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Novel therapeuties for the treatment of experimental model of relapsed allergic asthma

and

a new mouse model for experimental acute asthma

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Abstract

Allergic asthma is a chronic disease of the airways with patients developing symptoms including coughing, wheezing, shortness of breath, rapid breathing and tightening of the chest. The most effective medication for asthma are glucocorticoids but there are asthma patients who are resistant to steroids. In this first part of these studies, we tested the efficacy of two compounds in a mouse model of allergic asthma exacerbation. We found that the two compounds were as effective as glucocorticosteroids and may be useful in the future as adjunct, steroid-sparing drugs. The second part of these studies focused on comparing allergens in acute allergic asthma in two different strains of congenic mice, BALB/c and B6. We found significant differences in the ability of animals from these strains to respond to distinct allergens. In one case, the T_H1-type B6 mouse strain responded by developing much more severe allergic disease than the T_H2-type BALB/c mouse strain.

Zusammenfassung

Asthma ist eine komplexe chronische Krankheit. Die Symptome sind durch Husten (zumeist in der Nacht oder Frühmorgens), Atemlosigkeit, Engegefühl in der Brust, Atemschwierigkeiten, Überempfindlichkeit und chronische Entzündung der Lungen und durch wechselhafte Rückfälle gekennzeichnet. Die wirksamste Medikation gegen Asthma sind glucocorticoid-haltige Medikamente, manche Asthmapatienten, sprechen aber auf einer Behandlung mit Glucocorticoiden nicht an. Die erste Zielsetzung meiner Diplomarbeit war die Erprobung zweier glucocorticoid-freier Wirkstoffen gegen Asthma an einem Maus-Modell mit rückfälligen Mäusen. Unsere Ergebnisse bezüglich der zwei Komponenten zeigten, dass die glucocorticoid-freie Wirkstoffe ebenso effektiv waren, wie der glucocorticoid-hältige Standatd-Wirkstoff. Aus diesem Grund wären sie in der Zukunft als Glucocorticoid-Ersatz verwendbar.

Die zweite Zielsetzung meiner Diplomarbeit war der Vergleich verschiedener Allergene für akutes allergisches Asthma in BALB/c und C57BL/6 (B6) Mäusen. Unsere Ergebnisse für das neue Sensibilisierungsprotokoll für akutes, allergisches Asthma in Mäusen zeigten signifikante Unterschiede in der Immunantwort auf die Allergene. Der T_H1-Mausstamm (B6) entwickelte eine sehr viel stärkere allergische Erkrankung als die T_H2-Mausstamm (BALB/c).

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Chapter 1.

1.1. Socioeconomic impact of asthma

1.1.1. Etiology of asthma [2, 3, 14]

Asthma is a problem affecting a large number of populations which causes children to miss school and adults to miss time at work. Asthma also causes a high level of morbidity and mortality in the word. [2] A population with a broad range of psychological and social problems has the highest risk of getting asthma. Asthma has not only a physical effect; it also has a psychological and social effect. Asthma patients need to learn how to control their disease. Being aware of one´s loss of health can be an additional stress factor. It could be shown that individuals having a supportive family are better in successfully controlling their asthma. The costs for the treatment of allergic asthma are very high, mainly caused by emergency and hospital treatment.

1.1.2. Medical definition [5]

In most cases of asthma, the first symptoms occur during childhood in a subgroup of children. Asthma causes functional deficits in the airway increasing with age leading to persistent asthma symptoms in the adult life. Asthma is a difficult chronic disease characterized by variable and renewing symptoms, airflow obstruction, bronchial hyperresponsiveness and permanent inflammation. The interaction of these symptoms determines the clinical manifestation, the severity and the treatment of asthma.

During an asthma attack, many cells and cellular elements play a part: these cells are mast cells, eosinophils, neutrophils (in sudden onset emergencies, fatal exacerbations, smoker and occupational asthma), T lymphocytes, alveolar macrophages and epithelial cells.

The symptoms are renewing episodes of coughing (particularly at night or early in the morning), wheezing, breathlessness and chest tightness. These episodes are characterized by variable airflow obstruction, often reversible spontaneously or with treatment

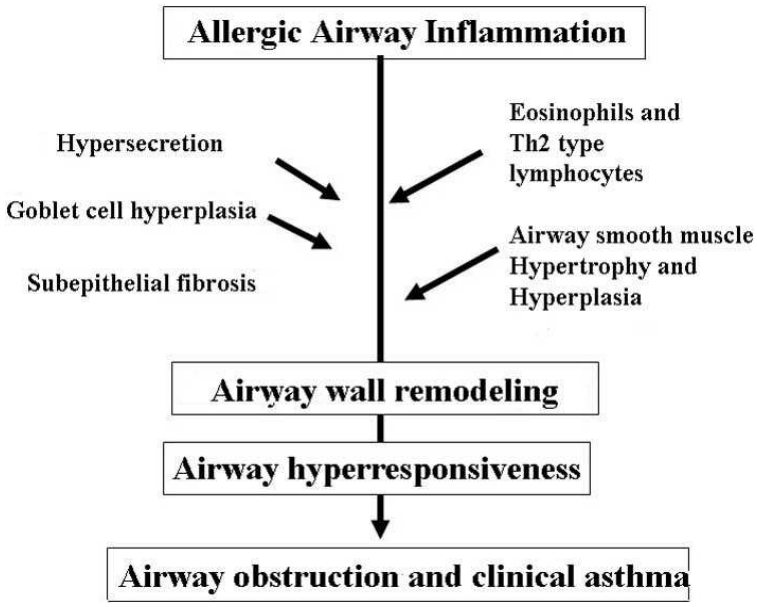


Fig. 1. Features of asthma **[95]**

1.1.3. Diagnosis of asthma [16, 23]

The key symptoms of asthma are wheezing and high-pitched whistling sounds when breathing out, especially in children. The history of any of the symptoms: coughing (worse at night), renewing wheeze, renewing difficulty in breathing and renewing chest tightness. More indicator factors: Symptoms appear or worsen by exercise, viral infections, inhalant allergens (e.g. house dust mites, pollen, animals with fur), irritants (tobacco, wood smoke, chemicals), changes in weather, strong emotional expression (laughing, crying), stress and a menstrual cycle.

Remodeling of airways may occur. Reversibility of airflow limitation may be incomplete in some patients. Persistent changes in airway structure occur, including sub-basement fibrosis, mucus hypersecretion, injury to epithelial cells, smooth muscle hypertrophy, and angiogenesis.

Recent studies provide insights on different phenotypes of asthma that exist. Different manifestations of asthma may have specific and varying patterns of inflammation (e.g., varying intensity, cellular mediator pattern, and therapeutic response). Further studies will determine whether different treatment approaches benefit the different patterns of inflammation.

1.1.4. Classification of asthma [16, 23]

Based on National Heart Blood and Lung Institute guidelines, severity of asthma is clinically classified, dependent on the renewing of the symptoms, forced expiratory volume in one second (FEV_1) and the peak expiratory flow rate. Asthma is also classified in regard to whether the symptoms are triggered by allergens as atopic (extrinsic) or as nonatopic (intrinsic) asthma.

1.1.4.1. Classification of asthma based on severity [16, 23]

Mild intermittent Asthma

- Coughing, wheezing, chest tightness or difficulty breathing occur 3 to 6 times a week
- Sudden outbreaks with varying intensity
- Symptoms during nighttime occur less than twice a month
- No symptoms between asthma attacks
- Lung function test FEV_1 is equal or above 80% of normal values
- Peak flow less than 20% variability AM-to-AM or AM-to-PM, day-to-day

Mild persistent asthma

- Coughing, wheezing, chest tightness or difficulty breathing 3 to 6 times a week
- Sudden outbreaks may influence the level of activity
- Symptoms during nighttime occur less than 2 to 4 times a month
- Lung function test FEV_1 is equal or above 80% of normal values
- Peak flow less than 20-30% variability AM-to-AM or AM-to-PM, day-to-day

Moderate Persistent Asthma

- Daily symptoms of coughing, wheezing, chest tightness or difficulty breathing
- Symptoms during nighttime occur 5 or more times a month
- Lung function test FEV_1 is above 60% but below 80% of normal values
- Peak flow more than 30% variability AM-to-AM or AM-to-PM, day-to-day

Severe Persistent Asthma

- Symptoms of coughing, wheezing, chest tightness or difficulty breathing are continual
- Symptoms often occur during nighttime
- Lung function test FEV_1 is less than or equal to 60% of normal values
- Peak flow more than 30% variability AM-to-AM or AM-to-PM, day-to-day

1.1.4.2. Classification of asthma based by type [16, 23]

Classical asthma

This type of asthma is described by wheezing and airway bronchospasm with eosinophils in the mucus. This type reacts well to bronchodilators and inhaled steroids.

Cough-variant asthma (first described in 1972)

This type of asthma is a common condition on small children; one third will develop classical asthma. This type is dry cough linked to a very sensitive cough-reflex; reacts well to bronchodilators.

Eosinophilic bronchitis

An isolated chronic cough linked type with eosinophils in sputum (adults), without wheezing or airway bronchospasm; it develops a fixed airway disease, e.g. chronic bronchitis, emphysema. This cough is not reacting to bronchodilators but is ameliorated with inhaled corticosteroids.

Allergic asthma

This type of asthma is triggered by antigens. It is also called atopic (extrinsic) asthma.

1.2. Allergic asthma [16, 102]

Allergic asthma is the most common type of asthma; about 90% of children with asthma have allergies, compared to adults of which 50% have allergies. The inhalation of allergens cause asthma and the symptoms associated with allergic asthma.

The patients with allergic asthma are hypersensitive to allergens to which they have become sensitized. When these allergens get into the airway, the immune system overreacts. In patients with allergies the IgE production is too vigilant, even on otherwise harmless substances, and reacts with the release of histamine resulting in inflammation and swelling of the

airways. These become overflowed with thick mucus and the muscles around the airways tighten (bronchospasm).

Allergic asthma is mediated by the activation of mast cells, sensitized by IgE antibodies. Allergic asthma is called extrinsic, because of the clear identification of an exogenous irritating antigen in most cases. [102] Many factors predispose to development of allergic asthma. The most important factor above all is the repeated exposure to allergen.

The common allergens of allergic asthma are substances small enough to be inhaled deeply into the lungs, e.g. windblown pollen from trees, grasses, weeds, mold spores or fragments, animal dander from hair, skin, feather and saliva, dust mite feces and cockroach feces. Allergic reactions can also be caused by scratches with the allergen (causing itchy, red skin), getting it in the eyes (causing itchy, red eyes), or eating it, which in rare cases can cause a life-threatening anaphylactic shock (including severe asthma attack). Allergic asthma symptoms can be worsened by irritants which trigger an asthma attack, even though they do not cause an allergic reaction. These irritants include: tobacco smoke, smoke from a fireplace, candles, incense, or fireworks; air pollution, cold air, especially vigorous exercise in cold air; strong chemical odors or fumes, perfumes, air fresheners or other scented products and dusty workplaces. The two most common (and recommended) tests are pricking the skin with a tiny amount of the allergen and measuring the size of the red bumps 20 minutes later, or a blood test (RAST or allergen-specific IgE levels). [16]

In chronic asthma the pulmonary changes include:

- Thickening of the basement membrane of the bronchial mucosa
- Hypertrophy of the bronchial smooth muscle
- Hypertrophy of the bronchial mucous glands
- Eosinophils, chronic inflammatory cells in the bronchial wall, with a substantial increase in the number of mast cells
- Presence of mucus containing large number of eosinophils in the bronchi. [102]

Allergic or non-allergic asthma have common symptoms: coughing, wheezing, shortness of breath, rapid breathing and tightening of the chest.

1.2.1. Environmental Control and Allergic Asthma [85]

A crucial part of controlling allergic asthma is to limit the exposure of the patients to allergens: Patients of allergic asthma should avoid going outside when the pollen counts are high and should keep the windows closed, even wearing a HEPA filter mask while they are outside. Patients should also avoid house dust mites. House dust mites live in fabrics and carpets, heavy curtains, upholstered furniture, mattresses, pillows and box springs. They should be wrapped with allergen-proof covers. Indoor humidity (relative humidity above 40%) could also turn into a problem at home because of mold growth, cockroaches and house dust mites. [16]

1.3. Introduction of the immune system [9]

The immune system has two main components, the innate or nonspecific immune system and the adaptive or specific immune system. The innate immune system is our first defense line against the invasion of microorganisms or foreign antigens. The adaptive immune system functions as the second, more specific line to defend invading microorganisms or foreign antigens (allergens) and also has a memory function against invading antigens, usually with the function to protect us against renewing infections with the same agents.

Each of the major components of the immune system has humoral and cellular subdivisions by which they act. Although the innate and adaptive immune systems are different in function they also interact with each other (components of the innate immune system have an influence on the adaptive immune system and vice versa).

All immune cells originate from the bone marrow and include myeloid (neutrophils, basophils, eosinophils, macrophages and dendritic cells) and lymphoid cells (B and T lymphocytes and natural killer cells). These cells differentiate along different pathways. The myeloid progenitor (stem) cells in the bone marrow develop to erythrocytes, platelets, neutrophils, monocytes/macrophages and dendritic cells. The lymphoid stem cells develop to B cells, T cells and natural killer cells. For the development of T cells, the precursor T cells have to migrate to the thymus where they differentiate into two different types of T cells: the $CD4^+$ T helper cells and the $CD8^+$ T pre-cytotoxic cells. Then two types of T helper cells are produced in the thymus: the T_H1 cells, which are needed for the $CD8^+$ pre-cytotoxic cells to differentiate into

cytotoxic T cells, and T_H2 cells which are needed for B cells to differentiate into antibody secreting plasma cells.

The major role of the immune system is to distinguish between self and nonself, for protection of the host organism from foreign, invading pathogens and to eliminate modified or altered cells (e.g. malign cells). A disease only occurs if the rate of the foreign antigen (allergen) is high enough to compromise the immune system.

During inflammation, which is the immune response, local discomfort and collateral damage to healthy tissue can occur as a result of the toxic agents produced during the immune response. In some cases the immune response may be directed against self tissues resulting in an autoimmune disease.

1.3.1. Response of the innate immune system to environmental antigens [9, 103]

The mechanisms of innate immunity are present before foreign antigens or allergens enter the host and are also present before the development of the adaptive immune response.

Innate immunity has three important properties. It is the first response it protects the host and in most cases fights the microbes or foreign Ags. The mechanisms of the innate immunity are also often used in adaptive immune response. For example, in cell-mediated immunity cytokines of antigen specific T lymphocytes activate macrophages, a major effector mechanism of innate immunity. Furthermore, in humoral immunity antibodies of B lymphocytes use two effector mechanisms of innate immunity for defense: phagocytose and the complement system.

Cells of the innate immune system [9]

- Mast cells
- Phagocytes (Macrophages, Neutrophils, Dentritic cells)
- Basophils and eosinophils
- Natural killer cells

The epithelial cell layer represent and actively interact, (as the primary interface with the environment and the front line defense against injury) with pathogens or environmental irritants, both in healthy and allergic individuals.

Although allergens are not infectious in nature, they contain bio-organic substances,

actively recognized by PRRs as TLRs. There is a suggestion, that allergens do have PAMPs that interact with various PRRs (including TLR2, TLR4, TLR9). Several of the common allergens (cockroach extracts, pollen) have protease activity, changing the barrier function of the epithelium by disrupting the epithelial tight junctions. The disruption of the tight junctions allows the allergens to enter to the underlying cells-including DCs. [103]

Professional Antigen-Presenting Cells (APC)

- Dendritic Cells
- Follicular Dendritic Cells
- Mononuclear Phagocytes
  - Bone marrow progenitors
  - Blood monocytes
  - Tissue Macrophages

1.4. Adaptive Immune system [9]

1.4.1. Cells of the adaptive immune system

The cells of the adaptive immune system are circulating in the blood and lymph nodes; they are also present as scattered cells in all tissues.

Lymphocytes

Lymphocytes are the only cells in the body capable of specifically recognizing and distinguishing different antigenic determinants. They are responsible for the two defining characteristics of the adaptive immune response: specificity and memory.
Lymphocytes consist of distinct subsets that are morphologically indistinguishable but different in their functions and protein products.

Classes of lymphocytes are the following: B-Lymphocytes are the antibody producing cells; T-Lymphocytes are the mediators of cellular immunity. T-Lymphocytes consist of subsets: helper T-Lymphocytes (T_H) cytotoxic T-Lymphocytes (CTLs) and NK-cells with the major function in the innate immunity[9].

1.4.2. The central role of T_H cells in immune responses [48, 9]

After T_H cells recognize specific antigens presented by an antigen presenting cell (APC), they are able to activate several key immune processes including selection of appropriate effector mechanisms, induction of proliferation of appropriate effector cells and enhancement of functional activity of other cells (e.g. macrophages, granulocytes, NK cells).

Four subpopulations of T_H cells are known: ***naive T_H0*** cells: when naive T_H0 meet an antigen in secondary lymphoid tissues, they are able to differentiate into inflammatory ***T_H1*** cells, helper ***T_H2*** cells or pathogenic ***T_H17*** cells which are different in the cytokines they produce.

Cytokines produced by T_H1 cells activate macrophages and also play a role in the generation of cytotoxic lymphocytes (CTL), leading to a cell-mediated immune response. Cytokines produced by T_H2 cells help to activate B cells, leading to antibody production.

T_H17 cells differentiate in response to IL-1, IL-6, IL-23 (TGF-beta is important for differentiation in mice but not in humans). T_H17 produce IL-17 which enhances the severity of some autoimmune diseases.

It is equally important that every population of differentiated T_H cells is able to inhibit other populations of T_H cells.

1.4.3. Types of antigen presenting cells (APC) for $CD4^+$ T helper Lymphocytes [9]

$CD4^+$ T cells are effector cells of cell-mediated immunity and provide stimuli that are important for the proliferation and differentiation of B lymphocytes.
Only few cell types that express MHC II molecules can function as APCs for $CD4^+$ lymphocytes.

Dendritic cells (DC)

DCs are related to mononuclear phagocytes, they are present in lymphoid organs, in the epithelia of the skin, gastrointestinal and respiratory tracts and in most parenchymal organs. All DCs originate from bone marrow precursors and are called myeloid DCs.

Immature DCs are located in the epithelia of the skin, the gastrointestinal and the respiratory system. The function of DCs is to capture protein antigens (allergens) and transport them to draining lymph nodes. During their migration to the lymph nodes DCs mature and become extremely efficient at presenting antigens (allergens) and stimulating naive T cells.

Mature DCs reside in the T cell zones of the lymph nodes and in this location they display allergens to the immature T cells.

Macrophages

Macrophages have numerous lysosomes, similar to PNMs. Macrophages have a typical kidney shaped nucleus; they are also detectable by the cell surface marker ***CD14***. Macrophages are APCs that actively phagocytose large particles; therefore they play an important role in presenting antigens. In the effector phase of cell-mediated immunity activated effector T cells recognize antigens on phagocytes and activate the macrophages. Although most macrophages express low levels of MHC II molecules, much higher levels are induced by IFN-gamma.

B Lymphocytes

B lymphocytes use their antigen receptors to bind and internalize soluble protein antigens and present processed peptides derived from these proteins to T_H cells.

1.4.4. Capture and Presentation of protein antigens in vivo [9]

The common routes which foreign antigens, e.g. allergens, use to enter a host are the skin and the epithelia of the gastrointestinal and respiratory systems. The skin and all epithelia contain numerous lymphatic capillaries that drain lymph from invaded sites onto the regional lymph nodes. The lymph drained from the skin, mucosa and other sites contains a mixture of all soluble and particular antigens from the tissues.

Lymph nodes that are interposed along lymphatic vessels act as filters; they collect the lymph at numerous points before it reaches the blood. Antigens that enter the bloodstream may be similarly sampled by the spleen. The innate immune system is also stimulated and develops reactions during which inflammatory cytokines are produced. Therefore the dendritic cells become activated.

Immature dendritic cells reside in epithelia and tissues capture protein antigens, begin to process the proteins into peptides capable of binding to MHC molecules. Then the dendritic cells lose their adhesiveness for epithelia, they begin to express the chemokine receptor CCR7, specific for chemokines in the T cell zones of the lymph nodes.

The chemokines attract the dendritic cells bearing antigens into the T cell zone of the

regional lymph nodes. Naive T cells also express CCR7 and this is why naive T cells migrate to the same regions of the lymph nodes where antigens bearing dendritic cells are concentrated. Dendritic cells mature during the migration to the lymph node, during that time they express high levels of class II MHC molecules with bound peptides as well as costimulators required for T cell activation – they develop into potent antigen-presenting cells (APCs): the process of maturation can be reproduced in vitro with cytokines TNF and GM-CSF and microbial products such as endotoxin. Antigens may also enter the lymph nodes in soluble form; they can be extracted by antigen-presenting cells (APCs) such as dendritic cells, B cells and macrophages.

Through the lymphnodes recirculating naive $CD4^+$ T cells encounter these APCs. $CD4^+$ T cells that are specific for the displayed peptide-MHC II-complex get activated: an immune response is initiated.

T-Lymphocytes play a central role in all adaptive immune responses against protein antigens. In cell-mediated immunity $CD4^+$ T cells activate macrophages to destroy phagocytosed microbes, $CD8^+$ T cells kill cells infected with intracellular microbes. In humoral immunity $CD4^+$ T helper cells interact with B lymphocytes and stimulate their proliferation and differentiation. Both, the induction phase and the effector phase of T cells are triggered by the specific recognition of antigens.

Certain APCs present antigens to naive T cells during the recognition to initiate immune responses. Some APCs present antigens to differentiated T cells during the effector phase to trigger the mechanisms that eliminates the antigens (Ags).

1.4.5. Properties of antigens (allergens) recognized by lymphocytes [98]

B cells and T cells recognize different antigens in different forms. The majority of the T cells recognizes and responds to foreign peptide Ags only when the Ags are in association with MHC products expressed on the surface of APCs (not in soluble form). APCs are cells which capture and display the Ags to T cells. T cell mediated immune responses are usually induced by protein antigens: Some T cells are specific for small chemical haptens: e.g. dinitrophenol, urushiol and b-Lactams of penicillin antibodies. T helper cells (T_H cells) recognize proteins associated with MHCII class molecules. A small population of T cells recognizes low molecular

weight nonpeptide antigens: these induce NK-T cells and gamma-delta T cells.

In comparison B cells bind antigens (allergens) in soluble form: Proteins in conformational, denatured or proteolysed forms, Nucleic acids, Polysaccharids, Some lipids, Small chemicals (haptens). Therefore B cells and secreted Ags bind soluble Ags (or allergens) in body fluids as well as exposed cell surface antigens.

1.5. Antigens (allergens) [98, 99, 101]

An allergen (antigen) is a substance that rather causes an allergic response than an immune response. [99]. Antigens are usually common environmental proteins and chemicals to which the most individuals do not produce specific IgE or develop potentially harmful reactions, as developed in genetically susceptible individuals.

Many different chemicals, either natural or synthetic are allergic. Complex natural-organic chemicals, especially proteins cause ***Antibody-mediated allergy***. Simple organic compounds, inorganic chemicals and metals often cause ***T-cell mediated allergy***. [101]. All immediate hypersensitivity reactions share common features, although they differ in the types of the antigens which cause these reactions and in their clinical and pathologic manifestations. Exposure to allergens is either artificial (e.g. by injection) or natural. Natural exposure includes inhalation, ingestion and contact with the eyes.

The typical sequences of events in hypersensitivity have in common:

- the re-exposure to an antigen
- activation of Th2 cells specific for the antigen and production of T_H2 antibodies,
- binding the antibodies to the Fc-receptors of mast cells
- triggering of the mast cells to release mediators
- the subsequent pathologic reactions.

The binding of IgE to mast cells is also called sensitization.

<u>The nature of allergens [98, 101, 103]</u>

Allergens are always proteins or are bound to proteins, the atopic person is chronically exposed to, because only proteins induce T-cell responses. Recent studies suggested that allergens activate the innate immune response, either through their intrinsic enzymatic activity or through

activation of pattern-recognition receptors (PRRs) on mucosal epithelial cells or APCs directly. Allergens from diverse sources have enzymatic activity, which may bias the immune response to a T_H2 phenotype. [103]

The structure of allergens [98]

There are no structural characteristics of proteins which could be definitely predicted to cause allergies, but some features are typical of many common allergens:

- Low molecular weights
- High solubility in body fluids
- Glycosylation:
  e.g. anaphylactic response to foods are typically caused by highly glycosylated small proteins.
- Some enzymes triggering immediate hypersensitivity:
  e.g. cysteine protease of the house dust mite and phospholipase A2 in bee venom.
- Some drugs when they chemically react with amino acid residues in self proteins to form ***hapten-carrier conjugates***.

