**Thesis**

# Immunopathology of Asthma

submitted by

**Adrian Mathias Moser**

Mat.Nr:0210651

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Supervisor

**Prof. Dr. Irmgard Lippe**

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## Zusammenfassung

**Hintergrund:** Allergische Erkrankungen wie zum Beispiel atopisches Asthma sind sehr häufig und ihre Inzidenz steigt. Einblicke in die Mechanismen der Erkrankung könnten neue Möglichkeiten zur Intervention bringen. Prostaglandin D2, produziert von Mastzellen, könnte zur eosinophilen Entzündung in allergischem Asthma beitragen. Chemoattractant receptor homologous molecule expressed on Th2 cells (CRTH2), vermittelt die Rekrutierung von Th2 Zellen, eosinophilen und basophilen Granulozyten zu Entzündungsherden. In meiner Diplomarbeit habe ich darum den Gebrauch von CRTH2 Antagonisten in einem in vivo Maus-Model für allergische Atemwegsentzündung untersucht.

**Methoden:** Die Mäuse wurden in vier Gruppen eingeteilt: OVA (Ovalbumin-sensibilisiert und provoziert) Mäuse mit und ohne Antagonist und Kontrollen. Die Messung der Lungenfunktion wurde nach Sensibilisierung und Provokation mit zwei Methoden durchgeführt, uneingeschränkte Ganzkörperplethysmographie (WBP) und direkte Atemwegswiderstandsmessung mit FlexiVent. Bronchoalveoläre Lavage (BAL) und Vollblutproben wurden analysiert um Zellreaktionen zu begutachten.

**Resultate:** OVA Mäuse verglichen zu den Kontrollen zeigten in der bronchoalveolären Lavage signifikante Erhöhung von eosinophilen und neutrophilen Granulozyten, Lymphozyten und Makrophagen. Die OVA+ CAY Gruppe war signifikant höher im Atemwegswiderstand (FlexiVent) verglichen mit den Kontrollen. Der CRTH2 Antagonist zeigte keinen signifikanten Einfluss auf die Zellzahl in der BAL oder im Vollblut, aber zeigte erhöhten Atemwegswiderstand in der OVA+ CAY Gruppe im Vergleich zu den Kontrollen. Messung mit der WBP brachte keine signifikanten Ergebnisse.

**Schlussfolgerung:** Die Resultate zeigten unklare Daten in Bezug auf den Effekt des Antagonisten auf die Atemwegsentzündung in vivo und auch gegensätzliche Ergebnisse der zwei Messmethoden. Darum bin ich in der Diskussion auf die Methoden eingegangen, mit der Schlussfolgerung, dass die WBP kritisch betrachtet werden sollte. Weiters habe ich chemischen Antagonismus und einen ganzheitlicheren Zugang zu allergischen Erkrankungen gegenübergestellt, der Infektionen verwendet, um immunregulatorische Mechanismen zu aktivieren.

## Abstract

**Background:** Allergic diseases such as atopic asthma are very common and the incidence is rising. Insights into the mechanisms of this disease bring new possibilities of intervention. Mast cell derived prostaglandin D2 may contribute to eosinophilic inflammation in allergic asthma. Chemoattractant receptor homologous molecule expressed on Th2 cells (CRTH2), mediates recruitment of Th2 cells, basophils and eosinophils to inflammatory sites. Therefore I investigated in my thesis the use of CRTH2 antagonists (CAY) in vivo in a mouse model of allergic airway inflammation.

**Methods:** Mice were divided into four groups: OVA (ovalbumin-sensitized and challenged) mice with and without antagonist and controls. The measurement of lung function was performed after sensitization and challenge with two methods, unrestrained whole body plethysmopgraphy (WBP) and direct invasive resistance measurement with FlexiVent. Bronchoalveolar lavage and whole blood sample measurements were also performed to examine cell reactions.

**Results:** OVA mice compared to controls showed significant elevation of eosinophils, neutrophils, lymphocytes and macrophages in the bronchoalveolar lavage (BAL) fluid. Also, OVA + CAY was significantly higher in resistance (FlexiVent) compared to controls. Eosinophils in OVA+ vehicle were significantly elevated in the whole blood sample compared to the controls. The CRTH2 antagonist showed no significant influence on cell counts in BAL or whole blood samples, but exhibited elevated resistance in the OVA + CAY group compared to controls. Measurement with whole body plethysmopgraphy showed no significant results at all.

**Conclusions:** The results showed unclear data concerning the effect of the antagonist on airway inflammation in vivo and contrasting outcomes of measurement methods. Therefore I critically discussed the used methods, showing a reconsideration of the use of WBP. Furthermore, I contrasted chemical antagonism to a more holistic treatment of allergic diseases, using targeted infection, which induces immunoregulatory mechanisms in the host.

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## Introduction

Atopic diseases like asthma are very common and the number of patients is rising. Immunological disorders around the complex phenomenon are rarely understood and therapeutic intervention is still a problem. In consequence of the enormous expansion of knowledge in immune-mediated diseases, there are a lot of new targets, which transform the possibilities of symptomatic treatment into causal intervention.

General Background

Allergy as a term was first used by von Pirquet (1906) as an altered immune reactivity (Ying, Zhang et al. 2006). Nowadays the term is used for the clinical expression of atopic diseases like asthma, rhinitis, eczema and food allergy. Atopy is defined as an elevation of allergen-specific IgE antibodies and susceptibility towards sensitization by ordinary exposure to environmental challenges. Atopic asthma itself, as I use the term in my thesis, denominates extrinsic asthma, being defined as an exaggerated response of the immune system to external substances. This immune response creates a complex inflammation in the airway system, which leads to serious tissue damage resulting in a chronic disease with airway hyperresponsiveness, inflammation and remodeling of the structure in airways. Also, the reversible airway obstruction is a typical sign. The clinical expression of asthma is reflected in symptoms like coughing, dyspnea, wheezing, chest tightness and reduction of the FEV1 (forced expiratory volume in 1 second). The different immunological cells, which are included in the massive inflammation response, are particularly lymphocytes, mast cells and eosinophils. The interactions between these cell types are now in the spotlight of research work (Ying, Zhang et al. 2006).

Immunological mechanisms in the early-phase response (EPR)

In the last years immunological research gained possibilities to get access to the world of inflammation. This caused an explosion of knowledge in the area of allergic disorders. Nevertheless, the coherencies between the different cell lines, their affectations and appreciations are still not clear.

In the case of asthma we have to discuss different types of hypersensitivity reactions (Ying, Zhang et al. 2006).

The major hypersensitivity reaction in asthma is the type 1 (also called anaphylactic hypersensitivity). In atopic asthma the early-phase response to extrinsic allergens is the type 1 reaction, which is mediated by mast cells and their mediators like histamine and leukotrienes (see Figure 1.1) in a sensitized individual (Holgate, Church et al. 2006).

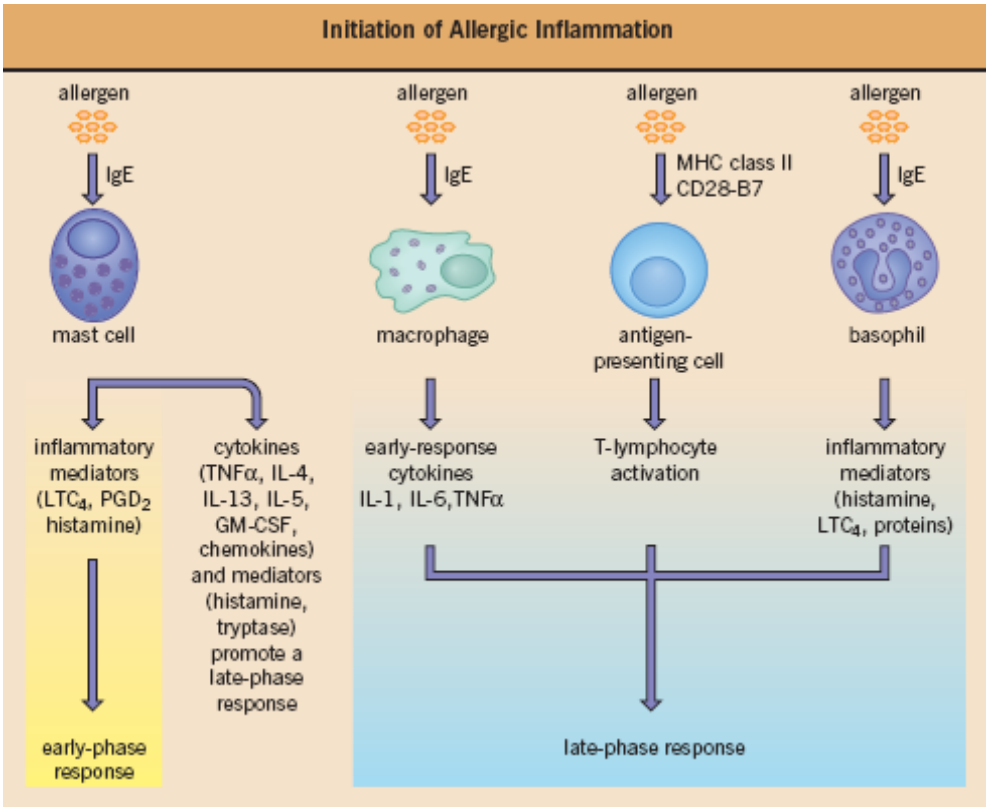



**Figure 1.1 Initiation of allergic inflammation:** Mast cell degranulation products promote the early-phase response and stimulate the late-phase via PGD2. Macrophages, lymphocytes, basophils and eosinophils contribute to the late-phase. (This figure was published in the book Allergy, Volume 3, Stephen T Holgate, Martin K Church, Lawrence T Lichtenstein, Page 397, Copyright Elsevier 2006(Holgate, Church et al. 2006))

This early-phase is defined by a symptom development in 10-15 minutes and a maximum peak within 30 minutes. The major point of developing the early-phase response is the sensitization of the individual and the production of IgE antibodies leading to the coating of tissue mast cells and circulating basophils. The production is very complex and is an important part of the pathway leading to allergic diseases. The processing of allergens depends on the activity and efficiency of antigen uptake by antigen-presenting cells (APC).

After editing, the APC´s present the antigen to T cells, which are divided into several groups due to their secretion profile. The major subsets are T helper cells (Th1, secreting y-Interferon) and Th2 cells, secreting interleukin 4, 5, 10 and 13 (IL 4, 5, 10, 13). The role of these T cells in the development of immune-mediated diseases is still unclear. Some data reflect the theory of Th2 polarization leading to allergy (Holgate, Church et al. 2006). Activation of Th2 cells stimulates the production of IgE antibodies. After stimulation of B cells via IL-4, secreted IgE binds to the surface of target cells like mast cells and circulating basophils. Binding is mediated by the very important FcεRI-receptor expressed as the complete complex αβγ2 on mast cells and basophils (Holgate, Church et al. 2006). Furthermore, the αγ2 heterotrimer is expressed on human APC´s like dendritic cells, monocytes and macrophages. If a sufficient quantity of the antigen is inhaled, it cross-links the coated IgE antibodies on the surface of mast cells and basophils leading to degranulation. The major mediators are as mentioned histamine, leuktriene C4 (LTC4), tryptase and prostaglandin D2 (PGD2). As a consequence, the histamine receptors are stimulated and they produce vasodilatation with a postcapillary venule leakage, constriction of the smooth muscle cell in the bronchial airways and secretion of mucus, resulting in the symptoms of asthma (Holgate, Church et al. 2006). This whole cascade represents the early-phase of inflammation, in which the mediators LT`s and histamine play a key role. These substances are also secreted by other cell lines like eosinophils, platelets and monocytes in IgE-dependent mechanisms. This reflects the complex interactions between the different cell lines (Ying, Zhang et al. 2006).

Late-phase response

As mentioned above, the asthmatic inflammation response is rationed in the early-phase and late-phase response, whereby the late-phase reaches its maximum at 6-12 hours later and resolves after 24 hours. This phase is characterized by influx of inflammatory cell lines such as eosinophils, CD4+ Th2 cells, mononuclear cells and basophils, recruited from the circulation being activated by different mediators as for instance PGD2 (see Figure 1.2).

These cells release different kinds of pro-inflammatory mediators and contribute to the epithelial damage and bronchial hyperresponsiveness (BHR). Cytokines are a major factor in the initiation and perpetuation of inflammatory processes. Under normal circumstances, the endothelial cells in blood vessels in the lungs do not express adhesion molecules such as vascular adhesion molecule 1 (VCAM-1) or intercellular adhesion molecule 1 (ICAM-1). The consequence is that customarily circulating leukocytes cannot adhere and gain access to lung tissue. After inhalation and sensitization of an individual, the mediators released by activated mast cells such as histamine promote expression of ICAM-1 and VCAM-1. So the late-phase is strongly associated with the early-phase, although sometimes the early-phase is not recognized by the sensitized individual (Holgate, Church et al. 2006).

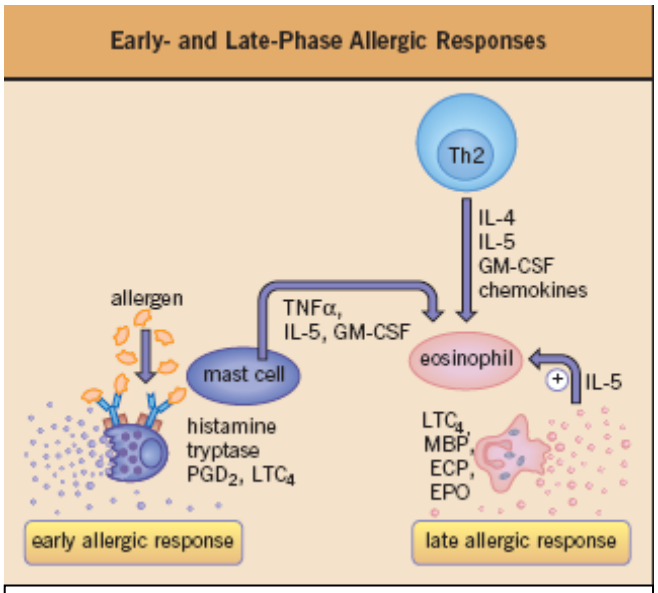



**Figure 1.2 Early- and late-phase-response:** Cytokines, chemokines and effector substances of early- and late-phase. (This figure was published in the book Allergy, Volume 3, Stephen T Holgate, Martin K Church, Lawrence T Lichtenstein, Page 332, Copyright Elsevier 2006(Holgate, Church et al. 2006))

After influx in the late-phase, the different cell lines of the late-phase response can damage tissue with different cytotoxic elements like reactive oxygen metabolites, major basic protein (MBP) and eosinophil derived neurotoxin (EDN) (Ying, Zhang et al. 2006).

## Inflammatory cells in allergic asthma

The complex phenomenon of allergic disorders is illustrated by the fact that nearly all kinds of immune cells are involved in the process of tissue damage, some of them act directly through damaging tissue, and some indirectly through mediating cell interactions.

### Neutrophils

The primary function of neutrophils is the defense against infection and tissue injury. Therefore, they need to get access into tissues very easily through expression of adhesion proteins on their cell surface. The major integrins, which are now in the spotlight of research, are endothelial leukocyte adhesion molecule 1 (ELAM-1), lymphocyte function-associated antigen 1 (LFA-1) and ICAM-1(Ying, Zhang et al. 2006). They also possess a variety of receptors for a wide range of chemotactic agents like IL-8 and LTB4. The role of neutrophils in allergic disorders is still unclear, although studies reported that they appear early after allergen challenge of skin and nose (Varney, Jacobson et al. 1992). But in contrast, the number is the same as in controls of asthma (Azzawi, Bradley et al. 1990). Nevertheless, neutrophil lysosomal granules contain different types of enzymes such as proteinases, collagenase, and lysozyme. These enzymes seem to have digestive effects on intracellular bacteria, but can also cause tissue damage after liberation in inflammation responses (Holgate, Church et al. 2006).

### Monocytes and macrophages

Macrophages represent the mature part of the mononuclear phagocyte system (MPS) and embody a central point in the whole immunological defense of the body. Released from the bone marrow as promonocytes and differentiated to monocytes in the blood, they are represented in every human tissue as macrophages, which are more “activated” and show more efficiency concerning phagocytosis. The main function of the MPS is to present antigens to stimulate T cell activation and to release soluble mediators that regulate immune and inflammatory processes (Holgate, Church et al. 2006).

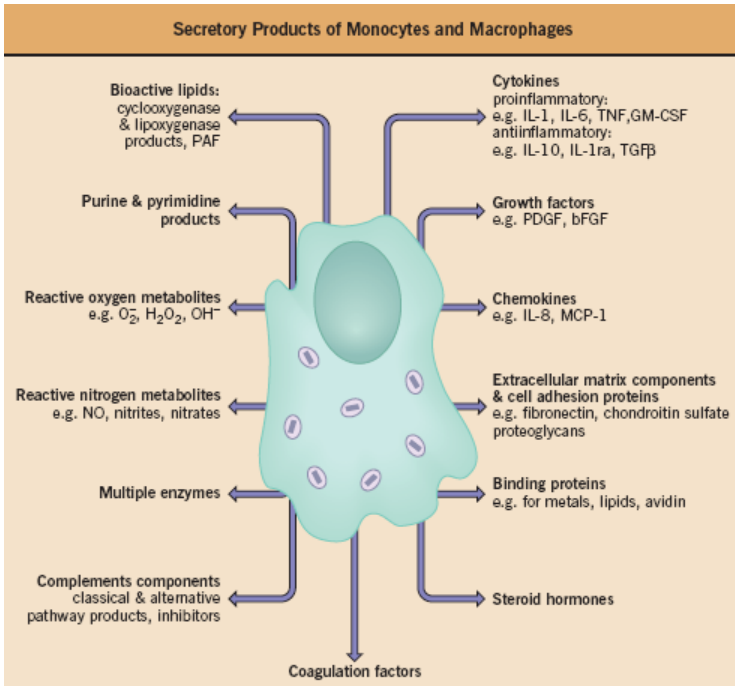



**Figure 1.3 Secretory products of monocytes and macrphages:** The secretory products of macrophages and monocytes are representing the multifactorial intervention of these cells. (This figure was published in the book Allergy, Volume 3, Stephen T Holgate, Martin K Church, Lawrence T Lichtenstein, Page 371, Copyright Elsevier 2006(Holgate, Church et al. 2006))

Therefore the number of different receptors reflects the enormous importance of the macrophages in activation and accomplishment of immunological responses. Both, monocytes and macrophages, carry a great number of different receptors on their cell surface, to recognize foreign antigens and allergens. Toll-like receptors and scavenger receptors are both highly specific for soluble ligands and offer a great possibility to activate the immune system via antigen presentation. Fc binding receptors recognize Fc portions of IgG1, IgG3, IgA, IgE, IgM allowing to process with opsonized antigens. Thus, they represent a link between the unspecific and specific immune response (Holgate, Church et al. 2006; Ying, Zhang et al. 2006). The pivotal role of macrophages in immunological phenomena is due to their capabilities to activate other immune cells through interleukins and other cytokines and to receive feedback signals from other cell types as well (see Figure 1.3) (Holgate, Church et al. 2006).

