contact. As a consequence, a lack of T_r leads to a defect in the control of inflammation, which in turn increases the incidence of allergic and autoimmune diseases. Green arrows indicate increase/upregulation, red arrows indicate decrease/downregulation.*

The population of T_r cells can be divided into different subsets. T_r cells that develop as a normal part of the immune system are defined as natural T_r cell. Another subtype was defined as adaptive T_r cells, as they develop in response to a particular antigenic encounter. In addition to the difference in their development, these subtypes differ in their mechanism of action, the dependence on T cell receptor, and co-stimulatory signaling [132]. It is suggested that adaptive T_r cells mediate their suppressive effects in a cytokine-dependent manner, whereas natural T_r cells deploy their function by a cytokine-independent mechanism, which involves direct interactions with responding T cells or antigen-presenting cells [133;134]. The suppressive function of T_r cells is supported by a study conducted in a model of mouse airway disease. This study reported that T_r cell activation with heat-killed *Mycobacterium vaccae* resulted in IL-10 and TGF-ß release which protected against airway inflammation [135]. Main effects of IL-10 are inhibition of macrophage activation and antimicrobial effector functions, as well as co-stimulatory activity and production of pro-inflammatory cytokines. In addition, IL-10 upregulates anti-inflammatory IL-1 receptor antagonists, inhibits the differentiation of dendritic cells, and suppresses inflammatory chemokine and matrix metalloproteinase production. Furthermore, IL-10 downregulates expression of leukocyte adhesion molecules, suppresses T cell proliferation, inhibits T_H1 cytokine production and induces antigen-specific anergy in CD4^{+} T cells (reviewed in [136]).

In summary, since 1989, when D. P. Stachan first postulated the hygiene hypothesis, as a theory to explain the increase of atopy and asthma, this hypothesis has been redefined and supplemented. Currently, environmental factors during early life and genetic background are thought to dictate the susceptibility to the development of allergic responses and lead to the inability to muster anti inflammatory control mechanisms, which is seen as the main reason for the increase in allergic diseases.

4. Biology of Toll-like receptors

The term "Toll-like" receptor (TLR) originates from the similarity to the protein coded by the "Toll" gene identified in *Drosophila* in 1985 by Christiane Nüsslein-Volhard [137]. TLRs are a fundamental part of the innate immune system as they are the main receptors used for sensing viral and bacterial infections and activating the subsequent immune responses. Currently, 11 TLRs have been described in humans and 13 in other mammals. Of these, TLR1, TLR2, TLR5, TLR6, TLR10 and TLR11 are expressed on the cell surface, whereas TLR3, TLR7, TLR8 and TLR9 are located in endosomes [138]. These TLRs are able to bind a variety of microbial patterns derived from bacteria, viruses, parasites and fungi, molecules known as "pathogen associated molecular patterns" (PAMPs). PAMPs can be categorized in three broad categories; nucleic acids, lipids/lipopeptides and proteins (Table 3).

Table 3.   Toll-like receptors and their ligands

Toll-like receptor	Ligands	Molecule class of ligand	Source of ligand
TLR1	Triacyl lipopeptides, Porin PorB	Lipopeptides	Bacteria, mycobacteria
TLR2	Lipoteichoic acid, Porins, Zymosan	Lipopeptides, lipids	Gram-positive bacteria, Fungi
TLR3	Poly (I-C)	dsRNA	Viruses
TLR4	Lipopolysaccharides	Lipopeptides, lipids	Gram-negative bacteria
TLR5	Flagellin	Protein	Bacteria
TLR6	Diacyl lipopeptides, Zymosan	Lipopeptides	Mycoplasma, Fungi
TLR7	Imiquimod, R-848	ssRNA	Synthetic compounds, Viruses
TLR8	R-848	ssRNA	Synthetic compounds, Viruses
TLR9	Unmethylated CpGs	DNA	Bacteria, Viruses, yeast
TLR10	N.D.	N.D.	N.D.
TLR11	Profilin	Protein	Parasite, Toxoplasma gondii

R848 = imidazoquinoline resiquimod, CpG = short single-stranded synthetic oligodeoxynucleotides containing motives of cytosine followed by a guanine, N.D. = not determined; reviewed and published in [139-142].

All TLRs are type I transmembrane glycoproteins consisting of three domains; the extracellular domain, which binds the ligand, the transmembran domain, which anchors the glycoprotein, and the cytoplasmic domain, which initiates the intracellular signaling. The extracellular domain, either on the cell surface or in intracellular compartments, contains leucine-rich repeat (LRR) motifs which form a horseshoe like structure with a ligand binding pocket. The last 200 amino acids in the cytoplasmatic domain are conserved throughout all different TLRs. This amino acid region is known as the Toll/IL-1 (TIR) domain and is characterized by three highly homologous regions called Box-1, -2, and- 3 [40]. When a ligand binds to its respective TLR, a dimerisation of two receptors is induced. The ligand-bridged dimerisation of extracellular domains is suggested to initiate intracellular signaling by approximation of the two cytoplasmatic TIR domains. Activation of the TIR domains results in conformational changes enabling the recruitment of cytosolic adaptor molecules. To date, four different primary adaptor molecules are known; myeloid differentiation primary-response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP or MAL), TIR-domain-containing adaptor protein inducing IFN-ß (TRIF or TICAM1) and TRIF-related adaptor molecule (TRAM or TICAM2) [143]. Different patterns of gene expression can be explained by the diversity of adaptor molecules and the selective recruitment of transcription factors by individual TLRs.

Table 4.   *Toll-like receptors with adaptor molecules and transcription factors*

Toll-like receptor	recruited adaptor molecules	MyD88 as adaptor	Transcription factors activated
TLR1	MyD88	Yes	NF-κB
TLR2	MyD88, TIRAP	Yes	NF-κB
TLR3	TRIF	No	IRF-7, IRF3
TLR4	MyD88, TIRAP,TRAM,TRIF	both pathways	IRF-7, NF-κB, IRF3
TLR6	MyD88, TIRAP	Yes	NF-κB
TLR7	MyD88	Yes	IRF-7, NF-κB
TLR9	MyD88	Yes	IRF-7, NF-κB

TRIF = TIR-domain-containing adaptor protein inducing IFN-ß, TRAM = TRIF-related adaptor molecule, TIRAP = TIR-domain-containing adaptor protein, MyD88 = myeloid differentiation primary-response protein 88, NF-$_K$B = nuclear factor kappa-light-chain-enhancer of activated B cells. IRF-7 = Interferon regulatory factor 7. From references [144;145].

Upon activation, MyD88 molecules form a dimer and recruit IL-R1 associated kinases (IRAKs) and tumor necrosis factor receptor associated factor 6 (TRAF6). TRAF6 induces the activation of the serine/threonine kinase TAK1 through K63-linked polyubiquitination. The heterodimer p50/p65, also called nuclear factor kappa-light-chain-enhancer of activated B cells (NF-$_\kappa$B), is activated by the IκB kinase (IKK) complex or mitogen-activated protein (MAP) kinases. NF-$_\kappa$B induces transcription of pro-inflammatory cytokines IL-6, IL-8, IL-12 and TNF-α. The second type of transcription factors is the family of Interferon regulatory factors (IRFs). Target genes activated by different IRFs encode for IL-6, IL-12, TNF-α (activated by IRF5), IFN-α (activated by IRF7), RANTES, IP-10 and MCP1 (activated by IRF3). TLR signaling results in mediator release, prominently IFNs, TNF-α, IL-1, IL-6, IL-10, IL-12, and many different chemokines. However, TLR activation can also lead to cell differentiation, proliferation or apoptosis. The eventual effect of TLR activation depends on the TLR activated, the frequency of ligand binding and activation of the TLR, and the cell type on which the TLR was activated.
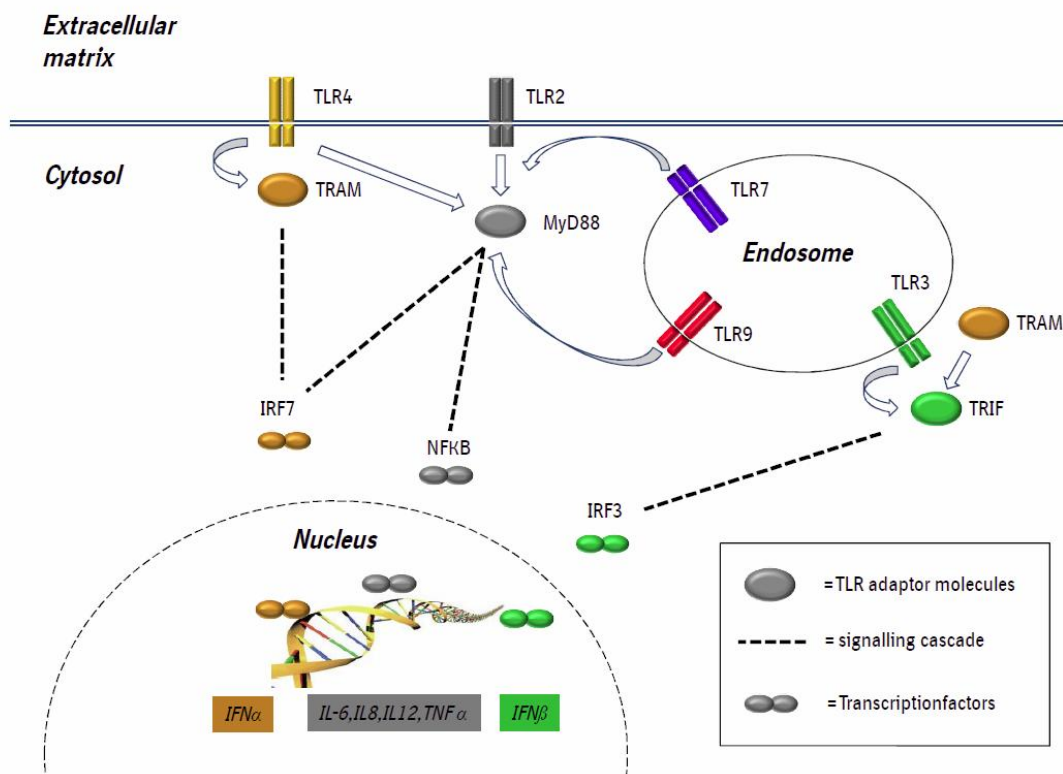
Fig. 3. Model for TLR-transmitted activation of cytokine production. Upon activation, TLRs recruit intracellular adapter proteins which are able to activate an intracellular signaling cascade ultimately leading to the translocation of activated transription factors into the nucleus. This leads to the transcription of pro-inflammatory cytokines. Depicted are five TLRs with extracellular domains on the cell surface or in endosomes. TLRs can either transmit a signal via MyD88, which is the case for TLR2,7 and 9, or via MyD88 independent transducer such as TRIF, which is the case in TLR3 activation. TLR4 is the only receptor that can signal through both pathways.

5. TLR agonists and asthma

Over the past decades, considerable improvements in the management of asthma have been achieved through improved diagnosis and the wider use of drugs with increased specificity and potency. The most frequently used medication includes bronchodilators, muscarinic antagonists, corticosteroids, leukotriene inhibitors and to a lesser extent antihistamines. These therapeutics are generally well tolerated and can normally control symptoms of the disease effectively. However, all these therapeutics fail to prevent acute exacerbations, long-term loss of lung function and most importantly, are aimed to control symptoms but do not induce curative effects. Of further concern are side effects. Normally, the most frequently used therapeutic class of corticosteroids are well tolerated when inhaled and used in low concentrations, but treatment can also result in unwanted side effects, especially when corticosteroids are given orally and for a longer period of time. The most common reported side effects of short-time oral corticosteroids usage are nausea, rapid heartbeat and loss or gain of appetite. Prolonged use over a period of 3-4 weeks can result in increased blood pressure, opportunistic infections, reduction of own corticoid production, development of diabetes, amyotrophia (loss of muscle mass), growth retardation, and osteoporosis [146;147]. Furthermore, patients suffering from the severe form of asthma often respond only poorly or not at all to steroid treatment. Thus there is a major need for new therapeutics and novel approaches to enable more efficient treatment of asthma patients.

Because TLRs mediate strong innate immune responses, TLRs have been considered as targets for therapeutic intervention in cancer, infectious diseases and atopic diseases such as asthma [148]. In asthma the immunological basis for potential therapeutic benefits are the immune-modulatory, or regulatory properties of TLR activation. As mentioned before, TLR activation can modulate the immune response via induction of a T_H1 response. The hopes are that the induced T_H1 response is able to counterbalance the dominant T_H2-type immune response in asthma, for example by the release of cytokines such as IL-12 and IFN-γ. It has also been suggested that TLR activation could attenuate the T_H2-type immune responses by activation of immune-regulatory T cells and induction of the anti-inflammatory cytokine IL-10 [149]. Furthermore, it has been suggested that TLR activation could suppress T_H2-type immune responses by induction of apoptosis in T_H2 effector-, or T_H2 immune response promoting cells [150]. These effects can be beneficial for both the prevention of the development of the disease, or for therapeutic effects in established disease. In asthma, mainly TLR agonist 2, 3, 4, 7/8 and 9 have been investigated.

TLR2: Epidemiologic studies focusing on early-life development of asthma and living conditions reported lower incidence of asthma for children growing up on farms. These studies were carried out in North America and Europe and consistently found that children living on a farm showed lower prevalence of hay fever, asthma and IgE-mediated reactivity to local allergens [151-155]. In line with the hygiene hypothesis, a rural area, in contrast to an urbanized area, is associated with increased exposure to microorganisms and their products. Interestingly, the lower incidence of asthma was found to correlate with increased levels of *TLR2* gene expression on blood cells of children growing up on farms [156]. Furthermore, genetic analysis suggest that *TLR2* polymorphism is strongly associated with the frequency of asthma and it was concluded that genetic variation in TLR2 is a major determinant of the susceptibility to asthma and allergies in children of farmers [157]. It was further hypothesized that a more-frequent TLR2 activation, leading to a subsequent up-regulation of TLR2 transcription and expression, could protect against the development of asthma. In addition to protective effects of TLR2 activation in the early development of the disease, it was also reported that TLR2 agonist administration could ameliorate established allergic airway inflammation in a murine model of asthma [158]. Hence, it was suggested that the effects were mediated by the promotion of a T_H1 response and not via regulatory T cells [158]. In concordance with this was a study in which a TLR2 agonist was protective against allergic inflammation in mice and TLR2-deficient mice exhibited greatly enhanced levels of serum IgE and lung eosinophilia [159]. As a potential mediator for the protective effect, the neutrophil-derived matrix metallopeptidase 9 (MMP9) was suggested. Contrary to these findings, it was reported that activation of TLR2 induced a T_H2 immune response and promoted experimental asthma in mice [160], which was thought to be mediated by activation of DCs [161]. It was also shown that TLR2 activation in human DCs lead to the release of the IL-12 inhibitory p40 homodimer, which subsequently favored T_H2 development [162]. Furthermore, activation of TLR2 on murine pulmonary mast cells resulted in the release of leukotrienes and asthma-related mediators such as IL-4, IL-5, IL-6 and IL-13, which was also seen in human mast cells activated by the TLR2 agonist zymosan [163]. In summary, there is a strong body of evidence supporting a protective effect of TLR2 activation during childhood. On the other hand, activation in established disease is less well understood and both exacerbating and ameliorative effects have been reported.

TLR3: Unlike TLR2, TLR3 belongs to the class of nucleic acid binding TLRs and is able to recognize double-stranded RNA (dsRNA). TLR3 was found to be essential for the maintenance of lung tissue integrity after viral infections. Viral infections, especially rhinovirus, respiratory syncytial virus (RSV) and influenza infections are the most prominent candidates for causing exacerbations in asthma [164]. Data from human lung epithelial cells indicate that TLR3 mediates the immune response to influenza A [165;166] and rhinovirus [167] infections. *In vitro* antibody-mediated blocking of TLR3 lead to increased replication of rhinoviruses in lung epithelial cells [167]. It was further shown that in TLR3-deficent mice, RSV infection led to elevated levels of IL-5, IL-13 and mucus in the airways [168]. According to these findings, it was concluded that TLR3 is essential for sensing viral presence and for the onset of the cellular immune response that leads to reduction of the viral load and subsequent to inhibition of exacerbations. In line with this are findings that in an experimental asthma model, the activation of TLR3 exerted preventive and suppressive effects on the disease, which were mediated by the additive effects of IL-12 and IL-10. Hence, it was reported that the TLR3 activation during the sensitization phase prevented the production of IgE and abolished airway hyperresponsiveness and allergic airway inflammation [169]. After these findings, it was somewhat surprising that TLR3 knock-out mice, despite having a higher viral production in the lungs, exhibited an unexpected higher rate of survival after influenza A infection [170]. There is also data reporting that activation of TLR3 in a mouse model of lung allergic exacerbation significantly increases airway hyperresponsiveness, lung inflammation and T_H2 response. It was furthermore reported that these symptoms are associated with an infiltration of eosinophils, myeloid DCs, and T lymphocytes into the lung [171]. It was also shown that long-term exposure to TLR3 agonist poly(I:C) alone was able to impair lung function in a murine model [172]. As a potential explanation for these somewhat contrary results, it was suggested that during viral infection inflammatory signaling pathways in some cases induce a protective response of the host, whereas in other cases viruses can utilize these pathways in order to enhance their replication. These findings suggest that TLR3 activation modulates the local inflammatory response and the ultimate effect is dependent on the immunological state of the host and the type of virus. Furthermore, it was published that some viruses such as Influenza A or RSV have developed strategies to inhibit TLR3 signaling by binding or cleaving of downstream signaling transducers adaptor TRIF/TICAM-1 [173;174]. In summary, the exact role of TLR3 in asthma is not known and seems to strongly depend on the type of virus and the host or the model used.

TLR4: In contrast to TLR3, much more data is reported on the role of TLR4 in asthma. TLR4 was the first human TLR for which the respective ligand, bacterial lipopolysaccharide (LPS), was identified. Since then, the TLR4 receptor and TLR4 activation has been the focus of extensive studies, especially as LPS is a major inducer of septic shock. Data from epidemiologic studies in humans indicate that exposure to LPS can protect from development of allergic asthma. Analogous to TLR2 activation, early life exposure to LPS is also thought to induce tolerance or to contribute to desensitization in allergic individuals [175-177]. In line with these suggestions, TLR4 activation sufficiently boosted protective immunity in preclinical models. It was published that in experimental asthma, microbial LPS modulated mucosal tolerance by inducing allergen-specific IgG1 production and distinct effector CD4+ T cells with a mixed regulatory/T_H1 phenotype [178]. Another animal study showed attenuation of eosinophilic inflammation after low dose administration of endotoxins [179]. In contrast to this finding it was reported that house dust mite allergen induced asthma like inflammations via TLR4 activation in a murine model [180]. In these experiments, TLR4 activation on epithelial cells caused production of the pro-allergic cytokines TSLP, GM-CSF, IL-25 and IL-33. This study concluded that TLR4 activation is necessary for induction of house dust mite induced allergic airway inflammation. The seemingly contradicting bidirectional capacity of TLR4 activation to either exacerbate or ameliorate asthma seems to depend on the developmental stage of disease and the concentration of TLR4-activating agonists. The data in the literature suggest that low concentrations of LPS and exposure in early life both have preventive effect on allergy and asthma. In contrast, LPS exposure at a later stage when allergen sensitization has already been established, may exacerbate and promote the inflammatory responses [179;181].

In allergic diseases TLR4 agonists are also used as a T_H1-inducing adjuvant in allergen specific immunotherapy (SIT). An example of this therapeutic approach is the administration of TLR4 agonist with chemically modified allergens [182]. The TLR4 agonist monophosphoryl lipid A was used in clinical studies in combination with modified allergens treating patients with grass pollen seasonal allergic rhinitis. In this study allergen combined with TLR4 agonists showed efficacy and were well tolerated [183-185]. However, the exact contribution of the TLR4 agonist was not determined as a head-to-head comparison between MLP/allergen and allergen-only setting was not performed.

TLR7/8: TLR7 and TLR8 recognize single-stranded RNA but can also be activated by small-molecular weight compounds such as imiquimod, resiquimod (R848) and guanosine analogues. Animal studies have shown that the activation of TLR7/8 can suppress the development of asthma and allergic responses by induction of a T_H1 cytokine profile [186;187]. In mice, topic application of R848 after sensitization reduced IL-4 and IL-5 production whereas IL-12 and IFNγ levels were increased. In addition, numbers of eosinophils and lymphocytes were significantly reduced [187]. It was published that systemic application of R848 before allergic sensitization was able to inhibit airway hyperresponsiveness and allergen specific IgE production. Interestingly, it was reported that the systemic application reduced production of both T_H2 and T_H1 cytokines and prevented recruitment of inflammatory cells [188]. It was further reported that these effects were not seen in MYD88-deficient mice. A similar study in mice confirmed a preventive effect on experimental asthma by TLR7 activation [169]. In this study, the TLR7 agonist was also able to prevent the secondary response and it was stated that these effects were IL-10 and IL-12 mediated. It was also published that resiquimod was able to inhibit goblet cell hyperplasia and smooth muscle cell thickening in a rat chronic model of asthma [189]. In a mouse model of chronic asthma the TLR7 agonist imiquimod significantly inhibited chronic inflammation, persistent airway hyperreactivity and airway remodelling. Imiquimod also reduced levels of total serum IgE, T_H2 cytokines and expression of TGF-β1 in lung lavage [190]. At present, there are no reports about human studies using TLR7 or TLR7/8 agonists for the treatment of asthma.

TLR9: The endosomal TLR9 is activated by DNA oligodeoxynucleotides bearing unmethylated CpG motifs, which are prevalent in bacterial but not in vertebrate genomic DNAs. The first evidence for immunological activity of DNA fractions was reported in 1984 when DNA fractions of *Mycobacterium bovis* (bacillus Calmette- Guérin) were used for antitumor application by Tokunaga et. al. [191]. This finding led to the discovery of the immunostimulatory properties of bacterial DNA [192;193]. This effect could be allocated to unmethylated CpG motifs of the bacterial DNA [194]. It was then discovered that DNA is recognized by TLRs and the respective receptor for CpG was found when TLR9-deficient mice did not show any response to CpG DNA [195]. Since this discovery, TLR9 agonists such as CpG DNA and synthetic CpG oligodeoxynucleotides have been considered for therapeutic applications in infectious disease, vaccination, cancer therapy, allergy and asthma

[196]. In atopic disorders including allergies, allergic rhinitis and asthma, CpG containing sequences have been tested in mice, rats, a monkey model of allergic asthma, as well as in first clinical studies. Before TLR9 was identified as a functional sensor for CpG, DNA containing CpG sequences had already been tested in a murine model of allergic inflammation, and were proposed as a novel form of active immunotherapy in allergic diseases [147]. In this study, Broide et al. showed that CpG reduced airway hyperresponsiveness to inhaled methacholine and inhibited eosinophilia in both blood and airways when given before allergen exposure in pre sensitized mice. Furthermore, it was reported that the oligonucleotides decrease levels of the T_H2 related cytokines IL-5 and GM-CSF while increasing the T_H1 related cytokines IL-12 and IFN-γ. Another study published shortly afterwards highlighted the essential role of IFN-γ, as CpG administration in IFN-γ knockout mice failed to inhibit eosinophila. In addition this study showed a reduction of IL-4 and allergen specific IgE-producing cells, as well as the formation of an allergen specific T_H1 (rather than T_H2 cell) memory response due to CpG administration [197]. The potential use for CpG in the treatment of human asthma was further supported by studies investigating CpG in the context of established allergic responses. Furthermore, it was published that intra-dermal deliver of ragweed pollen linked to CpG reversed established allergic responses in the murine lung [198]. Interestingly, suppression of T_H2 cytokines in this setting did not occur but a shift in the antibody profile from a T_H2-directed IgG1 response to a T_H1-directed IgG2a response was reported. Another study showed that the CpG induced suppression of IgG1 and IgE is IFN-γ independent and that the CpG mediated activation of T-bet in B cells is causative for the Ig switch [199]. Furthermore, publications claimed that CpG exhibited suppressive effects on airway hyperresponsiveness and allergic inflammation by impaired dentritic cell migration and induction of a strong allergen-specific CD8+ T cell response [200;201]. In a very recent study, it was proposed that treatment with CpG dampens T cell activation and effectively reduces airway inflammation by impairing dentritic cell-mediated antigen transport from the lungs to the lymph nodes [169;200]. More importantly, it could be shown that CpG attenuated airway hyperreactivity and airway remodeling in rhesus monkeys with experimentally induced allergic airways disease [202]. In this study, CpG was able to reduce airway hyperresponsiveness twofold compared to sham-treated monkeys. Airways from immunostimulatory oligonucleotide-treated monkeys exhibited thinner reticular basement membranes, fewer mucous cells, fewer eosinophils, and reduced numbers of mast cells when compared to sham-treated allergic monkeys. Also, an initial phase II clinical study of a CpG in a randomized, double-blind, placebo-controlled group of 40 subjects with mild intermittent

asthma has been conducted [203]. In this study, each participant received 36 mg of the CpG containing immunostimulatory sequence (ISS) or placebo by once weekly nebulization over a period of 4 weeks. ISS application showed pharmacological activity by inducing a T_H1 response, monitored by the induction of IFN-regulated genes in peripheral blood and sputum cells. In the study, ISS administration showed a good safety profile and was well tolerated, but disappointingly, ISS treatment showed no efficacy in attenuating or improving asthma. Compared with placebo, no attenuation of the early or late decrease in forced expiratory volume could be measured. Also, no reduction in allergen-induced sputum eosinophils and no reduction of T_H2-related gene expression were found in sputum cells. It was suggested that the lack of clinically relevant efficacy might be dose related or that the induced upregulation of IFN-α, IFN-γ, and IFN-inducible genes alone is not sufficient to provide a therapeutic benefit. More promising results have been published for a phase I/IIa clinical trial using CpG as adjuvant in allergen-specific immunotherapy. In this therapy 21, patients suffering from house dust mite (HDM) allergy were treated and it was reported that symptoms of rhinitis and allergic asthma were significantly reduced [204]. Currently, clinical trials related to both asthma and allergic disease are being conducted by different companies including Dynavax, Idera/Novartis, Coley pharmaceutical/sanovis-aventis and Cytos Biotechnologies. These studies assess the safety and efficacy of both CpG monotherapy or when combined with allergens in immunotherapy [205;206].

In conclusion, since their discovery in 1990, TLRs have come into focus as potential targets for treating allergic diseases and asthma, and conducted preclinical studies have reported disease-suppressive effects. Despite this progress, some important questions still need to be addressed, which TLR has the highest efficacy for asthma therapy, what are the exact underlying mechanisms, how strong is the TLR-induced pro-inflammatory component and can this inflammation be tolerated by the patient? The answer to these questions will greatly aid in the development of a TLR-targeting therapeutic approach for the treatment of asthma.

MATERIAL AND METHODS

1. Instruments

Hardware	Typ/Model	Manufacturer
Aerosol distribution system	10LPM	Buxco
Automatic dye machine	Bond max	Leica
Bias Flow regulator		Buxco
Cell counter and differentiator	XT1800 iVeT cell analyzer	Sysmex
Centrifuge	Multifuge 3 S-R	Thermo
Centrifuge	Micro 22R	Hettich
Cover slide machine	Midrom CTMG	Thermo
Dermal biopsy puncher	diameter 8 mm	Miltex
Eppendorf shaker	Mix Mate	Eppendorf
Fluorescence-activated cell sorter	LSRII	BD
Fine point lamp		Schott
Freezer (-20°C)	Comfort	Liebherr
Freezer (-80°C)	Ultra Low	Sanyo
Magnetic stirrer	MR3000	Heidolph
Microtom	HM355	Microm
Microscope	Axioskop2	Zeiss
Multiplex reader	Luminex	Biorad
Multiplex reader		Meso Scale Discovery
Nebuliser	Nebuliser Control 10	Buxco
Pipettes		Eppendorf
Paraffin blocking station	Histocenter 2	Shandon
Photometer	Spectra max 190	Molecular Devices
Plate reader	Power Wave HT	Biotek
Microplate shaker	Titramax 101	Heidolph
Plate washer	Elx405	Biotek
Refrigerator	Profi line	Liebherr
Scales	Scout pro	Ohaus
Staining machine	Mira Stainer	EMD
Special accuracy weighing machine	AT250	Mettler
Small rodent bias flow supply	10LPM	Buxco

Slide Scanner	Mirax Scan	Zeiss
Tissue homogeniser	Fast prep	MP
Vacuum infiltration processor	Tissue Tek VIP	Sakura
Vortex	Reax 2000	Heidolph
Water bath	16801	Medax

2. Consumables

Article / usage	Manufacturer
32G needle	Hamilton Company
Biopsy processing cassettes M508-2	Simport
Complete protease inhibitor cocktail tablets	Roche Diagnostics
Cryo vials 2ml, 1ml	Nalgene
F96 maxisorp immuno plate 442404	Nunc
Helipur / disinfectant	Braun
IV indwelling cannula (0.9 x 25 mm, 36 ml/min)	Braun
Lysis matrix tubes D (1.4 mm, ceramic)	MP
Microscope slides superfrost ultra plus / histology	Thermo scientific
Microvette500 Z-gel / blood serum collection	Sarstedt
Needles (0.45 x 25mm, 0.6 x 25mm)	Braun; Terumo
Petri dish	Nunc
Polypropylene conical tubes (50 ml, 15 ml)	Falcon
Safe lock tubes (5 ml, 2 ml, 1.5 ml, 500µl)	Eppendorf
Surgical disposable scalpel	Braun
Syringes luer-lok tip (10 ml, 5 ml, 1 ml)	BD Bioscience

3. Chemicals and reagents

Name	Manufacturer
Albumin egg	Serva
Al(OH)$_3$	Pierce
BM blue POD substrate precipitating	Roche Diagnostics
Bovine serum albumin	Sigma-Aldrich
Complete protease inhibitor cocktail tablets	Roche Diagnostics Guard´s
EDTA	Promega
Fast green	Clintech
Forane/Isoflurane	Abbott
Formamide/Sigma Ultra	Sigma-Aldrich
Glacial acetic acid	Sigma-Aldrich
Papanicolaou stain	Sigma-Aldrich
Periodic acid	Sigma-Aldrich
Picro sirius red	Clintech
Phosphate buffered saline	BioWhittaker
RED refine	Leica
Sulphuric acid 96%	Sigma-Aldrich
Streptavidin-horseradish peroxidase	BD Pharmingen
Triton X-100	Sigma-Aldrich
Tween 20	Fluka

4. Buffers and solutions

Name	Composition	Manufacturer
Bond™ polymer refine red detection		Leica
Bond™ primary antibody diluent		Leica
Bond™ wash solution		Leica
Eosin solution	1% solubilized in deionionized water	Sigma-Aldrich
Fast green staining solution	20% in PBS	Clintech
Giemsa solution	15 ml Giemsa solution	Merck
	65 ml Weise buffer	Merck
	220 ml Aqua ultrapure	Lonza
HCL EtOH solution	1% HCL solubilized in 70% EtOH	Sigma-Aldrich

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Hank´s salt solution		Biochrom AG
Hematoxylin	100 ml Eosinsolution	Merck
	7 ml phloxin solution	Merck
	10 ml glacial acetic acid	Merck
	780 ml 96% EtOH	Merck
Lavage buffer	0.0012% EDTA	Sigma-Aldrich
	1% BSA	Sigma-Aldrich
	Protease inhibitor	Roche
	solubilized in Hank´s salt solution	Biochrom AG
May Grünwald solution		Merck
Pentobarbital sodium salt	10% solubilized in PBS	Merial
Periodic acid solution	0.8 %	Sigma-Aldrich
Phosphate buffered saline		Lonza
Phosphate buffered formalin	4% Formalin solubilized in PBS	Fluca/ Lonza
Phloxin solution	2.5% solubilized in Deionized water	Sigma-Aldrich

5. Kits

Name	Manufacturer
Collagen I	Contrex
Collagen IV	Exocell
Mouse IL-4 ELISA set	BD Bioscience
Mouse IL-5 ELISA set	BD Bioscience
Mouse IL-13 ELISA set	BD Bioscience
Mouse ultrasensitive Th1/Th2 multiplex	MSD
Mouse ultrasensitive proinflammation multiplex	MSD
Sircol collagen assay	Biocolor
Mouse TGF-ß Platinum ELISA	eBioscience

6. Antibodies

Antibodies	Source /Catalogue number
Anti–IL-10 receptor (α–IL-10R) monoclonal antibodies, 1B1.2	*
Anti-IFN (α-IFNγ) monoclonal antibodies, XMG1	*
Biotin rat anti-mouse IgE	BD Pharmingen/553419
Biotin rat anti-mouse IgG1	BD Pharmingen/553441
Biotin rat anti-mouse IgG2a	BD Pharmingen/553388
Mouse anti-human α-smooth muscle actin	Dako AS Denmark
Mouse anti-chicken egg ovalbumin IgE	Serotec MCA 2259
Mouse anti-chicken egg ovalbumin IgG1	Sigma/A6075
Mouse anti-chicken egg ovalbumin IgG2a	Dianova/HYB 094-07-02
Purified mouse IgE	BD Pharmingen/557079
Purified mouse IgG1 κ isotype	BD Pharmingen/557273
Purified mouse IgG2a κ isotype	BD Pharmingen/553454
Rat anti-mouse IgE	BD Pharmingen/553413
Rat anti-mouse IgG1	BD Pharmingen/553440
Rat anti-mouse IgG2a	BD Pharmingen/553446)

* = α–IL-10R and α-IFNγ antibodies were generously provided by Prof. Dr. Edgar Schmitt (Mainz, Germany).

7. Allergens

The following allergens were used: OVA (salt free Albumin egg, Serva, Heidelberg, Germany), extracts from house dust mite (HDM, Mite, House Dust, *Dermatophagoides pteronyssinus,* Greer Laboratories, Lenoir, N.C., USA), and extracts from cockroach (CRA, Cockroach, German, *Blattella germanica,* Greer Laboratories, Lenoir, N.C., USA). For sensitization allergens were solubilized in 100 µl phosphate buffered saline (PBS, Biowhittaker, Köln, Germany) and absorbed to 100 µl $Al(OH)_3$ (Imject Alum, Rockford, USA). A total volume of 200µl was administered. For provocation/challenge OVA was either nebulized 1% OVA solution or administered intratracheal. All other allergens were given intratracheal, solubilised in 50 µl PBS.

8. TLR agonists

Toll-like receptor	ligand used for activation
TLR2	LTA-SA
TLR3	poly(I:C)
TLR4	LPS-EK
TLR7	R848
TLR9	CpG-ODN

For activation of murine TLR2, TLR3, TLR4, TLR7, and TLR9 the respective agonists were used; lipoteichonic acid from Staphylococcus aureus; LTA-SA, synthetic analogue of double stranded RNA; poly(I:C), lipopolysaccharide from E.coli K12; LPS-EK, small synthetic antiviral imidazoquinoline compound; R848 and synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides; ODN1826. All TLR agonist were purchased from InvivoGen, San Diego, USA.

