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Mechanisms of allergen-specific immunotherapy and new concepts for the therapy of hypersensitivity reactions in the context of house dust mite allergy

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Abbreviations

AIT, Allergen-specific immunotherapy

Alum, Aluminum hydroxide / Al(OH)3

APC, Antigen-presenting cell

BAL, Bronchoalveolar lavage

BALF, Bronchoalveolar lavage fluid

CCL, Chemokine ligand

CD, Cluster of differentiation

Cys-LT, Cysteinyl leukotriene

DC, Dendritic cell

Der p, Dermatophagoides pteronyssinus

Der f, Dermatophagoides farinae

EAACI, European Academy of Allergy and Clinical Immunology

EGF, Epidermal growth factor

ELISA, Enzyme-linked immunosorbent assay

FACS, Fluorescence-activated cell sorting

FcεR, Fc epsilon receptor

FGF, Fibroblast growth factor

FoxP3, Forkhead box P3

GATA3, GATA-binding protein 3

GM-CSF, Granulocyte-macrophage colony-stimulating factor

HBGF, Heparin-binding growth factors

HD-extract-AIT, High-dose HDM extract AIT

HDM, House dust mite

IFN-g, Interferon gamma

IgE, Immunoglobulin E

IGF, Insulin-like growth factor

IgG, Immunoglobulin G

IgM, Immunoglobulin M

IL, Interleukin

ILC2, Group 2 innate lymphoid cell

i.n., Intranasal

i.p., Intraperitoneal

LD-allergoid-AIT, Low-dose HDM allergoid AIT

LPS, Lipopolysaccharide

MCT, MicroCrystalline Tyrosine

MHC-II, Major histocompatibility complex II

MMP, Matrix metalloproteinases

MPL, Monophosphoryl lipid A

NGF, Nerve growth factor

NLPR3, NLR family pyrin domain containing 3

NPC2, Niemann-Pick Disease Type C2

PAR, Protease activated receptor

PDGF, Platelet-derived growth factor

PHA, Phytohaemagglutinin

s.c., Subcutaneous

SCF, Stem cell factor

SCIT, Subcutaneous immunotherapy

sIgE, Specific immunoglobulin E

sIgG, Specific immunoglobulin G

SLIT, Sublingual immunotherapy

SNP, Single nucleotide polymorphism

SPF, Specific pathogen free

ST2, Suppression of tumorigenicity 2

TGF, Transforming growth factor

TNF, Tumor necrosis factor

tIgE, Total immunoglobulin E

tIgG, Total immunoglobulin G

Th1, T helper 1

Th2, T helper 2

Th17, T helper 17

TLR, Toll-like receptor

TNF-a, Tumor necrosis factor–alpha

Treg, Regulatory T cell

TSLP, Thymic stromal lymphopoietin

VEGF, Vascular endothelial growth factor

1 Abstract

Allergen-specific immunotherapy (AIT) is currently the only potentially curative treatment for IgE-mediated type I hypersensitivity reactions that induces long-term tolerance. For more than 100 years, AIT is used to treat allergies by modifying the disease course. The mechanisms of successful AIT are not fully understood yet and the treatment efficacy varies between different types of allergy. House dust mites (HDMs) represent a major source of indoor allergens associated with allergic rhinitis, atopic dermatitis and allergic asthma. Safe and efficient treatment strategies of HDM-allergic patients are of major clinical relevance because they are also exposed to natural HDM contact during the immunotherapy treatment course.

In this study, a new, physiological, murine HDM allergen-specific immunotherapy model based on intranasal sensitization (i.n.) as well as subcutaneous (s.c.) therapy was established. Here, allergy-induced and protective AIT mechanisms were investigated which are sometimes hard to address in human studies. The new murine model resembles human HDM allergy in many aspects: the typical HDM allergy-driven eosinophilic and T helper 2 cell (Th2) infiltration into the lungs, decreased lung dynamic compliance, reduced mucus hypersecretion, HDM-specific immunoglobulin E (IgE) induction as well as elevated Th2-type cytokine secretion by lung-resident lymphocytes, lymph node cells and splenocytes. In addition, the model also displays important hallmarks of successful AIT in humans such as the induction of HDM specific immunoglobulin G (sIgG) antibodies while specific IgE (sIgE) levels do not immediately decrease.

Beside conventionally used HDM extracts, chemically modified HDM extracts, so called HDM allergoids, got into the focus of the development of new treatment strategies and allergy vaccines. The new murine AIT model was used to investigate the therapeutic potential of HDM allergoids and adjuvants. This study revealed that AIT with low-dose HDM allergoids in

combination with the depot adjuvant MicroCrystalline Tyrosine (MCT) and the T helper 1 cell-inducing (Th1) adjuvant monophosphoryl lipid A (MPL) has the potential to promote Th1 mechanisms and robust humoral immunoglobulin G (IgG) response counterbalancing the HDM allergy-driven Th2 immune response in a comparable manner to high-dose HDM extract AIT. Furthermore, the study demonstrated that not only allergen content, but also the adjuvant dosage of MPL plays a crucial role regarding the anti-inflammatory capacity of AIT.

Additionally, *ex vivo* stimulation experiments revealed that HDM allergoids with their chemically modified IgE epitopes are less potent to induce the allergen-mediated Th2 immune response in human peripheral blood mononuclear cells (PBMCs) compared to HDM extract. Moreover, the potential of the adjuvant MPL to shift the allergy-induced Th2 immune response towards a Th1 cytokine milieu could be demonstrated.

In summary, this doctoral thesis provides a side-by-side comparison of high-dose HDM extract- and low-dose HDM allergoid-based AIT and reveals that low allergen doses can induce cellular and humoral mechanisms counteracting Th2-driven inflammation by using HDM allergoids in combination with the depot adjuvant MCT and dose-adjusted Th1-inducing adjuvant MPL. Future therapeutic approaches may use low-dose allergoid AIT to drive cellular tolerance and adjuvants to modulate humoral, potentially protective immune responses.

2 Zusammenfassung

Die Allergen-spezifische Immuntherapie (AIT) ist derzeit die einzige potenziell kurative Behandlung von IgE-vermittelten Typ-I Hypersensitivitätsreaktionen, welche langfristig Toleranz induziert. Seit über 100 Jahren wird die AIT dazu genutzt Allergien durch den Eingriff in den Krankheitsverlauf zu behandeln. Die Mechanismen einer erfolgreichen AIT sind bisher nicht vollständig verstanden und der Behandlungserfolg variiert zwischen verschiedenen Allergiearten. Hausstaubmilben (HDMs) stellen eine Hauptquelle für Innenraumallergenen dar, welche mit allergischer Rhinitis, atopischer Dermatitis und allergischem Asthma in Verbindung gebracht werden. Sichere und effiziente Behandlungsstrategien HDM-allergischer Patienten sind von großer klinischer Bedeutung, da diese auch während des Behandlungsverlaufs der Immuntherapie dem natürlichen Kontakt zu HDM ausgesetzt sind.

In dieser Studie wurde ein neues, physiologisches, murines HDM Allergen-spezifisches Immuntherapie Model etabliert, welches auf intranasaler (i.n.) Sensibilisierung und subkutaner (s.c.) Therapie beruht. Dabei wurden allergieinduzierte und protektive AIT Mechanismen untersucht, welche in humane Studien oftmals schwer zu adressieren sind. Das neue murine Model spiegelt in vielen Punkten die Mechanismen humaner HDM Allergie wider: Die typische HDM allergievermittelte Infiltration von Eosinophilen und Typ2-T-Helferzellen (Th2) in die Luge, eine reduzierte dynamische Compliance der Lunge, die reduzierte Hypersekretion von Schleim, die Induktion von HDM-spezifischem Immunglobulin E (IgE) und eine erhöhte Sekretion von Th2 Cytokinen durch Lungenlymphozyten, Lymphknotenzellen und Milzzellen. Zusätzlich dazu bildet das Model wichtige Kennzeichen einer erfolgreichen AIT im Menschen ab, wie die Induktion HDM-spezifischer Immunglobulin G (sIgG) Antikörper während spezifische IgE (sIgE) Level nicht unmittelbar absinken.

Neben konventionell genutzten HDM Extrakten, rücken chemisch modifizierte HDM Extrakte, sogenannte HDM Allergoide, in den Fokus der Entwicklung neuer Behandlungsstrategien und Allergie Vakzinen. Das neue murine AIT Model wurde dazu angewendet das therapeutische Potential von HDM Allergoiden und Adjuvantien zu untersuchen. Diese Studie offenbarte, dass AIT mit niedrigdosierten HDM Allergoiden in Verbindung mit dem Depot-Adjuvant MicroCrystalline Tyrosine (MCT) und dem Typ1-T-Helferzellen-induziernenden (Th1) Adjuvant monophosphoryl lipid A (MPL) das Potential hat Th1 Mechanismen und eine solide humorale Immunglobulin G (IgG) Antwort auszulösen, die der HDM allergievermittelten Th2 Immunantwort vergleichbar zu hochdosierter HDM Extrakt AIT entgegenwirken. Außerdem zeigte die Studie, dass nicht nur der Allergen Gehalt, sondern auch die Dosierung des Adjuvant MPL eine essentielle Rolle in Hinsicht auf die anti-inflammatorische Eigenschaft der AIT hat.

Zusätzlich dazu zeigten ex vivo Stimulationsversuche, dass HDM Allergoide mit ihren chemisch modifizierten IgE Epitopen im Vergleich zum HDM Extrakt die allergenvermittelte Th2 Immunantwort in humanen mononukleären Zellen des peripheren Blutes (PBMCs) weniger stark induzieren. Des Weiteren konnte das Potential des Adjuvants MPL nachgewiesen werden die Allergen-induzierte Th2 Immunantwort in Richtung eines Th1 Cytokinmilieus zu verschieben.

Zusammenfassend liefert diese Doktorarbeit einen genüberstellenden Vergleich von hochdosierter HDM Extrakt und niedrigdosierter HDM Allergoid AIT und sie veranschaulicht, dass geringe Allergendosen zelluläre und humorale Mechanismen induzieren können, die der Th2-induzierten Inflammation entgegenwirken, wenn HDM Allergoide in Kombination mit dem Depot Adjuvant MCT und dem in der Dosis angepassten Adjuvant MPL verwendet werden. Zukünftige therapeutische Konzepte könnten niedrigdosierte Allergoid AIT dazu nutzen, um zelluläre Toleranz zu induzieren und Adjuvantien, um die humorale, möglicherweise protektive Immunantwort zu modulieren.

3 Introduction

3.1 Allergic diseases

Allergic diseases are one of the most common chronic diseases and include atopic eczema/dermatitis, asthma, allergic rhinitis and rhinoconjunctivitis, food allergy, and venom allergy [1]. The term allergy was first postulated by Clemens von Pirquet in 1906 and he wanted to generally describe changes in the body's reactivity during the first contact of the immune system with an antigen [2]. Today, the word "allergy" is restricted to specific immunologic hypersensitivity reactions against harmless foreign antigens [2]. Although allergy was seen as a rather rare disease at the beginning of the 20th century, today, it is estimated that in 2025 about half of the European population will be affected [3].

3.2 Immunological mechanisms of allergic inflammation

In 1963, Philip Gell and Robert Coombs published a classification to characterize the different types of hypersensitivity reactions [4]. Within the last 60 years the knowledge of the underlying immune mechanisms of allergic reactions increased and their simple classification scheme is controversially discussed nowadays. Nevertheless, Gell and Coombs's scheme is still used and often referred to as a basic concept to describe hypersensitivity reactions. Their classification divides allergies into four pathophysiological types, the immediate hypersensitivity or anaphylaxis (type I), antibody-mediated cytotoxic reactions (type II), immune complex-mediated reactions (type III), and the delayed hypersensitivity (type IV) [5]. Type I reactions are immediate hypersensitivity or anaphylactic reactions [6] and patients suffer from one or a combination of the following clinical features: Asthma, rhinitis, conjunctivitis, urticaria, angioedema or cardio-respiratory (anaphylactic) shock [5]. Allergen-induced

crosslinking of receptor-bound IgE molecules activates mast cells and basophils, followed by degranulation and the release of inducers of type I allergy such as histamines, thromboxanes, leucotrienes and prostaglandins [5]. Type II reactions, such as agranulocytosis, thrombocytopenia and immune-allergic hemolytic anemia, are caused by cytotoxic antibodies, mainly immunoglobulin M (IgM) and IgG [7]. Antibodies are directed against antigens on individual's own cell surface followed by cytotoxic action by killer cells or complement-mediated cell lysis [5]. Type III reactions, such as allergic alveolitis in the lungs (farmer's lung), cutaneous vasculitis or serum sickness are also mediated by IgM/IgG binding to soluble antigen, followed by the elimination of circulating infectious agents by immune complexing and phagocytosis [5]. Allergic contact eczema, drug exanthemas or photocontact allergy are examples for Type IV reactions against contact allergens and are T cell-mediated. T effector cells are activated by antigen presenting cells followed by activation of macrophages and inflammatory response [5]. Following chapters will mainly focus on the immunological mechanisms of the Th2- and IgE-mediated type I hypersensitivity reaction displaying the pathomechanisms of the most common allergic diseases, such as house dust mite allergy. Here, the mechanism of allergic immune response can be divided into different stages: The allergen sensitization, the acute allergic inflammation against specific allergens and the chronic inflammation after repeated allergen exposure [8].

3.2.1 Sensitization phase

The sensitization phase to an allergen reflects the allergen's ability to induce a Th2-driven immune response, in which Interleukin-4 (IL-4) and IL-13 mediate allergen-specific IgE production by promoting immunoglobulin class-switch recombination in B cells (Figure 1) [8]. There are different routes of allergen exposure such as inhalation (pollen, spores, …), injection (venom), ingestion (foods) or skin contact. Then, allergens can either be sampled by dendritic cells (DCs) and enter tissues through disrupted epithelium (not shown) or, for some allergens

with protease activity, can gain access to submucosal dendritic cells by cleaving epithelial-cell tight junctions. After allergen uptake, DCs migrate to local lymph nodes and present processed allergen peptides via major histocompatibility complex II (MHC-II) molecules to naïve cluster of differentiation 4^+ ($CD4^+$) T cells [9]. In the presence of IL-4, potentially derived from different sources, such as basophils, mast cells, eosinophils, natural killer T cells and T cells, the naïve antigen-specific T cells differentiate into Th2 cells. This process is mediated by the receptor-ligand engagement of Notch at the surface of T cells with Jagged on dendritic cells. The mechanism of Th2 cell polarization is not only induced by the cytokine IL-4 but also promoted by other circumstances such as production of the Th2 cytokines IL-5 and IL-13 by group 2 innate lymphoid cells (ILC2s) in response to epithelium–derived IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) [10]. Here, in context of house dust mite allergy, inhaled house dust mite allergens and body compounds can act on airway epithelial cells via surface receptor signaling such as toll-like receptor 4 (TLR4), inducing the downstream release of cytokine danger signals, including IL-25, IL-33, chemokine ligand 20 (CCL20), TSLP and granulocyte-macrophage colony-stimulating factor (GM-CSF) [11-13] promoting the Th2 cytokine environment. Upon allergen contact Th2 cells produce IL-4 and IL-13 and via ligation of co-stimulatory molecules (CD40 with CD40 ligand, CD80 / CD86 with CD28) B cells undergo immunoglobulin class-switch recombination and IgE is secreted, enters the lymphatic vessels and is then distributed systemically via the blood. Allergen-specific IgE binds to the high-affinity receptor for IgE (FcεRI) on mast cells and basophils, sensitizing them to initiate an allergic immune response under allergen re-exposure. In contrast, the initial sensitization phase does not induce allergic symptoms. Various factors determine whether exposure with an allergen results in clinically significant sensitization and influence the susceptibility to develop allergic reactions. On the one hand, the individual's genotype can play a crucial role and here, over 100 loci harboring variations associated with increased risk for asthma and allergic phenotypes were identified [14-16]. For example, studies revealed that defects in epithelial

barrier function such as mutations in FLG coding for filaggrin increase the propensity of sensitization to allergens and developing atopic dermatitis by altered tissue barrier function [17-19]. Allergens encountered in the upper airways can contribute to the development of systemic immune responses that result in allergic disease at other sites exposed to that allergen (e.g. in the lungs) [8]. Furthermore, single nucleotide polymorphisms (SNPs) in genes coding for the epithelial cell-derived cytokine TSLP, IL-33 and its receptor ST2 (suppression of tumorigenicity 2) play a role in asthma and atopy susceptibility [20-23]. Moreover, other Th2-associated cytokines such as IL-4 and its receptor were identified as candidate genes for atopic health outcomes [24, 25]. Nevertheless, the genetic background is not the only factor contributing to an individual's propensity to develop allergic diseases. In 1989, David Strachan published the "hygiene hypothesis" [26]. He postulated that the cleaner environment and advanced living standards in western countries are the reason for an increased risk of developing atopic diseases because of the reduced contact with parasitic infections, other pathogenic and non-pathogenic microorganisms and their products. In Strachan's hypothesis, such infections contribute to an appropriate control of potentially harmful immune responses by various regulatory T cell (Treg) populations [27]. Reduced exposure to infections, for example with helminths, accompanied with increased contact to common environmental allergens, can further increase the propensity for genetically predisposed individuals to develop allergic reactions [28-30]. Building on Strachan's "hygiene hypothesis", in the last few years the "biodiversity hypothesis" in connection with allergic diseases was postulated [31, 32]. One important key player is the increased use of antibiotics in western countries leading to a reduced diversity of the body microflora and an increased risk for developing asthma [33]. Furthermore, the biochemical characteristics of allergens and other agents can enhance the sensitization process during allergen exposure. For example, allergens with proteolytic activities, such as Der p 1, a major house dust mite (HDM) allergen, can reduce epithelial barrier function or other proteases can hydrolyze CD23, CD25, CD40 and DC-SIGN followed by T cell activation [34].

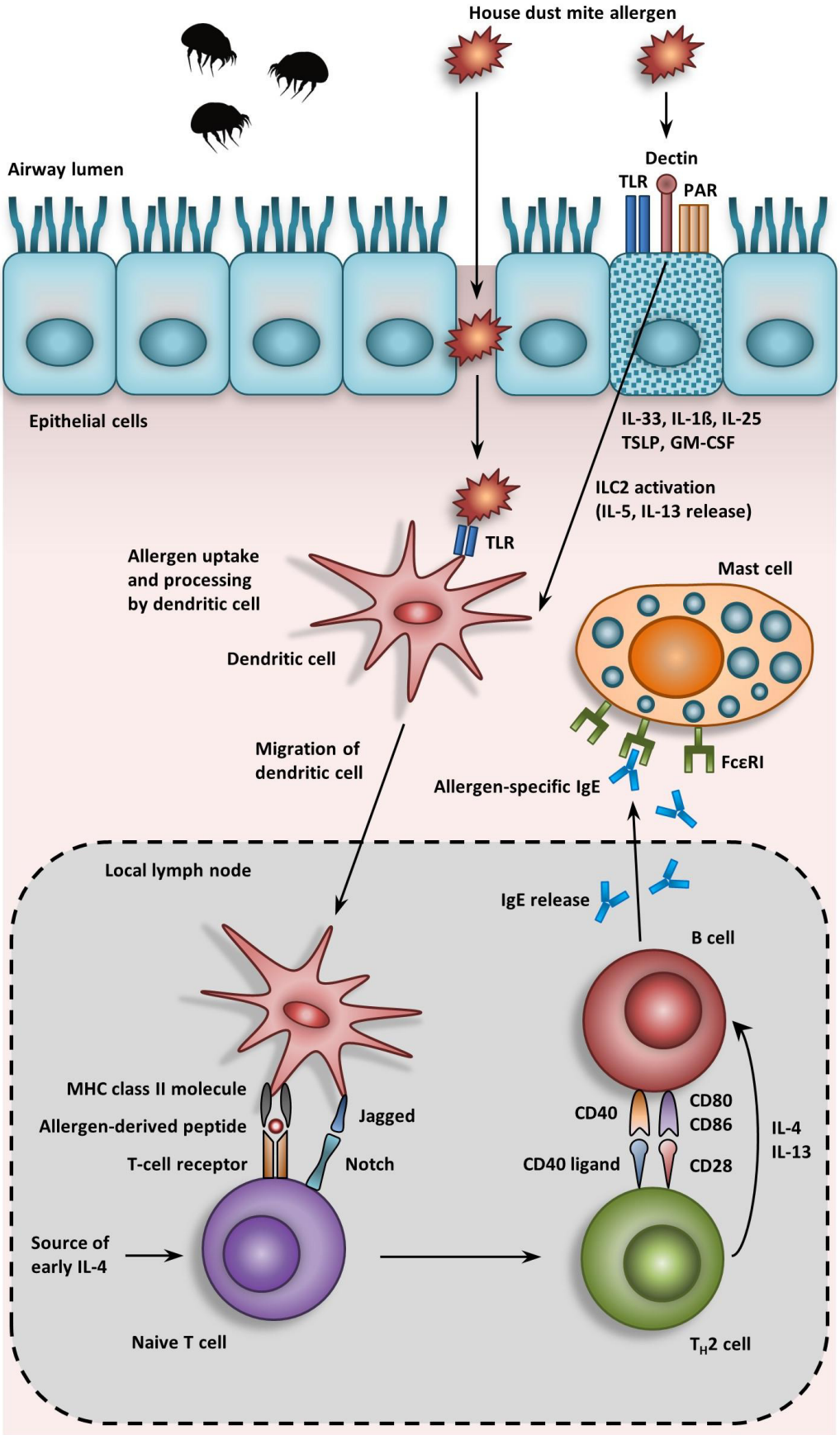

Figure 1: Sensitization phase

Figure is adapted from Galli *et al.* (2008)

Moreover, basophil activation by proteases followed by TSLP and IL-4 release promotes the initial sensitization for the development of Th2 cell- and IgE-mediated immune reactions [35, 36]. Other agents such as TLR ligands play a crucial role in the sensitization process. Lipopolysaccharides, which can usually promote Th1 cell immune response, enhance the development of Th2 cell immune response under low-level exposure [37, 38]. Other substances are chitin as inducer of IL-4 expression, found in many allergen sources, as well as other environmental pollutants [39-41]. Taken together, many factors such as host genotype, type of the allergen, allergen concentrations and the other co-stimulatory agents affect the likelihood of developing clinically relevant sensitization to allergens followed by allergic reactions.

3.2.2 Allergic inflammation

Allergic inflammation is divided into three main temporal phases: An early or acute phase induced within minutes after allergen contact, a late phase reaction occurring within hours and a chronic tissue inflammation triggered by persistent or repetitive local allergen exposure.

3.2.2.1 Early phase response

The early phase response or IgE-mediated type I immediate hypersensitivity reaction occurs within minutes after allergen exposure. Reactions can be local, such as acute asthma attacks, rhinitis and conjunctivitis or systemic leading to severe anaphylaxis (Figure 2) [8]. Allergen-specific IgE is bound to the high affinity FcεRI on the surface of mast cells or basophils. Allergen-induced IgE crosslinking mediates the aggregation of FcεRI followed by an intracellular signaling process that results in the secretion of biologically active products, mainly cytoplasmic granules, lipid-derived mediators, and synthesized cytokines, chemokines and growth factors [42] (Figure 2). The rapid secretion of these mediators contributes to vasodilation, bronchoconstriction, increased vascular permeability and mucus production. Furthermore, allergen-induced IgE-crosslinking on mast cells contributes to the late phase response by promoting the recruitment of pro-inflammatory leukocytes [8].