Cytokines like IL-2, IL-3, IL-4, tumor necrosis factor-α (TNF-α) and Interferon γ (IFN-γ) can activate macrophages or modulate their functions. IFN-γ is a highly potent activator and is especially secreted by Th1 cells.

The contribution of active macrophages to airway inflammation is suggested due to their secretory products like reactive oxygen metabolites, bioactive lipids like cyclooxygenase (COX) products and pro-inflammatory cytokines, especially IL-1 and IL-6, IL-8 and TNF-α acting as a promoter for the allergic process by enhancing recruitment and adhesion of immune cells to the inflammatory site (Holgate, Church et al. 2006; Ying, Zhang et al. 2006). Although it seems that the MPS has a pro-inflammatory role in asthma, alveolar macrophages could have an anti-inflammatory role through suppressing antigen-presenting function of local dendritic cells and T cells. Without this control mechanism, the lungs would be a very vulnerable organ, because of the exposure to foreign antigens and the large number of memory T cells, which would trigger an exaggerated immune response (Holgate, Church et al. 2006).

Eosinophils

This term comes from ability to bind acidic dye eosin and was named by Paul Ehrlich in 1879. The role of eosinophils in allergic diseases became more and more interesting in the last years and now there are strong indications that they play a major part in the late-phase response of asthma. Normally, eosinophils account for less than 5% of the circulating white cells and in the past they were associated with anti-inflammatory properties. Since realizing that their granule proteins like MBP, eosinophil cationic protein (ECP), EDN or eosinophil peroxidase (EPO) are also toxic to mammalian cells, the view changed and their impact on inflammation has gotten more and more into the spotlight of scientists. Interestingly, eosinophils produce a great amount of different mediators from cytokines, chemokines, and enzymes to mentioned basic proteins. Together with the detection that accumulation of eosinophils is allied with several diseases like allergic bronchial asthma and allergic rhinitis, the conclusion of pro-inflammatory characteristics is clear. The eosinophils rest in uninflamed tissue and peripheral blood to avoid unintended tissue damage.

So the major point of eosinophil inflammation is recruitment into target tissues through chemoattraction, adhesion and migration (Ying, Meng et al. 1999; Holgate, Church et al. 2006; Ying, Zhang et al. 2006). Eosinophils own a lot of receptors on their cell surface, mediating adherence to tissues.

Very late antigen 4 (VLA-4) and LFA-1 play an important role in immigration and therefore also for inflammation response binding through tissue receptors such as VCAM-1, mucosal addression cell adhesion molecule 1 (MadCAM-1) and ICAM-1 (see Figure 1.4). The critical cytokine to mediate chemotaxis in eosinophil recruitment seems to be IL-5, which enhances mature eosinophil function due to differentiation of the cell for chemotaxis, adhesion and cytotoxity (Holgate, Church et al. 2006).

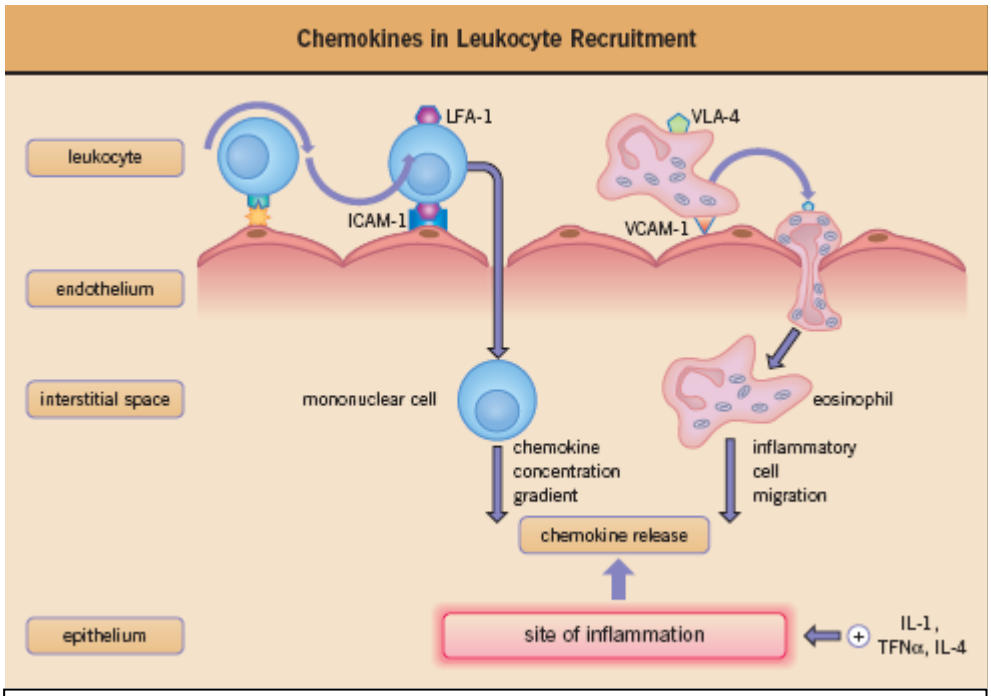



**Figure 1.4 Chemokines in leukocyte recruitment:** Adhesion molecules are expressed after chemokine stimulation. Leukocytes then gain access to target tissues and promote allergic inflammation there. (This figure was published in the book Allergy, Volume 3, Stephen T Holgate, Martin K Church, Lawrence T Lichtenstein, Page 339, Copyright Elsevier 2006(Holgate, Church et al. 2006))

Therefore IL-5 stands in the focus of novel therapeutic options, due to anti-IL-5 antibodies significantly inhibiting blood and sputum eosinophilia in asthmatics and expression of extracellular matrix proteins.

Another critical point in eosinophil inflammation response is chemokine receptor 3 (CCR3), which is expressed on eosinophils, activated Th2 cells and basophils. Chemokines binding to this receptor are for example eotaxin-1, eotaxin-2, and eotaxin-3 and macrophage chemoattractant protein 2. The inhibition of this receptor seems to be a novel approach and reduces eosinophil recruitment in murine models (Holgate, Church et al. 2006).

The contribution of eosinophils to allergic inflammation response appears in the late-phase. After the early-phase response stimulated via histamine, PGD2 and other cytokines, eosinophils are recruited and then damage tissues with mediators as mentioned above. Their presence is correlated with BHR, mucus secretion and smooth muscle contraction. After chemotaxis and adherence through molecules as explained above, eosinophils are now activated and their normal life span is prolonged from 2-5 days to 14 days, illustrating the elevated number of eosinophils in allergic tissues. In addition they produce a strong amount of cysteinyl leukotrienes like LTC4, which amplify the typical bronchial reactions mucus secretion, airway hyperresponsiveness and smooth muscle contraction. Additionally to all other secretions, activated eosinophils release typical cytokines and leukotrienes, reflecting their role as the major producer of leukotrienes in allergic inflammation (LTC4). The cytokine releasing profile includes TGF-α (transforming growth factor alpha), TGF-β1, IL-1α, IL-3, IL-5, IL-8, IL-10, IL-13, TNF-α and some chemokines (Phipps, Ying et al. 2002; Ying, Zhang et al. 2006). The combination of pro-inflammatory and anti-inflammatory cytokines shows their important role in perpetuation of tissue damage and also in inducing repair mechanisms through TGF-β (Phipps, Ying et al. 2002).

Mast cells

Mast cells present the most important cell in mediating the early-phase response to allergen or antigen presence. The most fascinating fact is that mast cells release histamine, which mediates the typical type 1 hypersensitivity reaction with vessel dilatation, capillary permeability and smooth muscle contraction, very fast, I.E. after activation through cross-linking IgE antibodies, in about 2 to 5 minutes (Bradding, Walls et al. 2006). Other products like heparin, chondroitin sulphates and neutral proteases are also released (see Fig.1.5).

About 15 minutes later they produce a strong amount of newly generated mediators like PGD2 and LTC4, which are typical eicosanoid-derived products. Interestingly, the secretion of these newly generated mediators represents the interface of the early-phase and late-phase response due to the fact that PGD2 activates eosinophils, basophils and Th2 cells, via the receptors prostanoid receptor 1 (DP1) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2).

These substances and receptors in this phase are very interesting for therapeutic intervention (Holgate, Church et al. 2006).

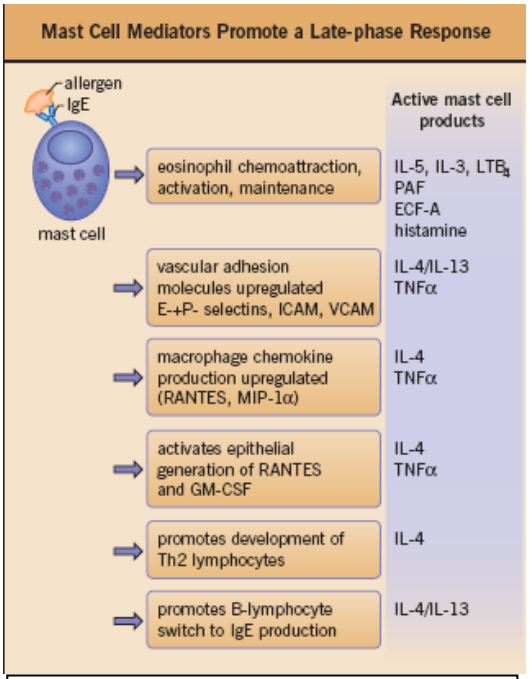



**Figure 1.5 Mast cell mediators promote a late-phase response:** ECF-A, eosinophil chemotactic factor A; MIP, macrophage inflammatory protein; PAF, platelet-activating factor. (This figure was published in the book Allergy, Volume 3, Stephen T Holgate, Martin K Church, Lawrence T Lichtenstein, Page 398, Copyright Elsevier 2006(Holgate, Church et al. 2006))

Mast cells are divided into two major groups: $MC_T$ phenotypes, which contain only tryptase and represent the with the immune system associated mast cells and $MC_{TC}$ phenotypes, which contain tryptase and chymase represent the non-immune system associated mast cells being related to functions like angiogenesis and tissue remodeling. Both types express the FcεRI-receptor, which is the major receptor for IgE-dependent immune reactions. This high affinity receptor is responsible for binding IgE-antibodies to the surface and through cross-linking of IgE the mast cell gets activated by biochemical pathways. Mast cells are not only producers of mediators for typical allergic symptoms. They are also responsible for the initiation and coordination of many local processes in inflammation, which are adjusted by different cytokines.

IL-4 is involved in switching B lymphocytes to IgE production, IL-5 is a strong chemoattractant for eosinophils and TNF-α plays a critical role in the whole inflammation response including induction of adhesion molecules and chemokines (Bradding, Walls et al. 2006).

Basophils

Basophils share a lot of similarities with mast cells due to their expression of the FcεRI-receptor and their large intracytoplasmic histamine-containing granules. Binding and cross-linking of IgE antibodies lead to degranulation of granule-derived and preformed mediators. Nevertheless, basophils are cells with other chores. The secretion of LTC4 is much higher than in eosinphils and secretion of IL-4 seems to be the major source of IL-4 in inflammatory responses, acting as an early activator. Basophils are also allied with the late-phase response in allergic inflammation, decoyed through PGD2 and their CRTH2-receptor (Ying, Zhang et al. 2006).

Lymphocytes

Lymphocytes play an essential role in all immunological activities. They represent the interface between innate and acquired immunity due to their capabilities to process antigens, and to modulate and to perform further responses like specific antibody production.

Lymphocytes are divided into B cells and T cells. B cells are derived from the bone marrow and their purpose is to recognize foreign antigens via the B-cell receptor (BCR). After processing the antigen, they can differentiate to antibody-producing plasma cells or can present the antigen to T cells (see Figure 1.6). Thereby naïve B cells (co-expressing IgM and IgD isotypes on their cell surface) recognize antigens and are able to proliferate into IgM-producing plasma cells resp. can differentiate into B memory cells (expressing single isotype BCR like IgM, IgG, IgA…) through maturation and Ig-class switching (from IgM to IgG). Memory B cells can differentiate very fast into IgG-producing plasma cells and therefore mediate the secondary-phase response to antigens.

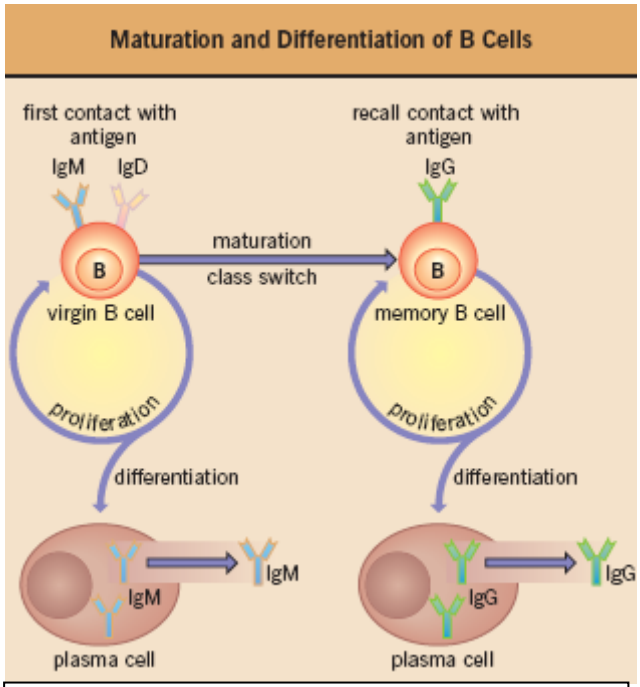



**Figure 1.6 Differentiation and maturation of B cells:** Differentiation and maturation of B cells after antigen contact to memory B cells. Memory B cells are mediating IgG response to secondary antigen contact. (This figure was published in the book Allergy, Volume 3, Stephen T Holgate, Martin K Church, Lawrence T Lichtenstein, Page 316, Copyright Elsevier 2006(Holgate, Church et al. 2006))

The presentation of antigens with major histocompatibility complex class II (MHC II) to T cells is only possible after internalizing and processing the antigen.

In this reaction, the MHC II molecule interacts with the cluster of differentiation antigen 4 (CD4+) of the T helper cell. The main function of B cells in allergic diseases is the antigen presentation and of course the production of IgE antibodies after class-switching, which is one of the crucial points in allergic immunology, presumably mediated by IL-4 (Holgate, Church et al. 2006).

T cells have been in the spotlight of investigations for a long time and their role in different kinds of immune-mediated diseases is barely understood, but one thing is clear: They represent a major cell in inflammation. T cells are also derived from bone marrow, but then get “educated” in the thymus to differentiate into mature T cells, to be able to recognize foreign antigens via their T cell receptor (TCR) and interact with MHC class I and II molecules of APC`s (see Figure 1.8) (Roitt and Delves 2006).

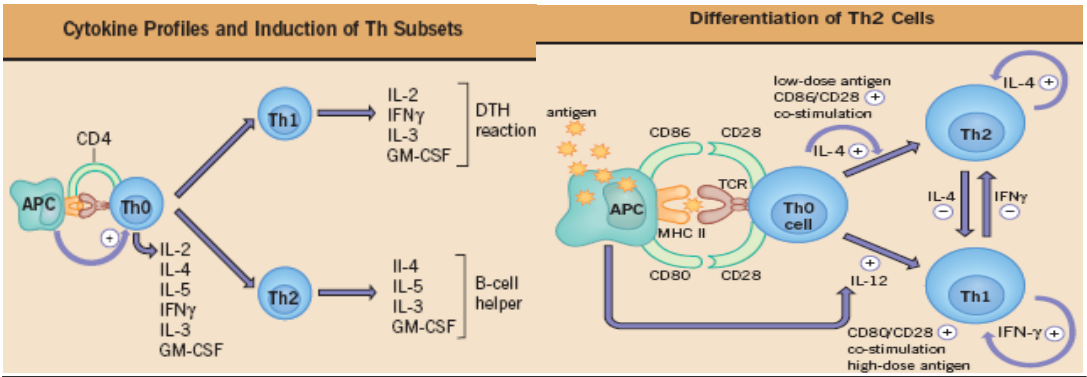



**Figure 1.7 Cytokine profiles and induction Th subsets and Figure 1.8 Differentiation of Th2 cells:** Differentiation of T cells from naive cells to Th1 and Th2 subsets. The secretion profile is responsible for denomination. The major cytokine of Th1 cells (IFN-γ) inhibits the production of Th2 cytokines (IL-4) and vice versa. (This figures were published in the book Allergy, Volume 3, Stephen T Holgate, Martin K Church, Lawrence T Lichtenstein, Page 318 and 333, Copyright Elsevier 2006 (Holgate, Church et al. 2006))

T cells are divided into different subsets, according to their interleukin secretion profile and due to their surface markers (see Figure 1.7). The differentiation from virgin T cells to memory T cells takes place after primary antigen exposure. Their function is to store antigen information to stimulate the secondary phase immune response. After secondary exposure they turn into mature T cells, acting as potent regulators and effector cells in inflammatory processes (Holgate, Church et al. 2006).

CD4+ surface receptors are expressed by T helper 0 (Th0) (secreting IL-2, IL-4, IFN-γ, IL-3), Th1 (IL-2, IFN-γ, IL-3) and Th2 cells (IL-4, IL-5, IL-6, IL-10, IL-13). Th1 and Th2 polarization, known as the Th1/Th2 hypothesis, is still a model for describing the origin of delayed-type of hypersensitivity versus allergic disorders, although the hypothesis has still a lot of flews in it (Steinman 2007). The major function of CD4+ T cells is antigen presentation and secretion of modulating interleukins, which drive specific responses such as antibody production. A critical point of allergic diseases, as mentioned above, is the production of IgE antibodies. T helper cells release mediators like IL-4, which are suspected to stimulate the immunoglobulin switching to IgE in naïve B cells. In asthmatic patients, the number of CD4+ cells was increased in the bronchoalveolar lavage (BAL) and decreased in the peripheral blood, suggesting the influx of T cells into lung tissue during inflammation (Kay 2006). Summarizing this data, it appears that T cells act as critical attendants in the pathogenesis of allergic asthma by stimulating B cells and by secreting cytokines which amplify the inflammatory response.