9. Animals

Female C57Bl/6, IL10-/- deficient (C57Bl/6 background), and BALB/c mice were purchased from Charles River (Sulzfeld, Germany). If not explicitly stated otherwise, all experiments were carried out with female BALB/c mice. At the onset of the experiments, animals were between 8 and 12 weeks of age, with a weight between 18-20g. Animals were kept in a pathogen-free animal facility, temperature was kept at 22 °C with a max. deviation from normal conditions of 20-25°C, and a humidity of 55% with a max. deviation from normal conditions of 45-65%. Animals had free access to food and water. Care and use of experimental animals conformed to the 'Guide for the Care and Use of Laboratory animals' published by the National Institute of Health (NIH publication 85–23, revised 1985), and the study was approved by the local governing authority.
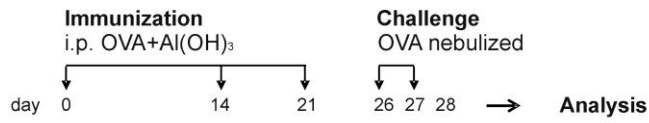
10. Treatment protocols

Mice were sensitized via intraperitoneal injection of 20 µg OVA solubilised in 100 µl phosphate buffered saline (PBS) and adsorbed to $Al(OH)_3$ (100 µl, Alum, Pierce, Rockford, USA). Mice sensitized with house dust mite or cockroach received 2.5 µg of extract again solved in 100 µl PBS and adsorbed to $Al(OH)_3$. The total volume of 200 µl was administered to animals on day 1, 14 and 21.

In the model of acute lung inflammation (Fig. 4a) mice were challenged with 1% OVA

aerosol on day 26 and day 27. For aerosol challenge 200 mg OVA solubilised in 20 ml PBS with 5 µl Triton was nebulized. During challenge mice received air with 5% CO_2 at an airflow rate of 2.5 L/min. First, mice received 7 min air/CO_2 mixture, then 20 min of challenge to aerosolized OVA followed by 7 min period in which no OVA was nebulized. All other allergens were given intratracheal. In the models of chronic inflammation (Fig. 4c, d) mice were challenged twice a week for 7 weeks by intratracheal application of 20 µg of the respective allergen or allergen extract solubilised in 50 µl PBS. Prior to intratracheal application, mice were anesthetized with 3.5 % isofluran at an airflow rate of 3 L/min. Vehicle controls were treated intraperitoneal with PBS and Al(OH)$_3$, and were challenged with PBS aerosol or received intratracheal administration of PBS. Mice were sacrificed 24 hours after the last OVA exposure. For administration, TLR agonists were solubilised in 50 µl PBS and administered at the indicated time point (Fig. 5a-d). The OVA and vehicle controls all received a sham administration of PBS at the same point of time as the other groups received TLR agonists. When mice were challenged on the same day, the application of TLR agonists was conducted one hour prior to OVA exposure. If dose of application was not explicitly stated mice received a dose of 1mg TLR agonist/kg animal bodyweight. In the acute preventive model (Fig. 5a) TLR agonists were administered intra-peritoneal together with OVA during sensitization. In two different acute protective models TLR agonists were either administered once 4 days prior to OVA challenge (Fig. 5c) or one hour prior to OVA challenge on day 26 and 27 (Fig. 5b). In the acute therapeutic model TLR agonists were administered on day 29 followed by an additional OVA exposure on day 33 (Fig. 5c). In the chronic model TLR agonists were first administered after 3 weeks of challenge on day 47 and 48. Following TLR agonists were administered each week twice 1 hour prior to allergen challenge.
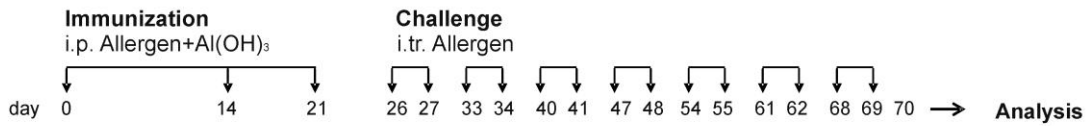
(a) Model of acute inflammation I

(b) Model of acute inflammation II
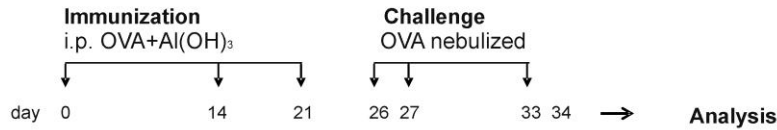
(c) Model of chronic inflammation
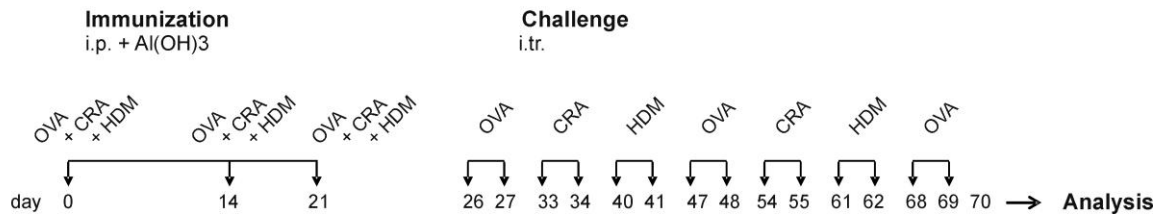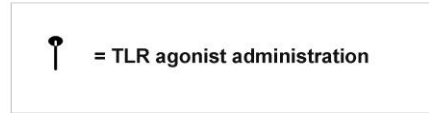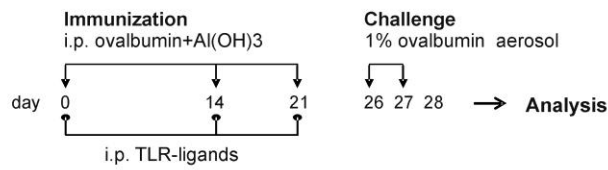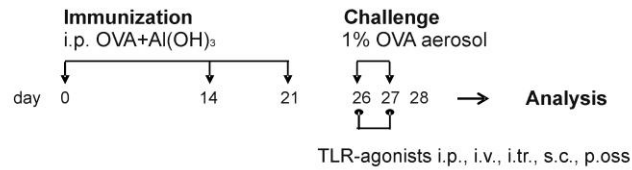

(d) Triple allergen combination model

Fig. 4. Treatment protocols for allergic lung inflammation models, termed: (a) acute I, (b) acute II, (c) chronic, and (d) triple allergen combination. OVA = purified ovalbumin, HDM = crude extract of house dust mite, CRA = crude extract of cockroach.

(a) Preventive TLR agonist administration

(b) Protective TLR agonist administration

(c) Therapeutic TLR agonist administration in model of acute allergic inflammation
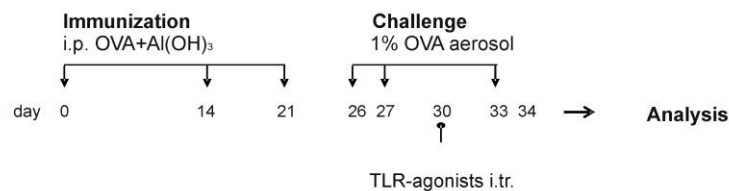
(d) Therapeutic TLR agonist administration in model of chronic allergic inflammation

Fig. 5. TLR agonist administration protocols, termed (a) preventive, (b) protective, (c) therapeutic in model of acute inflammation, and (d) therapeutic in model of chronic lung inflammation. In the chronic model animals were treated either by intratracheal administration of TLR agonists or by per os administration of the corticosteroid dexamethasone.

11. Anti–IL-10 receptor and anti-IFN-γ neutralizing antibodies

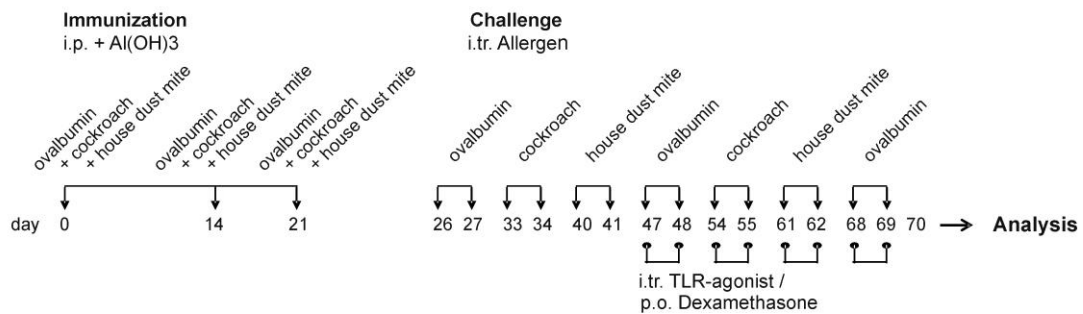Anti–IL-10 receptor (α–IL-10R) monoclonal antibodies 1B1.2 and anti-IFN-γ monoclonal antibodies XMG1 were generously provided by Prof. Dr. Edgar Schmitt (Mainz, Germany). BALB/c mice were treated with 250 µg/mouse (intraperitoneal) of anti–IL-10R or anti-IFN-γ monoclonal antibodies or, as controls, with 250 µg/mouse (intraperitoneal) of purified rat serum IgG monoclonal antibodies (Sigma, Steinheim, Germany). All antibodies were administered on day 25, one day prior to TLR application and OVA exposure.

12. Bronchoalveolar lavage

24 hours after the last OVA exposure mice were sacrificed by intraperitoneal injection of 10 % Pentobarbital sodium salt (Nacoren, Merial GmbH, Hallbergmoos, Germany), solved in phosphate buffered saline. Following a cannula was inserted into the trachea and the lung was flushed with 0.8 ml of buffer twice. Bronchoalveolar lavage (BAL) was performed with lavage buffer consisting of 0.0012 % 0,5M EDTA (Promega, Madison, USA) and protease inhibitor (Complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics GmbH, Mannheim, Germany, 1 tablet per 10 ml lavage buffer) in Hank´s salt solution (Biochrom AG, Berlin, Germany).

13. Measurement of total and differential cell counts

Total cell counts in BAL fluid and blood was determined by means of a Sysmex XT1800 iVeT cell analyzer (Sysmex Europe GmbH, Norderstedt, Germany). For cell differentiation and quantification, cytospin analyses were performed. 200 µl of BAL were spun down on microscope slides for 1 min at 500 rpm. In the next step, slides were dried for at least four hours and a standard Pappenheim staining was performed. In brief, slides were incubated for 5 min in May Grünwald solution, then stained for 20 min in Giemsa solution followed by two washing steps in Weise buffer, each one for the duration of one minute. For the final step slides were incubated for 2 min in tab water. A total number of 300 cells per slide were differentiated into macrophages, eosinophils, lymphocytes, and neutrophils by standard morphological criteria via microscopy. The percentages of cells were then used together with the measured concentration of leukocytes/ml and the total volume of BAL to calculate total counts of single cell populations.

14. Lung preparation

24 hours after the last OVA exposure mice were sacrificed by intraperitoneal injection of 10 % Pentobarbital sodium salt (Nacoren, Merial GmbH, Hallbergmoos, Germany) solved in phosphate buffered saline. Lungs were then extracted and the left lobus of the lung was cut vertically from the anterior to posterior axis directly above the main bronchus entry. Following this, the lobus was fixed in 4% formalin for at least 24 h. In the following step formalin was removed and lung tissue was embedded in paraffin wax using the Tissue Tek VIP Vacuum infiltration processor (Sakura, Finetek, USA). Afterwards lung were placed in paraffin blocks with the cut side down. Paraffin lung slices were cut with a thickness of 3 μm from anterior to posterior direction and transferred to microscopy slides.

15. Immunoglobulin analysis

If not stated otherwise, blood samples were collected 23 hours after the last challenge via retro-orbital bleeding of anesthetized mice (3.5 % isofluran at an airflow rate of 3 L/min). Samples were incubated for 30 min at room temperature for blood clotting and then centrifuged at 14.000 rpm for 20 min. Supernatant was collected and levels of total or OVA-specific IgE and IgG1 were measured using standard ELISA technique. All incubation steps were conducted on a microplate shaker (Titramax 101, Heidolph, Schwabach, Germany) with 500 rpm. All washing steps were conducted with an automated plate washer (Elx405, Biotek, Winooski, United States) and if not stated otherwise included three washing cycles.

a) Measurement of total Ig levels: For measurement of serum concentrations the different Ig subtypes, rat anti-mouse IgE, IgG1, IgG2a were used as primary antibodies for coating of 96 well plates. Purified mouse IgE, IgG2a κ isotype, and IgG1 κ isotype were used to produce standard dilutions. As second antibodies biotin rat anti-mouse IgE, IgG1, and IgG2a were applied for detection. All reagents were purchased from BD Biosciences (BD Biosciences, Erembodegem, Belgium). For analysis of different Immunoglobulins standard ELISA technique were applied. In brief, 50 μl of respective coating antibody was added to 96 well plates in a final concentration of 5μg/ml and incubated over night at 4°C. After a subsequent washing step plates were blocked with 100 μl of PBS containing 1% BSA for three hours at room temperature in order to avoid unspecific binding of antibodies to plate surface. Afterwards, either standards or samples were applied in a volume of 50 μl to the wells and incubated over night at 4°C. After a washing step 50 μl of the respective biotinylated antibody in a final concentration of 5μg/ml was applied to each well and incubated for two hours on the

shaker. After a final washing step 50 µl of streptavidinperoxidase were applied to each well and plate was incubate in the dark for 30 min at room temperature. Then plate was washed twice before 50 µl of detection reagent was applied. The color development was stopped with 50µl 2M Sulphuric acid after 5-10 min and absorbance was analyzed at a wavelength of 420 nm. Serum dilutions normally ranged from 1:10 to 1:50 for IgE, from 1:50 to 1:16000 for IgG2a, and from 100.000 to 500.000 for IgG measurement. Standards were applied in duplicates, concentration ranging from 1250 ng/ml to 1.7 ng/ml for IgE, and from 200 ng/ml to 3.125 ng/ml for IgG2a and IgG1.

b) Measurement of OVA-specific Ig levels: For coating each well was filled with 50 µl of OVA-solution in a concentration of 20 µg/ml and plates were incubated over night at 4°C. Then, plates were washed and blocked and ELISA was conducted according to the protocol for OVA-specific IgE, OVA-specific IgG1 or OVA-specific IgG2a using the previously mentioned incubations times, reagents, volumes and serum dilutions for the corresponding Ig-subtype.

16. Measurement of cytokines by ELISA and multiplex technology

In acute models, the amount of cytokines and chemokines were measured in bronchoalveolar lavage (BAL) fluids. In chronic models, cytokines and chemokines were measured in lung homogenates. The cytokines IFN-γ, IL-1β, IL-2, IL-4, IL-5 , IL-10, IL-12 (total), IL-17, and TNF-α were determined using 9-PlexBase mouse pro-inflammatory or T_H1/T_H2 cytokine/chemokine multiplex assay (Meso Scale Discovery, Gaithersburg, Maryland, USA) or 96 well plate mouse cytokine/chemokine premixed multiplex lincoplex KIT assays (LINCO, St. Charles, USA) according to the manufacturers´ instructions. In brief, fluorescently labelled microspheres coated with cytokine-specific capture monoclonal antibodies were incubated overnight at 2-8° C with 25 µl of lavage or homogenate. All incubation steps were conducted on a microplate shaker (Titramax 101, Heidolph, Schwabach, Germany) with 500 rpm. After two washing steps, biotinylated detection monoclonal antibodies were added and incubated for 60 min at room temperature, followed by 30 min of incubation with streptavidin-phycoerythrin. All washing steps were conducted with an automated Plate washer (Elx405, Biotek, Winooski, United States) and if not mentioned otherwise, included 3 washing cycles. After three washing steps sheath fluid was added and the plates were analyzed using a Bio-Plex reader (Bio-Rad, München, Germany). Standard curves and concentrations were calculated with BioPlex Manager 3.0 software. In preventive and therapeutic models amounts of IL-4 and IL-5 in the whole lung lavage were

measured by standard ELISA, using BD Biosciences mouse IL-4 and BD Biosciences mouse IL-5 ELISA sets (BD Biosciences, San Diego, USA) according to the manufactures instructions. In brief, plates were coated with 100 µl of capture antibody diluted in coating buffer and plates were incubated over night at 4° C. After a washing step plates were blocked with 200 µl assay diluents and incubated for one hour at room temperature. After a subsequent washing step 100 µl of standard in duplicates, ranging from 500 pg/ml to 7.8 pg/ml and samples were applied to the wells and incubated for two hours at room temperature. A washing step, this time with 5 washes was conducted prior to application of 100 µl of working detector, consisting of detection antibody and horseradish peroxidase and incubated for one hour at room temperature. The final washing step, this time with 7 washes, was followed by addition of 100 µl of substrate solution and a incubation phase of 30 min at room temperature in the dark. In the final step 50 µl of stop solution were applied to each well and absorbance was analyzed with a wavelength of 45n nm.

TGF-β was measured with mouse TGF-beta1 Platinum ELISA (Bender MedSystems GmbH, Vienna, Austria) according to manufacturers` instructions. In brief, 20 µl of each sample was pretreated with 180 µl of assay buffer and 20 µl of 1N HCL and incubated for one hour at room temperature. Then samples were neutralized by addition of 20 µl of 1N NaOH. Before applying samples and standards, provided pre-coated plates were washed and TGF-β standard dilutions ranging from 2000.0 to 31.3 pg/ml, and 60 µl of sample together with 40 µl of assay buffer were transferred to the wells and incubated for two hours at room temperature. Plates were then washed 5 times and 100 µl of Biotin-conjugate were added to each well and plates were incubated in the dark for 1h at room temperature. Plates were then washed 5 times and 100 µl of streptavidin-horseradish peroxidase was added and plates were incubated for one hour at room temperature. Plates were then washed 5 times and 100 µl of TMB substrate solution was added and plates were incubated for about 30 min at room temperature. The enzyme reaction was then stopped by adding 100 µl of stop solution and absorbance was analyzed at a wavelength of 420 nm.

17. Histological stainings

For analysis of pathological changes in lung tissue, slices were stained using standard tissue staining protocols. Directly before staining, slides were baked for 45 min at 65 °C. Hematoxylin and eosin (H&E) reagent (Merck, Darmstadt, Germany) were used for staining of inflammatory infiltrates, periodic acid-Schiff (PAS) reagents (Sigma-Aldrich GmbH, Steinheim, Germany) for goblet cells and mucus production. To visualise smooth muscle

cells, α-smooth muscle actin antibody (Alpha smooth muscle actin, Rabbit polyclonal anti human sma, abcam, ab5694, Cambridge, UK) was used. To detect collagen, a pico-sirius red staining was performed. Stained sections were visualised using a Mirax Scan microscope scanner (Zeiss, Göttingen, Germany).

Periodic acid-Schiff staining protocol

Step 1:	Periodic acid	5:00 min
Step 2:	Tap water	6:00 min
Step 3:	Deionized water	0:10 s
Step 4:	Tap water	9:00 min
Step 5:	Schiff-reagent	12:00 min
Step 6:	Tap water	5:00 min
Step 7:	Deionized water	0:10 s
Step 8:	Hematoxylin solution	4:00 min
Step 9:	Deionized water	0:10 s
Step 10:	HCL EtOH solution	0:30 s
Step 11:	Tap water	6:00 min
Step 12:	EtOH 70%	0:10 s
Step 13:	EtOH 96%	0:10 s
Step 14:	EtOH 100%	2:00 min
Step 15/16:	Xylol	each 1:00 min

Hematoxylin and eosin staining protocol

Step 1:	Hematoxylin	8:00 min
Step 2:	Deionized water	0:10 s
Step 3:	HCL EtOH solution	0:30 s
Step 4:	Tap water	9:00 min
Step 5:	EtOH 80%	0:10 s
Step 6:	EtOH 96%	0:10 s
Step 7:	Eosin-Phloxin	0:20 s
Step 8:	EtOH 96%	0:20 s
Step 9:	EtOH 96%	0:20 s
Step 10/11:	EtOH 100%	each 0:30 s
Step 12/13:	Xylol	each 0:30 s

Smooth muscle actin staining protocol

Step 1:	SMA primary antibody		15:00 min
Step 2-4:	Bond Wash Solution	each	0:10 s
Step 5:	Polymer AP		30:00 min
Step 6:	BondWash Solution		2:00 s
Step 7:	Bond Wash Solution		2:00 s
Step 8:	Deionized water		0:10 s
Step 9:	Mixed RED Refine		5:00 min
Step 10:	Mixed RED Refine		5:00 min
Step 11/12:	Deionized water	each	0:10 s
Step 13:	Deionized water		0:10 s
Step 14:	Hematoxylin		3:00 min
Step 15:	Deionized water		0:10 s
Step 16:	Bond Wash Solution		0:10 s
Step 17:	Deionized water		0:10 s

Picro Sirius red staining protocol

Step 1,2:	Deionizied Water	each	5:00 min
Step 3:	Sirius Red		60:00 min
Step 4:	Deionizied Water		0:10 s
Step 5:	Deionizied Water		0:10 s
Step 6:	Fast Green solution		30:00 min
Step 7:	Deionizied Water		0:10 s
Step 8:	EtOH 70%		0:10 s
Step 9:	EtOH 80%		0:10 s
Step 10:	EtOH 90%		1:00 min
Step 11/12:	EtOH 100%	each	2:00 min
Step 13:	Xylol		5:00 min
Step 14:	Xylol		30:00 min

18. Histological analysis of airway remodeling

For quantification of pathological effects the main bronchus area of the lung was selected (Fig. 6a). The degree of inflammation and fibrosis in the peribronchial and perivascular tissue surrounding the bronchus was evaluated. For quantification of mucus production and release the epithelial cell layer and intra bronchial airway was evaluated. Intensity of inflammation and amount of secreted mucus was scored manually by semi-quantitative analysis via double-blinded studies. For this, HE and PAS stained lungs were rated with the following key; 0 = no visible inflammation or mucus production, 1 = slight inflammation or slight mucus production, 2 = moderate inflammation or moderate mucus production and release, 3 = strong inflammation or strong mucus production and release. For quantification of fibrosis, smooth muscle thickening and mucus production automatic analyses were performed using imaging solutions based on the software Halcon (MVTec Software GmbH, München, Germany). For quantification of fibrosis, the compactness (C) of red stained collagen in the peribronchial and perivascular tissue was measured (Fig. 6b). Collagen was stained by pico-sirius staining. Calculation: C was defined as:

$$C = \frac{L^2}{4F\pi}$$

With L: length of the contour; F: area of the region.

In case of empty regions compactness value was considered 0. Thickening of airway smooth muscle was quantified by assessing of thickness and total area of the muscle layer surrounding the main bronchus (Fig. 6c). These parameters were assessed by automated measurement with the software Halcon. In brief, border of airway space (lumen) and epithelium was marked on computer. From each of these marked points in 200-300 µm distances a second outline was created. For quantification only the stained tissue inside this region was assessed. Following this, auxiliary lines were drawn from bronchus to this second limitative line. The thickness was defined as the distance between the intersections of the contour. Following the mean ± standard error of means (SEM) of all measured lengths were calculated and diagrammed. For quantification of mucus, epithelial layers of the PAS stained lung slices were defined by delimiting the area from lumen to sub epithelial tissue (Fig. 6d, green lines). Following this step, areas of epithelial layers and total red stained areas were quantified. Amount of mucus was then given in ratio area mucus to area epithelial cell layer. For mucus plugging, a lung was considered plugged when 75% of the main bronchus area was filled with mucus and cell detritus, or if the main bronchus area was filled 50 to 75% and at least one additional bronchiole area was filled to 75% with mucus and cell detritus.
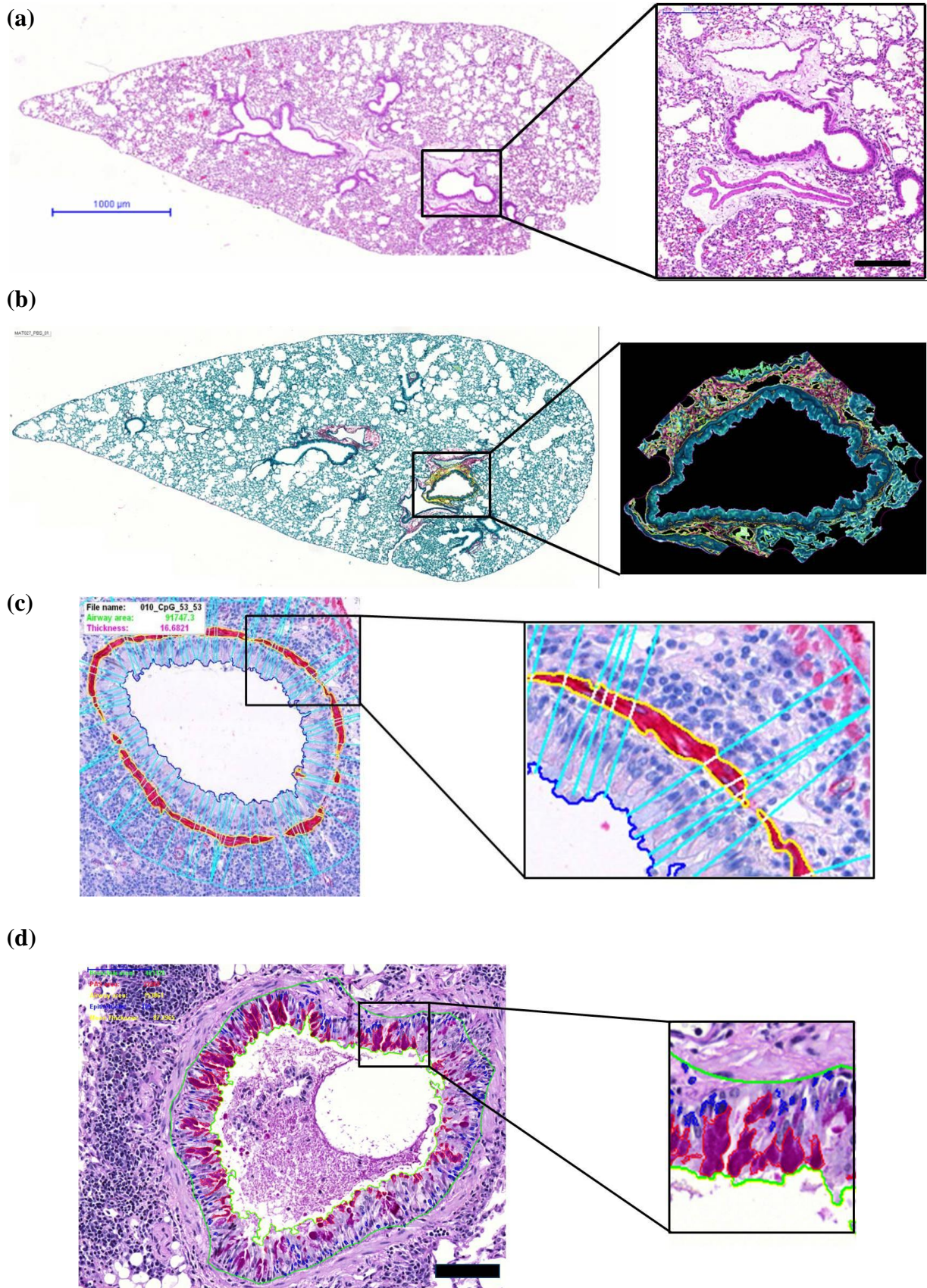
Fig. 6. Shown are lung regions and stainings used for analyses of lung pathology. Cross sections of hole left lung with perivasular and peribronchial areas surrounding main bronchus were used for analyses of inflammatory cell influx (a), compactness (b), smooth muscle thickening (c), goblet cell hyperplasia and mucus production (d). Scale bars; (a) blue =1mm, black = 200µm. (d) = 100µm.

19. Collagen analysis

Salt soluble collagen was measured with Soluble Collagen Assay (Biocolor, Carrickfergus, United Kingdom). For Soluble Collagen Assays, 50 µl supernatant of homogenized lung tissue was analyzed according to the instructions provided by the manufacturer. In brief, 1 ml of Sircol dye was added to samples and shaken for 30 min at 700 rpm. Subsequently, samples were centrifuged and supernatant discarded. Precipitates were dried and resolved in 1 ml Alkali reagent and transferred to 96 well plates. Levels of collagen were measured with wavelength set to 540 nm. Results were calculated in µg collagen per mg of lung weight, using the provided collagen standard solution as reference. Collagen type I, II and IV were measured using a mouse collagen I ELISA kit (Chondrex, Philadelphia, USA), mouse collagen III ELISA kit (Cusabio, Newark, USA) and a mouse collagen IV ELISA kit (Chondrex, Philadelphia, USA). All kits were used according to the instructions provided by the manufacturer using standard ELISA techniques. In brief, for measurement of collagen type I, plates were coated with rat IgG monoclonal antibodies specific to native mouse type I collagen and incubated at 4° C overnight. After a washing step a serial dilution of collagen I standard ranging from 2.5µg/ml to 0.08µg/ml and undiluted samples in total volume of 100µl were added and incubated for two hours at room temperature. After another washing step 100µl of detection antibody solution were added and plate was incubated for two hours at room temperature. After the next washing step 100µl of strepavidin peroxidase solution was added and plate was incubated for one hour at room temperature. Following a last washing step, 100µl OPD solution were added and plate was incubated for 30 min at room temperature and then reaction was stopped with 50µl of sulphuric acid and optical density was assessed with a wavelength of 490 nm. Collagen III was measured with plates pre-coated with mouse collagen III specific antibody. Using serial dilution of the provided standard, the detection range of the ELISAs ranged from 3.12 pg/ml to 200 pg/ml. 100µl of each sample or standard was added to the plates and incubated by 37° C for 2h. Liquid was then removed and 100µl of Biotin-antibody was added and incubated by 37° C for one hour. After a washing step 100µl of HRP-avidin working solution were added and plate was incubated for 1h at 37° C. After an additional washing step 90µl of TMB substrate were added to each well and incubate for 15 min at 37° C. In the last step 50µl of Stop solution were added to each well an optical density was assessed with a wavelength of 450 nm. Collagen IV was measured with a competitive indirect ELISA. Serially diluted of mouse collagen IV standard was used in the concentration ranging from 10-0.009 µg/ml. 50 µl of sample were added to 50 µl of rabbit anti-murine collagen IV antibody and incubated overnight at room temperature. Following, plates were

washed and 100 µl of goat anti-rabbit IgG HRP conjugate was given into each well and incubated for one hour at room temperature. Afterwards, plates were washed and 100 µl of developer was added and absorbance was determined at 450 nm after 30 min incubation time at room temperature. Because the assay is a competitive one, color intensity is inversely proportional to the logarithm of mouse collagen IV concentration of the sample.

20. Measurement of airway hyperreactivity

Airway hyperreactivity was assessed by means of non-invasive whole body plethysmography or by invasive measurement of resistance and compliance. Non-invasive measurement was conducted in conscious unrestrained mice and airway hyperreactivity was assessed determining enhanced pause (Penh) values after methacholine (MCh) –induced airflow obstruction. Mice were placed in a body plethysmograph (type PLY4211, Buxco Research system, Wilmington, USA) and breathing induced changes in pressure were measured. After recording baseline values, mice were exposed to aerosolized MCh (Sigma Aldrich, Steinheim, Germany) in dose steps of 0.6, 2.5, 5, 12.5, 25, and 50 mg/ml under continuation of lung function recording. For MCh exposure 200 µl of the respective dose was nebulized for 2 min followed by a 2 min period of lung function recording. During experiments airflow was kept at 1L/min. PenH values were calculated as described by Hamelmann et al. [207].

$$PenH = (PEP/PIP) \times ((Te - RT)/RT),$$

Te = expiration time; RT = relaxation time, PEP = peak of expiratory pressure; PIP = peak of inspiratory pressure.

The invasive measurement was conducted in narcotized mice receiving artificial respiration. Airway hyperreactivity was assessed 23 hours after the final challenge by measuring of airway resistance and compliance. Animals were anesthetized with an intraperitoneal injection of 0.5 mg/kg Medetomidin, 5 mg/kg Midazolam, and 0.05 mg/kg Fentanyl solved in PBS. 15 min after anaesthesia airway hyperreactivity was assessed in intubated mice receiving artificial ventilation with a maximal stroke volume of 10mL/kg and a maximal pressure of 30 cm H_2O. Mice were placed in supine position in a body plethysmograph (type AER 1103, Buxco Research system, Wilmington, USA), and the basic parameters pressure [cm H_2O], flow [ml/sec] were continuously recorded during experiment using fine point software (Buxco Research system, Wilmington, USA). The software directly calculates the functional

parameter "Resistance" [cm H_2O/ml/sec] and "Compliance" [ml/ cm H_2O] from the assessed basic parameters. After recording of baseline values, the mice were exposed to 10 µl of MCh nebulized in dose steps of 0.065, 2.5, 5, 12,5 mg/ml. Absolute values of resistance and compliance were collected for the respective group and represented in a curve. Resistance and compliance were further assessed by measurement of area under the curve (AUC) of the total value graph.

21. Detection of FOXP3+ cells by flow cytometry

24 hours after the last challenge, bronchoalveolar lavage was collected. Total numbers of leukocytes in lavage were measured and differentiated via Sysmex. ACK Lysis Buffer (Lonza, Basel, Switzerland) was used for red blood cell lysis and cells were washed and further processed in PBS supplemented with 0.5% BSA. The cell surface was stained with PerCP-Cy5.5 conjugated anti-mouse CD4 antibody (BD Biosciences, Heidelberg, Germany). For detection of intracellular FOXP3 antigen, the Mouse/Rat FOXP3 staining set (eBioscience, San Diego, USA) was used according to the manufactures instructions. In brief, $1x10^6$ cells were used for the staining of surface marker CD4, CD8, CD25. After the staining step, cells were washed in PBS, resuspended in 1 ml fixation solution and incubated for three hours in the dark. Another washing step was followed by blocking with Fc block at 4°C for 15 minutes. Following, antimouse/rat FOXP3 (FJK16s) antibody was added and cells were incubate at 4°C for 30 minutes in the dark. Fluorescence was analyzed on a LSRII Cytometer (BD Biosciences, Heidelberg, Germany) and quantification was performed by BD FACS DIVA Software 5.0. Gates were set on lymphocytes based on forward and side scatter properties, quadrants were defined using isotype controls.

22. Measurement of active cutaneous anaphylaxis

For measurement of active cutaneous anaphylaxis mice received an intravenous application of 200 µl 1% Evans blue solubilised in PBS, 24 hours after the last challenge. Following this, mice were anesthetized and received 5 µl of PBS with 5 µg OVA intravenous into the right ear by means of a 32G needle (Hamilton Company, Reno, NV) attached to a 10 µl syringe. For the negative control the left ear received 5 µl of PBS intravenous. 28 minutes after injection mice were anesthetized and sacrificed via cervical dislocation. A defined area of ear tissue (8 mm diameter) was punched from both ears and transferred to separate 2 ml eppendorf tubes. For Evans blue extraction tissue samples were then incubated with 300 µl formamide at

65° C for 24 hours at 450 rpm on a shaker. After dye extraction samples were cooled to room temperature and a total volume of 200 µl was transferred to a 96 flat bottom plate where dye concentration was measured at a wavelength of 620 nm.

23. Statistical analysis

The results are expressed as mean ± SEM. Statistical differences between different groups were evaluated by One-way ANOVA or unpaired t-test. One-way analysis of variance together with the Dunett post test was used for comparisons of all groups vs. the OVA group. In airway hyperreactivity measurement statistical differences of area under the curve (AUC) between vehicle control group and OVA treated animals were evaluated with unpaired t-tests, whereas methacholine response curves were compared with Two-way ANOVA. A P-value of < 0.05 was defined as statistically significant.