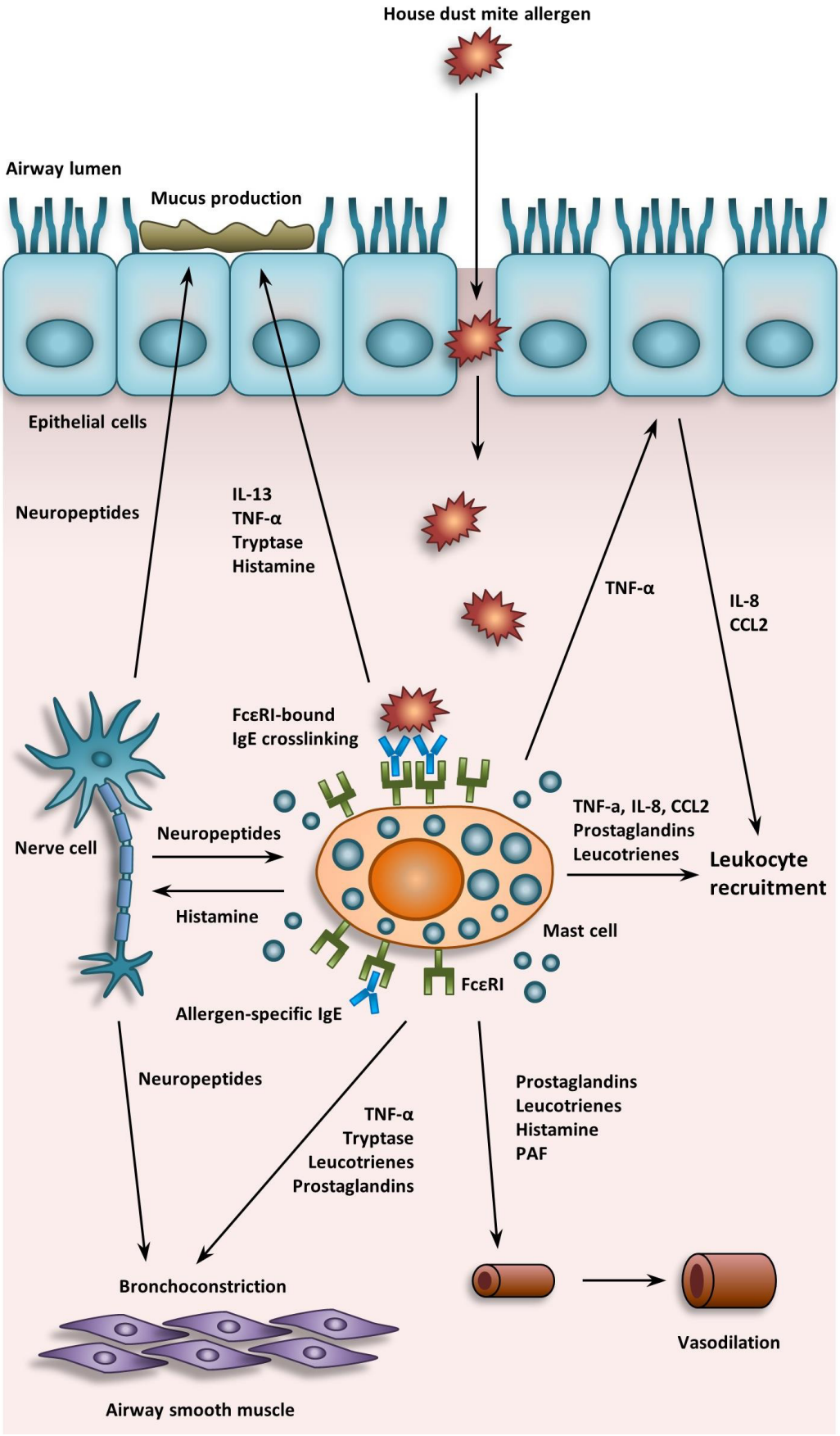

Figure 2: Early phase of allergic inflammation
Figure is adapted from Galli *et al.* (2008)

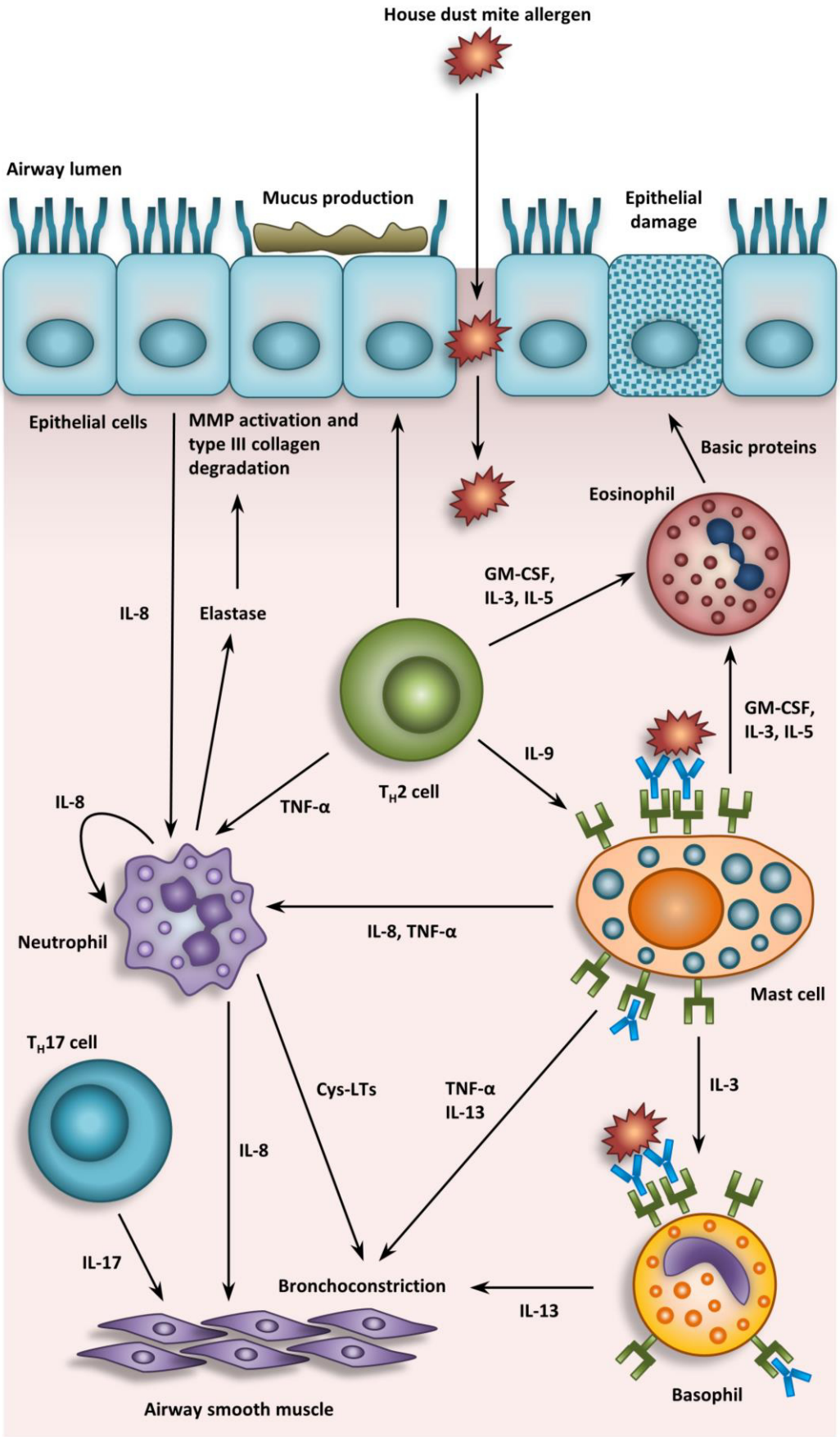

Figure 3: Late phase of allergic inflammation
Figure is adapted from Galli *et al.* (2008)

3.2.2.2 Late phase response

The late phase response occurs about 2-6 h after allergen exposure with a peak after 6-9 h and resolves within 1-2 days (Figure 3) [8]. It is initiated by the release of pro-inflammatory mediators by mast cells after allergen-induced IgE crosslinking accompanied with the local recruitment and activation of Th2 cells, eosinophils, neutrophils, basophils and other leucocytes (Figure 3). Some mast cell populations have the ability to rapidly release pre-stored mediators such as tumor necrosis factor–alpha (TNF-α) [43]. Mast cell-derived products can directly or indirectly influence immune cells via pro-inflammatory cytokine release including dendritic cells, T cells and B cells but also structural cells including vascular endothelial cells, epithelial cells, fibroblasts, smooth muscle cells and nerve cells [8]. In contrast, mast cells can also have immuno-suppressive and anti-inflammatory functions via IL-10 production which suppresses T cell proliferation and the expression of pro-inflammatory cytokines [44]. Furthermore, resident or recruited T cells that recognize allergen-derived peptides contribute to the development of late phase reactions [45]. At the early stage of response, Th2 cells mediate the recruitment of eosinophils and neutrophils [9, 46] and promote mucus production via IL-13 [47]. Furthermore, ILC2s are involved in the recruitment of eosinophils via IL-5 production [48]. Then, elastase released by neutrophils promotes the activation of matrix metalloproteinases (MMPs) and the degradation of type III collagen. Basic proteins secreted by eosinophils injure epithelial cells and in addition, other mediators produced by recruited or tissue-resident cells induce bronchoconstriction [8].

3.2.2.3 Chronic allergic inflammation

Persistent or repetitive contact to allergens, the individual is sensitized to, accompanied with large numbers of innate and adaptive immune cells at the affected site can lead to dysregulated and prolonged tissue inflammation resulting in altered count, phenotype and function of structural cells in the affected tissue (Figure 4) [8]. The process how persistent or repetitive allergen exposure promotes the change from early phase and late phase reactions to

chronic allergic inflammation is not fully understood yet. In chronic asthma, inflammation affects all airway wall layers. Innate immune cells including eosinophils, basophils, neutrophils and monocyte-macrophage lineage cells as well as adaptive immune cells such as Th2 cells, other types of T cells, and B cells, accumulate in the affected tissue. Furthermore, increased numbers of mast cells and basophils occur activated by allergen-induced IgE crosslinking. Pro-inflammatory mediator release leads to complex interactions between tissue-resident and recruited innate and adaptive immune cells, epithelial cells, blood and lymphatic vessels, nerves and structural cells including fibroblasts, myofibroblasts and airway smooth muscle cells [8, 49]. Repetitive and persistent epithelial injury can be elevated by exposure to pathogens or environmental factors. Repair response mechanisms can lead to formation of an epithelial-mesenchymal trophic unit (EMTU) and a sustained Th2 cell-associated inflammation [50, 51]. This promotes the additional sensitization to other allergens or allergen epitopes (e.g., via upregulation of the expression of co-stimulatory molecules on dendritic cells). In atopic dermatitis and allergic rhinitis, as well as in asthma, chronic allergic inflammation leads to airway tissue remodeling [8, 52, 53]. This results in long-term tissue changes and substantial alterations in the epithelial barrier function. Strong functional impairment of the lung is characterized by thickening of the airway walls such as epithelium, lamina reticularis, submucosa and smooth muscle, increased deposition of extracellular-matrix proteins including fibronectin, and type I, III and V collagen, and goblet cell metaplasia, which is associated with increased mucus production [8].

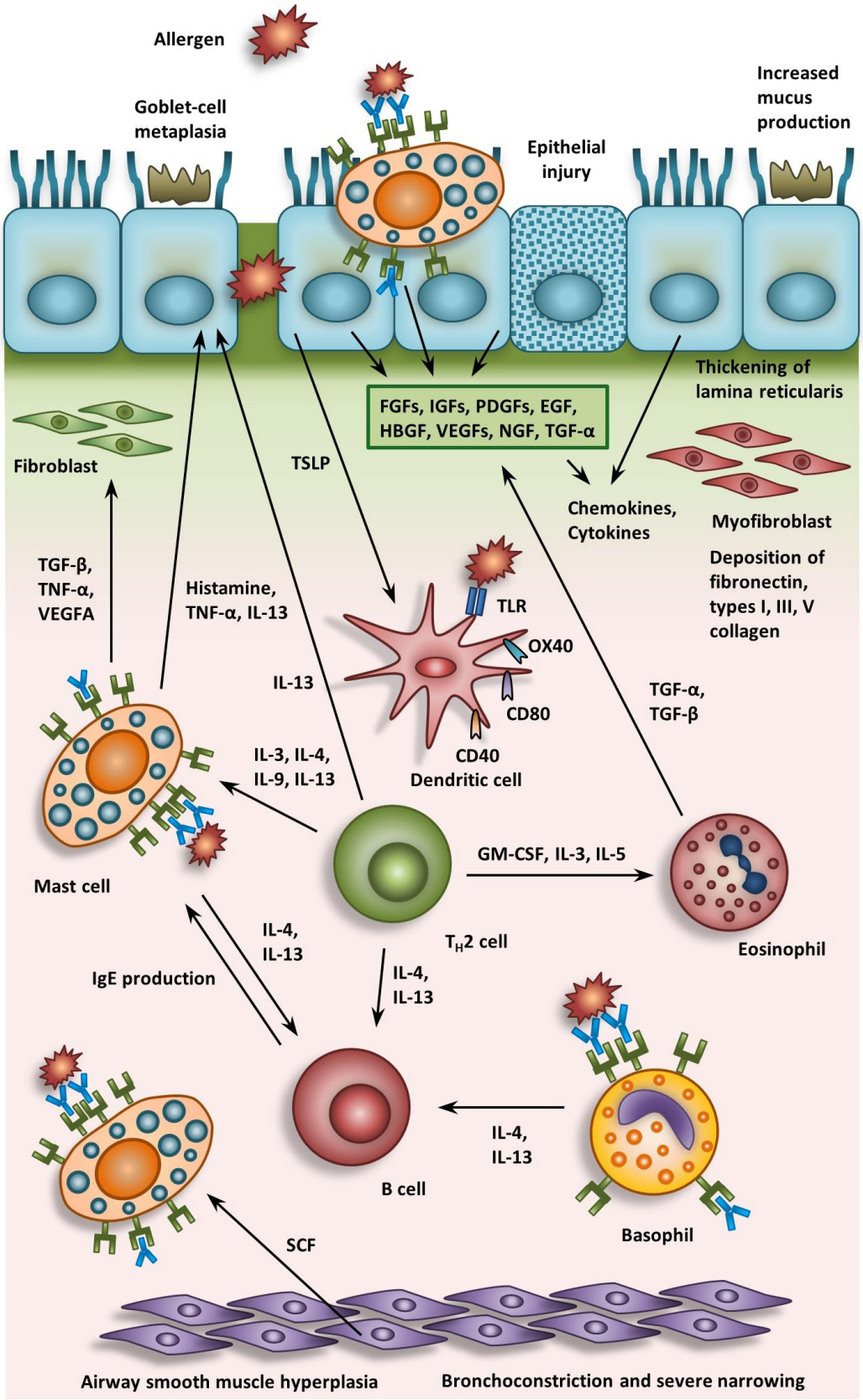

Figure 4: Chronic allergic inflammation
Figure is adapted from Galli *et al.* (2008)

3.3 House dust mite allergy

House dust mites represent a major source of indoor allergens associated with allergic rhinitis, atopic dermatitis and asthma and the development of type I hypersensitivity reactions mediated by allergen-specific IgE [54]. About 1-2% of the world's population are affected by HDM allergy, which is equivalent to 65 to 130 million individuals worldwide [55]. The HDM species *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f) are the principal sources of sensitizing HDM allergens in Europe [55, 56]. To date, 39 HDM allergen groups are annotated in the World Health Organization and International Union of Immunological Societies (WHO/IUIS) allergen database [57]. In detail, 33 include identified *Der f* allergens and 23 include identified *Der p* allergens, characterized by molecular profile and activity. The identification and characterization of HDM allergens, particularly the detection and naming of novel HDM allergens, is clinically relevant for the diagnosis and treatment of HDM-induced allergic diseases. HDM allergens from group 1 and 2 (i.e., Der p 1 and Der f 1, and Der p 2 and Der f 2) as well as group 23 (Der p 23 and Der f 23) are the major allergens in context of HDM allergy. Due to the considerable sequence and structure homology within the HDM allergen groups (80-90% within group 1 and 2 from Der p and Der f), HDM-specific IgE are usually not species-specific and cannot distinguish between allergens from the same group derived from different HDM species [58]. Group 1 and 2 allergens bind over 50% of HDM sIgE and induce HDM sIgE in about 80% of mite-sensitized patients [59]. Der p 1 and Der f 1 were characterized as cysteine proteases [60, 61] and Der p 2 and Der f 2 are part of the Niemann-Pick Disease Type C2 (NPC2) secretory protein family [62, 63]. Recently, Der p 23 and Der f 23, peritrophin-like proteins, were added to the group of major HDM allergens [64-66]. Der p 23 has been demonstrated to react with sIgE from 74% of Der p-allergic patients [65]. Most of the remaining IgE binding proportion is related to contributions from the HDM allergen groups 4, 5, 7, and 21 [67]. Whereas, HDM allergen groups 3, 8, 10 and 20 are reported

to induce rather low titers [59]. Some HDM allergens are widely cross-reactive among other invertebrates and linked to food allergy to crustaceans and snails. Der p 10 is thought to be responsible for the cross-reactivity to shrimps [68, 69] and Der p 4, 5 and 7 for the cross-reactivity to snails [70]. Interestingly, more than 95% of the allergens accumulating in HDM species are associated with faecal particles [71, 72]. House dust mite-derived particles are often found in pillows, mattresses, carpeting and upholstered furniture, float into the air when anyone vacuums, walks on a carpet or makes the bed and the mites settle down when the disturbance is over. Therefore, safe and efficient treatment strategies of HDM-allergic patients, who cannot evade natural exposure to HDM during initiation of allergen-specific immunotherapy (AIT), are of major clinical relevance [73].

3.4 Allergen-specific immunotherapy

Up to now there are two different main therapeutic approaches to treat allergic diseases. First, the use of therapeutic agents which suppress allergic inflammation accompanied with decreased allergic symptoms. Here, drugs such as antihistamines, inhaled corticosteroids, agonists of β-adrenergic receptors, omalizumab and others are used for allergy treatment. These can be effective in many patients, but not in all, have to be applied permanently and are not appropriate as causal treatment strategies [74]. The second beneficial approach is allergen-specific immunotherapy (AIT) which aims to induce a sustained allergen-specific tolerance [75-77]. It has been in use for over 100 years [78] and the following part will mainly focus on the immunological AIT mechanisms. AIT is the only potentially curative and long-lasting treatment of IgE-mediated type I hypersensitivity reactions. It is effective in reducing symptoms of venom-induced anaphylaxis, allergic asthma and rhinitis [79]. Currently, subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) are the two types of AIT in clinical practice but also several other new treatment strategies were evaluated in clinical trials within the last few years [73]. Although AIT has a long and successful clinical history, the exact

mechanisms are still not fully understood. Nevertheless, there are some mechanistical hallmarks of action (Figure 5). The course of successful AIT can be classified into four groups [75-77]. First, the rapid desensitization of mast cells and basophils by allergens leading to a decreased tendency for allergic symptoms and systemic anaphylaxis. The second part is the induction of allergen-specific regulatory T and B cells accompanied with the production of different mediators, cytokines, chemokines, and growth factors that could reduce inflammation or promote tissue repair such as IL-10 and transforming growth factor β (TGFβ) [80-82]. These cytokines have several suppressive effects on cells of the innate and adaptive immune system such as allergen-specific T cell subsets like Th2 cells [75]. The third part includes antibody isotype regulation with an early increase of specific IgE level, which later decreases and a continuous increase of specific IgG4 levels. A rise of blocking IgGs during allergen-specific immunotherapy prevents IgE-allergen-complex binding to mast cells, B cells as well as DCs and is associated with a decrease of allergic symptoms. Increased IgG4 levels are an important marker for successful therapeutic outcome [75, 83, 84]. The fourth group takes place after months of AIT and decreases numbers and pro-inflammatory mediator release of tissue mast cells and eosinophils [75]. Improved understanding of the underlying mechanisms of successful AIT is required for the development of novel treatment strategies to modulate immune response.

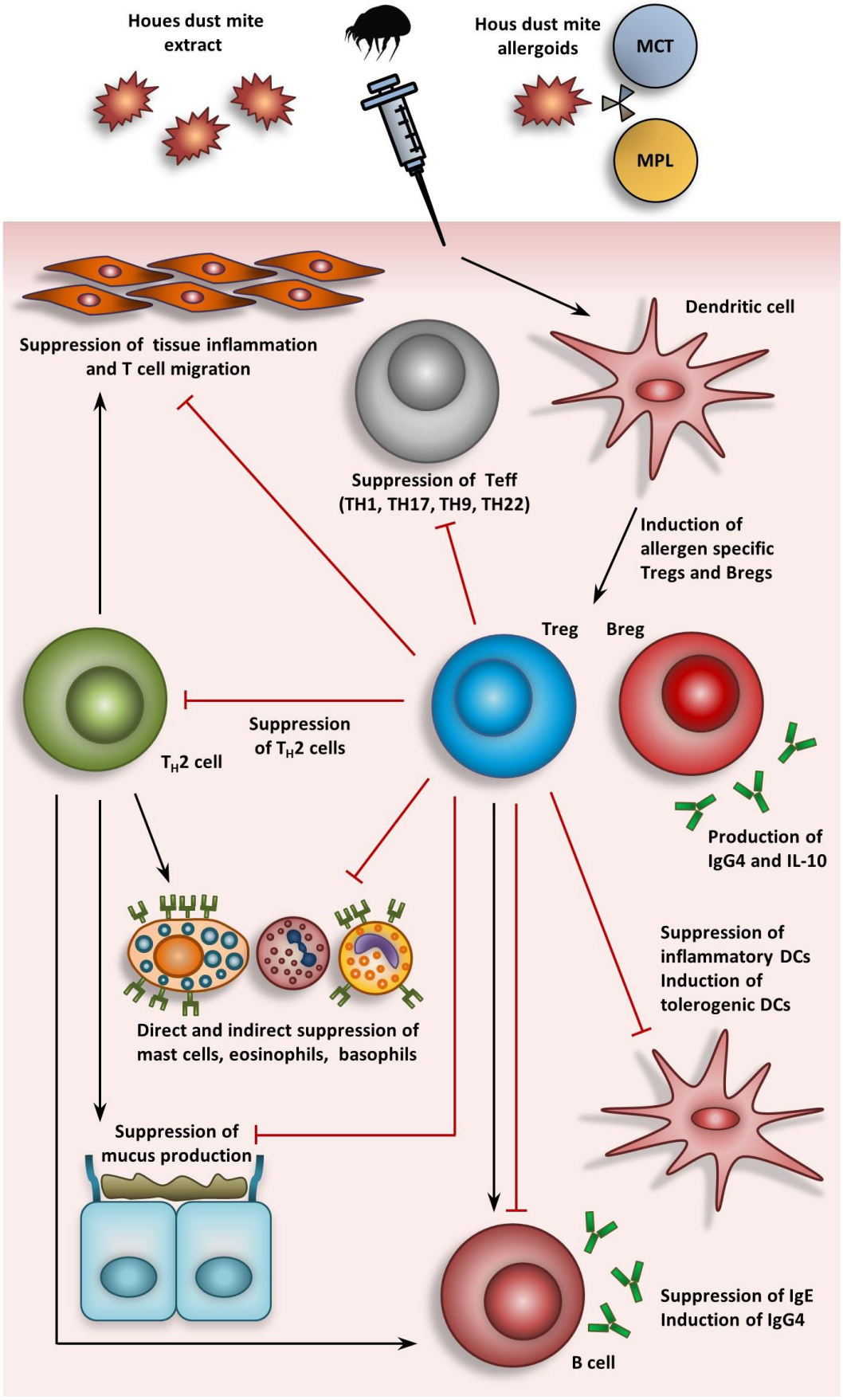

Figure 5: Hallmarks of AIT

Figure is adapted from Mark Larché, Cezmi A. Akdis, Rudolf Valenta (2014)

3.5 Native and chemically modified extracts (allergoids) in AIT

Conventional treatment strategies such as subcutaneous and sublingual allergen-specific immunotherapy with allergen extracts have proven efficacy [79, 85]. Nevertheless, AIT to a range of aeroallergens such as grass pollen, tree pollen and house dust mites also showed to induce drug-related adverse events [73, 86-88]. In addition, in context of HDM AIT, it was shown in 1993 that the efficacy of HDM extracts depend on the quantity of injected allergens. Here, production and composition of therapeutic allergen extracts are a critical issue. Analyses of commercially available extracts have revealed striking variabilities in their composition [89, 90]. The content ratio between the major HDM allergens Der p 1 and 2 can vary considerably and furthermore, in some extracts important allergens are even missing [91]. Within the last decades, allergy treatment research focused on developing and modifying AIT addressing its efficacy while maintaining or improving its safety profile to overcome drawbacks of conventional extract application. New AIT approaches of molecular forms such as recombinant wild-type or native-like allergens, T cell epitope-containing peptides, recombinant hypoallergens, nucleic acid-based vaccines, CpG-conjugated allergens and peptide carrier-based B cell epitopes came into focus of allergy treatment research [92]. Another AIT approach is the allergoid concept [93]. Modification of HDM allergens by formaldehyde or glutaraldehyde leads to a reduction of reactive B cell epitopes followed by decreased IgE binding, while T cell epitopes and immunogenic effects remain unaltered [93-95]. The clinically efficacy of allergoids was shown in several AIT studies and one of the first was published by Norman *et al.* in 1981 [96-101]. Notably, according to guidelines of the European Academy of Allergy and Clinical Immunology (EAACI), both modified and unmodified allergen extracts are recommended for subcutaneous AIT of allergic rhinocunjunctivitis for short-term benefit [102]. Furthermore, subgroup analyses comparing the combined symptom and medication

score (short term) for AIT in the context of allergic rhinoconjunctivitis found a clear benefit from allergoids and suggest (but not confirm) a benefit from unmodified preparations [103].