Re-exposure to the same allergen is necessary for the development of an allergic response, because IgE isotype switching and sensitization of mast cells with IgE must occur before a hypersensitivity reaction to an antigen follows.

1.5.1. Capture and Presentation of allergens [98]

Dentritic cells in epithelia capture the antigens (allergens), transport them to draining lymphnodes process them and present peptides to T cells. The T cells differentiate into the T_H2 subset of effector cells. Differentiated T_H2 cells promote switching to IgE mainly through the secretion of IL-4. Atopic individuals have a larger number of allergen specific IL-4-secreting T cells in their circulation, than non-atopic individuals. Then IL-5 secreted by T_H2, activates eosinophils, which is involved in many immediate hypersensitivity reactions. Epithelial cells are stimulated by IL-13 (e.g. in the airways) to secrete increased amounts of mucus (an excessive mucus production is also a common feature). T_H2 cells recruited to sites of immediate hypersensitivity, express chemokine receptors CCR4 and CCR3 –the chemokines that bind to

these receptors are produced by many cell types at sites of immediate hypersensitivity reactions e.g. epithelial cells.

1.5.2. Activation of B cells [98]

Under the influence of CD40 ligand and cytokines, mainly IL-4 produced by T_H2, the B cells undergo heavy chain isotype switching and produce IgE. Allergen specific IgE produced by B cells enters the circulation as bivalent antibody, and binds to Fc receptors on tissue mast cells, so that these cells are sensitized and ready to react to a subsequent encounter with the allergen. Circulating eosinophils and basophils also are capable to bind IgE.

1.5.3. Method of administration of the antigen (allergen) [9]

Dose

The administration dose of an antigen is a critical factor in the immunogenicity of the antigen because above or below a certain dose the immune response will not be optimal.

Route

The route of the antigen administration influences the nature of the immune response.

Adjuvants

Adjuvants are substances which are able to amplify the immune response to an immunogen.

1.5.4. Antigenic determinants [9]

1.5.4.1. Determinants recognized by B cells

Composition

The antigenic determinants of B cells are the antibodies they produce. B cells have cell surface-bound immunoglobuline as receptors with the same specificity as the immunoglobulin produced by these B cells after their activation. These antibodies are built in a linear manner or by primary sequence of the residues in the polymer of determinants. There are also antibodies created by secondary, tertiary or quaternary structures of the molecule, so-called conformational determinants.

Size

Usually antigenic determinants are small; they are limited to approximately 4 to 8 residues of amino acids or sugars.

1.5.4.2. Determinants recognized by T cells

Composition

The antigenic determinants, which are recognized by T cells, are determinants built by the primary sequence of amino acids in proteins; this is why T cells require antigens presented by antigen presenting cells (APCs) after the APC has proteolytically degraded the antigens in smaller peptides. T cells recognize polysaccharides bound to a major histocompatibility complex (MHC) molecule. The complex consists of an MHC molecule and a peptide recognized by the T cell.

Size

Usually antigenic determinants are limited to the size of approximately 8 to 15 amino acids.

1.6. Immunoglobulins [9]

Immunglobulins are built of glycoprotein molecules, produced by plasma cells as a reaction to an immunogen, and they act as antibodies.

1.6.1. Function of the immunoglobulins

Allergen (antigen) binding

The antigen binding is the primary role of the immunoglobulin and usually can result in protection of the host but not in the case of hypersensitivity reactions. Immunoglobins are specific; they bind to one antigen or a few closely related antigens by a binding domain which is a specific antigenic determinant to these antigens.

Effector functions of the antibodies

Immunoglobulins mediate effector functions in hypersensitivity reactions: the secretion of biologically active mediator molecules and the binding to a number of cell types which have receptors for the binding of antibodies—phagocytic cells, mast cells, basophils and platelets—

resulting in the performation of some functions.

1.6.2. Human immunoglobulin classes and subclasses [9] important in allergic asthma

IgG

All IgGs are monomers (7S immunoglobulin), the subclasses are different in the disulfide bonds and length of the hinge region. IgG is the most diverse immunoglobulin. It is able to carry out all functions of the immunoglobulin molecules, implies that IgG is the main serum in blood (75 %) and in the extravascular spaces. IgG is the only immunoglobulin capable of crossing the placenta (the transfer is mediated by a receptor on placental cells for the Fc-region of the IgG.

At the binding to cells: subclass IgG1 binds on Macrophages, monocytes, PMN´s and some lymphocytes. The consequence of this binding is a better internalization of antigens, which means that IgG1 Abs are good opsonins for phagocytosis for humans as well as for mice.

IgE

IgE exists only as monomer; it has an extra domain in the constant region. IgE does not fix complement but binds very tightly to Fc-receptors on basophils and mast cells, even before interaction with an antigen. IgE is involved in allergic reactions as a consequence of binding on basophils and mast cells.

As a result those cells release pharmacological mediators which cause allergic symptoms. Eosinophils have Fc-receptors on their surface for IgE. Upon binding of the eosinophils on IgE-coated helminthes the parasite will be killed.

1.7. Hypersensitivity reactions [9] important in allergic asthma

For hypersensitivity a pre-sensitized (immune) state of the host is essential. Based on the mechanisms and duration of development, hypersensitivity reactions are classified into four types: type I, type II, type III and type IV. In many cases the clinical disease may involve more than one type of reaction. In case of allergic asthma only hypersensitivity reactions I and IV are important. The mast cell (tissue) and basophils (peripheral blood) are the main effector cells for acute reactions. T cells and eosinophils are important in persistent asthma.

1.7.1. Type I hypersensitivity

Type I hypersensitivity is ***antibody-mediated;*** it is also defined as immediate or anaphylactic hypersensitivity. The reaction may trigger a range of symptoms from minor inconvenience to lethal symptoms. The reaction may include skin (urticaria, eczema), eyes (conjunctivitis), nasopharynx (rhinorrhea, rhinitis), bronchopulmonary tissues (asthma) and the gastrointestinal tract (gastroenteritis). The reaction usually takes 15 to 30 minutes from time of exposure to the antigen, sometimes the reaction is delayed by 10 to 12 hours.

The main cellular components in this type of hypersensitivity are mast cells or basophils further enlarged by platelets, neutrophils and eosinophils; a biopsy of the hypersensitivity reaction shows mainly mast cells and eosinophils. It has been demonstrated that individuals with type I hypersensitivity preferentially produce more T_H2 cells. T_H2 cells secrete IL-4, IL-5 and IL-13; in most cases it triggers an Ig-switch to IgE.

Diagnostic tests for immediate hypersensitivity contain skin tests (prick and intradermal), measurement of total IgE and specific IgE antibodies against the expected allergen.

The late phase follows 6-12 hours after antigen exposure. The characteristics of the late phase are a more prolonged reaction with infiltrations by neutrophils, eosinophils, basophils, lymphocytes and macrophages. The symptoms of the late phase are indurate, erythrematous, painful reactions in the lung, and a more prolonged deterioration in airflow, compared to rapidly reversible bronchoconstriction in the immediate or early phase of allergic asthma. Chronic reactions, e.g. hay fever and persistent asthma are associated with chronic tissue infiltration with mast cells, macrophages, T cells, B cells, eosinophils and smooth muscle hyperplasia. [102]

1.7.2. Type IV hypersensitivity

The type IV hypersensitivity is also called delayed type hypersensitivity (DTH).
Type IV hypersensitivity differ from other hypersensitivity reactions in two important points:

- The activation is due by T-cell activated macrophages rather than by antibodys.
- The hypersensitivity starts after a latent period of several hours and the peak is at 48-72 hours after exposure to the antigen. [99]
- In contrast to immediate hypersensitivity, there are no tissue specifity.

Type IV hypersensitivity reactions appear 1-3 days after allergen re-exposure.

In this ***cell mediated type of hypersensitivity*** activated macrophages are the ultimate effector cells. T cells promote activation and accumulation of macrophages.

The induction (sensitization) of DTH involves many natural and synthetic proteins (polypeptides and glycopeptides) and autocoupling haptens, but not polysaccharides.

The precise mechanism of DTH is unknown. The interaction of macrophage processed/presented antigen interacts with specific receptors on $CD4^+$ T cells. The interaction leads to formation of sensitized $T_H1$cells. One part of these sensitized cells become memory cells. T_H1 cells secrete cytokines which stimulate cytotoxic T cells, generating direct damage, recruiting and activating monocytes and macrophages. The main lymphokines in delayed hypersensitivity are monocyte chemotactic factors, IL-2, IFN-gamma, TNF-alpha/beta etc. The lesions are characterized by induration and erythema.

Table 1. Comparison of types of hypersensitivity I and IV [9, 100]

	type-I (anaphylactic)	type-IV (delayed type)		type-I	type-IV (delayed type)		
antigen	Exogenous	tissues & organs	Immune-reactant	IgE (Ab)	T_H1 cells	T_H2 cells	CTL
Response time	15-30 minutes	48-72 hours	Antigen	soluble Ag	soluble Ag	soluble Ag	Cell assot. Ag
appearance	weal & flare	erythema and induration	Effector Mechan.	Mast cell activation	Macroph. activation	IgE prod. Eosin.act. Masto-cytosis	Cyto-toxicity
histology	basophils and eosinophil	monocytes and lymphocytes					
examples	allergic asthma, hay fever, systemic anaphlyaxis	tuberculin test, granuloma	examples	allergic asthma, hay fever, syst. Anaph.	Contact dermat. Tuberculin reaction	Chronic asthma, chronic all. rhinitis	Contact dermatitis

1.7.3. Contribution of the biological system [9]

Genetic factors

Not all substances are immunogenic in all species (i.e. responders and nonresponders). The IgE levels in serum are under genetic control; the trait for low level of IgE is dominant. Abnormally high levels of IgE synthesis and the connected atopy often run in families. The full inheritance pattern may be multigenic. Within the same family the atopy may be targeting variable organs, or it may be present to various degrees in different members of the same family. All these individuals will show higher levels of plasma IgE, than normal individuals. If both parents have atopy, there is a 75% chance for their children to develop atopy. If one parent has atopy, there is a 50% chance, but 38% of atopy-patients have no parental history of atopy. [99]

Age

Age also has an influence: normally, very young and very old individuals are affected.

1.7.4. T and B cells are involved in immediate hypersensitivity reactions [99]

The activation of IgE-secreting precursor cells is T-cell dependent. T_H2 cell derivated IL-4 causes isotype switching to IgE, it promotes also eosinophil recruitment. Atopic individuals may have more allergen-specific IL-4 producing T_H2 cells than normal individuals, the magnitude and duration of IgE responses are determined by cross regulation between antagonistic IL-4 and T_H1-cell derived IFN-gamma signals. Another source of IL-4 (on this way maybe of IgE) is mast cells. Normally IgE has the lowest concentration of the five classes of antibodies in the plasma. The normal adult level of IgE is about 250 ng / ml of blood, severely allergic individuals have about 700 ng/ml. IgE assotiates either with high-affinity receptors FcεRI on mast cells and basophils or low-affinity receptors: FcεRII (or CD23) on eosinophils, B cells and macrophages.

1.7.5. Role of mast cells in immediate hypersensitivity [98]

The IgE sensitizes mast cells and basophils. The main effector cell is the mast cell. Mast cells are grouped in two subpopulations that vary in the mediators they produce. These are mucosal mast cells (gut, lungs), and connective tissue mast cells (associated with blood vessels).

The type of allergen induced IgE response depends on the activated type of the mast cell. Intravenously or subcutaneously administered allergens activate connective tissue mast cells. This mast cells release histamine and other mediators. Systematically release cause anaphylaxis, locally release cause wheal and flare. Monomeric IgE alone does not trigger mediator release, only when allergen encounter IgE on mast cells or basophils surface and it bridges several IgE-molecules and cross-links the corresponding receptor sites. Once activated, mast cells and basophils degranulate. The release of mediators from mast cells starts within 15-20 sec after activation. [99] Ingested or inhaled allergens activate mucosal mast cells, with histamine and other mediator-release in the upper respiratory tract or the intestinal tract.

The activation of tissue mast cells (or peripheral basophils) leads to release of preformed and newly synthetized effector molecules: including histamine, heparin, serotonin and arachidonic acid, which is in the late phase converted by other cells into prostaglandins and leucotrienes, responsible for the late phase inflammation reactions. On the one hand, histamine stimulates the smooth muscle of arterioles to dilate, because of increased blood flow (erythema). On the other hand, histamine stimulates the smooth muscle of pulmonary bronchi to contract. Prostaglandin E (PGE) is a potent dilator of the bronchial smooth muscle, Prostaglandin F (PGF) is a potent constrictor. Leucotriene-E4 (slow-reacting substance of anaphylaxis) causes later vasoreactions. Leucortiene-B4 is a chemoattractant for acute inflammatory cells.

The mast cell degranulation is increased in its effect by PAF (platelet activation factor) which generates platelet aggregation and the release of histamine, heparin and vasoactive amines. Further the ECF-A (eosinophil chemotactic factor of anaphylaxis) and the neutrophil chemotactic factor attract eosinophils and neutrophils which release hydrolytic enzymes causing necrosis. Mast cells also synthetize IL-1, IL-3, IL-4, IL-5, GM-CSF and TNF-alpha.

Mast cells, basophils and eosinophils are the effector cells of immediate hypersensitivity reactions and allergic disease. All three have cytoplasmic granules contains the major mediators of allergic reactions, they all produce lipid mediators and cytokines that induce inflammation.

Properties of mast cells [98]

All mast cells rise from progenitor cells in the bone marrow. Progenitor cells migrate to the peripheral tissues as immature cells and differentiate there. Mature cells circulate in the whole body, but predominantly near blood vessels, lymph organs, nerves and beneath epithelia. Human mucosal mast cells predominate in intestinal mucosa and alveolar spaces in the lung. Human connective mast cells are found in the skin and intestinal submucosa.

In an individual allergic to a particular antigen, a large amount of IgE specific for a certain antigen, are bound to mast cells (through the FcεRI-receptor). They cross link sufficient the mast cells. This cross-linking triggers mast cells activation. In contrast, in non atopic individuals IgEs associated with mast cells are specific for ***many different*** antigens; all of them cause only low levels of IgE production. This means, that no single type of antigen will cross link enough to activate mast cells.

The activation of mast cells results in:

- Secretion of the preformed contents of their granules by exocytosis
- Synthesis and secretion of lipid mediators
- Synthesis and secretion of cytokines (Il-4, IL-5, IL-6, TNF-gamma).

1.7.6. The role of eosinophils in hypersensitivity reactions [98]

Macrophages and neutrophils are not target cells in allergic reactions, but they are the main producer of prostaglandins and leucotrienes. Another blood cell type in allergic reactions without target functions are eosinophils. Norman individuals have 1-3% eosinophils circulating in their blood, while atopic individuals have 10-20%.

Chemotactic factors, released by mast cells, attract eosinophils to the allergic inflammatory site. Eosinophils rise in the bone marrow, GM-CSF, IL-3 and IL-5 promote eosinophil maturation from myeloid precursors, after maturation eosinophils circulate in the blood. IL-5 increases the maturation of eosinophils from the bone marrow precursors; IL-5 also enhances the eosinophils to release their granule contents. IL-4 (T_H2 cell cytokine) may have an influence on the expression of adhesion molecules for eosinophils. The granules of eosinophils contain lysosomal hydrolases (major basic protein, eosinophil cationic protein) and eosinophil

peroxidase. Activated eosinophils produce and release lipid mediators (PAF, prostaglandins and leucotrienes (LTC_4 and their derivatesLTD_4 and LTE_4). Eosinophils are abundant in the inflammatory infiltrates of late-phase reactions and they may also have a role in the pathologic processes in allergic diseases.

1.8. Pathological features of (allergic) asthma

1.8.1. Airway inflammation in allergic asthma [96]

All structures of the bronchi are affected in asthma. It is known that inflammation and remodeling in large and small airways play a key role in the pathophysiology of asthma. But asthma has varying phenotypes: some patients suffer from a chronic, low grade of asthma with sputum containing eosinophils. Other patients show fewer of the chronic asthma symptoms, but they have acute, sudden asthma attacks that may be life threatening. Other patients only develop asthma symptoms from exercise.

In patients suffering from fatal asthma attacks, more thickening of the subepithelial basement membrane and a significant higher amount of eosinophils in the medium and larger airways occurs than in patients with non fatal asthma.

1.8.2. Airway remodeling and airway hypersensitivity [10, 17, 18, 20, 21, 22, 26, 29, 47, 49, 50, 51, 52, 53, 64, 65, 68, 77]

In asthma and COPD patients the airway wall is thickened to the smooth muscle layer due to inflammatory changes: the bronchial glands develop an increase in size (hypertrophy and hyperplasia of granular structures in the mucosa, often with increased sputum production and often associated with intraluminal exudates), further infiltration with inflammatory cells (eosinophils, neutrophils, lymphocytes etc.), and inflammatory edema due to vasodilatation with higher hydrostatic pressures, increased vascular enlargement and vascular permeability and the excretion of osmotic active substances and the accumulation of mucus at the inner surface of the airway. This is going hand in hand with a smaller internal diameter and increased airway resistance by the swelling of the adventitia on the outside of the smooth muscle layer.

The shortening of the smooth muscle shows the degree of the narrowing of the airway lumen and an increase in airway resistance.

Airway sensitivity and airway reactivity are the two major symptoms of AHR [20, 22, 10, 49, 50, 51, 52, and 53]. Interestingly, airway sensitivity is correlated to eosinophil counts but not to airway thickness. And airway reactivity negatively corresponds with airwall thickness but not with the eosinophil number. The grade of airway thickening is correlated to the severity of the disease and airflow obstruction. Airway thickening weakens airway reactivity in patients with asthma. These results may be important for the treatment of airway remodeling.

Airway thickening may cause airway hyperresponsiveness (AHR), an essential symptom of asthma, which results in worsening airway narrowing response to allergens, exercise in some cases, histamine or methacholine challenge. [17].

1.8.3. Inflammatory cells in Asthma [4, 12, 59, 71]

There are various cells involved in allergic asthma, characterized by the inflammatory infiltrate of the asthmatic airway. These cells contain:

- Inflammatory cells: T cells and myeloid cells e.g. eosinophils, mast cells, basophils, alveolar macrophages and neutrophils.
- Structural cells, as fibroblasts and smooth muscle cells
- Epithelial cells with an important role in the interaction between air and submucosa.

The interaction between these cells and their products mediate the inflammatory response. The inflammatory cells release a part of the interacting vasoactive mediators, toxic metabolites and cytokines, all involved in acute and chronic bronchoconstriction. The initial response to an allergen in sensitized individuals is controlled by products associated with mast cell activation, particularly histamine, prostaglandin D2 (PGD2), leucotriene C4 (LTC4) and tryptase. A few hours after antigen uptake, inflammatory cells are recruited from the circulation; these cells are T cells, neutrophils, eosinophils, basophils and monocytes. Recent clinical studies have shown that IgE and CysLTs, (cystenyl leucotrienes) play important roles in the most early and late physiologic responses to irritation of the bronchi in the allergic airway and the recruitment of inflammatory cells.

Epithelium

The regeneration of the epithelium of the airways in asthmatic patients appears to be normal but show an increased number of goblet cells. The ciliated cells are swollen, vacuolated and often cilia are lost.

The epithelial cells are activated because they release a higher amount of fibronectin, prostaglandin E2 and endothelin; present increased expression of membrane markers, cytokines or chemokines. During an asthma attack epithelial cells can be activated by IgE-dependent mechanisms or by pro-inflammatory mediators (e.g. histamine). Activated epithelial cells release a wide pattern of mediators, cytokines or extracellular matrix proteins able to induce bronchial obstruction, inflammation and airway remodeling.

Lymphocytes

T lymphocytes belong to the second major cell type of the mixed cell infiltrates from patients' airways. Most of them are $CD4^+$ and their number correlates with the severity of asthma. T cells get activated in response to antigen stimulation or during acute asthma exacerbations. And they produce high levels of cytokines. $CD4^+$ cells are further divided in three subtypes, according to their surface cell markers and functions, in Th1, Th2 and Th3 cells. After allergen challenge many allergen specific Th2 cells are in the bronchoalveolar lavage fluid or bronchial biopsies. The Th3 cells play an important role in immune tolerance.

Mast cells

Normally mast cells are the only resident cells in the airway. They are present on the surface and within the airways, and they are well positioned to respond to an initiative stimulus on the way of the IgE binding on the receptor $F_{CE}RI$. Within a few minutes after allergen uptake mast cells are degranulated – shown directly by electron microscopy and immunohistochemistry and indirectly by their released mediators: histamine, tryptase, prostaglandins D2 (PGD2), and LTC_4. These products are potent bronchoconstrictor and may induce vascular permeability. It has also been shown that the severity of asthma is significantly correlated to the level of tryptase or histamine in the bronchoalveolar lavage. [71]

Eosinophils

Patients with symptomatic asthma show activated eosinophils in their airways. Patients with chronic asthma have increased number of activated eosinophils in bronchial biopsies; these eosinophils are located beneath the basement membrane. A broad correlation between the activation of eosinophils and the severity of asthma has been found. The mucosa damage in chronic asthma is related to cytotoxic and pro-inflammatory mediator release from activated eosinophils. The products causing the damages are reactive oxygen species, cytotoxic granule, vesicular proteins e.g. major basic protein (MBP), eosinophil cationic protein, eosinophil peroxidase, eosinophil derived neurotoxin, as well as cytokines, chemokines and pharmacologically active mediators. Eosinophils from asthma patients have an overexpression of LTC_4 compared to healthy controls.

Alveolar macrophages

Macrophages carry out accessory cell functions by antigen presentation and providing secondary signals (e.g. IL-1) required for the differentiation and proliferation of specific lymphocytes. These might play a role in the sensitization of the airways in response to further exposures of antigens. Although the role of resident macrophages in the initiation of a immune response remains unclear because dendritic cells, and blood monocytes are better antigen-presenting cells, airway macrophages may play a role in airway inflammation through the low-affinity receptor FcεRII (for IgE) – increased in asthmatic patients in relation to healthy people. Macrophages also release lysosomal enzymes, leucotrienes B4, LTC4, PGD2 and superoxide anion and other inflammatory mediators e.g. the platelet-activating factor, prostaglandin F2a, and thromboxane. These mediators may play a role in the development of bronchoconstriction or in inflammatory changes, including cell recruitment and altered vascular permeability.

Mesenchymal cells

Fibroblasts, endothelial cells and smooth muscle cells are all activated and develop a complex cellular network, which directly regulates the development of the inflammation and regulation in the airways. These changes may lead to the development of airway remodeling, and dependent on the grade of the airway remodeling may stimulate permanent tissue destruction. [4, 12]

1.8.4. Inflammatory Mediators of Asthma [31, 98]

Asthma is a complex chronic inflammatory disease of the airways involving the activation of many inflammatory and structural cells, all of them release inflammatory mediators resulting in the typical pathophysiological changes of asthma (Barnes, 1996a). Inflammatory mediators are secreted cell products, exerting functional effects. In addition, novel mediators of asthma, such as the cytokines, have been identified. To date 50 different mediators have been identified in asthma. Advances in this field have been greatly assisted by the development of potent and specific inhibitors that either block the inflammatory mediator receptors or inhibit mediator synthesis

Cellular Origin of Mediators [31]

These include mast cells, macrophages, eosinophils, T lymphocytes, dendritic cells, basophils, neutrophils and platelets. It is now increasingly recognized that structural cells may also be important sources of mediators in asthma. Airway epithelial cells, smooth muscle cells, endothelial cells, and fibroblasts are all capable of synthesizing and releasing inflammatory mediators (Levine, 1995; Saunders *et al.,* 1997; John *et al.,* 1997). And this may explain how asthmatic inflammation persists even in the absence of activating stimuli.

Mediators derived from mast cells [98]

These mediators are divided into preformed mediators (biogenic amine and granule macromolecules) and newly synthesized mediators (lipid derived mediators and cytokines).

Biogenic amine [98]

Biogenic molecules are stored in cytoplasmic granules. They are also called vasoactive amines. In human mast cells the only mediator of this class is histamine. Binding of histamine to endothelium causes cell contraction, which leads to leakage of plasma into the tissues. Histamine stimulates endothelia cells to synthesize vascular smooth muscle cell relaxant, e.g. prostacyclin (PGI_1) and nitric oxide, which cause vasodilation. Histamine also induces contraction of intestinal and bronchial smooth muscle.