24. Software

Mircosoft Office 2007, GraphPad Prism Version 5.03, Reference manager version 11, CorelDRAW Version X3 were used for analysis and presentation of data, Mirax Viewer (Carl Zeiss MicroImaging GmbH, Germany) was used for histological analyses, Halcon machine vision software (MVTec Software GmbH München, Germany) was used for automated histological quantification, Fine point software (Buxco Research Systems; Wilmington, North Carolina, USA) was used to acquire, evaluate, and analyze pulmonary parameters in airway hyperreactivity measurement.

RESULTS

This study was designed to analyze the suppressive effects of TLR activation on allergic inflammation, airway hyperreactivity and airway remodeling, and to determine the level of TLR-induced proinflammation. For this purpose, the five TLR agonists LTA, poly(I:C), LPS, R848 and CpG, which activate the toll-like receptors 2, 3, 4, 7 and 9, respectively, were administered in mouse models of allergic asthma. In order to identify the effects of TLR activation on different developmental stages of asthma, TLR agonists were given a) preventatively in non-sensitized mice exhibiting no allergic inflammation, b) protectively in mice that were sensitized but not challenged exhibiting no pulmonary inflammation, and c) therapeutically in sensitized and challenged mice that exhibiting established pulmonary allergic inflammation. The study also included experiments of TLR agonists administered via different routes in both presensitized and untreated mice. The therapeutic potential of the different TLR agonists was further tested in a model of severe chronic asthma to assess effects of TLR activation on chronic inflammation and symptoms associated with the severe chronic form of the disease. Allergic inflammation, hyperreactivity and airway remodeling were assessed by measurement of cellular influx of eosinophils, allergen-specific or total immunoglobulin levels in serum, cytokine levels in the lung, airway hyperreactivity to methacholine, and identification of pathological changes in lung tissue by histological staining of lungs.

1. TLR agonist-mediated suppression of allergic responses is associated with increased innate inflammation in the airways

The first experiments in this thesis were conducted to evaluate suppressive effects of TLR agonists on allergic responses in mouse models of acute pulmonary inflammation. To identify essential mediators for suppression of allergic inflammation, levels of cytokines in the lungs were quantified. In addition, knockout mice and blocking antibodies were used to evaluate the role of cytokines in TLR-mediated suppression of allergic responses. In the models used, TLR agonists were administered intratracheally. The different TLR agonists were applied in different concentrations and in respect to allergen challenge at different application time points. After sensitization with OVA, mice received an intratracheal administration of the respective TLR agonist, followed by a pulmonary allergen challenge with OVA. TLR agonists were applied in a protective manner shortly before allergen challenge, or therapeutically in mice that had already received an allergen challenge (Fig. 5b, c). To

measure influence of TLR activation on asthma, cellular influx, levels of cytokines, histology, and airway hyperreactivity were determined and compared to negative and positive control groups. Most of the following results have been published under the title; "TLR agonist mediated suppression of allergic responses is associated with increased innate inflammation in the airways" [Duechs et al., Pulm Pharmacol Ther. 2011 Apr; 24(2):203-14].

1.1 Effects of TLR agonists in a protective setting on the development of allergic inflammation in the lungs

To test the pro-inflammatory and anti-asthmatic effects of TLR activation, the five TLR agonists LTA, poly(I:C), LPS, R848, and CpG were administered in the 28 day OVA-allergen asthma model (methods, Fig. 4a), which has been used in previous studies to address effects on allergic responses in mice [208]. TLR agonists were administered in a protective manner shortly before pulmonary allergen challenge. For the assessment of concentration-dependent effects, different groups of mice were treated with 0.001, 0.01, 0.1 or 1.0 mg of TLR agonist per kg body weight. The TLR agonists were given intratracheally one hour before allergen exposure on day 26 and 27 (methods, Fig. 5b). At the end of the experiment, influx of macrophages, neutrophils and eosinophils were analyzed.

The figure 7 shows that all agonists, with the exception of R848, increased the number of total macrophages in the bronchoalveolar lavage (BAL) dose-dependently. A similar effect was seen for the recruitment of neutrophils into the lung as increasing concentrations of TLR agonists correlated with increasing numbers of neutrophils. Interestingly, at a dose of 0.1 mg/kg, administration of R848 also induced a very weak neutrophilia. However, there was no increase in macrophage number at any concentration. Surprisingly, in this study only poly(I:C), R848 and CpG suppressed the development of allergen-induced airway eosinophilia, whereas TLR2 and TLR4 agonists were reported to show suppressive effects as well [209-212]. In contrast, in the present study a significant increase in the recruitment of eosinophils was detected when lower doses of either LTA- or LPS were used. Histological analysis confirmed the overall lower inflammation detected in the poly(I:C), R848 and CpG treated mice in comparison to the OVA controls and LTA and LPS treated animals, which showed the strongest inflammation (Fig. 8). Analysis of cytokines in BAL showed that all tested agonists, again with the exception of R848, induced the production of the pro-inflammatory cytokines and chemokines IL-6, IL-1b, TNF-α and reduced the levels of IL-4 and IL-5 in the whole lung lavage (Fig. 9). Most of the observed effects were dose-dependent.

IL-13 was reduced in the mice treated with CpG, R848, LPS and LTA. No difference in the amount of IFN-γ was found in any of the OVA treated groups. Interestingly, CpG was the only agonist that induced the production of IL-10. The cytokines IL-12, IFN-α, and IFN-ß could not be detected in any of the whole lung lavage samples.

It was then analyzed whether the TLR agonists had suppressive effects on the development of airway hyperreactivity. To assess airway hyperreactivity, conscious unrestrained mice were exposed to increasing concentrations of methacholine while enhanced pause (PenH) data were continuously recorded. Figure 10 shows that LPS, as well as the highest concentration also R848, significantly reduced PenH levels. The question of whether the PenH parameter really reflects airway hyperreactivity is under hot debate and recently PenH has fallen more and more into disrepute as it does not directly assess lung function, but rather measures a potentially correlating effect [213-215]. Therefore, the experiment was repeated using only the highest dose of 1 mg of TLR agonist per kg body weight and airway hyperreactivity was assessed by direct measurement of airway resistance in anesthetized and intubated mice. Increase in airway resistance, and therefore in the work of breathing, is one of the main symptoms of asthma. Resistance is defined as the opposition of driving pressure to the rate of air flow. The most important variable contributing to an increase in resistance is the radius of the airways. Hence, narrowing of airways caused by smooth muscle constriction and/or mucus production can be assessed by measurement of resistance. These measurements, conducted by M. Braun in the lab of HG Hoymann at the Fraunhofer Institute Hannover, showed that only R848 significantly reduced airway resistance (data not shown, see Duechs et al., Pulm Pharmacol Ther. 2011 Apr;24(2):203-14, [216]).
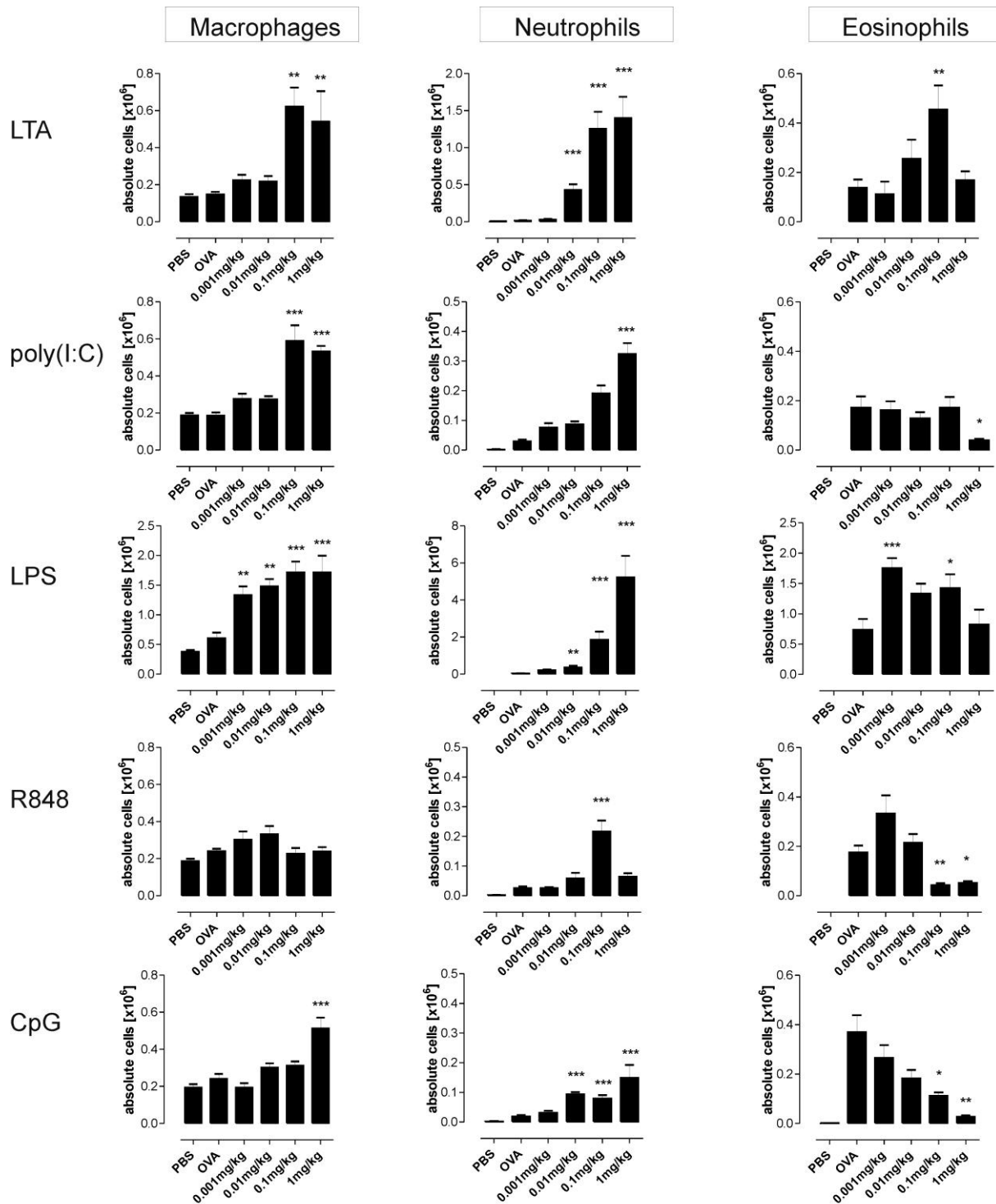
Fig. 7. TLR agonist administration one hour prior to OVA challenge suppresses allergen-induced eosinophilia and induces airway neutrophilia in a model of acute allergic asthma. *Mice were either treated with vehicle (PBS), the allergen OVA, or with allergen and different concentrations of the TLR agonists LTA, poly(I:C), LPS, R848 and CpG. Total number of macrophages, neutrophils and eosinophils in bronchoalveolar lavage were determined 24 hours after the final OVA exposure. Data are presented as mean ± SEM of 8 mice/group. * = p<0.05, ** = p<0.01, *** = p<0.001, in comparison to the OVA group. Experiments were repeated once showing similar results. [Duechs et al., Pulm Pharmacol Ther. 2011 Apr;24(2):203-14]*
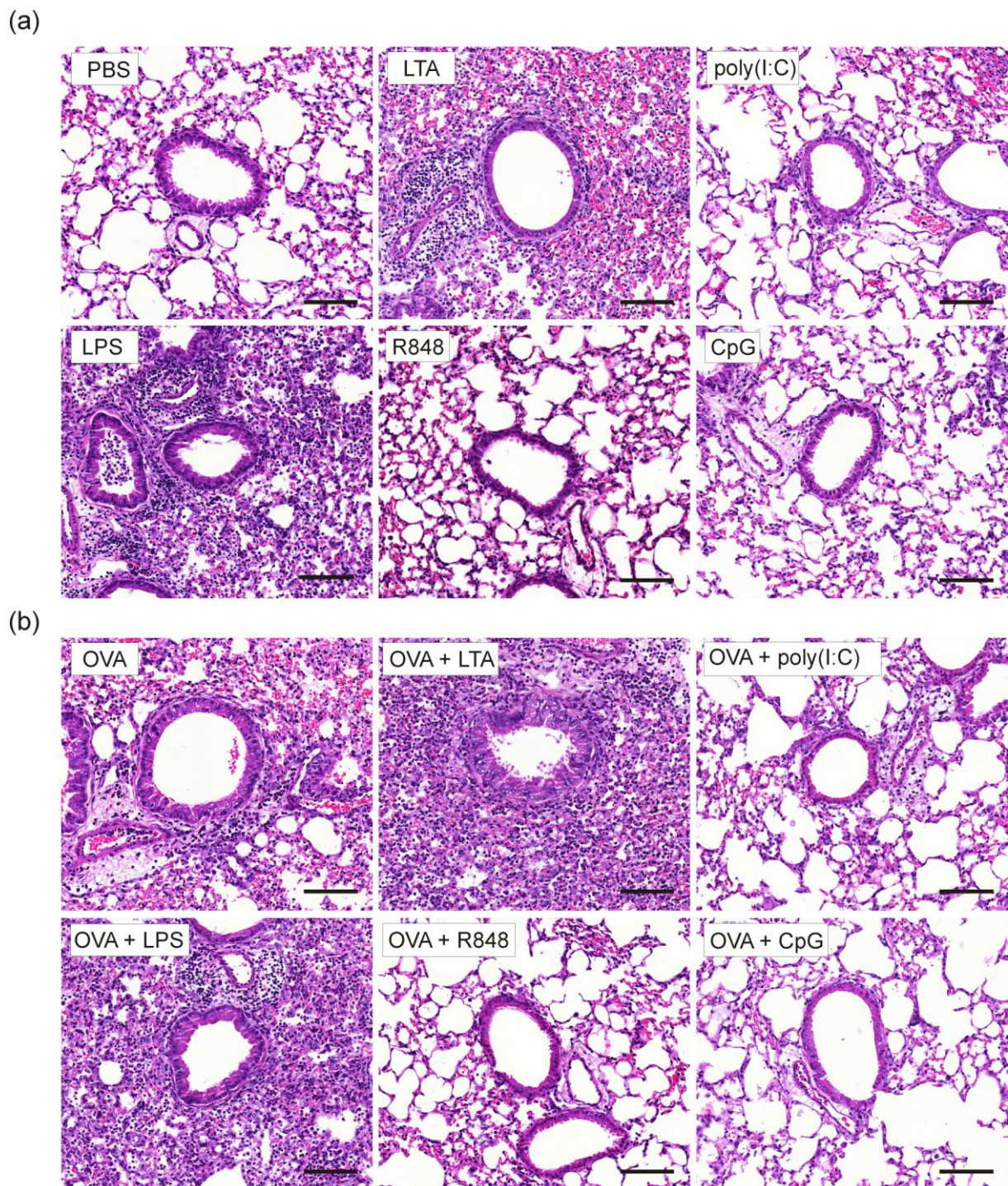
(a)

(b)

Fig. 8. Treatment with the TLR agonists poly(I:C), R848 and CpG reduces allergen induced cellular influx, wherease administration of the TLR agonists LTA and LPS enhances allergen induced cellular influx in lung tissue. *Shown are H&E and PAS stainings of the lungs from mice treated with TLR agonist and OVA. Lung tissues were obtained from naïve mice treated with the TLR agonists alone (a) or from mice sensitized and exposed to OVA and treated with different TLR agonists (b). Tissues were stained with haematoxylin and eosin, and examined by light microscopy. Scale bar = 100µm. Shown are representative examples of 8 mice/group. [Duechs et al., Pulm Pharmacol Ther. 2011 Apr;24(2):203-14]*
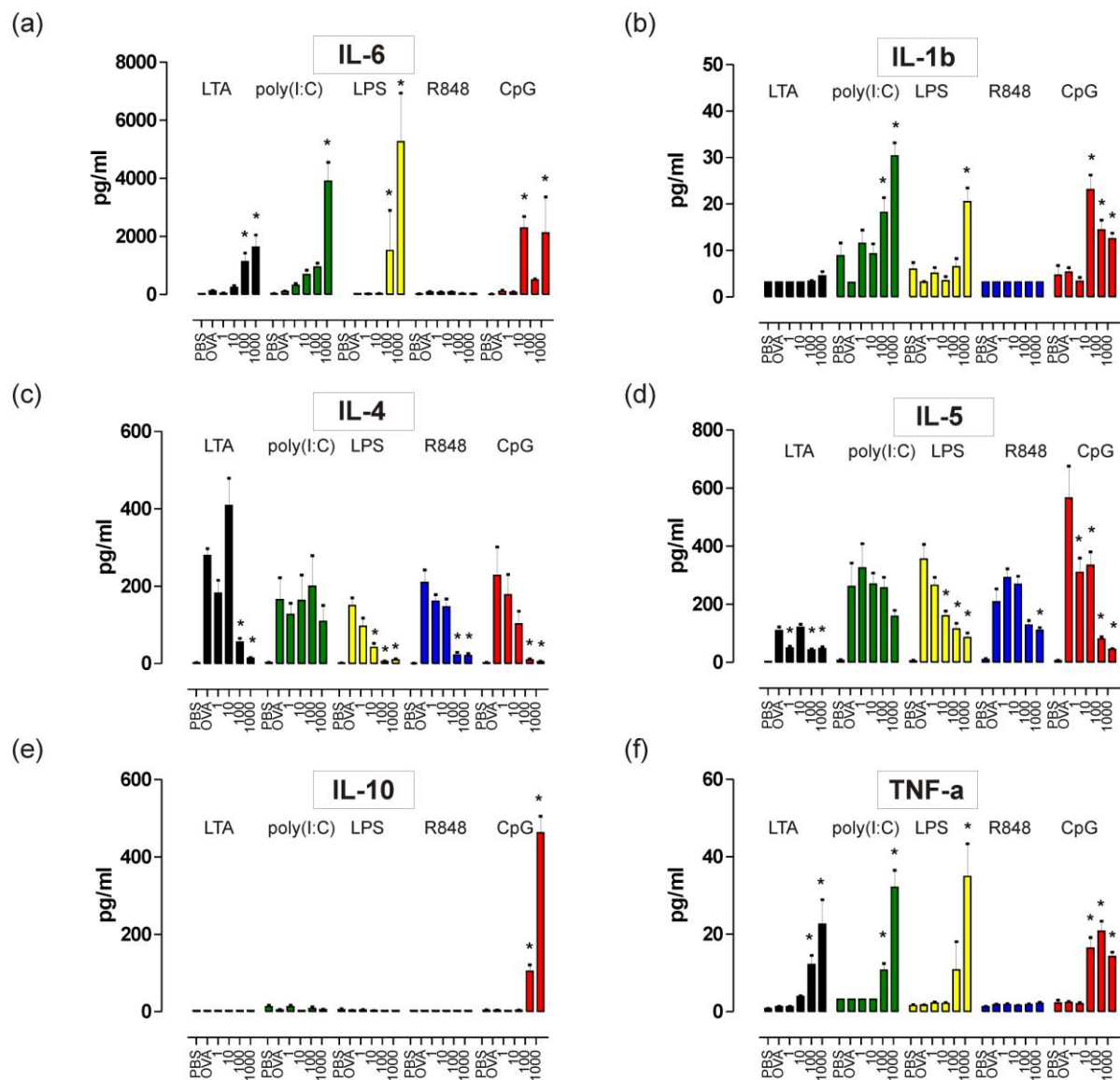
Fig. 9. In a model of acute allergic asthma, TLR agonist treatment reduces T_H2 cytokines and increases pro-inflammatory cytokines and chemokines in the airways. *The level of the different mediators were measured in BAL fluid, which was collected 24 hours after the final OVA exposure (■ = LTA, ■ = poly(I:C), ■ = LPS, ■ = R848 , ■ = CpG). Data are presented as mean ± SEM of 8 mice/group. * = p<0.05, in comparison to the OVA group. [Duechs et al., Pulm Pharmacol Ther. 2011 Apr;24(2):203-14]*
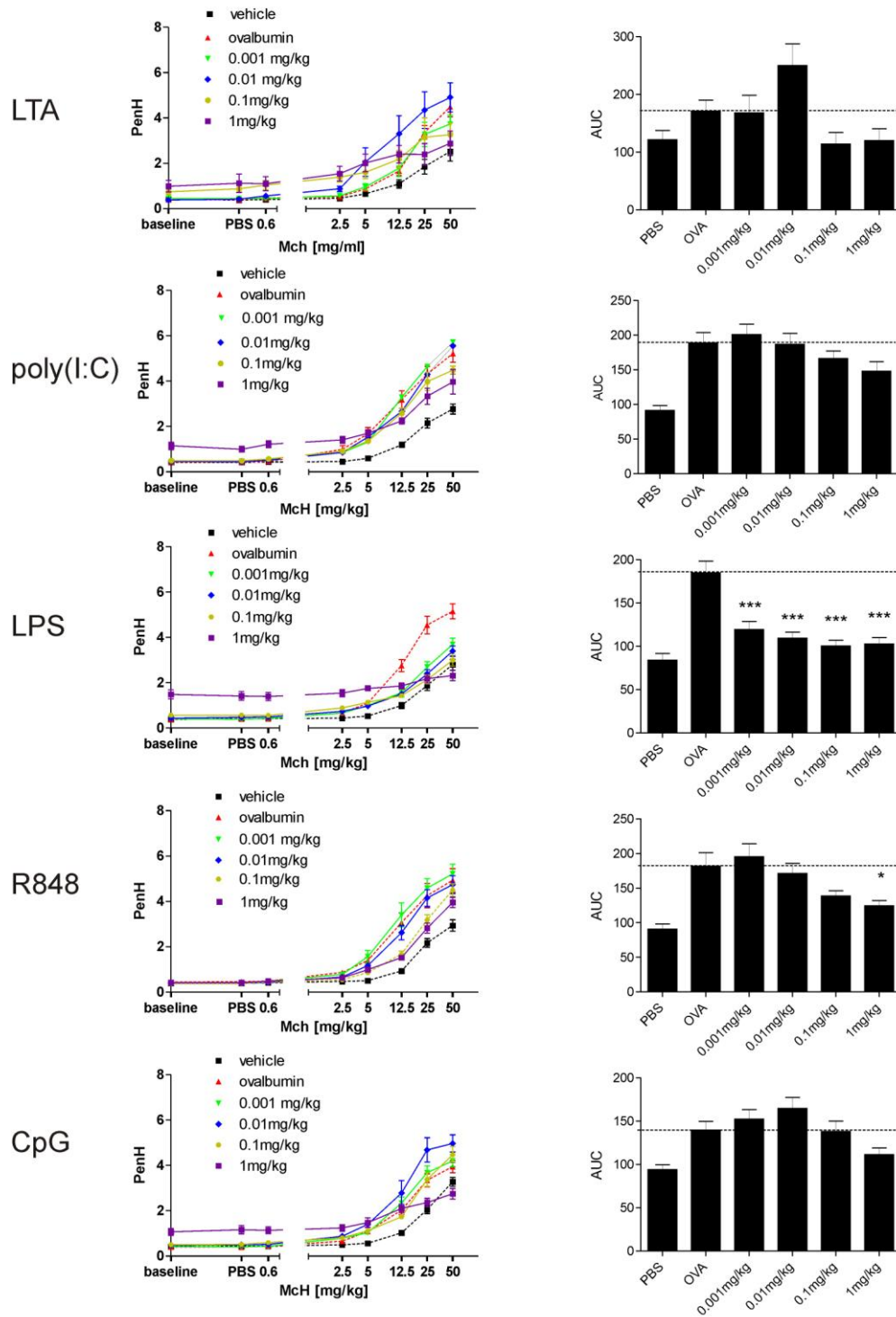
Fig. 10 . Treatment with LPS and R848 significantly reduces OVA-induced increase in PenH. *Mice were first exposed to nebulized PBS, followed by increasing doses of methacholine. Enhanced pause values (PenH) were determined after each exposure period (a) and further analyzed by calculation of the area under the curve (b). Data are presented as mean ± SEM of 9-16 mice/group. * = p<0.05, ** = p<0.01, *** = p<0.001, in comparison to the OVA group.*

(a)

(b)

(c)

Fig. 11. When given four days prior to allergen challenge, TLR agonists inhibit influx of eosinophils and production of IL-4. *(a) Number of inflammatory cells and (b) level of IL-4 and IL-5 in the BAL. (c) Invasive measurement of airway resistance. Data are presented as mean ± SEM of 8 mice/group. * = p<0.05, ** = p<0.01, *** = p<0.001, in comparison to the OVA group. [Duechs et al., Pulm Pharmacol Ther. 2011 Apr;24(2):203-14]*
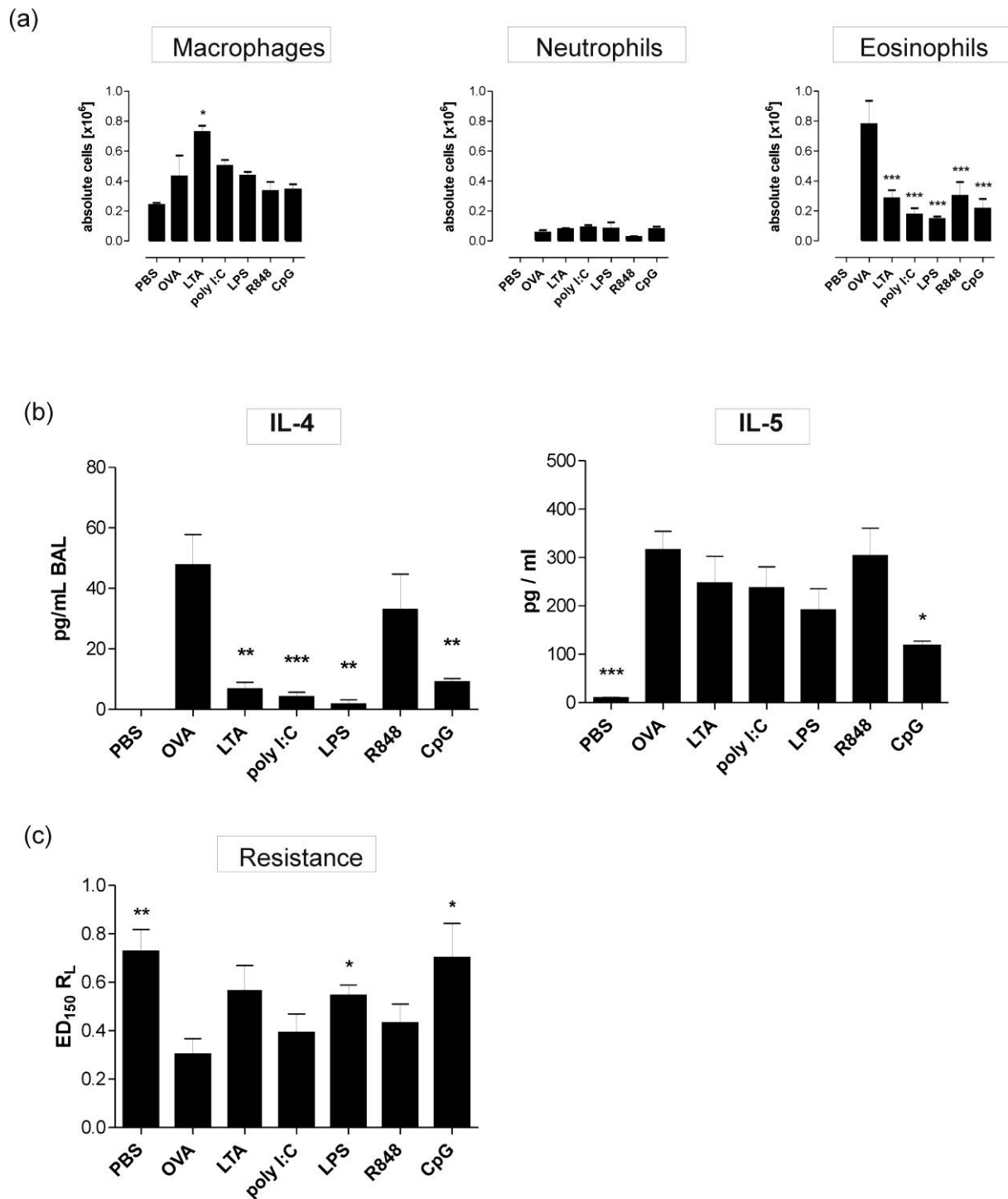
As mentioned before, previous reports showed that both LTA and LPS were able to reduce airway eosinophilia in mice [210-212]. As this effect was not seen in the present study, the five TLR agonists were administered using a different protocol. In this model only the highest dose of the respective TLR agonists (1 mg/kg) were administered once, four days before the allergen exposure.

In this experimental setting now all of the TLR agonists inhibited the development of airway eosinophilia (Fig. 11a). LPS was the only TLR agonist that induced a significant increase in macrophage numbers at this time point. In contrast to TLR agonist administration directly before the allergen challenge, no neutrophilia was detected (Fig.7 and Fig. 11a). The surprising lack of neutrophilia in this model might be explained by the longer time period between TLR agonist administration and analysis of cellular influx. This would suggest that in all cases a neutrophilia is induced but that this is at a later time point no longer detectable. Supporting this view are kinetic studies showing that the peak of neutrophilic influx in the lung is observed 24 hours after TLR application and no or only few neutrophils are present five days after the application (data not shown). When levels of cytokines in BAL were assessed, R848 was the only TLR agonist which did not reduce levels of IL-4. In addition, R848 did not reduce levels of IL-5. All other agonists significantly reduced IL-4 levels with CpG being the only one that significantly reduced IL-5 levels as well (Fig. 11b). Interestingly, in this setting LPS and CpG, but not R848, showed reduced airway hyperreactivity when resistance was assessed (Fig. 11c).

1.2 Suppressive effects of TLR agonists are not dependent on IL-10, IFN-γ or enhanced numbers of T regulatory cells in the airways

Previous studies suggest that the suppressive effects of different TLR agonists on the development of allergic responses may be associated with the production of IFN-γ, IL-10 or activation of FOXP3+ T_r cells [138;149;206;217-219]. To evaluate if these factors are essential for the observed suppression, experiments were repeated using an IL-10 knock-out strain and specific antibodies blocking either IL-10 receptors (α-IL-10 R) or binding to IFN-γ (α- IFN-γ). Furthermore, the amount of T_r cells in the lungs of TLR agonist-treated mice was assessed.

In the experiments using knock-out mice and antibodies, the agonists poly(I:C), R848 and CpG - which showed a significant reduction of T_H2 response in previous experiments - were applied at a concentration of 1 mg/kg body weight. The IL-10 deficient mice were bred on the C57Bl/6 background and the experiment was conducted in both the knock-out and the background strain as control. IL-10 receptor and IFN-γ blocking antibodies were tested in BALB/c mice. In these experiments animals received a single treatment of antibody, one day before the first TLR administration. For the knock-out and antibody studies, cellular influx of macrophages, neutrophils and eosinophils were assessed. For measurement of T_r cells, FACS analysis of BAL was conducted, identifying populations of CD4 and FOXP3 positive cells.

Figure 12a shows that the suppressive effects of the three tested TLR agonists were similar in both the control and IL-10 deficient mice. Furthermore, treating OVA-immunized/exposed and TLR agonist-treated BALB/c mice with anti-IFN-γ or anti-IL-10 receptor-neutralizing antibodies showed that the suppressive effects appeared not to be dependent upon either IFN- γ or IL-10 (Fig. 12b). Analysis of FOXP3+ Tr cell numbers in the whole lung lavage of OVA and TLR agonist-treated mice showed that the suppressive effects of poly(I:C), R848 and CpG were not associated with increased numbers of FOXP3+CD4+ T cells (Fig. 13). Interestingly, both LTA- and LPS-treated mice showed increased numbers of T_r cells in the bronchoalveolar lavage, albeit without reducing the overall number of eosinophils, as seen in Figure 7. Surprisingly, the lung lavage of R848-treated mice exhibited a dramatically reduced number of CD4+ and CD4+ FOXP3+ T cells.

Fig. 12. IL-10 and IFN-γ mediated signaling is not necessary for TLR agonist-mediated suppression of airway eosinophilia. *(a) C57Bl/6NCrl, C57Bl/6NCrl IL-10-/-, and (b) BALB/c mice were sensitized and exposed to OVA and treated with different TLR agonists as indicated in materials and methods (Fig. 5b). (b) On day 25, BALB/c mice received an intraperitoneal injection of either anti-IL-10R or anti-IFN-γ neutralizing antibodies. Number of cells in the whole lung lavage was determined 24 hours after the last exposure. Data are presented as mean ± SEM of 8 mice/group. * = p < 0.05, ** = p < 0.01, *** = p < 0.001, in comparison to the OVA group. [Duechs et al., Pulm Pharmacol Ther. 2011 Apr;24(2):203-14]*
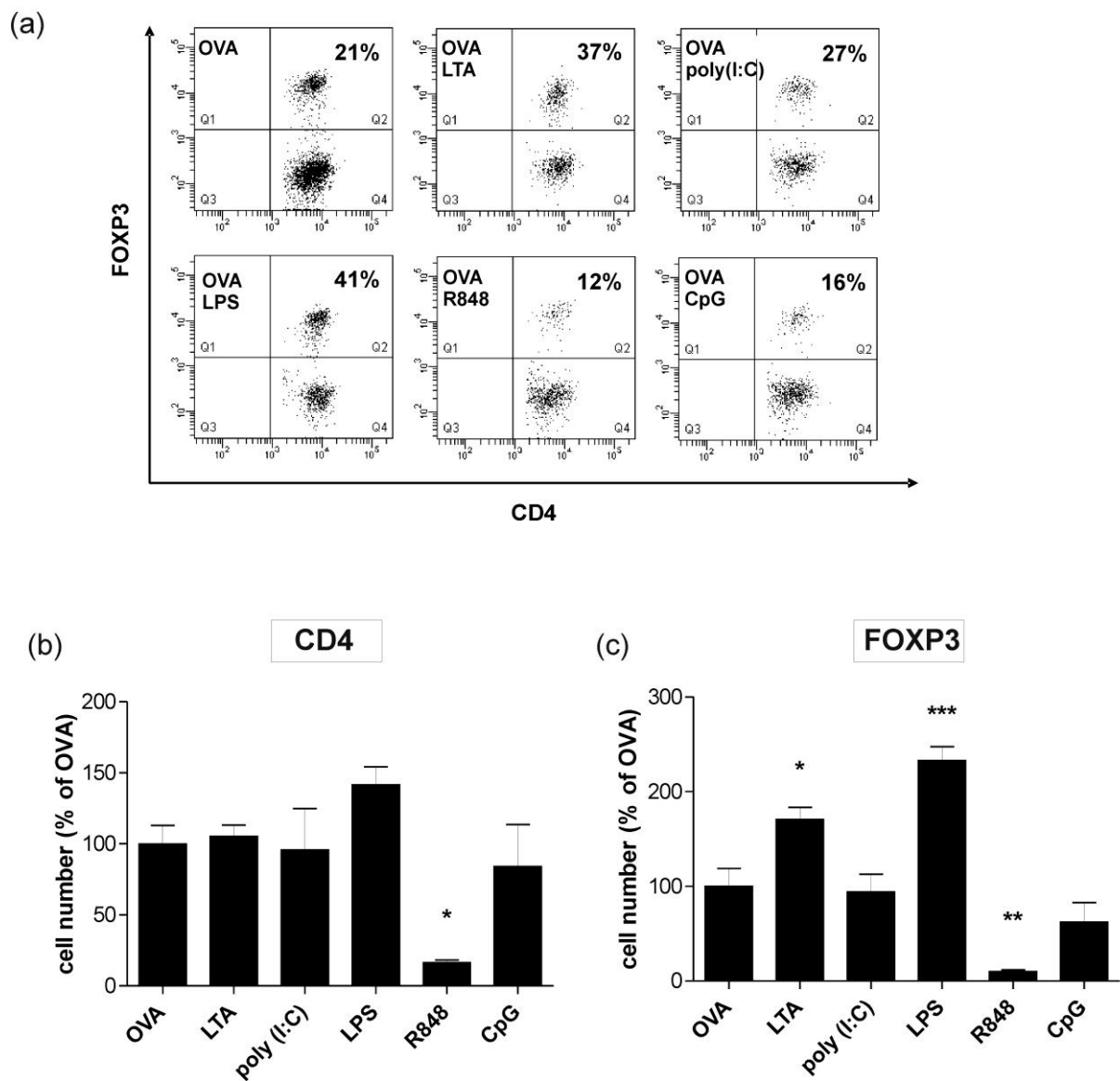
Fig. 13. Suppressive effects of TLR agonist on eosinophilia do not correlate with increased numbers of T_r cells. *(a) Shown are representative examples of whole lung lavage cells stained for CD4+ surface and FOXP3 intracellular expression. Total numbers of CD4+ and CD4+/FOXP3+ cells in the whole lung lavage (% of OVA control) are shown in (b) and (c), respectively. BAL cells were stained as described in materials and methods (chapter 2.20). Data are presented as mean ± SEM of 4 samples/group (whole lung lavage cells from 4 mice/group were pooled (n = 2/group) from 2 separate experiments (total of n = 4). * = p < 0.05, ** = p < 0.01, *** = p < 0.001, in comparison to the OVA group. [Duechs et al., Pulm Pharmacol Ther. 2011 Apr;24(2):203-14]*

1.3 TLR agonists suppress allergen-induced eosinophilia when administered therapeutically

The experiments above clearly show that some, but not all of the tested TLR agonists reduced the development of allergen-induced T_H2 responses in the lung when administered before allergen exposure. In this protective experimental setting no lung inflammation has occurred prior to the treatment with the TLR agonists. To analyze the effects of TLR agonist administration in established lung inflammation, an additional experiment with a therapeutic application of agonists was conducted.