3.6 Adjuvants in allergy treatment

In allergy treatment, adjuvants are substances or compounds that are co-administered with the native or modified allergen extract and can increase allergen immunogenicity or/and modulate the elicited immune response [104]. Over the years, adjuvants have been applied in allergy vaccines to enhance and improve the efficacy and safety profile of allergen-specific immunotherapy treatments [105, 106]. Adjuvants for allergy vaccines are classified into two different generations: The first generation describes molecules that represent vehicles for allergen binding and can co-stimulate innate cells such as antigen-presenting cells. The second generation of adjuvants are specific immunomodulatory substances modulating either immune cells or immunological pathways [107]. Over years, aluminum hydroxide (alum) represents a conventional and first generation adjuvant which is used in most subcutaneous allergen-specific immunotherapy applications [108, 109]. Beside its depot function [110], alum was shown to enhance antigen uptake by antigen-presenting cells (APCs) [111] and NLR family pyrin domain containing 3 (NLRP3) inflammasome activation [112, 113]. Generally, alum is classified as safe in terms of acute local or systemic side effects, but different reports suggest chronic aluminum toxicity [114, 115]. Studies showed that alum can mediate Th2 immune response [110] and furthermore, there a some concerns about the accumulation of alum in the tissue during AIT [109]. Hence, MicroCrystalline Tyrosine (MCT) with its distinct crystalline particle size and needle-like morphology is an alternative first generation depot adjuvant to alum [116]. Both are comparably effective in inducing antigen-specific IgG and T cell responses. Hence, MCT is a suitable alternative to alum-based AIT vaccines because of its biodegradable and biocompatible character and less induction of IgE and IL-4 secretion than alum [116]. The

pollen AIT vaccine Pollinex® Quattro Ragweed showed the potential of combining the depot adjuvant MCT with short ragweed pollen allergoids and the second generation adjuvant monophosphoryl lipid A (MPL) [98-100]. MPL is the lipid A portion of *Salmonella minnesota* lipopolysaccharide (LPS) from which the (R)-3-hydroxytetradecanoyl group and the 1-phosphate have been removed by successive acid and base hydrolysis [117]. MPL and LPS act via TLR4 signaling and induce similar cytokine profiles, but MPL is at least 100-fold less toxic [118]. MPL was the first TLR4 agonist included in a licensed human vaccine and is associated with Th1 cytokine production by DCs via IL-12 followed by increased interferon gamma (IFN-γ) production [107, 119, 120]. Studies revealed that saccharide structures with exposed C–H groups like the 2-deoxy-2-aminoglucose in backbone of MPL can interact with the aromatic groups of L-tyrosine through C–H···π [121, 122] (Figure 6). The mechanism involved in MCT + MPL adjuvancy are still not fully understood, but studies revealed a synergistic attenuation of IgG and modulation of the Th1/Th2 imbalance in allergy. The clinical efficacy and synergistic properties for the use of the MCT and MPL adjuvant system in combination with allergoids in AIT has been proven [98, 123, 124].

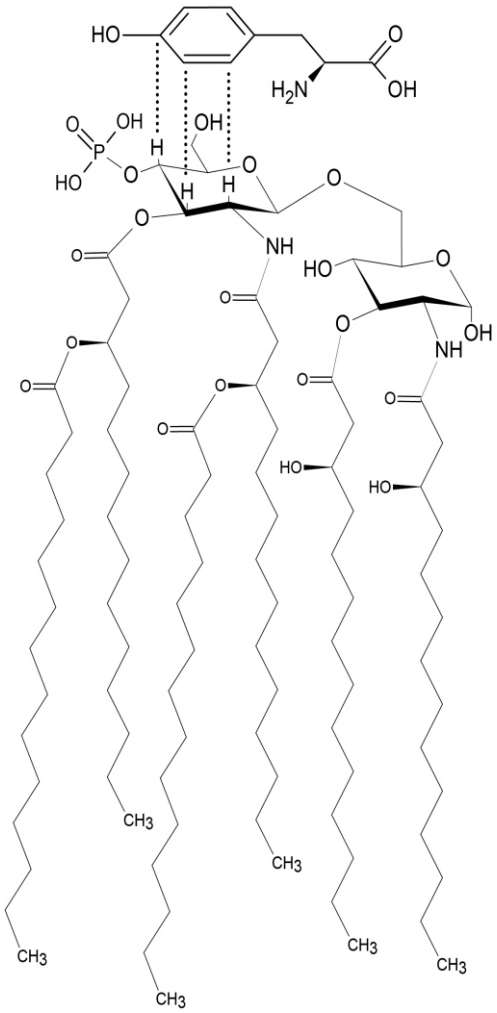

Figure 6: Adsorption of MPL to L-tyrosine
Figure adapted from Bell *et al.* (2015)

4 Aims of the project

In context of the investigation of mechanisms of allergen-specific immunotherapy and new concepts for the therapy of hypersensitivity reactions, the ultimate goal of this dissertation was to compare the therapeutic efficacy of chemically modified HDM allergens, so called allergoids, with native HDM extract in a new, physiological, murine HDM allergen specific immunotherapy model. Moreover, the immunological effects of the adjuvants MicroCrystalline Tyrosine and monophosphoryl lipid A on allergoid-based AIT should be investigated and translated to human HDM-allergy research.

Aims

- Establishment of a new, physiological, murine HDM AIT model based on intranasal (i.n.) sensitization and subcutaneous (s.c.) immunotherapy.
- Application of the established murine AIT model for the investigation of the therapeutic potential of low-dose HDM allergoid AIT (LD-allergoid-AIT) compared to high-dose HDM extract AIT (HD-extract-AIT). Broad characterization of therapeutic effects on HDM allergy-induced cellular and humoral immune response.
- Examination of the immunological effects of adjuvants MCT and MPL on low-dose HDM allergoid AIT approach.
- Investigation of dose-response effects of adjuvant MPL in combination with low-dose HDM allergoid + MCT AIT.
- Translation of the therapeutic properties of native HDM extract and HDM allergoids in combination with the adjuvant MPL to human HDM allergy research. *Ex vivo* stimulation assays with human PBMCs.

5 Methods

5.1 Allergen extracts and allergoids

GMP-grade freeze-dried native extract of *D. farinae* and *D. pteronyssinus* were supplied by Allergy Therapeutics and produced from GMP (Ph. Eur.)-grade raw material (>99% purity) of freeze-dried *D. farinae* and *D. pteronyssinus* (bodies and faeces). Following media extraction and purification by clarification, the extract was diafiltered, concentrated and clarified prior to sterile filtration and then freeze-dried. Biochemical characterization included total protein content, SDS-PAGE, total allergenicity and individual allergen content measured against biologically standardized in-house reference preparations (Quality Assurance Units/vial).

GMP-grade modified allergen extracts (allergoids) were supplied by Allergy Therapeutics. Following purification by clarification, the extract was diafiltered, modified with glutaraldehyde, diafiltered, sterile filtered, and then filled and analyzed. Biochemical characterization included total protein content, total allergenicity and individual allergen content measured against biologically standardized in-house reference preparations (Quality Assurance Units/vial).

4% (w/v) MicroCrystalline Tyrosine® (40 mg /mL) was supplied by Allergy Therapeutics, used to formulate vaccines at point of use, targeting a 2% (w/v) final concentration for all formulations. A sterile aqueous form of 1mg/mL MPL® was supplied by Allergy Therapeutics, manufactured in accordance to the Ph. Eu. monograph 2537. This was used to formulate allergoid+MCT formulations to explore the dose-dependent effects of MPL (12.5, 25, 50 and 100 µg).

Allergoids + 2% MCT (Acarovac Plus®) and allergoids + 2% MCT + 50 µg MPL (Acarovac MPL®) were supplied by Allergy Therapeutics, GMP manufactured.

5.2 Animal procedures

5.2.1 Laboratory animals

Female 5-6 week old C57BL/6 mice were purchased from Charles River (Sulzefld, Germany). Animals were kept in individually ventilated cages (VentiRack, Biozone, Margate/UK) under specific pathogen free (SPF) conditions. Experiments were started with 6-7 week aged mice. All experiments were performed under federal guidelines for the use and care of laboratory animals and approved by the government of the district of Upper Bavaria (Reference number of ethical approvals for all performed animal experiments is ROB-55.2-2532.Vet_02-12-156). In vivo experiments were performed in room 6103, E-Streifen (KTH), Zentrale Versuchstierhaltung des Helmholtz Zentrum München, Ingolstädter Landstraße 1, 85764 Neuherberg.

5.2.2 Animal handling

Anesthesia

Mice were anesthetized by intraperitoneal (i.p.) injection of 10% Ketamine/2% Xylacin in PBS. 100 µl of preparation was injected per 10 g body weight. For short-term anesthesia, Sevoflurane was used.

Blood sampling

Blood was collected via penetrating the retro-orbital sinus. Up to 15% of the calculated blood volume was collected from Sevoflurane anesthetized mice. Blood was collected in Microvette® serum tubes, centrifuged at 8000 *g* and stored at -80 °C.

Euthanasia

Mice, which had to undergo bronchoalveolar lavage (BAL) analysis, received an overdose of anesthetic leading to a death without regaining consciousness.

Intranasal application

Mice were anesthetized with Sevoflurane. Immediately 10 µl of allergen solution was slowly administered to each nostril using a 10 µl pipette.

Intraperitoneal injection

Intraperitoneal injection was performed in the lower right quadrant of the abdomen.

Subcutaneous injection

Subcutaneous administration was done into the loose skin over the neck between the shoulders.

5.2.3 Murine models of allergic diseases

Allergic and AIT-treated mice were i.n. sensitized with 1 µg (total protein content) HDM extract (1:1, Der p and Der f) in 20 µl PBS on three consecutive days followed by i.n. challenges with 1 µg HDM extract on days 7, 13 and 19 (Figure 1A). Non-allergic mice received 20 µl PBS i.n.. AIT was performed with s.c. injections of either 220µg HDM extract, or 1 µg (total protein content) HDM allergoids (1:1, Der p and Der f), or 1 µg HDM allergoids combined with 2% (v/v) MCT or 1 µg HDM allergoids combined with 2% (v/v) MCT and 50µg MPL (or with 12.5µg, 25µg and 100µg MPL in the experiments addressing dose-dependent effects of MPL) in 200 µl PBS on days 14, 17 and 21. Not AIT-treated allergic and non-allergic mice received s.c. injections of 200 µl PBS. The time points for injections were adapted from existing protocols of murine AIT (22, 23). All mice were challenged i.n. with 10µg HDM extract on days 29, 30, 31 and 32 and euthanized at day 35 for analysis (day 33 for lung function measurement).

5.2.4 Lung function analysis

Dynamic compliance after methacholine (Mch, Sigma-Aldrich, Taufkirchen, Germany) challenge was measured 24 h after the last HDM challenge in intubated, mechanically ventilated animals (Data Sciences International (DSI), New Brighton, MN, USA), as previously described [125]. Briefly, animals were anesthetized by an intraperitoneal injection of ketamine (100mg/kg) and xylazine (5mg/kg) in PBS. After cannulation of the trachea and starting mechanical ventilation, the animals were challenged with increasing methacholine concentrations, using an in-line nebulizer (5 µl Mch solution in PBS delivered for 30 seconds

at the following concentrations: 0, 5, 10, 20 and 40 mg/ml). Data were recorded using the FinePoint software v2.4.6. The lowest values of dynamic compliance were recorded every 5 seconds during the data-recording interval set at 3 min after each Mch level. The mean value of this parameter measured during each recording interval was then calculated. The heart rate of each animal was continuously monitored using an electrocardiography device connected with three subcutaneous electrodes throughout the entire experiment. Respiratory system resistance was not monitored due to the known lack of response in C57BL/6 mice [126].

5.3 Cell Biology

5.3.1 Isolation of bronchoalveolar lavage fluid cells

For isolation of BAL cells, the chest of euthanized mice was opened, the trachea cannulated, airways lavaged five times with 0.8 mL PBS and BAL cells analyzed via fluorescence-activated cell scanning (FACS).

5.3.2 Isolation of lung resident lymphocytes

For isolation of tissue-resident lymphocytes, lungs were removed, chopped into approximately 1 mm x 1 mm sized pieces and digested in 3 mL RPMI containing 1 mg/mL collagenase and 100 µg/mL DNAse. Digestion at 37 °C was stopped after 30 minutes by the addition of 15 µl 0.5 M EDTA. Digested lungs were mashed through a 70 µm cell strainer. Next, cells were pelleted (400 g, 4 °C, 5 min), resuspended in 5 mL 40% percoll in RPMI (v/v) solution, underlayered with 5 mL 80% percoll solution and centrifuged (1600 g, RT, 15 min, without brake). Lymphocytes were collected from the interphase, washed with PBS and stored on ice.

5.3.3 Isolation of lymph node cells

Cervical lymph nodes were removed from mice and collected in a 15 mL tube with 5 ml RPMI. Lymph nodes were mashed through a 70 µm cell strainer into a 50 mL tube, washed with 5 mL RPMI (400 g, 4 °C, 5 min) and stored on ice.

5.3.4 Isolation of spleenocytes

Spleens were removed from mice and collected in a 15 mL tube with 5 ml RPMI. Spleens were mashed through a 70 µm cell strainer into a 50 mL tube, washed with 10 mL PBS and pelleted (400 g, 4 °C, 5 min). For removing erythrocytes, spleen cells were resuspended in 1 mL ACK-lysis buffer and incubated at room temperature for 2 minutes. 7 mL PBS were added and cells filtered through a 70 µm cell strainer. Cells were pelleted (400 g, 4 °C, 5 min) and resuspended in 3 mL RPMI complete, 1% glutamine, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% Na-pyruvat. After adding 10 mL PBS, splenocytes cells were washed twice with PBS and stored on ice.

5.3.5 Counting of isolated cells

Cell numbers were determined using a BD Accuri C6 flow cytometer. Cells from BAL fluid and lymph nodes were counted without dilution and cells from spleen and lung were diluted 1:10. Cells in a final volume of 50 µl PBS were transferred to FACS tubes and 1 µl cell counting master mix was added (0.5 µl anti-CD45-FITC, 0.5 µl propidium iodide diluted 1:10 with PBS). After incubation for 5 minutes at 4 °C, cells were counted and diluted for *ex vivo* restimulation assays.

5.3.6 *Ex vivo* restimulation of lung lymphocytes, lymph node cells and splenocytes

Flat bottom 96-well plates were coated with 2 µg/ml anti-CD3 and 2 µg/ml anti-CD28 in PBS and incubated at 4 °C overnight. Wells were washed with PBS. Lymph node cells and splenocytes were plated out in duplicates at a density of $2x10^5$ and lung lymphocytes at a density of $1x10^5$ cells per well in 200 µl RPMI complete. Cells were incubated for 72 h at 37 °C, 5%

CO2 in a humidified atmosphere. Supernatants were collected and stored at -80 °C for further analysis.

5.3.7 Histology

For the histological analysis, after BAL the lungs were excised and the left lobe fixed in 4% buffered formalin and embedded in paraffin. Sections of 4 µm thickness were stained with hematoxylin-eosin (H&E) and periodic acid Schiff (PAS).

5.3.8 Isolation and *ex vivo* stimulation of human peripheral blood mononuclear cells

PBMCs were isolated from five healthy controls, six HDM-allergic patients and four HDM-allergic patients that received SCIT by density gradient centrifugation (Lymphoprep) according to manufacturers' protocol. The study was approved by the local ethics committee of the Faculty of Medicine of the Technical University of Munich and all patients and volunteers had given written informed consent to draw blood samples. $2x10^5$ cells from each donor were seeded in 200 µl RPMI complete medium in 96-well flat bottom plates and stimulated with 1 µg/ml HDM extract w/o 10 µg/ml MPL, 1 µg HDM allergoids w/o 10 µg/ml MPL or 10 µg/ml MPL alone for five days at 37°C and 5% CO_2. 10 µg/ml LPS and phytohaemagglutinin (PHA) served as positive control. Supernatants were collected and stored at -80 °C for further analysis and cells were analyzed via FACS.

5.4 Molecular biology and protein chemistry

5.4.1 Flow cytometry analysis

Single cell suspension of isolated murine lung-resident lymphocytes were stained with live/dead aqua in PBS for 10 min, washed with FACS buffer (PBS, 1 % FCS) (centrifuged at 450 g, 5 min, 4 °C), stained for 20 min with extracellular antibody mix (Table 6) and washed twice with FACS buffer (centrifuged at 450 g, 5 min, 4 °C). Cells were fixed with

eBioscience™ Forkhead box P3 (FoxP3)/Transcription kit (Thermo Fisher Scientific) and washed twice in fixation/permeabilization kit buffer (centrifuged 980 g, 5 min, 4 °C). Fixed cells were stained for 30 min with intracellular GATA-binding protein 3 (GATA3)/FoxP3 antibody mix (Table 6), washed with fixation/permeabilization kit buffer (centrifuged at 980 g, 5 min, 4 °C), washed with FACS buffer (PBS, 1 % FCS) (centrifuged at 980 g, 5 min, 4 °C) and analyzed by FACS, FACSDiva software and FlowJo software.

For intracellular IFN-γ staining, single cell suspension of isolated murine lung-resident lymphocytes were stimulated with 20 ng/ml phorbol myristate acetate and 1 µg/ml ionomycin (1 h, 37 °C, 5% CO_2) followed by addition of 5 µg/µl Brefeldin A (3 h, 37 °C, 5% CO_2). Cells were washed twice with PBS (at 450 g, 5 min, 4 °C) and then live/dead aqua, extracellular and intracellular IFN-γ staining were performed (described above).

Murine BAL cell suspension was washed twice with PBS (at 450 g, 5 min, 4 °C), stained with 40 µl of a master mix of fluorochrome-conjugated antibodies for 30 min (Table 7) and after washing twice (at 450 g, 5 min, 4 °C) with FACS buffer (PBS, 1 % FCS) analyzed using FACS, FACSDiva software and FlowJo.

Stimulated human PBMCs were washed with PBS (centrifuged at 450 g, 5 min, RT), 50 ng/ml phorbol myristate acetate and 1 µg/ml ionomycin (1 h, 37 °C, 5% CO_2) followed by addition of 5 µg/µl Brefeldin A (3 h, 37 °C, 5% CO_2). Cells were washed with PBS (at 450 g, 5 min, 4 °C) and then live/dead aqua, extracellular and intracellular staining were performed (described above)(Table 8).

5.4.2 Measurement of immunoglobulin levels in blood serum

For total IgE quantification, plates were coated with IgE ELISA MAX™ Capture Antibody (200X) and detected with Mouse IgE ELISA MAX™ detection antibody and ELISA MAX™ Avidin-HRP according to manufacturers' protocol. Sera were diluted 1:600 with PBS for the measurement of tIgE.

Measurement of Der p- and Der f-specific IgE and IgG1 levels was done by coating the plates with mouse IgE ELISA MAX™ capture antibody, or 50 µl of 12.5 µg/ml polyclonal anti-mouse IgG1 antibody and detection with 50 µl biotinylated *D. pteronyssinus* or *D. farinae* HDM extract. Detection was done with ELISA MAX™ Avidin-HRP. Sera were diluted 1:10 with PBS for the measurement of sIgE and 1:300 for the detection of sIgG1. Analyses of total IgG1, IgG2a, IgG2b and IgG3 were done with Legendplex Immunoglobulin Isotyping Panel according to the manufacturers' protocol and analyzed by FACS.

5.4.3 Measurement of cytokines in culture supernatants

Measurement of cytokine levels in the supernatants of anti-CD3- and anti-CD28-restimulated cells from murine lungs, lymph nodes and spleens or in the bronchoalveolar lavage fluid (BALF) were performed using BioLegend's LEGENDplex murine T Helper Cytokine Panel according the manufacturers' protocol and analyzed by FACS.

Measurement of cytokine levels in the supernatants of stimulated human PBMCs were performed using BioLegend's LEGENDplex human T Helper Cytokine Panel and human Cytokine Panel 2 according the manufacturers' protocol and analyzed by FACS.

5.5 Data analysis and statistics

Gaussian distribution was tested by D'Agostino & Pearson omnibus normality test. Gaussian and non-Gaussian distributed results were further analyzed by unpaired t-test and Mann-Whitney test, respectively. Lung function parameters were analyzed by two-way analysis of variance with Tukey's multiple comparison test. The scoring for mucus hypersecretion and inflammatory cell infiltrate was analyzed by one-way analysis of variance with Tukey's multiple comparison test. P-values of ≤0.05, ≤0.01, ≤0.001, and ≤0.0001 are shown as *, **, ***, and ****, respectively (GraphPad Prism).