Granule proteins and Proteoglycans [98]

Neutral serine proteases like tryptase and chymase play a role in tissue damage in immediate hypersensitivity reactions. The function of these proteins in vivo are not known, but in

vitro they suggest important biologic effects e.g. tryptase cleaves fibrinogen and it also activates collagenase, resulting in tissue damage; chymase plays a role in degradation of epidermal basement membrane and stimulate mucus secretion. Proteoglycans composed of polypeptide core and multiple unbranched glycosaminoglycan side chains with a strong net negative charge to the molecules. Within the granules they act as storage matrices for positively charged biogenic amines, proteases and other mediators and prevent their accessibility to the rest of the cell. The Proteoglycans release the mediators at different rates after granulocyte exocytosis. They may control the kinetics of immediate hypersensitivity reactions in this way.

Lipid mediators [98]

The result of mast cell activation results in rapidly de novo synthesis and release of lipid-derived mediators effecting blood vessels, bronchial smooth muscle and leucocytes. The major arachidonic acid-derived mediator produced by the cyclooxigenase pathway in mast cells is prostaglandin D_2 (PGD_2). When released, PGD2 binds to receptors on smooth muscle cells and acts as a vasodilator and as a bronchoconstrictor; it also stimulates neutrophil chemotaxis and accumulation at inflammatory sites.

The most important arachidonic acid-derived mediators produced by the lipooxigenase pathway are the leucotrienes, especially $LTTC_4$ and its degradation products LTD_4 and LTE_4. Mast cell derived leucotrienes bind to specific receptors on smooth muscle cells and stimulate prolonged bronchoconstriction.

A third type of lipid mediator produced by mast cells is the platelet-activating-factor (PAF). PAF has direct bronchoconstricting actions and also causes retraction of endothelial cells and relaxation of vascular smooth muscle. In individuals with a genetic deficiency of PAF hydrolase develop asthma in early childhood. PAF maybe has an importance in late-phase reactions, activating inflammatory leucocytes, but in this case the major source of PAF could be basophils or vascular endothelial cells – stimulated by histamines or leucotrienes.

Mediator Receptors [31]

The receptors for many inflammatory mediators have the typical seven-transmembrane domain structure expected for G protein-coupled receptors. For non cytokine mediators, inflammatory receptors are often coupled through G proteins (Gq and Gi), leading to

phosphoinositide (PI) hydrolysis, but it is increasingly recognized that other pathways may also be activated, including the complex mitogen-activated protein (MAP) kinase pathways, that are involved in more long-term effects of mediators. Cytokine receptors work through complex pathways, including MAP kinases and other protein kinases, resulting in the activation of transcription factors. Transcription factors regulate the expression of many genes, including inflammatory genes themselves.

Mediator Effects [31]

Inflammatory mediators produce many effects in the airways, including bronchoconstriction, plasma exudation, mucus secretion, neural effects and attraction and activation of inflammatory cells. Although the acute effects of mediators have been emphasized, there is increasing recognition that mediators may result in long-lasting structural changes in the airways, also caused by the release of inflammatory mediators. These changes may include fibrosis resulting from the deposition of collagen, seen predominantly under the epithelium even in patients with mild asthma. The airway smooth muscle layer is also thickened in asthma, and this is likely the result of increases in the number of smooth muscle cells (hyperplasia) and increases in their size (hypertrophy) (Knox, 1994). There may be proliferation of airway vessels (Kuwano *et al.,* 1993) and of mucus-secreting cells. There may also be changes in the innervations of the airways.

Involvement of Mediators in Asthma [59]

Thus, mediators or their metabolites may be detected in plasma (e.g., histamine), urine (e.g., LTE4) or, more likely, the airways (in biopsies, bronchoalveolar lavage fluid, induced sputum, or exhaled air). However, this does not necessarily mean that the mediator plays any important role in asthma.

The best evidence for the involvement of a mediator in asthma is obtained with the use of specific blockers. These may be drugs that block the synthesis of the mediators (e.g., 5-LO inhibitors) or drugs that block their receptors (e.g., antihistamines). Although it is unlikely that blockade of a single mediator will be entirely effective in controlling asthma, there is accumulating evidence that some mediators are more important than others. PAF receptor antagonists are of no obvious clinical benefit in asthma (Kuitert *et al.,* 1993), but cysteinyl-LT

(cys-LT) receptor antagonists have considerable clinical effects (O'Byrne *et al.,* 1997) [59]. The role of a mediator in asthma may be difficult to assess when the mediator has a long term effect on airway function. It is easy to measure the effect of a mediator on airway smooth muscle, but it is more difficult to determine its effect on airway microvascular leakage and mucus secretion. It may be even more difficult to determine the role of a mediator on chronic inflammatory effects, such as airway smooth muscle proliferation and fibrosis that may develop over many years. However, prevention of the long term consequences of asthmatic inflammation, such as irreversible airway narrowing, may be an important goal of asthma therapy, and it is necessary to devise methods to investigate how mediators may affect these long term consequences of asthma. Asthma has a characteristic clinical pattern, and the histological appearance of asthma is very similar among patients, even when there are differences in asthma severity or in whether or not the asthma is allergic. However, it is likely that there are differing mechanisms of asthma among patients and that different patterns of inflammatory mediators are involved. This suggests that mediator antagonists would have different effects in different patients.

Late phase reaction [98]

The immediate hypersensitivity reaction is followed 2-4 hours later by late phase reactions with accumulation of inflammatory cells including eosinophils, basophils, neutrophils and T_H2 cells. The inflammation has its maximum by about 24 hours. Mast cell granules carry cytokines, including TNF, which is able to upregulate endothelial expression of leucocyte adhesion molecules like E-selectin and intercellular adhesion molecule-1 (ICAM-1). Mast cell degranulation may lead to induction of adhesion molecules on vascular endothelial cells, promoting the recruitment of leucocytes into the tissues. The late-phase reaction may appear without a detectable preceding immediate hypersensitivity reaction, e.g. bronchial asthma and eczema. In disorders like this, there may be a little mast cell activation, the cytokines, inducing the late-phase reaction may be produced mainly by T_H2 cells.

Chronic Inflammation [31]

Although in the past much attention has been paid to acute inflammatory responses (such as bronchoconstriction, plasma exudation, and mucus hypersecretion) in asthma, it is being increasingly recognized that chronic inflammation is an important aspect of asthma (Redington

and Howarth, 1997). This chronic inflammation may result in structural changes in the airway, such as fibrosis (particularly under the epithelium), increased thickness of the airway smooth muscle layer (hyperplasia and hypertrophy), hyperplasia of mucus-secreting cells, and new vessel formation (angiogenesis). Some of these changes may be irreversible, leading to fixed narrowing of the airways. These chronic inflammatory changes are mediated by the secretion of distinct mediators. These factors include cytokines and growth factors. Cytokines are a large group of protein mediators that play a critical role in determining the nature of the inflammatory response and its persistence. They play a key role in the pathophysiological changes in chronic asthma and are increasingly recognized as important targets for treatment.

1.8.5. Cytokines in asthma [22, 78,79]

The first indication of cytokine involvement in the pathology of asthma came in the early 90´s. Th2 cell cytokines (IL-3, IL-4, IL-5, and GM-CSF) are significantly upregulated after antigen challenge in asthmatics, in comparison with healthy individuals [22]. IL-13 is associated with IgE synthesis, chemoattraction of eosinophils, mucus hypersecretion, fibroblast activation and the regulation of the smooth muscle function. IL-9, another Th2 cytokine is also upregulated and associated with airway hyperresponsiveness, mucus hypersecretion, eosinophil function, IgE-regulation, and upregulation of the Ca^{2+} – activated chloride channel.

Mast cells

Mast cells also participate in the inflammatory changes in asthma through cytokines in response to IgE-dependent stimuli. Mouse cells produce a response of IgE-dependent stimuli Th2 cytokine: IL-3, IL-4, and IL-5. Human lung mast cells produce IL-4, IL-5, and IL-13 in vitro, mucosal biopsy specimens from asthmatic patients have been positively stained by immunohistochemical means for IL-4, IL-5, IL-6 and TNF-alpha in mast cells. The mast cells are the main immediate source of TNF-alpha for IgE-dependent reactions.

Macrophages

IL-1, TNF-alpha, IL-6 and GM-CSF are pro-inflammatory cytokines released from macrophages. GM-CSF may induce endothelial cell activation, cellular recruitment and prolongation of eosinophil survival. IL-6 and TNF-alpha may be a response to IgE-dependent

stimulation. Macrophages also release histamine-release factors which act on the basophils and mast cells by their reaction with surface IgE.

Eosinophils

Th2 cytokines IL-3, IL-5 and GM-CSF are important in regulating eosinophil priming, activation and prolonged survival in the tissue. IL-16 is a strong T cell and eosinotactic cytokine and is an important product of bronchial epithelial cells; it has also been shown to be a feature of human bronchial asthma. IL-16 acts on the $CD4^+$ receptors expressed on the surface of eosinophils to activate their chemotactic activity. IL-5, produced by mononuclear cells under stimulation of (mite) antigen; is also a critical factor for eosinophil terminal differentiation.

1.8.6. Regulatory cells in Asthma [8]

The lung represents a unique mucosal environment, with the primary function of gaseous exchange. Therefore a large surface area is exposed to the external environment, continuously exposed to airborne antigens. The epithelium plays a central role via the production of molecules such as mucins, surfactants, complement products and antimicrobial peptides (reviewed in Holt et al., 2008).

In addition, a unique population of macrophages termed alveolar macrophages, resident outside the body in the airway spaces, should also play an important role with the exhibition of potent phagocytic and antimicrobial functions (Holt et al., 2008). Finally, they play a major role in sequestering antigens from dendritic cells, but they have also been proposed to be immunosuppressive by downregulating local dendritic and T cell activation (Holt et al., 2008).

Asthma is characterized by T helper cell 2 (Th2) type inflammation leading to airway hyperresponsiveness and tissue remodeling. The Th2 cell-driven inflammation represents an abnormal response to harmless airborne particles. Normally these reactions are suppressed by regulatory T cells, which maintain airway tolerance. The anti-inflammatory cytokine IL-10 plays there a central role. In allergic asthma a breakdown in the cytokine transforming growth factor b (TGF-beta) regulatory mechanism is observed.

Regulatory T cells (T-reg cells) are essential in the maintenance of immunological tolerance to ‘‘self’’ and in the regulation of the immune response to infectious organisms

(pathogens and commensals). The primary functions of T-reg cells in the airways are to limit the inflammatory consequences of infection and to maintain tolerance to harmless, inhaled aeroallergens. T-reg cells represent a major pathway to contribute to the maintenance of immune homeostasis in the airways. Major populations of regulatory T cells studied in the context of pulmonary health and asthma are the natural thymic-derived CD4+Foxp3+ T-reg cells, and peripherally antigen-induced adaptive CD4+ Treg cells, which comprise both Foxp3-positive and -negative populations [32].

Anti-inflammatory cytokines like IL-10 and TGF-b (Li et al., 2006; O'Garra et al., 2008), are known inhibitory mechanisms; Inhibitory molecules such as CTLA-4 and PD1 are also contributing (Vignali et al., 2008). Although CD4+ Treg cells have been focused, any cell capable to secrete inhibitory cytokines may have a potential to regulate the immunological tolerance, including CD8+ T cells, NK cells, god T cells, B cells, mast cells, and various APC populations (O'Garra et al., 2008).

1.9. Asthma medication

1.9.1. Current asthma medications [12, 25, 44, 45 , 55, 57]

Asthma is a lifelong disease, it cannot be cured but with the right treatment most asthma patients are able to have a normal life. Asthma has two cornerstones in the treatment: airway inflammation and acute bronchoconstriction. As shown by research, reducing and preventing inflammation is very important in preventing asthma attacks, hospitalization or even death from asthma.

Anti-inflammatory drugs

These drugs currently prevent asthma attacks. They decrease swelling and mucus production in the airways, and as a result, the airways are less sensitive towards allergens.

Bronchodilators

These drugs work by relaxing the tightening muscle bands around the airways. They also help to clear mucus from the lungs because in the opened airways the mucus moves better and can be coughed out more easily.

Asthma prevention or long term control medications

Long-term asthma medications are very important in the treatment and the prevention of asthma attacks by impeding inflammation and swelling of the mucous membrane lining in the airways of the lungs. Their effect is in most cases reached by a daily application and from time to time they should be controlled and, if necessary, readjusted as well.

Inhaled Corticosteroids, recommended for persistent asthma

Inhaled corticosteroids are a very effective anti-inflammatory treatment: it reduces inflammation of the airway walls, blocks allergic immune response and prevents infiltrates in the tissue of the airways. Inhaled corticosteroids are applied by a metered dose inhaler with a spacer, to prevent entering of the drug into the digestive system.

- Fluticasone (Flixotide, Flovent Diskus, Flovent HFA),
  This medication decreases the inflammation of nasal passages or bronchial tissue;
- Budesonide (Pulmicort Flexhaler),
  it prevents and reduces inflammation in the airways by regular usage
- Beclomethasone (Becotide, Qvar, Asmabec),
  is used as an asthma attack preventing medication for the treatment of chronic asthma, reaching its peak effect after two weeks. It is not used to treat asthma attacks. It is hypothesized to substitute oral steroids in patients.
- Mometasone (Asmanex),
- Flunisolide (Aerobid),

Bronchodilator

- Ventolin (albuterol):
  is used for the treatment of asthma, chronic bronchitis and emphysema. The drug relaxes the smooth muscle in the lungs and dilates the airways to ease breathing.
- Volmax
  Volmax is an oral albuterol sulfate (salbuterol), it is a sympathonimetic bronchodilators, a class of anti-histaminic agents. It is a medication to prevent and treat bronchospasm in patients with reversible obstructive airway disease and exercise-induced bronchospasm.
- Salmeterol is a bronchodilator Serevent Inhaler

- Severent Accuhaler

  Similar to albuterol *(Proventil®* or *Ventolin®),* bitolterol *(Tornalate®),* pirbuterol*(Maxair®)* terbutaline *(Brethaire®).* (short-acting medicaments) It has no immediate effect, though, allowing a dose of two servings daily instead of four, as is usual for other short-acting medicaments.

- Atrovent

  Atrovent is a treatment for patients with asthma, emphysema and chronic obstructive bronchitis; it meliorates air flow by relaxing the muscular tubes in the lungs. It acts quickly after use but it reaches its maximum effect only after 2 hours.

Leucotriene modifier [12, 25, 44, 45, 55, 57]

Leucotriene modifiers are alternative drugs to steroids and mast cell stabilizers. Their effect is the blocking of leucotriene responsible for asthma symptoms. These medications prevent asthma symptoms for up to 24 hours.

- montelukast (Singulair),
- zafirlukast (Accolate),
- zileuton (Zyflo, Zyflo CR).

Long-acting beta agonists (LABAs)

LABAs are bronchodilators; their effect is the dilatation of the constricted airways and the reduction of the inflammation. These medications should be combined with inhaled corticosteroids.

- salmeterol (Serevent Diskus)
- formoterol (Foradil Aerolizer)

Combination inhaler

These medications consist of a LABA with a corticosteroid.

- Seretide, Advair MDI, Advair Discus:

  contains fluticasone propionate and salmeterol They synergistically control asthma.

- Symbicort: contains budesonide and formoterol
- COMBIVENT®: ipratropium bromide and albuterol sulfate: a bronchodilator.

Theophylines

Theophylines are anti-inflammatory, non-steroidal bronchodilators in the shape of a daily pill, liquid or intravenous drug used for the prevention of nighttime-symptoms. This medication is used for asthma patients who are difficult to observe or suffer from with severe attacks.

- Uniphyl
- Theo-Dur
- Slo-Bid
- Theo-24

Short-term or quick-relief (rescue) medications

These medications are for the rapid relief of symptoms during an asthma attack or before exercise. They have an effect within minutes for several hours, relaxing the muscles around the bronchial tubes and provide a reliable relief of symptoms.

- Salbutamol (Ventolin, Airomir, Asmasal),
- Terbutaline (Bricanyl),
- Fenoterol(Berotec)
- albuterol (ProAir HFA, Ventolin HFA, others),
- levalbuterol (Xopenex HFA)
- pirbuterol (Maxair Autohaler)
- Ipratropium (Atrovent, Combivent & Duovent)
- Anticholinergics, bronchodilators applied in addition to short-acting beta-agonists, when needed, or even as an alternative to short acting beta-agonists.

Oral and intravenous corticosteroids

These medications cure airway inflammation by severe asthma, but they are used only short-term to relieve severe asthma symptoms.

Quick-relief asthma medications include

- short acting beta 2 agonists,
- ipratropium
- oral and intravenous corticosteroids

1.9.2. Treatment of allergic asthma [87]

Allergic asthma is the most widely-spread type of asthma, concerning children as well as adults. While childhood asthma is linked to allergies in most cases, only half of asthma cases in adults have prevalence to allergic asthma. The symptoms of allergic asthma are similar to the non-allergy asthma symptoms, with the difference that allergic asthma attacks are provoked by specific allergens and the contact with the specific allergen causes the cells of the body to excrete histamines to defend the foreign substances. Caused by the histamines, nose linings and airway start to swell; this causes constriction of the air passage inhibiting air flow through the lungs. Asthma symptoms can be controlled well by avoiding allergens because the reaction is only triggered by the specific allergens the patients are sensitized to. Patients of allergic asthma have to defend two diseases at the same time; they need anti-allergic treatment (each time they get in contact with the allergen) as well as an asthma treatment like corticosteroids or bronchodilators to ease breathing.

Allergic asthma medications

These medications contain oral, nasal antihistamines, decongestants and corticosteroids. The short-term allergic asthma treatment can remain the same as usual, with bronchodilators and corticosteroids. Additionally, anti-allergic treatments are necessary, as well, e.g. antihistamines and anti-inflammatory drugs:

- inhaled corticosteroids
- bronchodilator
- antihistamines,
- nasal decongestants
- immunotherapy vaccines.

Allergy shots (immunotherapy)

These medications are administered once per week during a period of a few months, then as an injection once per month for a three-to-five-year period. These medications, if given continually over time, have an allergy-reducing effect to the specific antigen.

Omalizumab (Xolair)

This medication is a long-acting allergy treatment of monoclonal anti-IgE antibodies, applied in form of an injection, in a 2-to-4-week interval, when inhaled steroids for asthma have failed to control asthma symptoms, for people with moderate to severe asthma who also have allergies. The function of this medicament is to convert the immune reaction to the allergen.

1.9.2.1. Allergy medications

Antihistamines [12]

- Claratyne/Claratin
  used in treatment of symptoms of perennial and seasonal associated allergies, e.g. sneezing, nasal discharge and itching as well as itching and burning in the eyes.
- CARINASE/Claradin D
  Sustain 12 hours remedy from allergic rhinitis (hay fever) symptoms. Each tablet contains a decongestant (pseudoephedrine sulfate) and anti-histamine (Loratadine).

- Zyrtec
  used in form of tablets containing Cetrizine Hydrochloride 10 mg, an anti-allergic cure against hay fever during summer and spring caused by airborne pollens.

Nasal Spays

Rhinolast/Astelin

Rhinolast is an anti-histamine nasal spray which prevents allergic reactions by the nose caused by pollens, cat and dog hairs, house dust mites or other allergens. Each dose contains 0.14 mg of azelastine hydrochloride.

Current and future developments

Many drugs for asthma have been developed on the basis of inhibition of allergic airway inflammation and bronchial smooth muscle contraction. Recent studies have proved that airway remodeling is a new potent target in asthma medication. In order to this findings, it would be very important to determine the target molecules of the remodeling process, and to evaluate the compounds in the development of new drugs in the treatment of asthma.

1.9.3. Novel anti-inflammatory treatments [90, 91]

A lot of new medications are currently been developed. Some of them are [94]:

- NF-kappaB Inhibitors
- Statins
- Macro-steroids
- PDE4 Inhibitors
- Modulation of T_h1/T_h2 Balance
- Monocyclic aroylpyricinones
- Pyrimidyl Sulphone

1.10. Compounds for testing in Allergic asthma in mice

1.10.1. Tiotropium bromide [1, 2]

Tiotropium bromide is a drug, already in use for maintenance treatment of COPD (chronic obstructive pulmonary disease). Our reason to test Tiotropium bromide was to test if allergic asthma could be a new indication. Tiotropium is an analog of Atropine (an agent of belladonna), an anticholinergic agent of the parasympatholitica group. An anticholinergic agent is a substance that blocks the neurotransmitter acetylcholine in the central and peripheral nervous system. Tiotropium is a quadrennial ammoniac salt which increases the lipophilie; it binds to the receptor subtypes muscarinic acetylcholine receptor M1-M5, especially to M3. Muscarinic receptors are G-protein coupled acetylcholine receptors, which are situated in the plasma membranes of neurons. M3 receptors are located on many places in the body, as well in the lungs. The M3 receptor mediates the increase in intracellular calcium, which typically causes contractions of smooth muscles for example bronchoconstriction. Tiotropium is a muscarinic receptor antagonist, acting mainly on M3 muscarinic receptors in the airways; it produces smooth muscle relaxation, through the mechanism of reversible and competitive occupation of the receptor subtypus M3, which has a bronchoconstriktiv effect. The dissociation of Tiotropium from the M3-receptor is

much slower than the dissociation of e.g. Atropine or ipratropium. Tiotropium has a half life time of 5-6 days and its effect is the dilatation of the bronchi by patients with COPD (Cronical obstructive pulmonary disease). It is used to prevent bronchospasm attacks and it has a sustain effect over 24 hours. But it is not useful for already begun attacks, because the bronchodilutative effect starts after Tiotropium inhalation. COPD is characterized by shortness of breath (dyspnea), persistent cough, sputum or mucus production, wheezing, chest tightness and tiredness. After inhalation of Tiotropium, the primary effect is local on the bronchi, with an effect of significantly decreased dyspnea and COPD-exacerbations.

The effect of Tiotropium is also the increase of potential for expiratory flow, increase of the ability for expiration, increasing oxygen concentrations on inspiratory reserve volume and increase of the exercise indurations (for a mean of 21% compared with COPD-patients with placebo). The mobility of COPD- or asthma-patients is also restricted because of short breathing; Tiotropium increases the mobility and life qualities of these patients [1].

Tiotropium bromide ‘‘Spiriva‘‘is administered through oral inhalation of a manually pierced capsule in a piercing chamber. The dose of Tiotropium, used in different clinical studies, is 18µg once a day. Two preparates of Tiotropium bromide are in use: Spiriva® HandiHaler®. (in Austria, Germany) and Favint (in Austria).

The benefits of Tiotropium are that it is metabolized in the liver and that the metabolic rate is very low: 20-25 % of the dosis is excreted with the urine, 75-80% of Tiotropium is unchanged and renal excreted and the effect of bronchial dilution lasts over a year without tolerance effects.

The safety and efficacy of Tiotropium (Spiriva) was proved in a four-year landmark, multinational, multi-centre, placebo-controlled, randomized, double-blind clinical trial with 5,993 patients with COPD from 37 countries across the globe. The study showed that Tiotropium does not increase the risk of death, cardiovascular death, myocardial infarction and stroke and it is also associated with a decreased risk of adverse events in these organs [2].

Because of the similarity of symptoms between COPD and asthma patients, namely shortness of breath and lack of oxygen, because of bronchial constriction and also decreased mobility by patients with asthma, Tiotropium may also be useful for the treatment of asthma.

1.10.2. The compound AAC [28, 35, 38, 39, 81, 88, 89]

AAC is a combination of N-acetyl cysteine and gold salt, which may have a combined effect of the both substances. There are only few trials using gold salts as treatment. In these trials gold is administered intramuscular for the treatment of severe rheumatoid arthritis. In other trials gold treatment was orally applied for asthma patients for 20 weeks. The trial has shown to decrease the frequency of asthma attacks as well as the reactivity to methacoline and the reduction of corticosteroid requirement. But there was no difference in peak expiratory flow (PEF) or the number of asthma exacerbations between the treated and the placebo group. A recent double-blind trial on non-corticosteroid dependent asthma patients showed a significant reduction in bronchial responsiveness to methacoline in gold treated patients, with a dose of 3 mg twice per day.

Gold has anti-inflammatory effects; it inhibits the IgE-mediated excretion of histamine and LTC4 from basophils and mast cells. Moreover, it has been shown, that guinea pig tracheal smooth muscle cells incubated with gold have a weaker immune response to histamine and specific antigen. The first placebo controlled trial of intramuscular gold was done by Muranaka et al in 1979. After 30 weeks the gold treated group suffered from fewer symptoms, therefore they needed less medication. The side effects of gold treatment are dermatitis, stomatitis or proteinuria [28, 39, and 81].