In the therapeutic setting the TLR agonists were administered intratracheally on day 30, two days after the second OVA exposure followed by a final allergen exposure on day 33 (methods, Fig. 5c). For this experiment the TLR agonists were administered in doses of 1 mg/kg body weight, as these doses have shown the greatest suppressive effect in the protective application setting. Figure 14a shows that all the TLR agonists, with the exception of LTA, significantly reduced the development of airway eosinophilia. No increase in neutrophil numbers was observed in any of the treated mice. However, LTA and LPS treated mice showed a significant increase in total macrophage numbers. All TLR agonists reduced IL-4 levels in the lung lavage (Fig. 14b). LPS and CpG showed the strongest reduction of IL-4, almost falling to the background level in untreated mice. IL-5 levels were also significantly reduced by therapeutic administration of all TLR agonists with the exception of the poly(I:C) treated mice. These experiments show that TLR agonists are able to reduce allergic inflammation in an established and ongoing allergic inflammation.
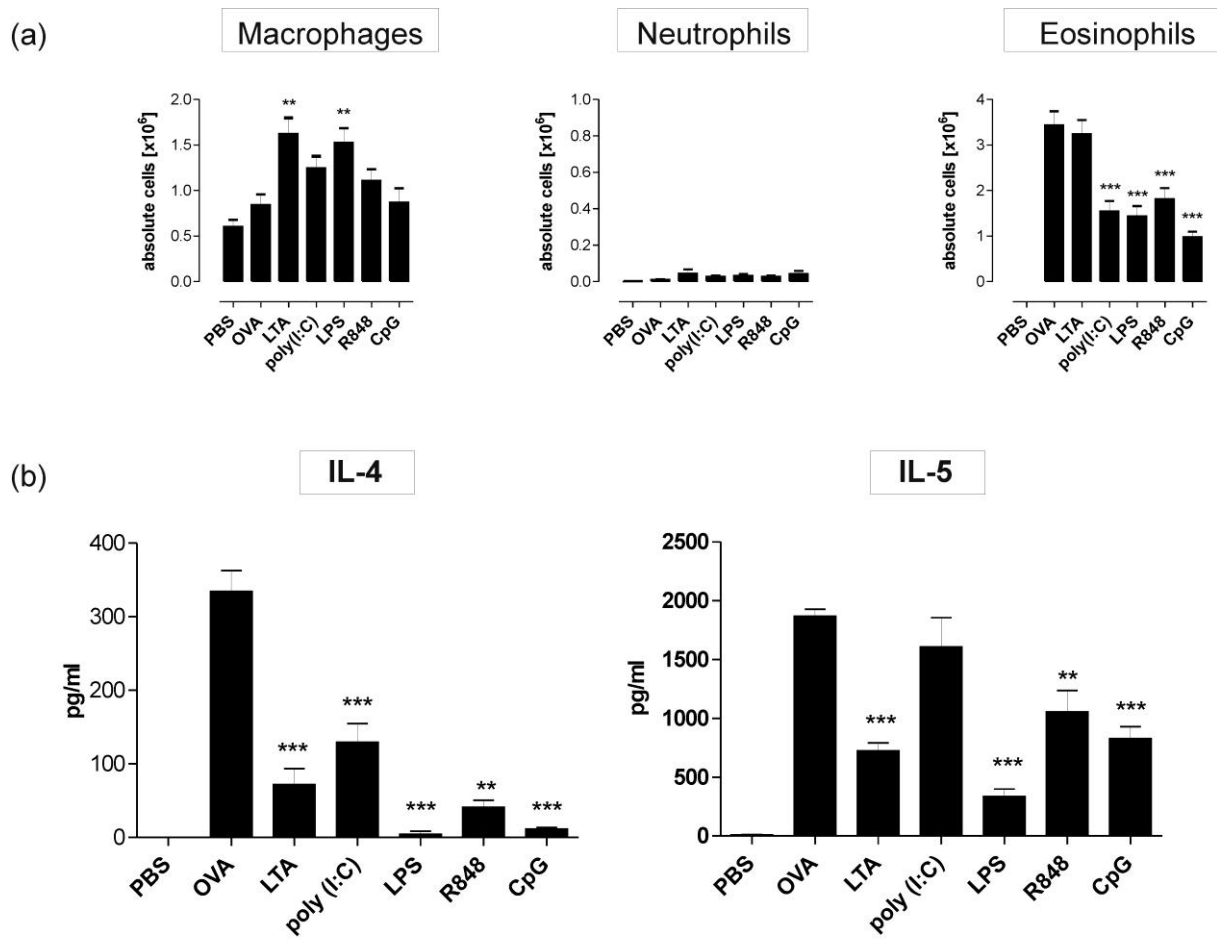
*Figure 14. Therapeutic administration of TLR agonists suppresses allergen-induced eosinophilia and IL-4 production in the airways. BALB/c mice were sensitized, exposed to OVA and treated with different TLR agonists as indicated in materials and methods (Fig. 5c). (a) Lung lavage was collected 24 hours after the last exposure and the number of inflammatory cells as well as (b) the levels of IL-4 and IL-5 in the bronchoalveolar lavage were determined. Data are presented as mean ± SEM of 8 mice/group. ** = p < 0.01, *** = p < 0.001, in comparison to the OVA group. [Duechs et al., Pulm Pharmacol Ther. 2011 Apr;24(2):203-14]*

2. Application of TLR agonists LPS and CpG during allergen sensitization prevents the development of allergic airway responses and induces an allergen-specific T_H1 response

In the previous experiments it was shown that in established allergy TLR agonists are able to protect against the following allergen-mediated acute inflammation in the lung. In the next part of this thesis experiments were conducted to evaluate if TLR activation during allergen sensitization can prevent the development of allergic response to subsequent allergen challenges. The basis for these experiments are epidemiologic studies which compared children growing up in either a microbe-rich or a more aseptic surrounding. These studies reported a correlation of environmental exposure to microbes to the probability of developing asthma. These findings led to the suggestion that early-life infections, resulting in frequent activation of TLRs, can protect against the development of allergic diseases like asthma. The aim of the following study was to investigate if TLR agonist/allergen combination can suppresses the development of allergic airway responses. Furthermore, which agonists is most effective and to clarify if the suppression is accompanied by the induction of an allergen-specific T_H1 response. Therefore, TLR agonists and allergen were administered together intraperitoneally during the sensitization phase in the 28 day OVA model of acute allergic asthma. Doses of 0.0025, 0.025, 0.25 and 2.5 mg of TLR agonist per kg body weight were given together with 1 mg/kg OVA and the adjuvant $Al(OH)_3$. Mice received the co-administration on day 0, 14 and 21 (materials and methods, Fig. 5a). After the sensitization phase mice were challenged on day 26 and 27 and sacrificed 24 hours after the final challenge. To measure TLR-induced effects on the development of allergic responses, cellular influx, levels of cytokines and immunoglobulins were assessed and compared to the negative and positive control groups. Effects were further evaluated by histology and active anaphylactic response measurement.

Analysis of cellular influx showed that none of the TLR agonists induced an increase in macrophages and that, with the exception of R848, all TLR agonists induced an increase of neutrophils, which was highest in CpG- and LPS-treated animals. Allergen-induced lung influx of eosinophils was significantly reduced by CpG, LPS, and the highest concentration of LTA. The decrease of eosinophils induced by CpG and LPS was dose-dependent (Fig. 15). Histological analysis of the peribronchial and perivascular tissue surrounding the main bronchus supported the previous findings in the BAL that LPS and CpG were most effective in reducing the OVA induced cellular influx.
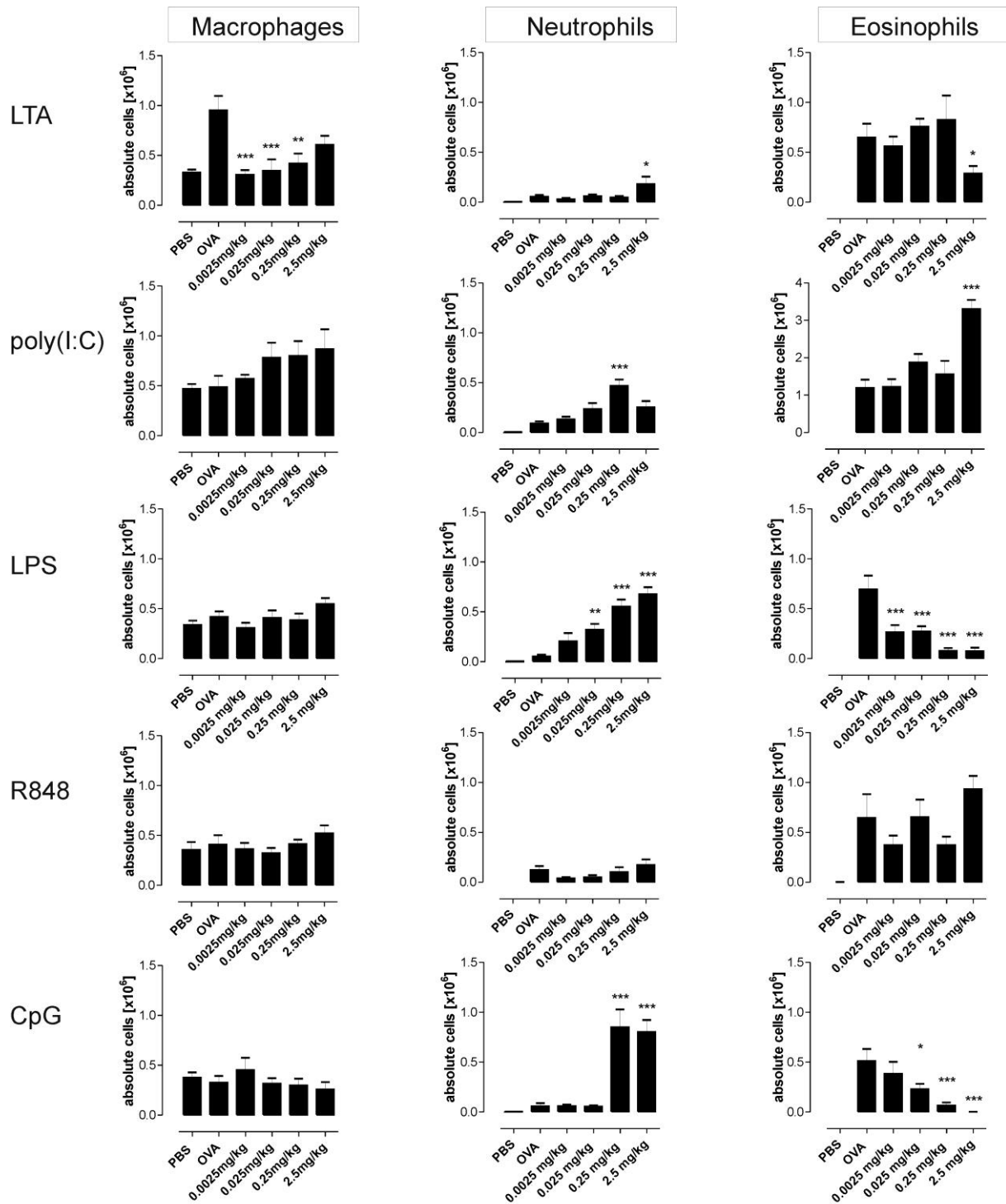
Fig. 15. Co-administration of allergen with either LTA, LPS or CpG during the sensitization phase inhibits development of allergen-induced lung eosinophilia. *(a) Mice received an intraperitoneal co-administration of OVA and TLR agonist on day 0, 14 and 21. On day 26 and 27 mice were exposed to nebulized OVA. (b) Total numbers of macrophages, neutrophils and eosinophils in BAL were measured 24 hours after the final OVA exposure. Cell counts are presented as mean ± SEM of 8 mice/group. * = p<0.05, ** = p<0.01, *** = p<0.001, in comparison to the OVA group. Experiments were repeated once showing similar results.*
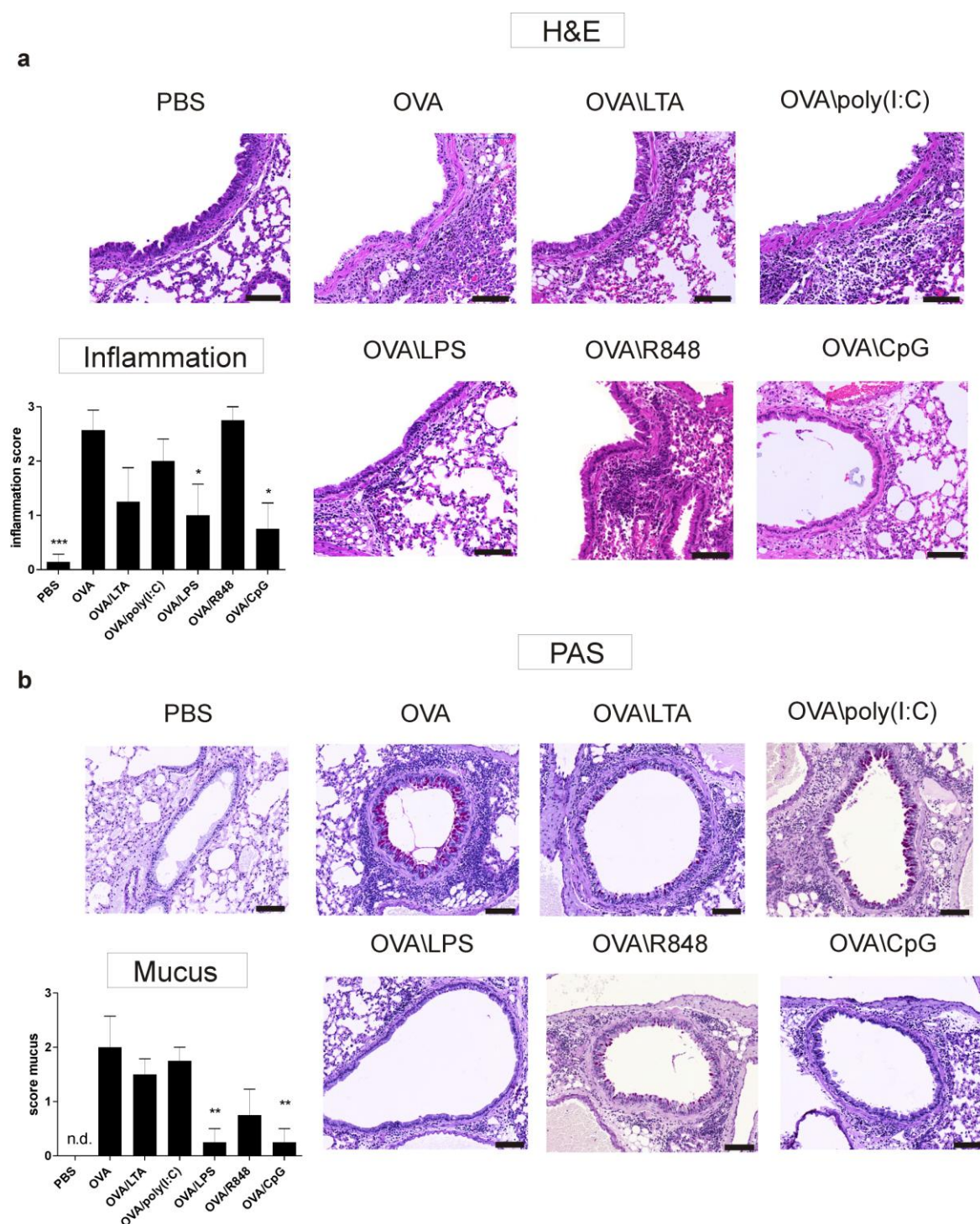
Fig. 16. Co-administration of allergen with LTA, LPS and CpG during the sensitization phase inhibits allergen-induced cell influx and mucus production. *Histological analysis of the lungs from OVA and TLR agonist treated mice. Inflammation and mucus were scored by semiquantitative double-blinded analysis of lung sections. Lung tissues were obtained from naïve mice treated with PBS, mice sensitized and exposed to OVA and mice treated with OVA plus TLR agonists. (a) Tissues were stained with haematoxylin and eosin (H&E) or by (b) periodic acid Schiff staining, and examined by light microscopy. Intensity of inflammation and mucus was scored by two independent observers (0 = no inflammation or mucus, 1 = slight inflammation or mucus, 2 = moderate inflammation or mucus, 3 = strong inflammation or mucus). Scale bar = 100µm. Inflammation and mucus scores are presented as mean ± SEM of 8 mice/group. * = p<0.05, ** = p<0.01, in comparison to the OVA group.*

Histological analysis showed that LTA induced slight suppressive effects on inflammatory cell influx. In contrast, no reduction of cell influx was detected in poly(I:C) and R848 treated animals (Figure 16a). Staining for mucus and goblet cell metaplasia showed that co-administration of poly(I:C) or R848 with allergen exhibited only marginal effects on mucus production and hyperplasia. In contrast, LPS and CpG completely prevented mucus production and reduced goblet cell hyperplasia (Figure 16b).

To further assess allergic inflammation, cytokine levels in BAL, immunoglobulin levels in serum, and subcutaneous anaphylactic reaction to allergen were measured. Cytokine measurements in the BAL showed that treatment with TLR agonists, with the exception of R848, significantly inhibited allergen-induced increase in IL-5 levels. Furthermore, CpG, poly(I:C), and LPS significantly reduced IL-4 levels, with CpG and LPS showing the strongest concentration-dependent inhibition (Fig. 17).
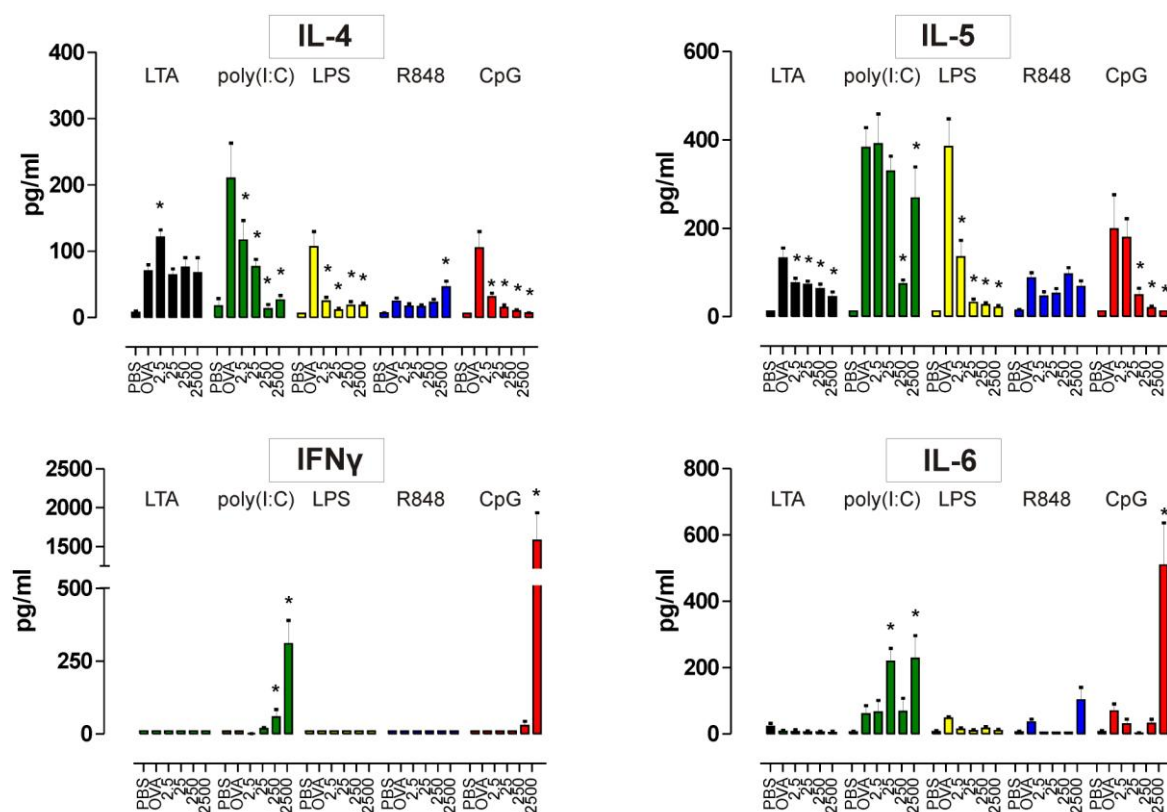

*Fig. 17. Administration of TLR agonists during the sensitization phase prevents allergen-induced increase of T_H2 cytokines. Levels of T_H1 and T_H2 cytokines were measured in BAL. Data are presented as mean ± SEM of 8 mice/group. * = p<0.05, in comparison to the OVA group.*

Levels of allergen-specific IgE and IgG2a in the serum of TLR agonist-treated mice were compared to levels in control mice. Analysis of OVA-specific IgE levels showed that all administered TLR agonists significantly reduced immunoglobulin levels (Fig. 18a). The only exception was seen in poly(I:C) treated mice, in which the decrease was not statistically significant. The strongest reduction was seen in animals treated with CpG followed by R848-treated animals. Furthermore, all TLR agonists significantly increased levels of OVA-specific IgG2a in the serum, albeit to different extents (Fig. 18b). In comparison to the control group, levels of OVA-specific IgG2a increased three to four fold in LTA- and LPS-treated mice. R848 treatment resulted in a 20 times higher level and poly(I:C) and CpG treatment induced a level over 100 times higher then levels measured in the control group.
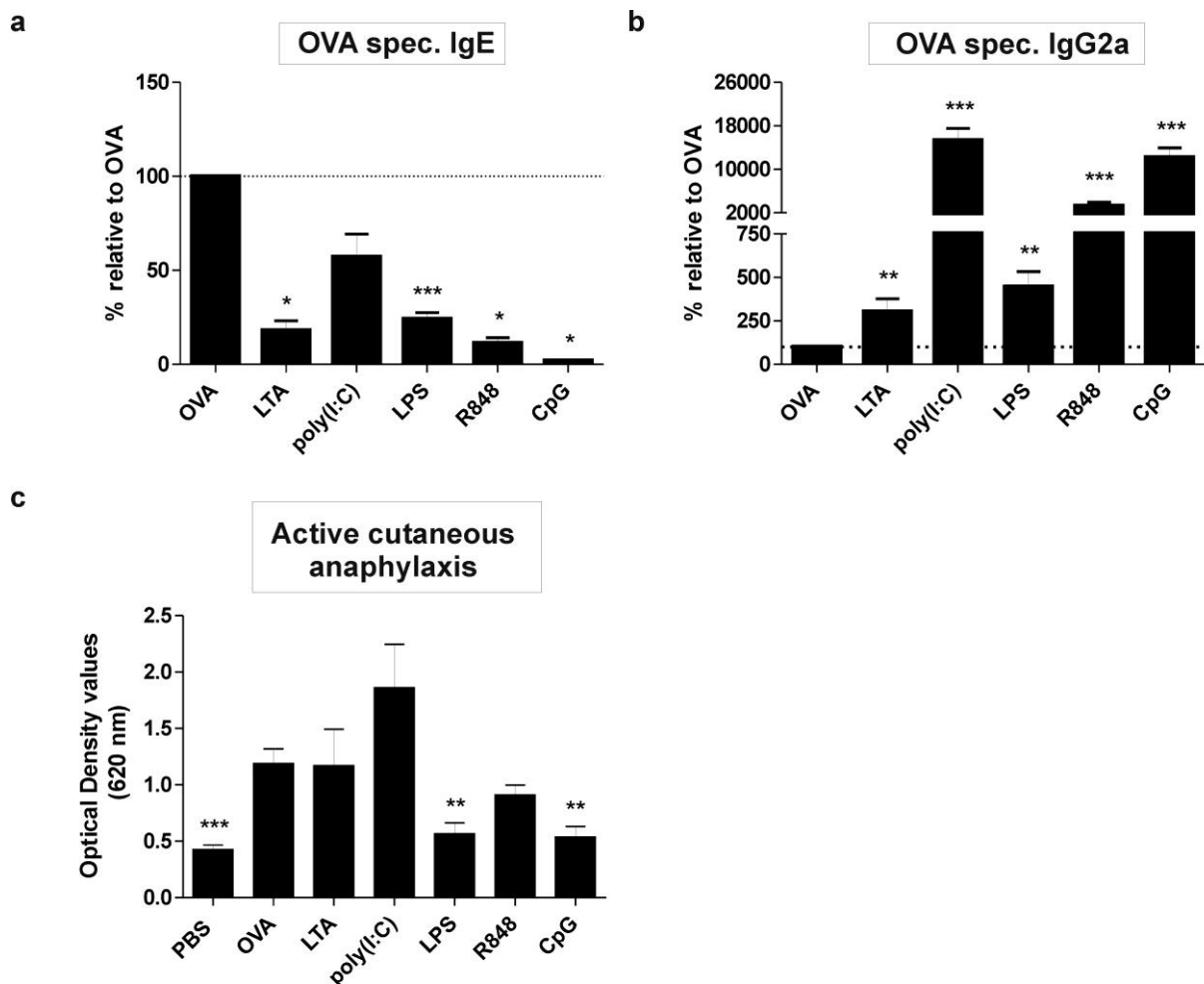

Fig. 18. TLR agonists induce an immunoglobulin switch from allergen-specific IgE to allergen-specific IgG2a. *(a) Levels of allergen-specific IgE and (b) IgG2a were measured in serum. (c) Allergen-dependent active cutaneous anaphylaxis was measured by allergen-induced vascular leakage. Vascular leackage was assessed by quantification of evans blue, given intravenously shortly before allergen application. After extraction, concentration of evans blue in tissue was assessed by measurement of absorption. Data are presented as mean ± SEM of 8 mice/group. * = p<0.05, ** = p<0.01, *** = p<0.001, in comparison to the OVA group.*

To test if the application of TLR agonists can prevent cutaneous anaphylaxis, the experiment above was repeated and active cutaneous anaphylaxis to OVA was tested in the TLR agonist-treated animals. Figure 18c shows that co-administration of allergen with either LTA, poly(I:C) or R848 did not reduce induction of anaphylactic reaction. In contrast, LPS and CpG both completely prevented allergic anaphylactic reaction to sensitivity of non-sensitized control mice.

3. Establishment of a novel mouse model for severe chronic asthma

In the previous experiments it was shown that TLR agonists administered either preventatively or therapeutically can prevent the development of allergic T_H2 responses, as well as reduce ongoing allergic T_H2 responses in the lung. In the present thesis it was further studied whether TLR activation can affect the severe chronic form of asthma. However, a problem of current mouse models is that they often fail to display important characteristics of the severe form of human asthma, thus limiting the potential to assess the effects of TLR agonist administration. Hence, a new mouse model with a distinct pathology, more comparable to the situation in human severe chronic asthma, was developed.

A problem with current mouse models of chronic asthma is the decrease of inflammation after repeated allergen challenge. In addition, models often show only a marginal degree of remodeling in the airways. Thus, experiments were designed to compare different allergen models for their ability to elicit airway inflammation, airway hyperreactivity, and most importantly, airway remodeling in a chronic setting. OVA allergen is by far the most frequently used allergen and induces a robust allergic pulmonary inflammation in mice. The first chronic OVA model was established and published over 12 years ago by Temelkovski et al. [220;221]. However, OVA is not a relevant factor in human asthma and in particular for OVA models, long-term challenges have been reported to induce tolerance and down-regulation of inflammation and decrease in airway hyperreactivity [222;223]. Therefore, alternative allergens with a greater clinical relevance such as house dust mite (in figures referred to as HDM) and cockroach extracts (in figures referred to as CRA) have been used in murine asthma models and were reported to induce asthma-relevant pathology [224;225].

In order to establish a model with a clinically relevant pathology, the allergens OVA, cockroach and house dust mite were compared in a chronic asthma model. In these experiments mice received three intraperitoneal administrations of the respective allergen during the sensitization phase. Mice were then challenged intratracheally twice weekly over a period of 7 weeks with the respective allergen (methods, Fig. 4d). In an additional group mice received a combination of the three allergens OVA, cockroach, and house dust mite during the sensitization phase, thus inducing allergy against the three allergen extracts. This triple allergy model was developed as it was reported that patients with a pre-established allergy are more susceptible to developing additional allergies, plus additional allergies have been shown

to exacerbate the clinical situation and multiple allergies dramatically lower the chance for successful treatment [226]. In this novel model the triple allergen sensitization was followed by alternating challenges of one single allergen a week. The alternation of allergens was carried out in order to minimize induction of potential allergen-induced tolerance, a process leading to a decrease of disease symptoms, which has been reported for murine models using chronic allergen exposure [227-229]. For comparison of different models, inflammation in the lung, airway hyperreactivity and airway remodeling were assessed. Inflammation was assessed by cellular influx and measurement of cytokine levels, whereas airway hyperreactivity was measured invasively assessing resistance and compliance. The compliance describes the elasticity of the lung at any given time during actual movement of air and is calculated by the change in volume and the respective change in pleural pressure. Asthma, and especially lung fibrosis, is associated with a decrease in pulmonary compliance. Airway remodeling was assessed by quantification of mucus release, airway smooth muscle thickening, and collagen deposition as indication for fibrosis.

3.1 Triple allergen combination treatment induces the strongest eosinophilia, highest levels of IgE and T_H2-cytokines and an increase in T_H1-cytokines in the lung

To assess inflammatory responses in the lung, BAL samples were taken 24 hours after the final allergen challenge and cell influx into the lung was quantified by assessing total numbers of cells, macrophages, eosinophils, and neutrophils. Levels of T_H2 and T_H1 cytokines were measured in lung homogenate, levels of total IgE and total IgG1 were measured in serum.