CD8+ T cells were originally described as “suppressor” cells and are now known as typical MHC I restricted antigen recognition cells, mediating cytotoxity. They play an essential role in targeting neoplastic and virally infected cells expressing novel antigens. Regarding asthma, the role of CD8+ cells is not clear, but it appears that they contribute more to the late-phase via Th1-like mechanisms in driving tissue damage. Some recent findings show that the secretion of IFN-γ by CD8+ cells influences the Th2 cytokines in the pathogenesis of asthma in young people (Ying, Zhang et al. 2006).

Mediators of allergic response

Allergic inflammation is like all other inflammation processes a combination of different elements such as cells and mediators. The interesting thing about mediator substances is that they stimulate and modulate the different pathways of cellular actions. Consequently they gain interest due to the possibility to interfere in these actions via inhibiting those substances with therapeutic agents or stimulate others to modify the reactions of the complex systems in inflammation. A lot of new and old therapeutic agents aim at receptors of these substances and thereby influence the allergic process.

Additionally, they represent the key point of switching from early-phase reactions to late-phase response, as they mediate the recruitment of late-phase cells like eosinophils, basophils and T cells in allergic disorders. In that case it is necessary to differentiate between early-phase and late-phase mediators. The typical early-phase substances are histamine, cysteinyl leukotrienes (LTC4, LTD4) and prostaglandins (PGD2), nevertheless promoting also the perpetuation of disease into late-phase (Holgate, Church et al. 2006).

Histamine

The major mast cell product in the first response to IgE cross-linking is histamine. It is released with other substances like PGD2 and LTC4. Histamine mediates its effects (bronchoconstriction, vasodilatation, capillary leakage) through special receptors ($H_1$-$H_4$), whereas the $H_1$ receptor is responsible for the contraction of airway and gastrointestinal smooth muscle, hypotension, pruritus, pain, vascular permeability and tachycardia.

Therapeutic intervention with antihistamines showed that histamine plays a major role, although not all symptoms were attenuated. Other experiments elucidated that histamine, together with leukotrienes, plays an essential role for bronchoconstriction in the lower airways in asthma (Holgate, Church et al. 2006).

Leukotrienes

In the production of cysteinyl leukotrienes the 5-lipoxygenase plays a major role due to oxidation from arachidonic acid. The first step of synthesis is mediated by the phospholipase A2, which separates arachidonic acid from phospholipid. The 5-lipoxygenase then generates the leukotrien $LTA_4$, a prestage of $LTC_4$ and $LTD_4$. $LTC_4$ and $LTD_4$ are known under the denomination cysteinyl leukotrienes, also designated as slow-reactive substances of anaphylaxis (SRS-A). Mast cells, eosinophils and alveolar macrophages have the enzymatic structure to produce these leukotrienes. The degradation product of $LTC_4$, $LTD_4$ is $LTE_4$, which is less active but measurable in urine after secretion in active inflammation. Increased $LTE_4$ levels have been found after allergen challenge in the early-phase, reflecting the importance of leukotrienes in early-phase response.

The major sources of these mediators are activated mast cells, although eosinophils take over the production in the late-phase. The main effects of leukotrienes are bronchoconstriction (resolving after 1-2 hours), elevation of microvascular permeability and mucus secretion, all of them being associated with the early-phase response to allergen exposure (Holgate, Church et al. 2006). LT´s act via their receptor $CysLT_{1,}$ which is expressed by eosinophils, mast cells, macrophages, interstitial cells and smooth muscle cells. Antagonists like montelukast selectively block $CysLT_1$ and therefore attenuate asthma symptoms in the early-phase and surprisingly also in the late-phase, showing the interesting role of LT`s in allergic inflammation (Biernacki, Kharitonov et al. 2005).

Prostaglandin D2

This crucial mediator has gained the interests of scientists in the last years due to its role in initiation and perpetuation of allergic diseases, acting as a messenger substance interfacing early- and late-phase response (Schratl, Royer et al. 2007). PGD2 is derived from arachidonic acid by the cyclooxygenases (COX`s) and the PGD2-synthase. The unstable intermediates like PGG2 and PGH2 are then processed to PGD2 by the PGD2-synthase. The major source of production is the activated mast cell during allergic response, but other cells like Th2 cells or dendritic cells produce PGD2 at levels far lower (Pettipher, Hansel et al. 2007). The mechanism of mediating inflammation and modulating cells is due to different receptor types, leading to its biological effects (see Fig.1.9).

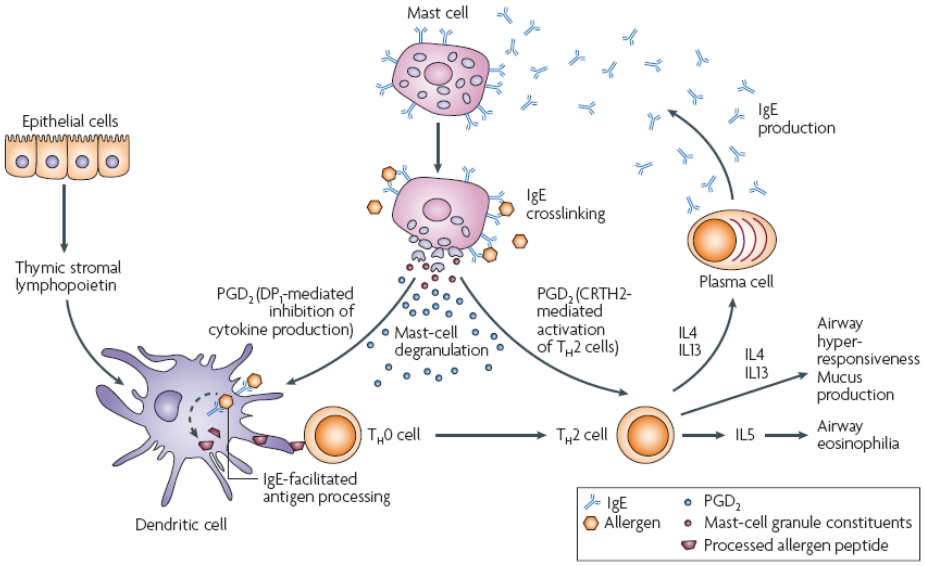



**Figure 1.9 PGD2 effects:** PGD2 production of mast cells leads to DP1 and CRTH2-mediated Th2-polarization. IgE cross-linking on the mast cell surface leads to degranulation of mast cells. PGD2 stimulates DP1 on dendritic cells and inhibits the cytokine production, which promotes Th1 cells (IL-12). Therefore Th2 cells are stimulated and produce IL-4, which again stimulates plasma cells to secrete IgE. (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery (Pettipher, Hansel et al. 2007), copyright 2007)

The two receptors of PGD2 are denominated d-type prostanoid DP1 and DP2 (also called chemoattractant receptor-homologous molecule expressed on Th2 cells, CRTH2) (Pettipher, Hansel et al. 2007). Various effects of PGD2 (vasodilatation, recruitment of eosinophils and Th2 cells, modulating Th2 cytokines and bronchoconstriction) are mediated by these receptors and the contribution to allergic disorders is very complex and sometimes paradox. DP1 receptors are distributed in the bronchial smooth muscle, the vascular smooth muscle, Th1 cells, dendritic cells, platelets and the central nerve system (Pettipher, Hansel et al. 2007).

Therefore, DP1 mediates vasodilatation and bronchodilatation in airways and contributes to increased nasal blood flow after allergen exposure. Interestingly, showing these bronchoprotective properties, DP1 deficient mice show a diminished response to low dose antigen exposure and reduction of Th2 cytokines, eosinophil presence, mucus production, representing a crucial but paradox role in allergic response.

It seems that DP1 has an influence in regulating Th2-dependent airway inflammation and it is presumed that its distribution is provides the answer. As mentioned above, DP1 receptors are expressed by dendritic cells and Th1 cells. Activation of DP1 leads to inhibition of cytokine production in these cell types (Pettipher, Hansel et al. 2007). Apart from that, DP1 activation doesn`t affect Th2 cytokine production. Hence, scientists assume that inhibiting Th1 cytokines via DP1 leads to Th2 cytokine production, because of the contradistinctive role of Th1/Th2 cytokines. So on the one hand PGD2 has an pro-inflammatory role in allergic diseases via Th2 induction, on the other hand PGD2 inhibits Th1-dependent mechanisms, indicating an anti-inflammatory role in the delayed-type of hypersensitivity (Trivedi, Newson et al. 2006). PGD2 is produced by mast cells predominantly after allergen exposure, showing its importance in the early-phase response. The distinctiveness is shown by the interface between early-phase and late-phase response due to the fact that the CRTH2 receptor is distributed in various immune cells like Th2 cells, eosinophils and basophils. PGD2 activates CRTH2 and stimulates the recruitment of these cell types and therefore perpetuates the pathophysiology of allergic inflammation (see Figure 1.9).

CRTH2 is responsible for the respiratory burst and degranulation of eosinophils, induction of pro-inflammatory cytokines in Th2 cells and as mentioned above, the general chemotaxis of effector cells in allergic asthma (Schratl, Royer et al. 2007). Apart from that, CRTH2 mediates the independent release IL-4, IL-5 and IL-13 (Xue, Gyles et al. 2005) in Th2 cells. Investigations on CRTH2-effects have been supported by the discovery of effective antagonists and agonists, which illuminated CRTH2 role in inflammation response. For example, selective agonists of CRTH2 and PGD2 enhanced in vivo allergic responses (Spik, Brenuchon et al. 2005). This encouraging data is now in the spotlight of scientists because of the possibility of intervention with antagonists, which could inhibit the CRTH2 mediated inflammation response. Clinical trials with Ramatroban, a non-selective antagonist of CRTH2, in asthmatic patients, showed an inhibited airway hyperresponsiveness to metacholine, although this effect was attributed to antagonism to another PGD2 receptor named thromboxane receptor (TP) (Aizawa, Shigyo et al. 1996).

Interestingly, the two distinct receptors DP1 and CRTH2, although having some opposing actions, work together in promoting allergic responses via Th2 cells. DP1 activation stimulates Th2 cytokine production and CRTH2 stimulation promotes the recruitment of Th2 cells to target tissues. Therefore it could be interesting to inhibit both pathways in combined experiments (Pettipher, Hansel et al. 2007). The third PGD2 receptor is, as mentioned, the thromboxane receptor, which is expressed by bronchial and vascular smooth muscle cells and platelets. Therefore activation leads to potent bronchconstriction, overlaying the DP1-mediated bronchodilatation effect of PGD2, vasoconstriction and platelet aggregation. This effect is also antagonized with Ramatroban and other selective antagonists (Magnussen, Boerger et al. 1992). Taken together, it is now clear that PGD2 acts as a potent promoter and regulator of allergic responses by polarizing Th2 cells. Its receptors, CRTH2 and DP1, mediate the various effects and therefore represent a therapeutic target. There is a growing number of potent and selective CRTH2 antagonists and results of clinical trials will show their benefit in the treatment of allergic diseases (Pettipher, Hansel et al. 2007).

Cytokines

When we take a look at the mechanism of immunological disorders, we recognize the strong influence of mediators in the whole regulation of processes. As I mentioned above, cytokines play a crucial role by mediating cell recruitment, activation and regulation. There are many different types of cytokines and the following is just an overview.

Cytokines are extracellular signaling molecules with one important function: regulation of inflammatory processes via binding to specific cytokine receptors on the cell surface. They mediate this function by paracrine (stimulation of neighbor cells), autocrine (self-stimulation) and endocrine (distant cells in other organs) effects. Thus, they orchestrate the initiation and perpetuation of immune-mediated diseases like asthma. The major problem for pharmacologists and immunologists in the past was that the complex interaction of cytokines was barely understood.

Nowadays, after investigations concerning the function of single cytokines, it seems: Inhibition of one cytokine could be the key to therapeutic achievement, as we see it in TNF- inhibiting substances, but pro and anti-inflammatory properties of just one single cytokine make therapeutic intervention very difficult and represent the redundancy of cytokines in inflammation (Holgate, Church et al. 2006). Some important cytokines in allergic inflammation are:

IL-1

This cytokine induces upregulation of adhesion molecules and therefore promotes leukocyte recruitment to target tissues. High levels were found in asthmatic airways, but IL-1 antibodies were disappointing in clinical trials (Holgate, Church et al. 2006).

IL-4

IL-4 plays a central role in allergic disorders, because of mediating the IgE-switch, Th2 polarization and adhesion molecule expression. IL-4 antibodies did ameliorate BHR and inhibited eosinophil recruitment to lung tissue in mouse models, but did not show a clinical benefit in trials. IL-4 mediates its biological effects via the IL-4 receptor, which is expressed by a lot of immune cells (Holgate, Church et al. 2006).

IL-5

Beside other chemotactic factors for eosinophil recruitment, IL-5 plays a main role in eosinophil response. Antibodies to IL-5 reduced eosinophils in sputum and blood by over 95% and in airways by over 55%, because of their role in promotion of growth, differentiation and survival of eosinophils. Therefore, antibodies gained interest in therapy (Holgate, Church et al. 2006).

IL-10

This cytokine is a crucial factor in immune-mediated disease, due to the fact that it has anti-inflammatory properties via inhibiting synthesis of TNF, IL-5 and other pro-inflammatory cytokines(Holgate, Church et al. 2006). IL-10, secreted by so-called T regulatory cells (CD4+, CD25+), inhibits Th2 cytokines production and airway hyperresponsiveness and therefore attracts interest regarding therapeutic intervention, also because of its induction of antigen-specific T cell tolerance. Perhaps IL-10 plays a key role in the mechanism of specific immunotherapy (Akdis, Blesken et al. 1998).

IL-12

IL-12 was first thought to be associated with IL-2, synergizing its effect of increasing cytotoxic lymphocyte responses and inducing IFN-γ synthesis. IL-12 is secreted by monocytes, macrophages, B lymphocytes and dendritic cells. It inhibits IgE production via the IL-4 pathway and may play an important role in inhibiting inappropriate IgE synthesis (Chung 2001). The administration of IL-12 antibodies to asthmatic patients had severe side-effects like cardiac arrhythmia and therefore may not be a target for asthma therapy (Holgate, Church et al. 2006).

Chemokines

Chemokines are involved in several immune responses and play an essential role in the chemoattraction of immune cells. They consist of low molecular weight proteins expressed by different immune cells and tissues.

The classification is due to their consistence of cysteine and amino acid, whereas C is cysteine and X stands for amino acid. They are divided into four groups: CXC, CC, C and CXXXC (also CX3C).

The CC chemokines are expressed on the typical allergic cell types such as eosinophils, basophils, lymphocytes, macrophages. CXC chemokines are expressed by neutrophils (Holgate, Church et al. 2006).

The induction of chemokine release is stimulated by bacterial products, IL-1, TNF-α and viral infections within one hour and they represent a major link between unspecific and specific responses (by activating T cells).

The production of chemokines by immune cells and tissues such as the airway epithelium leads to gradient of concentration between airways (high) and blood vessels (low). This stimulates the immigration of effector cells to the target lung tissue by changing the affinity of adhesion molecules from low affinity to high affinity, mediating the adhesion process and therefore the inflammation response. Thereby, several chemokines bind to different receptors and mediate their chemotactic function as well as hematopoiesis, angiogenesis and cell growth. These receptors are also divided into CC chemokine receptor CCR (expressed by macrophages, eosinophils, basophils) and CXC receptors CXCR, expressed by neutrophils and lymphocytes (Holgate, Church et al. 2006) (see Fig. 1.10).

The role of chemokines in allergic inflammation, especially CC chemokines, is to attract important inflammatory cells like eosinophils, basophils, and lymphocytes. The possibility of using chemokine receptors as a target for modulating the inflammatory response has the interest of scientists. Of special interest is the CCR3 receptor due to its expression by many immune cells such as eosinophils, basophils and activated Th2 cells.

Different chemokines activate CCR3 on eosinophils (eotaxin-1, eotaxin-2, eotaxin-3, macrophage inflammatory protein 1 MIP-1, regulated on activation normal T cell expressed and secreted RANTES) and mediate the eosinophil infiltration into airways in allergic asthma (Holgate, Church et al. 2006).

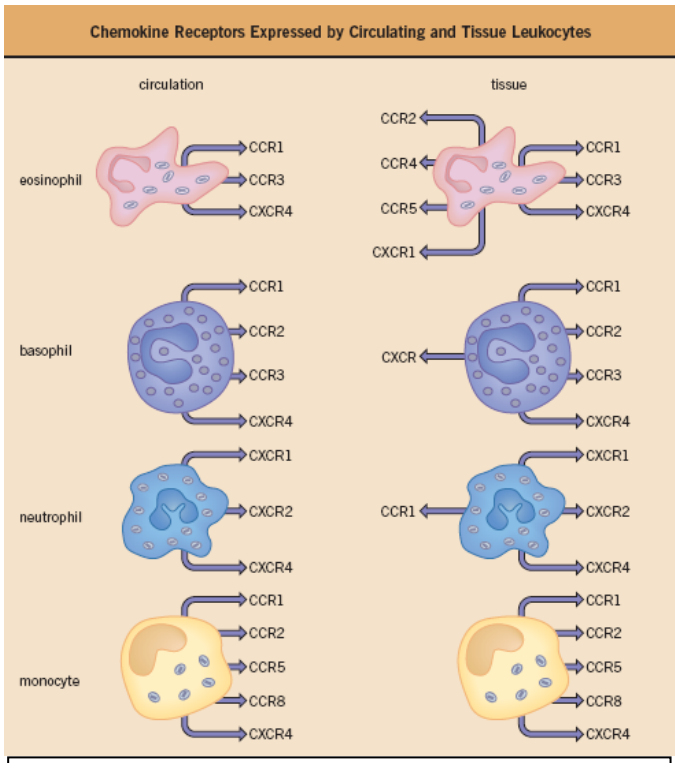



**Figure 1.10 Chemokine receptors expressed by circulating and tissue leukocytes:** The different types of expressed receptors CXCR and CCR by inflammatory cells. (This figure was published in the book Allergy, Volume 3, Stephen T Holgate, Martin K Church, Lawrence T Lichtenstein, Page 340, Copyright Elsevier 2006 (Holgate, Church et al. 2006))

In several mouse models of CCR3 antagonists, a beneficial role has been shown by inhibiting eosinophil recruitment to lung tissues, whereas the clinical benefit of this effect is to be investigated in clinical trials. Taken together, chemokines play an essential role in allergic diseases, but the impact of inhibiting only these pathways is not clear and thus still remains in the focus of immunological research (Holgate, Church et al. 2006).

## Methods and Materials

Measurement of airway hyperresponsiveness in conscious animals

In my thesis, I used a relatively new system to measure airway hyperresponsiveness (AHR) in conscious animals, especially mice and rats. AHR is related to elevated IgE production and eosinophil lung infiltration. Therefore AHR represents a good parameter to evaluate bronchial hyperresponsiveness and asthmatic responses to allergen challenge in mice (Hamelmann, Schwarze et al. 1997).