N-acetyl cysteine (NAC) is the pre-acetylated form of cysteine. It naturally occurs in food and it is a powerful antioxidant that plays a role in protecting cells against oxidative stress. It has been shown, that NAC is able to suppress HIV replication, to protect against cell damage occurring by chemotherapy, radiation therapy, to be mucolytic by bronchopumonary diseases as cystic fibrosis, chronic bronchitis, asthma and pneumonia. It has also been shown, that NAC has a supporting effect on the immune system to fight diseases. It is free available in health food shops in many counties, it has neither a sedative or stimulant effect. Moreover it is safe with and well tolerated. But in asthma patients it causes bronchospasm after inhalation or oral uptake [35, 38, 88, and 89].

1.11. Mouse models of experimental asthma [2, 60, 93]

The experimental models of allergic asthma are well-proven for almost 10 years. They provide many chances to study the disease pathogenesis and to study and develop new treatments. [93] The aim of these mouse models is to study the allergic immune response, the clinical features of the disease, as it gives us a view in the pulmonary pathophysiology, and allow us to create a theory about the effect of tested, new medications.

While mouse models barely recreate all the symptoms of human allergic asthma after sensitization and respiratory tract challenges with an allergen, the wild type mice develop clinical symptoms closely mimicking allergic asthma, known as eosinophilic lung inflammation, airway hyperresponsiveness (AHR), increased IgE and IgG1, mucus hypersecretion and airway remodeling sometimes.

Although these mouse models are well-proven, there are discrepancies between mouse and human physiology:

- allergic asthma patients have methacholine-induced AHR even if they are free of symptoms, in mice only transient methacholine-induced AHR occurs only after allergen exposure.
- The chronic allergen exposure in patients with allergic asthma induces chronic allergic asthma, while repeated challenges with the allergen in sensitized mice leads to suppression of the disease.
- IgE and mast cells in humans lead to early and late phase allergic immune responses, in mice both are not needed for the development of allergic asthma.

However, two mouse models are to date the best approach to study allergic asthma and to develop new treatments for the disease.

An innovative mouse model is developed in our laboratory to the existing mouse models, which are the intranasal (i.n.) sensitization and aerosol challenge with the allergen, instead of intraperitoneal (i.p.) sensitization and aerosol challenge of the mice with the allergen, with the intranasal way of sensitization being closer to the natural way of sensitization to the allergen. We compared two mouse stains and two different components. On the one hand we tested OVA of chicken egg, the standard component to induce asthma with a high-cost compound. The other

compound was low-fat milk, which also induces allergic asthma to different proteins in the low-fat milk and is much less cost-intensive than OVA. The two different mouse stains were BALB/c and C57B6 (B6) mice, which have different ways of immune responses to allergens. While BALB/c mice have a T_H2 immune response, the B6 mice have a T_H1 immune response to the allergen.

1.12. Aims

There were two aims in this project. The first aim was to compare the effectiveness of two compounds on allergic asthma during disease exacerbation in mice with a (standard) glucocorticosteroid treatment. The second aim was to determine whether distinct Th1-type, B6 and Th2-type BALB/c mice responded similarly to different allergens.

Chapter 2

2. Materials and methods

2.1. Mice, equipment and materials

Mice used for the experiments

- 4-week-old female BALB/c AnNCr mice purchased from Charles River, Germany, for the experiments with the relapsed asthma model, treated with Tiotropium or AAC.
- 4-week-old female BALB/c and female B6AnNCr mice, purchased from Charles River, Germany, for the experiments with the new acute asthma model induced with i.n. sensitization with OVA or low fat milk.

Materials for sensitization and challenge with the allergen

- Syringes and needles for anesthesia
- pipettes and tips for i.n. challenge
- Solutions/suspensions for sensitization/challenge
- Mice
- Extra cages to store sensitized/challenged mice
- 70% Ethanol

Materials for the aerosol challenge of the mice

- Nebulizer
- Plastic chambers and tubes for the nebulizer
- Aerosol
- 1% (wt/vol) OVA (in 1xPBS (Gibco, Cat# 14190)

Materials for Assessment

Materials for anesthesia of the mice

- Syringes 1ml (Soft-Jekt, Lot# OD190048)
- Needles 0.6 mm (BD Microlance ^{TM} 3, Lot# 070509)
- Rompun 2% (Bayer, Xylazin-hydrochloride, Ch-B KPO3L8D)

- Ketanest S 25mg/ml (Pfizer, Esketamin-hydrochorid, Cat# 0036126,ONP002-1)
- Autoclaved water
- Prepared Rompun-Ketanest solution
- Extra cage for already anesthetized mice

Materials for cardial punction

- Racks with marked Eppendorf-Tubes for blood samples
- Anesthetized mice
- Syringe and needles
- 70% Ethanol
- Cork plate
- Needles for fixing the anesthetized mice
- Scissors
- Forceps (2)
- Bags for biohazard material

Materials for collecting BAL fluid

- Racks with marked Eppendorf-Tubes for BAL fluid samples
- 1xPBS (Gibco, Cat# 14190, Lot#792641)
- Ice
- Syringe and pediatric i.v. catheter
- Cork plate
- Needles for fixing the anesthetized mice
- Anesthetized mice
- Scissors and clamps
- Forceps (2)
- Bags for biohazard material

Materials for collection of lungs

- Cassettes for the lungs (Simport, Lot#93508265)
- Syringe and pediatric i.v. catheter

- 4% PFA
- Cork plate
- Needles for fixing the anesthetized mice
- Anesthetized mice
- Scissors and Clamps
- Forceps (2)
- Bags for biohazard

Materials for preparation and counting BAL cells with Trypan blue

- Eppendorf-Tubes with BAL fluid samples
- Neubauer chamber
- 96 well racks
- Trypan blue solution 0.4% (Sigma, Lot#44K2394)
- Pipettes and tips for the pipettes
- 70% Ethanol

Materials for cytospin slides

- Eppendorf-Tubes with BAL fluid samples
- 2 marked cytospin slides for each sample
- Pipettes and tips for the pipettes
- Metal clamps for cytospin slides
- Cytospin plastic funnel attached to the cytospin clamps
- Cytospin 4 centrifuge (Thermo Scientific Machine)

Materials for preparation of sera from blood

- Racks with Eppendorf-Tubes with blood samples
- Racks with Eppendorf-Tubes for sera samples
- Pipettes and tips for the pipettes
- Biocentrifuge HeraeusSepatech

Materials for staining techniques

Materials for staining of cytospin slides with Thermo Shandon KWIK DIFF for differential cell counting:

- KWIK DIFF staining set: contains solution 1, 2 and 3, all in one kit, (Thermo Electron Corporation, Cat# 9990700 Lot#177)
- Distilled water
- Cytospin slides
- Racks for the slides
- Glass chamber for each step of the staining
- Timer

Materials for deparaffinisation and rehydratation of the lung samples

- 0-Xylene (c8h10 Fluka, Chemika, Lot# 1428451, cat# 95663)
- Ethnol Absolute (AustroAlco Österr. Alkoholhandels GmbH, 2104 Spillern, 5L)
- Slides with lung samples
- Racks for the slides
- Glass chamber for each step of the staining

Materials for staining of lung sections with Hematoxilin and Eosin (H&E)

- Ethanol Absolute (AustroAlco Österr. Alkoholhandels GmbH, 2104 Spillern, 5L)
- Hematoxilin (Sigma Aldrich Chemie GmbH, Lot#108K4343)
- Eosin Y: (Eosin Y solution alcoholic, Sigma-Aldric, Lot# 118K4356)
- Fuming HCl (HCl, 37% Merck, Ref: K32970717.1000)
- Mounting media: Eukitt (Fluka , Lot#1432836, Charge# C106)
- Slides with lung samples
- Racks for the slides
- Glass chamber for each step of the staining
- Timer

Materials for Staining of lung sections with PAS

- 0-Xylene (Fluka, Chemica, Ref: 95690)
- Ethnol Absolute (AustroAlco Österr. Alkoholhandels GmbH, 2104 Spillern, 5L Schüsselnr.55351)
- Sodium disulfite (Merck KgaA Lot# HX821018)
- Fuming HCl (HCl, 37% Merck, Cat# K33557117, Lot#430)
- Periodic acid (FLUKA Chemica, 25g Lot# 380218/1)
- Schiff´s reagent (Merck KgaA Lot#HX879521)
- Papanicolaus solution 1a Harrys Hematoxilin solution (Merck, Lot#HX879521)
- N-butyl acetate (Merck,$C_6H_{12}O_2$, Lot#K24240052 738)
- Mounting media: Eukitt (Fluka , Lot#1432836, Charge# C106)
- Slides with lung samples
- Racks for the slides
- Glass chamber for each step of the staining
- Timer

Materials for Staining of lung sections with LUNA:

- Hematoxilin ($C_{16}H_{14}O_6$ x H_2O Merck, Lot# K21882538)
- Ethanol
- Ferric chlorite (Iron (III) chloride hexahydrate) $FeCl_3$*$6H_2O$ (Sigma, ot# F2877-250G, Batch# 035KO180)
- Distilled water
- Fuming HCl cat# K33557117 Lot#430
- Biebrich Scarlet (Ponceau S practical grande, Sigma, Lot# 086K0160)
- Lithium carbonate (Li_2 CO_3, Merck, Lot# K36982880 713)
- Slides with lung samples
- Racks for the slides
- Glass chamber for each step of the staining
- Timer

Materials for ELISA

- Materials for ELISA for OVA-specific IgG1
- Iwaki ELISA 96 well plate, flat bottom, code 3801-096
- 1xPBS, Gibco, Cat# 14190, Lot#792641)
- 10xPBS, Gibco, Cat# 12400, Lot#753896
- Distilled water
- Albumin from chicken egg white (OVA) grade V, Sigma-Aldrich, Cat# A-5503, lot. 40K7070
- Tween 20, Bio-Rad, Cat# 170-6531, Lot# 164973A
- Bovine Serum Albumin (BSA), Sigma-Aldrich, Cat# A2153, Lot# 70H0184
- Sera of the mice
- Secondary Antibody: goat anti mouse IgG1 biotin, Southern biotechnology assotiates Inc. USA, Lot# G046-T456) in 1:5000 (in 1% BSA/1xPBS) dilution
- Streptavidin-HRP, Southern biotechnology associates Inc. USA, Lot#D126-786H in 1:8000 (in 1%BSA) dilution
- Washing buffer (1L 10xPBS + 9L distilled water + 10 ml Tween20)
- Blocking buffer (2% BSA in 1xPBS)
- Carrier buffer (1% BSA in 1xPBS)
- TMB Substrate (3.3´, 5.5`-tetramethylbenzide Cat# 555214, Lot#62130)
- Stop solution: 0.18M H_2SO_4 in distilled water

Materials for ELISA for milk-specific IgG1

- Iwaki ELISA 96 well plate, flat bottom, code 3801-096. Lot#0191001)
- 1xPBS, Gibco, Cat# 14190
- 10xPBS, Gibco, Cat# 12400
- milk
- Tris(hydroxymethyl)-aminomethan (Merck, Lot# 8382A016 744)
- NaOH 10 Molar
- Distilled water

- Tween 20, Bio-Rad, Cat# 170-6531, Lot# 164973A
- Bovine Serum Albumin (BSA), Sigma-Aldrich, Cat# A2153, Lot# 70H0184
- Sera of the mice
- Secondary Antibody: goat anti mouse IgG1 biotin, Southern biotechnology associates Inc. USA, Lot# G046-T456) in 1:5000 (in 1%BSA) dilution
- Streptavidin-HRP, Southern biotechnology associates Inc. USA, Lot#D126-786H in 1:8000 (in 1%BSA) dilution
- Washing buffer (6.06 g Tris + 1L distilled water + 2 ml Tween20)
- Blocking buffer: 0.5% Tween20 (2, 5 g of Tween20 in 500 ml 1xPBS –adjusting of the buffer to pH8). This is used also for carrier buffer
- TMB Substrate (3.3´, 5.5`-tetramethylbenzide Cat# 555214, Lot#62130)
- Stop solution: 0.18M H_2SO_4 in distilled water
- H_2SO_4 2N (Roth, Ch.050152977

Substances used for experiment setups

Substances used for setting up the experiment for a model of asthma relapse treated with Tiotropium or AAC

- Albumin from chicken egg white (OVA) grade V, Sigma-Aldrich, lot. 40K7070
- 1xPBS, Gibco, Cat# 14190
- Tiotropium **or** AAC depending on the experiment
- Dexamethasone as standard substance

Substances in the experiment

- Albumin from chicken egg white (OVA) grade V, Sigma-Aldrich, lot. 40K7070
- Low fat milk
- 1xPBS, Gibco, Cat# 14190

2.2. Methods

2.2.1. In vivo experimental methods

2.2.1.1. Efficacy of Dexamethasone and Tiotropium in model of OVA-induced asthma relapse in BALB/c mice

Recovered BALB/c mice from the OVA-induced acute asthma model were used for these experiments. Recovered mice have developed acute allergic asthma after sensitization and aerosol challenge with OVA and then they were left for 3 months for recovery (see description bellow).

Generation of mice recovered for acute allergic asthma

All mice, except those from the naive control group, were sensitized with OVA by i.p. injection of 10 µg of OVA in 0.2 ml 1xPBS, on days 0 and 21 and then aerosol-challenged with 1% (wt/vol) OVA in PBS on days 28 and 29 (acute asthma model in BALB/c mice) for 60 minutes, twice a day for two consecutive days and then left for three months for recovery.

Experimental design for testing Tiotropium efficacy

Table 2. Overview of the treatment with Tiotropium bromide

Mouse	Boost (day 125)	Treatment (days 132-136)			Challenge (days 135 and 136)	Assesment Day 139
		Subst.	Dose	Route		
Naive mice	-	-	-	-	-	-
Dexamethasone (standard substance)	10 µg of OVA / 0.2 ml PBS i.p.	Dexamethasone	1 mg/kg, 2x/day	i.n.	Aerosol 1% (wt/vol) in PBS 2x/day.	Blood, BAL fluid, lungs
Tiotropium bromide		Tiotropium bromide	1mg/kg, 2x/day	i.n.		
PBS (asthmatic mice)		PBS	-	i.n.		

After three months of recovery, blood from recovered BALB/c mice was taken for ELISA for OVA-specific IgG1 antibody determination. Only mice with elevated titers of OVA-specific IgG1 (titer from 1: 312500 minimum) were included in the experiments to decrease the variability in the results due to excluding non responder mice.

The responder mice received (day 125) an OVA-Boost of 10µg OVA/0.2 ml PBS intraperitoneal. These three groups were (on days 132, 133, 134, 135 and 136) treated intranasal, one group with 1 mg/kg Dexamethasone (as standard group), the second group with the tested substance Tiotropium, 1 mg/kg and the third group with PBS, twice a day. On the last two days (days 135 and 136), all treated mice were aerosol-challenged with 1% (wt/vol) OVA in PBS, than recovered for one hour and treated intranasal with Dexamethasone, Tiotropium or PBS.
72 hours after the last treatment, all mice were assessed.

2.2.1.2. Efficacy of AAC in model of OVA-induced asthma relapse in BALB/c mice

Table 3. Overview of the treatment with AAC

Mouse	Boost (day 125)	Treatment (days 132-136)			Challenge (days 135 and 136)	Assesment Day 139
		Subst.	Dose	Route		
Naive mice	-	-	-	-	-	-
Dexamethasone (standard substance)	10 µg of OVA / 0.2 ml PBS i.p.	Dexamethasone	1 mg/kg, 2x/day	i.n.	Aerosol 1% (wt/vol) in PBS 2x/day.	Blood, BAL fluid, lungs
AAC		AAC	10mg/kg, 2x/day	i.n.		
PBS (asthmatic mice)		PBS	-	i.n.		

This experiment was done also on recovered mice, generated as described in previous chapter. After three months of recovery, blood from recovered BALB/c mice was taken for ELISA for OVA-specific IgG1 antibody detection. Only BALB/c mice with elevated titers of OVA-specific IgG1 (titer 1: 312500 minimum) were included. These BALB/c mice with elevated

titers of Ova-specific IgG1 were used (for the standard group and the AAC-tested group of the mice) to boost the OVA-specific IgG1 level with OVA. To decrease the variability in the experiments only mice with elevated levels of OVA-specific IgG1 were used.

The standard Dexamethasone-treated group, the AAC-treated and the PBS-treated group of the mice were given an OVA-Boost of 10µg OVA/0.2 ml PBS intraperitoneal (on day 125). These groups were treated intranasally twice a day on days 132, 133, 134, 135 and 136 with 1 mg/kg Dexamethasone, AAC 10 mg/kg or PBS, respectively. On the last two days (days 135 and 136), the mice were aerosol-challenged with 1% (wt/vol) OVA in PBS.
72 hours after the last treatment, the mice were assessed.

2.2.1.3. Experimental protocol for asthma model with intranasal (i.n.) administration of OVA or milk in BALB/c or in B6 mice

Table 4. Overview of the acute asthma model induced by intranasal administration of allergens in BALB/c and B6 mice

Mouse group	Σ	Taking blood from tail for OVA/milk specific IgG1 (day 0)	Challenge (days 0, 2, 4, 14, 16, 18)	Assessment Day 21
BALB/c OVA	5	From all mice on day 0 before starting the sensitization	50 µg of OVA in 50 µl 1x PBS	Blood, BAL fluid, lungs
BALB/c milk	5		50 µg of milk in 50 µl 1x PBS	
B6 OVA	5		50 µg of OVA in 50 µl 1x PBS	
B6 milk	5		50 µg of milk in 50 µl 1x PBS	

On the first day of the experiment (day 0) the mice were anesthetized and blood was collected from the tail of each mouse to obtain sera as naive control for OVA- or milk specific IgG1-titer. After blood sampling either OVA or milk (depending on the study group) was administered intranasal to the still anaesthetized mice. The dose of OVA or milk was 50µg/50µl 1xPBS per mouse. The challenge with milk or OVA was done on experimental days 0, 2, 4, 14, 16 and 18. The mice were assessed after 72 hours after last OVA or milk challenge.

2.3. Sampling procedures

2.3.1. Collection of sera

The blood of the mice was collected twice: the first time before starting the ongoing experiment by extracting a few drops of blood from the tail vein and at the end of the experiment by heart puncture taking 1 ml of blood from each mouse. For blood collection from the tail vein, each tail was briefly (~ 25 sec.) heated by holding it against an infrared lamp. For blood collection by cardial puncture, every mouse was anesthetized until loss of all reflexes occurred.

For cardiac punction a 10 ml syringe with a 0, 6 µl needle was used to extract blood. The needle was attached to the second upper thorax rib; a vacuum was induced by pulling the stem of the syringe and by inserting a needle into the tissue. The syringe was slowly moved to find a heart ventricle. When the needle reached the heart-ventricle a slow blood flow was visible. The collected blood, kept at room temperature, was centrifuged at 13.000 rpm for 8 minutes to obtain the sera.

The sera were stored at -20°C until used in the ELISA for OVA- or milk-specific IgG1.

2.3.2. Collection of bronchoalveolar lavage fluid (BAL) fluid

After blood sampling by cardial punction, a tracheotomy was performed and a tracheal catheter (a pediatric i.v. catheter) was clamped into the trachea, to harvest bronchoalveolar fluid (BAL). The lungs were filled first with 0.4 ml of 1 x PBS and the fluid collected in an Eppendorf tube. The procedure was repeated for another two times using 0.3 ml of 1 x PBS each, for a total end volume of 1 ml of BAL fluid.

The BAL fluid sample has to be used freshly. It was harvested the same day; a small amount of the BAL fluid was used to determine the total cell number and the total leukocyte cell number including their viability by using a Neubauer´s Chamber. For counting total cell number a Zeiss microscope and the 10x magnification was used. For viability analysis the leucocytes were stained with Tryphan blue. After counting the total cell number and the viability analysis, the BAL fluid was applied for preparation of cytospin slides used for total cell countings of macrophages, eosinophils, neutrophils and lymphocyte cells.

2.3.3. Collection of lungs

After the bronchoalveolar lavage fluid was collected from the lungs, the lungs of the mice were excised, put in a preparation cassette and then fixed with 4% PFA. After preparation, the lungs were embedded in paraffin.

2.4. Sample preparation of the Bronchoalveolar lavage fluid (BALF) and lungs

2.4.1. Preparation of cytospin slides

The cytospin slides were prepared using glass slides. Approximately 200 µl of bronchoalveolar lavage fluid (BAL fluid) were used for every cytospin slide to reach an aproximal amount of 10^5 cells per slide. For the centrifugation a Cytospin 4 (Thermo Shandon) centrifuge at 800 rpm for 3 min was used. The cytospin slides were then air dried and stained with KWIK DIFF Thermo Shandon to determine differential cell counts.

2.4.2. Preparation of paraffin sections of the lungs

The paraffin-embedded lungs were stored for 3 hours, or over night at -20°. The freezed lung samples were cut in 3 µm paraffin sections with a Microm HM400. The samples were cut from the middle of the lungs, because there are the most bronchi to be localized. The lung sections were attached to glass slides and air-dried over night.
The paraffin-embedded lung sections of 3 µm were then stained with:

- Hematoxilin and Eosin (H&E) for morphological evaluation
- Periodic-acid-Shiff stain (PAS) for mucopolyssacharide staining, which reflects mucus production by respiratory epithelial goblet cells.
- LUNA staining to grade the eosinophilic infiltration

2.5. Staining techniques

2.5.1. Staining of BAL cells with Trypan blue for cell viability analysis

For viability analysis mainly a concentration of 1:1 (BAL fluid: Trypan blue) was used. For the 1:1 dilution 50 µl of Bronchoalveolar fluid was mixed with 50 µl of Trypan blue. The stained fluid was used immediately after mixing, because Trypan blue stains the leucocytes but kills them as well. For cell counting a Neubauer´s chamber was used on the microscope with a 10x magnification. The cell number should be as high as possible to count them easily.

If the concentration of the leucocytes is too high, a concentration of 1:3 dilution (25 µl bronchoalveolar lavage fluid + 75 µl of Trypan blue) or 1:10 dilution (10 µl Bronchoalveolar lavage fluid + 90 µl Tryphan blue) was used.

Calculation of the total cell number:

$$\text{Number of counted cells} \times \text{Dilution factor} \times 10^4$$

2.5.2. Staining of cytospin slides (BAL fluid) with Thermo Shandon KWIK DIFF for differential cell counting

The aim of the staining method is to stain the cells from bronchoalveolar lavage fluid (BALF) for differential cell counting. First the slides were dipped ten times, slowly (one dip per a second) in solution 1, then ten times in solution 2, then ten times in solution 3 and then washed in distilled water with three dips. After washing the slides were air-dried. After drying the slides were used for differential cell counting,

2.5.3. Staining of lung sections with Hematoxilin and Eosin (H&E)

The aim of the staining of the 3-µm-thick lung sections is the analysis of cell infiltrates around blood vessels, airways and in the parenchyma. The slides with the 3-µl-thin paraffin sections were placed at 65°C for at least 40 min to remove the paraffin through melting. After heating, the lung samples were placed in Xylene for 2x10 min to remove the melted paraffin from the sample.

After the removal of paraffin, the slides were placed in alcohol gradients of 95% for 1 minute, 70% for 1 minute and 50% for 1 minute to rehydrate the samples. After rehydratation, the lung samples were incubated in Hematoxilin (Sigma Aldrich Hematoxilin solution, Lot#106K4352) for 5 minutes. After the incubations time the color of the lung tissue should be deep purple. The slides were then washed in tap water and incubated for 3 to 6 seconds in 1% (v/v) HCl water; this removes the Hematoxilin excess from the tissue.

The Eosin is dissolved in 90% Ethanol. Therefore, the tissue should be dehydrated in an ethanol gradient of 50% for 30 seconds and 70% for 30 seconds and incubated in Eosin Y for 30 seconds. The tissues were to be dehydrated again for better conservation of the samples. The dehydratations steps are the following: 50% ethanol for 30 seconds, 70% ethanol for 30 seconds and 95% ethanol for 30 seconds, then air dried and covered with mounting media and cover slips.