6 Material

6.1 Media and buffers

Table 1: Media and buffers

Medium / Buffer	Substances
ACK lysis buffer	1.5 M NH_4Cl, 100 mM $KHCO_3$, 10 mM Na_2EDTA, ddH_2O, pH 7.4
Anesthesia	100mg/kg ketamine, 5mg/kg xylazin, 8,9 ml PBS
RPMI complete	RPMI, 10% FCS, 1% glutamine, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% Na-pyruvat
ELISA washing buffer	PBS, 0,05% Tween20
ELISA coating buffer	5,2 g $NaHCO_3$, 1,78 g Na_2PO_4, on 500 ml dH_2O. pH 9,5
ELISA blocking buffer	PBS, 1 % BSA
ELISA substrate buffer	1-Step™ Ultra TMB-ELISA
ELISA stop solution	2 M H_2SO_4

6.2 Animal experiments

Table 2: Material for animal experiments

Substance	Manufacturer
HDM allergoids	Allergy Therapeutics
HDM extract	Allergy Therapeutics
Isofluorane	Piramal Healthcare
Ketamine	Medistar
MCT	Allergy Therapeutics
Methacholine	Sigma-Aldrich
MPL	Allergy Therapeutics
PBS	Thermo Fisher Scientific
Sevofluorane	Piramal Healthcare
Xylacine	WDT

6.3. Cell biology

Table 3: Material for cell biology

Substance	Manufacturer
anti-CD3	BD Bioscience
anti-CD28	BD Bioscience
anti-CD45-FITC	eBioscience
Collagenase A	Sigma-Adrich
Deoxyribonuclease I	Sigma-Adrich
EDTA	Sigma-Aldrich
FCS	Sigma-Aldrich
Glutamine	Thermo Fisher Scientific
$KHCO_3$	Merck
Lymphoprep	Stemcell Technologies
Na_2EDTA	Merck
Na-pyruvat	Thermo Fisher Scientific
NH_4CL	Sigma-Aldrich
Non-essential amino acids	Thermo Fisher Scientific
PBS	Thermo Fisher Scientific
Pen/Strep	Thermo Fisher Scientific
Percoll	GE Healthcare
Propidium iodide	Sigma-Adrich
PMA / Ionomycin	Sigma-Adrich / Biomol
Brefeldin A	Sigma-Adrich
RPMI	Thermo Fisher Scientific

6.4 Cytokine and immunoglobulin measurements

Table 4: Kits for cytokine and immunoglobulin analyses

Kit	Manufacturer
Human T Helper Cytokine Panel	BioLegend
Human Cytokine Panel 2	BioLegend
Mouse Immunoglobulin Isotyping Panel	BioLegend
Mouse T Helper Cytokine Panel	BioLegend

Table 5: ELISA material for immunoglobulin analyses

Substance	Manufacturer
anti-mouse IgG1 antibody	Citeq Biologics
Biotinylated *D. pteronyssinus* extract	Citeq Biologics
Biotinylated *D. farinae* extract	Citeq Biologics
BSA	Sigma-Aldrich
H_2SO_4	Merck
IgE ELISA MAX™ capture antibody	BioLegend
IgE ELISA MAX™ avidin-HRP	BioLegend
Na_2CO_3	Sigma-Aldrich
$NaHCO_3$	Merck
PBS	Thermo Fisher Scientific
1-Step™ Ultra TMB-ELISA	Thermo Fisher Scientific
Tween20	Sigma-Aldrich

6.5 Flow cytometry

Table 6: Antibodies for FACS analyses of murine lymphocytes isolated from lung

Marker	Fluorochrome	Clone	Manufacturer
Extracellular			
CD3ε	FITC	145-2C11	BD Bioscience
CD4	AF700	RM4-5	BioLegend
CD45	APCeF780	30-F11	eBiosciences
ST2	BV421	DIH9	MDBioproducts
Intracellular			
FoxP3	PerCP-Cy5.5	FJK-16s	eBiosciences
GATA3	eF660	TWAJ	eBiosciences
IFN-γ	PE-Cy7	XMG1.2	eBiosciences

Table 7: Antibodies for FACS analyses of murine BAL cells

Marker	Fluorochrome	Clone	Manufacturer
CD4	A700	RM4-5	BioLegend
CD8	FITC	53-6.7	BD Bioscience
CD11b	BV711	M1/70	BioLegend
CD11c	PE-Cy7	N418	Biolegend
CD45	PerCP-Cy5.5	30-F11	BioLegend
CD206	PE-Dazzle594	C068C2	BioLegend
F4/80	APC-e780	BM8	eBiosciences
NK1.1	APC	PK136	eBiosciences
Ly-6G	PacificBlue	1A8	Biolegend
Siglec-F	PE	E50-2440	BD Bioscience
Live/dead aqua fixable cell stain	405nm	—	Thermo Fisher

Table 8: Antibodies for FACS analyses of human PBMCs

Marker	Fluorochrome	Clone	Manufacturer
Extracellular			
CD3	FITC	OKT3	BioLegend
CD4	VioGreen	M-T466	Miltenyi
CD8	BV570	RPA-T8	BioLegend
CXCR5	APC	J252D4	BioLegend
CD25	BV421	BC96	BioLegend
Intracellular			
CD4	VioGreen	M-T466	Miltenyi
Foxp3	PerCP/Cy5.5	PCH101	eBioscience
IFN-γ	BV711	B27	BD Bioscience
IL-4	PE/Cy7	8D4-8	BD Bioscience
IL-10	Pe/Dazzle594	JES-9D7	BioLegend
IL-17A	PE	N49-653	BD Bioscience

6.6 Histology

Table 9: Material for histology

Substance	Manufacturer
Ethanol, 99,9% denatured	Helmholtz
Eosin Y 1% aqueous solution	Bio Optica
Formaldehyde	AppliChem
Mayers hematoxylin	Bio Optica
Paraplast X-tra	Leica
Periodic acid	VWR
Mounting Medium	Pertex
Schiff's reagent	Sigma-Aldrich
Xylene	J.T.Baker

6.7 General material and equipment

Table 10: Consumables

Consumable	Type	Manufacturer
96 well plate	Nucleon delta-treated, flat-bottom	Thermo Fisher Scientific
96 well plate	TC surface, u-bottom	Thermo Fisher Scientific
96 well plate	U-bottom, non-sterile	Thermo Fisher Scientific
96 well plate	V-bottom, polypropylen	Greiner Bio-One
Cell strainer	70 µm	Greiner Bio-One
FACS tube	Microtube	Greiner Bio-One
Gloves	L	Kimtech / Meditrade
Needles	Disposable hypodermic needle	Braun
Serum tube	Microvette	Sarstedt
Serological pipettes	1 ml, 2 ml, 5 ml, 10 ml, 25 ml	Sarstedt
Syringes	1 ml	Braun
Reaction tube	0.2 ml, 0,5 ml, 1,5 ml, 2 ml, 5 ml	Sarstedt
Pipette tips	10 µl, 200 µl, 300 µl, 1000 µl	Sarstedt
Tubes	15 ml, 50 ml	Sarstedt

Table 11: Software

Software	Software Publisher
i-control	Microplate reader software, TECAN
Endnote	Clarivate Analytics
FinePoint software v2.4.6	DSI
BD FACSDIVA	BD Bioscience
FlowJo V10	FlowJo, LLC
BD FACSDIVA	BD Bioscience
LegendPlex Analysis Software	BioLegend
Microsoft Office	Microsoft

Table 12: Equipment

Equipment	Type	Manufacturer
Flow cytometer for cell counting	Accuri C6	BD Pharmingen
Flow cytometer	LSR Fortessa	BD Pharmingen
Centrifuge	5424R	Eppendorf
Centrifuge	5810R	Eppendorf
CO_2 incubator	Galaxy 170 S	New Brunswick
Freezer (-20 °C)	Premium Nofrost	Liebherr
Freezer (-80 °C)	V570 HEF	New Brunswick
Fridge (4 °C)	MediLine	Liebherr
Neubaur counting chamber	-	Brand
pH meter	pHenomenal 1100L	VWR
Pipettes	PeqPette	Peqlab
Vortex	Vortex genie	Scientific industries
Vortex	Vortex 1	IKA
Microplate washer	Hydrospeed Tecan	Tecan
Microscope	Modell MS 5	Leica
Shaking incubator	Innova 42	New Brunswick
Accu-jet pro	-	Brand
Thermomixer	Thermomixer C	Eppendorf
Biological safety cabinet	BioVanguard	Telstar
Pulmanory Function test	-	Buxco
Histo Embedder	-	Leica
Tissue processing system	- TP1020	Leica
Electronic rotary microtome	HM 340E	Microm

7 Results

7.1 Establishment of a new physiological murine HDM AIT model

In humans, local allergy-driven and protective AIT mechanisms in the airways and in lymphoid organs are hard to address. Therefore, aim of the thesis was to establish a new, physiological, murine HDM AIT model based on alum-free i.n. sensitization and subsequent s.c. AIT and to investigate the potential of this new murine model to resemble human allergy-driven inflammation mechanisms. In contrast, commonly used murine HDM AIT models are based on i.p. sensitization combining the allergen with the adjuvant alum and are not reflecting the natural sensitization route to HDM allergens in humans [127-130]. Here, the establishment process is based on a commonly used HDM allergy model with an initial i.n. sensitization with 1 µg HDM extract followed by repeated 10 µg HDM extract i.n. challenge after one week (Figure 7 A) [131]. First, the sensitization process of the mice against the HDM allergens was improved. Therefore, the initial sensitization time points were increased to three days resulting in a more consistent pro-inflammatory BAL cell infiltration after HDM extract challenge (Figure 7 B). Then, the model protocol was elongated from 15 days up to 35 days and three s.c. AIT applications introduced at day 14, 17 and 21. The first HDM AIT model was characterized by a consistent sensitization profile but within the HDM-allergic group, which did not receive AIT, no challenge-induced serum IgE levels could be detected at the end of the experiment (Figure 7 C). This observation indicated that the time course between initial sensitization and final challenge was too long to induce a sustained allergy-driven IgE response. Based on previous studies from our lab [132], we introduced weekly i.n. challenge timepoints with 1 µg HDM extract to overcome drawbacks of the first HDM AIT model (Figure 7 D). The following chapters will mainly focus on the characterization of the newly established murine HDM AIT model based on i.n. sensitization and s.c. immunotherapy.

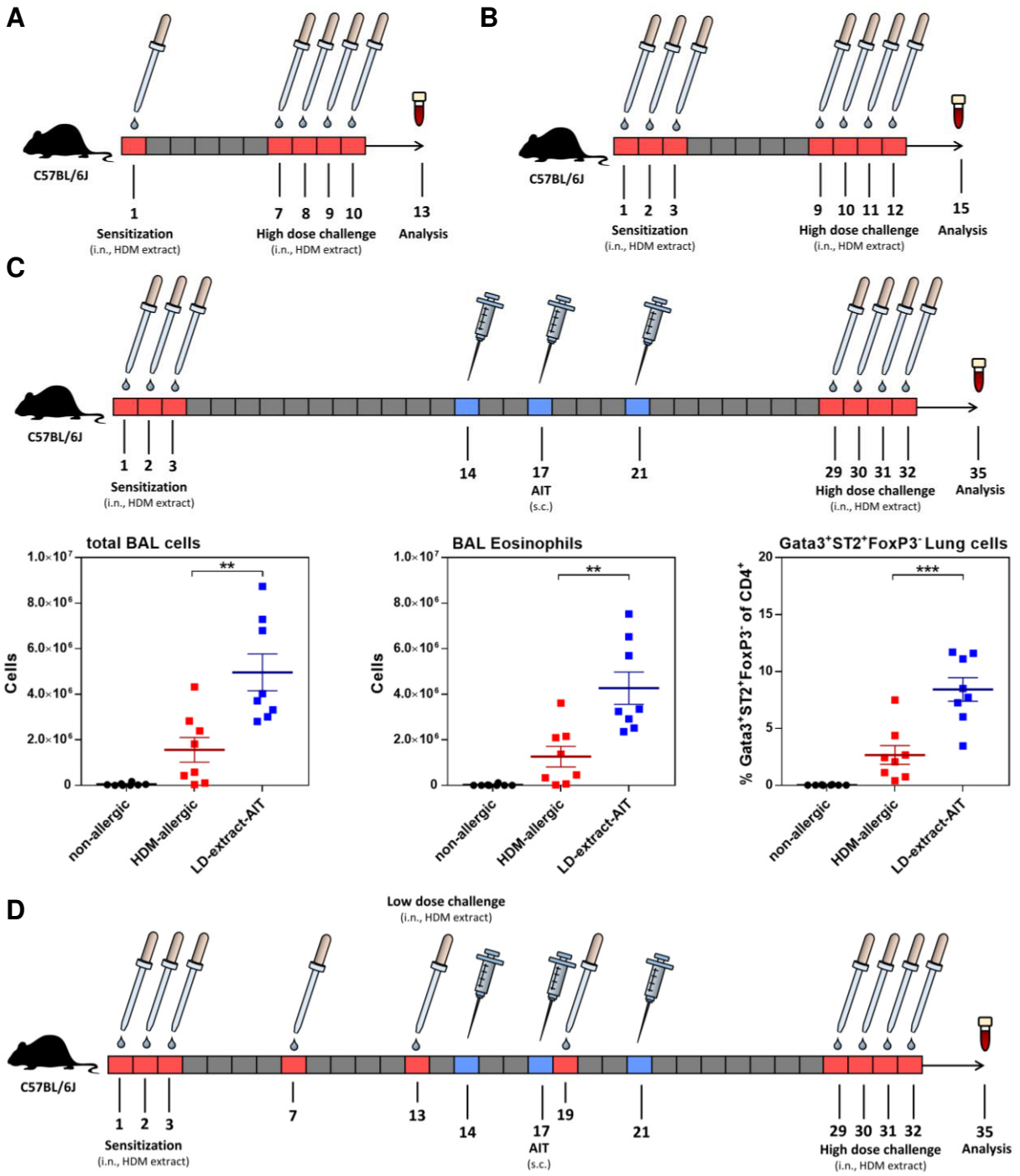

Figure 7: Establishment of a new physiological murine HDM AIT model

A HDM allergy model; one initial i.n. sensitization with 1 µg HDM extract, four final high-dose i.n. challenges with 10 µg HDM extract. **B** Modified HDM allergy model; three initial i.n. sensitization with 1 µg HDM extract, four final high-dose i.n. challenges with 10 µg HDM extract. **C** HDM AIT model; three initial i.n. sensitization with 1 µg HDM extract, three s.c. AIT applications, four final high-dose i.n. challenges with 10 µg HDM extract. Measurement of total BAL cells, BAL eosinophils and $Gata3^{+}ST2^{+}FoxP3^{-}CD4^{+}$ cells in the lung. Data are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively. **D** Final HDM AIT model; three initial i.n. sensitization with 1 µg HDM extract, weekly low-dose i.n. challenges with 1 µg HDM extract, three s.c. AIT applications, four final high-dose i.n. challenges with 10 µg HDM extract.

7.2. Therapeutic effects of low-dose HDM allergoid and high-dose HDM extract AIT

After refinement and establishment of a new murine house dust mite AIT model based on adjuvant-free i.n. sensitization and s.c. immunotherapy, the therapeutic potential of the application of low-dose (LD; 1 µg) HDM allergoid in comparison with high-dose (HD; 220 µg) HDM extract was investigated. Additional allergen challenges before and during AIT were introduced to mimic the unavoidable allergen contact of HDM-allergic individuals (Figure 8).

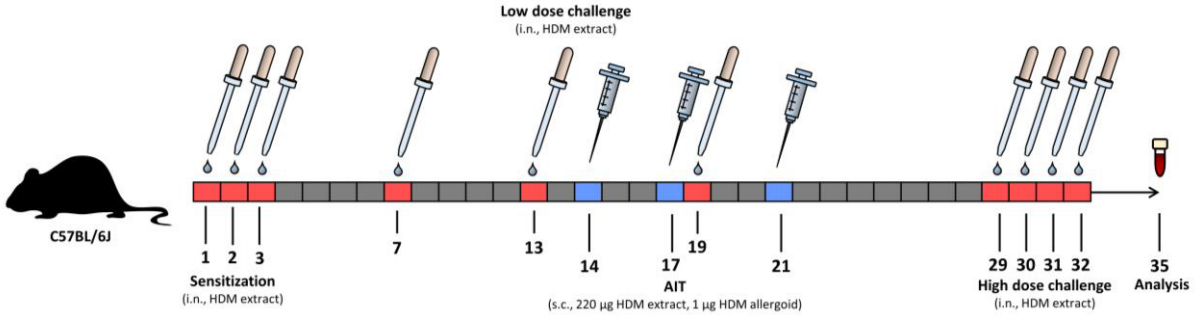

Figure 8: Murine model for low-dose HDM allergoid AIT and high-dose HDM extract AIT
Mice (C57BL/6J) were treated as follows: Mice from the non-allergic control group only received high dose challenge with HDM extract at the end, all other treatments were done with PBS; the allergic control group received sensitization, low-dose challenge, high dose challenge with HDM extract and PBS-AIT; high-dose HDM extract AIT group received sensitization, low-dose challenge, high dose challenge and AIT with HDM extract; low-dose HDM allergoid AIT group received sensitization, low-dose challenge, high dose challenge with HDM extract and AIT with HDM allergoids.

7.2.1 Effects of LD-allergoid-AIT and HD-extract-AIT on lung inflammation

As expected, in the HDM-allergic mice group, initial sensitization with HDM extract, followed by weekly low-dose challenge and final high-dose challenge led to a significant higher (significance not shown) and robust rise of total BAL cells in the HDM-allergic group compared to the non-allergic group (Figure 9 A). Regarding BAL cell subtypes, eosinophils were the major type of infiltrating cells, followed by increased numbers of $CD8^+$, $CD4^+$ cells as well as neutrophils. According to recent HDM AIT models by other research groups [127], s.c. AIT with HD-extract led to a significant decrease of total BAL cells as well as all different BAL cell subtypes. Interestingly, s.c. application of LD-allergoid with a 220-fold lower protein content

revealed comparable protection against the HDM challenge-induced infiltration of proinflammatory cells in the BAL (Figure 9 A).

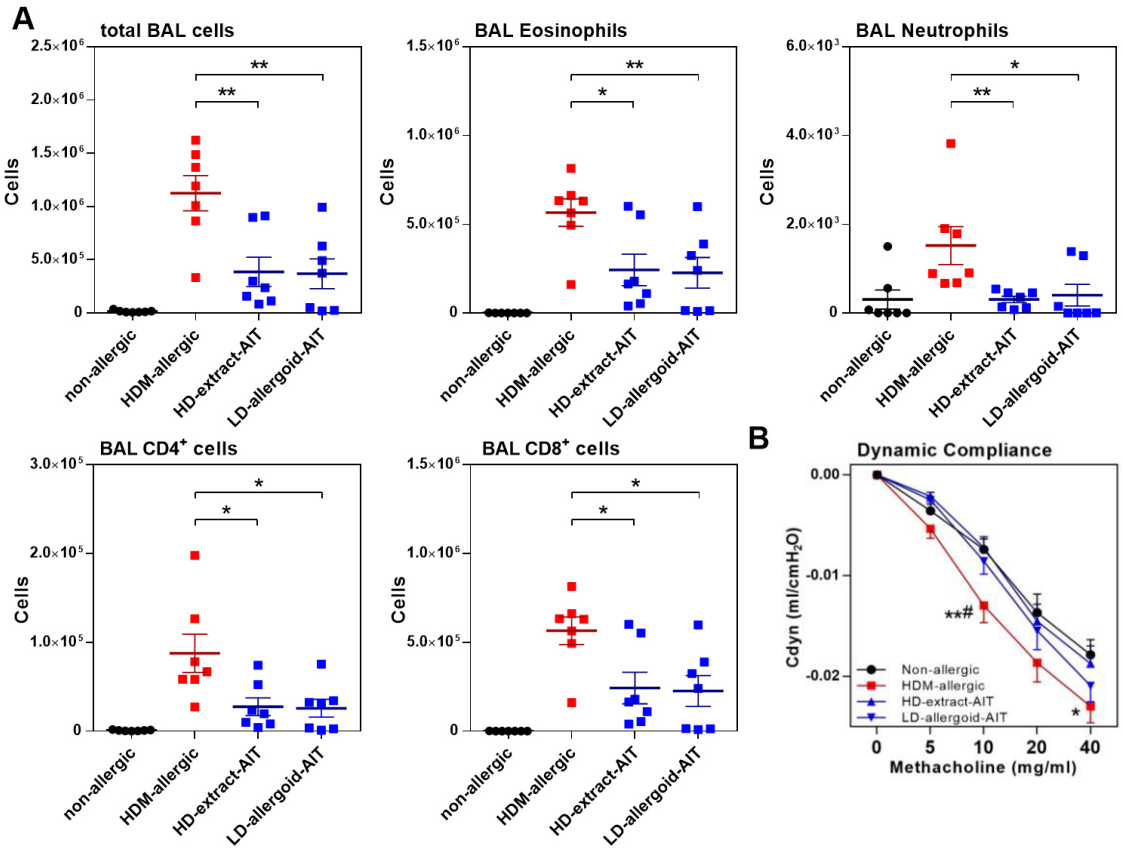

Figure 9: Reduction of inflammatory BAL cell infiltration and restoration of lung dynamic compliance under AIT

A Count of total BAL cells as well as eosinophils, neutrophils, $CD4^+$ and $CD8^+$ cells in the BAL fluid. Data are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively. **B** Measurement of dynamic compliance of the lungs in response to increased methacholine concentrations. Lung function parameters were analyzed by two-way analysis of variance with Tukey's multiple comparison test.

Moreover, HD-extract-AIT significantly improved lung dynamic compliance and a obvious trend towards lung function recovery could be achieved also by LD-allergoid-AIT (Figure 9 B). As expected, differences in airway hyperresponsiveness, as a hallmark of successful human AIT, were not observed in the C57BL/6J mouse strain (data not shown) [126]. Next, the cytokine pattern in the BAL fluid was analyzed. In HDM-allergic mice levels of IL-4, IL-5 and TNF-α were significant increased (significance not shown) compared to the non-allergic group (Figure 10 A). Both therapeutic strategies, LD-allergoid AIT as well as HD-extract-AIT, reduced the amount of all three cytokines in the BAL fluid. Especially, IL-5 levels were

significantly decreased by both treatments, whereas levels of IL-4 as well as TNF-α were only significantly decreased by LD-allergoid-AIT. No difference in IL-17A levels could be detected. Therefore, the results indicated a rather Th2-mediated inflammation regarding BAL fluid cytokine levels. In addition, isolated lung-resident lymphocytes from all mouse groups were further characterized. Here, lung resident cells were characterized by extracellular and intracellular markers as well as *ex vivo* restimulated for the measurement of cytokine release. First, lymphocytes of differently treated mice were isolated from lung tissue and analyzed by flow cytometry (Figure 10 B). HDM-allergic mice showed a significantly higher frequency (significance not shown) of $FoxP3^+$ and $GATA3^+ST2^+FoxP3^-$ $CD4^+$ T cells compared to mice from the non-allergic control group. The increase of lung-resident $GATA3^+ST2^+FoxP3^-$ Th2 cells found in allergic mice was neither reverted by HD-extract-AIT nor by LD-allergoid-AIT. In contrast, the number of $FoxP3^+CD4^+$ T cells, which was elevated in HDM-allergic mice, was reduced by both treatments. Furthermore, the frequency of IFN-γ^+CD4^+ T cells was significantly decreased in HDM-allergic mice compared to non-allergic mice displaying the allergy-induced shift towards a Th2-dominated phenotype. A trend towards reversion of this effect could be achieved by both AIT strategies. Then, after *ex vivo* anti-CD3/anti-CD28-restimulation of lung lymphocytes, cytokine levels in the culture supernatants were analyzed (Figure 10 C). Here, levels of secreted Th2-type cytokines IL-4, IL-5, IL-9 and IL-13 were significantly increased in the HDM-allergic group. Overall, all four cytokines were downregulated by HD-extract-AIT (not significant for IL-9) and LD-allergoid-AIT (not significant for IL-13). Moreover, both AIT regimens significantly reduced the elevated IL-10 levels found in the HDM-allergic group. Levels of IL-17A, IL-17F, IL-22, TNF-a, IFN-g, IL-6 and IL-2 were not altered in both treatment groups.

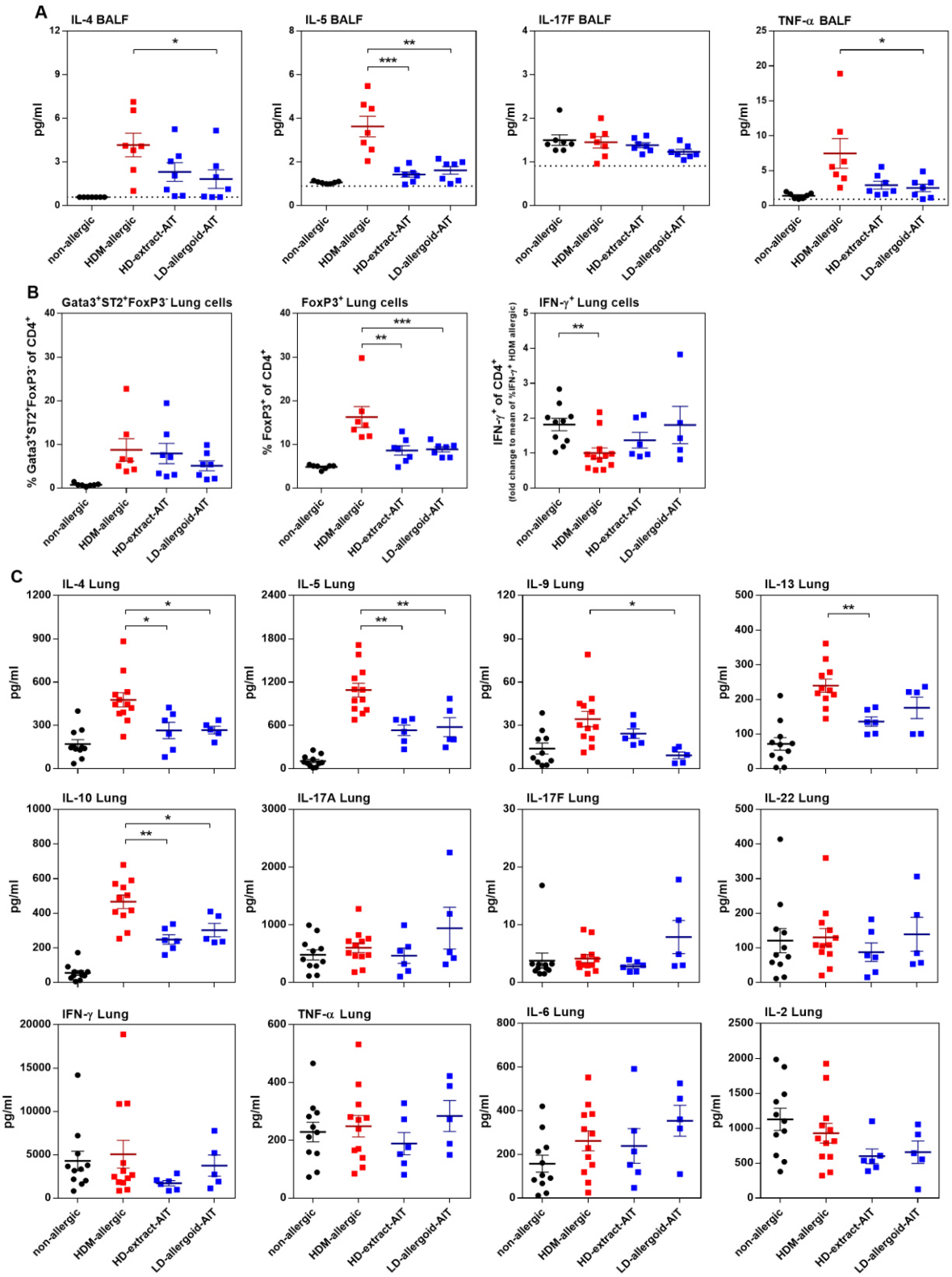

Figure 10: Effects of LD-allergoid-AIT and HD-extract-AIT on BALF cytokines, lung-resident lymphocytes, and cytokine secretion of restimulated lung lymphocytes

A Cytokine milieu in the BAL fluid **B** Analysis of lung-resident lymphocyte populations and IFN-γ$^+$CD4$^+$ T cells. **C** Cytokine release from lung-resident lymphocytes after anti-CD3/anti-CD28-restimulation *in vitro*. Data are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively.