In the past, there were invasive measurements, which were timeconsuming and technically demanding. The new model of measurement is a noninvasive whole body plethysmography (WBP) in conscious, unrestrained, and nonanaesthesized animals using the $P_{enh}$ parameter (enhanced pause, explanation follows).

Concept of WBP from BUXCO Systems

The measurement in the WBP is an addition of different changes in air volume in the chamber. Two processes cause an air volume change in the chamber with an animal: The first process is the heating and humidification of the air after coming into the lungs of the mouse, also called conditioning ($\Delta V_C$), the second is the resistance of the air stream ($\Delta V_R$). Like Drorbaugh and Fenn analyzed in 1955 that the inspiration of the animal leads to an expansion of the thorax and the air gets heated and humidified in the lungs by body temperature and saturated by temperature (Drorbaugh and Fenn 1955). The increase of the volume due to expansion of the chest is greater than the volume of the air removed from the chamber during inspiration (Lomask 2005).

In experimental conditions the temperature is 23°C and humidity is 40%, so that the tidal volume can be computed with the following equation (Lomask 2005) (Figure 2.0):

$$P_b \ \alpha \ \Delta V_c = V(T,H) - V$$

$$= V\left[\frac{T_a(P_{atm} - \rho_c)}{T_c(P_{atm} - \rho_a)} - 1\right]$$

$T_c$ = Chamber Temperature
$H_c$ = Chamber Humidity
$T_a$ = Animal Temperature
$H_a$ = Saturated Humidity at Body Temperature
$\rho_c$ = Vapor Pressure in Chamber
$\rho_a$ = Vapor Pressure in Animal
Ta and Tc are in ° Kelvin.

At end inspiration, $\Delta V_C = K_c V_T$

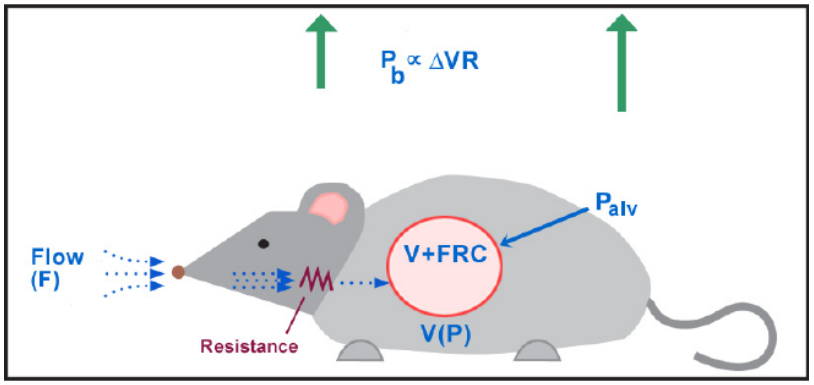
where $V_T$= tidal volume.

**Figure 2.0 Tidal volume equation:** Heating and humidification of inspired air creates a change in volume and pressure in the chamber ($\Delta V_C$); Pb = pressure in the box.(Lomask 2005) (Published with permission of Morton Lomask, copyright Lomask 2005)

The changes between temperature and humidity in expiration are smaller, therefore the conditioning is less. The expired air has body temperature in the lungs during expiration process, temperature decreases and leaves the nose with approximately 30°C (Lomask 2006).

The second process of air volume changing in the chamber is the resistance of the air stream. The air stream follows physical laws as it passes the lung, like Ohm`s law. R=V/I represents the major effect on the air stream, whereas R= resistance, V=Voltage, I= Current. Hence, resistance gets high, if the voltage is high or the current is low. This reflects the point as we need it in allergic disorders, where the current lowers down because of bronchoconstriction and the production of mucus, leading to barrier in the airways (Lomask 2005).

The whole inspiration process follows a complex system of different actions: The air stream follows a negative pressure, thus the thorax expands and creates a negative alveolar pressure $P_{alv}$, to pull air in through the airway resistance (Lomask 2006, Lomask 2005) (see Figure 2.1).

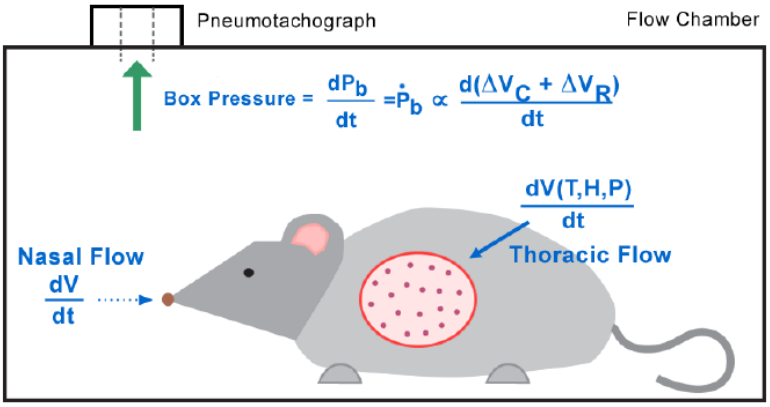



$$P_b \propto \Delta V_R = K_r \times P_{alv} \times (FRC + V) = K_r \times R \times F \times TGV$$

**Figure 2.1 Measurement in the WBP:** The measurement of the airflow (F), Resistance (Ohm´s Law) and the TGV ( inspired volume and functional residual capacity FRC) is leading to the change in volume ($\Delta V_R$).(Lomask 2005) (Published with permission of Morton Lomask, copyright Lomask 2005)

Negative pressure ($P_{alv}$) in the lungs expands the air, residing in the lungs, denominated thoracic gas volume (TGV). Volume change is directly proportional to the product of $P_{alv}$ and TGV and therefore by applying Boyle`s Law, it is possible to compute the change in volume $\Delta V_R$, $\Delta V_R = P_{alv} * TGV / (P_{atm}-P_a)$ (Lomask 2005).

Putting together the two processes, the volume change and therefore the pressure change depends on $\Delta V_C$ and $\Delta V_R$, but due to the fact that $\Delta V_C$ is 0.02 ml and $\Delta V_R$ is 0.002 ml, computed by BUXCO Systems, pressure change is denominated by $\Delta V_C$ (Lomask 2006). This pressure change is now used to measure the volume change (as computed above) and is represented in the parameter PWBP (Pressure in the box or $P_b$, pressure whole body plethysmograph). The PWBP shows the net volume change due to respiration (Lomask 2006, Lomask 2005). Another parameter in the chamber measurements is the flow whole body plethysmograph (FWBP). It measures the net volume change in relation to time ($P_b / \Delta t$). Measuring the difference between thoracic displacement and nasal air volume outcome is reflected in $P_b$. The mathematical relationship between the two plethysmographs is shown in Figure 2.2 (Lomask 2006, Lomask 2005).




$$\dot{P}_b \;\alpha\; \Delta\dot{V}_c + \Delta\dot{V}_R = K_c\frac{dV}{dt} + K_r\frac{d[P_{alv}\times(FRC+V)]}{dt}$$

**Figure 2.2 Measurement in the FWBP:** In the FWBP there is a pneumotachograph in the chamber and measures the PWBP in relation to time.(Lomask 2005) (Published with permission of Morton Lomask, copyright 2005)

*"The conditioning component in the FWBP is the derivative of the conditioning component in the PWBP. It is now proportional to the animal's flow, rather than to its inspired volume. And likewise the resistance component in the FWBP is the derivative of the resistance component in the PWBP. In the FWBP, two things happen at end inspiration. Firstly, the conditioning component, which is now proportional to flow, is zero. (This is very different from the PWBP, where the conditioning component is a maximum at end inspiration.) Conditioning, which may be dominant in other regions of the FWBP waveform, is minimal at the zero flow crossings. The box pressure of the FWBP now is proportional to R x TGV x ΔF/Δt. Since $P_{alv}$ = R x F, ΔPb/Δt may also be expressed as TGV x $\Delta P_{alv}/\Delta t$ at the flow zero crossings. Secondly, ΔF/Δt is typically large at the zero crossings. (This does not apply to treatments, exposures, or challenges which induce a pause at end inspiration). The transition from an inspiratory to an expiratory thoracic flow in the mouse from –1 ml/sec to +1 ml/sec, may take 10 milliseconds, producing a value for ΔF/Δt of 200 ml/sec/sec. Thus at flow zero crossings, not only is the conditioning term small, but the compressive term is greatly enlarged. In the region of the animal's zero flow crossings, the resistance component of the FWBP waveform dominates, even if conditioning dominates in the rest of the waveform.*

*Understanding this separation of regions is crucial in the controversy over the application of the FWBP to airway reactivity measurements.*

*Those who claim that the WBP waveform is dominated by conditioning, and therefore cannot be used as the basis for resistance information, have not recognized that resistance dominates in the axis crossing regions of the FWBP waveform." (Lomask 2005)*

Waveforms

Information about the different volumes and measurements is processed as waves and therefore show the development of airway conditions (Lomask 2006, Lomask 2005) (see Figure 2.3).

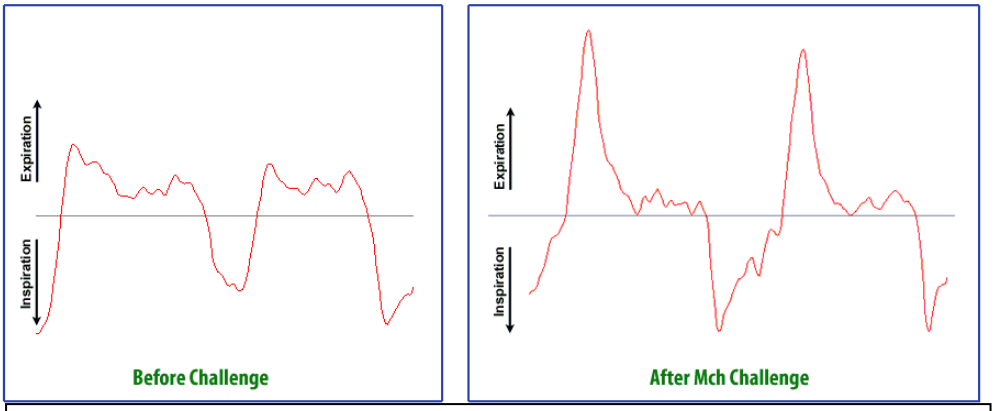
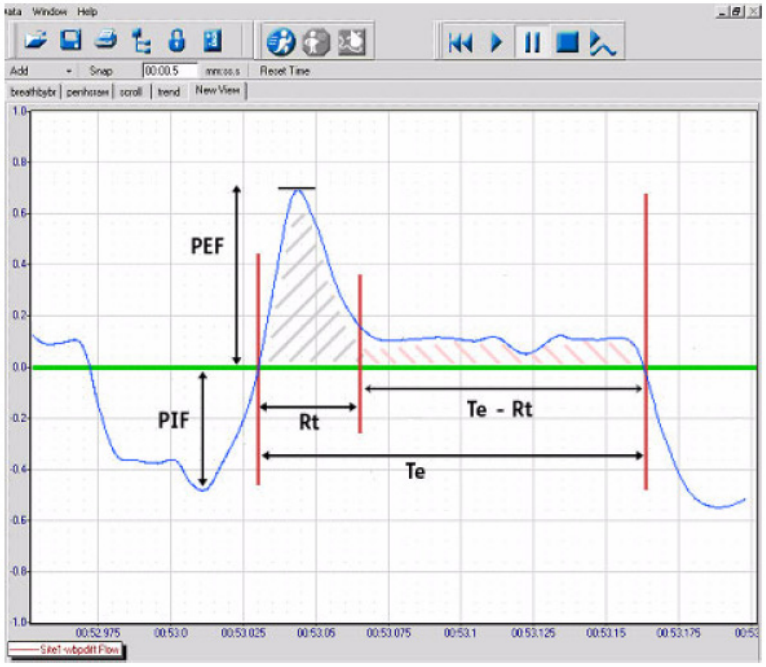



**Figure 2.3 Waveforms:** The challenge of mice with metacholine (a potent parasympathomimetic, and thus a bronchoconstrictor) shows different waveforms. After challenge the peak is much higher. (Lomask 2005) (Published with permission of Morton Lomask, copyright Lomask 2005)

The FWBP waveform displays a flow, which is produced by chest movement and nasal flow. Thus, it is necessary to compare these two signals, to know how they combine in the waveform. After constriction, the nasal flow lags the thoracic flow, due to the efforts of the rib cage and the nasal flow following the whole process (Lomask 2006). *"The time delay between the nasal flows and the thoracic flows at the zero flow crossing is related to specific airway resistance, sRaw (product of airway resistance and TGV)" (Lomask 2005)*

Penh (enhanced pause)

After recognizing that the early expiratory peak in the FWBP waveform is dominated by resistance, it should be possible to measure resistance by these waveforms. Waveforms can be divided into the early region, which shows the transition from inspiration to expiration, and the late region. Rt (Figure 2.4) shows the early region and therefore represents 65 % of the expiratory area, whereas the late region shows 35 %. This setting, whereby Rt is controlled by the operator, produced good correlations between Penh and resistance (Hamelmann, Schwarze et al. 1997). *"Penh and Pause quantify the degree of resistance by comparing the average height of the early region to the average height of the late region. Penh and Pause are nondimensional. It is important to understand that the term "Pause" is not apneic pause. "Pause" is a term used to describe the plateau-like appearance of the late expiratory region of the FWBP waveform during constriction. It is not a period of zero animal flow. (Lomask 2005) "*




PIF = Peak Inspiratory Height
PEF = Peak Expiratory Height
Te = Expiratory Time
Rt = Time to expire 65% of the "volume"
Pause = Te/Rt-1
Penh = PEF/PIF x Pause

$$Penh = \frac{PEF}{PIF} \times \left( \frac{Te}{Rt} - 1 \right)$$

**Figure 2.4 Penh as a marker of resistance.** (Lomask 2005)
(Published with permission of Morton Lomask, copyright 2005)

In figure 2.4, Pause is expressed by Te/Rt-1. Therefore, after constriction of the airways, the waveform in the FWBP changes into an emphasized early expiratory peak now configuring a larger percentage of the whole expiratory area showing an increase of the Pause (Lomask 2006). *"The ratio of peak expiratory height/peak inspiratory height also responds to constriction. In the expiratory phase of the FWBP waveform, conditioning is less dominant than in the inspiratory phase; hence changes in constriction will be more visible in the expiratory phase. The addition of the PEF/PIF factor enhances the response of Pause to constriction. The product PEF/PIF x Pause is defined as Penh. (Lomask 2005)"*

In animal models of constriction in the airways, the bronchoconstriction affects the waveform of the FWBP, building a peak in the early expiratory phase. The prominence of peak correlates with the degree of constriction, and Penh visualizes the peak as a manifestation of specific airway resistance (sRaw). Therefore it can be used as a valid marker for airway responsiveness to allergen challenge in mice (Dohi, Tsukamoto et al. 1999).

Animals

Male BALB/c mice were obtained from the Institute Oddelenie Toxikologie Chown Laborato'rnych Zvierat UEF SAV at Dobra Voda, Slovakia. The protocol for ovalbumin sensitization and lung function measurement has been approved by Animal Experiment Committee of the Austrian Federal Ministry of Science and was used in the laboratory. An animal license for the current study was applied.

Study protocol

BALB/c male mice were divided into four groups (n=10-19): control + CAY, control + vehicle (no active agent), OVA + CAY and OVA + vehicle. Controls were not sensitized, but received the same amount of saline in alum. Group OVA + CAY and OVA + vehicle were sensitized with ovalbumin in alum (20 µg OVA emulsified in 173µl aluminum hydroxide Al $(OH)_3$ (Alum inject; Pierce Chemical, Rockford, Ill; USA) intraperitoneally on days 0, 7. The groups control + CAY and control + Vehicle were sham-sensitized with the same amount of saline in alum.

On days 20-27, the mice of the groups OVA + CAY and control + CAY were treated twice daily with CAY10471 (CAY, CRTH2 antagonist) 10mg/kg orally by a feeding tube while the two other groups received a vehicle instead of CAY. Aerosol challenge with ovalbumin (OVA) (1%) was performed on the two OVA groups on days 21, 23 and 25 by ultrasonic nebulization. The two other groups were treated in the same way, except that they breathed aerosolized saline instead of OVA (1%). Airway challenge was performed with a chamber, which the mouse was put into. Then OVA (1%) was aerosolized by a nebulizer and delivered continuously into the closed chamber. On day 26 measurement of the airway hyperresponsiveness in unrestrained animals was performed by using WBP (Buxco, Troy, N.Y., USA, comment follows). On the next day, measurement of resistance with the flexiVent ventilator was performed (Scireq, Montreal, PQ). Mice were ventilated with a tidal volume of 8ml/kg at a rate of 450 breaths per minute after having been anesthetized with pentobarbital 50mg/kg intraperitoneally. Increasing concentrations of metacholine were used from 0-100 µg/ml being applied via aerosol. Thereby, airway hyperresponsiveness was quantified via measuring resistance. After the resistance measurements, the mice were killed and BAL/blood samples were performed (see Fig. 2.5).

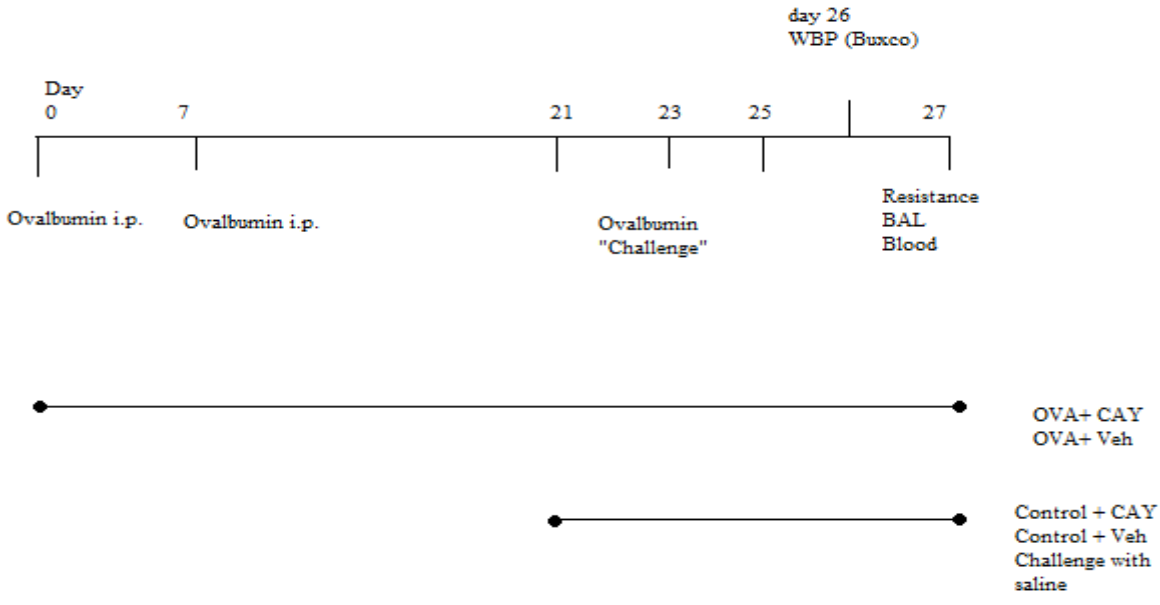



**Figure 2.5 Study protocol:** OVA groups were immunized with ovalbumin on days 0 and 7 and then challenged with ovalbumin as aerosol on day 21, 23 and 25. Controls received saline in alum i.p. on day 0, 7 and were then challenged with saline also on day 21, 23 and 25. On Day 26 measurement in the WBP (Buxco) took place. On day 27 the mice were anaesthetized and their lung function was measured with FlexiVent. Afterwards, the mice were killed and BAL/blood samples were performed.