In comparison to sham-treated animals, sensitization and chronic intratracheal application of OVA, cockroach, and house dust mite extracts induced significant influx of eosinophils, neutrophils and macrophages (Fig 19 b, c, d). Mice treated with house dust mite extracts induced the lowest influx of respective cells (Fig 19a). The highest counts of total cells, eosinophils, and macrophages were detected in mice treated with the triple allergen combination (in figures referred to as "Combi" group).
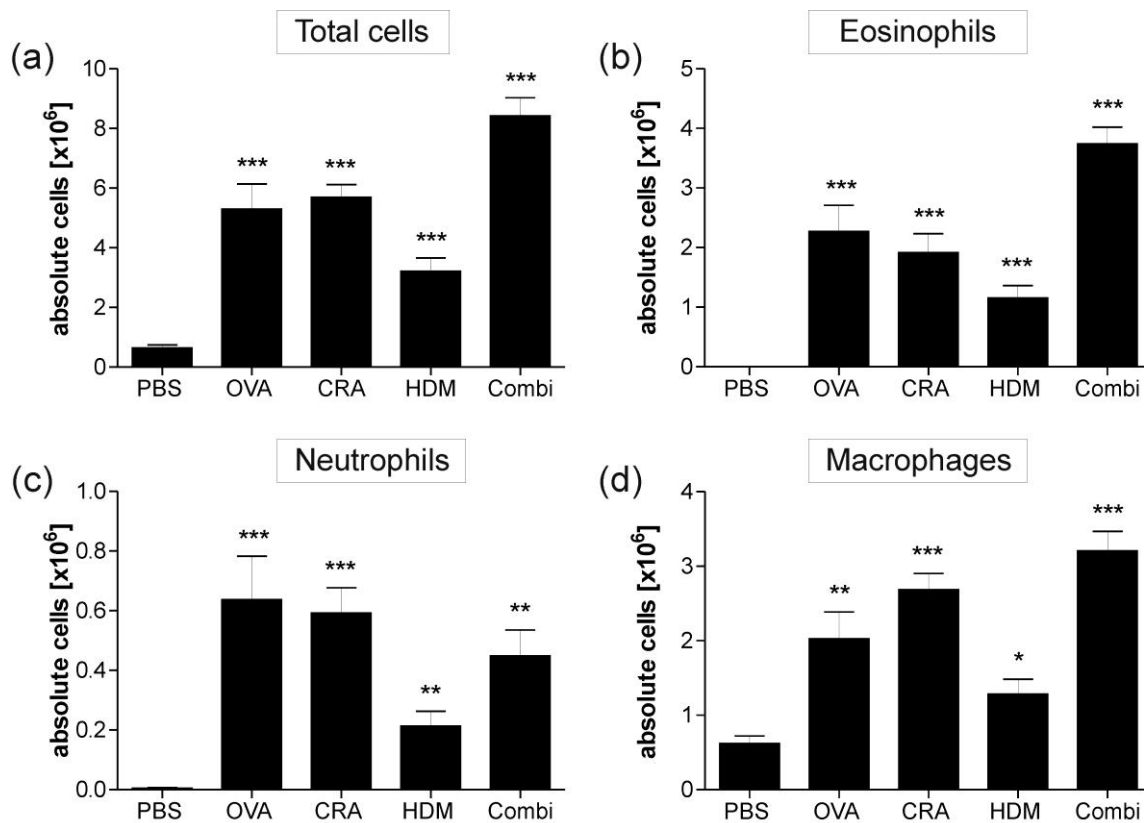
Fig. 19. The triple allergen combination model induces the highest influx of macrophages and eosinophils. *Absolute numbers of (a) total cells, (b) eosinophils, (c) neutrophils and (d) macrophages measured in brochoalveolar lavage (BAL) from mice treated with phoshate buffered saline (PBS), OVA (OVA), cockroach (CRA), house dust mite (HDM) or the triple allergen combination model (Combi). The unpaired one-way ANOVA (* = p < 0.05; ** = p < 0.01, *** = p < 0.001) was used to compare differences between allergen treated mice and PBS control mice. Data are presented as mean ± SEM, n = 8-14 mice/group.*

The levels of the T_H2 cytokines IL-4, IL5, IL-9 and IL-13 were measured in the homogenate of lung tissue. Results show that the allergen models OVA, house dust mite and cockroach all induced a comparable and significant increase in IL-4 and IL-5 levels (Fig. 20a, b). However, the triple allergen combination model resulted in levels of IL-5 and IL-4 that were three fold higher then these seen in the single allergen models. Compared to sham-treated animals, no significant increase in IL-9 levels could be measured and only the triple allergen combination model treatment lead to significant increase in IL-13 levels (Fig. 20c). In addition, significant elevated levels of the pro-inflammatory cytokines IFN-γ, IL-12, and IL-2 were measured in the homogenate of animals receiving the triple allergen combination treatment (Fig. 20d, e, f). The levels of TGF-ß1 and IL-17 were measured, as it was suggested that these cytokines play a role in airway remodeling [230-234]. Only the house dust mite and the triple allergen combination model showed significantly elevated TGF-ß1 levels (Fig 20g), when compared

to sham-treated control mice. In contrast, a significant increase in IL-17 was measured for all models with the OVA model exhibiting the highest levels of IL-17 (Fig. 20h). Each of the allergen models induced a strong increase in total IgE level. The highest level of IgE was measured in the triple allergy combination model, which exhibited a level three fold higher than that of the OVA model (Fig. 21a). The highest level of IgG1 was measured in the OVA model, followed by the triple allergen combination model (Fig. 21b).
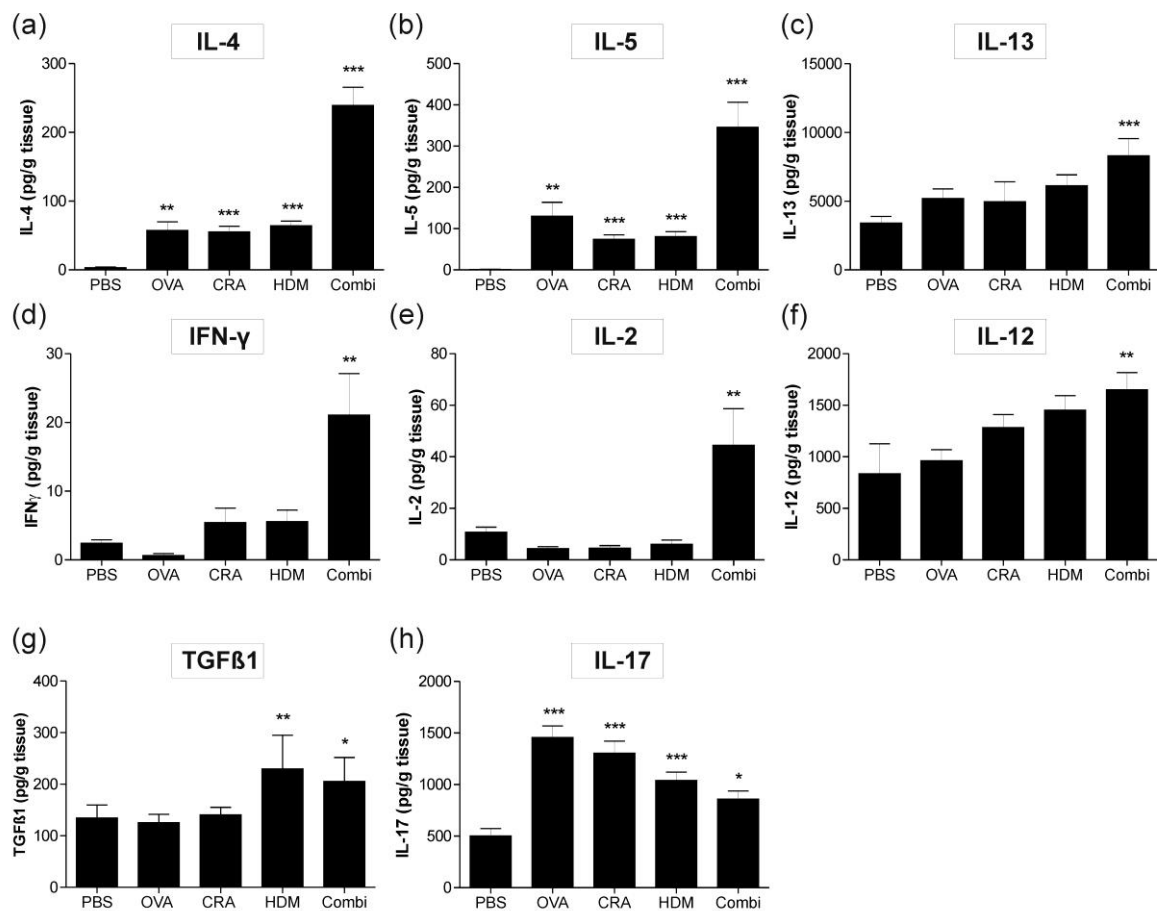

Fig. 20. The triple allergen combination model induces the highest increase in T_H2 cytokines in lung tissue. *Cytokines measured in lung homogenate: (a) IL-4, (b)IL-5, (c) IL-13, (d) IFNγ, (e) IL-2, (f) IL-12 (g) TGF-β1 and (h) IL-17. Data are presented as mean ± SEM, n = 8-14 mice/group and * = p < 0.05; ** = p < 0.01, *** = p < 0.001 in comparison with PBS control mice.*
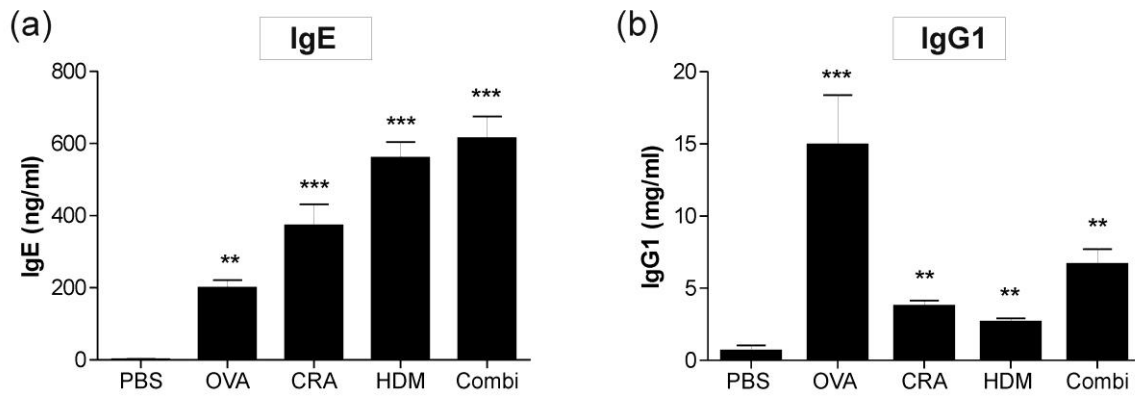
*Fig. 21. Chronic allergen treatment leads to increased IgE and IgG1 levels. Levels were measured in serum of blood collected 23 hours after the final allergen challenge. Data are presented as mean ± SEM, n = 8-14 mice/group and * = p < 0.05; ** = p < 0.01, *** = p < 0.001, in comparison with PBS control mice.*

3.2 Chronic allergen treatment leads to increased airway hyperreactivity

For assessing airway hyperreactivity, PenH was measured 23 hours after the final allergen challenge in conscious unrestrained mice by means of whole body plethysmography. Mice were exposed to increasing concentrations of nebulized methacholine whilst changes in pressure were assessed for calculation of PenH levels. Airway hyperreactivity was further assessed by direct invasive measurement of resistance and compliance in mice exposed to increasing concentrations of nebulized methacholine, as described earlier.

After methacholine challenge, all groups of mice developed a significant increase in PenH levels compared to untreated mice (Fig. 22a). For further comparison of groups, the area under the curve (AUC) from the PenH graphs was assessed for each animal. The analysis of AUC showed that all allergen models induced a comparable increase in PenH (Fig. 22b). For invasive measurement of resistance and compliance mice were anesthetized, intubated and artificial respiration was applied. Mice were then challenged intratracheally with nebulized methacholine with the following concentrations: 0.635, 2.5, 5.0 and 12.5 mg/ml. The first significant increase in resistance was measured at a methacholine dose of 2.5 mg/ml in the animals receiving the triple allergen combination treatment. At the concentration of 12.5 mg/ml, the triple allergen combination, cockroach, house dust mite, but not OVA treatment showed significantly increased airway resistance (Fig. 22c).
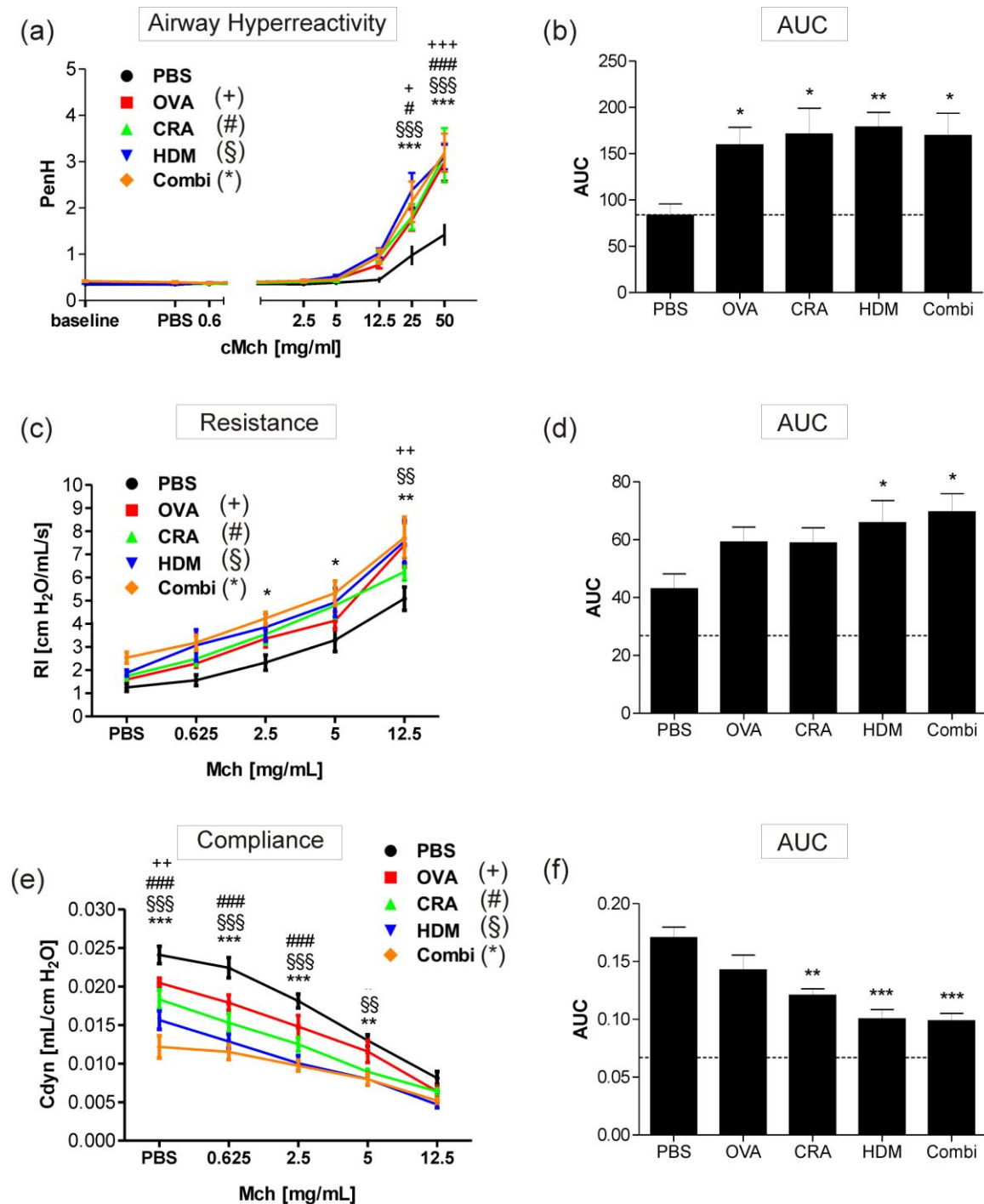
Fig. 22. The triple allergen combination model induces the highest airway hyperrereactivity in mice. *Airway hyperreactivity was analyzed 23 hours after the final allergen challenge on day 70. Assessed were either PenH(a) in unrestricted spontaneously breathing mice, or resistance (c) and compliance (e) in narcotized mice receiving artificial ventilation. Airway hyperreactivity was also presented by analysis of area under the curve (AUC), calculated for (b) PenH, (d) resistance and (f) compliance. Data are presented as mean ± SEM, n = 8 mice/group, analyzed with means of Two way ANOVA in comparison with PBS control mice. Indexes for significances: PBS vs OVA= +, PBS vs CRA = #, PBS vs HDM = §, PBS vs Combi = *.*,+ = p < 0.05; **,++, §§, ## = p < 0.01, ***,+++, §§§, ### = p < 0.001.*

Measurement of compliance showed that all chronic models significantly decreased baseline compliance. Methacholine challenge of mice treated with OVA showed no statistically significant differences to untreated mice. Stronger effects were seen in the cockroach, house dust mite, and the triple allergen combination model, which significantly reduced compliance up to a methacholine concentration of 5 mg/ml. House dust mite and triple allergen combination induced the strongest compliance in this chronic setting.

In summary, the airway hyperresponsiveness measurements indicated that chronic treatment with OVA induced the weakest and treatment with the triple allergen combination induced the severest hyperresponsiveness.

3.3 Chronic allergen treatment induces structural changes in lungs with the triple allergen combination model showing strongest effects

As mentioned before, the main differences between the mild and the severe chronic form of asthma are the more pronounced and permanent structural changes in the airways. These changes involve extensive cellular influx of immune cells into alveolar tissue, changes in the epithelial layer such as goblet cell hyperplasia, mucus production, as well as airway smooth muscle thickening and fibrosis, which is defined by increased extracellular matrix deposition. All these changes are collectively referred to as airway remodeling and as mentioned before, unresolved airway inflammation is thought to be the driving process causing this airway remodeling. As the analysis of cellular influx and cytokines showed strong inflammation in the models, the question was do the different allergens also induce airway remodeling and which of the allergens induces the strongest pathology.
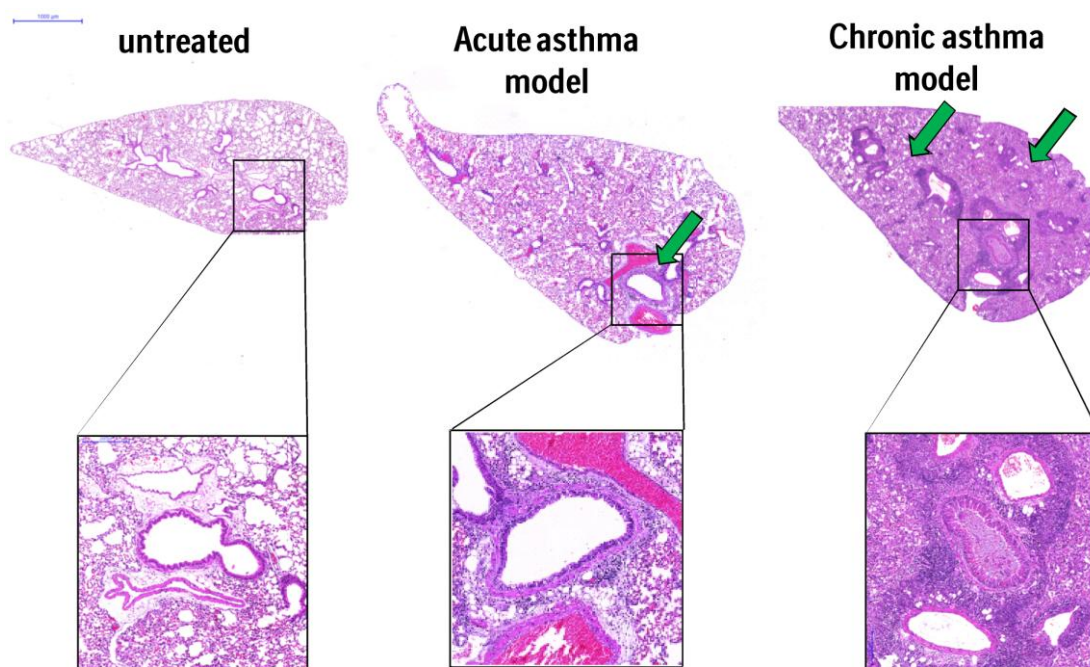

Fig. 23. Chronic allergen challenge induces a severe pathology affecting not only peribronchial but also alveolar tissue. Depicted are the lungs of a mouse receiving sham-PBS treatment (untreated), a mouse that received OVA treatment in the 28 day asthma model (acute asthma model, see Fig. 5a), and a mouse that received the chronic treatment of the triple allergen combination model (see Fig 5e). Lungs were stained with the hematoxylin and eosin as described in materials and methods. Scale bar = 1mm. Section are representative pictures of experiment with n = 8-14 mice/group.

Lungs from mice of acute and chronic models differed strongly in the extent and localization of the influx of inflammatory cells. In acute models, inflammatory cell influx is largely restricted to the peribrochial and perivascular area at the main bronchus, whereas in the chronic model the immune cells infiltrate the alveolar tissue as well (Fig. 23). Histological

analysis of HE stained lung sections showed a strong influx of immune cells, confirming the influx measured in BAL (Fig. 24). Sections of HE-stained main bronchus area showed that the triple allergen combination treated animals exhibited the highest influx of immune cells in the peribronchial and perivascular tissue (Fig. 24a). In all models chronic allergen exposure resulted in goblet cell hyperplasia and mucus hyper-production (Fig. 24b). Processes such as cellular influx, airway remodeling, smooth muscle increase and mucus production affect the structure of the lung, and as direct consequence must lead to an increase of the lung weight. Despite being an unspecific parameter, the lung weight can be used as an indicator of strong pathological changes in the lungs of treated mice. Therefore, wet weight of the left lung was assessed after mice were sacrificed and the lung was extracted. Interestingly, chronic allergen exposure in all models caused a significant increase of around 30% in the weight of lungs from treated mice. The highest average lung weight was measured in the triple allergen combination model group, in which treated lungs were 1.7 times heavier then lungs from untreated animals (Fig. 31a).

The first remodeling parameters that were directly assessed were changes in the epithelial layer of the main bronchus. Analysis of lung sections showed that treatment with allergens resulted in goblet cell hyperplasia and hyper-secretion of mucus. Mucus was assessed by PAS staining of lung sections and automated analysis of stained intracellular mucus in the epithelial layer of the main bronchus. The area of mucus was then calculated in relation to the area of epithelial layer. Histological analyses (Fig. 24b) and quantification of mucus area (Fig 25b) showed that OVA treated animals exhibited the weakest hyperplasia and induced the lowest amount of mucus. The highest amounts of intracellular mucus in goblet cells were measured in animals treated with extracts of house dust mite and cockroach (Fig. 25b). In severe chronic asthma, acute respiratory failure is a severe exacerbation leading to life threatening situations. In the majority of the cases this is due to extensive mucus release into the bronchial airway lumen, plugging bronchial tubes and inhibiting the exchange of oxygen and carbon dioxide. Analysis of bronchial tubes showed that OVA treatment did not lead to mucus plugging; in contrast, approximately 35% of the lungs of cockroach extract treated mice exhibited plugged bronchial tubes. Comparable to cockroach extract treatment, approximately 36% of house dust mites extract treated animals showed mucus plugging and triple allergen combination treatment resulted in 61% of lungs with plugged bronchial tubes (Fig. 25c).
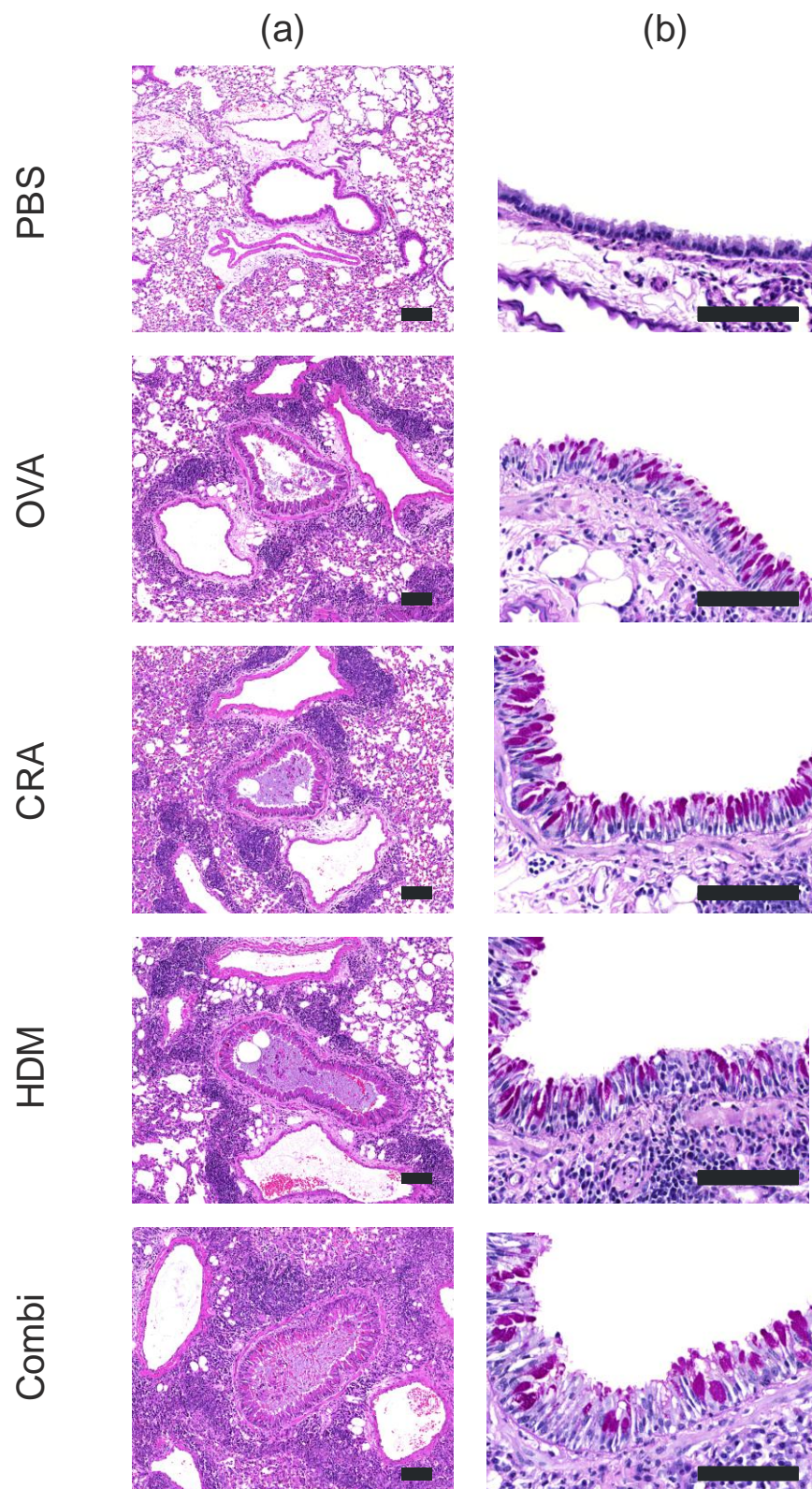
Fig. 24. Histological staining visualizing inflammatory cell influx and goblet cell hyperplasia shows that all models induce inflammation and mucus production, albeit to different degrees. *Shown are representative photomicrographs of (a) haematoxilin/eosin-(H&E) stained main bronchus area and (b) Periodic acid-Schiff staining of the epithelial cell layer in the main bronchus. Scale bar = 100 µm. Pictures show representantive sections of the lungs from 8-14 mice/group.*

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Fig. 25. Chronic allergen treatment increases lung weight and mucus production. *(a) Wet weight of left lung was measured 24 hours after the final challenge. (b) Mucus production was quantified from PAS-stained sections of the main bronchus. (c) Mucus plugging was assessed by quantification of plugged bronchial tubes per lung taking one PAS-stained whole lung sections of each mouse. Data are presented as mean $\pm$ SEM, n = 8-14 mice/group for lung weight and mucus quantification, * = p < 0.05; ** = p < 0.01, *** = p < 0.001, in comparison with PBS control mice, n.d. = not detectable.*
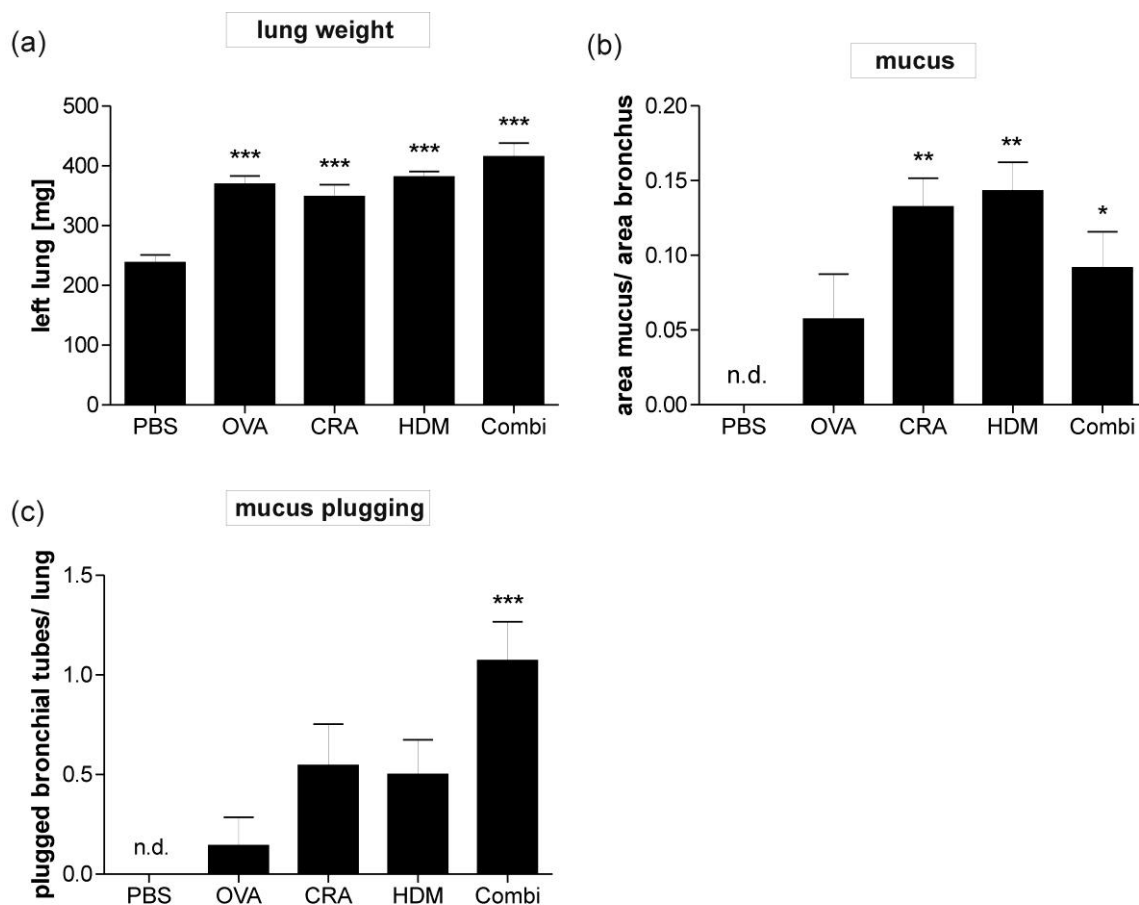
Thickening of airway smooth muscle was assessed by staining lung sections with α-actin smooth muscle antibody. For comparison of smooth muscle layers, only the smooth muscles surrounding the main bronchus at a defined position in the lung were analyzed. Histology showed that compared to sham-treated mice, all models induced thickening of smooth muscle layers, albeit to different degrees (Fig. 26a). Histological sections were further assessed by automated measurement of the smooth muscle area and mean smooth muscle thickness (Fig. 27a, b).

Quantification of total smooth muscle area showed a significant increase in the groups of cockroach, house dust mite and triple allergen combination, however the measured increase in the OVA treated animals was not statistically significant (Fig. 27a). The strongest increase

was measured in house dust mite and the triple allergen combination model. Triple allergen combination treatment also led to the strongest thickness of the airway smooth muscle, which was highly significant compared to untreated mice (Fig. 27b). Here, cockroach and house dust mite extract induced a significant increase in thickness as well, whereas in the OVA model again no statistically significant increase was measured. A further important symptom of airway remodeling is fibrosis. In fibrosis the deposited extracellular matrix consists mainly of different types of collagen. In this study fibrosis was visualized using pico-sirius red staining of collagen in lung sections. In the stained sections, collagen was quantified via automated analysis of compactness, which was used as a factor for quantification of the accumulation and the constriction of subepithelial collagen. Measurement of compactness was applied only to the peribrochial region of lungs (see methods, 6b). Furthermore, amount of collagen was assessed in homogenized lung tissue by measurement of total collagen (by means of biochemical quantification) and measurement of collagen I and IV (by means of ELISA).

Histological staining showed lowest fibrotic chances in mice treated with OVA (Fig. 26b). No significant differences of house dust mite, cockroach or triple allergen combination model treated animals could be detected from optical comparison of stained lung. Biochemical and ELISA-based quantifications of total collagen and collagen I in lung homogenate showed that a significant increase was induced by all allergen models (Fig 27 c, d). Collagen IV was not significantly increased in OVA-treated mice, whereas treatment with house dust mite and cockroach extract showed significant elevated (Fig 27e). The highest rise was seen in the triple allergen combination model. In this model the measured levels of collagen IV were around three times higher than levels measured in the untreated control group. Automated analysis of histological stained peribrochial area showed that all chronic models induced increased collagen deposition. The model showing the highest compactness of collagen fibers was again the triple allergen combination model (Fig. 27f).

Taken together, all data consistently show that the triple allergen combination model elicits high mucus production, the strongest smooth muscle thickening, and the most pronounced fibrosis, thus inducing the strongest airway remodeling. Therefore, this model exhibits the most severe pathology and is best suited for the preclinical testing of TLR agonists.
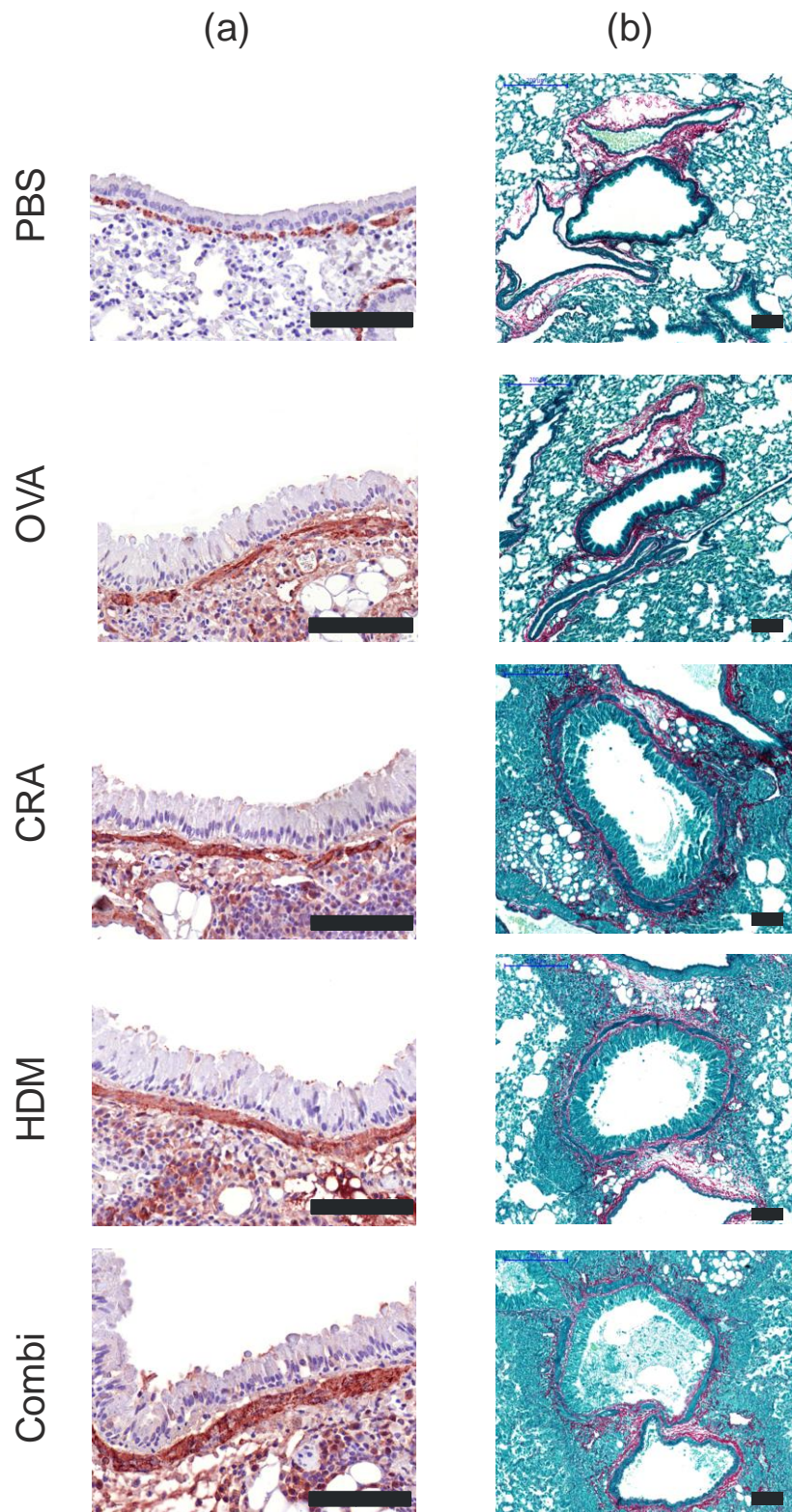
Fig. 26. Chronic treatment induces airway smooth mucle thickening and airway fibrosis. *(a) Shown are sections of the main bronchus of lungs stained for smooth muscle actin, or (b) main bronchus area stained with sirius red for visualizing subepithelial collagen deposition. Scale bar = 100 μm. Pictures show representantive sections of the lungs from 8-14 mice/group.*
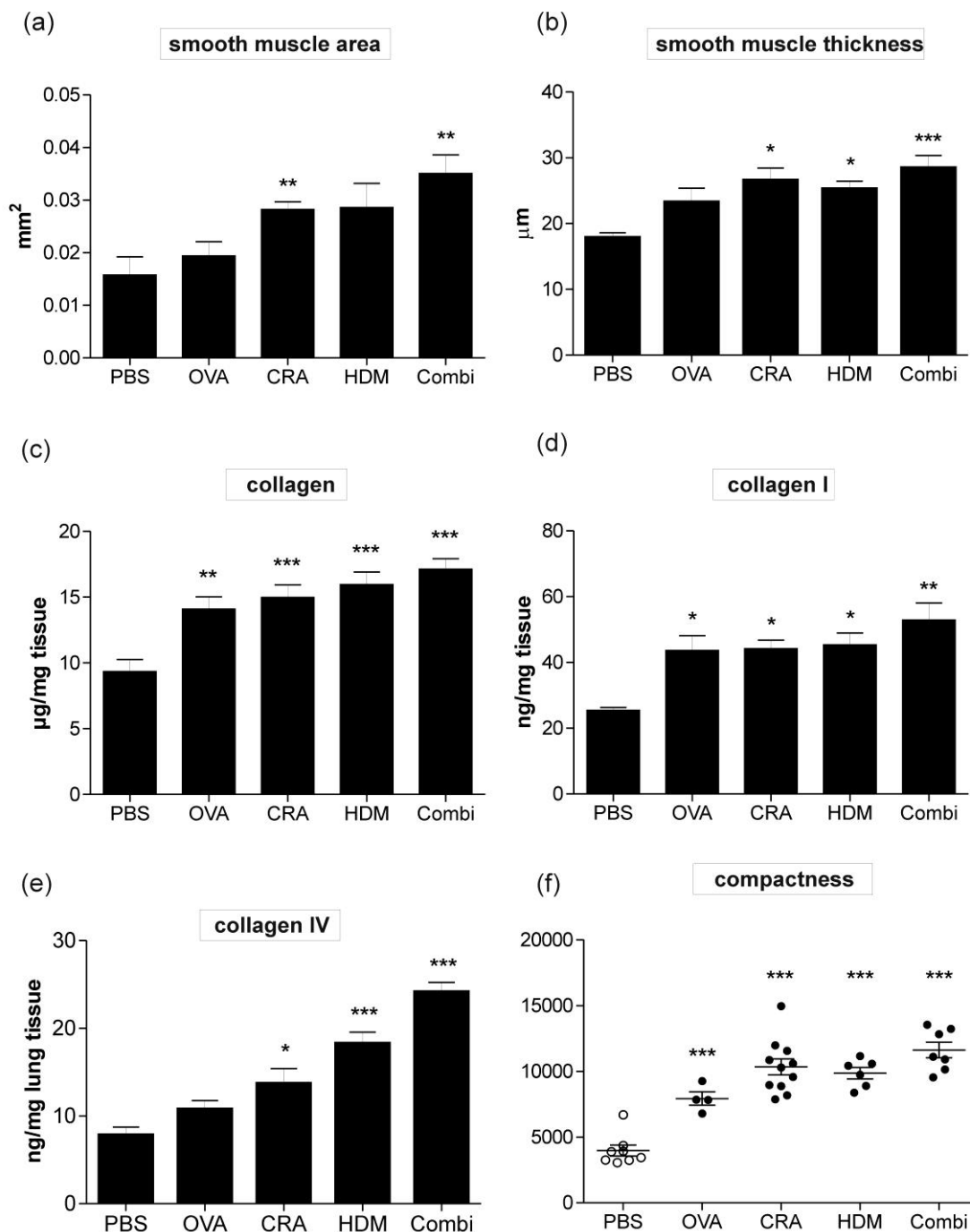
Fig. 27. The triple allergen combination model induces the strongest smooth muscle thickening and the strongest fibrosis. *(a) Smooth muscle thickening was analyzed by quantification of area and mean thickness of smooth muscle actin layer (Fig. 7c). Using lung homogenate, total collagen (c) was assessed by biochemical measurement. Collagen I (d) and collagen IV (e) were assessed by ELISA. (f) Compactness of collagen stained area was assessed to quantify collagen in peribronchial area of picro sirius red stained lungs (Fig. 7b). Data are presented as mean ± SEM. Results were measured in 8 -14 mice/group with * = p < 0.05; *** = p < 0.001, in comparison with PBS control mice.*

4. Effects of TLR agonists and dexamethasone on T_H2 inflammation, hyperreactivity and airway remodeling in triple allergen combination model

The previous results showed that the triple allergen combination model induced strong pathological changes in the lung tissue, closely mimicking pathological aspects of human severe chronic asthma. Furthermore, it was shown that TLR agonists can efficiently prevent or dampen asthma like symptoms in models of acute allergic inflammation [158;202;216;235]. The following experiments were conducted to analyze how activation of different TLRs affects the pathology in a model of chronic inflammation representing the more severe form of the disease. Therefore the five TLR agonists were administered in the triple allergen combination model and asthma relevant parameters were assessed. In addition, effects of the TLR agonists were directly compared to oral treatment with a corticosteroid, mimicking a standard clinical treatment for severe chronic asthma.