Furthermore, histological analyses confirmed the observed results regarding an AIT-mediated reduction of the allergy-induced airway inflammation, as lungs of both therapeutic groups displayed decreased peribronchiolar and perivascular inflammatory infiltration and mucus hypersecretion (Figure 11 A and B). Whereas lungs of the HDM-allergic group were characterized by a high inflammatory cell infiltrate accompanied with mucus hypersecretion.

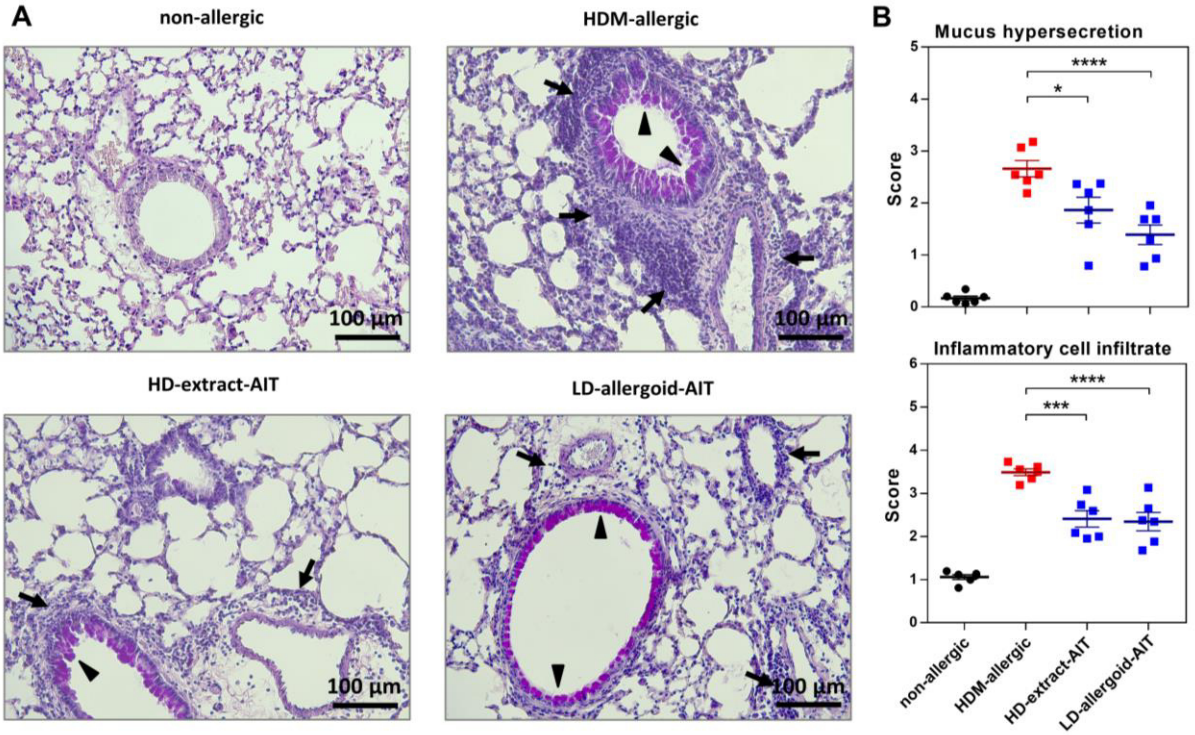

Figure 11: Reduced inflammatory cell infiltrate and mucus hypersecretion under AIT

A Representative lung histology specimen for non-allergic, HDM-allergic, HD-extract AIT and LD-allergoid AIT, taken three days after the last HDM challenge stained with periodic acid-Schiff. Arrows, inflammatory cell infiltrate; arrowheads, mucus hypersecretion. **B** Scoring for mucus hypersecretion and inflammatory cell infiltrate. Data were analyzed by one-way analysis of variance with Tukey's multiple comparison test.

7.2.2 Modulation of cytokine milieu of restimulated cells from lymphoid organs by LD-allergoid-AIT and HD-extract-AIT

Secreted cytokines of *ex vivo* anti-CD3/anti-CD28-restimulated cells from draining lymph nodes as well as splenocytes were analyzed after LD-allergoid-AIT and HD-extract-AIT. First, cytokine levels in culture supernatants from restimulated cervical lymph node cells were measured (Figure 12 A). Both treatment strategies resulted in a significantly decreased IL-5 and IL-13 secretion. HD-extract-AIT induced slightly, but not significantly, higher IL-4 levels and slightly lower IL-9 levels compared to LD-allergoid-AIT-treated and HDM-allergic mice. Secretion of IL-10 was reduced in both treatment groups, but only significant in the LD-allergoid-AIT group. Secretion of T helper 17 cell (Th17) cytokines IL-17A, IL-17F and IL-22 and other cytokines such as TNF-α and IL-6 were significantly decreased by both treatment strategies. Furthermore, reduced IFN-γ levels were significant lower in the LD-allergoid AIT but not in the HD-extract-AIT group. Secretion of IL-2 was restored in both AIT groups compared to the non-allergic control.

In addition, cytokine release of *ex vivo* anti-CD3/anti-CD28-restimulated splenocytes was analyzed (Figure 12 B). Both treatment strategies decreased the levels of IL-17A, IL-17F, IL-22, TNF-α and IFN-γ (IFN-γ only significant for LD-allergoid-AIT). Th2-type cytokines were also reduced in the supernatants of restimulated splenocytes following AIT, but effects were less pronounced than in restimulated cells from cervical lymph nodes. Levels of other cytokines such as IL-17A, IL-17F, IL-22, TNF-α, IL-6 and IL-2 were not altered.

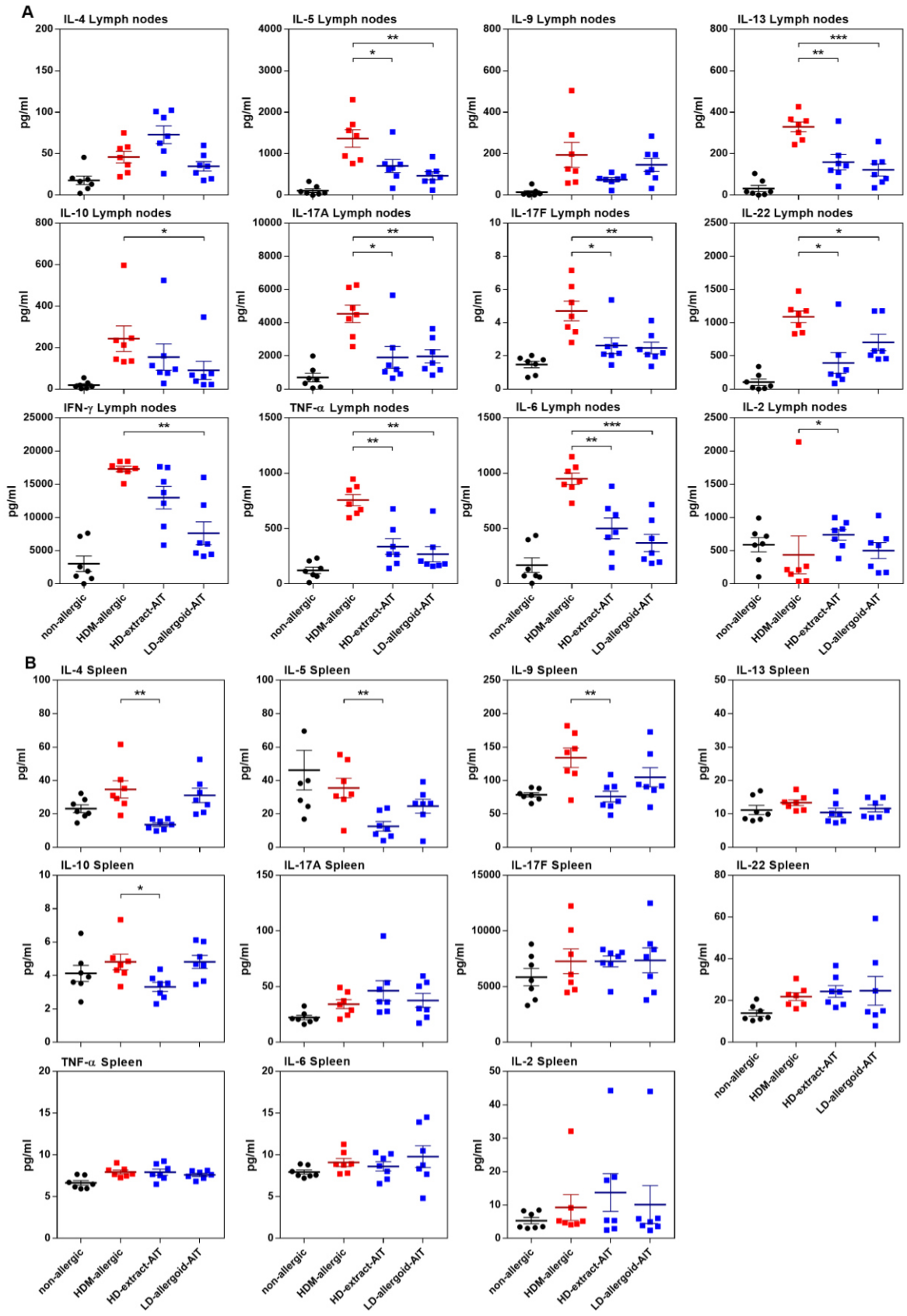

Figure 12: Effects of LD-allergoid-AIT and HD-extract-AIT on cytokine secretion of restimulated lymphoid organ cells

A Supernatant cytokine concentration of aCD3/aCD28-restimulated cervical lymph node cells. **B** Levels of secreted cytokines of aCD3/aCD28-restimulated splenocytes. Data are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively.

7.2.3 Therapeutic effects of LD-allergoid-AIT and HD-extract-AIT on the humoral immune response

On humoral level, serum concentrations of several immunoglobulins were detected (Figure 13). Here, LD-allergoid-AIT as well as HD-extract-AIT had no influence on the elevated total Der p-specific IgE (sIgE) serum levels of HDM-allergic mice, whereas total IgE (tIgE) levels were significantly lower in the LD-allergoid-AIT compared to the HD-extract-AIT group. While HD-extract-AIT induced high levels of total IgG1 (tIgG1), LD-allergoid-AIT failed to induce tIgG1. In contrast, both therapies led to an induction of Der p-specific IgG1 (sIgG1) levels, although changes were only significant in the HD-extract-AIT group. In addition, Der f-sIgE and -sIgG1 antibodies were detectable in a comparable manner (data not shown). Moreover, total IgG2c levels were slightly decreased in both AIT groups and total IgG2b levels in the LD-allergoid-AIT group while total IgG3 levels were not affected. Total IgM levels were decreased in both treatment groups.

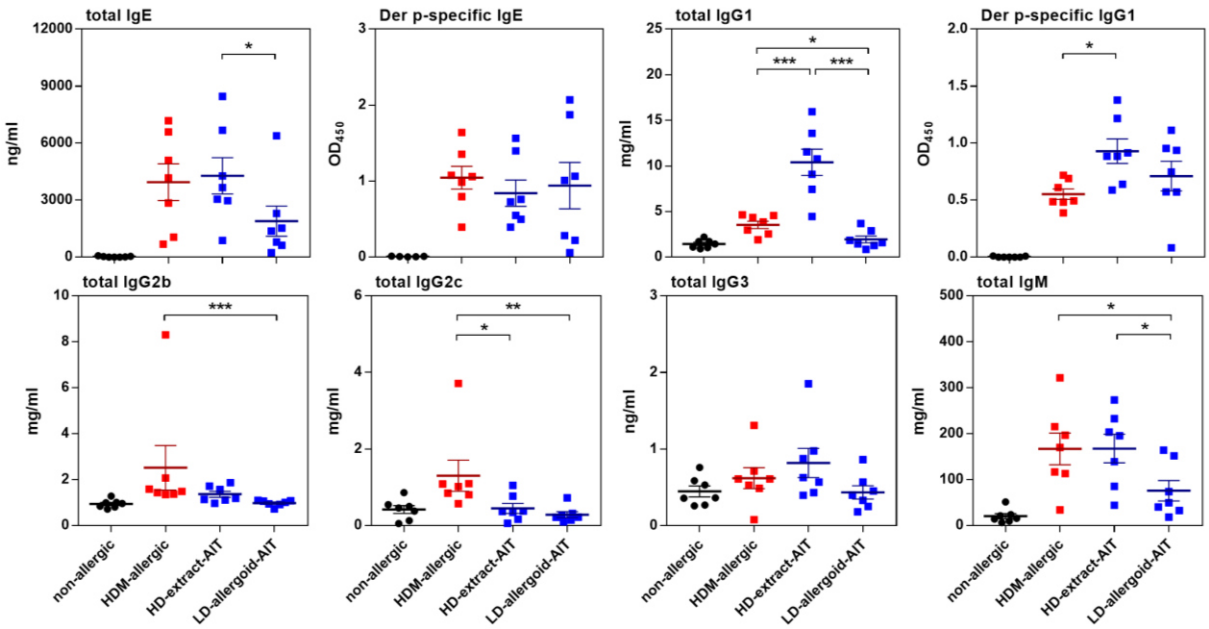

Figure 13: Analysis of serum-immunoglobulin levels after LD-allergoid-AIT and HD-extract-AIT

Levels of total IgE, Der p-specific IgE, total IgG1, Der p-specific IgG1, total IgG2b, IgG2c, IgG3 and IgM are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively.

7.3. Beneficial modulation of LD-allergoid-AIT by the adjuvants MCT and MPL

The therapeutic potential of combining low-dose (LD; 1 µg) HDM allergoid AIT with the depot-adjuvant MCT and the Th1-inducing adjuvant MPL was investigated (Figure 14).

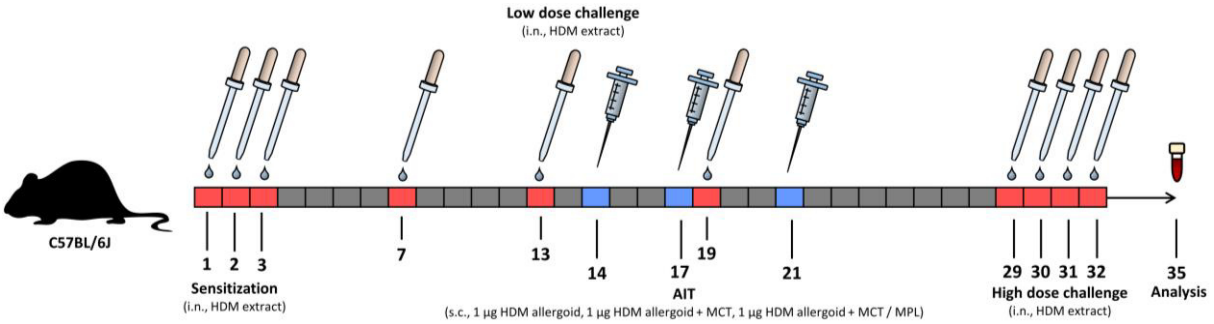

Figure 14: Murine model for low-dose HDM allergoid AIT w/o MCT/MPL
C57BL/6J were treated as follows: Mice from the allergic control group received sensitization, low-dose challenge, high dose challenge with HDM extract and PBS-AIT; the three AIT groups received sensitization, low-dose challenge, high dose challenge with HDM extract and AIT with low-dose HDM allergoids, low-dose HDM allergoids + MCT or low-dose HDM allergoids + MCT + MPL.

7.3.1 Effects of MCT and MPL on LD-allergoid-AIT-mediated protection against allergy-induced lung inflammation

First, the impact of LD-allergoid-AIT + MCT and LD-allergoid-AIT + MCT + MPL compared to LD-allergoid-AIT alone on infiltrating BAL cell count was investigated. All three treatment strategies had comparable effects on the reduction of total BAL cells, and BAL eosinophils, neutrophils, $CD4^+$ and $CD8^+$ T cells (Figure 15 A). Here, the additional use of the depot adjuvant MCT alone as well as in combination with the Th1-inducing adjuvant MPL revealed no further improvement in the protection against allergen challenge-induced BAL cell infiltration compared to LD-allergoid-AIT alone. Furthermore, all three AIT groups showed improved lung dynamic compliance compared to HDM-allergic mice (Figure 15 B). Here, a significant effect was achieved only in the LD-allergoid-AIT + MCT + MPL group.

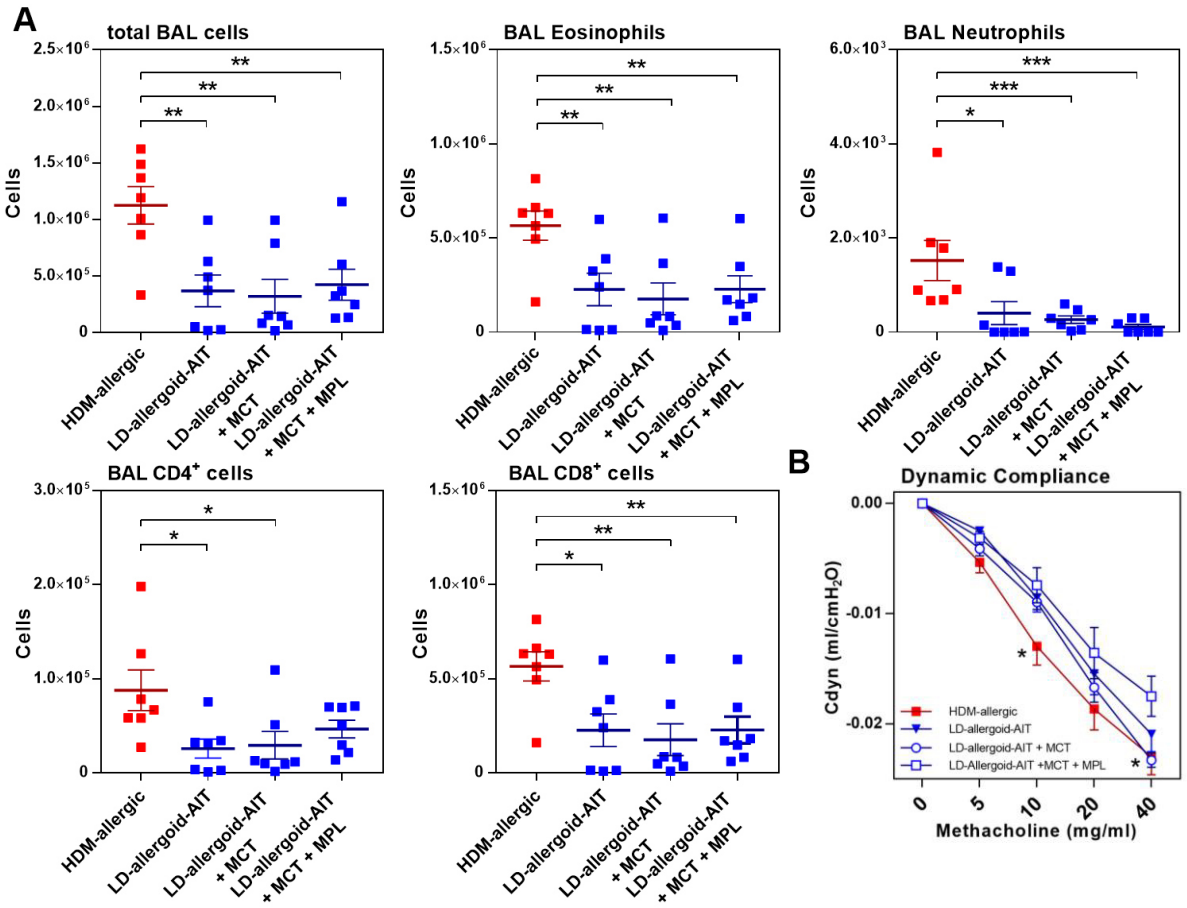

Figure 15: Reduction of inflammatory BAL cell infiltration and restoration of lung dynamic compliance under LD-allergoid-AIT w/o MCT/MPL

A Count of total BAL cells as well as eosinophils, neutrophils, $CD4^+$ and $CD8^+$ cells in the BAL fluid. Data are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively. **B** Measurement of dynamic compliance of the lungs in response to increased methacholine concentrations. Lung function parameters were analyzed by two-way analysis of variance with Tukey's multiple comparison test.

In addition, the levels of IL-4, IL-5 and TNF-α in the BAL fluid were measured (Figure 16 A). All three therapeutic strategies showed a tendency towards a reduced amount of all three cytokines in the BAL fluid. Here, the addition of MPL further decreased IL-4, IL-5 and TNF-α levels compared to LD-allergoid-AIT + MCT alone. While LD-allergoid-AIT alone had only a minor effect on the number of lung-resident $GATA3^+ST2^+FoxP3^-$ Th2 cells, these cells were significantly reduced in mice treated with LD-allergoid + MCT or LD-allergoid + MCT + MPL, whereas lung-resident $FoxP3^+CD4^+$ T cells were equally reduced in all treatment groups (Figure 16 B).