Before taking readings, boxes were calibrated with a rapid injection of 1ml air within a present interval of time. The injected air penetrates the chamber and leaves the chamber through the pneumotachographs in the wall of the chamber to the atmosphere. The mice were then put into the chamber and baseline readings were taken and averaged for 3 min. Thereafter, compressed air was passed through a flow meter adjusted to 2,5 ml/sec for 2,5 minutes and was then aerosolized with an integrated nebulizer. The nebulizer was filled periodically with 200 µl increasing concentration of metacholine, a potent parasympathomimetic drug mediating bronchoconstriction. The concentrations were 0,1,3,10,30,100 mg/ml metacholine. The concentration of metacholine was compared to the Penh parameter of the WBP, which is an indicator for resistance (Lomask 2006). For quantification of the concentration/Penh relation, the results of the provocation tests were expressed by concentration-response curve (see results, table 1).

BAL (bronchoalveolar lavage) and blood samples

Analysis of leukocyte numbers in peripheral blood and bronchoalveolar fluid was performed as described (van Rijt, Kuipers et al. 2004). 48 hours after the last ovalbumin challenge, mice were sacrificed by an anesthetic overdose and the blood was sampled by cardiac puncture. Bronchoalveolar lavage was performed with 3x1 ml of Dulbecco PBS (without $Ca^{++}$ und $Mg^{++}$) + 1mM EDTA (18mg/50ml) + Hepes 1:100 0,5ml filled up to 50ml buffer (basic fertilizer solution 1mM) + 5 drops NaOH filled up to 50ml buffer. The lungs were dissected, cannulated and 3x1 ml (as mentioned above) PBS were injected to scour the lungs. After massaging the lungs, the recovery was approximately 2, 5 ml. Red blood cells were lysed using ammonium chloride lysis buffer. Absolute leukocyte numbers were determined using a Neubauer hemacytometer and Kimura stain, while differential counts were obtained by a four-color flow cytometry. To this end, the cells were stained with anti-mouse monoclonal antibodies against MHCII (FITC), CD11c (APC), CD3 and B220 (CyChr), and CCR3 (PE). To prevent nonspecific binding to Fc receptors, a 2.4G2 blocking was added to the monoclonal antibody mix.

Lymphocytes were identified as cells with low forward and side scatter expressing CD3 or B220 and B cells were distinguished from T cells by MHCII expression in the B220/CD3-high gate.

Granulocytes were recognized as highly granular (high side scatter) cells with low staining for B220/CD3, and within this gate eosinophils were distinguished from neutrophils as cells expressing the eotaxin receptor CCR3. Alveolar macrophages were distinguished as large MHCII/CD11c-high cells. Monocytes were identified as MHCII-high/CD11c-low cells in the high forward scatter region (Heinemann 2006).

Chemicals

Ovalbumin (OVA) obtained from the company Sigma was an albumin from chicken egg white with grade V (minimum 98 % in agarose gel electrophoresis). Ovalbumin was used as mentioned above for sensitizing the mice by intraperitoneal injection of 100µl solution (from 20µg OVA and 173µl Al $(OH)_3$. For the OVA challenge we used 1% OVA solution in 0.9% NaCl.

Al $(OH)_3$ was used as an adjuvant to promote an immunological response via activating APCs at the site of antigen delivery (Roitt and Delves 2006).

Acetly-β-metacholine chloride was also obtained from the company Sigma and was used as a solution for WBP (Buxco) to promote bronchocontriction. The concentrations were 1, 3, 10, 30, 100 mg/ml dissolved in 0, 9 % NaCl.

CAY10471 was obtained from the company Eubio, a Ramatroban - Analog. We used the dose 10 mg/kg. The basic fertilizer solution was 20mg CAY10471 + 486µl 0.5 M $Na_2HPO_4$ warmed up until it was dissolved, then 114µl O.5 M $Na_2HPO_4$ + 400µ aqua destillata was administered. This solution was dissolved 1:20 in 0.5 % methylcellulosis and from that, 200µl (10mg/kg) were given per os with a feeding tube. The CRTH2 antagonists Cay10471 (TM30089), (+)-3-[[ (4-fluorophenyl) sulfonyl]methylamino]-1,2,3,4-tetrahydro-9H-carbazole-9-acetic acid, were prepared as described (Ulven and Kostenis 2005). Mice were anaesthetized with pentobarbital 50mg/kg. The solution was prepared with 60mg pentobarbital + 0.2ml ethylenglycol + 0.1 ml ethanol 97% filled up to 1ml with aqua destillata.

| **Flow Cytometry:** | | |
|---|---|---|
| **Antibodies** | **Company** | **Order number** |
| (PE)-labelled CCR3 | www.rndsystems.com | FAB 729P |
| (APC)-labelled CD11c | www.bdbioscience.com | 550261 |
| (CyChr)-labelled AB against CD3 (145-2C11) | www.bdbioscience.com | 553065 |
| (CyChr)-labelled AB against B220 RA3-6B2 | www.bdbioscience.com | 552772 |
| MHCII-FITC 2G9 | www.bdbioscience.com | 553623 |
| 2.4G2 blocking reagent | www.bdbioscience.com | 553141 (0,1mg)<br>553142 (0,5 mg) |

Antibody Mix (all dissolved in PBS)

| | |
|---|---|
| 2.4 G2 | 6µg/ml (blocking) |
| MHCII FITC | 2,5 µg/ml |
| CCR3 Cy-Chr | 625 ng/ml |
| CD3 Cy-Chr | 10 µg/ml |
| B220 Cy-Chr | 2 µg/ml |
| CD11c APC | 2 µg/ml |

Statistical Analysis

Statistical Analysis was performed using one-way ANOVA followed by student Newman-Keuls post-test as well as Kruskal-Wallis one-way Analysis of Variance on Ranks (Sigmastat, SPSS Inc., USA). A value of $p < 0.05$ was considered significant.

## Results

The Relationship of the Penh parameter and metacholine

All four groups of mice were taken to WBP on day 26, after getting challenged with OVA or saline on day 21, 23 and 25. Pretreatment was Ovalbumin or saline intraperitoneally on days 0 and 7. Each mouse was measured with ascending metacholine concentrations (0, 1, 3, 10, 30, 100 mg/ml, see Table 1).

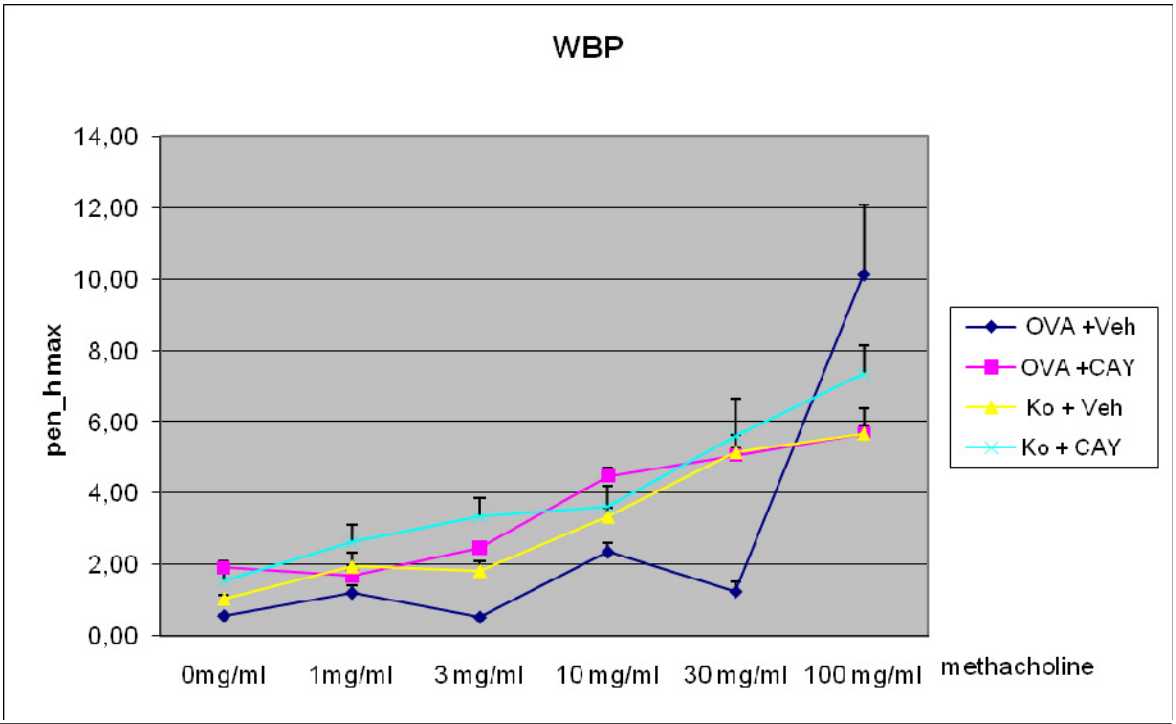



**Table 1 WBP results** Penh compared to metacholine concentration measured with the WBP (Buxco). Although the curve shows an elevated value at 100mg, the increased Penh was not significantly elevated.

Thereby, no significant difference was measured between the different groups, also showing no significant changing in the sensitized (OVA) and non-sensitized (Ko) mice. In the OVA + veh group there was an increase of Penh at 100mg/ml metacholine, but the wide range of values (standard error of the mean) showed no significant elevation compared to the controls.

Measurement of Resistance with FlexiVent

Interestingly, the results in measuring the resistance directly with FlexiVent were totally different from what expected. As shown in table 2, the OVA + Cay group had the strongest resistance from the beginning. When OVA+CAY, was compared to controls + vehicle, OVA + CAY was significantly ($p<0.05$) higher in resistance in 0, 1, 3, 10, 30, and 100 µg/ml metacholine. OVA + vehicle compared to controls + vehicle was significantly higher in 1, 3, 10 and 30 µg/ml metacholine.

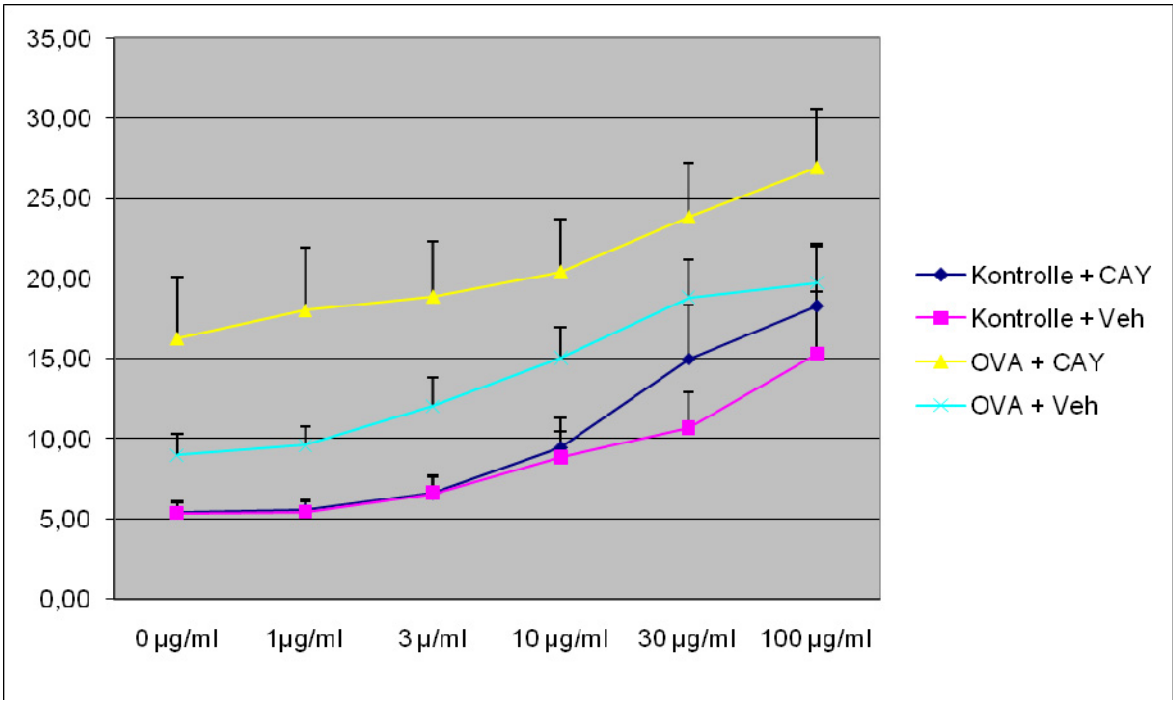



**Table 2 FlexiVent results:** Measurement of resistance with FlexiVent. Significant values were measured comparing OVA+ Cay and controls + vehicle group, and OVA+ vehicle compared to controls+ vehicle.

BAL

When comparing different groups a lot of significant values became obvious. Eosinophils were significantly increased in the OVA groups compared to the controls, but nevertheless CAY did not significantly reduce eosinophils in the BAL (OVA+CAY versus OVA+ veh). Neutrophils were significantly increased in OVA groups compared to control + vehicle. Lymphocytes were increased in the OVA groups compared to the controls.

Interestingly, macrophages were also increased in the OVA groups compared to the controls and in addition CAY inhibited macrophages significantly in the OVA groups (OVA+CAY versus OVA+ veh).

Monocytes were significantly increased in the OVA groups too, when compared to the controls (see Table 3 and 4).

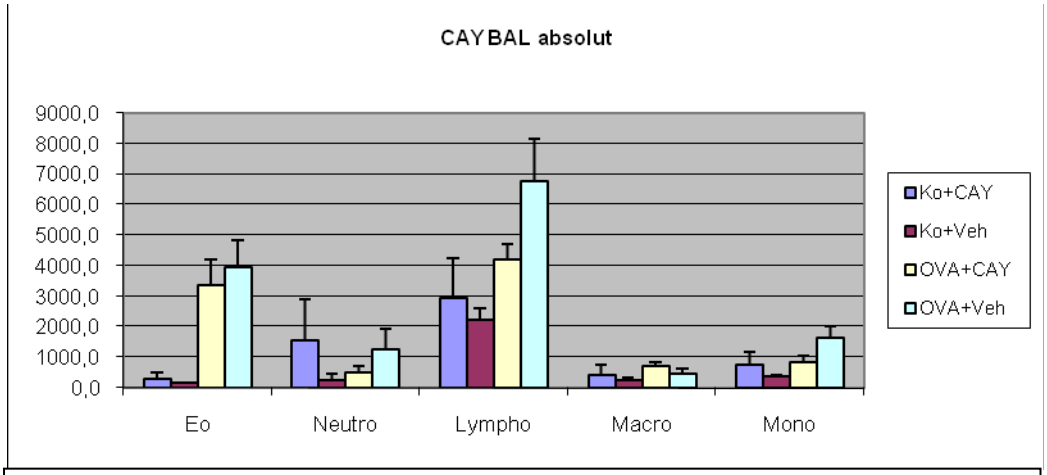



**Table 3 BAL absolute:** BAL results shown in absolute number of cells. CAY significantly inhibited macrophages only.

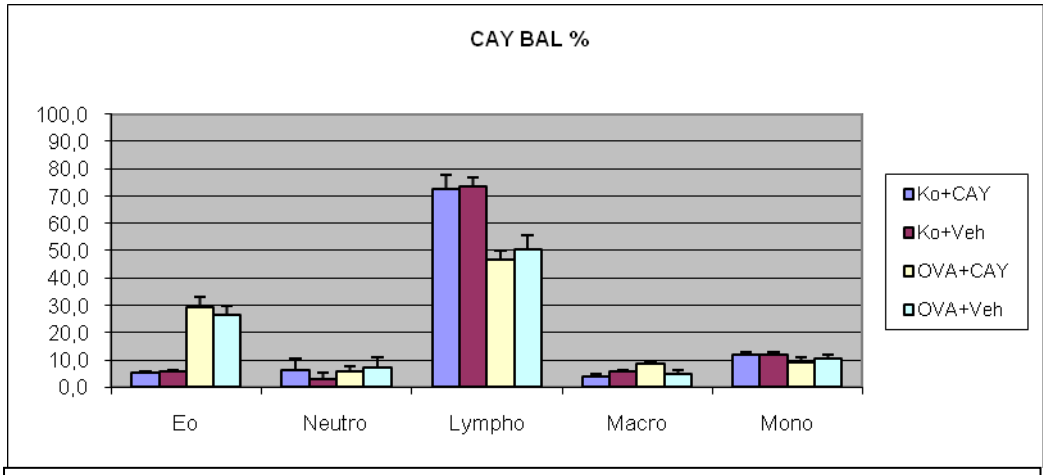



**Table 4 BAL in percentage**.

Whole-Blood

In the whole-blood samples, eosinophils were significantly increased in the OVA + Veh group compared to the controls + Veh. Macrophages were also significantly increased in the OVA+ Veh group compared to the controls + Veh. All other values were not significant.