4.1 In the model of severe asthma TLR agonists poly(I:C), R848 and CpG reduce chronic allergic airway inflammation

To analyze the effect of TLR activation in chronic severe asthma, agonists were administered in the triple allergen combination model. Animals were sensitized with the combination of allergens and then mice were challenged for a total period of seven weeks with allergens. Therapeutic applications of TLR agonists and steroid were started after the third week of challenge was completed (methods, Fig. 5d). In the present study, protective and therapeutic application of TLR agonists CpG and R848 showed the highest efficacy in reducing allergic responses. LTA, poly(I:C), and LPS on the other hand induced the strongest increase in lung neutrophilia, suggesting an unfavorable safety profile for chronic administration. Thus, the TLR agonists CpG and R848 were chosen for application in the triple allergen combination model. Corticosteroids are a very potent class of therapeutics and the most frequently anti-inflammatory drugs used for asthma therapy in clinics. Hence, a group treated with dexamethasone was included in the experiment to compare the efficacy of TLR agonists and steroid side by side. Dexamethasone and TLR agonists were then given starting in the fourth week of allergen challenge, one hour prior to each challenge. Mice received a dose of 1 mg/kg of TLR agonist intratracheally, whereas dexamethasone was administered orally in a dose of 1 mg/kg. At the end of the study, cellular influx, levels of cytokines, levels of immunoglobulins, airway hyperreactivity, and airway remodeling were assessed.

Analysis of cell influx showed that all TLR agonist significantly reduced eosinophils, whereas the steroid dexamethasone did not. R848 treatment reduced eosinophilia by 55%, but the strongest reduction was seen in CpG treated animals, showing a reduction of 91%. While the treatment with dexamethasone and R848 did not affect macrophages, CpG induced a significant increase (Fig. 28d). The two TLR agonists tested induced an increase of neutrophils, this was however not statistically significant for either agonist (Fig. 28c).
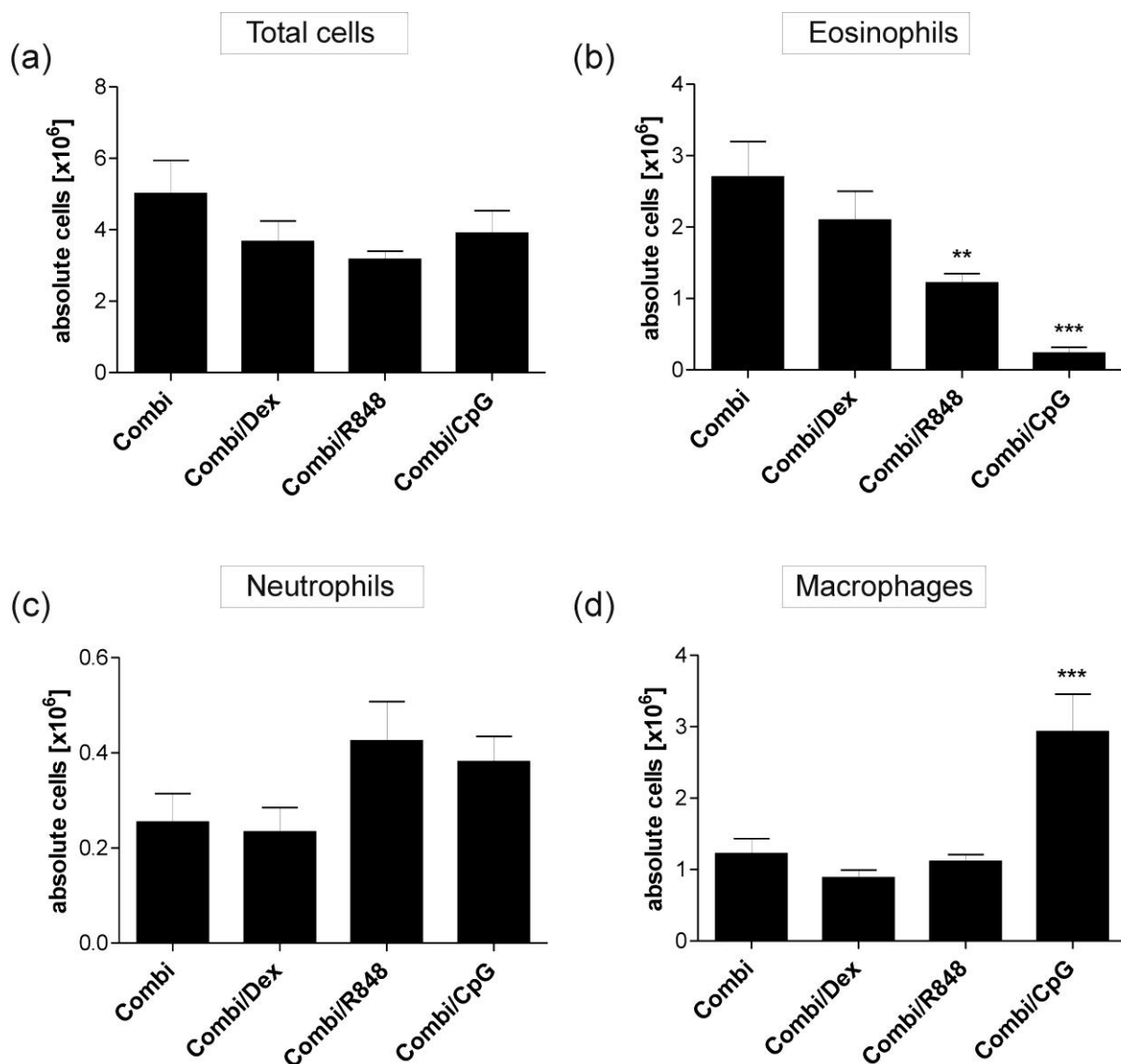
Fig. 28. TLR agonist administration significantly reduces eosinophilic lung influx in the triple allergen combination model, while treatment with the steroid dexamethasone does not. *Absolute numbers of (a) total cells, (b) eosinophils, (c) neutrophils and (d) macrophages were measured in BAL. The PBS group received only the triple allergen combination treatment, other groups were treated with the steroid dexamethasone (DEX), TLR7 agonist R848 or TLR9 agonist CpG. An unpaired one-way ANOVA (*p = < 0.05; ** = p < 0.01, *** = p < 0.001) was used to compare differences between allergen-treated mice and PBS control mice. Data are presented as mean ± SEM, n = 8-14 mice/group.*

Analysis of T$_H$2 cytokines in lung homogenate showed that CpG strongly reduced the levels of both, IL-4 and IL-5, whereas R848 and dexamethasone did not (Fig. 29 a, b). In line with the results seen in the acute model, administration of CpG again induced an increase in IL-10 levels (Fig. 29c). Also the levels of the pro-inflammatory cytokines IFN-γ and IL-12 were highest in the group receiving CpG treatment (Fig. 29 d, f). Administration of TLR agonists did not affect TGF-β1 or IL-17 levels (Fig. 29 g, h). Interestingly, CpG treatment also induced a significant increase in IL-1 β levels, which was not seen in the R848 treated groups (Fig. 29 i). Measurement of IgE showed that neither treatment with one of the TLR agonists nor dexamethasone reduced IgE levels in serum (Fig. 30a). Measurement of IgG2a showed that the only significant increase in IgG2a level was elicited by R848 treatment, this level was four times higher than that of the immunoglobulin in the untreated control group (Fig. 30b). For IgG1, it was again only the R848-treated group that exhibited a significant rise (Fig. 30c).
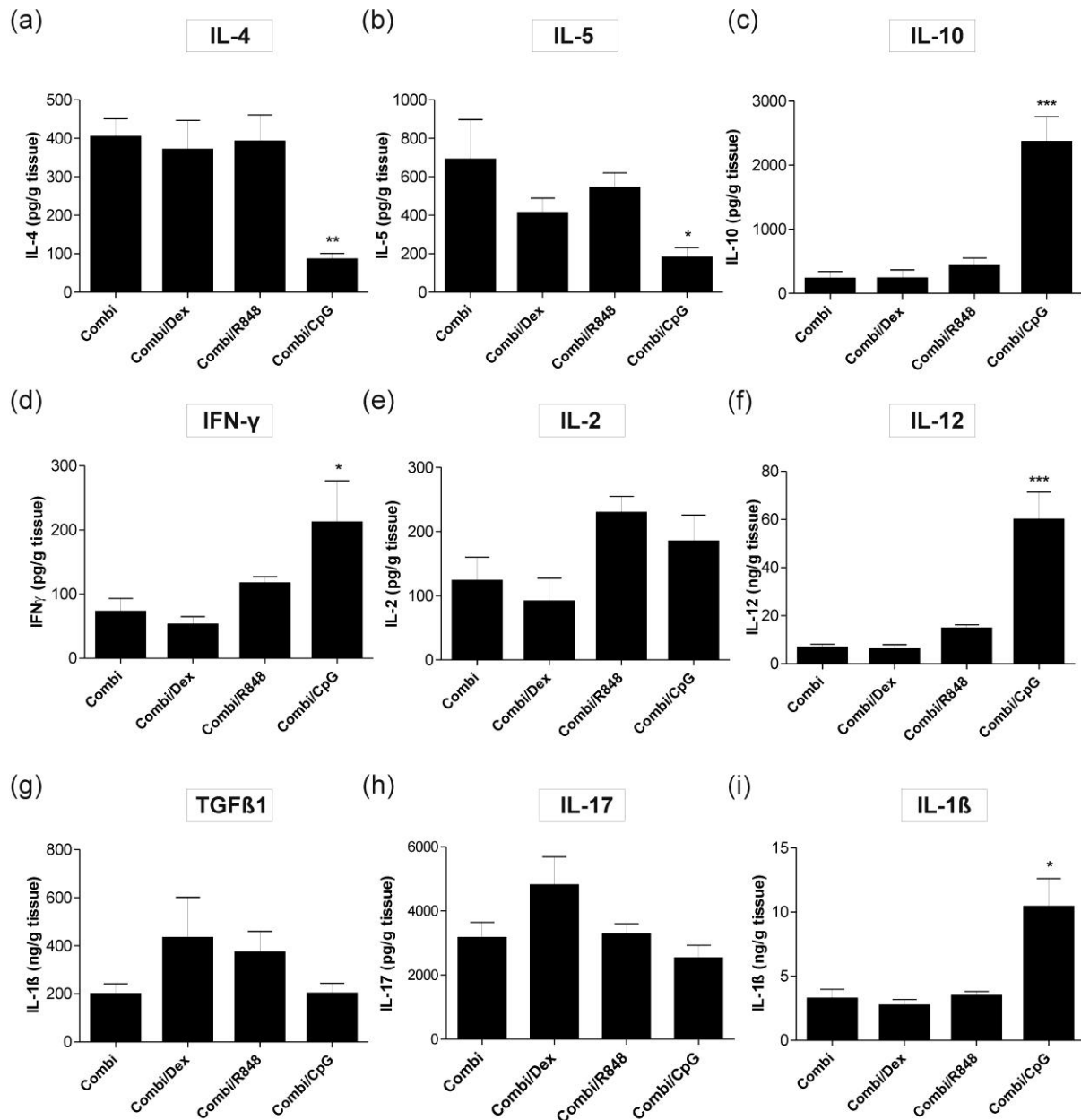
Fig. 29. In the triple allergen combination model, administration of TLR agonist CpG, but not R848 or dexamethasone, significantly decreased T_H2 while increasing T_H1 cytokines in the lung. *Cytokines measured in lung homogenate: (a) IL-4, (b) IL-5, (c) IL-10, (d) IFNγ, (e) IL-2, (f) IL-12, (g) IL-1ß, (h) IL-17, and (i) IL-1ß. Data are presented as mean ± SEM, n = 8-14 mice/group and * = p < 0.05; ** = p < 0.01, *** = p < 0.001 in comparison with mice receiving sham PBS treatment.*
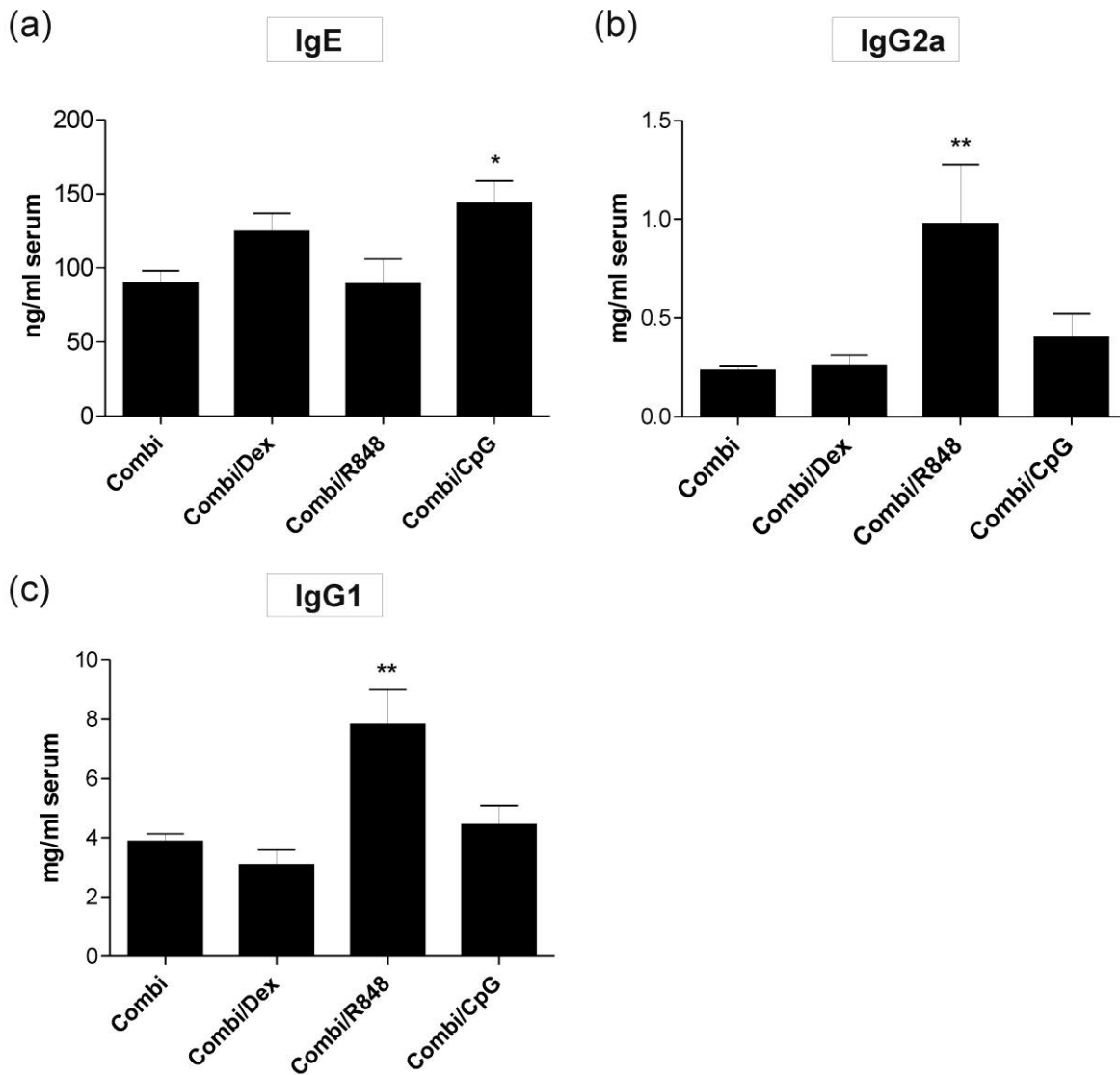
Fig. 30. TLR agonist treatment in the triple allergen combination model does not reduce amount of total IgE in serum. *Data are presented as mean ± SEM, n = 8-14 mice/group and * = p < 0.05; ** = p < 0.01, *** = p < 0.001 in comparison with mice receiving a sham PBS treatment (Combi).*

4.2 Administration of TLR agonist R848 and CpG does not reduce airway hyperreactivity in the triple allergen combination model

In the acute models, application of the TLR agonists R848 and CpG reduced allergen induced airway hyperreactivity. Therefore, these agonists were now applied in the triple allergen combination model to assess if the reduction could also be induced in a model of severe chronic inflammation showing pronounced pathological changes in the lung. Airway hyperreactivity was evaluated via invasive measurement of airway resistance and compliance.
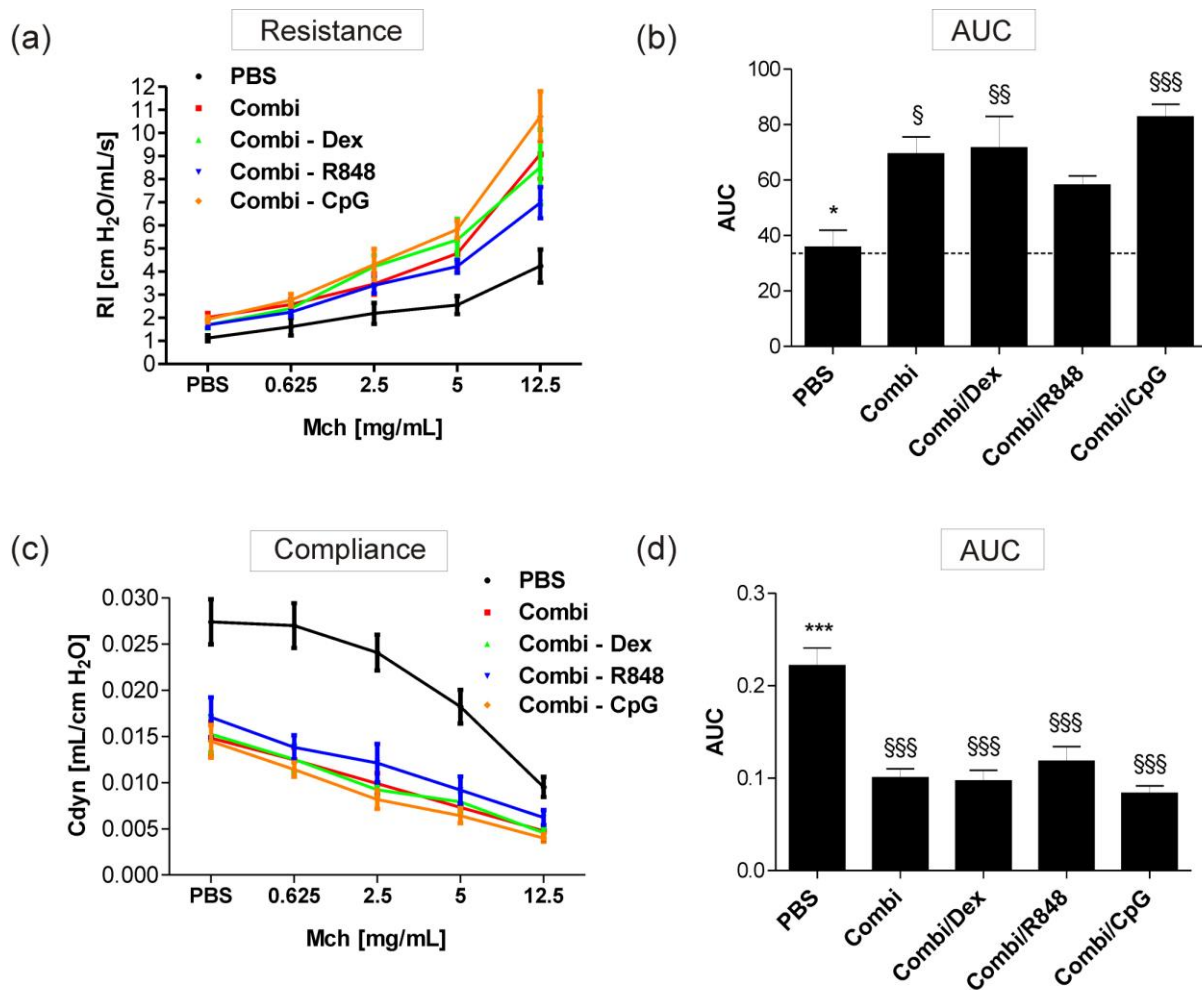
Fig. 31. Neither dexamethasone, CpG, nor R848 treatment significantly reduced allergen induced airway hyperreactivity in the triple allergen combination model. *Groups treated with dexamethasone, R848 and CpG were directly compared to the group receiving triple allergen combination and sham PBS treatment (Combi). Airway hyperreactivity was analyzed 24 hours after final challenge on day 70, assessing (a) airway resistance and (b) compliance in intubated narcotizised mice. Data were further analyzed using area under the curve (AUC, b, d). Data are presented as mean ± SEM, with n = 8 mice/group. Data were analyzed by Two way ANOVA, comparing groups to mice receiving Combi treatment only (a, c), or one way ANOVA (b, d) comparing groups to mice receiving Combi treatment only and sham-treated control group. Indexes for significances; * = compared to Combi only treated control group, § = compared to vehicel treated control group (PBS). *,§ = p < 0.05; **,§§ = p < 0.01, ***,§§§ = p < 0.*

Artificial ventilation was shown to activate neutrophils and pro-inflammatory cytokines in both clinic and preclinical models, which was also seen in the present study (data not shown). Therefore, airway hyperreactivity had to be measured in a separate experiment in which cellular influx and cytokines were not assessed. Analysis of resistance and compliance showed that neither the steroid dexamethasone, nor R848 or CpG, significantly reduced allergen-induced airway hyperreactivity (Fig. 31). In previous experiments, conducted in

models of acute allergic inflammation, R848 exhibited strong positive effects on airway hyperreactivity (Fig.10, [216]). Despite exhibiting no statistically significant reduction in this chronic model, it was noticeable that at all measured methacholine concentrations R848-treated mice exhibited the highest compliance and the lowest increase of resistance. This was also seen for baseline measurement. AUC analyses also showed the lowest and highest levels of resistance and compliance, respectively, for R848 treated animals. However, these effects were again statistically not significant, when compared to the triple allergen combination control group.

4.3 CpG treatment reduces mucus production and collagen deposition whereas R848 and dexamethasone show no effect on the pathology of severe chronic asthma

To assess if TLR agonist application affects airway remodeling, lung sections were stained either for analysis of cell influx, mucus production, smooth muscle thickening, or fibrosis. Hematoxilin-eosin stained sections were analyzed to assess cellular influx of immune cells in lung tissue (Fig. 32a). Histological sections showed that both steroid and TLR agonist treatment slightly reduced the cellular influx into the alveolar tissue. In contrast, in the peribronchial and perivascular area, no reduction in inflammatory cells was seen, regardless of whether steroid or TLR agonist was applied. Thus, histological sections showed only slight indications for reduced influx of immune cells. Assessment of the remodeling parameter mucus production showed that CpG-treated mice exhibited an articulate reduction of mucus. The mucus was assessed by comparison of the main bronchus area of lung sections (Fig. 32 b). Automated quantification of mucus confirmed the observed reduction of mucus showing significantly less mucus in lungs of CpG-treated mice. Dexamethasone treatment had no effect, whereas R848 treatment resulted in a slight but statistically not significant reduction. In this experiment 50% of the mice receiving triple allergen combination exhibited mucus plugging. This was also seen in 56% of the mice treated with R848. Interestingly, no mucus plugging was seen in mice treated with dexamethasone or CpG.
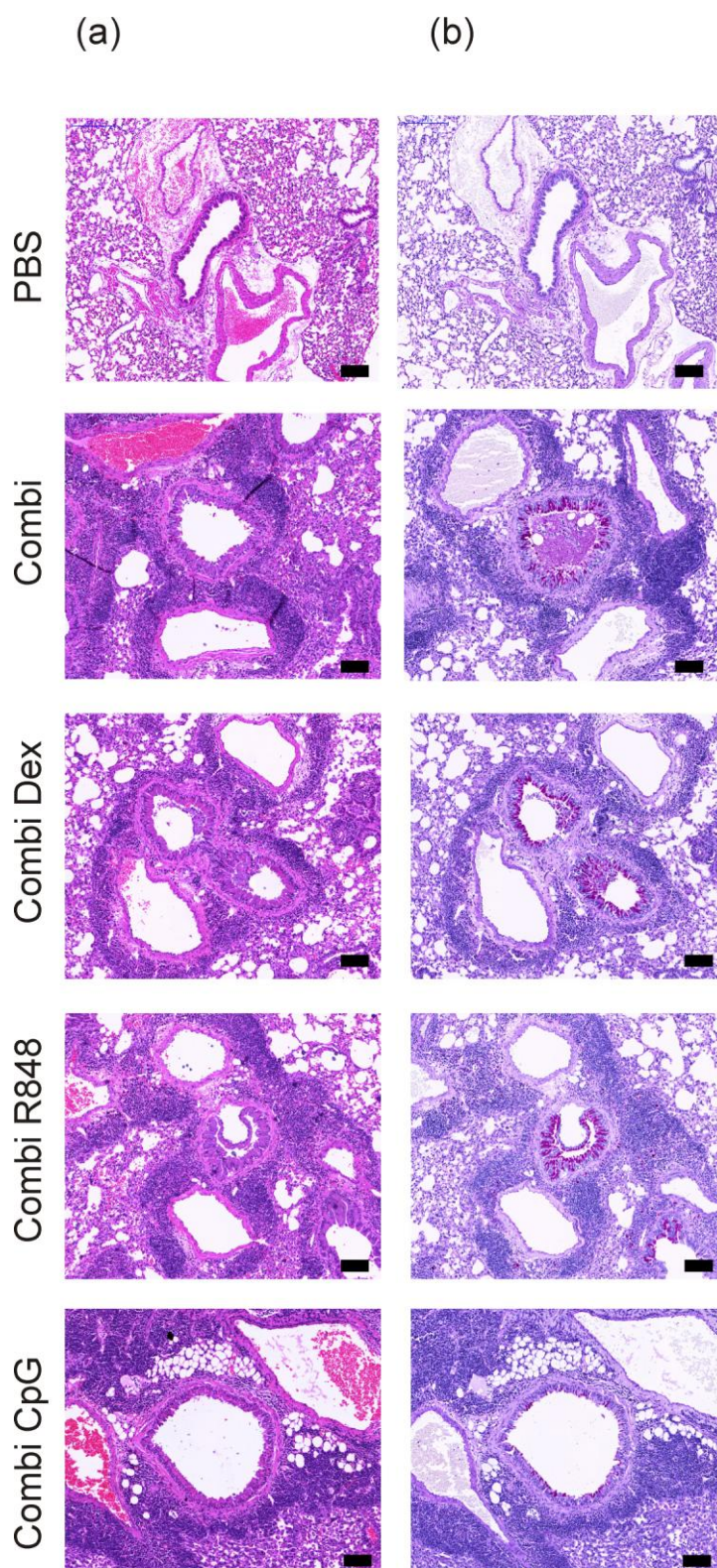
Fig. 32. In the triple allergen combination model treatment with dexamethasone, R848, and CpG induces only slight decreases in cellular influx, and only the treatment with CpG reduces mucus production significantly. *Histological staining of main bronchus lung area was used for visualizing inflammatory cell influx and goblet cell hyperplasia. Shown are representative sections of (a) haematoxilin/eosin and (b) Periodic acid-Schiff stained main bronchus area. Scale bar = 100 µm. Pictures show representantive sections of the lungs from 8-14 mice/group.*
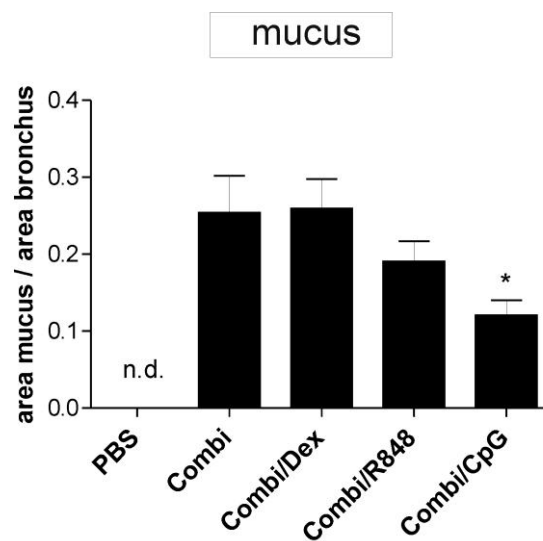
Fig. 33. TLR agonist CpG significantly reduces allergen-induced mucus production in the triple allergen combination model. *Area of mucus and bronchus was assessed by automated analysis of PAS stained main bronchus epithelial layer. Data are presented as mean ± SEM, n = 8-14 mice/group and * = p < 0.05; in comparison with mice receiving a sham treatment (Combi).*

Histological analysis of smooth muscle layers surrounding the main bronchus showed that the TLR agonists R848, CpG, and the steroid dexamethasone did not reduce smooth muscle thickening in the triple allergen combination model (Fig. 34a). Automated analysis of smooth muscle area and mean thickness of smooth layer also showed no effect of TLR agonist administration on smooth muscle thickening (Fig. 35 a, b). For analysis of fibrotic processes, lung was stained for collagen (Fig. 34b). Histological pictures showed indication of a potential reduction in subepithelial collagen for the TLR agonist CpG, which was less articulate for R848- or dexamethasone treated animals. No treatments reduced collagen IV, and only CpG treated mice showed significant reduction of the other two collagen subtypes I and III.