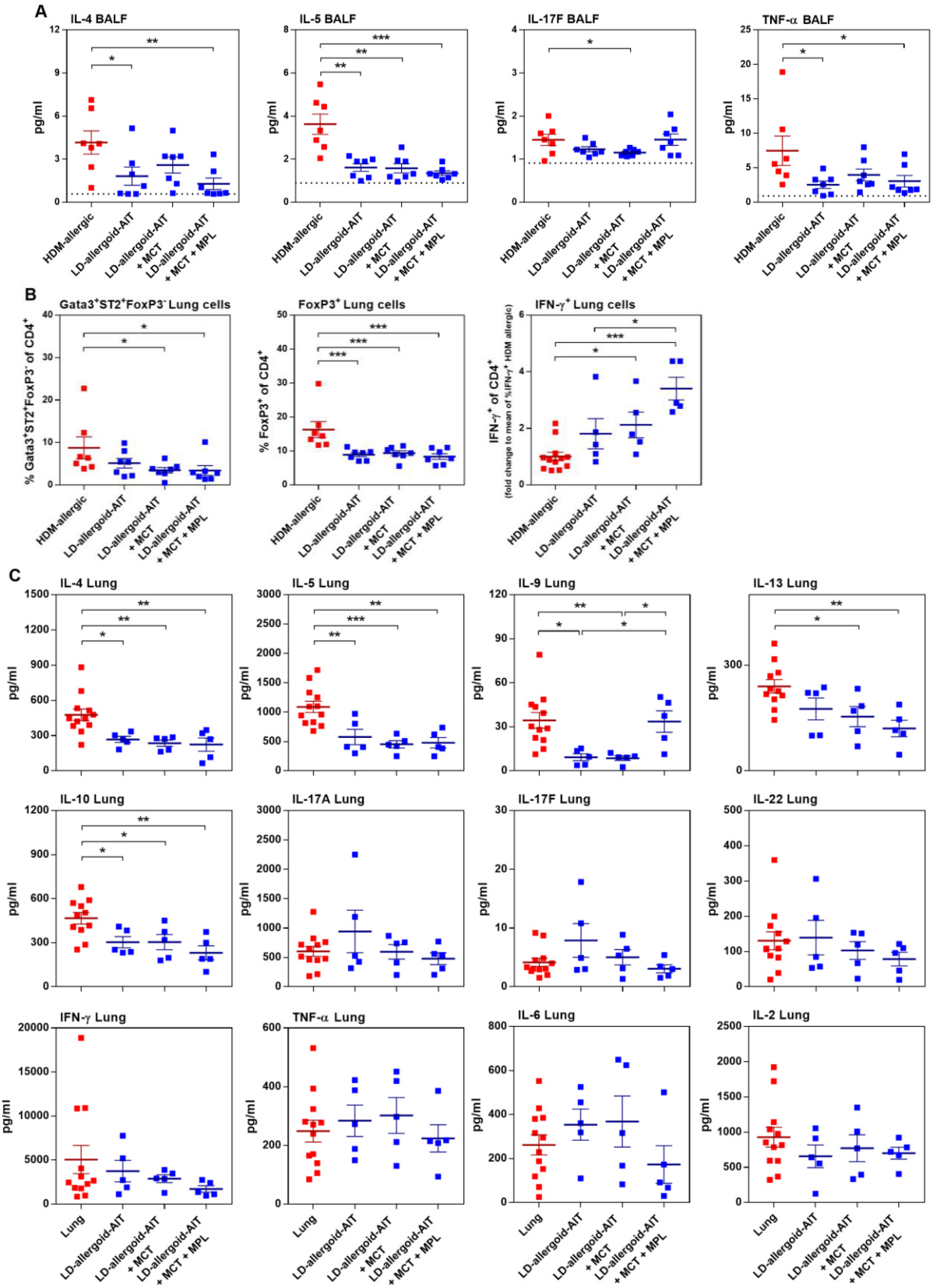

Figure 16: Effects of LD-allergoid-AIT w/o MCT/MPL on BALF cytokines, lung-resident lymphocytes, and cytokine secretion of restimulated lung-resident lymphocytes

A Cytokine milieu in BAL fluid **B** Analysis of lung-resident lymphocyte populations and IFN-γ$^+$CD4$^+$ T cells. **C** Cytokine release from lung-resident lymphocytes after anti-CD3/anti-CD28-restimulation *in vitro*. Data are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively.

While not effectively restored by HD-extract-AIT or LD-allergoid-AIT alone (Figure 10 B), the addition of MCT and MCT + MPL to the LD-allergoid-AIT formulation gradually increased the frequency of IFN-$\gamma^{+}CD4^{+}$ T cells in the lung compared to HDM-allergic mice (Figure 16 B). All three AIT strategies significantly decreased *ex vivo* secretion of IL-4, IL-5 and IL-10 of anti-CD3/anti-CD28-restimulated lung lymphocytes (Figure 16 C). Significant reduction of IL-13 secretion was only achieved by adding MCT or MCT + MPL to the formulation. While AIT with LD-allergoid and LD-allergoid + MCT also significantly reduced IL-9 secretion, this effect was reversed by the addition of MPL to the formulation. Other cytokines such as IL-17A, IL-17F, IL-22, IFN-γ, TNF-α, IL-6 and IL-2 were not altered. Additionally, all three treatment strategies comparably reduced peribronchiolar and perivascular inflammatory infiltration and mucus hypersecretion (Figure 17 A and B).

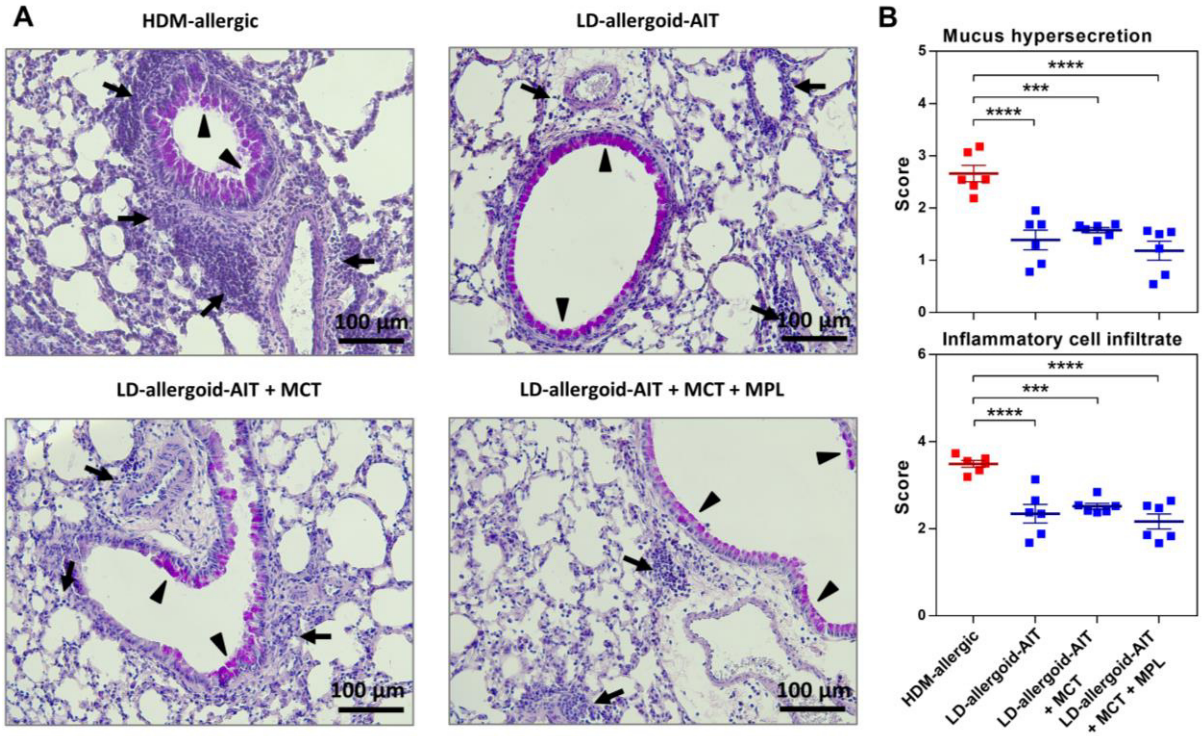

Figure 17: Reduced inflammatory cell infiltrate and mucus hypersecretion by LD-allergoid-AIT w/o MCT/MPL
A Representative lung histology specimen for HDM-allergic, LD-allergoid AIT, LD-allergoid AIT + MCT and LD-allergoid AIT + MCT + MPL, taken three days after the last HDM challenge stained with periodic acid-Schiff. Arrows, inflammatory cell infiltrate; arrowheads, mucus hypersecretion. **B** Scoring for mucus hypersecretion and inflammatory cell infiltrate. Data were analyzed by one-way analysis of variance with Tukey's multiple comparison test.

7.3.2 Modulation of the cytokine secretion of restimulated cells from lymphoid organs by adding MCT/MPL to LD-allergoid-AIT

Secreted cytokines of *ex vivo* anti-CD3/anti-CD28-restimulated cells from draining lymph nodes as well as splenocytes were measured. Compared to allergic mice, the level of IL-13 in supernatants of restimulated lymph node cells was significantly reduced in all three treatment groups (Figure 18 A). IL-5 levels were significantly lower in the LD-allergoid-AIT as well as in the LD-allergoid AIT + MCT groups and IL-9 levels in the LD-allergoid AIT + MCT group. IL-10 levels were lower in all treatment groups, but only significant in the LD-allergoid-AIT group, while IL-4 levels were unchanged compared to allergic controls. All treatment regimens had comparable effects on the reduction of secreted IL-17A, IL-17F, IL-22 and TNF-α from lymph node cells. Interestingly, the reduced IFN-γ production, observed in LD-allergoid-AIT- compared to HD-extract-AIT-treated mice, was reverted by the addition of MCT to the AIT formulation with no additional effect of MPL.

In addition, cytokine release of *ex vivo* anti-CD3/anti-CD28-restimulated splenocytes was measured (Figure 18 B). Again, the effects of all three AIT strategies on reduced Th2-type cytokine secretion from restimulated splenocytes were less pronounced compared to restimulated lymphocytes. Levels of other secreted cytokines such as IL-17A, IL-17F, IL-22, TNF-α, IL-6 and IL-2 were not altered.

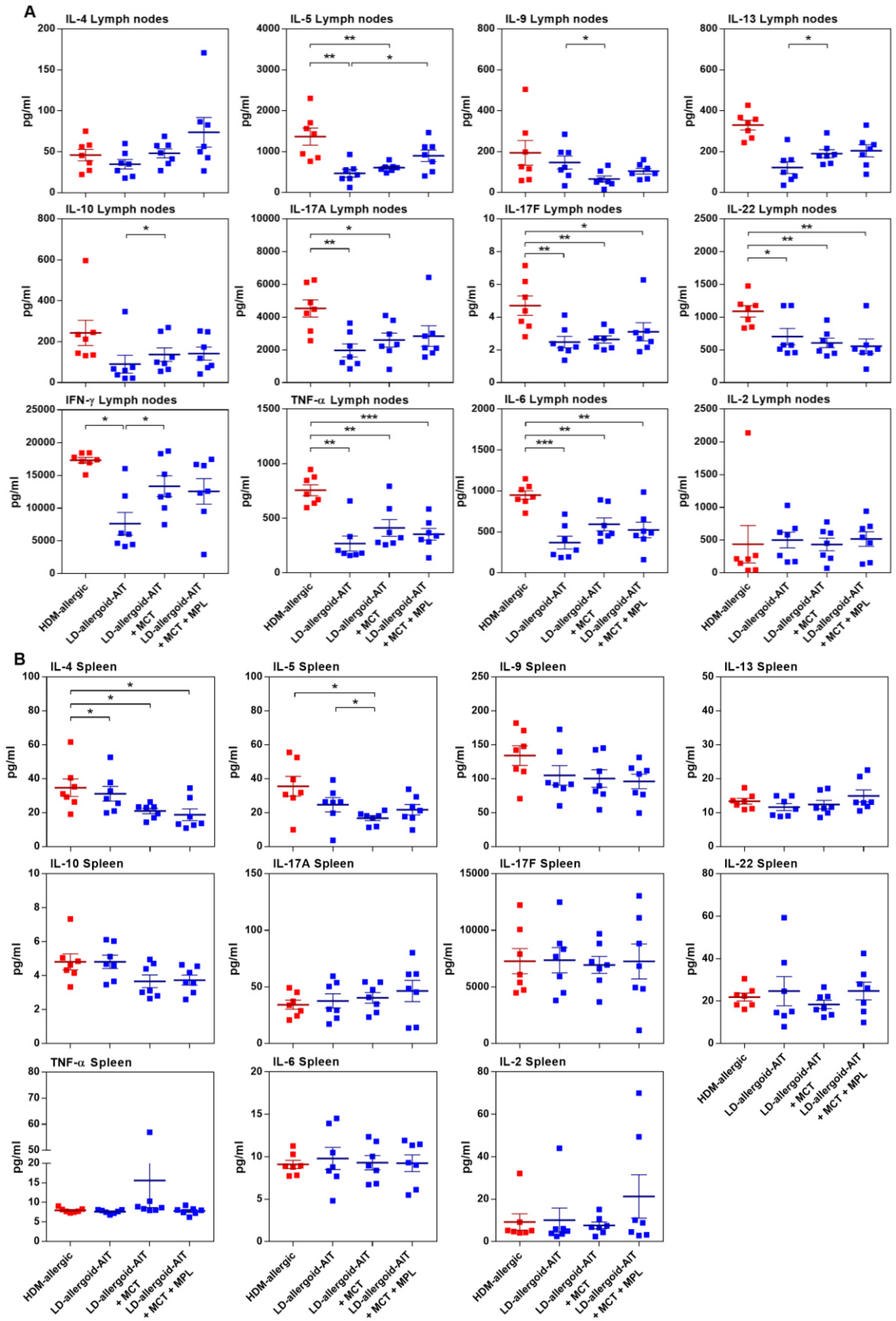

Figure 18: Effects of LD-allergoid-AIT w/o MCT/MPL on cytokine secretion of restimulated lymphoid organ cells

A Supernatant cytokine concentration of aCD3/aCD28-restimulated cervical lymph node cells. **B** Levels of secreted cytokines of aCD3/aCD28-restimulated splenocytes. Data are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively.

7.3.3 Modulatiory effects on the humoral immune response by the addition of MCT/MPL to the LD-allergoid-AIT

On humoral level, serum concentrations of different immunoglobulins were measured (Figure 19). Total IgE and Der p-sIgE levels were not affected by any of the three treatment regimens. While LD-allergoid-AIT alone was not able to induce a significant tIgG1 response, the addition of MCT as well as of MCT + MPL to the formulation iteratively increased tIgG1 levels significantly. Although Der p-sIgG1 levels were comparably elevated in all three treatment groups, changes were only significant in the LD-allergoid-AIT + MCT + MPL group. While total IgG2b and IgM levels were gradually increased by MCT and MCT + MPL, total IgG2c levels were comparable in all three treatment groups and lower compared to allergic controls. Levels of tIgG3 antibodies revealed a significant induction by the addition of MPL to the formulation.

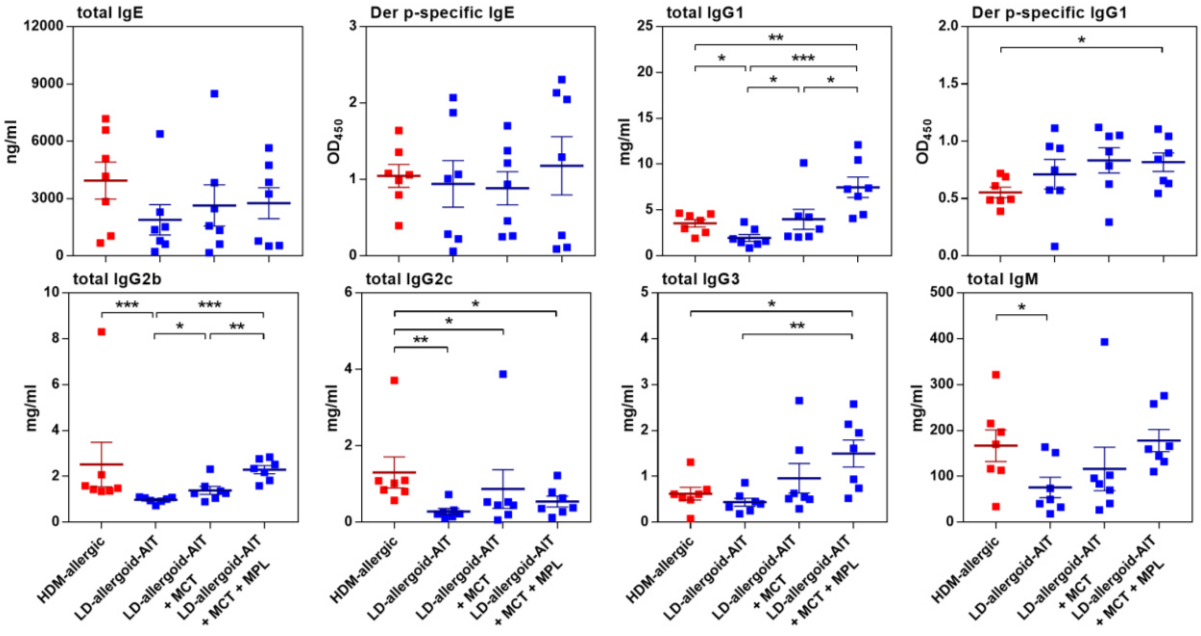

Figure 19: Analysis of serum-immunoglobulin levels after LD-allergoid-AIT w/o MCT/MPL

Levels of total IgE, Der p-specific IgE, total IgG1, Der p-specific IgG1, total IgG2b, IgG2c, IgG3 and IgM are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively.

7.4. Dose response effects of MPL in MCT-based HDM allergoid AIT

As not only the allergen, but also the adjuvant dose may have crucial influence on the anti-inflammatory capacity of AIT formulations, it should be investigated whether the newly established murine HDM AIT model is also appropriate to address dose-response effects of increasing MPL concentrations (12.5 µg, 25 µg, 50 µg or 100 µg) in combination with low-dose (LD; 1 µg) HDM allergoid AIT + MCT (Figure 20).

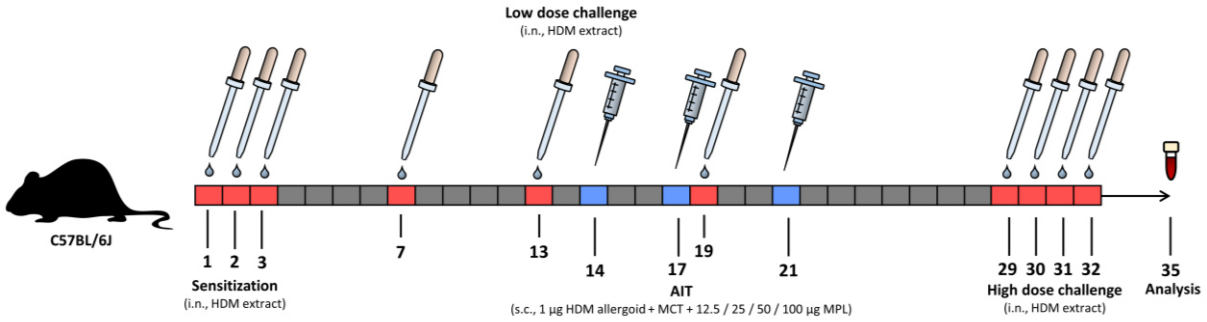

Figure 20: Murine model for low-dose HDM allergoid AIT + MCT combined with increasing MPL concentrations
Mice (C57BL/6J) were treated as follows: Mice from the non-allergic control group only received high dose challenge with HDM extract at the end, all other treatments were done with PBS; the allergic control group received sensitization, low-dose challenge, high dose challenge with HDM extract and PBS-AIT; Low-dose HDM allergoid AIT + MCT + 12.5 / 25 / 50 / 100 µg MPL groups received sensitization, low-dose challenge, high dose challenge with HDM extract and AIT with HDM allergoids + MCT + 12.5 / 25 / 50 / 100 µg MPL.

7.4.1 Effects of different MPL doses on LD-allergoid-AIT + MCT mediated protection against allergy-induced lung inflammation

First, effects of increasing MPL concentrations in combination with LD-allergoid-AIT + MCT on BAL cell infiltration were investigated (Figure 21). LD-allergoid-AIT with 12.5µg, 25µg and 50µg MPL resulted in a significant decrease of total BAL cells as well as BAL eosinophils, $CD4^+$ and $CD8^+$ cells. This beneficial effect was less pronounced in mice treated with 12.5µg MPL and, interestingly, nearly completely reversed applying 100µg MPL. Comparable trends could be observed regarding the amount of BAL neutrophils.

Furthermore, lymphocytes of the differently treated mice groups were isolated from lung tissue and analyzed by flow cytometry (Figure 22 A).

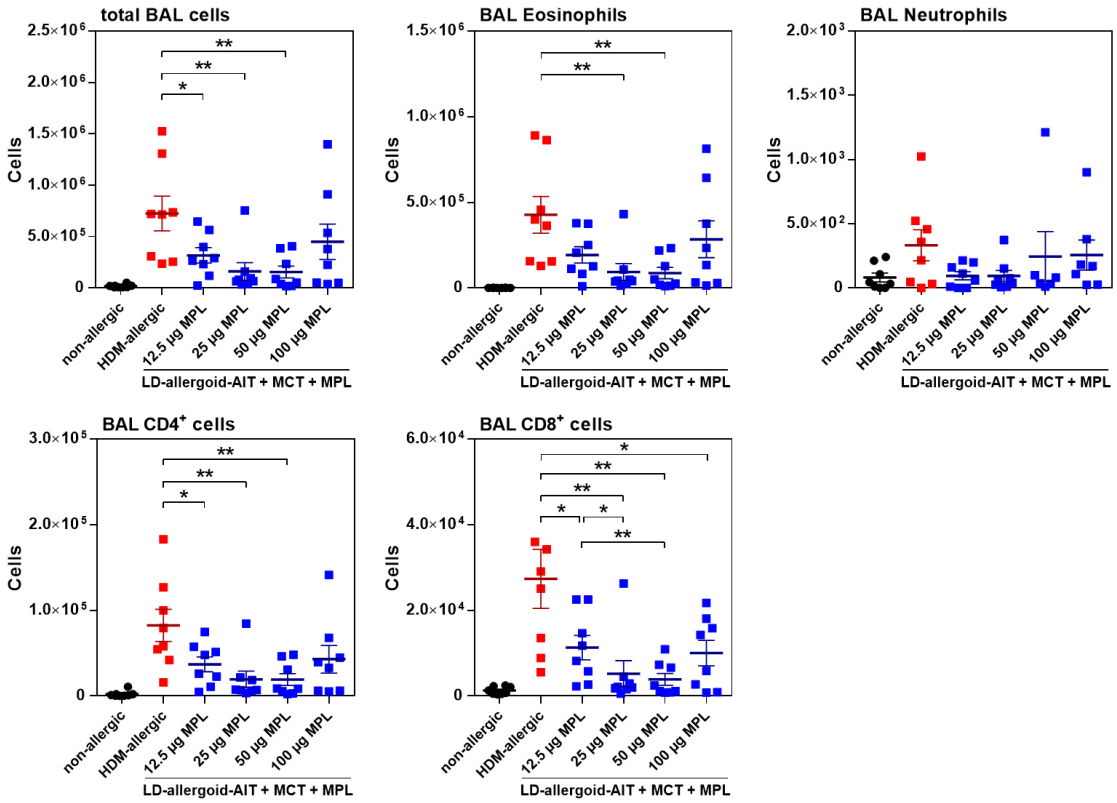

Figure 21: Effects of increasing MPL doses on LD-allergoid-AIT + MCT mediated reduction of inflammatory BAL cell infiltration

Count of total BAL cells as well as eosinophils, neutrophils, $CD4^+$ and $CD8^+$ cells in the BAL fluid. Data are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively.

The HDM challenge-induced increase of lung-resident $GATA3^+ST2^+FoxP3^-$ Th2 cells found in allergic mice was significantly decreased by LD-allergoid-AIT + MCT in combination with 25µg and 50µg MPL. The decreasing effect was less pronounced by using 12.5 and 100 µg MPL. Comparable trends could be observed for $FoxP3^+CD4^+$ T cells.

Furthermore, cytokine secretion of ex vivo anti-CD3/anti-CD28-restimulated lung-resident lymphocytes was analyzed (Figure 22). The most prominent effects on the reduction of IL-4, IL-5, IL-13, IL-6 and IL-10 levels in the supernatants were again achieved by using 25 µg or 50 µg in combination with the LD-allergoid-AIT + MCT. Whereas the use of 25 µg MPL led to a significant decrease of IL-17A and IL-17F levels, the use of 50 µg MPL significantly downregulated IL-22 and TNF-a secretion. Interestingly, using 25 µg and 50 µg MPL

significantly increased IFN-γ secretion compared to 12.5 µg and 100 µg MPL. IL-2 levels were not altered in all four treatment groups.