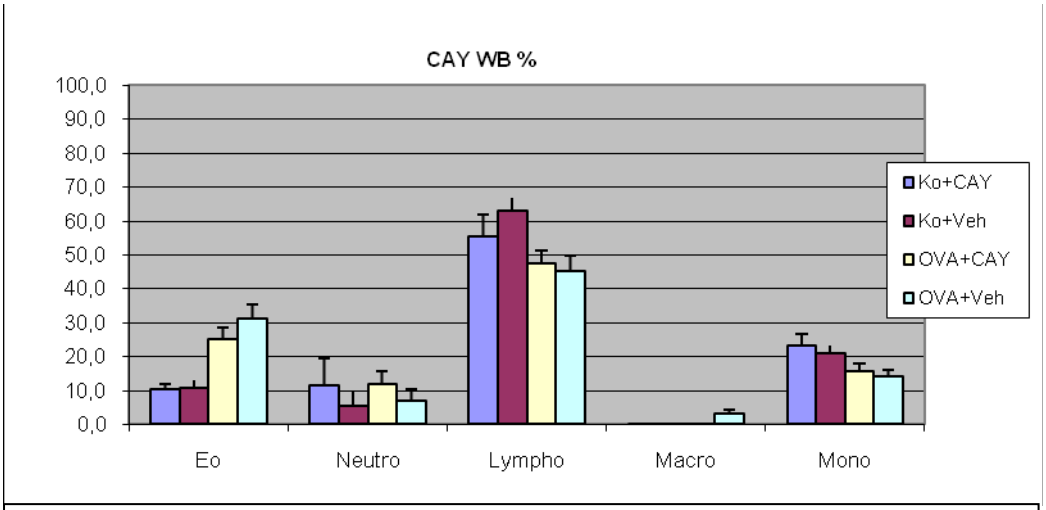



**Table 5 Whole blood sample results in percentage**.

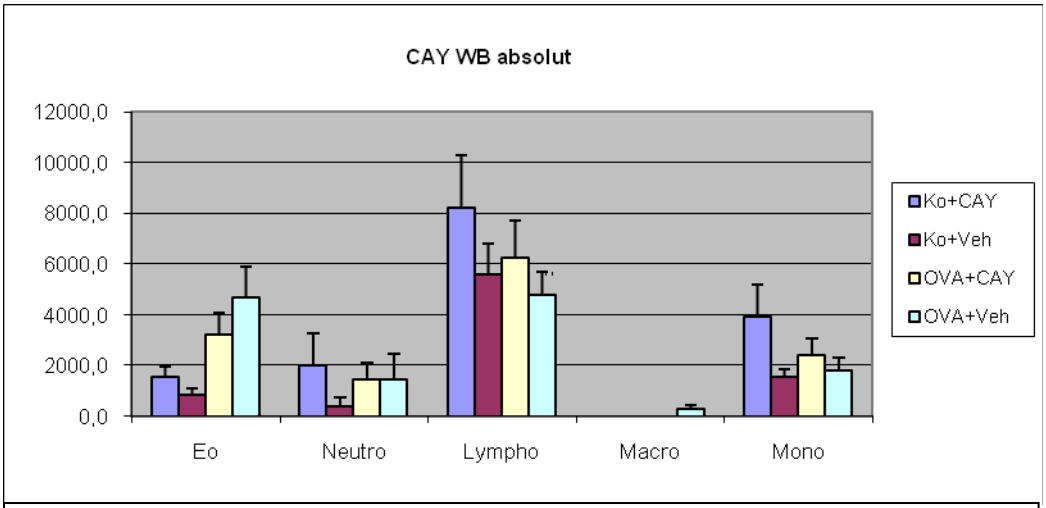



**Table 6 Whole blood sample results absolute.**

## Discussion

The results of the CRTH2 antagonist CAY10471 shown in Tables 1 to 6 were surprising. As mentioned, there was only a significant reduction of macrophages in the BAL in the CAY groups compared to the controls. The inhibitory effect of CRTH2 was not reflected in BAL and whole blood. The resistance measurement of OVA+CAY compared to controls+ vehicle showed unanticipated results (see table 2). Resistance was significantly higher in OVA + CAY than in the controls+ vehicle group. Also, comparing resistance of OVA+ CAY with OVA+ Vehicle showed higher results, but these were not significant. Resistance measurement showed a contrary result than expected due to highest airway resistance results in the treated CAY group. There has been no data published about eventual effects of pharmacokinetic metabolism of CAY, and the major question concerning the use of orally available antagonists is, if and how the metabolism changes the activity-profile of the substance. Crosignani et al. 2008 showed that a spiro-indolinone compound (CRTH2 antagonist) investigated with a structure-activity relationship (SAR) displayed that changes in a particular part of the molecule can lead to a reversal of functional activity, leading to an agonistic activity (Crosignani, Page et al. 2008). Hypothetically, methylisation and/or hydroxylation of CAY could change the molecule structure and form a new indomethacin-analog. This modification could promote an agonistic instead of an antagonistic effect of the substance and could be responsible for inducing airway hyperresponsiveness in the treated mice group, although the BAL and whole blood of the treated groups showed no increased cell numbers. Further investigations are needed to explore the pharmacokinetic effects on CAY.

Interestingly, compared to the controls, the OVA showed typical sensitization reaction: increased eosinophils, neutrophils, lymphocytes, macrophages, monocytes in BAL, and also increased eosinophils and macrophages in the whole blood sample. This reflects the response to the allergen challenge and activation of immunological cells, which tries to imitate the human disease. The intraperitoneal injection of ovalbumin, mostly together with a Th2 skewing alum, is commonly used to stimulate sensitization of the mouse to the new allergen.

After injection the mouse produces ovalbumin-specific IgE, which afterwards promotes the reaction to the ovalbumin-challenge inducing eosinophilic infiltration into the lung tissue and airway hyperresponsiveness (Kips, Anderson et al. 2003). The reaction is used to imitate the pathology of asthma and to get a proper possibility to investigate immunological disorders in airways. Mouse models of asthma show characteristic signs of histopathology such as eosinophilia and mucus cell hyperplasia (Persson, Erjefalt et al. 1997; Uller, Lloyd et al. 2006). Therefore it is common to use eosinophilia as a marker for allergic responses in mice models. Apart from that, it is very important to know that there are differences between the animal OVA-challenge model and the human disease atopic asthma. In 2008 Kumar et al reviewed the classical ovalbumin challenge in mice and the problems with mouse models, when compared to human disease. One consideration is the problem of BAL fluid measurements in challenged mice compared to human asthmatic BAL, whereby challenged mice may show 40-80 percent of eosinophils while with human BAL it is only 1-3% (Kumar, Herbert et al. 2008). Another problem is the question, of whether the number of eosinophils is related to airway hyperresponsiveness. Green et al. 2002 discovered a relationship between sputum eosinophils and asthma exacerbations. Asthma treatment strategies with the target of normalizing of sputum eosinophils reduce asthma exacerbations and admissions without using additional anti-inflammatory treatments (Green, Brightling et al. 2002). This data supports the theory of targeting eosinophils in asthma, due to the correlation between eosinophil numbers in sputum and asthma symptoms. In a recent study, the impact of lung remodeling during severe airway inflammation on respiratory mechanics has been investigated. OVA-challenged mice showed that eosinophilic and neutrophilic lung inflammation in light and electron microscopy correlated with elevated airway responses to metacholine and elevated cytokine levels (IL-4, IL-5, and IL-13) (Silva, Passaro et al. 2008). Summarizing this data, it is important to know the advantages and disadvantages of mouse models in imitating the human asthma disease. Results from a mouse model should always be seen critically, although these models provide the only possibility of investigating asthma pathology in vivo. Therefore, knowing the differences between animal models and the human disease is essential for evaluating new findings.

Conflicts in the Interpretation of Unrestrained WBP

Resistance measurements showed increased airway hyperresponsiveness in the OVA groups compared to the controls, also showing the sensitization reaction. Besides, the WBP did not reflect the measurement of resistance measured with flexiVent. In the WBP group there was no significant difference between the OVA and control groups, but in the flexiVent measurement there was a significant difference, reflecting contrary data.

The Penh parameter has been critically discussed due to its complex calculation and the possibility of having problems to correlate it with lung mechanics. Hamelmann et al. 1997 studied the mechanisms and kinetics of airway responsiveness to allergen-sensitized and allergen-challenged mice (Hamelmann, Schwarze et al. 1997). They used increasing Penh as a marker for airway obstruction and the response to inhaled metacholine in conscious, unrestrained mice after sensitization and airway challenge with metacholine. Increased Penh (correlated with airway responsiveness) was associated with elevated IgE production and eosinophil lung-tissue infiltration. Comparison of invasive pulmonary resistance measurement with Penh the day after WBP with the same mice also showed an increased response to metacholine. Pretreatment with a beta2-receptor agonist (albuterol) diminished the increased Penh value (Hamelmann, Schwarze et al. 1997). Thus: *"**The measurement of AR (airway responsiveness) in unrestrained, conscious animals provides new opportunities to evaluate the mechanisms and kinetics underlying the development and maintenance of airway hyperresponsiveness and to asses various therapeutic interventions**. (Hamelmann, Schwarze et al. 1997)"* Also, Dohi et al. 1999 proposed Penh as a marker for bronchial responses to a specific allergen and correlated Penh with eosinophilic bronchial inflammation, because there was a strong correlation between Penh and the number of eosinophils in bronchoalveolar lavage fluid ($p<0.0001$) (Dohi, Tsukamoto et al. 1999). This data suggested the possibility of using **Penh as a marker for airway hyperresponsiveness** and also for the grade of inflammation with eosinophil granulocytes.

However, Petak et al 2001 investigated the influence of hyperoxia on lung tissue and changes in lung mechanics in mice.

Thereby they discovered that after 60 hours of normobaric hyperoxia, lung tissue reacts with alveolar edema combined with increasing tissue damping and elastance. The comparison of low-frequency forced oscillations (LFOT) and WBP revealed a non-reaction of Penh regarding tissue and airway mechanical properties (Petak, Habre et al. 2001). *"This inconsistent relationship between LFOT parameters and WBP indexes suggest that the changes in the latter reflect alterations in the breathing pattern rather than in the mechanical properties. It is concluded that in the presence of diffuse lung disease,* ***Penh is inadequate for characterization of the mechanical status of the respiratory system****. (Petak, Habre et al. 2001)"*

Another investigation by Lundblad et al 2002 suggested a critical view of the Penh parameter. The group showed that the use of unrestrained whole body plethysmograpy (WBP) is only accurate if functional residual capacity and tidal volume are measured independently and the chamber gas is preconditioned to body temperature and humidity, because the major term to calculate airway resistance in WBP, Pb(t), depends on gas conditioning. When the air is preconditioned in the chamber, changes in Pb (t) are greatly reduced and compression and decompression of the gas is then also associated with lung resistance without many disturbances (Lundblad, Irvin et al. 2002). *"Our study also shows that, unless these various quantities can be either controlled or measured, UP is not likely to be of practical use as means of obtaining accurate measures of mechanical lung function.* ***This applies in particular to Penh, which we conclude should not be used as a means of assessing bronchial responsiveness****. (Lundblad, Irvin et al. 2002)"*

Adler et al 2004 examined the role of Penh and unrestrained WBP in different mouse strains. Immunized and control mice groups of BALB/c (n = 16) and C57BL/6 (n = 14) were put into a WBP chamber and ascending doses of metacholine were nebulized to induce a bronchial response and increase in resistance.

Parameters were measured while exposing mice to aerolized metacholine such as inspiratory time, expiratory time, total time, peak inspiratory pressure, pause, Penh and tidal volume. The day after, lung resistance and compliance were invasively measured in the same animals.

Thereby they found that different parameters of unrestrained WBP showed different correlations to the invasively measured resistance and that mice strain-type also had an influence on the level of correlation. For example compliance and Penh have a higher magnitude of correlation in BALB/c mice, compared to resistance measurement, than in C57BL/6 mice. Taking the parameter Penh, they showed that Penh is strain specific and behaves differently compared to BALB/c and C57BL/6 mice. Nevertheless, the Penh parameter correlates with the invasively measured resistance in BALB/c mice and can be therefore used for resistance measurement, although the relationship to other parameters like expiratory is similar for both strains (Adler, Cieslewicz et al. 2004). *" We conclude, therefore that Penh is not special compared with other more easily interpretable quantities derivable from Pb and that it is* ***inappropriate to use UP (unrestrained plethysmography parameters in general, and Penh specifically, as substitute variables for traditional, valid measures of lung mechanics. (Adler, Cieslewicz et al. 2004)****"*

The discussion about the use of Penh in measuring of airway resistance is a very long lasting however interesting dialog between scientists. In the year 2004, Bates et al. published a letter to the editor of the American Journal of Respiratory Cell and Molecular Biology. In this letter, a long list of internationally respected respiratory physiologists stated that Penh was related to ventilator timing and unrelated to airway resistance (Bates, Irvin et al. 2004). *"However tempting is the ease of using the unrestrained plethysmography,* ***Penh is not a measure of airway mechanics, and in the absence of confirmatory methods based on physical principles, should not be used to invoke terms such as "airway (hyper)reactivity" or "airway (hyper)responsiveness(Bates, Irvin et al. 2004)****". The increasing uncritical use of unrestrained plethysmography imparts a danger of mass-producing false experimental evidence in the search for mechanisms of respiratory disease. (Bates, Irvin et al. 2004)"*

In the year 2006 Morton Lomask wrote a paper to discuss the Penh parameter referring to the letter of Bates et al. in 2004. The interpretation of these findings and critics is very hard and therefore I contacted the author of the publication I used as a reference for my methods, Morton Lomask. He sent me the letter, which he had sent to the editor of the Journal of Applied Physiology to refer to the critics in the other publications (published with permission of Morton Lomask):

*"Letter to the Editor---Journal of Applied Physiology*

*I read with interest the debate between Dr. Bates and Dr. Mitzner (Point: Counterpoint Lung Impedance Measurements, etc.) on the proper way to measure respiratory mechanics. One point they both agreed upon was that enhanced pause (Penh) was "completely discredited." My interest in this debate is heightened by the fact that I made the observations that led to the creation of Penh.*

*Dr. Bates cites three references to support his conclusion about Penh (Lundblad et al. 2002; Adler et al. 2004; Bates et al. 2004). These references deserve some examination because they contain significant omissions and questionable conclusions in their assessment of Penh.*

*Lundblad et al., present a mathematical introduction to the respiratory signal from a whole body plethysmograph (WBP). They concluded that the effect of temperature and humidity changes (conditioning) far exceeded the effect of resistance on the respiratory signal from a WBP, and that therefore a useful measure or indicator of resistance could not come from that signal, and that therefore Penh was not related to resistance. Unfortunately their mathematical analysis described a waveform from a* ***sealed*** *WBP, which responds to volume changes created by respiration within the WBP. Penh is not derived from a sealed chamber. It is derived from a chamber with a pneumotachograph in its wall (FWBP). The FWBP waveform responds to the* ***flow*** *changes created by respiration within the chamber. It is like a large pneumotachograph which encloses the animal. If a syringe is emptied into it, the pressure within does not respond to the volume of the syringe, as it does with the sealed chamber, but rather to the speed at which the syringe is emptied.*

*If the authors had discussed the time derivative of the pressure signal of the sealed chamber, their argument would have been much more relevant to Penh measurements.*

*There is a major difference between the respiratory signal from a sealed chamber and the FWBP, both visibly and conceptually. The pressure within the FWBP is created by the difference between the nasal flow, and the flow due to chest movement (the thoracic flow), which caused the nasal flow. The nasal flow lags the thoracic flow by a time delay. When resistance increases, the time delay between the two flows increases. This produces a change in shape of the FWBP waveform. The change in shape is particularly significant and visible in the transition region between end-inspiration and start-expiration. A peak is present in that region, and it increases as the time delay between the nasal and thoracic flows increases; and the time delay between the two flows increases with constriction. None of this was addressed by the Lundblad paper, even though the characteristic peak at start expiration was described in a previous paper which validated Penh (Hamelmann et al. 1997). Lundblad et al. claimed that it is impossible to separate the conditioning and resistance effects from the respiratory signal within the WBP (except by removing conditioning by heating and humidifying the chamber to body conditions). That is true for the sealed WBP. It is not true for the FWBP, where the characteristic peak occurs in a zero flow region, where conditioning effects are much reduced. A fuller description of the waveform from the FWBP, including the mathematical basis of its presentation of resistance information, has been published recently (Lomask 2006).*

*Adler et al. described an experiment which showed a good correlation between Penh and traditionally measured resistance with Balb/C mice, but not with C57BL/6 mice. This experiment had the unusual outcome that the allergen sensitized and challenged C57BL/6 had a lower response to methacholine doses than the non-sensitized C57BL/6, as measured by traditional resistance. The authors used their own software for this experiment, using a sampling rate of 100/sec, and their own chamber. Whether their chamber incorporated a reference chamber to cancel noise is not clear. Taking samples 10 milliseconds apart is clearly inappropriate for measurements of the sharp, early expiration peak, and of the axis crossings; all of which is essential to the Penh measurement.*

*The sampling rate should be an order of magnitude greater. Interestingly, the authors commented on the great change in Penh if the axis crossing was "off" by one sample point.*

*Rather than recognizing that their sampling rate was inappropriate for this measurement, they suggested that Penh was inherently unreliable. In short, this study has several experimental issues which could impact significantly on the sensitivity of their measurements of Penh, and raise questions about their conclusions.*

*Bates et al. noted that Penh as an index of resistance was supported by some studies, but contradicted by others. They cited the theoretical arguments against Penh in the Lundblad paper, and noted that at the time there was no published theoretical basis for Penh. The omissions in the theoretical arguments of the Lundblad paper has been discussed above, and a theoretical and conceptual basis for Penh, and its connection to resistance, does exist now (Lomask 2006).*

*In summary, the paper commonly cited (Lundblad 2002) to invalidate the application of the WBP to resistance measurements did not assess a FWBP, from which Penh is derived, but a sealed chamber with different properties. The FWBP waveform contains resistance information, which stands out from the rest of the waveform.* ***A conceptual and theoretical basis for the application of the FWBP as an indicator of resistance has recently been provided*** *(Lomask 2006)."*

The newest paper concerning Penh was published in the European Respiratory Journal, where scientists express serious concern about the methods in a study using Penh as a measure of lung function (Lundblad, Irvin et al. 2007). In this study they investigated the reaction of OVA-sensitized mice to choline gavage and the reaction of the lung function using unrestrained WBP. In their critic, they propose that no papers using Penh as a measurement of airway hyperresponsiveness should be accepted." ***Penh has been thoroughly discredited as a measure of respiratory mechanics and reviewers, editors and journals should not accept any study that uses this quantity and claims it represents airway or pulmonary resistance (Lundblad, Irvin et al. 2007)***."

Taken together all discussions about Penh and lung function measurements, it is far beyond my competence to conclude whether Penh is actually a proper way of measuring or not.