In order to assess potential adverse effects of TLR agonist treatment, after the final challenge the body weight of mice was assessed (Fig. 36). Measurement showed that in comparison to untreated animals, the triple allergen combination model induced a significant weight loss. Noteworthy was that CpG-treated animals exhibited the lowest mean body weight of all groups. However this loss was statistically not significant when compared to mice treated only with triple allergen combination.
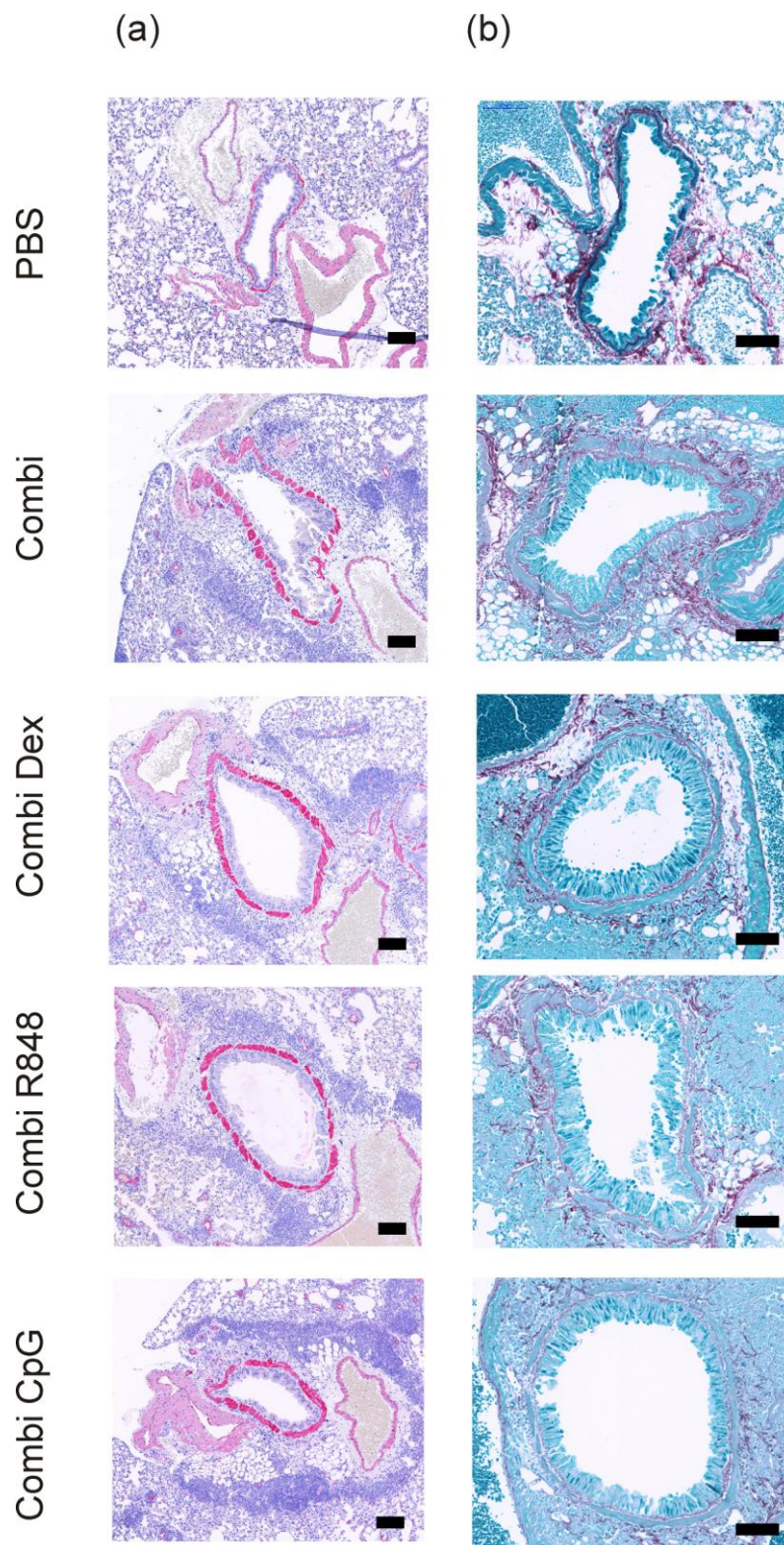
Fig. 34. Neither dexamethasone, nor TLR agonist treatment can reduce triple allergen combination model induced smooth muscle thickening.*(a)Lung sections were stained for alpha smooth muscle actin and (b) for extracellular collagen deposition. Shown are histological sections of the main bronchus and the peribronchial area surroundig the bronchus. Scale bar = 100 µm. Pictures show representantive sections of the lungs from 8-14 mice/group.*
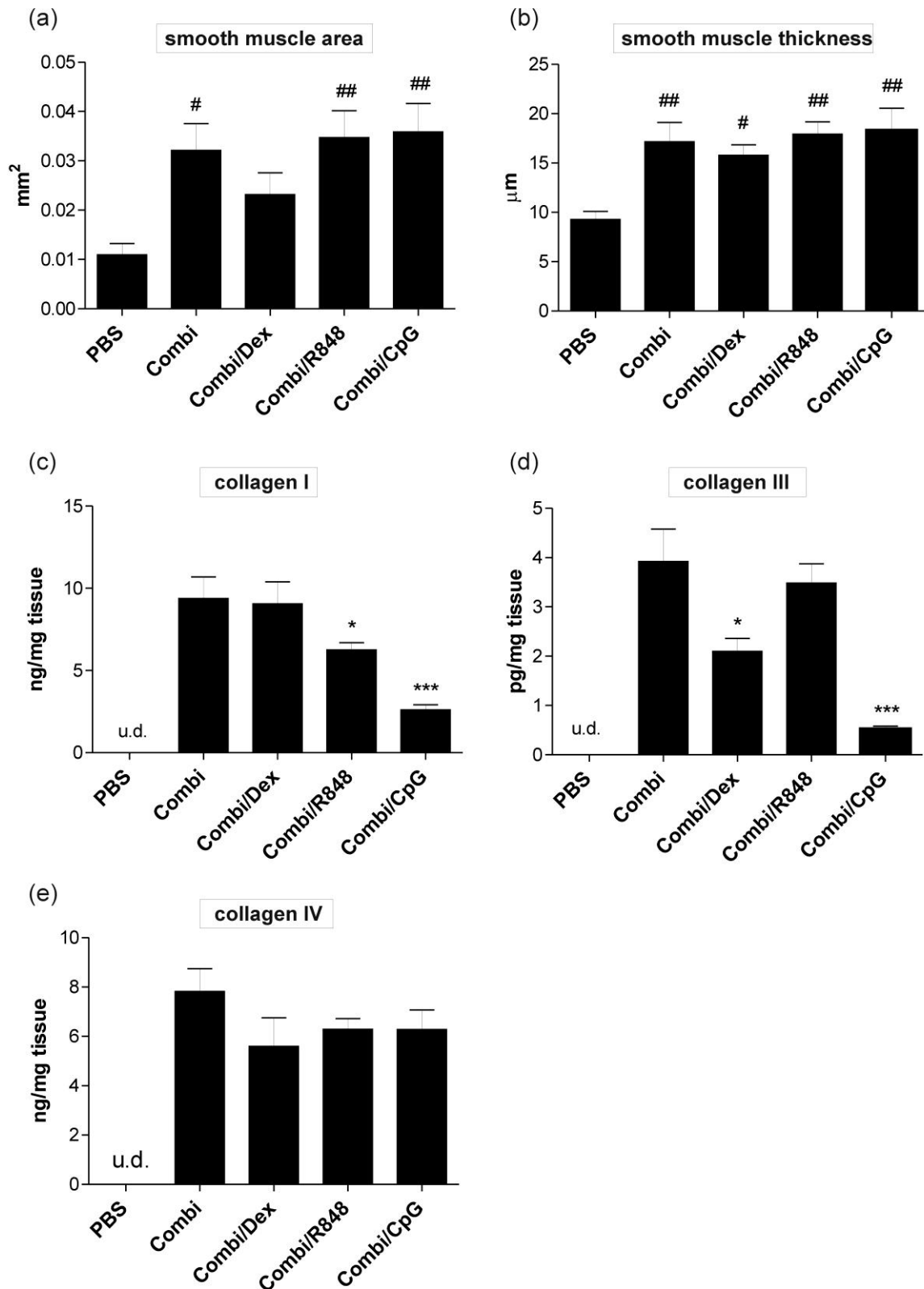
Fig. 35. Neither TLR agonist nor dexamethasone treatment decreases smooth muscle thickening, but CpG reduces fibrosis in the triple allergen combination model. *Measured were (a) the smooth muscle area and (b) the mean thickness of the smooth muscle layer surrounding the main bronchus. For assessment of fibrosis (c) collagen I, (d) collagen II and (e) collagen IV were measured. Data are presented as mean ± SEM with an n of 6-12 mice/group with # = P < 0.05;## =P < 0.001 in comparison with mice receiving no treatment (PBS), u.d. = under detection limit.*
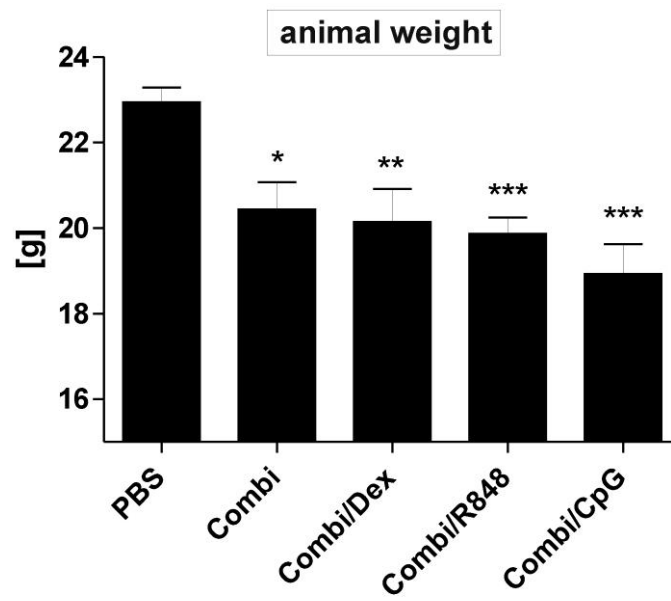
***Fig. 36. TLR agonist application does not inhibit triple allergen combination model induced weight
loss.*** *Data are presented as mean ± SEM of two independent experiments, each with an n = 8-14
mice/group. * = p<0.05, ** = p<0.01, *** = p<0.001 in comparison with mice receiving a sham PBS
treatment.*

5. Non-lung directed administration of TLR agonists can suppress allergen-induced eosinophilia without inducing lung neutrophilia

In the experiments shown so far, TLR agonists strongly reduced airway eosinophilia and T_H2 biased inflammatory responses in the lung. However, lung directed administrations of TLR agonists were always accompanied by an increase in airway neutrophil numbers (e.g. Fig. 7, 12, 15). This neutrophilia might limit the use of TLR agonists as therapeutics and a good safety profile should exhibit no increase of these cells in the lung. This raises the question whether the lung neutrophilia could be avoided without losing anti-asthmatic effects by administration via an alternative route of TLR administration. To analyze efficacy and safety of alternative administration routes, the 28 day murine OVA model of acute allergic inflammation was used (see methods, Fig. 4a). In this model, each of the five previously tested TLR agonists was administered on day 26 and 27, one hour prior to OVA challenge. Each TLR agonist was tested in a separate experiment in which the agonist was administered directly to the lung (intratracheally), intraperitoneally, intravenously, subcutaneously, or orally (per os). In all groups the TLR agonists were applied at a concentration of 1 mg/kg body weight. For negative controls mice were treated with phosphate buffered saline only, whereas positive controls received OVA treatment only. For analysis, BAL was collected and cellular influx and cytokine levels of IL-4, IL-5, IFN-γ, and IL-10 were measured.

Intratracheal administration of the TLR2 agonist LTA induced a strong neutrophilic influx, whereas LTA given via other application routes did not increase the numbers of neutrophils in the lung (Fig. 37). Furthermore, the lung-direct administration was not the most efficient route to reduce eosinophilia. Intratracheal administration of LTA resulted only in a marginal reduction of allergen-induced eosinophilia, whereas per os, intravenous and subcutaneous administration of LTA significantly reduced eosinophilic counts in the BAL. Interestingly, only intratracheal administration of LTA significantly lowered the levels of the T_H2 cytokines IL-4 and IL-5 in the lung (Fig. 38).
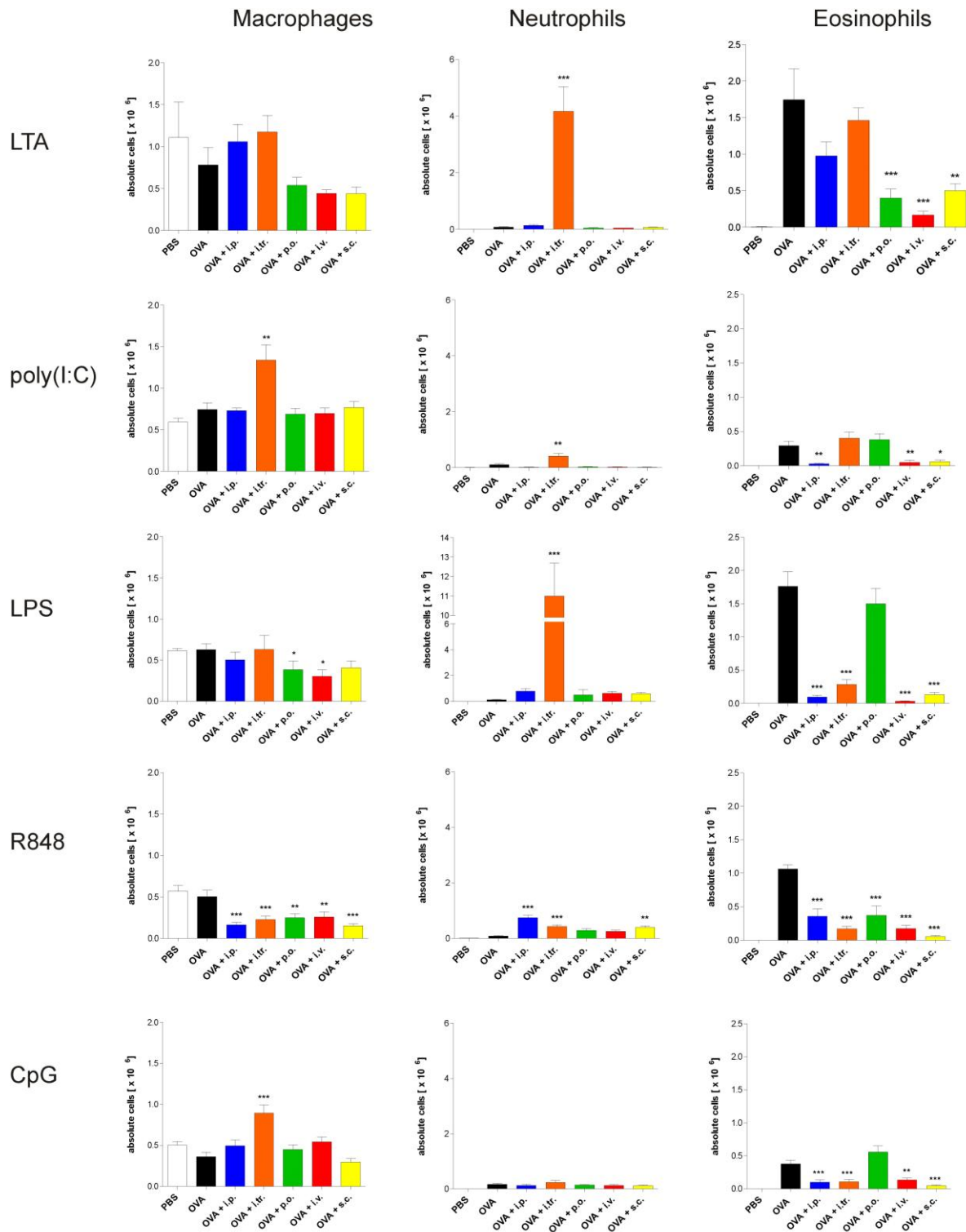
Fig. 37. Lung-directed administration of TLR agonists is not the most efficient application to reduce allergen-induced lung eosinophilia but induces lung neutrophilia. *Mice received either an intra-peritoneal (i.p)., intratracheal (i.tr.), oral (p.o.), intravenous (i.v.), or subcutaneous (s.c.) administration of TLR agonist on day 26 and 27, one day prior to OVA challenge (see Fig. 5b). Total numbers of cells were measured in BAL. Cell counts are presented as mean ± SEM of 8 mice/group. * = p<0.05, ** = p<0.01, *** = p<0.001, in comparison to the OVA group. Experiments were repeated once, showing similar results.*

For poly(I:C), again the only route of application that significantly increased neutrophils was the lung-directed intratracheal administration (Fig. 37). Aside from inducing lung neutrophilia, intratracheal administration of poly(I:C) had no significant effect on eosinophilic influx, whereas intraperitoneal, intravenous and subcutaneous administration significantly reduced eosinophilic numbers in the lung. Evaluation of cellular influx showed that a significant increase of macrophages was induced, but again only when poly(I:C) was given intratracheally. In contrast to intratracheal administration of LTA, intratracheal administration of poly(I:C) did not reduce IL-4 nor IL-5, but when administered intraperitoneally, intravenously or subcutaneously, poly(I:C) significantly reduced both cytokines (Fig. 38). As seen in previous experiments, intratracheal application of poly(I:C) induced an increase of IFN-γ; however, all other application routes of poly(I:C) did not increase the IFN-γ levels in the lung (Fig. 39).

When LPS was administered, only intratracheal application resulted in a statistically significant increase in numbers of neutrophils (Fig. 37). In line with earlier experiments conducted in this thesis, compared to the other TLR agonists, intratracheal application of LPS induced the strongest neutrophilic lung influx. Except given orally, which had no effect on eosinophilic influx, via all other application routes, LPS strongly reduced eosinophils in the lung. Measurement of IL-4 and IL-5 showed that independent of the route of administration, LPS significantly reduced both of these T_H2 cytokines (Fig. 38). Intratracheal application of LPS also induced an increase of IFN-γ and a reduction in IL-10 levels (Fig. 39). A reduction of IL-10 levels was also measured for intravenous and subcutaneous administration of LPS.

R848 given via different routes always induced an increase in neutrophil numbers in the lung (Fig. 37). Yet, compared to the increase induced by intratracheal application of LTA or LPS, this increase was much less pronounced. In addition, statistically significant increases were measured only for the intraperitoneal, intratracheal, and subcutaneous application of R848. Interestingly, R848 was the only TLR agonist that, independent of the administration route used always showed a strong and statistically significant reduction in numbers of eosinophils as well as a significant reduction in numbers of macrophages. Measurement of IL-4 and IL-5 showed that all routes of administration reduced both of these T_H2 cytokines significantly (Fig. 38). Administration of R848 had no effect on IFN-γ or IL-10 levels, regardless of the route of application which R848 was administered.
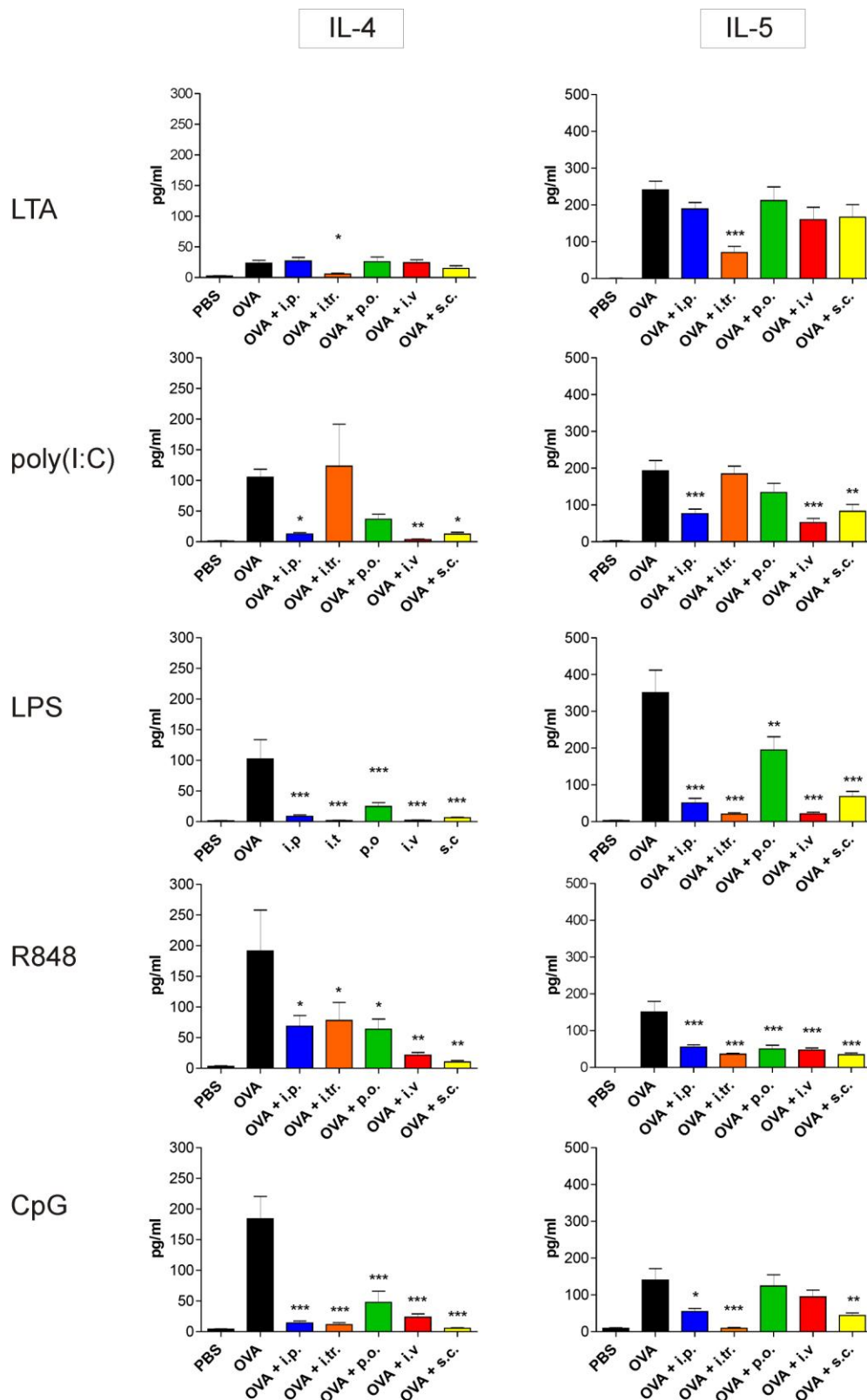
Fig. 38. TLR agonists-induced reduction of IL-4 and IL-5 levels in the lung depends on the route of application. *Mice received either an intra-peritoneal (i.p)., intratracheal (i.tr.), oral (p.o.), intravenous (i.v.), or subcutaneous (s.c.) administration of TLR agonist on day 26 and 27, one day prior to OVA challenge (see Fig. 5b). Levels of cytokines were measured in BAL, which was collected 24 hours after the final OVA exposure. Cytokine levels are presented as mean ± SEM of 8 mice/group.* * = p<0.05, ** = p<0.01, *** = p<0.001, as compared with the OVA group.*
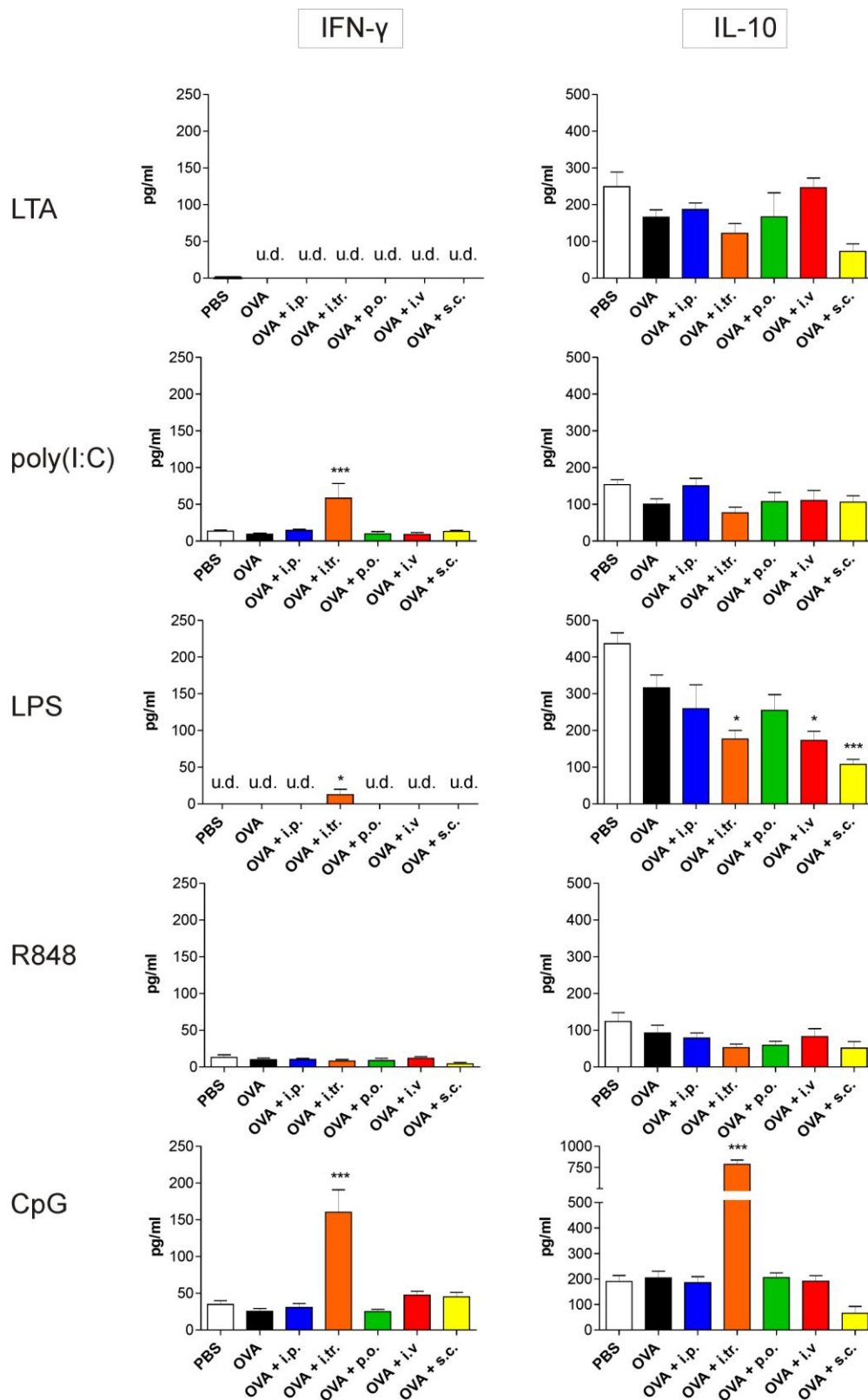
Fig. 39. Only lung-directed administration of poly(I:C) and CpG increase IFN-γ and IL-10 levels in the lung. *Mice received either an intra-peritoneal (i.p.)., intratracheal (i.tr.), oral (p.o.), intravenous (i.v.), or subcutaneous (s.c.) administration of TLR agonist on day 26 and 27, one day prior to OVA challenge (see Fig. 5b). Levels of cytokines were measured in BAL, which was collected 24 hours after the final OVA exposure. Cytokine levels are presented as mean ± SEM of 8 mice/group. * = p<0.05, ** = p<0.01, *** = p<0.001, as compared with the OVA group.*

Noteworthy, in these experiments CpG administration induced no increase of neutrophils independent of the application route used (Fig. 37). Nevertheless, CpG was highly effective in reducing eosinophilic counts, and only oral administration of CpG showed no effect on lung eosinophilia. An increase in macrophages numbers was measured, but only when CpG was given intratracheally. CpG administration always reduced IL-4 levels, but oral and intravenous administration did not significantly lower IL-5, whereas other application routes did (Fig. 38). Interestingly, intratracheal application of CpG induced the highest increase in both IFN-γ and IL-10 (Fig. 39).

Collectively, these experiments show that with the exception of R848, the TLR agonists LTA, poly(I:C), LPS and CpG all can reduced allergen-induced eosinophilia without causing neutrophilia in the lung. To assess systemic effects of TLR agonists, a separate experiment was conducted where untreated mice received the TLR agonists via the different routes of application. In these experiments an increase in the percentage of neutrophils and levels of TNF-α in the blood was observed. The increase was seen for all TLR agonists and for all application routes, with the exception of oral TLR administration, which had no effect on percentage of neutrophils. The strongest systemic increase in the neutrophil percentage was induced in R848 treated mice (data not shown). Interestingly, already six hours after application of TLR agonists TNF-α in blood had fallen to background levels (Fig. 40).
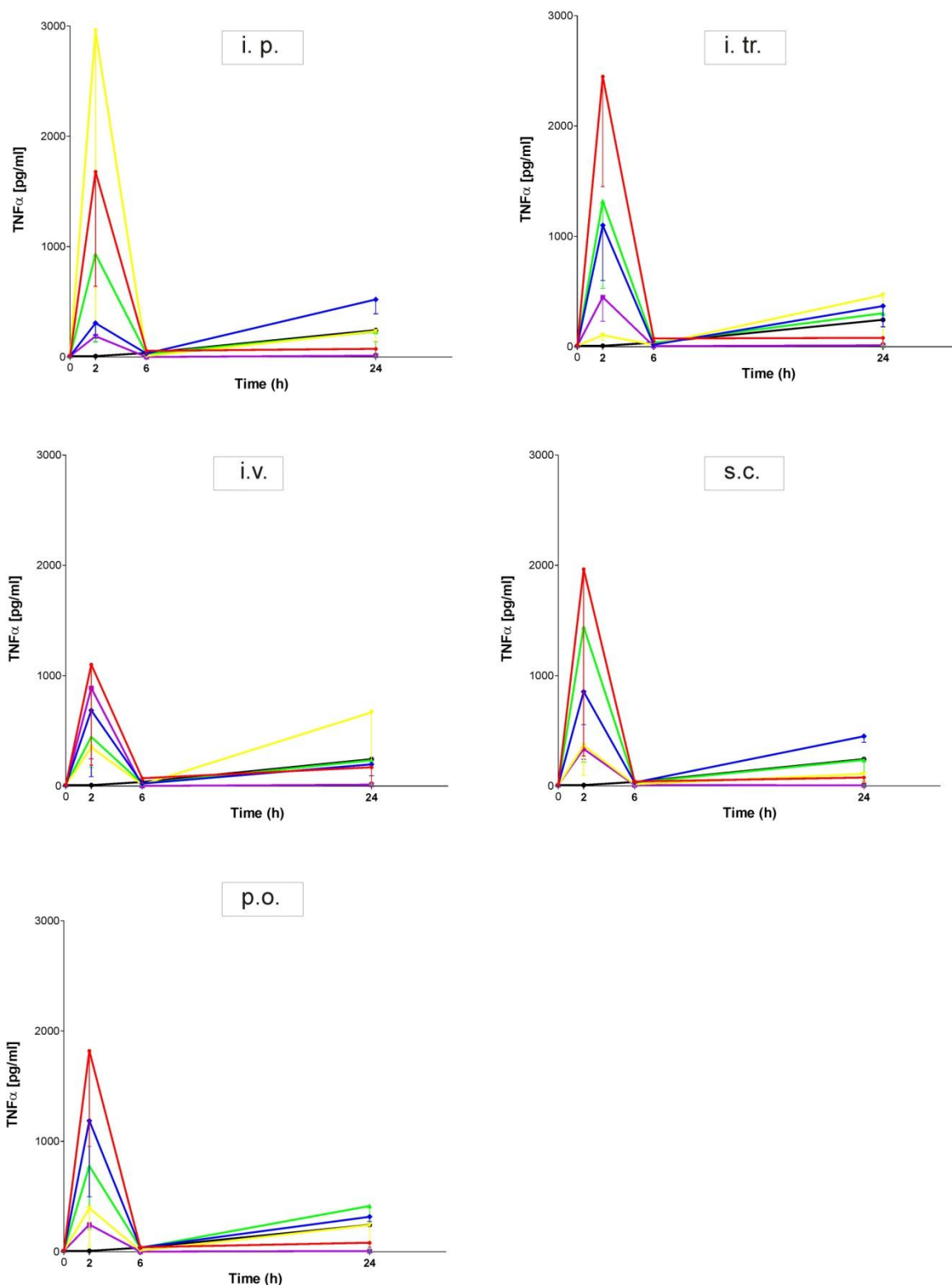
Fig. 40. All TLR agonists induce a fast and transient increase in serum TNF-α levels. *At the start of the experiments (time point 0h) serum was collected and pooled, and then TLR agonists were applied. Serum was then collected 2-, 6-, and 24 hours post treatment and the TNF-α level of each mouse was measured. Beside TLR application mice received no other pre-treatment. Levels of TNF-α are presented as mean ± SEM of 3 mice/group. (■ = untreated, ■ = LTA, ■ = poly(I:C), ■ = LPS, ■ = R848 , ■ = CpG)*

DISCUSSION

The aim of this study was to analyze the efficacy of TLR activation to treat allergic responses in mouse models of asthma and to assess TLR-induced pro-inflammatory responses. For this purpose, TLR2, TLR3, TLR4, TLR7, and TLR9 were activated with the respective TLR agonists LTA, poly(I:C), LPS, R848, and CpG. These agonists were administered parallel to allergen sensitization, before and during acute, or after established chronic allergic inflammation. Clinical relevant parameters were then assessed to define the individual preventive, protective, and therapeutic potential of each applied TLR agonist.

In the first part of the thesis, the TLR agonists were given directly before pulmonary exposure to the allergen in a setting of acute airway inflammation. These experiments showed that the intratracheal administration of CpG, R848, and poly(I:C), one hour before allergen exposure, dose-dependently suppressed the development of allergen-induced airway responses, which included decreased airway eosinophilia along with reduced IL-4 and IL-5 levels in lung lavage. The experiments showed that increasing concentrations of CpG and poly(I:C) resulted in a stronger suppressive effect on allergic inflammation than the other tested TLR agonists. These findings are in unison with previously published reports [149;219;236;237]. Furthermore, it was found that, with the exception of R848, all TLR agonists dose-dependently increased the numbers of total macrophages, neutrophils, and pro-inflammatory cytokines in the whole lung lavage. In contrast to other published reports [209;238-240], in the present study the application of LPS or LTA did not suppress the development of allergen-induced T_H2 responses in the lung or airway hyperreactivity. However, this discrepancy may be due to the differences in application protocols. Supporting this view is that in the present study LPS and LTA did show inhibition of airway eosinophilia when given four days prior to allergen challenge. This is strong evidence that the time point of LPS and LTA application is essential for the effects these two agonists elicit. More precisely, it seems that LPS and LTA may need more than 48 hours to fully develop their anti-asthmatic potential. Surprisingly, the application of R848 four days prior to allergen challenge resulted in the loss of R848s anti-allergic effects. This finding further emphasizes the importance of the administration time point of individual TLR agonists. It appears that the kinetics of the induction of protective factors differs strongly and that R848 and CpG mediate their effect faster than LTA and LPS. Another potential explanation might be based on the finding that only LTA- and LPS-treated mice showed increased amounts of FOXP3+ cells in the lung. It is possible that these cells need to be activated longer to develop suppressive effects on the T_H2

responses in the lung. A further surprising result was that TLR agonist-induced reduction of eosinophils did not always correlate with a reduction in IL-4 and IL-5 levels in the lavage fluid (as was the case for R848 and CpG). It is not clear why the effects are so different between the TLR agonists, although they signal through very similar signal transduction pathways leading to similar pro-inflammatory responses [138;241]. A potential explanation might be the differing distribution of TLRs on specific immune cells, which has been reported in mice (reviewed in [242]). Hence, these diverse effects might be caused by the different, cell type-specific expression of TLRs, leading to individual immune cell activation profiles for each TLR agonist.