2.5.4. PAS staining for paraffin tissue

3-µm thick sample cuts were deparaffinized and rehydrated as described for H&E staining. After rehydratation, the lung samples were incubated in 0,5% (v/v) periodic acid for 10 minutes, then washed in distilled water and put in Schiff´s reagent for 30 minutes at 60°C. After 30 min, the slides were expected to be purple. After staining, the slides were incubated in freshly prepared SO_2-water for 6 minutes, which fixes the stain. The slides were then washed in tepid hot water, for 15 minutes and placed in Papanicolaus solution 1a Harris Hematoxilin for 19 seconds. After washing, the slides pass the dehydratation steps with ethanol gradients of 50% ethanol, 70% ethanol and 95% ethanol, each step for 5 minutes. After the dehydratation, the slides were incubated in n-butyl-acetate for 5 minutes, then air-dried and covered, using mounting media and glass cover slips.

2.5.5. LUNA staining for eosinophils

3-µm thick sample cuts were deparaffinized and rehydrated as described for H&E staining. After deparaffinisation and rehydratation, the slides were incubated for 7.5 min in working solution C: 5 ml Solution B: (0.5 g Biebrich Scarlet + 50 ml dH_2O) added to each 45 ml of Solution A: (1g Haematoxilin + 100 ml 95% ethanol). After the working solution, the slides

were three times (fast) dipped in 1% acid alcohol solution (300 ml of 70% ethanol + 3 ml HCL), then the slides were washed under running water (with racks in glass chamber). After washing the slides were dipped 3-5 times in lithium carbonate (0.5 g lithium carbonate solved in 500 ml of dH_2O) until the color of the slides change from pink to blue, then the slides were washed under running water. After washing, the slides pass the dehydratation steps with ethanol gradients of 50% ethanol, 70% ethanol and 95% ethanol, each step for 5 minutes, air-dried and covered, using mounting media and glass cover slips.

2.5.6. Scoring system description for Luna, H&E and PAS

H&E:

The extent of inflammation in lung sections was graded using following scoring system:

- 0: no inflammation
- 1: if the inflammation reaches the central airway area
- 2: if the inflammation is visible on the middle airways
- 3: if the inflammation is extended to the periphery airways

Luna:

For Luna stained slides all eosinophils in whole tissue were counted.

PAS:

The mucus hyperproduction in the central airways on one lung tissue section was graded by estimating the percentage of mucus-producing cells in central airways. Following score were used:

- 0: no mucus producing cells in the airways
- 1: 0 – 20% of cells produce mucus
- 2: 21 – 40% of cells produce mucus
- 3: 41 – 60% of cells produce mucus
- 4: 61 – 80% of cells produce mucus
- 5: 81 – 100% of cells produce mucus

2.6. ELISA

2.6.1. ELISA for OVA-specific IgG1

The 96 well ELISA plates were coated with 50µl OVA solution per well (10µl/ml OVA solution = 0,5 µg OVA/50µl 1xPBS/well), put onto a rotating plate for 10 minutes, to remove bubbles from the wells and then incubated over night at 4°C for coating.

On the second day the OVA solution for coating was discarded, the plates washed three times with washing buffer (200µl/well). After washing, 200µl blocking buffer was added in each well to block the uncoated places in the wells and incubated for 2 hours at room temperature. Sera were diluted freshly in 1% BSA/PBS for the ELISA (in most cases a 1:500 dilution was used). After incubation, the ELISA plates were washed three times with 200µl washing buffer per well and the sera were pipetted on the following order: first 80µl of carrier buffer in each row, except the first row. The first row was leaved empty. To the first row 100 µl of the 1:500 diluted sera were added in duplicates. Then 20µl of the sera dilution from the first row was transferred into the second row and mixed. This titration process was continued until the penultimate row. The remaining 20µl of sera dilution after titration was discarded, because in the last row sera were committed, hence containing only carrying buffer. The plates were then incubated over night at 4°C.

On the third day the sample solution was discarded and the plates washed with 200µl washing buffer/well. After washing 100µl/well secondary antibody were added and incubated for two hours at 4°C. After incubation, the secondary antibody was discarded and washed with 200µl washing buffer per well. After washing 100µl of Streptavidin-HRP were added to each well, incubated for one hour at room temperature, then washed three times with washing buffer and 100µl of TBM were added to each well. After 10 minutes of TMB incubation, 100µl of 0.18 M H_2SO_4 stop solution were added to stop the reaction and the plates were analyzed with Spectra Max M5 max Pro 4.8.

2.6.2. ELISA for milk-specific IgG1

The 96 well ELISA plates were coated with 50µl milk solution per well (10 µl/ml milk solution = 0,5 µg milk/50µl 1xPBS/well), put for 10 minutes onto a rotating plate, to remove bubbles from the wells and then incubated over night at 4°C for coating.

On the second day the milk solution for coating was discarded, the plates washed three times with washing buffer (200µl/well). After washing, 200µl blocking buffer was added in each well to block the uncoated places in the wells and incubated for 2 hours at room temperature. Sera were diluted freshly in 1% BSA/1xPBS for the ELISA (in most cases a 1:500 dilution was used). After incubation, the ELISA plates were washed three times with 200µl washing buffer per well and the sera were pipetted on the following order: first 80µl of carrier buffer in each row, except the first row. The first row was left empty. To the first row 100 µl of the 1:500 diluted sera were added in duplicates. Then 20µl of the sera dilution from the first row was transferred into the second row and mixed. This titration process was continued until the penultimate row. The remaining 20µl of sera dilution after titration was discarded, because in the last row sera were committed, hence containing only carrying buffer. The plates were then incubated over night at 4°C.

On the third day the sample solution was discarded and the plates washed with 200µl washing buffer/well. After washing 100µl/well secondary antibody were added and incubated for two hours at 4°C. After incubation, the secondary antibody was discarded and washed with 200µl washing buffer per well. After washing 100µl of Streptavidin-HRP were added to each well, incubated for one hour at room temperature, then washed three times with washing buffer and 100µl of TBM were added to each well. After 10 minutes of TMB incubation, 100µl of 0.18 M H_2SO_4 stop solution were added to stop the reaction and the plates were analyzed with Spectra Max M5 max Pro 4.8.

2.7. Statistical analysis

Data are expressed as means ± SEM. The program used for statistical analysis was GraphPad Prism5. Statistics were done by Kruskal-Wallis Test followed by Dunn´s multiple comparison test, with a significance of $p = < 0,05$.

Chapter 3

3. Results

3.1. Effect of Tiotropium Bromide treatment

3.1.1. Effect of Tiotropium Bromide treatment on inflammatory cells in bronchoalveolar lavage fluid (BALF)

The bronchoalveolar lavage fluid of naive mice is defined by a high percentage of macrophages (> 80%) (Fig.3. page 61), no eosinophils (0 %) (Fig.5. page 62.), neutrophils (17%) (Fig.7. page 62) and lymphocytes (~ 1 %) (Fig.9. page 63). The bronchoalveolar lavage fluid of the asthmatic mice (PBS group) is characterized by a lower percentage of macrophages (> 60 %) (Fig.3. page 61), a highest possible percentage of eosinophils (40%) (Fig.5. page 62), a low percentage of neutrophils (5%) (Fig.7. page 62) and lymphocytes (4%) (Fig.9. page 63). The bronchoalveolar lavage fluid of mice treated with the standard substance Dexamethasone is characterized by a higher percentage of macrophages than in PBS-treated mice (~ 90%) (Fig.3. page 61), a low percentage of eosinophils (5%) (Fig.5. page 62), a low percentage of neutrophils (5%) (Fig.7. page 62), and a low percentage of lymphocytes (2%) (Fig.9. page 63). The bronchoalveolar lavage fluid of mice treated with the test substance Tiotropium is characterized by a higher percentage of macrophages than in PBS-treated mice (~ 90%) (Fig.3. page 61), a low percentage of eosinophils (8%) (Fig.5. page 62), a low percentage of neutrophils (5%) (Fig.7. page 62), and a low percentage of lymphocytes (1%) (Fig.9. page 63).

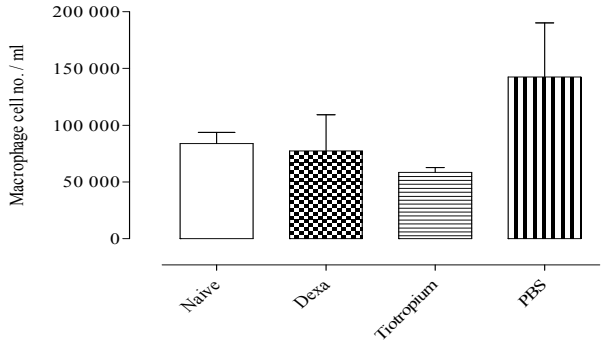


Fig.2. Cell number of macrophages in BAL fluid of BALB/c mice treated with Tiotropium bromide and Dexamethasone in comparison to untreated (naive and PBS) BALB/c mice. Data are expressed as means ± SEM.

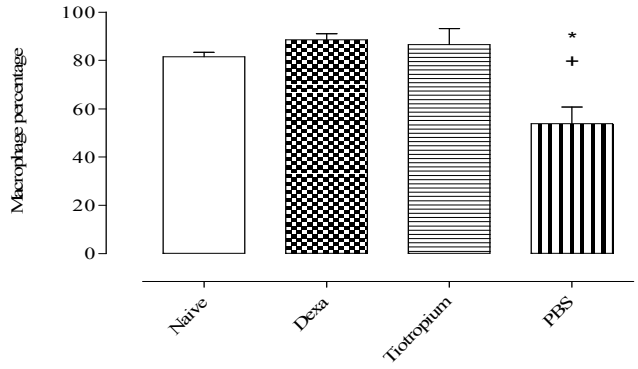


Fig.3. Percentage of macrophages in BAL fluid of BALB/c mice treated with Tiotropium bromide and Dexamethasone in comparison to untreated (naive and PBS) BALB/c mice (Star (*) designates the significant difference between Tio vs. PBS*, plus ($^{+}$) between Dexa vs.PBS^{+} groups). Data are expressed as means ± SEM. Statistics by Kruskal-Wallis Test followed by Dunn´s multiple comparison test, with a significance of p = < 0,05.

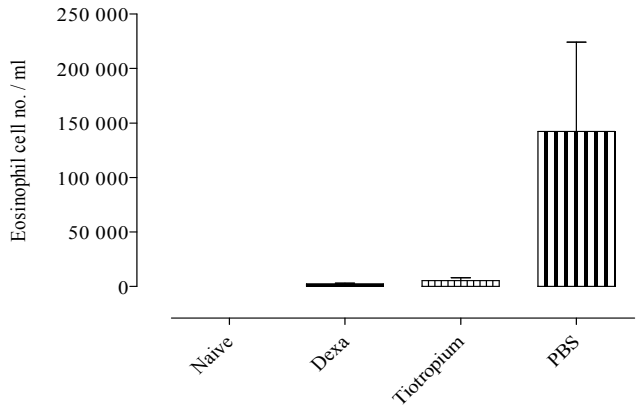


Fig.4. Cell number of eosinophils in BAL fluid of BALB/c mice treated with Tiotropium bromide and Dexamethasone in comparison to untreated (naive and PBS) BALB/c mice. Data are expressed as means ± SEM.

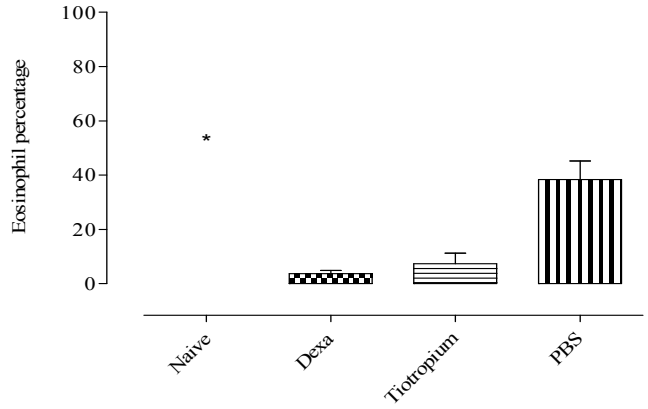


Fig.5. Percentage of eosinophils in BAL fluid of BALB/c mice treated with Tiotropium bromide and Dexamethasone in comparison to untreated (naive and PBS) BALB/c mice (Star (*) designates the significant difference between Naïve vs. PBS* groups). Data are expressed as means ± SEM. Statistics by Kruskal-Wallis Test followed by Dunn´s multiple comparison test, with a significance of p = < 0,05.

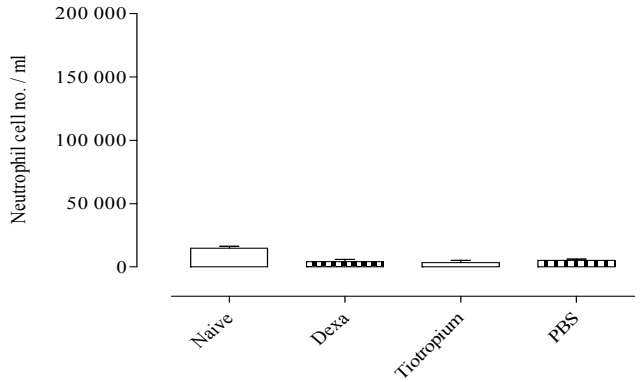


Fig.6. Cell number of neutrophils in BAL fluid of BALB/c mice treated with Tiotropium bromide and Dexamethasone compared to untreated (naive and PBS) BALB/c mice. Data are expressed as means ± SEM.

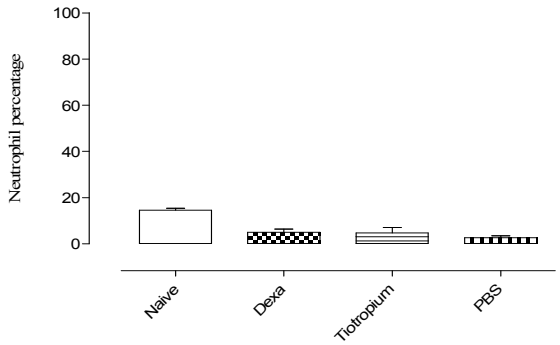


Fig.7. Percentage of neutrophils of BALB/c mice treated with Tiotropium bromide and Dexamethasone compared to untreated (naive and PBS) BALB/c mice. Data are expressed as means ± SEM.

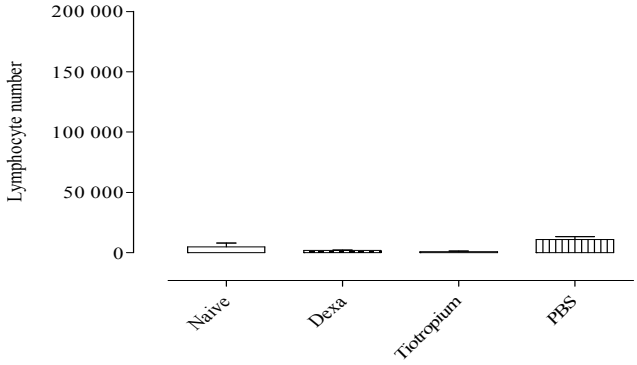


Fig.8.Cell number of lymphocytes/ lung of BALB/c mice treated with Tiotropium bromide and Dexamethasone in comparison to untreated (naive and PBS) BALB/c mice. Data are expressed as means ± SEM.

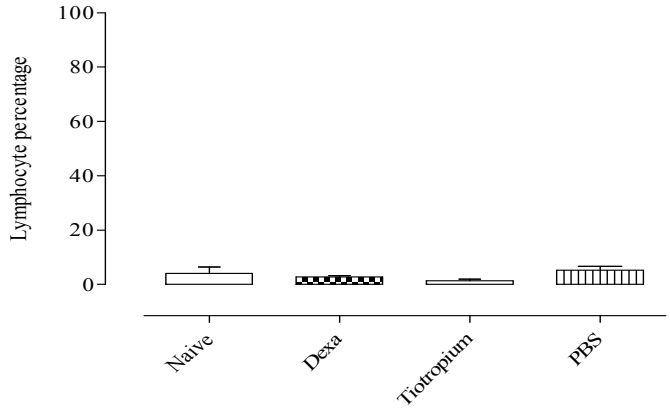


Fig.9. Percentage of lymphocytes/ lung of BALB/c mice treated with Tiotropium bromide and Dexamethasone in comparison to untreated (naive and PBS) BALB/c mice. Data are expressed as means ± SEM.

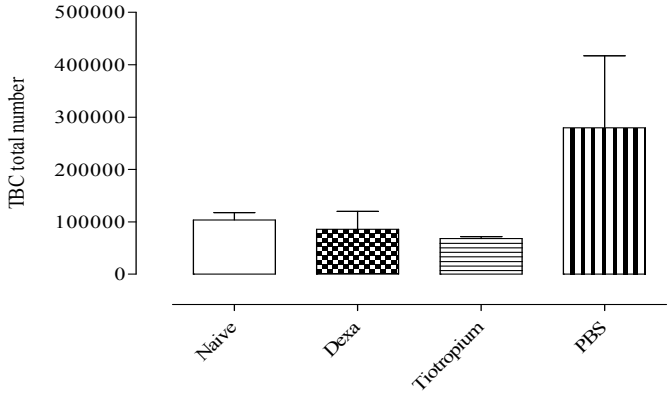
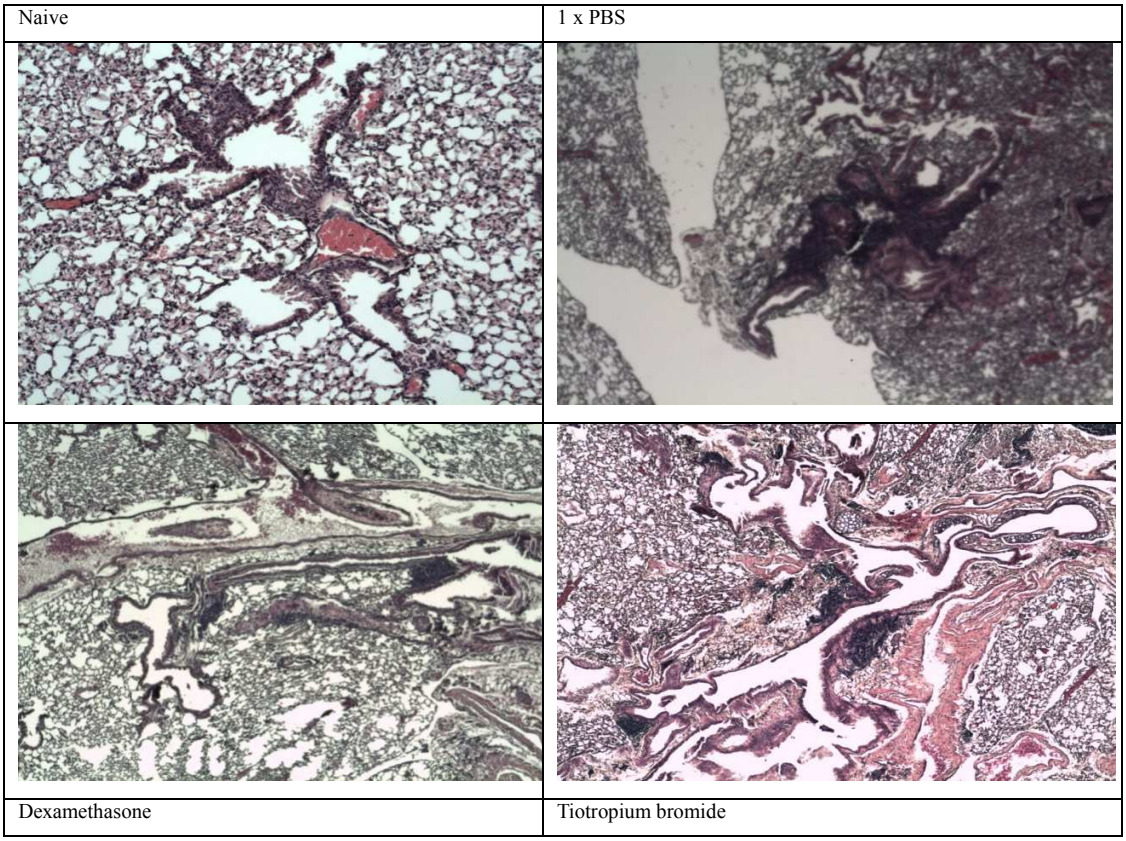


Fig.10. TBC total number in BAL fluid/lung of BALB/c mice treated with Tiotropium bromide and Dexamethasone in comparison to untreated (naive and PBS) BALB/c mice. Data are expressed as means ± SEM.

3.1.2. Effect of Tiotropium Bromide treatment on inflammation of the lung tissue

In the naive group, the histological staining methods applied to the lungs, as expected, failed to reveal signs of asthma. This is not the case in the other three groups of mice: the asthmatic group (PBS), the standard substance group (Dexamethasone) and the testing substance group (Tiotropium). The lung inflammation could not be detected in naïve mice. In contrast, strong infiltration of inflammatory cells was detected in the group of the asthmatic mice (PBS). Both Dexamethasone and Tiotropium markedly, albeit not significantly suppressed lung inflammation, compared to the PBS-treated group (**H&E**: Fig.11. page 65). In order to enumerate infiltrating eosinophils, slides were stained with Luna. As expected in naive mice only rare eosinophils were detected, while in the group of the asthmatic mice (PBS) numerous eosinophil were counted on the lung slices. As with lung inflammation, both treated groups had markedly, albeit not significantly decreased eosinophil number in lungs (**Luna**: Fig.12. page 66). The mucus production in the lungs varied within the groups. Most mucus was produced in the lungs of the asthmatic mice, followed by the group receiving the test substance (Tiotropium), followed by a non significant difference by the group treated with the standard substance (Dexamethasone). The group of the naive mice showed no visible mucus production (**PAS:** Fig.13. page 67).

A



B

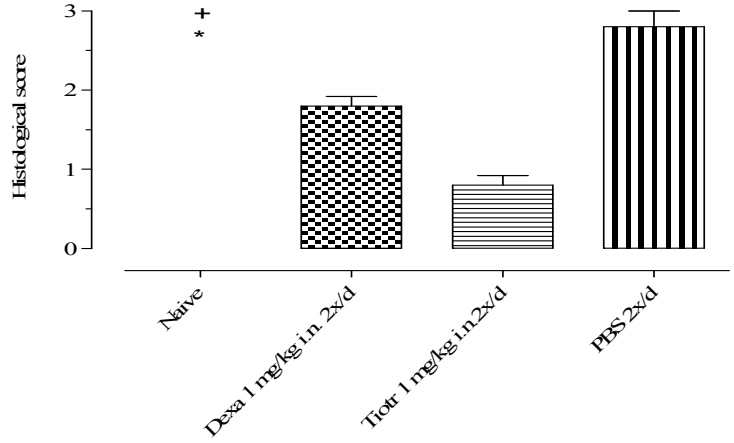
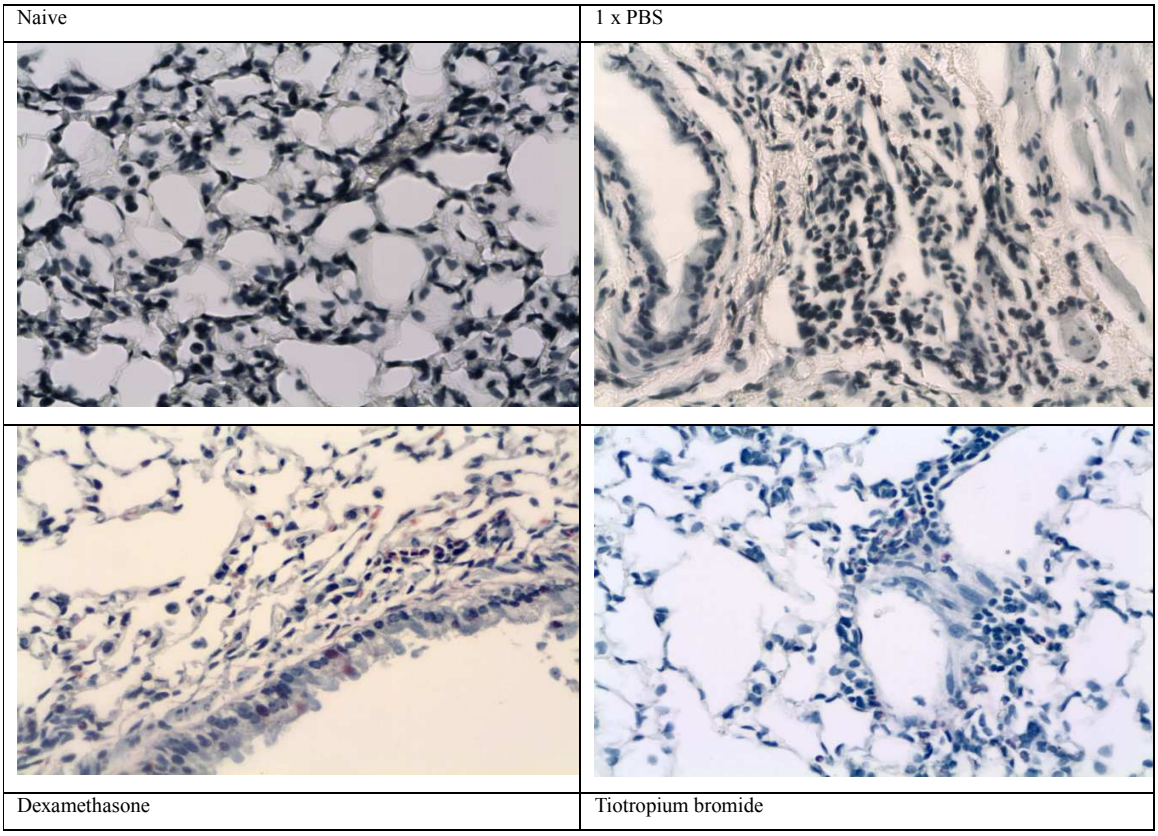


Fig.11. H&E: A. Representative photomicrographs of lung tissue sections of BALB/c mice with infiltrations of inflammatory cells in the lungs (original magnification x40) from healthy mice or mice sensitized and challenged with OVA, and treated with PBS, Dexamethasone or Tiotropium bromide, as indicated. **B**. Scores for lung inflammation in healthy control mice, or mice treated with PBS, Dexamethsone or Tiotropium bromide. Values are expressed as means ± SEM. Statistics by Kruskal-Wallis Test followed by Dunn´s multiple comparison test, significance of $p = < 0,05$. (Star (*) designates the significant difference between Naïve vs. PBS*, and plus ($^{+}$) between Tiotropium vs. PBS^{+} groups).