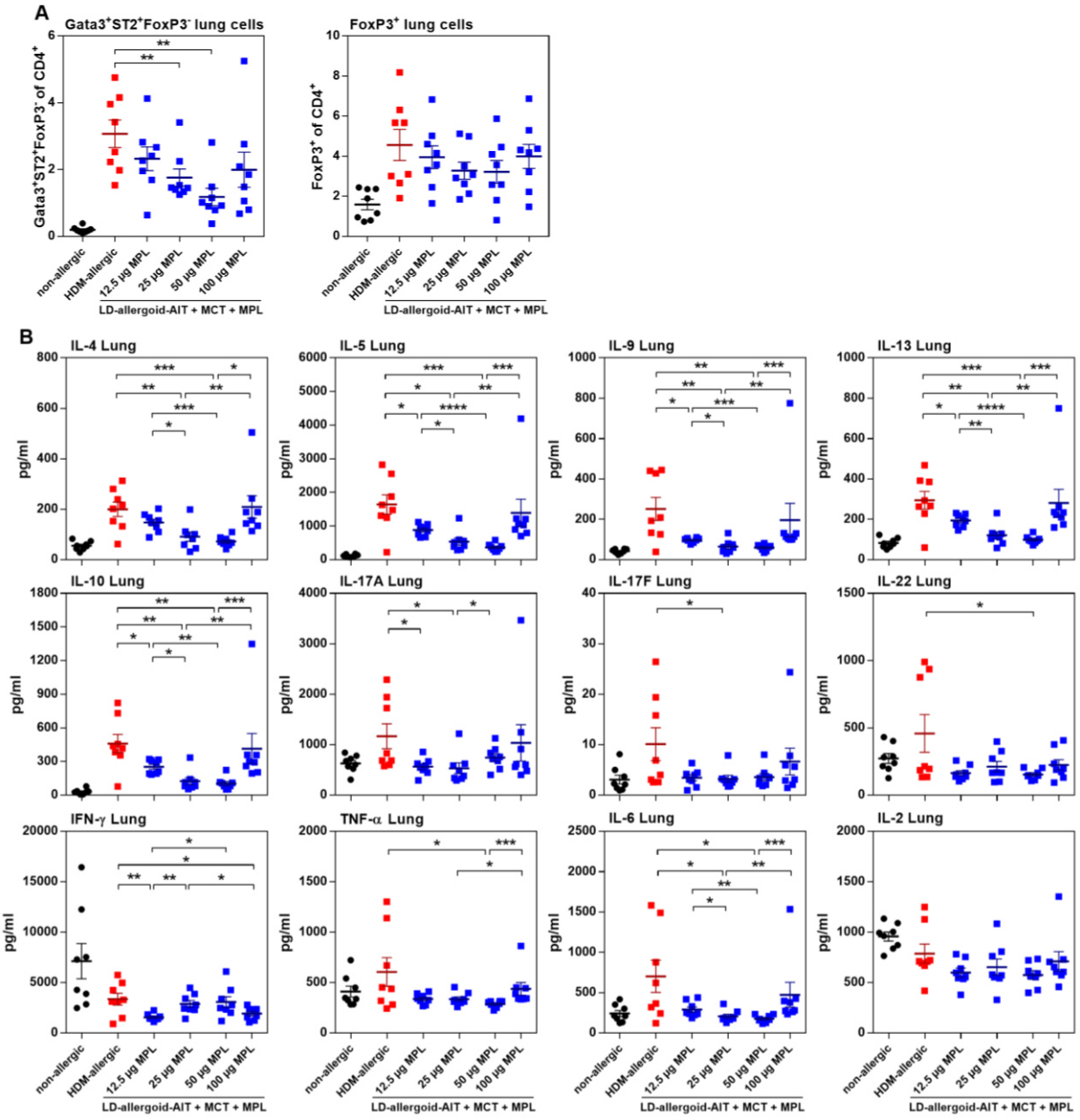

Figure 22: Effects of LD-allergoid-AIT + MCT + increasing MPL doses on lung-resident lymphocytes and cytokine secretion of restimulated lung-resident lymphocytes

A Analysis of lung-resident lymphocyte populations. **B** Cytokine release from lung-resident lymphocytes after anti-CD3/anti-CD28-restimulation *in vitro*. Data are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively.

7.4.2 Modulation of the cytokine secretion of restimulated cells from cervical lymph nodes by adding increasing MPL doses to the LD-allergoid-AIT + MCT formulation

Secreted cytokines of *ex vivo* anti-CD3/anti-CD28-restimulated cells from draining lymph nodes were measured. Here, again the addition of 25 µg and 50 µg MPL worked best to significantly reduce the secretion of IL-4, IL-5, IL-9, IL-13, IL-6 and IL-17A. Supernatant levels of IL-10, IL-22 and TNF-a showed similar trends. Interestingly, IFN-γ release of restimulated cervical lymph node cells was equally and significantly increased by the addition of 25 µg, 50 µg and 100 µg to the LD-allergoid-AIT + MCT compared to 12.5 µg MPL.

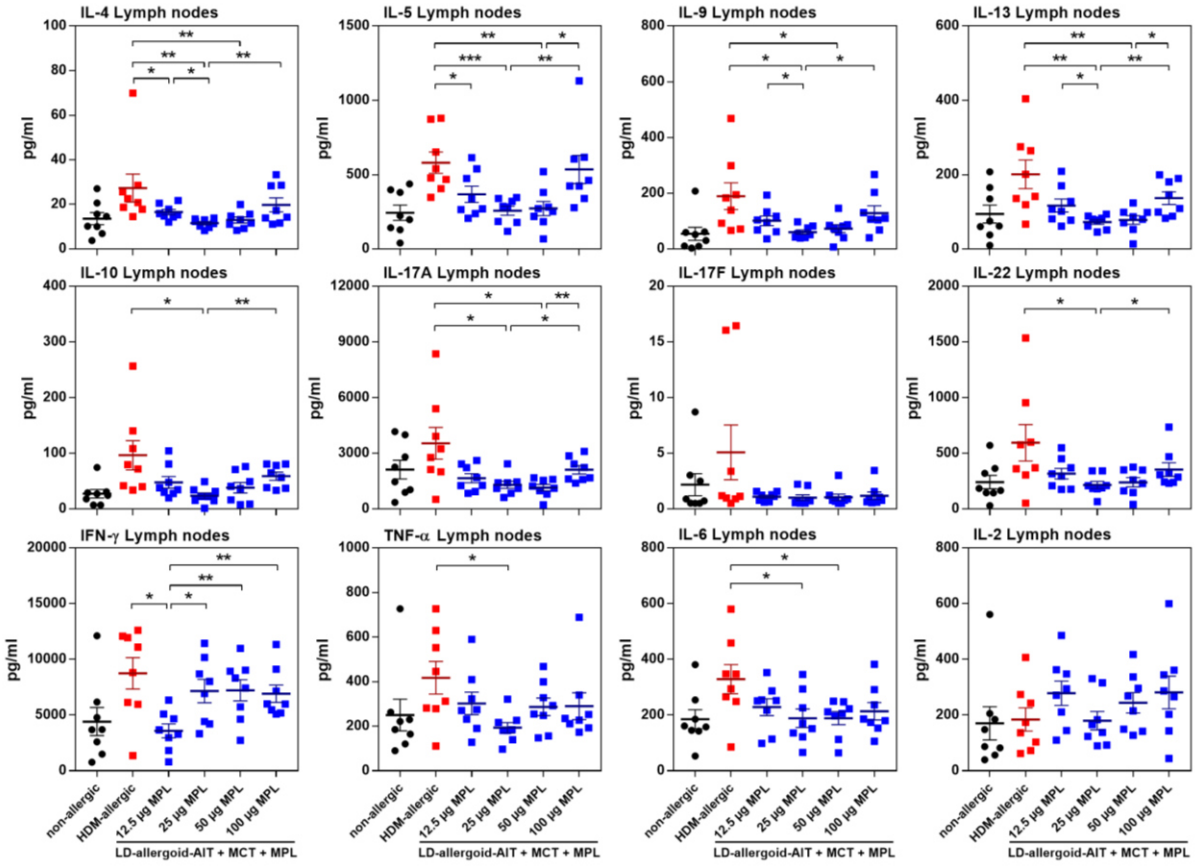

Figure 23: Effects of LD-allergoid-AIT + MCT + increasing MPL doses on cytokine secretion of restimulated lymph node cells

Supernatant cytokine concentration of aCD3/aCD28-restimulated cervical lymph node cells. Data are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively.

7.4.3 Modulatory effects on the humoral immune response by the addition of increasing MPL doses to the LD-allergoid-AIT + MCT formulation

On humoral level, concentrations of several immunoglobulins in the serum were analyzed (Figure 24). Here, tIgE and Der p-sIgE levels slightly increased by using lower MPL concentrations (12.5 µg and 25 µg) compared to HDM-allergic mice, this effect was reversed applying the two higher concentrations (50 µ and 100 µg). Most importantly, the use of 25µg and 50µg MPL combined with LD-allergoid-AIT + MCT in the formulation worked best for the induction of tIgG1 and Der p-sIgG1. Furthermore, a stepwise increase of IgG2b as well as IgG3 with adding increasing MPL doses could be observed. Serum level of total IgG2c and IgM was not altered by all treatment regimens compared to the HDM-allergic group.

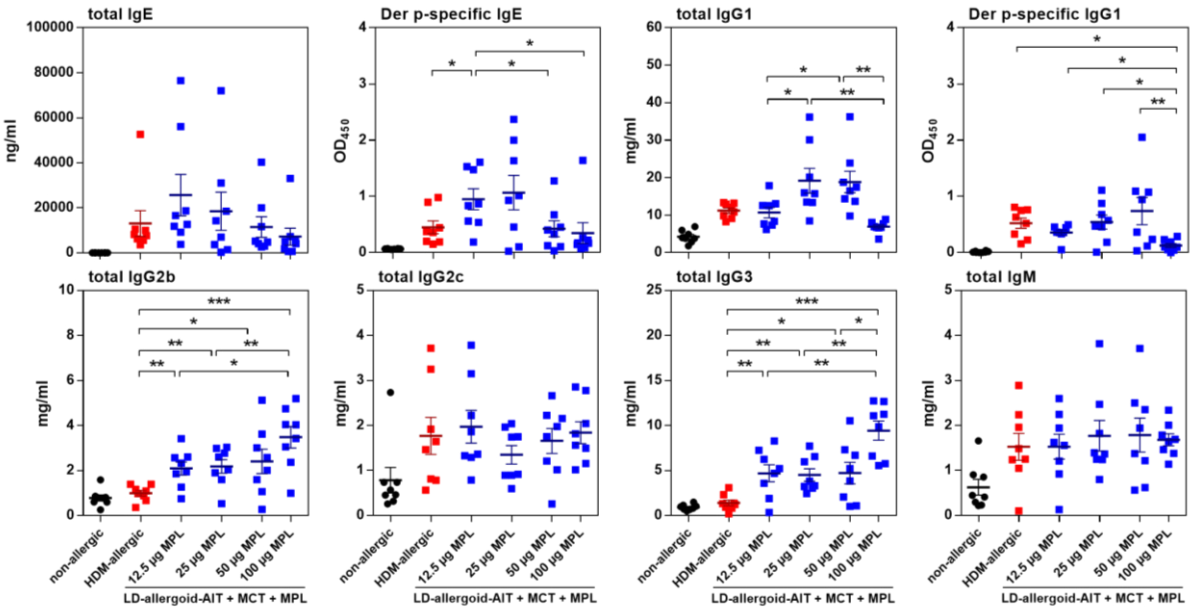

Figure 24: Analysis of serum-immunoglobulin levels after LD-allergoid-AIT + MCT + increased MPL doses
Levels of total IgE, Der p-specific IgE, total IgG1, Der p-specific IgG1, total IgG2b, IgG2c, IgG3 and IgM are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively.

7.5. Human PBMC stimulation with HDM extract, HDM allergoids and adjuvants

Another aim of the project was to translate the adjuvant-based allergoid AIT approach also to human allergy and allergy immunotherapy research. Therefore, PBMCs were isolated from five healthy controls, six HDM-allergic patients and four HDM-allergic patients that received SCIT. In an *ex vivo* stimulation assay, $2x10^5$ cells from each donor were stimulated with 1 µg/ml HDM extract w/o 10 µg/ml MPL, 1 µg HDM allergoids w/o 10 µg/ml MPL or 10 µg/ml MPL alone for five days. 10 µg/ml LPS and PHA served as positive control.

7.5.1 Modulation of human PBMC cytokine release by HDM extract or HDM allergoid w/o MPL stimulation

First, a broad characterization of secreted cytokines in culture supernatants was performed. HDM extract stimulation led to a significant increase of Th2 cytokine release such as IL-4, IL-5, IL-9 and IL-13 by PBMCs from HDM-allergic patients with SCIT. PBMCs from HDM-allergic patients without SCIT showed slightly lower Th2 cytokine secretion, whereas there was almost no Th2 cytokine release by PBMCs from healthy patients (except slight IL-4 and IL-9 secretion) (Figure 25). Stimulation with the mitogen PHA revealed the potential of PBMCs from all donors to release Th2 cytokines in the same manner. Interestingly, co-stimulation with MPL led to a significant decrease of HDM extract-induced IL-5, IL-9 and IL-13 levels (Figure 25). As expected, stimulation with MPL alone as well as with the LPS control did not induce Th2 cytokine secretion. In contrast to HDM extract, stimulation with HDM allergoids resulted in no alteration of secreted IL-4, IL-5, IL-9 and IL-13 levels (Figure 25). Furthermore, stimulation with MPL as well as with LPS led to a high IL-6 and IL-10 release. PBMC co-stimulation with HDM extract as well as HDM allergoids in combination with MPL showed a higher secretion of IL-6 and IL-10 than HDM extract or HDM allergoid stimulation alone. MPL-induced secretion of IL-6 and IL-10 was independent of the allergic status of the PBMC donors (Figure 25).

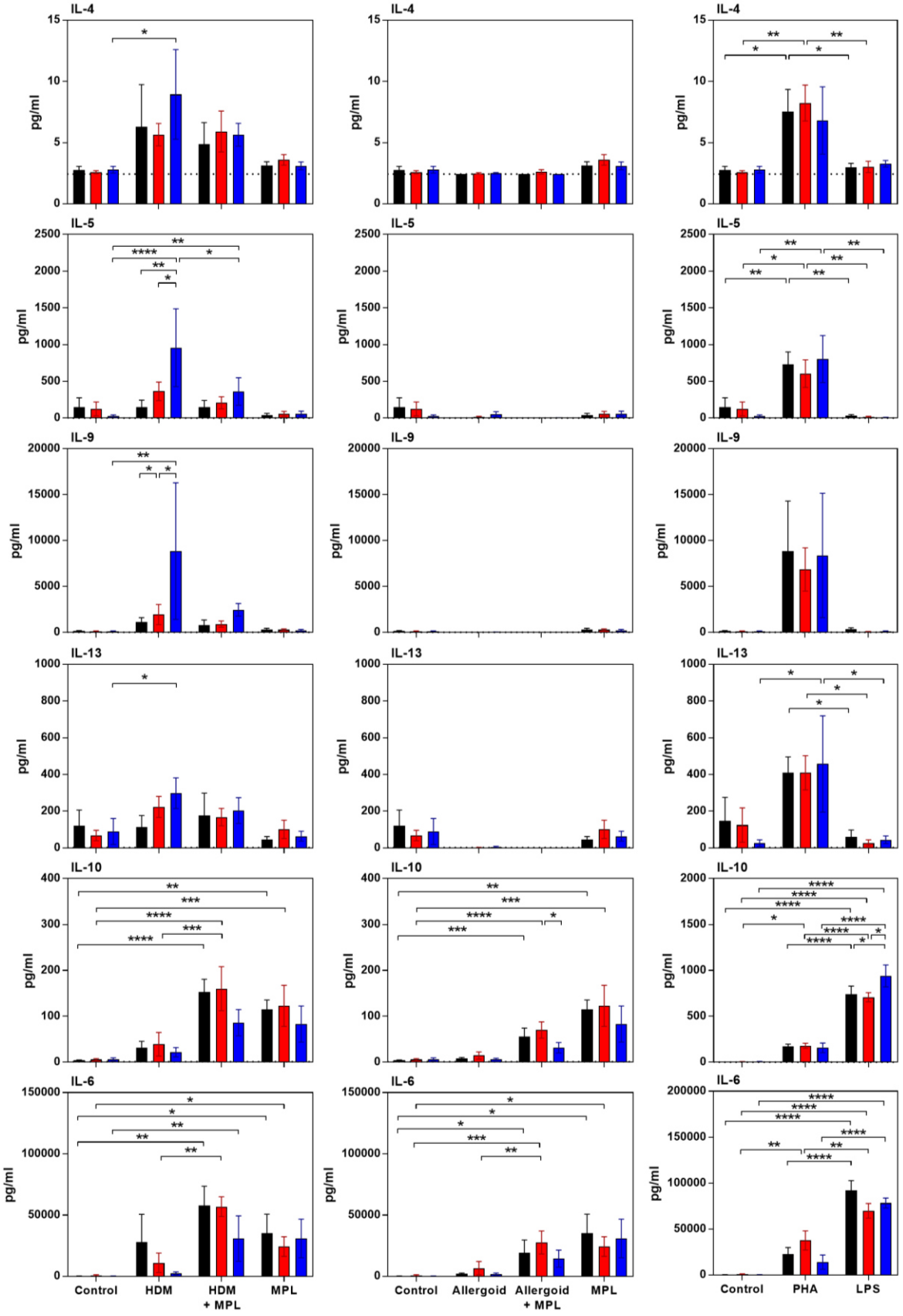

Figure 25: Reduction of HDM extract-induced Th2 cytokine secretion and elevation of IL-10 levels by MPL

Levels of supernatant Th2 cytokines IL-4, IL-5, IL-9, IL-13 as well as IL-10 and IL-6 secreted by stimulated human PBMCs from healthy donors (black bars), HDM-allergic patients (red bars) and HDM allergic patients with SCIT (blue bars). Data are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively.

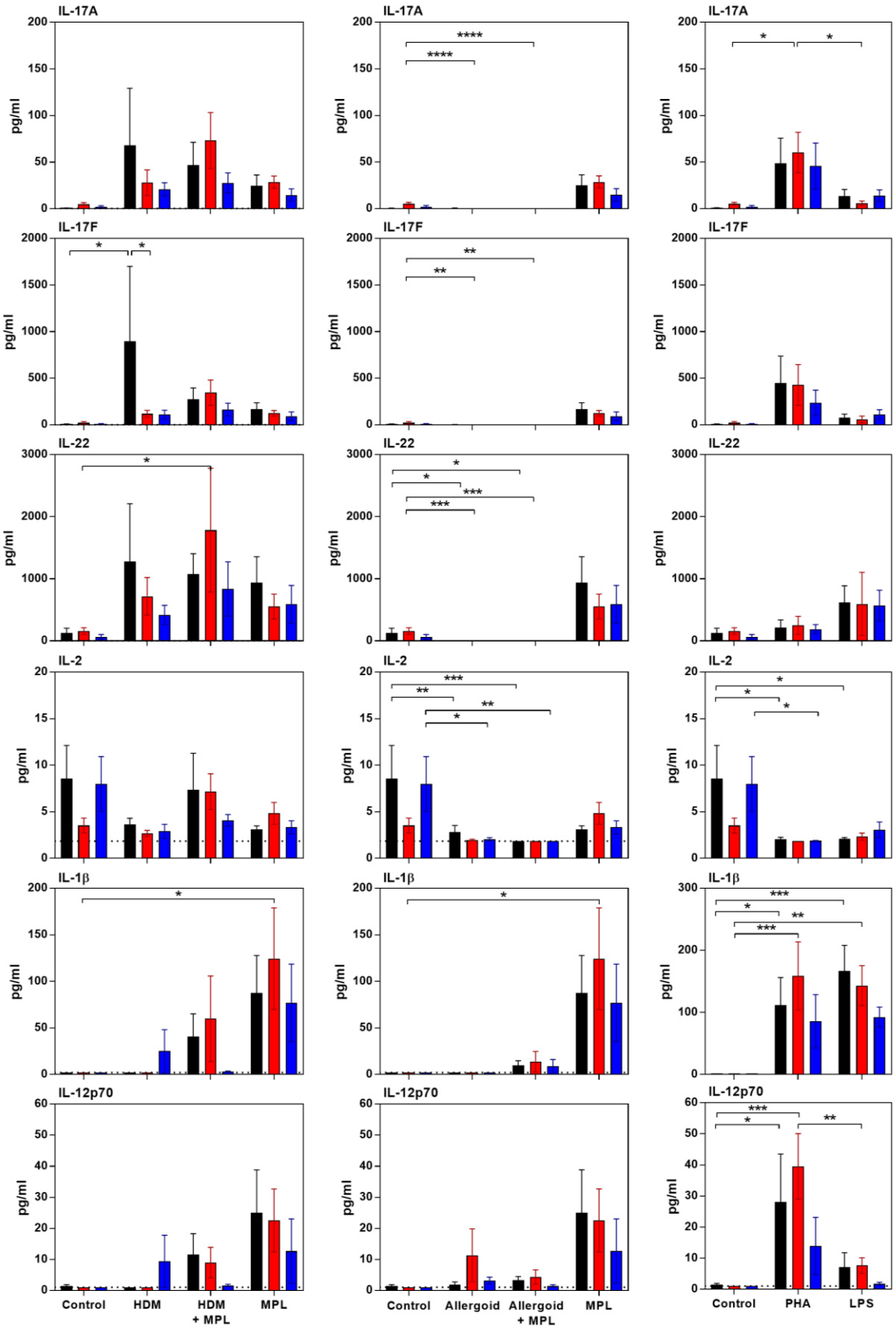

Figure 26: Induction of IL-1β and IL-12p70 levels by MPL

Levels of supernatant Th17 cytokines IL-17A, IL-17F, IL-22 as well as IL-2, IL-1β and IL-12p70 secreted by stimulated human PBMCs from healthy donors (black bars), HDM-allergic patients (red bars) and HDM allergic patients with SCIT (blue bars). Data are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively.

Regarding Th17 cytokine release, MPL as well as HDM extract stimulation of PBMCs led to increased levels of IL-17A, IL-17F and IL-22 (Figure 26). Although there were no differences in Th17 cytokine release by HDM extract-stimulated PBMCs from HDM-allergic patients w/o SCIT, secretion seemed a little bit higher by PBMCs from healthy controls. Combining HDM extract with MPL stimulation showed only slight and not significant alterations of Th17 cytokine levels. Stimulation with HDM allergoids w/o MPL did not result in IL-17A, IL-17F or IL-22 secretion. Furthermore, secreted IL-2 levels were a little bit higher in unstimulated PBMCs from healthy as well as HDM-allergic patients with SCIT (Figure 26). IL-1b and IL-12p70 levels showed similar patterns. Here, both cytokines were secreted by PBMCs from all donor groups after stimulation with MPL, LPS or PHA (Figure 26). The addition of MPL to HDM extract or HDM allergoids resulted in no significant alterations. Furthermore, stimulation with MPL led to an increase of IFN-γ and TNF-α. Combining the MPL stimulation with HDM allergoid resulted in an additional increase of TNF-α but not IFN-γ, whereas the combination with HDM extract increased both cytokines (Figure 27).

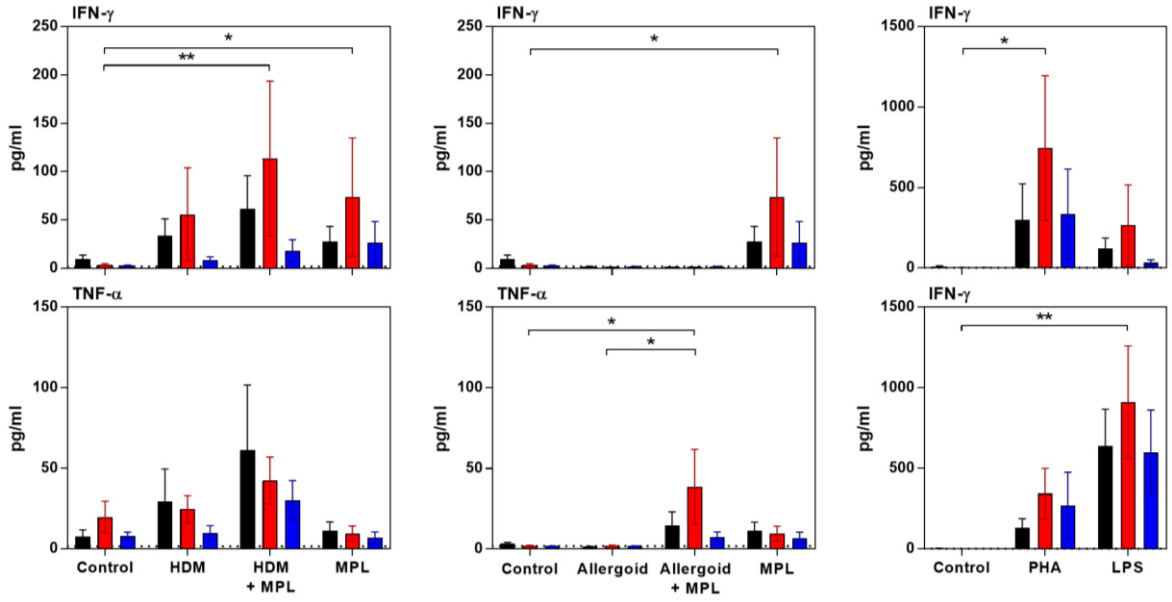

Figure 27: Th1-inducing properties of MPL

Levels of supernatant Th1 cytokines IFN-γ and TNF-α secreted by stimulated human PBMCs from healthy donors (black bars), HDM-allergic patients (red bars) and HDM allergic patients with SCIT (blue bars). Data are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively.