The high amount of published papers which criticize the Penh parameter should anyhow be taken seriously and perhaps promote further investigation and reevaluation of Penh as a main point of lung function measurement in modern airway inflammation research. The authors of the main critical papers against Penh published another scientific paper about unrestrained video-assisted plethysmography in 2008. Thereby they used unrestrained WBP plus a method of measuring lung volume changes with orthogonal video imaging of the thorax. This new method (unrestrained video-assisted plethysmography, UVAP) uses heated and humidified chambers to estimate specific airway resistance (sRAW). Bates et al. 2008 conclude that this new method is able to provide useful estimates of sRAW and breathing patterns in mice, offering an alternative measurement method of unrestrained lung functions (Bates, Thompson-Figueroa et al. 2008).

The role of CRTH2 in vitro and in vivo

Comprising our data in the OVA-challenge, the CRTH2 antagonist CAY10471 showed no significant inhibition of immunological cells in the response to allergen exposure. Interestingly, other studies showed a significant outcome of the use of CAY10471 (Royer, Schratl et al. 2007). Administration of CAY10471 inhibited PGD2 –induced eosinophil release from the bone marrow of guinea pigs and also chemotaxis of eosinophils of guinea pig bone marrow plus human peripheral blood eosinophils. CAY therefore blocks different ways of activating eosinophils: the chemotactic action of PGD2; the chemotactic priming and the PGD2- induced production of reactive oxygen species. This study showed for the first time that CRTH2 antagonists can block eosinophil release from the bone marrow and therefore can be used as a target in the treatment of allergic diseases (Royer, Schratl et al. 2007).

Uller et al. 2007 showed that Tm30089 (CAY10471), representing the CRTH2 antagonist, significantly reduces the peribronchial lung tissue eosinophilia and mucus cell hyperplasia induced by allergen-challenge in a mouse model.

Also, a comparison was made between ramatroban and Tm30089, which is structurally closely related to ramatroban but with a different receptor antagonizing profile (ramatroban blocks thromboxane receptor TP and CRTH2 and Tm30089 are selective CRTH2 antagonists.).

Uller et al 2007 showed the efficacy of a selective CRTH2 antagonist in a vivo mouse model, suggesting the use of Tm30089 as therapeutical approach to allergic diseases (Uller, Mathiesen et al. 2007).

The idea behind the use of CRTH2 antagonism is built on the observation that PGD2 and its metabolites are major substances in the mast-cell activation in allergic asthma in vivo (Dahlen and Kumlin 2004). Murray et al 1986 showed that allergen-challenge in stable chronic asthma patients triggers the production of PGD2 within 9 minutes, showing the role of PGD2 in the early-phase reaction of allergen-sensitized individuals. The BAL of these patients showed a 150-fold higher PGD2-level after allergen challenge (challenge with dermatophagoides pteronyssinus) (Murray, Tonnel et al. 1986). Naclerio et al 1983 showed for the first time that nasal allergen-challenge leads to an increased production of mediators like histamine and PGD2 in allergic humans. These findings directed the interests of the scientists more to the field of mediators and receptors of action (Naclerio, Meier et al. 1983). Nagata et al 1999 elucidated the role of the CRTH2 receptor as a selectively expressed receptor on Th2 cells (Nagata, Tanaka et al. 1999) and two years later Hirai et al 2001 demonstrated that PGD2 mediates chemotaxis in Th2 cells, eosinophils and basophils in a DP1 independent manner (Hirai, Tanaka et al. 2001). Clinical findings related to the CRTH2 receptor were also encouraging as for example another research group revealed that CRTH2+ positive lymphocytes were increased in the disease-related cutaneous lymphocyte-associated antigen-positive lymphocyte compartment showing a possible involvement of CRTH2+ lymphocytes in atopic dermatitis lesions (Iwasaki, Nagata et al. 2002). Spik et al 2005 also pointed out the role of the CRTH2 receptor in an in vivo model of asthma and atopic dermatitis in mice, and thus supported the idea of using antagonists of the CRTH2 receptor in diseases with eosinophil involvement (Spik, Brenuchon et al. 2005).

Since there now exist a lot of different studies displaying the important role of PGD2 mediated CRTH2 stimulation and eosinophilic airway inflammation (Almishri, Cossette et al. 2005) and since recent studies show the effect of CRTH2 antagonists in vitro and in vivo (Royer, Schratl et al. 2007; Uller, Mathiesen et al. 2007), further investigation for CRTH2 in vivo could clearly bring more information about the otions of using these substances ( see Figure 4.0 and Figure 4.1).

Nevertheless, another group already treated two patients suffering from eosinophilic otitis media with ramatroban successfully underlining the inhibiting role of ramatroban in eosinophil action (Wada, Uemaetomari et al. 2006).

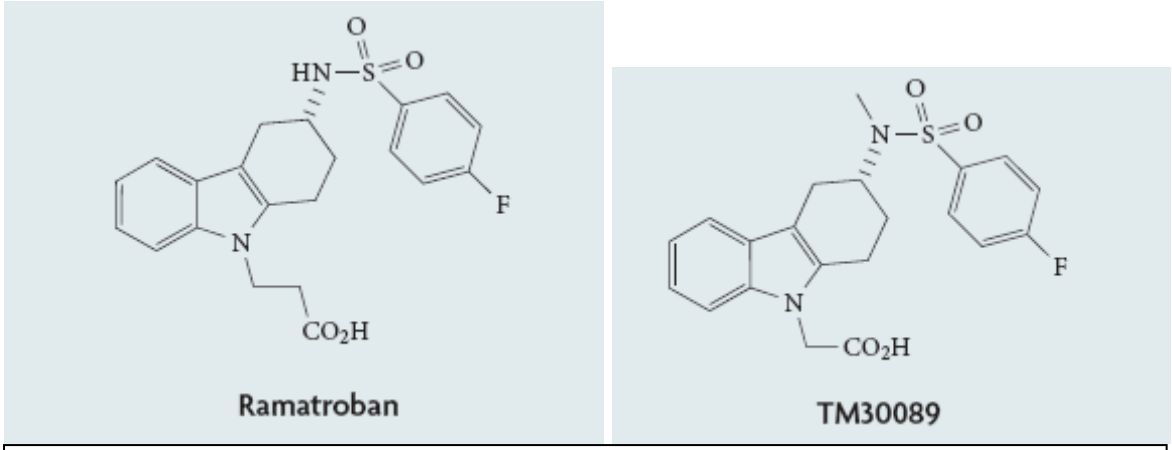



**Figure 4.0 Structure of Ramatroban and Figure 4.1 Structure of TM30089:** The structure of Ramatroban and the selective CRTH2 antagonist TM30089. (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery (Pettipher, Hansel et al. 2007), copyright 2007)

Putting together preliminary data concerning the use of CRTH2 antagonism, the in vitro models brought exciting results, showing the possibility of using these antagonists in vivo. Uller et al 2007, as mentioned above were the first using this antagonism in an eosinophilic airway inflammation mouse model and had interesting results to offer. The methods this group used were different to ours because they not only used BAL to get the quantity of eosinophils but also histological examination of mice lungs regarding tissue eosinophilia. Thereby TM30089 diminished tissue eosinophilia and interestingly also BAL eosinophilia, showing the expected results (Uller, Mathiesen et al. 2007).

As also discussed in this paper, it is very important to measure tissue eosinophils together with BAL eosinophils because of the interaction between the quantity of tissue cells and the loss of cells into the airway lumen. This effect can draw incorrect conclusions about the real tissue cell number and drug effects (Uller, Lloyd et al. 2006).Pettipher 2008 reviewed the roles of DP1 and CRTH2 receptor in allergic responses and presented the study of the effect of a compound of indole acetic acid series. These indole acetic acids were structurally non-related to ramatroban, but were also very effective as highly-potent and selective CRTH2 antagonists (Armer, Ashton et al. 2005). The effect of the in vivo use of these indole acetic acid series was fascinating. Sensitized guinea-pigs were challenged with an antigen aerosol and afterwards, BAL was taken. Increased leukocytes in the BAL were measured, strongly represented by eosinophils, monocytes and neutrophils. The pretreatment of the guinea-pigs with a selective CRTH2 antagonist attenuated the total number of leukocytes and eosinophils, representing an inhibitory effect of the antagonist (Pettipher 2008). These data also suggests further exploration of CRTH2 antagonism in in vivo models (see Figure 4.2).

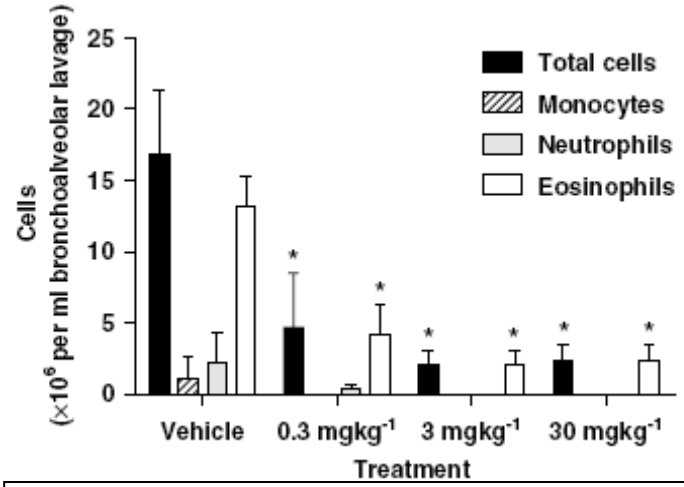



**Figure 4.2 CRTH2 antagonism in a guinea-pig model:** CRTH2 antagonist effect on airway inflammation in guinea-pigs. The antagonist was orally given one hour before and 12 hours after allergen exposure. n= 10 animals per group. P<0.01 compared to vehicle control. (performed by Aptuit Limited, Ricarrton, Scotland (Pettipher 2008).) (Reprinted by permission from Macmillan Publishers Ltd: British Journal of Pharmacology (Pettipher 2008), copyright 2008)

The relationship between the two PGD2 receptors DP1 and CRTH2

The two distinct receptors DP1 and DP2 (CRTH2) have always been in the spotlight of research due to their intervening role in the processes of allergic responses. Interestingly, DP1 is often designated as the anti-inflammatory and CRTH2 as the pro-inflammatory receptor concerning allergic inflammation, although recent studies have shown that there could be interplay between these receptors, regulating the processes after PGD2 activation in a complex way (Pettipher, Hansel et al. 2007). Investigation of allergic inflammation concerning CRTH2 is therefore always linked to the role of the DP1 receptor, as for example eosinophils, the major target for allergic diseases, express both receptors on their surface and interact with each other to regulate the response to PGD2. CRTH2 activation of eosinophils with PGD2 leads to morphology change, an increase in chemokinesis, and promotion of eosinophil degranulation. These effects were mediated by CRTH2 receptor due to the observance that DK-PGD (2), a selective CRTH2 agonist, stimulated these effects but not a DP1 agonist. The administration of the selective DP1 agonist delayed the onset of apoptosis in cultured eosinophils, showing a link between DP1 activation and survival (Gervais, Cruz et al. 2001). Furthermore, an other investigation showed that inhibiting DP1 with a selective DP1 antagonist (BWA868C) leads to an enhanced CRTH2 activation, also reflecting the opposing roles of these two receptors (Monneret, Gravel et al. 2001). Several in vivo studies of the DP1-receptor also showed the interesting but not yet clear role of DP1 in inflammation. Some of them reflected a pro-inflammatory role (Matsuoka, Hirata et al. 2000; Fujitani, Kanaoka et al. 2002; Torisu, Kobayashi et al. 2004) and some an anti-inflammatory property of DP1 (Arai, Takano et al. 2004; Spik, Brenuchon et al. 2005). Bringing DP1 and CRTH2 face to face, DP1 showed pro and anti- inflammatory properties in vivo whereas CRTH2 consistently showed a pro-inflammatory role in vivo (Kostenis and Ulven 2006).

A major target of PGD2-mediated influence in the allergic response could be dendritic cells. Faveeuw et al. 2003 showed that stimulation of the DP1 receptor with PGD2 inhibited the production of Th1 cell-driving IL-12 by murine splenic dendritic cells (Faveeuw, Gosset et al. 2003).

Hammad et al. 2003 also showed the inhibiting effect of BW245c, a DP1 agonist, on the migration of lung dendritic cells to draining lymph nodes and therefore inhibiting the proliferation of naïve T cells and the production of T cell cytokines IL-4, IL-10 and γ-IFN (Hammad, de Heer et al. 2003).

Dendritic cells also play an essential role in controlling T cell polarization to form Th1 or Th2 responses. Different types of dendritic cells can also induce different types of T cell cytokines. Faith et al. 2005 showed a functional plasticity of human respiratory mucosa dendritic cells (RTDCs), which is reflected in different kinds of secretion profiles for cytokines dependent on the trigger. In mixed lymphocyte cultures, RTDCs induced Th1/Th2 cytokine profiles in contrast to peripheral blood dendritic cells, which induced Th1cytokine profiles (Faith, McDonald et al. 2005). Another critical role for DP1 receptor in allergic diseases discovered Hammad et al. 2007 showing that inhalation of a DP1 receptor agonist suppressed the major features of asthma by targeting dendritic cell function via increasing proliferation of CD4+Foxp3+ regulatory T cells. Induction of these T cells inhibited inflammation in an IL-10 dependent way. Administration of both DP1 and CRTH2 agonists in OVA-sensitized BALB/c mouse models showed differences between the activation of the receptors compared to the control groups. OVA challenged mice receiving vehicle without agonist developed eosinophilia in BAL and peribronchial lung tissue as well as Th2 cytokine production in the mediastinal lymph nodes. The DP1 agonist (BW245C) strongly inhibited inflammation. Also, measurement of bronchial hyperresponsiveness (BHR) using the Penh parameter for correlation, showed attenuation after treatment with BW245C. The group therefore investigated specific responses of OVA-dendritic cells to BW245C in vitro. BW245C treated dendritic cells induced a reduction of IL-5 production in OVA-specific T cells compared to vehicle-treated T cells. Also, the IL-10 level and the number of regulatory T cells was elevated in the BW245C group, showing a change of the secretion profile (Hammad, Kool et al. 2007). Controversially, Matsuoka et al. 2000 created a DP1-deficiency mouse model and compared OVA-induced allergic responses in DP-/- mice to wild-type mice.

The IgE level was the same in both mice-types, but astonishingly Th2 cytokines, lymphocyte numbers and eosinophilic infiltration of the lungs were reduced in the DP1-deficiency groups, also failing to develop airway hyperreactivity (Matsuoka, Hirata et al. 2000). These data stayed completely against the findings of Hammad et al. 2007, who found that endogenous ligands binding on the DP1-receptor inhibited airway inflammation, as selective deficiency of hematopoietic PGD2-Synthase enhanced airway inflammation (Hammad, Kool et al. 2007). Furthermore, they probed the role of DP1-deficient dendritic cells and displayed that these deficient cells promote an intensified Th2 response in the lung (Hammad, Kool et al. 2007). *"On the one hand, DP1-mediated inhibition of IL-12 promotes Th2 polarization during the sensitization phase while suppression of dendritic cell function during the effector phase reduces activation of antigen-specific Th2 cells.(Pettipher 2008)"* The influence of the DP1 receptor on allergic inflammation responses is dependent on the phase in which PGD2 is produced and on the location (target organ vs. lymph node). The complexity of DP1 receptor involvement in allergic inflammation has to be investigated more deeply to get better insight whether the use of antagonists or agonists can lead to therapeutic improvement.

From Etiology to Therapy- “New” Approaches to Asthma

Immunological research in the field of asthma is, as immunological research in general, complex and divided into different approaches to investigate the system of inflammation. Some groups concentrate on mediator-research like histamine, PGD2, or leukotrienes. Other groups take a look at different cell types and their role in the development of asthma. Through the lavish search for literature for my thesis,I gained insight into new developments such as cytokine antagonists (Holgate and Polosa 2008), stimulation of receptors like PPARγ (Park and Lee 2008), and antibodies against IgE (Holgate, Djukanovic et al. 2005). The access path of these therapies derives from precise investigation of biological pathways and search for intervention possibilities. Another approach to allergic diseases is the question why allergic diseases and immune-mediated diseases have had an increasing incidence since the last century.

Investigations of possible etiologies of immune-mediated diseases have aslo led to support of the hypothesis that etiology could give a major hint regarding development of such diseases. (Guarner, Bourdet-Sicard et al. 2006) The results of immunological research in the field of asthma showed complex interactions between inflammation responses to allergens and susceptibility to develop these kinds of reactions. The link between modern lifestyle and the development of allergic diseases formed the term “hygiene hypothesis” (Strachan, Taylor et al. 1996).

Observing that the incidence of allergic diseases has risen since the late 19$^{th}$ century and nearly doubled in the 60´s and 70`s of the 20$^{th}$ century led to the idea of interactions between infections and allergic immune responses (Bach 2002). At first immunological explanation of this phenomenon was a reduced exposure to microorganisms, which were thought to stimulate Th1 cell responses and therefore inhibiting Th2 cell actions. This idea was connected to the Th1/Th2 hypothesis, which supported the model of Th1 mediated diseases like type-1 diabetes, multiple sclerosis, and Th2 mediated diseases (allergic) such as atopic asthma. Therefore the hypothesis promoted the idea that typical Th1 cytokines inhibit Th2 cytokines and vice versa.

New research has shown complex interactions between these cytokines, in which some of them promote and some inhibit pathways in inflammation, reflecting the more complex system behind T cell subtype-interactions (Steinman 2007). Putting together all these findings, the Th1/Th2 hypothesis should be seen critically because of data showing a more complex way of T helper cell interaction. Also, new cell types like Th17 cells could play a central role in the development of inflammation (Steinman 2007). Nevertheless the “hygiene hypothesis” still offers the possibility of explaining the growing number of patients by means of immune-mediated diseases like type-1 diabetes and allergic disorders using a more complex and holistic theory than the Th1/Th2 hypothesis. Recent investigations have shown a major role of the setup of immunological tolerance against common antigens and food proteins. These “suppressive” mechanisms could be mediated by APC´s, which are responsible for inducing the development from naïve T helper cells into distinct T cell subsets (see Figure 4.2). Thereby environmental factors also play an important role, such as for example microbial heat-shock proteins (van Eden, van der Zee et al. 2005).