A



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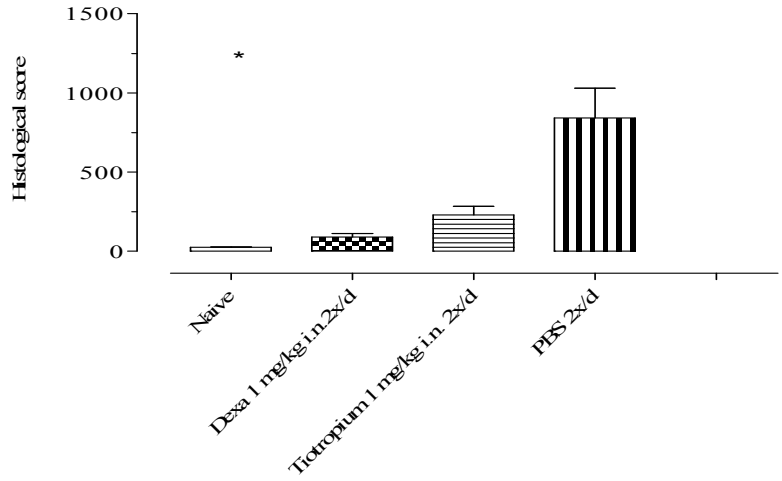
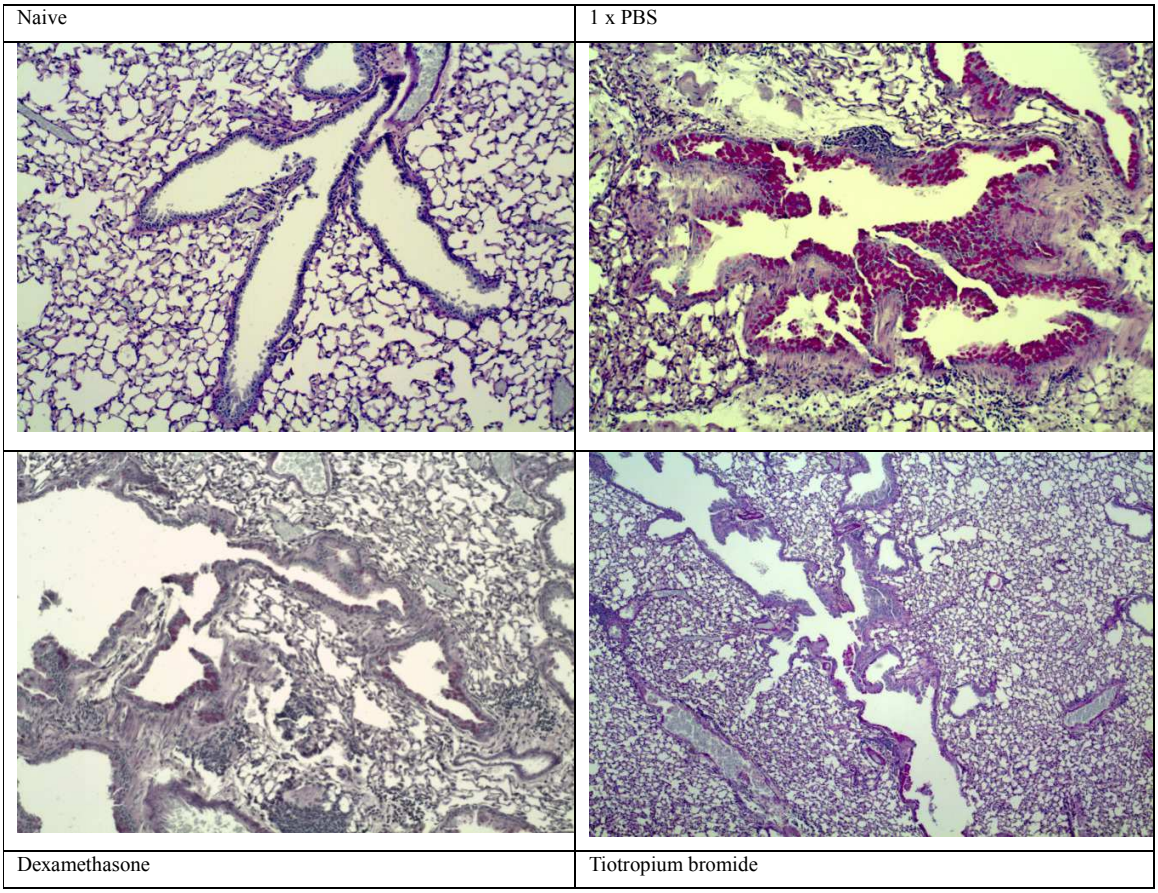


Fig.12. Luna: A. Representative photomicrographs of lungs tissue section of BALB/c mice with eosinophilic infiltration in the lungs (original magnification x400) from healthy mice or mice sensitized and challenged with OVA, and treated with PBS, Dexamethasone or Tiotropium bromide, as indicated. **B**. Scores for lung inflammation in healthy control mice, or mice treated with PBS, Dexamethsone or Tiotropium bromide. Values are expressed as means ± SEM. Statistics by Kruskal-Wallis Test followed by Dunn´s multiple comparison test, significance of p = < 0,05. (Star (*) designates the significant difference between Naïve vs. PBS* groups).

A



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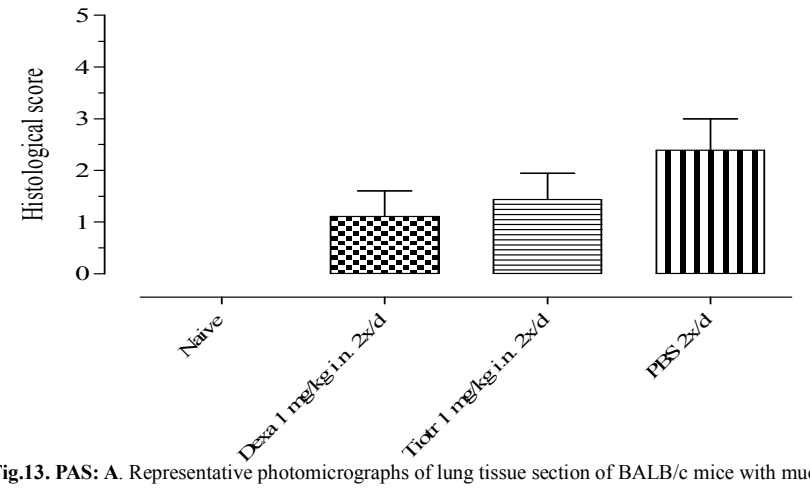


Fig.13. PAS: A. Representative photomicrographs of lung tissue section of BALB/c mice with mucus hypersecretion (original magnification x200) from healthy mice or mice sensitized and challenged with OVA, and treated with PBS, Dexamethasone or Tiotropium bromide, as indicated. **B**. Scores for lung inflammation in healthy control mice, or mice treated with PBS, Dexamethsone or Tiotropium bromide. Values are expressed as means ± SEM. Statistics by Kruskal-Wallis Test followed by Dunn´s multiple comparison test, significance of p = < 0,05. (No statistical significance).

3.1.3. Effect of Tiotropium Bromide on the titer of OVA-specific IgG1

Sera from all mice were taken before the start of the experiment and after the boost and treatment to determine the level of the OVA-specific IgG1. As previously described (in the setup of the experiment, page 58), only mice recovered from acute asthma with elevated titers of OVA-specific IgG1 were used, with the aim to decrease the variability within the experiment, excluding mice without asthma (no detectable or very low OVA-specific IgG1). Mice were divided into the groups:naïve, asthmatic (PBS), standard (Dexamethasone) and testing substance (Tiotropium) group. As expected, the sera of the naive mice had no detectable OVA-specific IgG1 antibodies. After boosing with OVA and aerosol challenge all mice had markedly increased OVA-specific IgG1 titer than after recovery (Fig.14. page 68). However, both Dexamethasone and Tiotropium only slightly decreased OVA-specific IgG1 titer in comparison to PBS treated group.

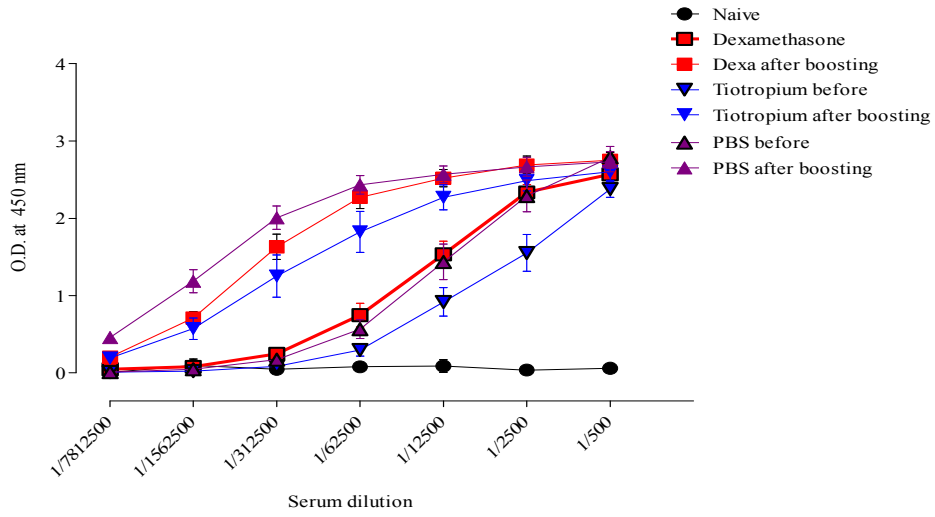


Fig. 14. OVA-specific IgG1 before starting the experiment ("before"-line) , means before challenging the mice with OVA and treatment with Tiotropium and after the experiment (boosting and treatment with AAC – "after"-line).

3.2. Effect of AAC treatment

3.2.1. Effect of AAC treatment on inflammatory cells in bronchoalveolar lavage fluid (BALF)

The bronchoalveolar lavage fluid of naive mice is defined by a high percentage of macrophages (> 80%) (Fig.16. page 70), no eosinophils (0 %) (Fig.18. page 71), a low percentage of neutrophils (18%) (Fig.20. page 71) and lymphocytes (< 3 %) (Fig.22. page 72). The bronchoalveolar lavage fluid of the asthmatic mice (PBS group) is characterized by a lower percentage of macrophages (~ 55 %) (Fig.16. page 70), a significant higher percentage of eosinophils than in naive mice (40%) (Fig.18. page 71), a low percentage of neutrophils (2%) (Fig.20. page 71) and lymphocytes (5%) (Fig.22. page 72). The bronchoalveolar lavage fluid of mice treated with the standard substance Dexamethasone is characterized by a higher percentage of macrophages than in naive mice (~ 90%) (Fig.16. page 70), a low percentage of eosinophils (3%) (Fig.18. page 71), a low percentage of neutrophils (4%) (Fig.20. page 71), and a low percentage of lymphocytes (2%) (Fig.22. page 72). The bronchoalveolar lavage fluid of mice treated with the test substance AAC is characterized by a higher percentage of macrophages than in naive mice (~ 78%) (Fig.16. page 70) but lower than in the treatment with Tiotropium, a low percentage of eosinophils (10%) (Fig.18. page 71) but higher as in the Tiotropium treatment (Fig.5. page 62), a low percentage of neutrophils (6%) (Fig.20. page 71) and a low percentage of lymphocytes (~ 3%) (Fig.22. page 72), which were not significant but higher than in the group treated with Tiotropium (Fig. 9. page 63).

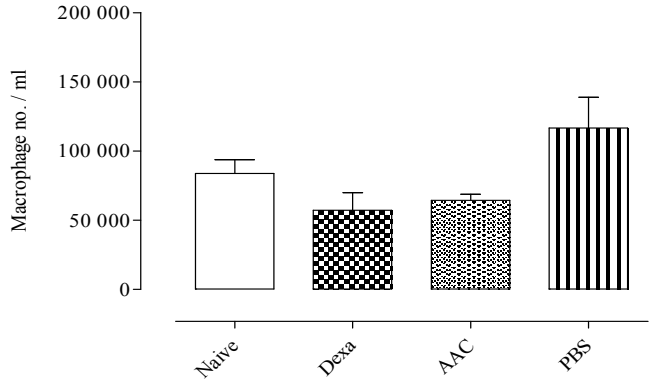


Fig.15. Cell number of macrophages in BAL fluid/lung of BALB/c mice treated with AAC and Dexamethasone in comparison to untreated (naive and PBS) BALB/c mice Data are expressed as means ± SEM.

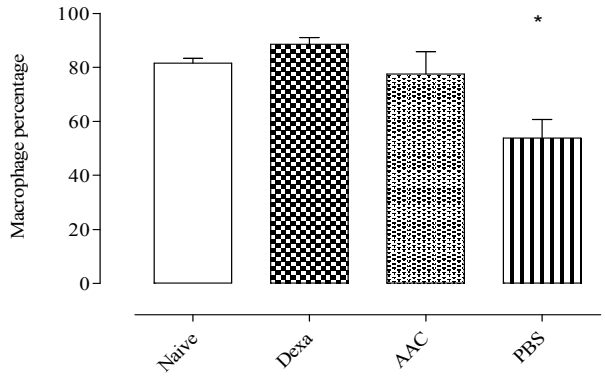


Fig.16. Percentage of macrophages in BAL fluid/lung in BALB/c mice treated with AAC and Dexamethasone in comparison to untreated (naive and PBS) BALB/c mice. (Star (*) designates the significant difference between Naïve vs. PBS* groups). Data are expressed as means ± SEM. Statistics by Kruskal-Wallis Test followed by Dunn´s multiple comparison test, significance of p = < 0,05.

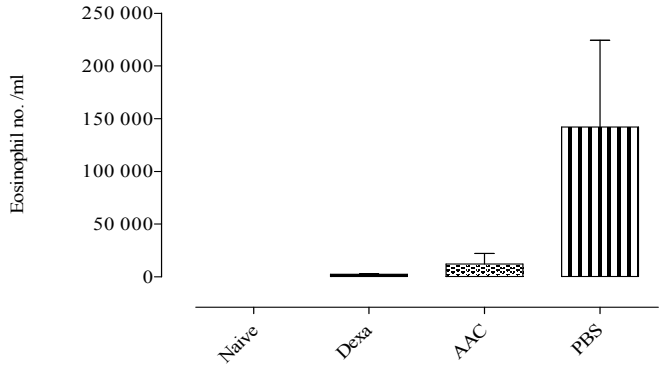


Fig.17. Cell number of eosinophils in BAL fluid/lung in BALB/c mice treated with AAC and Dexamethasone in comparison to untreated (naive and PBS) BALB/c mice. Data are expressed as means ± SEM.

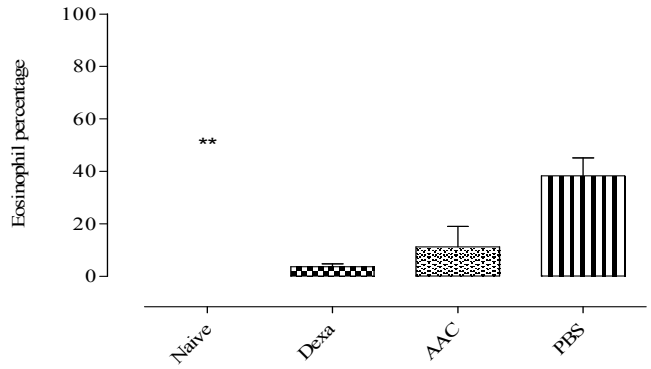


Fig.18.Percntage of eosinophils in BAL fluid/lung of BALB/c mice treated with AAC and Dexamethasone in comparison to untreated (Naive and PBS) BALB/c mice. (Stars (**) designate the significant difference between Naïve vs. PBS** groups). Data are expressed as means ± SEM. Statistics by Kruskal-Wallis Test followed by Dunn´s multiple comparison test, significance of p = < 0,05.

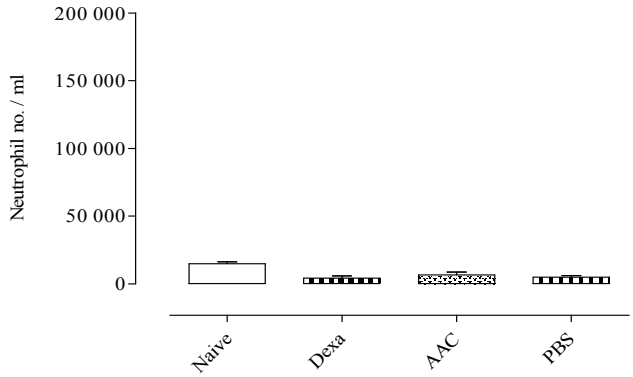


Fig.19. Cell number of neutrophils in BAL fluid per lung in BALB/c mice treated with AAC and Dexamethasone in comparison to untreated (naive and PBS) BALB/c mice. Data are expressed as means ± SEM.

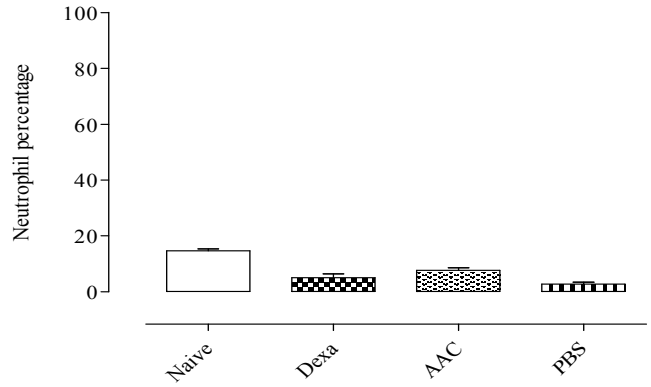


Fig.20. Percentage of neutrophils in BAL fluid of BALB/c mice treated with AAC and Dexamethasone in comparison to untreated (naive and PBS) BALB/c mice. Data are expressed as means ± SEM.

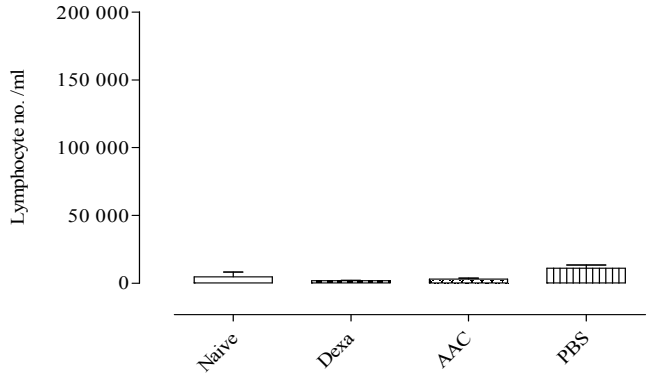


Fig.21. Cell number of lymphocytes in BAL fluid of BALB/c mice treated with AAC and Dexamethasone in comparison to untreated (naive and PBS) BALB/c mice. Data are expressed as means ± SEM.

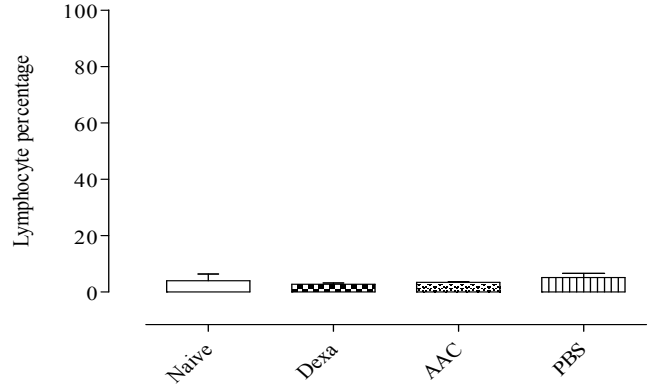


Fig.22. Percentage of lymphocytes in BAL fluid of BALB/c mice treated with AAC and Dexamethasone in comparison to untreated (naive and PBS) BALB/c mice. Data are expressed as means ± SEM.

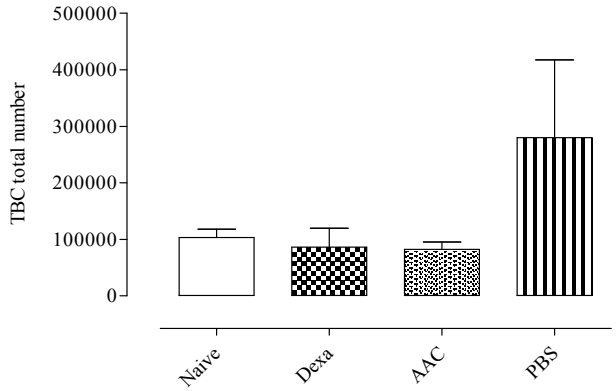
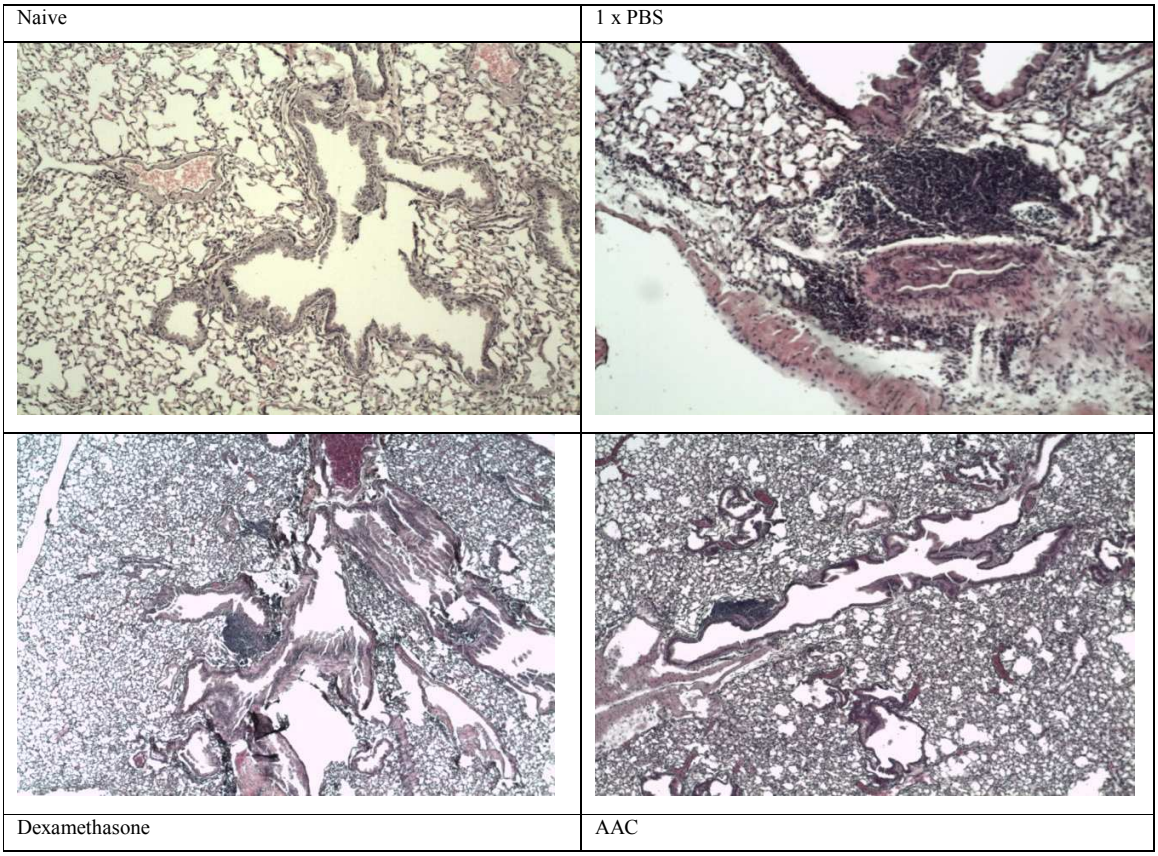


Fig.23. TBC total number in BAL fluid of BALB/c mice treated with AAC and Dexamethasone in comparison to untreated (naive and PBS) BALB/c mice. Data are expressed as means ± SEM.