7.5.2 Patterns of human IL-4$^+$CD4$^+$ and IFN-γ$^+$CD4$^+$ T cells under HDM extract or HDM allergoid w/o MPL stimulation

In addition, the amount of living IL-4$^+$CD4$^+$ as well as IFN-γ$^+$CD4$^+$ T cells within the restimulated PBMCs was measured (Figure 28). Here, the number of IL-4$^+$CD4$^+$ T cells after HDM extract stimulation reflected the patterns of secreted Th2 cytokines. A significant increase of IL-4 production could be observed in PBMCs from HDM-allergic patients with SCIT and could be clearly distinguished from PBMCs from healthy donors. PBMCs from HDM-allergic patients without SCIT showed slightly lower numbers of IL-4$^+$CD4$^+$ T cells. In both patient groups, adding MPL to the HDM extract stimulation resulted in a decreased amount of IL-4$^+$CD4$^+$ T cells comparable to unstimulated conditions. Here again, stimulation with HDM allergoids did not lead to an increase of IL-4$^+$CD4$^+$ T cells. Furthermore, the addition of MPL to the HDM extract stimulation increased the number of IFN-γ$^+$CD4$^+$ T cells compared to HDM extract stimulation alone, whereas stimulation with MPL in combination with HDM allergoids showed weaker effects.

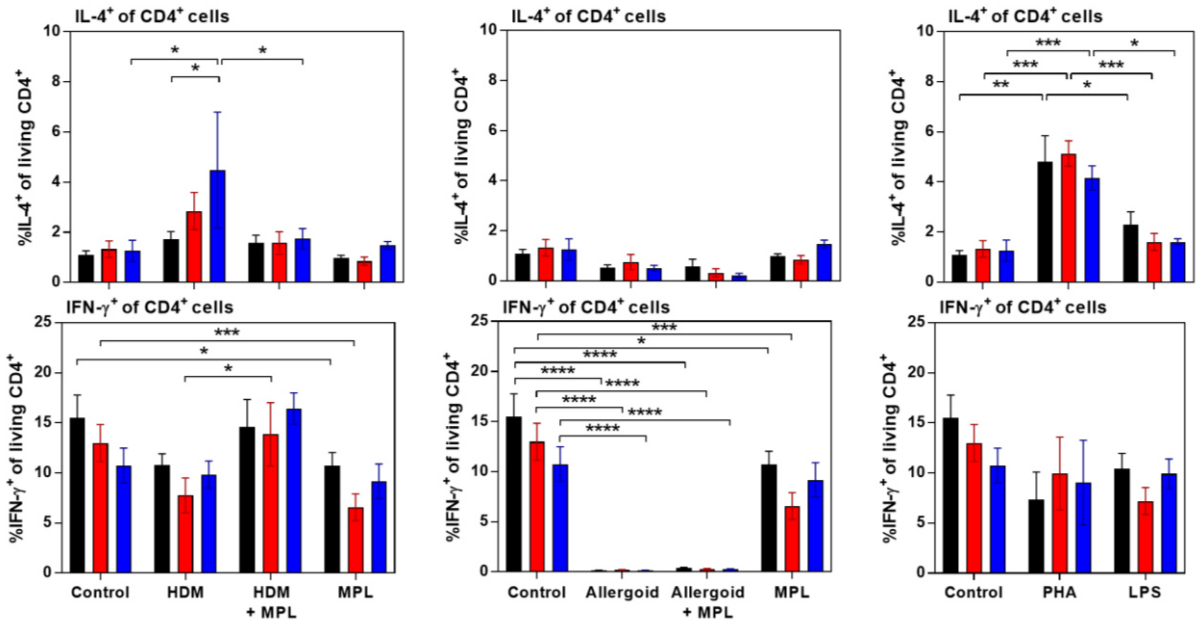

Figure 28: Reduction of intracellular IL-4 levels and induction of intracellular IFN-γ levels by MPL
Analysis of IL-4$^+$ and IFN-γ$^+$CD4$^+$ T cells within the stimulated human PBMCs from healthy donors (black bars), HDM-allergic patients (red bars) and HDM allergic patients with SCIT (blue bars). Data are given as means +/- SEM. Gaussian and non-Gaussian distributed results were analyzed by unpaired t test and Mann Whitney test, respectively.

8 Discussion

The aim of this doctoral thesis was to give a deeper insight into HDM allergy-driven and protective AIT mechanisms. Regarding the improvement of new allergy immunotherapy approaches, the therapeutic potential of low-dose HDM allergoid alone and in combination with the depot adjuvant MCT and the Th1-inducing adjuvant MPL in comparison to conventional high-dose HDM extract should be investigated. The first step of this project focused on the establishment of a new murine HDM AIT model based on alum-free i.n. sensitization and SCIT with the aim to resemble local allergy-driven and protective AIT mechanisms in the airways and in lymphoid organs, which are hard to address in human HDM allergy studies. Commonly used murine HDM AIT models are based on i.p. sensitization combining the allergen with the adjuvant alum [127-130]. A drawback of these models is that they are not reflecting the natural sensitization route to HDM allergens in humans and previous studies revealed broad unspecific alum-mediated immune activation [109, 110]. Therefore, the new model should bear the advantage of mimicking human HDM allergy in a more physiological way. Based on more realistic initial adjuvant-free i.n. sensitization followed by weekly and final i.n. challenge timepoints, this model mimics natural contact to HDM allergens and resembles in many aspects human allergic inflammation [55, 133-137]. Here, the model is characterized by the typical HDM allergy-driven eosinophilic and Th2 cell infiltration into the lung, decreased lung dynamic compliance, reduced mucus hypersecretion, tIgE and HDM sIgE induction as well as elevated Th2-type cytokine secretion by lung-resident lymphocytes, lymph node cells and splenocytes. In addition, the model also displays hallmarks of successful AIT in humans such as the induction of HDM sIgG antibodies while sIgE levels do not immediately decrease [138-140]. In contrast to human AIT, in murine AIT models based on alum-dependent i.p. sensitization, the sIgE response is nearly completely lost during treatment [141]. Within the last decade, allergy immunotherapy research focused on developing and modifying new AIT

strategies addressing its efficacy while maintaining or improving its safety profile [79]. Beside conventionally used HDM extracts, HDM allergoids got into the focus of the development of allergy vaccines [142]. HDM allergoids are allergen extracts that are chemically modified by glutaraldehyde or formaldehyde. The modification results in less reactive B cell epitopes and thus reduced IgE binding, while T cell epitopes and immunogenic effect remain unaltered [79, 143-146]. However, the tolerogenic potency of allergoids compared to natural allergen extracts is controversially discussed [147-149] and prior studies have questioned the efficacy of allergoids due to the modification process [150]. The present study demonstrates significant effectiveness of allergoids even at low-dose accessing a wide set of biomarkers. Notably, according to guidelines of the European Academy of Allergy and Clinical Immunology (EAACI), both modified and unmodified allergen extracts are recommended for SCIT of allergic rhinocunjunctivitis for short term benefit [102]. Furthermore, subgroup analyses comparing the combined symptom and medication score (short term) for AIT in the context of allergic rhinoconjunctivitis found a clear benefit from allergoids and suggest (but not confirm) a benefit from unmodified preparations [103].

In this study, the newly established murine model allowed the direct comparison of LD-allergoid-AIT with a 220-fold higher HD-extract-AIT regarding efficacy and immunological mechanisms. Preliminary experiments during model establishment showed that s.c. AIT with 1 µg HDM extract (LD-extract-AIT) displayed rather pro-inflammatory responses such as increased numbers of total BAL cells, BAL eosinophils and lung-resident $GATA3^{+}ST2^{+}FoxP3^{-}$ Th2 cells compared to allergic mice (Figure 7 C). Earlier studies in mice showed that high allergen doses induce elevated numbers of specific $CD8^{+}$ T cells [151] and Th1 cells in lungs, while low allergen dosage induces Th2 cells and pro-inflammatory mechanisms such as immune cell infiltration [152, 153]. Therefore, the applied dosage of HDM extract AIT was adapted to a concentration used in other murine studies [127, 128]. Regarding HDM allergen

content reduction in allergy vaccination the effects of LD-allergoid-AIT (1 µg allergoid) and HD-extract-AIT (220 µg extract) were compared and interestingly, both treatment strategies revealed comparable therapeutic potential. This similar control of inflammation of LD-allergoid-AIT and HD-extract-AIT might be explained by the destruction of conformational IgE epitopes. The associated FcεR-mediated uptake of allergoids may be impaired resulting in a decreased Th2 inflammation-promoting immune milieu, while immunogenicity is maintained [98, 146-149, 154-156]. Allergoids have been introduced for AIT by Marsh *et al*; who hypothesized that allergen preparations with reduced allergenicity would be beneficial in allergy treatment strategies [93].

In this thesis project, LD-allergoid-AIT and HD-extract-AIT demonstrated similar capacity to protect against infiltration of pro-inflammatory cells into the BAL fluid. Here, not only total BAL cells were equally reduced by both treatments compared to untreated HDM-allergic mice, but also BAL cell subtypes such as eosinophils, neutrophils, $CD4^+$ and $CD8^+$ cells. Studies of the time course of allergen challenge-induced leukocyte infiltration in human and experimental asthma revealed comparable infiltration kinetics of granulocyte subtypes [157]. Neutrophils peak about 18 h and eosinophils about 42 h after allergen challenge implicating a common role for these cells in human and murine asthma. In contrast, there are marked differences in lymphocyte kinetics between human and experimental asthma. In humans, T cell count peaks within the first days after allergen challenge indicating their important role in activation and differentiation of various effector cells [158]. In contrast, in the murine models, lymphocytes peak 14 days after allergen challenge, whereas granulocyte subtypes have already returned to baseline at that time point [157]. Here, in the established murine HDM AIT model, 3 days after HDM allergen exposure were chosen as analysis time point, that displayed the comparable capacity of LD-allergoid-AIT and HD-extract-AIT to protect against the infiltration of pro-inflammatory cells into the BAL fluid. Furthermore, both treatments restored lung dynamic

compliance although HD-extract-AIT was slightly more effective. In contrast, there were no AIT effects on lung resistance measurable. This observation confirms past studies, which revealed, that sensitized and challenged BALB/c mice show increased lung resistance and decreased dynamic compliance after methacholine challenge, whereas C57BL/6J mice - used in the present thesis project - only exhibit lower dynamic compliance but are characterized by higher infiltration of eosinophils in the BAL fluid after allergen exposure [126]. In addition, LD-allergoid as well as HD-extract treatment reduced HDM allergy-induced Th2 cytokines such as IL-4 and IL-5 in the BAL fluid as well as in lung lymphocytes. This reduced Th2-promoting milieu at local and systemic sites has previously been hypothesized to be beneficial for immunological tolerance induction [75]. Furthermore, the AIT-mediated reduction of TNF-α levels was previously described to control the recruitment of eosinophils, neutrophils, and T cells into the lung [159]. The AIT-mediated protective modulation of the pro-inflammatory cytokine milieu was not only observed locally in the lungs but also in *ex vivo* anti-CD3/anti-CD28-restimulated cervical lymph node cells as well as to some degree in splenocytes. Here, LD-allergoid-AIT and HD-extract-AIT controlled the HDM allergy-induced levels of IL-17 and TNF-α [138]. Furthermore, the observed decline of $FoxP3^{+}CD4^{+}$ T cells in lung tissue as well as the reduced IL-10 secretion by restimulated lung lymphocytes and lymph node cells is likely to be a result of the migratory behavior of the cells and, therefore, reflects the inflammatory process rather than the mechanism of tolerance induction [132, 160, 161]. Elevated numbers of Tregs were also observed in asthmatic patients in a phase of active inflammation indicating that rather the quality than the quantity of Tregs is decisive for the anti-inflammatory capacity [162]. Additionally, it was reported that IL-10 is critical for antigen-specific Th2 responses in mice. A mouse model of allergic dermatitis induced by epicutaneous ovalbumin (OVA) sensitization revealed that expression of eotaxin, IL-4 and IL-5 mRNA as well as infiltration of eosinophils was reduced in IL-10-/- mice [160]. Interestingly, a strongly reduced IFN-γ secretion of restimulated lymph node cells was induced by LD-allergoid-AIT.

This difference may originate from higher amounts of TLR-ligands contained in the HD-extract-AIT (chitin, LPS, …) [163, 164]. In contrast, both treatment strategies showed comparable effects on the amount of IFN-γ^+CD4^+ T cells as well as secreted IFN-γ levels of lung cells.

Beside the demonstrated, comparable local cellular effects of both treatment strategies, the newly established, murine HDM AIT model also allowed to study the modulation of the humoral immune response by LD-allergoid AIT compared to HD-extract-AIT and revealed major differences. LD-allergoid-AIT reduced tIgE but not HDM sIgE levels in murine blood serum whereas HD-allergoid AIT neither influenced tIgE nor HDM sIgE levels. Reasons for lower tIgE-induction properties of LD-allergoid-AIT might be the loss of conformational IgE-epitopes as well as reduced amounts of mite body components like chitin and other IgE-inducing factors or serine protease activity [137, 165]. Moreover, LD-allergoid-AIT induced lower total IgG3 and IgM levels compared to HD-allergoid-AIT whereas total IgG2b and IgG2c levels were slightly reduced by both treatment strategies. More interestingly, HD-extract-AIT led to a robust HDM sIgG1 and tIgG1 response whereas LD-allergoid-AIT slightly lower increased sIgG1 and did not alter tIgG1 levels. Notably, murine IgG1 is supposed to be the structural and functional homologue of human IgG4. Like human IgG4, it does not interact with C1q and also inhibits the binding of IgG2a, IgG2b and IgG3 to C1q and, hence, suppresses complement activation. Furthermore, both murine IgG1 and human IgG4 show low affinity to activating FcγRs and preferably interact with the classical IgG inhibitory receptor FcγRIIb [166]. In human allergy treatment, HDM AIT-mediated induction of sIgG4 is a hallmark of successful AIT, because of its potentially protective role [167]. Hence, the lack of robust tIgG1 immune responses might reflect suboptimal B cell activation of LD-allergoid-AIT, although the clinically more relevant HDM sIgG1 induction was comparable to HD-extract-AIT [167].

The observed potential B cell activation deficit may be compensated by higher allergoid doses or adjuvants. Hence, the immunological effects of the depot adjuvant MCT and Th1-inducing adjuvant MPL on LD-allergoid-AIT were investigated. The adsorption of allergoids and MPL to MCT has been previously characterized and MCT- and MPL-based formulations are already clinically approved [98, 122, 144, 145]. Here, in context of pollen allergy treatment, Pollinex® Quattro Ragweed can be mentioned, which contains ragweed pollen allergoids adsorbed to MCT and MPL [98]. In the present thesis project, the therapeutic approach of combining chemically modified extracts with the synergistic effects of the "first generation adjuvant" MCT and the "second generation adjuvant" MPL [107] should be investigated in the newly established murine model of HDM AIT. Study results revealed that HDM allergoid + MCT and HDM allergoid + MCT + MPL AIT formulations showed similar effects on the reduction of BAL cell infiltration as well as mucus hypersecretion and inflammatory cell infiltration in the lungs compared to LD-allergoid-AIT alone. Comparable patterns could be observed for BAL cell subtypes such as eosinophils, neutrophils, $CD4^+$ and $CD8^+$ cells. Here, the strong protective effects of LD-allergoid-AIT alone, which were comparable to HD-extract-AIT, could not be exceeded by the addition of both adjuvants. However, the addition of MPL to the LD-allergoid-AIT + MCT formulation was slightly more effective in restoring lung dynamic compliance as well as in the reduction of BALF IL-4 and IL-5 levels. In contrast to allergoid alone, adding MCT to the formulation led to significant reduction of the percentage of lung-resident Th2 cells, whereas MPL showed no additional effect. Strikingly, LD-allergoid-AIT treatment combined with MCT and MCT + MPL led to a stepwise increase of the number of IFN-γ^+CD4^+ T cells in the lung. Furthermore, higher levels of IFN-γ secretion by aCD3/aCD28-restimulated cervical lymph node cells. Here again, unspecific *ex vivo* restimulation was chosen to be able to monitor not only allergen-specific but also bystander and unspecific effects of LD-allergoid-AIT as well as of both adjuvants on the cytokine-producing capacity of the addressed immune cells. Previous murine studies showed that MCT triggers Th1-associated immune response

mechanisms more efficiently than alum, via modulating the recruitment of DCs, $CD4^+$ and $CD8^+$ T cells accompanied with IFN-γ as well as TNF-α production. In contrast, alum induced higher levels of IgE and IL-4 than MCT [116]. Furthermore, MPL significantly increases IFN-γ production of in vitro restimulated PBMCs in context of grass pollen allergy, postulating the induction of Th1-promoting mechanisms as a protection for allergy [119]. In the present LD-allergoid-AIT study, combining both adjuvants resulted in Th1-promoting conditions such as induction of IFN-γ^+CD4^+ T cells in the lung and IFN-γ secretion of restimulated cervical lymph node cells.

More strikingly, addition of MCT and MCT + MPL to the LD-allergoid-AIT formulation enhanced the humoral response and gradually increased tIgG1, tIgG2b and IgM levels. Importantly, combining LD-allergoid-AIT with MCT and MPL overcame potential drawbacks in tIgG1 induction of LD-allergoid-AIT alone and reached tgG1 levels comparable to HD-extract-AIT. Moreover, both adjuvant-based therapies equally increased HDM sIgG1 levels. These effects on IgG production are caused by the adjuvant-induced Th1-biased immune milieu [116, 120]. Significant levels of tIgG3 were only induced by MPL. This is in line with a study showing that LPS is able to induce B cell switching to IgG3 via TLR4 signaling [168]. In contrast to alum-based adjuvants, MCT and MPL showed no IgE-inducing properties, as demonstrated previously [116, 169].

Taken together, these findings demonstrate that combining LD-allergoid-AIT with the adjuvant MCT alone, or in combination with MPL has the potential to promote Th1-inducing mechanisms and robust humoral responses counterbalancing the allergic Th2 immune response.

Another part of the thesis project was to investigate, whether not only the HDM allergen, but also the adjuvant dose may have crucial influence on the anti-inflammatory capacity of the allergoid-AIT formulation [170, 171]. Therefore, the modulatory effects of different MPL concentrations on the efficacy of the AIT with LD-allergoid + MCT were addressed.

Interestingly, the doses of 25µg and 50µg of MPL were most effective in reducing allergen challenge-induced infiltration of total BAL cells, BAL eosinophils, $CD4^+$ and $CD8^+$ cells. Moreover, both concentrations revealed the most potent effects in decreasing Th2 cells as well as Th2 cytokine production by restimulated lung and lymph node cells. Furthermore, the use of 25µg and 50µg of MPL led to a robust induction of tIgG1 as well as HDM sIgG1 [120]. Notably, the increasing MPL doses gradually increased IgG3 levels [168]. Compared to 25 µg and 50 µg MPL, most of these effects were almost completely reversed by using the 100µg dose. While the 12.5µg dose seems to be too low for effective adjuvant effects, the 100µg dose had rather adverse effects on AIT outcome. These might be explained by altered TLR4 signaling mechanisms in higher doses. MPL acts via TRAM/TRIF-biased stimulation of TLR4 along with selective activation of p38 signaling followed by induction of adaptive immune responses and TRIF-dependent endotoxin tolerance. In contrast, LPS-mediated TLR4 stimulation causes MAL/MyD88 and TRAM/TRIF-dependent signaling events, which are followed by pro-inflammatory responses. Nevertheless, MPL is not completely devoid of MyD88 involvement, which might explain the observed dose-response and rather adverse effects by using 100 µg MPL [172]. Of note, the 50µg dose is also used in vaccines for human use [122].

Finally, another aim of the thesis project was to translate the obtained results to human allergy research. Therefore, PBMCs were isolated from healthy controls, HDM-allergic patients and HDM-allergic patients which received SCIT and *ex vivo* stimulation assays were performed. Interestingly and in contrast to HDM extract stimulation, HDM allergoids did neither induce intracellular production nor secretion of Th2 cytokines. This observation is according to the findings of other studies. The reduced reactivity of allergoids with human PBMCs to induce antigen presentation relies on the reduced IgE reactivity due to the modified IgE epitopes of allergoids [173]. The highest proportion of antigen-presenting cells within PBMCs such as B

cells uses immunoglobulin and FcR for antigen recognition and uptake. In contrast, DCs and macrophages exhibit a pronounced and reproducible T cell-stimulating capacity in the presence of allergoids [148, 173]. In human PBMCs, lymphocytes are in the range of 70–90 %, monocytes from 10-20 %, while DCs represent only 1–2 %. The frequencies of cell types within the lymphocyte population include 70–85 % $CD3^+$ T cells, 5–10 % B cells, and 5–20 % natural killer cells [174]. These results suggest that further allergoid T cell reactivity studies should rather concentrate on the use of more simplified assays with monocyte-derived DCs and subsequent naïve T cell co-culturing than PBMCs [148]. Nevertheless, the results from the present PBMC stimulation experiments revealed the Th1-inducing properties of MPL as well as its protective modulation of the HDM extract-induced Th2 response. Here, MPL stimulation alone induced the secretion of anti-inflammatory IL-10 and Th1 cytokines IFN-γ and TNF-α. Furthermore, cytokines IL-6, IL-12p70 were strongly induced and a slight increase of Th17 cytokines such as IL-17A, IL-17F and IL-22 was observed. In, contrast MPL did not elevate Th2 cytokine levels. This broad cytokine induction by MPL independent of the allergic status of the PBMC donors was confirmed by previous studies [175, 176]. Other studies suggested that the enhanced production of IL-10 by MPL contributes to its reduced toxicity compared to LPS [177]. More strikingly, combining MPL with HDM extract stimulation was able to counteract the allergen-induced Th2 cytokine response. The addition of MPL reduced the levels of secreted IL-5, IL-9 and IL-13 as well as the amount of IL-4^+CD4^+ T cells in PBMCs from allergic patients with and without SCIT. In contrast, the amount of IFN-γ^+CD4^+ T cells as well as IL-6, IL-10, IFN-γ and TNF-α secretion were further elevated. Of note, in the present study, the depot adjuvant MCT was not appropriate to perform PBMC stimulation assays due to its insoluble crystalline nature. In contrast, *in vivo* studies already revealed the potential of MCT to induce IL10, IFN-γ and TNF-α production [116]. Taken together, these findings demonstrate that allergoids with their modified IgE epitopes are less potent to induce IgE crosslinking and subsequent Th2 immune response in PBMCs compared to HDM extract. Moreover, the

potential of MPL to shift the allergen-induced Th2 immune response towards a Th1-promoting milieu could be demonstrated.

In summary, this thesis project provides a side-by-side comparison of high-dose HDM extract- and low-dose HDM allergoid-based AIT and demonstrates that low allergen doses can induce cellular and humoral mechanisms counteracting Th2-driven inflammation by using HDM allergoids in combination with the depot adjuvant MCT and dose-adjusted Th1-inducing adjuvant MPL. Future therapeutic approaches may use low-dose allergoid AIT to drive cellular tolerance and adjuvants to modulate humoral, potentially protective immune responses.

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Declaration

Parts of my thesis submitted for the degree of *Dr. rer. nat* in July 2021 were further published in “Immunological effects of adjuvanted low-dose allergoid allergen-specific immunotherapy in experimental murine house dust mite allergy” in the Journal Allergy in July 2021 [178]. Figures, sentences, small passages, and whole paragraphs adopted from the published article may be quoted verbatim without being highlighted/cited separately.

Furthermore, with this, I declare that I wrote the thesis with the title “Mechanisms of allergen-specific immunotherapy and new concepts for the therapy of hypersensitivity reactions in the context of house dust mite allergy” by myself and that I did not use other resources than those indicated. I did not undertake an unsuccessful graduation attempt and this thesis was not submitted to another institution before.

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