Which mechanisms are responsible for the TLR9, TLR3 and TLR7 agonist induced reduction of allergic responses? Previous publications have suggested relevant roles for several mediators such as IL-10, IL-12, IFN-α and -β, or IFN-γ [149;158;243-247]. Experiments performed in this study using IL-10 deficient mice, mice treated with antibodies binding to IL-10 receptor, or neutralizing IFN-γ suggest that IL-10 and IFN-γ are not responsible for the observed inhibitory effects. This result was unexpected for CpG, since this agonist induced a strong increase in IL-10 after the intratracheal application and IL-10 has been shown to have down regulating effects on both T_H1 and T_H2 cell activity [243-247]. Nevertheless, it is also possible that CpG and poly(I:C) exert their suppressive effect through a different, recently published mechanism involving both IL-10 and IL-12 [149], and that by blocking just one mediator, suppression can still be sustained. It was also published that type 1 IFNs are strong inhibitors of allergic responses [138;206;241;248]. However, neither IFN-α nor IFN-β could be detected in the lung lavage of TLR agonist treated mice, suggesting that these may not be responsible for the suppression of the allergic response. However, it can not be ruled out that they were induced in too small an amount to be detected by ELISA. The induction of regulatory T cells has also been hypothesized in TLR agonist mediated suppression of allergic disorders [206;241;248]. Very recently Van et al. published that R848 treatment reduced asthma symptoms in a murine model [249]. In this report they also proposed that the anti-asthmatic effect of R848 is T_r dependent and largely mediated through a TGF-β-dependent pathway. Surprisingly, in the experiments of the present study CD4+FOXP3+ T cell numbers in the whole lung lavage did not increase in the groups that showed reduced allergic responses, but T_r numbers were increased in LPS and LTA treated mice, which exhibited no reduced allergic responses. Consistent with the results in the present study, a report by Van et al. stated that IL-10 is not necessary for the suppressing effects of

R848. Van et al. also suggested that R848 induced an expansion of T_r populations in the lungs of mice. This finding could not be confirmed in the present study. In contrast, R848 treated mice exhibited strongly reduced numbers of CD4+ FOXP3+ T cells in the lavage. It can be suggested that this discrepancy is due to the method used for quantification, as according to the information given in the publication, Van et al. only measured the percentages of CD4+ FOXP3+ cells in the population of CD4+ cells, but did not assess the total amount of CD4+ and CD4+ FOXP3+ cells nor compare the total numbers of CD4+ FOXP3+ from R848 treated mice to numbers of R848 untreated mice. In the present study, first the total counts of leukocytes in the BAL were assessed followed by the measurement of the percentages in comparison to the total numbers of cells in the positive control group receiving only allergen treatment. Regarding the published data by Van et al, a potential R848-mediated decrease of total CD4+ cells might have occurred in their study as well; however this decrease would not have been detected as they did not quantify the total counts of T cells in the lavage. Nevertheless, it can not be excluded that the use of different mouse strains and application models might account for this discrepancy. In the experiments conducted for this thesis, the loss or lack of recruitment of CD4+ cells into the lung could be interpreted as one major reason for the reduced T_H2 responses detected in the airways. Confirming the results of the present study, another previously published work reported that a systemic administration of the TLR7 agonist R848 induced a lymphopenia in mice [250]. The induction of lymphopenia, reducing the overall T cell population, would also explain why R848 despite inducing only a very weak proinflammation but was still able to suppress T_H2- responses. Whether this is a specific effect or an off-target effect of R848 needs to be determined. Currently, it cannot be explained by which mechanisms the different TLR agonists mediate their suppressive effects. However, these results indicate that R848 most likely inhibits T_H2- responses by a different mechanism than for example CpG or poly(I:C).

When treating patients with atopic asthma, with the exception of seasonal asthma, all patients have ongoing T_H2 type inflammation in the airways [251]. With respect to possible future use in humans the effects of the different TLR agonists were analyzed in a therapeutic setting. Therefore, the TLR agonists were administered in mice that have previously been challenged with allergen, thus exhibiting an established allergic inflammation in the lung. In this study, with the exception of LTA, all TLR agonists significantly reduced the development of airway eosinophilia and IL-4 levels in the lung lavage. Interestingly, no increase in numbers of neutrophils was observed, whereas numbers of macrophages were elevated. This

surprising finding may again be explained by the time point of application, as in this model TLR agonists were applied four days to prior to analysis. It can be suggested that TLR agonists did induce neutrophilia in this model as well, which however at the later time point of analysis have already subsided to background levels. This is supported by kinetic studies conducted by Bozinovski et al. reporting that after LPS challenge neutrophilia peaked 24 hours post administration and was strongly reduced after 48 hours, eventually reaching background level 72 hours post treatment [252], which was also seen in experiments conducted the current study. In contrast, it was reported that macrophage numbers are still increasing 48 hours after challenge and reached their maximum 72 hours post treatment [252]. In line with this report, in the present study increased counts of macrophages, but not neutrophils, could be measured 72 hours after TLR agonist application. Importantly, in these experiments TLR agonist mediated suppression of eosinophilia was sustained even when the induced neutrophilia was no longer detectable in the lung. Therefore, a sustained neutrophilia is not required for TLR agonists mediated suppression of allergic inflammation. This suggests that a TLR agonist based therapy, suppressing eosinophilia, would not cause a constant neutrophilic influx into the lung.

Other novel approaches to treat asthma have focused on single different mediators of allergic inflammation, for example cytokines such as IL-4, IL-5 and IL-13 [253]. So far these approaches have not resulted in successful therapies, generally due to a lack of efficacy [254;255]. In this regard, TLR-based immunomodulation offers the advantage of a complete suppression of the underlying pathomechanism (T_H2 inflammation) rather then targeting one specific mediator. The immunomodulatory capacities of TLR agonists are also used to support the therapeutic approach of specific immunotherapy also called SIT. At present, SIT is the only disease-modifying therapy available for allergy sufferers [256]. The standard SIT involves either subcutaneous (SCIT) or sublingual (SLIT) application of increasing doses of purified allergen, most commonly over a period of up to $3 - 5$ years [257-259]. However, the efficacy of SIT, especially in more severe allergic disorders like asthma is still not satisfactory. In order to overcome this limitation, a current approach is to combine SIT with immunomodulators [260]. Co-administration of allergen and TLR agonists is one option to boost the suppression of the pathogenic T_H2 immune profile by induction of a protective T_H1 profile or regulatory processes [261-264]. The suggested mechanisms by which immunomodulator-combined SIT achieves clinical improvement are induction of Ig class switch, reduction of mast cell and eosinophilic homing, and enhancement of

immunosuppressive effects of regulatory T cells [265]. The Ig class switch leads not only to reduced IgE levels, but also to elevated levels of allergen-blocking IgG antibodies, in particular of the IgG4 class, which in contrast to IgE does not activate effector cells and antigen-presenting cells. In this regard, simultaneous application of allergen and TLR agonists may be an elegant approach to support SIT therapy, and first results have been encouraging. However, published preclinical SIT studies have used different TLR agonists for combination with allergen [184;266] and so far there has been no study directly comparing activation of different TLRs to identify which TLR agonist is most effective in SIT.

To address this question, in this thesis each of the TLR agonists LTA, LPS, poly(I:C), R848, and CpG were first applied intraperitoneal together with OVA/Al(OH)$_3$, subsequently TLR/allergen treated mice were exposed to nebulized OVA. The experiments showed that only the application of LPS and CpG significantly and dose-dependently reduced the development of airway eosinophilia. At the highest dose used, the suppressive effect of CpG was close to 100%. All of the TLR agonists reduced OVA-specific IgE levels and increased OVA-specific IgG2a levels. With an increase of more than 100 fold compared to OVA only treated mice, application of CpG and poly(I:C) exhibited the strongest increase in OVA-specific IgG2a levels. These two groups were also the only ones in which IFN-γ could be detected in the BAL, indicating enhanced T$_H$1-responses. None of the TLR agonists tested reduced airway hyperreactivity after methacholine challenge. Furthermore, IL-17 could not be detected in the BAL, suggesting that T$_H$17 cells were not induced by any of the TLR agonists, or only in small quantities. As mentioned before, previous reports suggest that infections and the application of TLR agonists, in particular LPS and LTA, can also exacerbate allergic responses [267-270]. Therefore, another aim of this study was to assess if the preventive administration of TLR agonists can provoke exacerbations of allergic responses. The results showed that the highest dose of poly(I:C) increased allergen-induced recruitment of eosinophils into the airways, whilst the highest dose of R848 increased levels of IL-4, and the highest dose of CpG, possibly due to the increased numbers of neutrophils, increased airway hyperreactivity. However, poly(I:C) and especially CpG showed a very pronounced reduction of other aspects of allergic responses including cytokine levels, IgE titers and anaphylaxis. Altogether, R848 or poly(I:C) seemed to induce no or only very weak effects on allergic responses. Taken together, the data suggest that the application of the different TLR agonists can prevent (CpG > LPS), reduce (LTA > poly(I:C)), or have no effect (R848) on the allergic T$_H$2 responses. Furthermore, TLR agonist differ in their induction of allergen-specific T$_H$1-

responses (CpG > poly(I:C) >> LPS > LTA > R848). How activation of TLR during sensitization affects the development of asthma is not entirely clear. In the literature mouse studies report divergent results. For example, Redecke et al. reported that TLR2 ligands bias the adaptive immune response towards a T_H2 phenotype and lead to aggravation of experimental asthma [160]. In contrast, Velasco et al. reported that activation of both TLR2 and TLR4 showed efficacy in preventing allergen-induced pulmonary responses [240]. The results from the present study support the findings reported by Velasco et al. showing that coadministration of an TLR2 or TLR4 agonists with allergen is able to prevent airway eosinophilia, T_H2 cytokine induction and IgE production. Why the reported results differ can currently not be explained and it can only be suggested that these differences might be due to model or concentration dependent effects. In comparison, reported effects of TLR9 activation by CpG are more consistent. First reports focusing on the potential of this agonist to prevent development of allergic reactions in mice, have already been published over 10 years ago [236]. Subsequent reports and the experiments of the present thesis confirm the potential of TLR9 activation to induce a strong immune deviation from T_H2 to T_H1 responses. In line with these findings, a first small clinical SIT trial with 20 patients suffering from house dust mite allergy, using the TLR9 agonist CpG as adjuvant, reported a good efficacy and safety profile [266].

Concluding, the suppressive effects of TLR agonists and allergen co-administration vary strongly and depend on the TLR agonist administered. The most effective treatment in the present study, completely preventing the later responses to allergen, was the activation of TLR4 and TLR9 by application of LPS and CpG. Interestingly, all published clinical SIT trials were conducted with agonists either activating TLR4 or TLR9 [183;266], which are also favoured by the data of the present study. In summary, this preclinical study suggests that the TLR9 agonist CpG has the strongest suppressive effects on the development of allergen-specific T_H2 responses and the strongest capacity to induce allergen-specific T_H1 responses, when directly compared with LPS, LTA, R848, and poly(I:C), and is therefore recommended for SIT therapy of allergic asthma by this study.

A further aim of this thesis was to determine if TLR activation can also affect the pathology of severe chronic asthma. In order to be able to measure potential TLR agonist-mediated effects, a good model of this form of the disease is essential. This mouse model must closely mimic essential aspects of the clinical pathology and show an acceptable therapeutic window. In current murine asthma models different allergens such as extracts of

cockroach, pollen, house-dust-mite, and most frequently, purified ovalbumin protein from egg white are administered to elicit allergic inflammation. However, in some of these models chronic allergen application induces a decline in cellular infiltration and asthma-associated cytokines in the lung as well as reduced airway hyperreactivity [222;223;271]. In addition, some models induced only marginal levels of airway remodeling. Hence, for testing effects of TLR activation in severe chronic asthma, one aim was to establish a model with a strong pathology comparable to human severe chronic asthma. Therefore, chronic models using the allergens OVA, cockroach, and house dust mite, were directly compared for their ability to induce severe asthma-like pathology. In contrast to other reports, no indications of the development of allergen tolerance were found in the present study [272;273]. Instead, chronic treatment with OVA, cockroach-, and house dust mite extract induced higher influx of eosinophils, increased levels in IL-4, IL-5 and resulted in strong hyperreactivity. It can be suggested that in chronic models with repeated allergen administrations the route of application is one decisive factor determining development of tolerance or decline in strength of the allergic response. In favor of this assumption is that in all reported cases of immune-tolerance [273;274], nebulized or intranasal application but never intratracheal application was used for challenge. Furthermore, nebulized or intranasal application of allergen inevitably leads to oral allergen contact of challenged mice. This oral contact in turn was shown to be sufficient to reduce allergic inflammation as well as airway remodeling [275-277]. Therefore, it can be suggested that the intratracheal application and avoidance of oral allergen contact is the reason why the models of the current study showed no immune tolerance or regression of inflammation. Though the different allergens induced comparable eosinophilia and levels of T_H2 cytokines, differences of models were seen in airway hyperreactivity and remodeling. Measurement of airway hyperreactivity showed that ovalbumin induced the lowest increase in hyperreactivity and a less pronounced airway remodeling when compared to cockroach and house dust mite extract treatment. Combining the three allergens in one model led to the severest pathology. In this model mice were first sensitized against a mix of OVA, house dust mite, and cockroach extracts. In the challenge phase the single allergens were given in alternating order, one allergen per week. This triple allergen treatment induced the strongest influx of eosinophils and the highest levels of IL-4, IL-5, and IL-13 in the lung tissue. This novel model also led to the highest airway hyperreactivity manifested by a significant decreased in compliance and elevated resistance of the lung. When assessing remodeling by histological analyses of lung sections and quantitative measurements in lung homogenate, the novel model showed the strongest fibrosis, a pronounced smooth muscle thickening and

increased mucus production. The triple allergen model also led to the most frequent mucus plugging of bronchioles, which in severe chronic asthma is a factor directly correlating with mortality. These experiments show that in the chronic setting OVA, cockroach, and house dust mite extracts can induce essential symptoms of the severe form of asthma, and that the induced effects of these extracts are quite comparable. The novel model, combining the three extracts, however induced the highest allergic inflammatory response and the strongest pathology. Therefore, this model is superior in mimicking essential features of severe chronic asthma and hence best suited for the testing of new treatment options.

There are only a few studies published that apply TLR agonists in rodent models of severe chronic asthma [278-280] and most of these studies focus on the question whether agonists can prevent symptoms of asthma when administration during sensitization [279] or during early development of disease [280]. In another approach, TLR agonist were given continuously starting with the first allergen challenge [281]. Hence, these studies are not designed to gain conclusive data about TLR treatment in an already established chronic inflammation. In the present study TLR agonists were applied in the newly developed triple allergen model after chronic inflammation was established. In these experiments R848 and CpG, the agonists showing the most promising profile in the acute models, were applied. Effects on the severe chronic form of the disease were directly compared with the corticosteroid dexamethasone. Interestingly, dexamethasone had no effect on allergic inflammation in this model, and showed no effects on airway hyperreactivity and remodeling. This result was quite surprising as in a similar chronic model, using OVA as allergen, the same concentration of corticosteroid exhibited significant effects [282]. As mentioned before, steroid treatment represents the most frequently used anti-inflammatory medication in asthma and efficiently decreases recruitment and activation of eosinophils in moderate asthma. In contrast to dexamethasone, R848 treatment significantly reduced eosinophils but had no effect on T_H2 cytokine levels. In all measured methacholine concentrations, R848 consistently showed the lowest airway hyperreactivity which however failed to reach statistical significance. When airway remodeling was assessed R848 treatment showed no effect. In the triple allergen combination model of severe asthma, treatment with the TLR9 agonist CpG showed the strongest reduction in allergen induced eosinophilia and reduced both, IL-4 and IL-5 levels. The decrease in T_H2 cytokines was accompanied by an increase in the T_H1 cytokines IFN-γ and IL-12. Only CpG induced an increase in IL-1β and in IL-10 levels. CpG treatment had no effect on airway hyperreactivity. Analysis of airway remodeling showed no

change in airway smooth muscle thickening but CpG showed significant effects on fibrosis in both, histology and in quantitative analysis of collagen I and III. As mentioned before, in the severe form of asthma extensive mucus production and mucus plugging directly correlates with increased mortality [283]. In the lungs of animals treated with CpG, no mucus plugging of the main bronchi was seen and the level of intracellular epithelial mucus was significantly reduced.

In summary, the TLR agonists R848, and most efficiently, CpG inhibited eosinophilic lung inflammation in a model of severe chronic inflammation. Furthermore, CpG significantly reduced allergen-induced T_H2 cytokines, fibrosis and secreted mucus. In the present study, R848 and especially CpG showed superior efficacy in direct comparison with oral corticosteroid treatment. Thus, the results of these experiments support a TLR9-based approach as a viable option for the treatment of severe chronic asthma which may presents a potential alternative to anti-inflammatory therapy with steroids.

Considering the results of the present thesis the most important question is how these data transfer to the human situation. It is obvious that mice and humans differ in their immunology as mice and men have developed divergent immune systems adapting to the demands of their specific environment [284]. As a consequence, mice do not spontaneously develop allergic diseases like asthma. In addition, so far mice models of asthma can only reflect one specific disease pattern of the diverse and heterogeneous population of asthmatics. Furthermore, mice have in some cases been poorly predictive for human disease, as several approaches to treat asthma, such as targeting IL-13, IL-4 or IL-5, showed efficacy in mouse but not in human studies (reviewed in [285]). Despite these disparities, mice and humans share a strongly conserved immunological background and the mouse models of asthma reflect important processes characteristic for asthma, which in the past aided greatly in the understanding of different aspects of the disease. Also, mice models have been predictive for other approaches, as for example in the development of the new drug classes of leukotriene modifiers and IgE directed IgG antibodies (omalizumab) [285] and are sensible to steroid treatment. Species specific disconnects are more likely to occur when a disease-related factor instead of the underlying mechanism is targeted. Therefore, the TLR agonist based approach might be less prone to show species-specific effects as it does not target one specific mediator but rather the entire asthma specific immune response. Furthermore, TLRs are highly conserved in mammals and between human and mouse only few major disparities in TLR immunology have been reported. These include TLR2 expression on peripheral blood

leukocytes, which in mice are expressed at a lower rate, when compared to their human analog [286]. TLR3 in mice is expressed by DC and macrophages, whereas human macrophages lack this receptor [287]. Also TLR9 is expressed by myeloid DCs, plasmacytoid DCs, macrophages, and B cells in mice, whereas in humans TLR9 expression is restricted to plasmacytoid DCs and B cells [288-290]. Despite these differences TLR agonists induce immune reactions have been reported to be very similar in both preclinical mouse models and human studies. For example it was reported that CpG–allergen complexes stimulate primary bone marrow cells to induce T_H1- rather than T_H2-type cytokines production in allergic subjects [291]. Furthermore, CpG application was reported to induce IFN-γ and IFN inducible genes in asthmatics [203], which was also reported in mice models. Overall, asthma mouse models may successfully predict principles of therapies for human asthma and a less specific and broader approach, such as TLR activation, might translate better to humans than more specific approaches. Nevertheless, it can not be excluded that TLR agonists might show different effects in preclinical tests and patients.

The results of the present study support the approach to use the immunomodulatory properties of TLR agonists to treat allergic inflammation. However, lung-directed application of TLR agonists resulted in increased neutrophils in the lung, raising the question if repeated pulmonary applications of TLR agonist are safe for human use. Neutrophils are key players in the defence against viral and bacterial infections and play an essential role in pulmonary host defence. In contrast to their important function in host defence, in asthma, an excess of neutrophils in the lungs was reported to correlate with the degree of nonspecific bronchial hyperreactivity in patients with severe persistent disease [292]. Overall, current data propose a disease-supporting role of neutrophils in the severe form of asthma [293-296]. Considering a potential clinical application of TLR agonists, the induction of a strong neutrophilic influx can therefore limit the use of such therapeutics. Yet, the present study showed that the TLR agonists induced lung neutrophilia can be prevented by using non lung-directed applications. TLR agonists given subcutaneous, intraperitoneal or intravenously, did not induce lung neutrophilia and were at least as effective as lung-directed administration in the inhibition of pulmonary allergic responses. In regard to a later clinical use of TLR agonists, the subcutaneous administration might be favorable, as it is safer and easier to conduct compared to intravenous and intraperitoneal application. Despite the absence of TLR induced pro-inflammation in the lung, all tested TLR agonists induced systemic proinflammation, seen by increase in the percentage of neutrophils and increase in TNF-α levels in the blood. The TLR

agonist CpG also induced increase in IFN-γ levels. TNF-α and IFN-γ are both factors essential for boosting the immune system but high levels of these cytokines can have severe pathological effects. Weight loss, lymphadenopathy or endotoxic shock are effects mediated or associated with TNF-α [195]. Increase in IFN-γ levels was reported to induce airway hyperreactivity, in a allergic mouse model using CpG for treatment [297]. These potential side effects must be considered when using TLRs. Nevertheless, different findings suggest that potential TLR agonist-induced adverse effects can be avoided. For example in the present thesis percentage neutrophils in the blood abated 24 hours after the administration of TLR agonists. Also the levels of TNF-α, which peaked two hours post application, returned to baseline levels after six hours. This indicates that TLR agonists-induced inflammation is a fast and transient process. On the other hand, TLR agonists strongly suppressed allergic inflammation even when given four days prior to allergen challenge. This long-lasting suppression of T_H2 responses suggests that it would be sufficient to give TLR agonists only once or twice a week. As a consequence of the fact that the agonists do not need to be given daily, potential TLR agonists-induced side effects may be kept at a low and for the patient tolerable level. It has also been published that some TNF-α mediated effects are likely to be mouse specific and are not to be expected in humans [298]. Third and most important, available information from clinical phase I trials using CpG and other TLR agonists, e.g. TLR7 or TLR11 agonists in tumor therapy or specific immunotherapy, indicate that a therapy with TLR agonists can be well tolerated in humans [299-302;302;303].

In conclusion, this study supports the approach to use the immunomodulatory potential of TLR activation as a treatment option in asthma. Judging from the data of this study, the most promising results are to be expected from TLR-based therapies combining CpG or LPS with allergen in specific immunotherapy. In regard to TLR agonist monotherapy, R848 and CpG showed the most promising profile. In severe chronic asthma, in which corticosteroids show only limited effects, CpG treatment might be a promising novel therapeutic approach.

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ABSTRACT

In the last decades, both the incidence and the severity of asthma have steadily increased. Although current medications can control mild to moderate asthma there is still a great unmet medical need to develop novel therapeutics for severe chronic asthma. Furthermore, available therapies only treat the symptoms but do not cure the disease. Immune modulation induced by TLR agonists may be a promising novel approach to effectively treat asthma as it targets the underlying immunopathology directly rather than one mediator alone.

The aim of this thesis was to investigate if the immunostimulatory properties of Toll-like receptor (TLR) agonists can be utilized to develop novel therapeutic intervention strategies for the treatment of asthma using murine models of allergic inflammation. For this purpose five different TLR agonists were tested in preclinical mouse models of acute and chronic asthma, both in preventive and therapeutic settings. Firstly, TLR-2, 3, 4, 7/8 and 9 agonists were delivered intratracheally at different doses before pulmonary allergen exposure in the asthma model of acute inflammation. TLR9 agonist CpG-containing oligodeoxynucleotides (CpG) > TLR7 agonist Resiquimod (R848) > TLR3 agonists poly(I:C) strongly reduced allergen induced airway eosinophilia and IL-4 levels in a dose-dependent manner. All TLR agonists increased neutrophil numbers, TLR4 agonist lipopolysaccharide (LPS) > TLR2 agonist lipoteichonic acid (LTA) > poly(I:C) > CpG > R848 and, with the exception of R848, the amount of pro-inflammatory cytokines in the airways. Suppressive effects were not dependent upon IFN-γ and IL-10 or associated with increased numbers of regulatory T cells in the airways. All TLR agonists, except LTA, similarly reduced airway eosinophilia and IL-4 levels when applied therapeutically after allergen challenge. These results show that the TLR agonists have different suppressive effects on T_H2 responses in the airways which further depend on the dose and the experimental setup in which they were tested. Interestingly, all agonists induced airway neutrophilia, albeit to different degrees, raising the question if TLR ligands are safe for human use when applied directly into the lung. Different TLR agonists are also being developed for human use as adjuvants combined with allergen in specific immunotherapy. Recent clinical data suggest that this may be achieved by induction of allergen-specific T_H1 responses. For this reason, the ability of different TLR agonists to induce allergen-specific T_H1 and suppress allergen-specific T_H2 responses in a preclinical setting was investigated in this thesis. Different doses of the TLR agonists were applied together with allergen, then mice were exposed to allergen aerosol. CpG > LPS >LTA dose-dependently strongly suppressed the development of airway eosinophilia with poly(I:C) and R848 having no effect. The decrease in eosinophilic numbers was associated with

increased neutrophils present in the airways. IL-4 and IL-5 levels in the bronchoalveolar lavage fluid were also decreased when poly(I:C), LPS, and CpG were used. All TLR agonists increased allergen-specific IgG2a, and with the exception of poly(I:C), reduced allergen-specific IgE levels in the serum. Cutaneous anaphylaxis to allergen was completely prevented when LPS or CpG were given as adjuvant. The strongest T_H1 responses were induced by CpG and poly(I:C), characterized by the presence of IFN-γ in the bronchoalveolar lavage and the highest allergen-specific IgG2a levels in the serum. This data supports approaches to use TLR9 or TLR4 agonists for human therapy as adjuvant in combination with allergen in novel specific immunotherapy formulations.

In the last part of the thesis, it was investigated if TLR activation can also affect the pathology of severe chronic asthma. In order to be able to measure potential TLR agonist-mediated effects, a novel mouse model was established. In this model, mice were first sensitized against a mix of ovalbumin, and extracts from house dust mite and cockroach. In the challenge phase, the single allergens were given in alternating order, one allergen per week. This triple allergen treatment induced a much stronger pathology in the lung than previous models published. Using this model, therapeutic administration of R848 or CpG reduced features of inflammation and remodeling. Both agonists showed superior effects to dexamethasone, with CpG being more efficient than R848. This result again supports a TLR9-based therapy as a viable option for the treatment of severe chronic asthma which may present a potential alternative for anti-inflammatory therapy with steroids.

Taken together, the results of this thesis support the use of TLR agonists to treat asthma. The most favorable efficacy/safety ratio is to be expected from TLR-based therapies combining TLR4 or TLR9 agonists with allergen in specific immunotherapy. In regard to TLR agonist monotherapy, R848 and CpG showed the most promising profiles, CpG particularly in a model of severe chronic asthma. However, since all TLR agonists used in this study also showed pro-inflammatory potential, the safety aspect of such an approach needs to be taken into account.

ZUSAMMENFASSUNG

In den letzten Jahrzehnten wurde für Asthma ein Anstieg der Neuerkrankungen und der schweren Krankheitsverläufe verzeichnet. Moderates Asthma ist heutzutage medikamentös gut kontrollierbar, jedoch besteht für schweres chronisches Asthma ein hoher medizinischer Bedarf an neuen, wirksamen Medikamenten. Des Weitern kontrollieren angewandte Therapien zwar Symptome, bieten aber keine Heilung. Ein vielversprechender Ansatz, mit dem Ziel den ursächlichen Krankheitsmechanismus zu inhibieren, ist die TLR Agonisten induzierte Immunmodulation.

Ziel der vorliegenden Arbeit war es, die Eignung von immunstimulatorischen Toll-like Rezeptor (TLR) Agonisten für neue Therapieansätze in allergischen Entzündungsmodellen zu untersuchen. Hierfür wurden fünf verschiedene TLR Agonisten in murinen Modellen von akutem oder chronischem Asthma, sowohl prophylaktisch als auch therapeutisch verabreicht. Als erstes wurden in einem Modell mit akuter Entzündungsreaktion verschiedene Konzentrationen der Agonisten für TLR 2, 3, 4, 7 und 9, vor der pulmonalen Allergenexposition intratracheal appliziert. Hier verminderten TLR9 Agonist CpG-Oligodesoxynukleotide (CpG) > TLR7 Agonist Resiquimod (R848) > TLR3 Agonist poly(I:C) konzentrationsabhängig die allergen-induzierte Eosinophilie in den Atemwegen. Alle TLR Agonisten erhöhten die Anzahl an Neutrophilen, am stärksten TLR4 Agonist Lipopolysaccharid (LPS) > TLR2 Agonist Lipoteichon Säure (LTA) > poly(I:C) > CpG > R848. Weiterhin erhöhten, bis auf R848, alle TLR Agonisten die Menge an pro-inflammatorischen Zytokinen in den Atemwegen. Die hierbei beobachteten suppressiven Effekte waren weder IFN-γ noch IL-10 abhängig und korrelierten auch nicht mit einer Erhöhung der pulmonalen regulatorischen T Zellen. Die therapeutische Gabe von TLR Agonisten nach Allergenexposition reduzierte ebenfalls die Eosinophilie sowie IL-4 in den Atemwegen. Diese Ergebnisse zeigen, dass sich die TLR Agonisten in ihrer suppressiven Wirkung stark unterscheiden, und dass ihre Wirkung zum einen von der verabreichten Konzentration und zum anderen von dem experimentellen Aufbau abhängig ist. Auffällig war, dass alle Agonisten, wenngleich in unterschiedlicher Ausprägung, eine Neutrophilie in den Atemwegen induzierten. Dies wirft die Frage auf, ob eine wiederholte pulmonale Gabe für den Menschen verträglich wäre.

Ein anderer Ansatz verwendet TLR Agonisten als Adjuvanzien für die Kombination mit Allergenen in der spezifischen Immuntherapie. Aktuelle klinische Ergebnisse deuten darauf hin, dass die TLR vermittelte Erhöhung der allergen-spezifischen T_H1Antwort die Effektivität der Therapie steigern kann. Deswegen wurden in der vorliegenden Arbeit die verschieden

TLR Agonisten auf ihre Fähigkeit hin untersucht allergen-spezifische T_H1 Antworten auszulösen und allergen-spezifische T_H2 Antworten zu unterdrücken. Hierfür wurde Allergen zusammen mit verschiedenen Konzentrationen der TLR Agonisten appliziert und anschließend die Mäuse Allergen-Aerosol ausgesetzt. Hier konnte eine starke, konzentrationsabhängige Unterdrückung der Atemwegseosinophilie, begleitet von einer Neutrophilie, bei CpG > LPS >LTA beobachten werden. Poly(I:C) und R848 zeigten keine Effekte. Auch wurde die Menge von IL-4 und IL-5 in der bronchoalveolaren Lavage durch poly(I:C), LPS, und CpG erniedrigt. Weiterhin reduzierten alle TLR Agonisten, mit der Ausnahme von poly(I:C), die Menge an allergen-spezifischem IgE im Serum. Die kutane anaphylaktische Reaktion gegen das Allergen wurde durch CpG- oder LPS-Adjuvans komplett verhindert. Die stärkste T_H1 Antwort, charakterisiert durch erhöhtes IFN-γ in der Lavage und die größte Menge an allergen-spezifischem IgG2a, wurde durch CpG und poly(I:C) ausgelöst. Diese Resultate unterstützen den klinischen Ansatz CpG als erfolgsversprechenden Adjuvants-Kandidaten für die Kombinationstherapie mit Allergen in der spezifischen Immuntherapie einzusetzen.

Im letzten Teil der vorliegenden Arbeit wurde untersucht ob die Aktivierung von TLRs auch den Krankheitsverlauf von schwerem chronischem Asthma beeinflussen kann. Hierfür wurde ein neues Mausmodel entwickelt, in welchem die Tiere zunächst gegen eine Mischung aus Ovalbumin-, Hausstaubmilben- und Schaben-Extrakt sensibilisiert wurden. Während der Expositionsphase wurde wöchentlich jeweils eines dieser Allergene gegeben. Im Vergleich zu bereits publizierten Modellen induzierte die Behandlung mit drei Allergenen ein deutlich schwereres Krankheitsbild in der Lunge. In diesem Model reduzierten die beiden TLR Agonisten CpG und R848 Faktoren des Atemwegumbaus und der Entzündung effektiver als das Steroid Dexamethasone, wobei CpG die höchste Effektivität aufwies. Dieses Ergebnis unterstützt ebenfalls eine auf TLR9 Agonisten basierende Therapie als einen vielverspechenden Ansatz für die Behandlung von schwerem chronischem Asthma auch als eine potentielle Alternative zur antiinflammatorischen Therapie mit Steroiden.

Zusammenfassend unterstützen die Resultate die Verwendung von TLR Agonisten für die Behandlung von Asthma. Die höchste Effektivität und Verträglichkeit ist für eine TLR Allergen Kombinationstherapie mit TLR4 oder TLR9 Agonisten in der spezifischen Immuntherapie zu erwarten. Für eine mögliche TLR Monotherapie zeigten R848 und CpG die besten Wirkungsprofile, für schwereres chronisches Asthma bevorzugt CpG. Hierbei muss jedoch stets berücksichtigt werden, dass TLR Agonisten auch selbst entzündliche Reaktionen hervorrufen können.

Curriculum Vitae

Dissertation

Since 09/2007   **Respiratory Diseases Research, Boehringer Ingelheim, Biberach**

Topic of Thesis:

Effects of toll-like receptor agonists on the pathogenesis of atopic

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Thesis committee:

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University

10/2001–05/2007   **Study of Biology at the University of Würzburg**

Main subjects studied:

Microbiology, Biochemistery and Biotechnology

Degree: in each case "sehr gut" (= very good; best possible degree)

Title of diplomathesis:

Construction and evaluation of *L. monocytogenes* strains as novel

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01/2004-06/2004   **Research project at Umeå University in Umeå, Sweden**

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Placements

05/2007-08/2007   **Placement in research division of the clinic and policlinic for dermatology, Würzburg, Germany**

Topic: Analysis of melanoma cell migration by confocal microscopy and live cell imaging

07/2005-09/2005   **Industrial Placement in the division of quality control at the company L&S AG, Bad Bocklet, Germany**

Project work: Efficacy analysis of different disinfectants on company specific surfaces

Training and courses

Since 06/2008   Associated member of the graduate school "Research Training Group 1331", Konstanz, Germany

05/2011   Animal models for therapeutic research
informa life science, Berlin

11/2010   New Drugs and Targets for Asthma and COPD
National Heart & Lung Institute, London

10/2010   2nd Autumn School, Current Concepts in Immunology, German Association of Immunology, Bad Schandau, Germany

01/2010   2. Allergie Akademie, German Association of Allergology and Clinical Immunology, Munich, Germany

09/2009–04/2010   Lecture and exam in "Pharmacology and Toxicology II", University of Konstanz, Germany

09/2009   2nd European Congress of Immunology, Berlin

03/2009   5. Spring school on Immunology, German Association of Immunology, Ettal, Germany

03/2009   21. Mainzer Allergie-Workshop**,** German Association of Allergology and Clinical Immunology, Mainz, Germany

06/2008   Advanced Scientific writing course, Biberach, Germany

07/2007   Course: animal welfare and experimental animal handling, University of Würzburg

Publications

[derived from the current thesis]

TLR-agonist mediated suppression of allergic responses is associated with increased innate inflammation in the airways
Matthias J. Duechs, Christian Hahn, Ewald Benediktus, Melanie Werner-Klein, Armin Braun, Hans Gerd Hoymann, Florian Gantner and Klaus J. Erb
Pulmonary pharmacology & therapeutics; 2011 Apr; 24(2):203-14.

Adjuvant effects of different TLR agonists on the induction of allergen-specific Th2 responses
Matthias J. Duechs, James Brunt, Florian Gantner, and Klaus J. Erb
(submitted)

[others]

Probiotic Escherichia coli Nissle 1917 suppresses allergen-induced Th2 responses in the airways
Bickert T, Trujillo-Vargas CM, Duechs M, Wohlleben G, Polte T, Hansen G, Oelschlaeger TA, Erb KJ. International Archives of Allergy and Immunology; 2009;149(3):219-30

Induced Syk deletion leads to suppressed allergic responses but has no effect on neutrophil or monocyte migration in vivo
Eva Wex, Thierry Bouyssou, Matthias J Duechs, Klaus J Erb, Florian Gantner, Michael P Sanderson, Andreas Schnapp, Birgit E Stierstorfer, and Lutz Wollin, European Journal of Immunology; 2011 Aug 10. (Epub ahead of print)

Posters:

14th International Congress of Immunology, Kobe, Japan, 22-27. August 2010
Title: **TLR-agonist mediated suppression of asthmatic-responses is associated with increased innate inflammation in the airways**
Matthias J. Düchs, Christian Hahn, Florian Gantner and Klaus J. Erb.

2nd Congress of Immunology, Berlin, Germany, 13-16. September 2009
Title: **Novel mouse model of chronic asthma**
Matthias J. Düchs and Klaus J. Erb.

Oral presentation:

21. Mainzer Allergie-Workshop, Mainz, 19.–20. March 2009
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German Association of Allergy and Clinical Immunology
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