3.2.2. Effect of AAC treatment on inflammation of the lung tissue

The lungs of the naive animals were, as expected, free of any visible sign of disease by our used histological staining methods to show asthma. This is not the case in the other three groups of mice: the asthmatic group (PBS), the standard substance group (Dexamethasone) and the testing substance group (AAC). The inflammatory cell infiltration **(H&E**: Fig.24. page 74) was absent from naïve mice, but was prominent in lungs of mice with asthma relapse (PBS group). Extent of lung inflammation was decreased in mice treated with standard substance Dexamethasone and to even greater extent in the mice treated with the test substance AAC (**H&E:** Fig.11. page 65). The group of the asthmatic mice (PBS group) had severe eosinophil infiltrations (**Luna**: Fig.25. page 75). The eosinophilic infiltration in the group of the tested substance (ACC), comparable to the Tiotropium treatment (**Luna**: Fig.12. page 66), was slightly higher than in the group of mice treated with the standard substance (Dexamethasone). The naive group of mice showed only little eosinophil infiltration in the parenchyma of the lungs. The most mucus (**PAS**: Fig.26. page 76) was produced in the lungs of asthmatic mice, followed by the group of the standard substance (Dexamethasone) and the group of the tested substance (AAC) (the mucus production was slightly lower than the one by the Tiotropium treatment (**PAS**: Fig.13. page 67). The group of the naive mice showed no visible mucus production.

A



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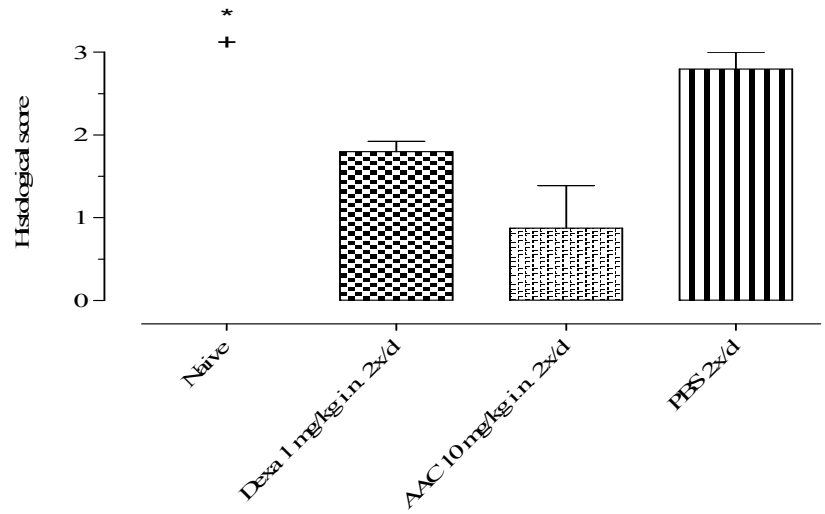
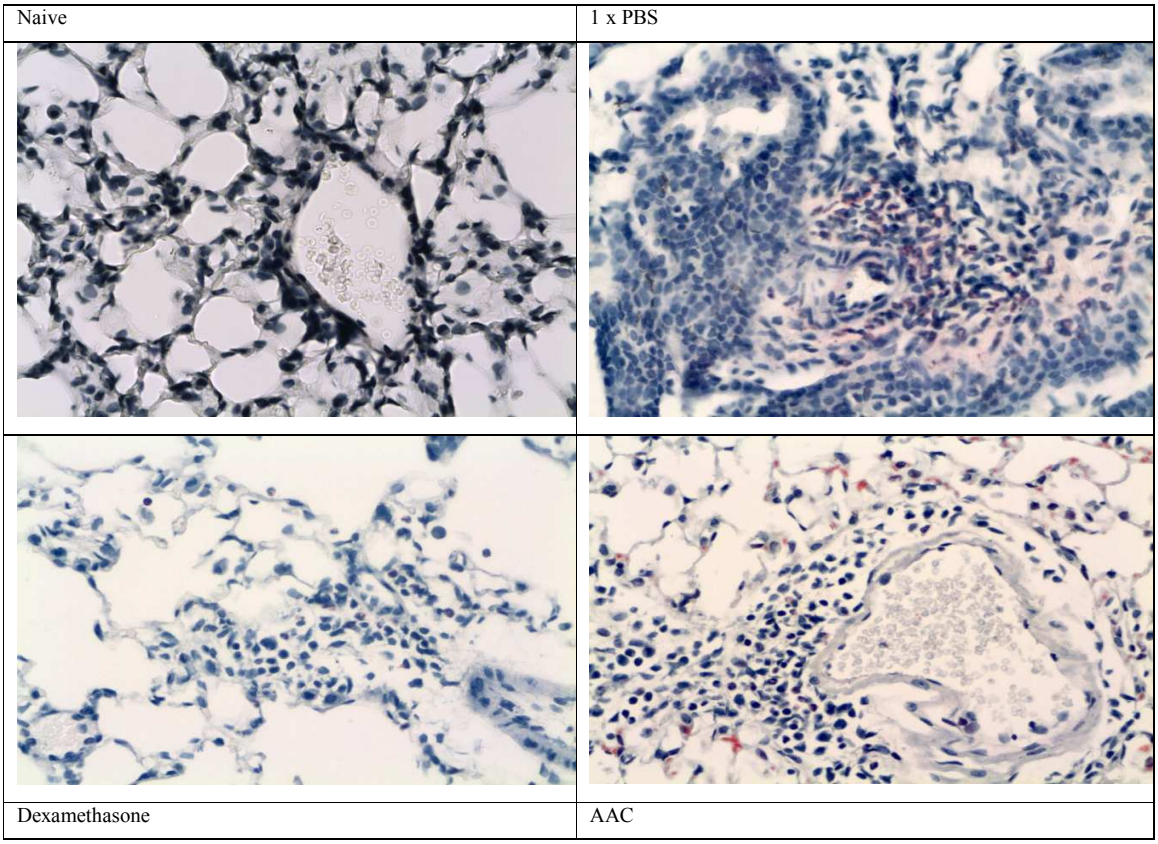


Fig.24. H&E: A. Representative photomicrographs of lung tissue section of BALB/c mice with infiltration of inflammatory cells in the lungs (original magnification x40) from healthy BALB/c mice or BALB/c mice sensitized and challenged with OVA, and treated with PBS, Dexamethasone or AAC, as indicated. **B**. Scores for lung inflammation in healthy control mice, or mice treated with PBS, Dexamethsone or AAC. Values are expressed as means ± SEM. Statistics by Kruskal-Wallis Test followed by Dunn´s multiple comparison test, significance of $p = < 0,05$. (Star (*) designates significant difference between PBS vs. Naïve*, and plus ($^{+}$) between PBS vs. AAC^{+} groups).

A



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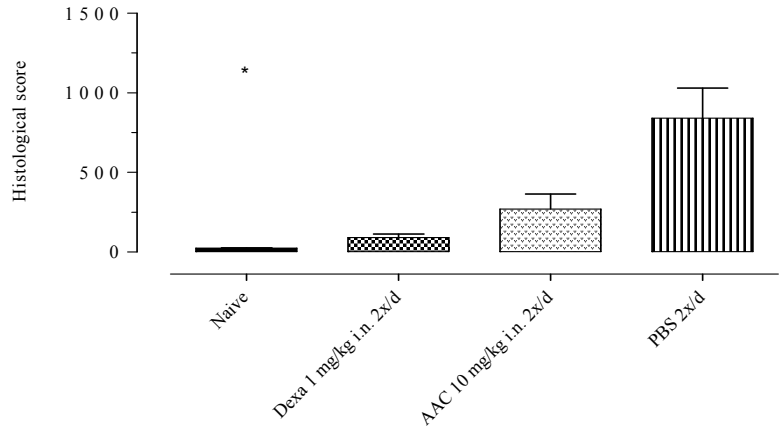
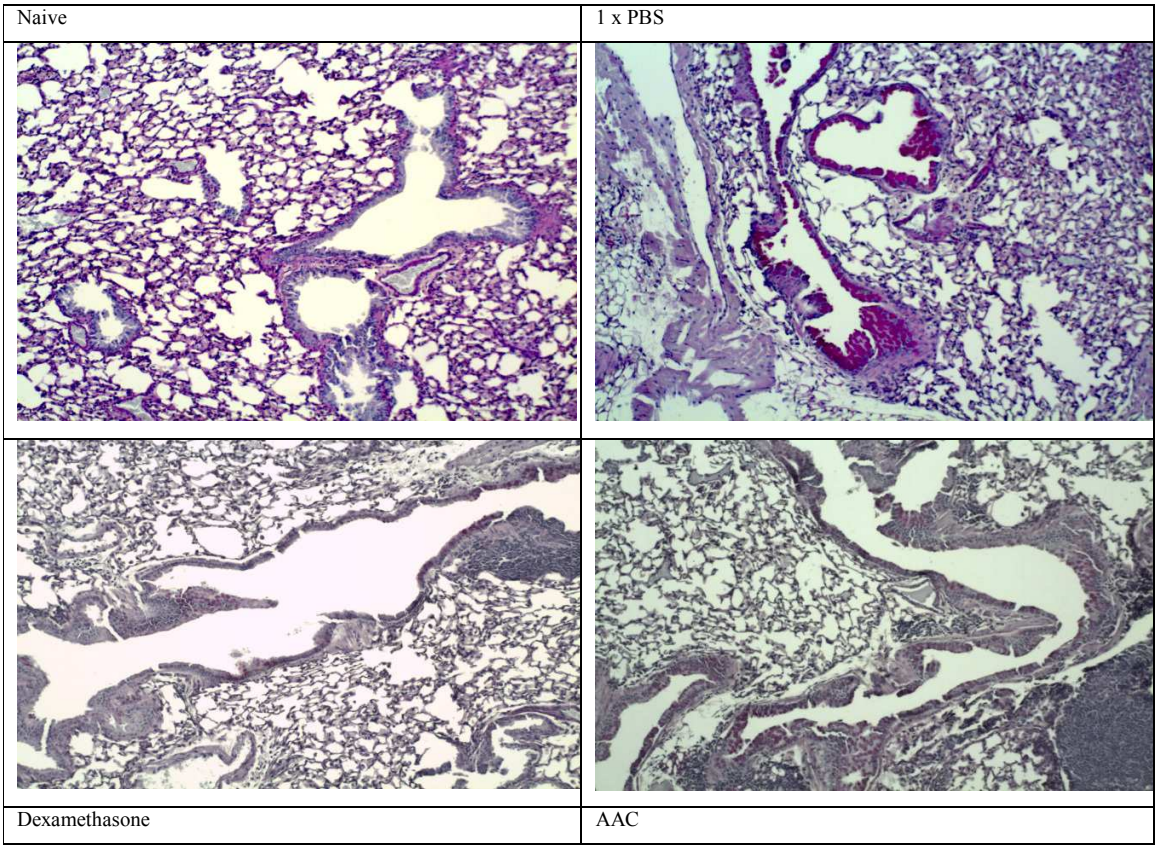


Fig.25. Luna: A. Representative photomicrographs of lungs of BALB/c mice with eosinophilic infiltration in the lungs (original magnification x400) from healthy mice or mice sensitized and challenged with OVA, and treated with PBS, Dexamethasone or AAC, as indicated. **B**. Scores for lung inflammation in healthy control mice, or mice treated with PBS, Dexamethsone or AAC. Values are expressed as means ± SEM. Statistics by Kruskal-Wallis Test followed by Dunn´s multiple comparison test, significance of p = < 0,05. (Star (*) designates significant difference between Naïve vs. PBS* groups).

A



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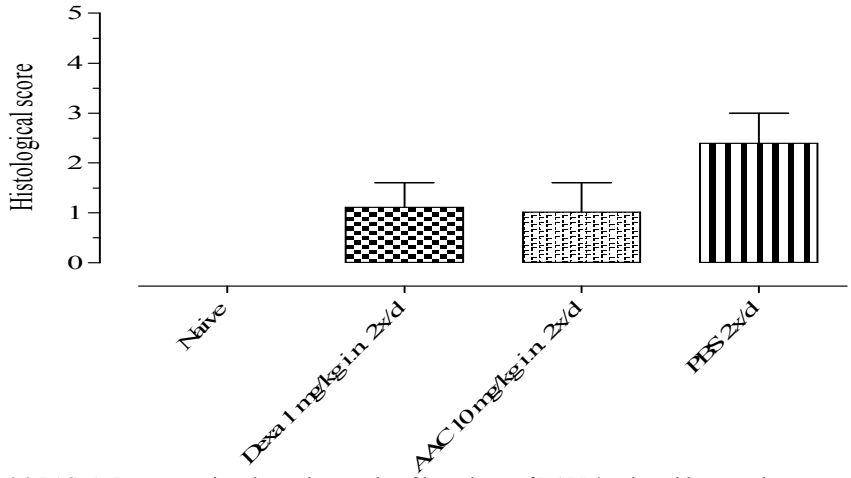


Fig.26. PAS: A. Representative photomicrographs of lung tissue of BALB/c mice with mucus hypersecretion (original magnification x200) from healthy mice or mice sensitized and challenged with OVA, and treated with PBS, Dexamethasone or AAC, as indicated. **B**. Scores for lung inflammation in healthy control mice, or mice treated with PBS, Dexamethsone or AAC. Values are expressed as as means ± SEM. Statistics by Kruskal-Wallis Test followed by Dunn´s multiple comparison test, significance of p = < 0,05. Mucus hypersecretion in lungs of BALB/c mice treated with AAC and Dexamethasone (No statistical significance).

3.2.3. Effect of AAC on the titer of OVA-specific IgG1 Ab

Sera from all mice were taken before the start of the experiment as well as after the boost and treatment to evaluate the level of the OVA-specific IgG1. As previously described only mice recovered from acute asthma but which also had an elevated titer of OVA-specific IgG1 were used. The mice were divided into the groups: naïve, asthmatic (PBS), a standard (Dexamethasone) and a testing substance (AAC) group. As expected, the sera of the naive mice had no detectable OVA-specific antibodies. Mice before allergen boost had moderate titer of OVA-specific IgG1 antibodies, and after the allergen challenge highest OVA-specific IgG1 titer. Neither Dexamethasone (Fig.14. page 68) nor AAC (Fig.27. page 77) treatment did not decreased titer of OVA-specific IgG1 antibodies.

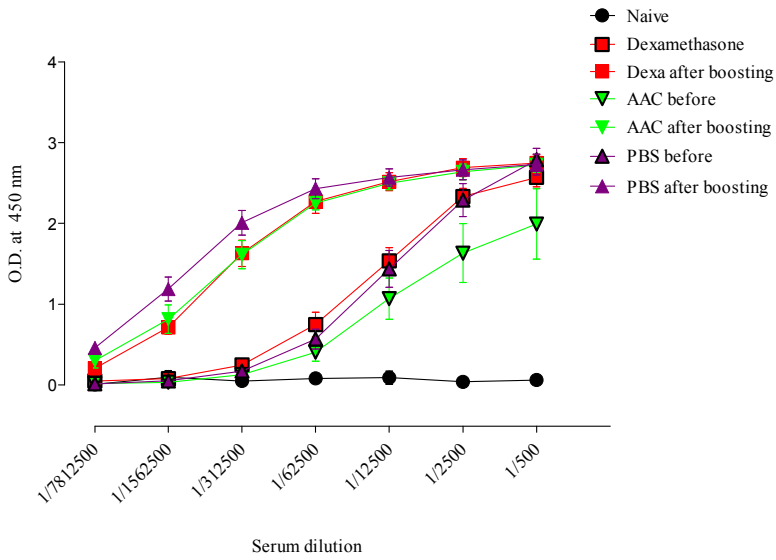


Fig. 27. OVA-specific IgG1 before starting the experiment ("before"-line) , means before challenging the mice with OVA and treatment with AAC and after the experiment (boosting and treatment with AAC – "after"-line)

3.3. OVA and milk as allergens in BALB/c and B6 mice

3.3.1. Comparing the effect of milk or OVA between BALB/c and B6 on inflammatory cells in airways

The bronchoalveolar lavage fluid of the OVA-sensitized BALB/c mice is characterized by a percentage of macrophages of ~ 60 % (Fig.29. page 79), a percentage of eosinophils of 42 % (Fig.31. page 80), a low percentage of neutrophils about 1% (Fig.33. page 80) and a percentage of lymphocytes about 3% (Fig.35. page 81). The bronchoalveolar lavage fluid of OVA-sensitized B6 mice is characterized by a higher percentage of macrophages than in OVA-sensitized BALB/c mice ~ 80% (Fig.29. page 79), a percentage of eosinophils about 20 % (Fig.31. page 80), a low percentage of neutrophils 1% (Fig.33. page 80) and a low percentage of lymphocytes of 3% (Fig.35. page 81). The bronchoalveolar lavage fluid of milk-sensitized group of BALB/c mice is characterized by a percentage of macrophages ~ 55% (Fig.29. page 79), an eosinophil percentage of 43 % (Fig.31. page 80), there were no detectable neutrophils (0%) (Fig.33. page 80) and a low percentage of lymphocytes of ~ 3% (Fig.35. page 81). The bronchoalveolar lavage fluid of the milk-sensitized B6 mice is characterized by a percentage of macrophages of about ~ 50 % (Fig.29. page 79), the lowest of all groups, a not significant but higher percentage of eosinophils than in all other groups (about 45%) (Fig.31. page 80), a low percentage of neutrophils ~1% (Fig.33. page 80) and a low percentage of lymphocytes ~5% (Fig.35. page 81).

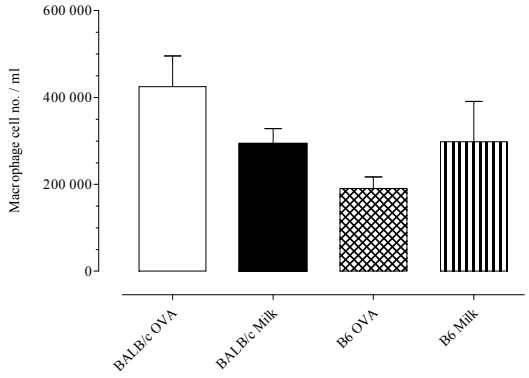


Fig.28. Comparison of the cell number of macrophages in BAL fluid between BALB/c and B6 mice sensitized with OVA or with milk. Data are expressed as means ± SEM. Data are from two individual experiments repeated under same conditions.

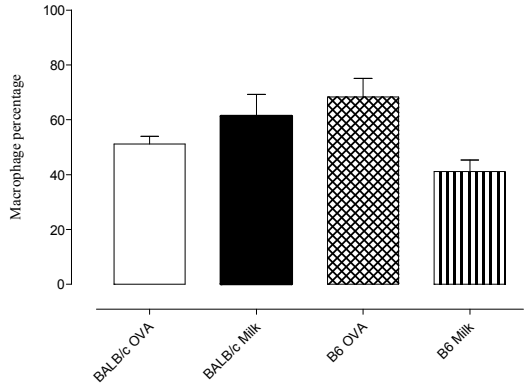


Fig.29. Comparison of the percentage of macrophages in BAL fluid between BALB/c and B6 mice sensitized with OVA or with milk (no statistical significance). Data are expressed as means ± SEM. Statistics by Kruskal-Wallis Test followed by Dunn´s multiple comparison test, significance of p = < 0,05. Data are from two individual experiments repeated under same conditions.

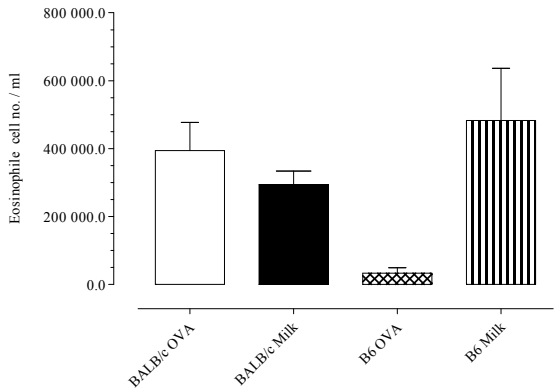


Fig.30. Comparison of the cell number of eosinophils in BAL fluid between BALB/c and B6 mice sensitized with OVA or with milk. Data are expressed as means ± SEM. Data are from two individual experiments repeated under same conditions.

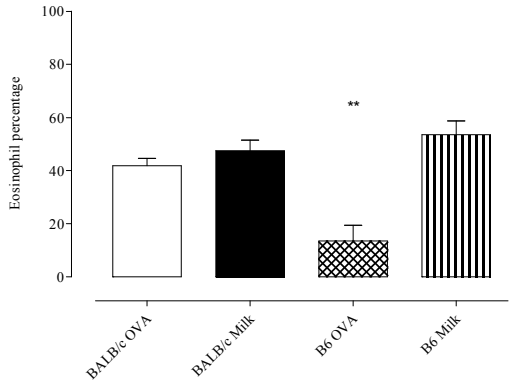


Fig.31. Comparison of the percentage of eosinophils in BAL fluid between BALB/c and B6 mice sensitized with OVA or with milk. (Stars (**) designate significant difference B6 Milk vs. B6 OVA** groups). Data are expressed as means ± SEM. Statistics by Kruskal-Wallis Test followed by Dunn´s multiple comparison test, significance of p = < 0,05. Data are from two individual experiments repeated under same conditions.

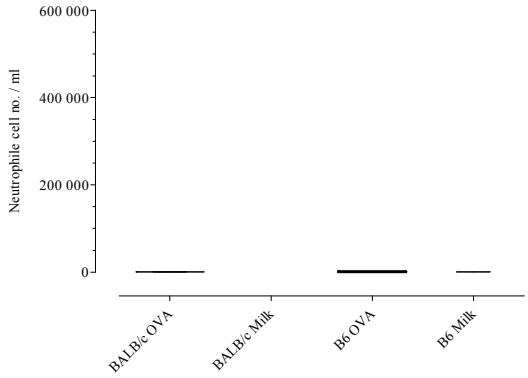


Fig.32. Comparison of the cell number of neutrophils in BAL fluid between BALB/c and B6 mice sensitized with OVA or with milk. Data are expressed as means ± SEM. Data are from two individual experiments repeated under same conditions.

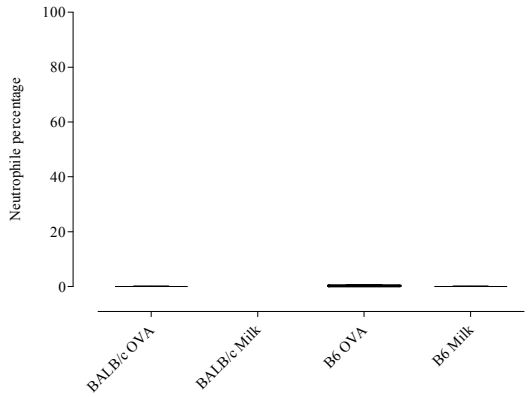


Fig.33. Comparison of the percentage of neutrophils in BAL fluid between BALB/c and B6 mice sensitized with OVA or with milk. Data are expressed as means ± SEM. Data are from two individual experiments repeated under same conditions.

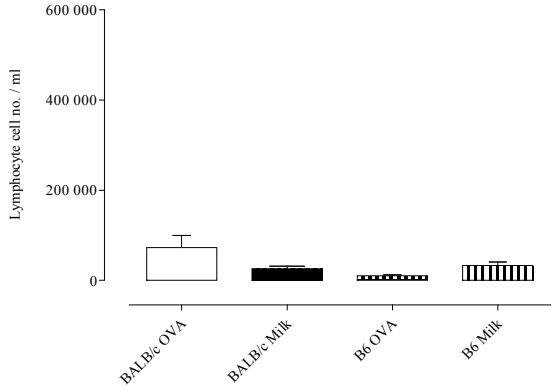


Fig.34. Comparison of the cell number of lymphocytes in BAL fluid between BALB/c and B6 mice sensitized with OVA or with milk. Data are expressed as means ± SEM. Data are from two individual experiments repeated under same conditions.

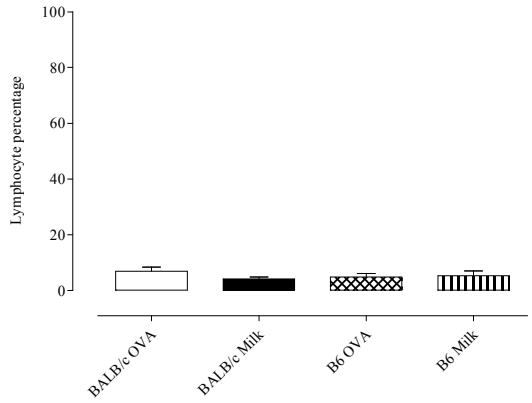


Fig.35. Comparison of the percentage of lymphocytes in BAL fluid between BALB/c and B6 mice sensitized with OVA or with milk. Data are expressed as means ± SEM. Data are from two individual experiments repeated under same conditions.

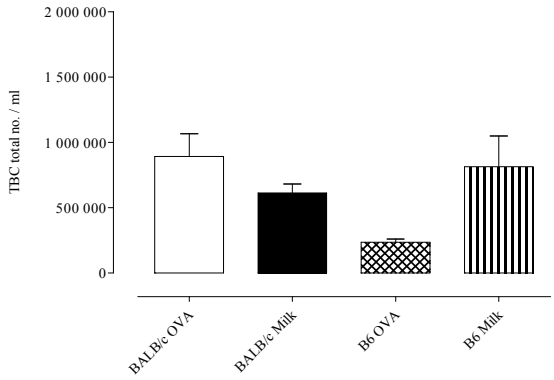
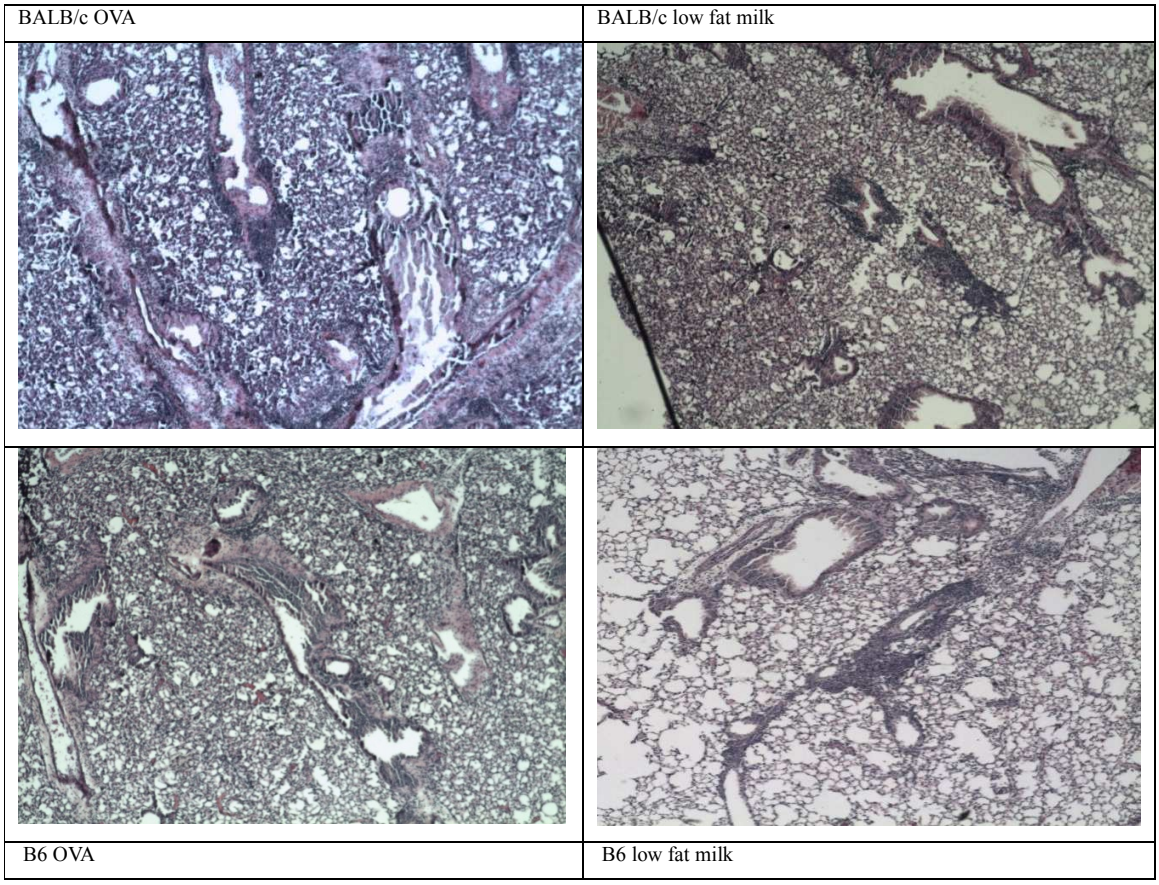


Fig.36. TBC total number in BAL fluid between BALB/c and B6 mice sensitized with OVA or with milk.
Data are expressed as means ± SEM. Data are from two individual experiments repeated under same conditions.

3.3.2. Milk vs. OVA as allergens in BALB/c and B6 on inflammation of the lung tissue

Regarding the values of cell infiltrates around blood vessels, airways and parenchyma (**H&E**: Fig.37. page 83): the milk-sensitized B6 mice showed the highest values in both trials, similar to the milk-sensitized BALB/c mice, followed by OVA-sensitized BALB/c mice. The lowest level of cell infiltrates around blood vessels, airways and parenchyma in both trials was found in the OVA-sensitized B6 group. The eosinophil infiltration in the lung parenchyma (**LUNA:** Fig.38. page 84) was at a maximum in the milk-sensitized B6 mouse group, followed with non-significant varying values (between first and second experimental approach – data not shown) by the milk-sensitized BALB/c group or OVA-sensitized BALB/c group. The eosinophil infiltration is significantly lower in the OVA-sensitized B6 mouse group compared to all other groups. The mucus production in the milk-sensitized B6 mouse group was between 60 -80 %, as well as in the milk sensitized BALB/c mouse group. The mucus overproduction in OVA-sensitized BALB/c mouse group was similar to the groups challenged with -milk at a constant 70% in both trials, as well as the OVA-sensitized B6 mouse group at 20% (**PAS:** Fig.39. page 85).

A



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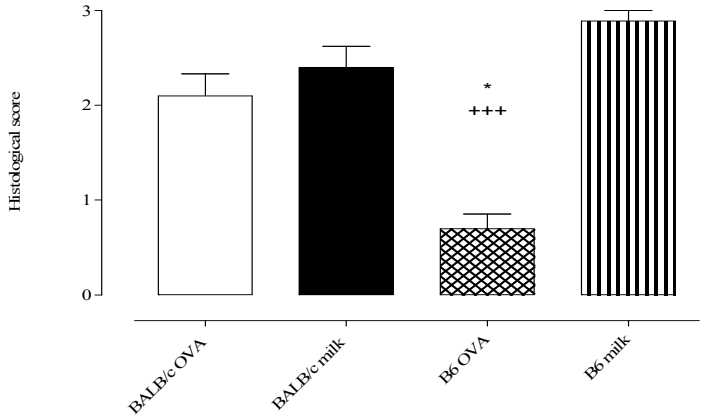
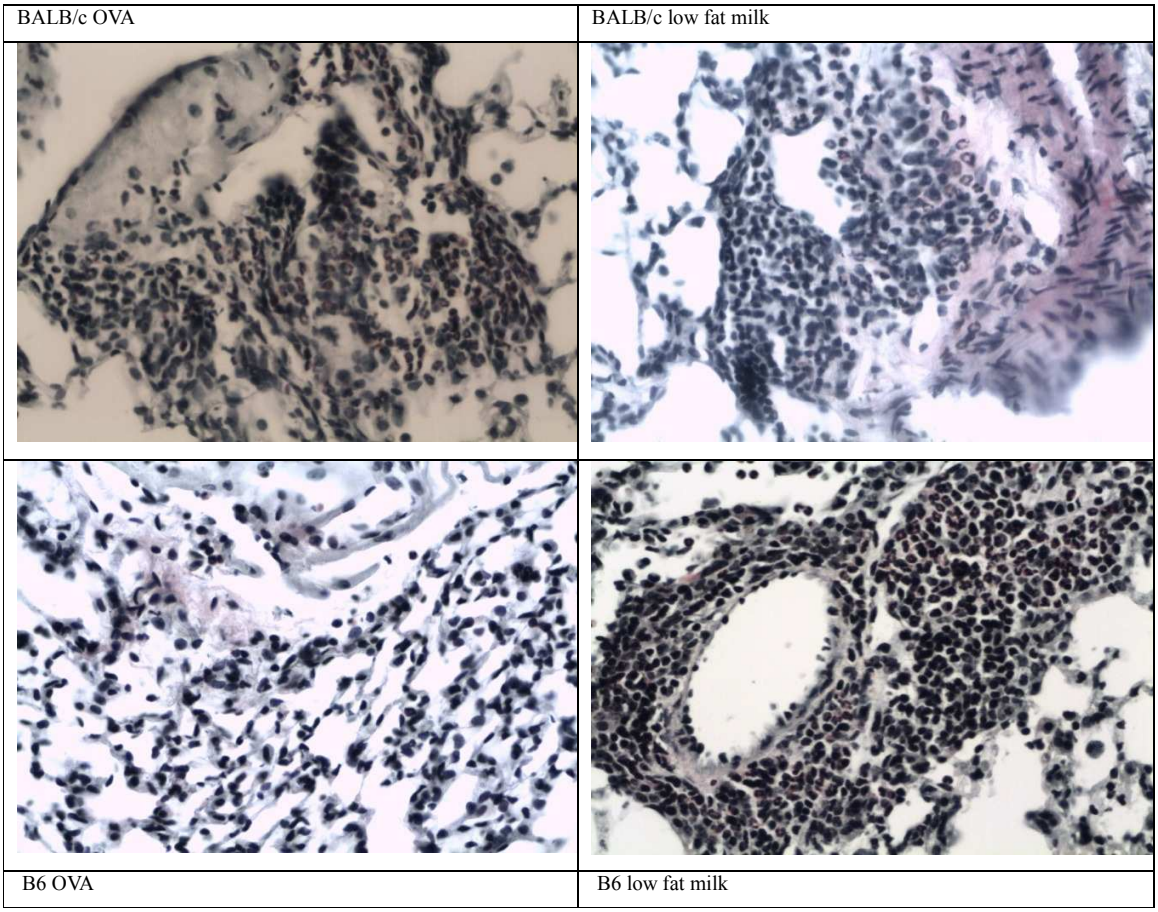


Fig.37. H&E: **A**. Representative photomicrographs of lung tissue section of BALB/c vs. B6 mice with infiltration of inflammatory cells in the lungs (original magnification x40) mice sensitized and challenged with OVA or low fat milk, as indicated for the acute asthma model. **B**. Scores for lung inflammation in BALB/c vs. B6 mice. Values are expressed as means ± SEM. Statistics by Kruskal-Wallis Test followed by Dunn´s multiple comparison test, significance of p = < 0,05. Data are from two individual experiments repeated under same conditions. (Star (*) designates significant difference between BALB/c OVA vs. B6 OVA*, and plus ($^{+++}$) between B6 OVA vs. B6 Milk^{+++}).

A



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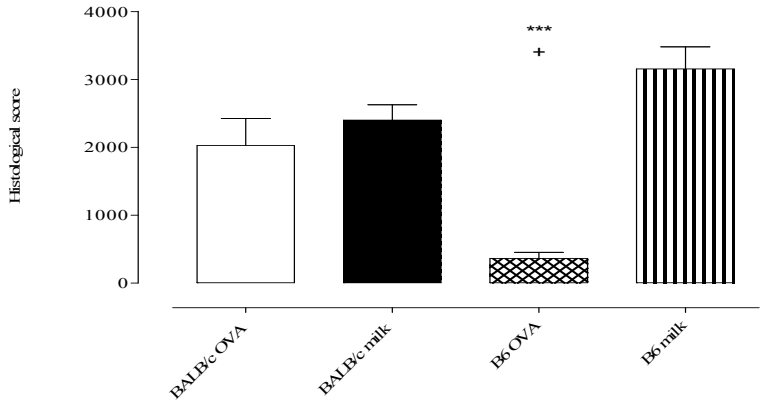
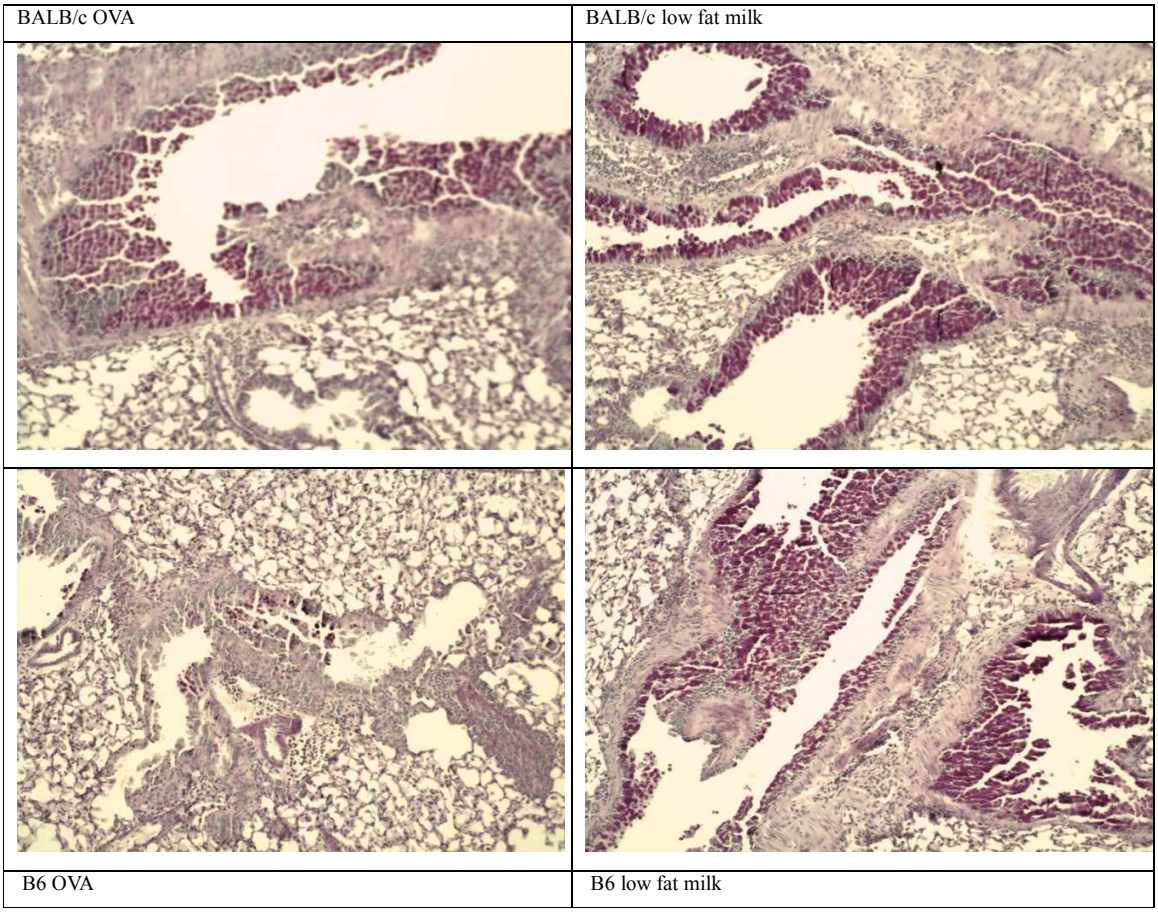


Fig.38. Luna: A. Representative photomicrographs of lungs of BALB/c vs. B6 mice with eosinophilic infiltration in the lungs (original magnification x400) mice sensitized and challenged with OVA or low fat milk, as indicated for the acute asthma model. **B**. Scores for lung inflammation in BALB/c vs. B6 mice. Values are expressed as means ± SEM. Statistics by Kruskal-Wallis Test followed by Dunn´s multiple comparison test, significance of p = < 0,05. Data are from two individual experiments repeated under same conditions. (Stars (***) designate significant difference between B6 OVA vs. B6 milk***, and plus ($^{+}$) between BALB/c OVA vs. B6 OVA^{+} groups).

A



B

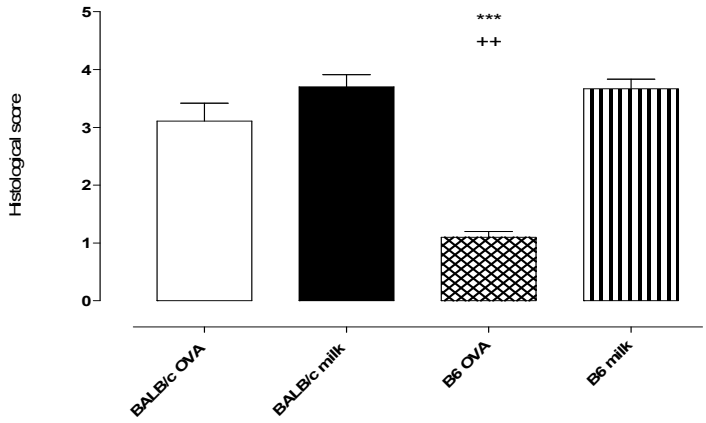


Fig.39. PAS: A. Representative photomicrographs of lung tissue of BALB/c vs. B6 mice with mucus hypersecretion (original magnification x200) mice sensitized and challenged with OVA or low fat milk, as indicated for the acute asthma model. **B**. Scores for lung inflammation in BALB/c vs. B6 mice. Values are expressed as means ± SEM. Statistics by Kruskal-Wallis Test followed by Dunn´s multiple comparison test, significance of p = < 0,05. Data are from two individual experiments repeated under same conditions. (Stars (***) designate significant difference between B6 OVA vs. B6 milk***, and plus ($^{++}$) between BALB/c OVA vs. B6 OVA^{++} groups).

3.3.3. Milk vs. OVA as allergens in BALB/c and B6 on OVA-specific IgG1

Sera from all mice for ELISA of OVA or milk-specific IgG1 were taken before the start of the experiment and set as naive IgG1. After sensitization with OVA of the chicken egg or milk, also sera from all sensitized mice were taken to determine the level of the OVA- or milk-specific IgG1 at the end of the experiment. As expected, no detectable OVA- or milk-specific antibodies were detected in sera taken before the start of experiment. In mice challenged with milk titer of milk-specific IgG1 molecules was comparable between two mice strains. In contrast, titer of OVA-specific IgG1 molecules was higher in BALB/c than in B6 mice. (Fig.40. page 86).

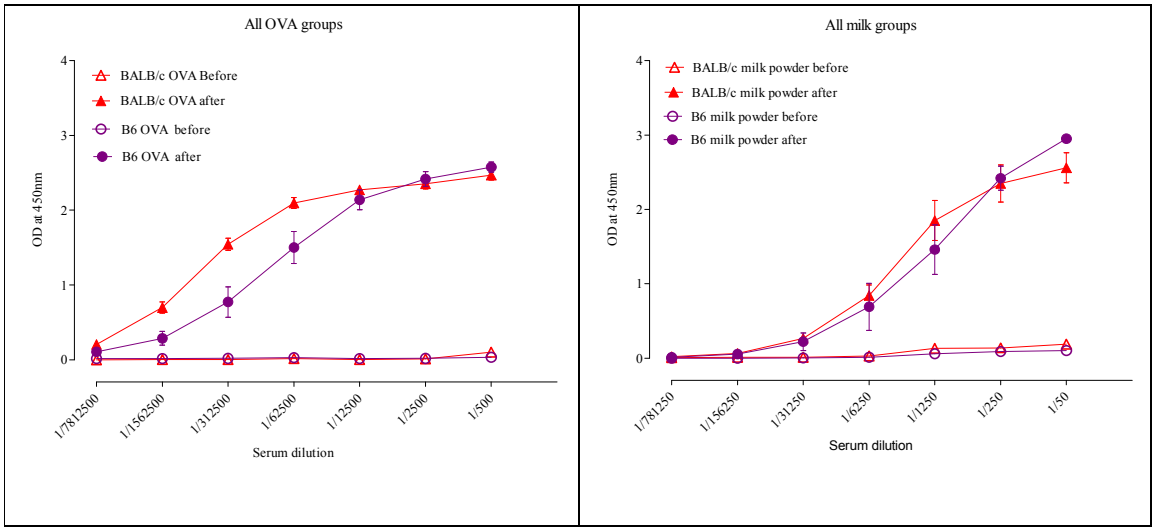


Fig.40. OVA- (first graph, left panel) or milk-specific IgG1 Ab (second graph, right panel) before the sensitization of BALB/c or B57BL6 B6 mice with OVA or milk (marked with before sensitization-lines = no detectable OVA or milk-specific IgG1) and after the experiment (after OVA or milk challenge – marked with "after sensitization-lines" on the graph)

Chapter 4

4. DISCUSSION

4.1. Tiotropium bromide and AAC effectiveness in allergic asthma

In these studies, we found that both Tiotropium and AAC were able to effectively reduce allergic asthma in mice. We found that they reduced airway and lung inflammation similar to the gold standard, glucocorticoid Dexamethasone. However, they were unable to reduce the levels of serum antigen-specific antibody. Both compounds reduce lung inflammation and mucus at the same level as Dexamethasone by approximately 50%. We would argue that this model of allergic asthma exacerbation is similar to steroid-resistant asthma in patients because steroids do not completely suppress disease. We conclude that these two compounds are as effective as steroids and would argue that they can be used as potential steroid-sparing drugs.

4.2. Milk vs. OVA as allergens in BALB/c or B6 mice

Comparing the standard OVA allergen in allergic asthma in mice with milk as an allergen, we observed that milk induces significantly more severe allergic disease in the Th1-type B6 mice compared to BALB/c mice. This is in contrast with OVA, which induces more severe disease in BALB/c mice compared to B6 mice. These findings are important because they indicate that there are significant differences in the allergens used in generating allergic asthma in mice and there are significant genetic differences between congenic strains mice. Most surprising is that the Th1-type mouse B6 develops more severe allergic disease compared to the Th2-type BALB/c mice. It is not yet clear what genetic differences between mice explain the findings and their responses to distinct allergens.

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List of abbreviations

Ab – Antibody

Ag – Antigen

AHR – Airway hyperreactivity

APC – Antigen presenting cells

ATP – Adenosine Triphosphate

BAL fluid – Bronchoalveolar fluid

cAMP – Cyclic Adenosine Triphosphate

CD - Cluster of Differentiation

CCL – Chemokine Ligand

CCR – Chemokine Receptor

COPD - Chronic Obstructive Pulmonary Disease

CTLs: cytotoxic T-Lymphocytes

Cys-LT - Cysteinyl-Leucotriene

DC - Dendritic Cells

ELISA - Enzyme-Linked Immunoabsorbant

FEV_1 - forced expiratory volume in one second

GM-CSF - Granulocyte macrophage colony-stimulating factor

HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A reductase

ICAM-1 - Inter-Cellular Adhesion Molecule 1

Ig - Immunoglobulin

IL – Interleukin

IFN – Interferon

LABA – Long acting β-Adrenoreceptor Agonists

LAK cells: Lymphokine Activated Killer cells

LPS – Lipopolysaccharide

LTC_4- Leukotriene C4

MBP – Major basic Protein

NAC - N-acetyl cysteine

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NFκB - Nuclear Factor-κB

Nk cells – Natural Killer cells

NO - Nitric Oxide

OVA – Ovalbumin

PAF - Platelet Activating Factor

PAMPs: pathogen associated molecular patterns

PBS - Phosphate Buffered Saline

PG – Prostaglandin

PRRs: pattern recognition receptors

T_H - helper T-Lymphocytes

TLR - Toll-Like Receptor

TNF - Tumor Necrosis Factor

T-reg - Regulatory T Cells

VCAM-1- Vascular Cell Adhesion Molecule 1

VLA-4 - Very Late Antigen-4

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