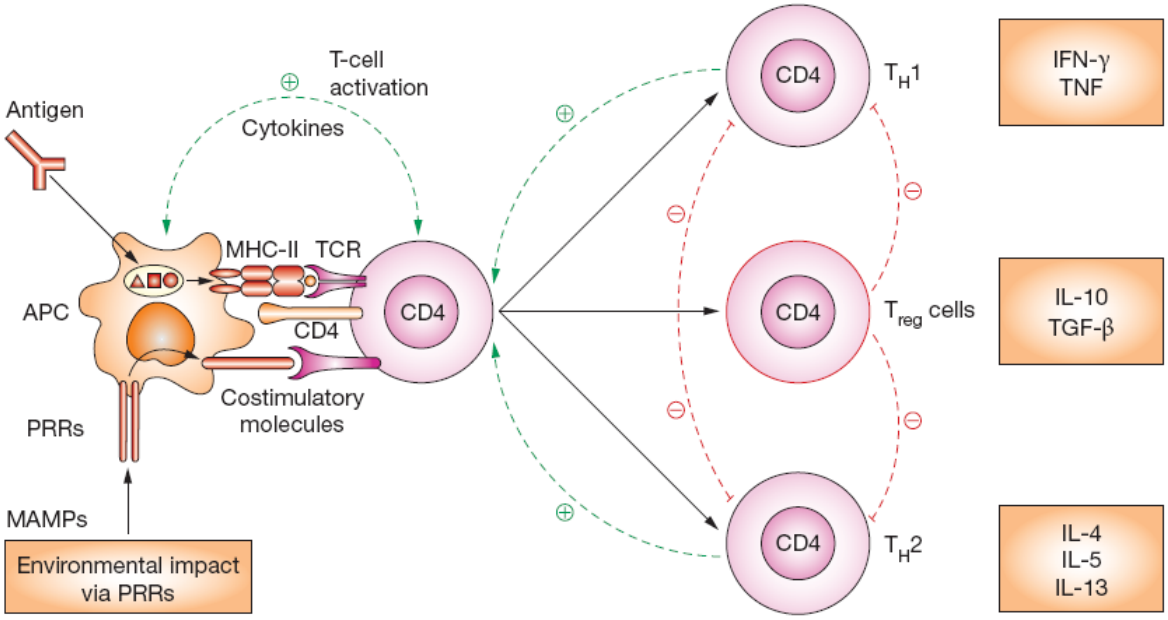



**Figure 4.3 Formation of Th subsets:** Formation of different kinds of CD4+ cells from naive into T cell subsets with characteristic cytokine-secretion profiles. The presentation of different antigens is crucial for induction signals forming different T cells. APC`s have to present and provide appropriate costimulatory signals like soluble factors. Therefore APC`s have a major but yet unknown role of induction of Treg cells(Guarner, Bourdet-Sicard et al. 2006). (Reprinted by permission from Macmillan Publishers Ltd: Nature Clinical Practice (Guarner, Bourdet-Sicard et al. 2006), copyright 2006)

Zuany-Amorim C et al. 2002 showed that administration of a mycobacterium vaccae-suspension induced a specific T cell subset, so-called T regulatory cells CD4 + CD45RB (lo) and suppressed eosinophilic airway inflammation. This effect was mediated by IL-10 and TGF-β (transforming growth factor beta) because antibodies against IL-10 and TGF-β totally inhibited the effect of T regulatory cells (Tregs) (Zuany-Amorim, Sawicka et al. 2002). Induction of Tregs could be a major source of developing a "normal" response to allergens and antigens, and perhaps plays a key role in all immune-mediated diseases (see Figure 4.3). It is not clear yet, if the "hygiene hypothesis" can be taken as an explanation for the raise of immune-mediated diseases but there are a lot of indications, which are leading to the rationale of supporting further investigations in this field (Guarner, Bourdet-Sicard et al. 2006). The results of in vivo models of environmental factors and their impact on immunological formation are exciting. Wilson et al. 2005 investigated the impact of helminthes on the expression of airway inflammation (see Figure 4.4).

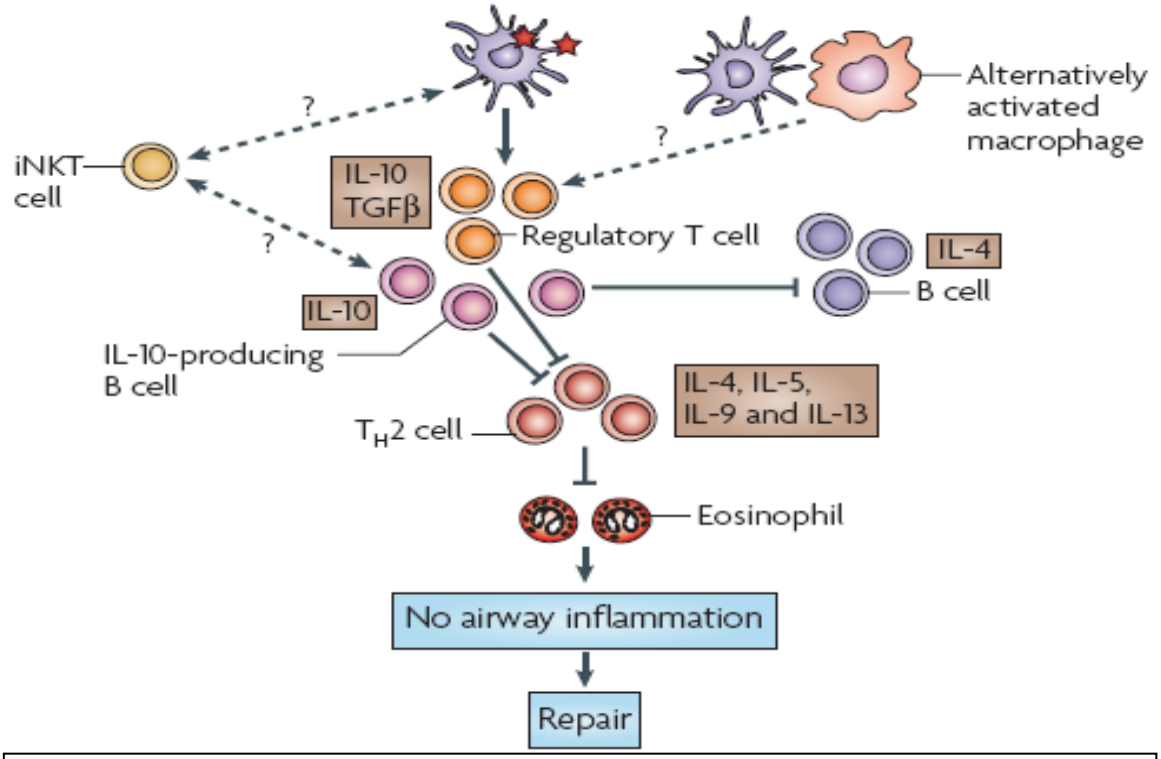



**Figure 4.4 The impact of helminth infection on allergic response:** Different types of cells cause inhibition in a helminth-infected individual (regulatory T cells, IL-10 producing B cells, invariant natural killer T cells (iNKT cell). Elevated IL-10, TGF-β and decreased levels of IL-5 prevent exacerbated Th2 response to allergens (Fallon and Mangan 2007). (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Fallon and Mangan 2007), copyright 2007)

Gastrointestinal infection with the nematode Heligmosomoides polygyrus was examined in an OVA-sensitized and challenged mouse-group type BALB/c and in a Der p1 house dust mite allergen group in C57BL/6 mice (Wilson, Taylor et al. 2005). The nematode was used according to Bashir et al. 2002 showing its allergy-protecting effect (Bashir, Andersen et al. 2002). Infected mice revealed significant changes in BAL fluids compared to the controls. Both groups had significantly reduced airway cellular infiltrates after challenge with OVA (BALB/c) or Der p 1 (C57BL/6) and reduced especially eosinophils and neutrophils. Examination of lung histology displayed a reduction of tissue inflammation peribronchially and perivascularly with a reduced number of goblet cells (mucus-producing cells). The intestinal infection with helminthes showed an inhibition of β-hexosaminidase, reflecting an inhibition of mast cell degranulation. A major target of this study was also the measurement of cytokines in reaction to the infection, to get insight to how this infection with helminthes polarizes the immune system. Typical cytokines for Th1 and Th2 responses were examined. IL-4 showed no diminished levels in infected mice, while IFN-γ was not detectable in both infected and uninfected mice. These data show that infection does not have an effect on the Th2 dominance in allergic responses. The most interesting observation was that IL-5 and eotaxin were both significantly diminished by infection (Wilson, Taylor et al. 2005). *"Reduction in these two key agents of mobilization and extravasation of eosinophils provides a mechanistic explanation for the dramatically reduced airway eosinophilia in infected mice.(Wilson, Taylor et al. 2005)"* The role of regulatory cytokines was elucidated by measurement of IL-10 and TGF-β, whereby only IL-10 was significantly elevated in infected C57BL/6 mice. In BALB/c mice TGF-β levels were elevated in the BAL, suggesting an important role of this cytokine in immune regulation during infection. Further investigation of the question which cell was responsible for mediating this suppressive effect seen in the BAL, showed that anti-CD25 antibodies totally restored inflammation reaction in the lung tissue, reflecting the important role of CD25+ positive cells in this response. Adoptive transfer of mesenteric lymph node cells (MLNC) from infected to uninfected animals transferred the protective effect seen in infected mice to the uninfected.

Interestingly, MLNC from infected mice showed elevated Foxp3 messenger RNA (mRNA), higher TGF-β levels and a generally higher number of CD4+CD25+Foxp3+ cells, reflecting the induction of T regulatory cells through the infection (Wilson, Taylor et al. 2005) (see Figure 4.3).

Summarizing this data, Wilson et al. 2005 underlined the role of the "hygiene hypothesis" in the development of allergic diseases by showing that infection with helminthes can suppress airway inflammation in a very complex and holistic shift of cytokine profiles, chemotactic profiles and cell interactions (Wilson, Taylor et al. 2005). Also, other studies showed the amazing impact of helminth infections on asthma (Araujo, Hoppe et al. 2004; Kitagaki, Businga et al. 2006; Mangan, van Rooijen et al. 2006) and interestingly, also the increase of IL-10 in response to infection (Kitagaki, Businga et al. 2006).

Observing that organisms have an impact on immunoregulation led to the "old friend hypothesis", first used by Rook et al. 2004, suggesting that different organisms including saprophytic mycobacteria, bifidobacteria, lactobacilli and helminthes, are recognized by the innate human immune system as innocuous (Rook, Adams et al. 2004) (see Figure 4.5). These organisms can induce specific and unusual maturation patterns of dendritic cells, which in turn can induce regulatory T cell responses (Adams, Hunt et al. 2004). Exposure to organisms, as mentioned above, is recognized by the immune system as harmless through different kind of pattern-recognition receptors like CARD15 or TLR2 (toll-like receptor 2). This exposure triggers immature dendritic cells to mature into regulatory dendritic cells, which can induce and drive T cell polarization. Permanent stimulation of regulatory DC´s can trigger the production of T regulatory cells in a way that produces amounts of IL-10 and TGF-β. This formation creates a background suppression or so-called "bystander suppression" (Guarner, Bourdet-Sicard et al. 2006). Regulatory dendritic cells are then also integrated in the recognition of self-antigens, allergens, and other epitopes. They are able to induce T regulatory cells, which also produce IL-10 and TGF-β in reaction to these "new" antigens and are therefore a major part of the immunoregulatory mechanism.

Interaction between DC´s and T cells might therefore be very interesting for further investigation due to a high amount of indication that they play a major role in different immune-mediated diseases (McKee and Pearce 2004).

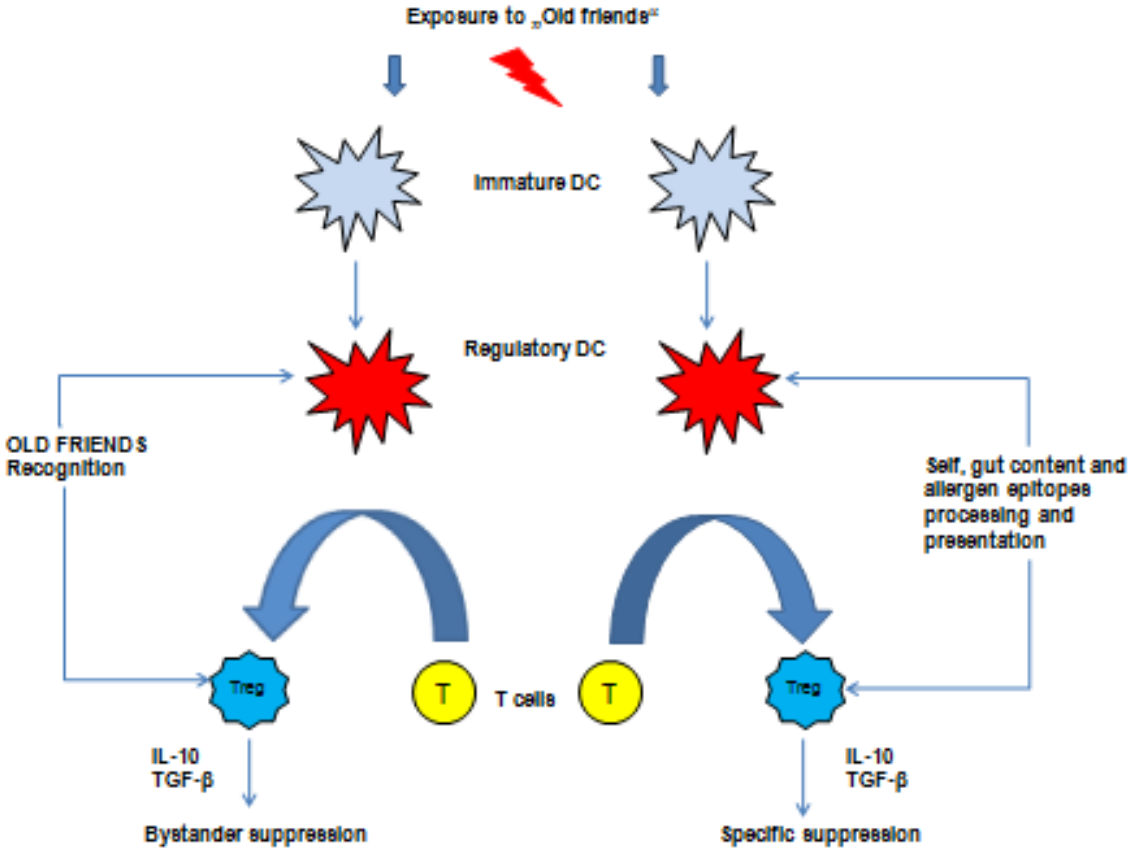



**Figure 4.5 "Old friends" Hypothesis:** Organisms are recognized by different pattern-recognition receptors on the surface of DC (dendritic cells) such as CARD 15 and TLR2 (toll-like receptor 2). These antigens promote immature DC´s to mature into regulatory DC´s, which stimulate the regulatory T cell reaction to the "old friends" permanently to induce a background suppression of the immune system to tolerate the organisms. Regulatory DC´s might also process and present other epitopes from self, gut content and allergens. This process drives specific immunoregulation (Guarner, Bourdet-Sicard et al. 2006).

Role of T regulatory cells

The role of T regulatory cells in asthma and allergic diseases is also very fascinating due to their fascinating nature to inhibit and modulate immune responses and actions (Maloy and Powrie 2001). As I have discussed above, Tregs appear to have a major role in modulation of allergy-suppressing processes. The response of Tregs to different kinds of triggers is extremely complex based on the fact that distinct cells have regulatory functions in the immune system. T regulatory cells are divided into different groups reflecting their origin and their abilities to influence immune responses (see Figure 4.6).

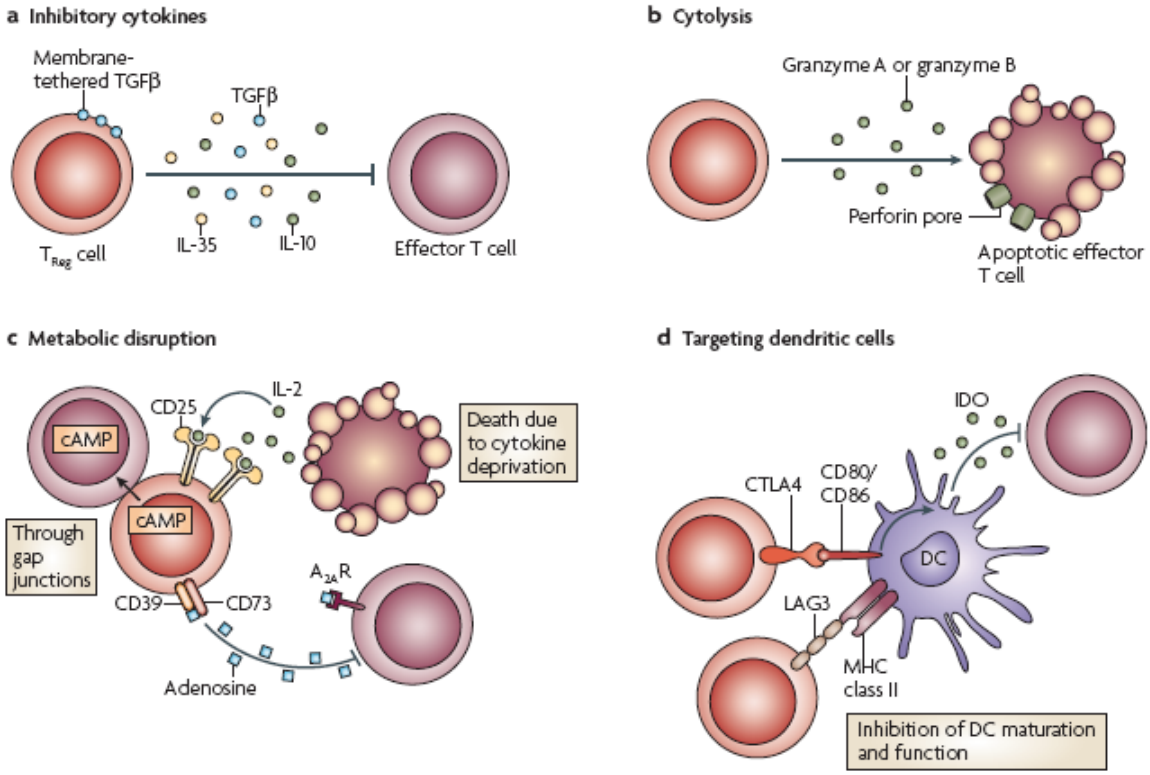



**Figure 4.6 Mechanism of Treg suppression:** The immunosuppressive effect of T regulatory cells is mediated through four different kinds of actions. Inhibitory cytokines are a major target of modern immunological research due to the fact that their role in asthma and allergy is still not clear and shows often contrary data (Hawrylowicz and O'Garra 2005). Activation of cytolysis via Granzym A has been newly investigated and is now also linked to T regulatory cells (Grossman, Verbsky et al. 2004). Metabolic disruption of T regulatory cells via the cytokine (IL-2)-deprivation-mediated apoptosis is a possible mechanism of T regulatory cells to modulate T cell function. The mechanism could be the deprivation of IL-2 (which is needed by normal T effector cells for survival) via the CD25 expression (Vignali, Collison et al. 2008). T regulatory cells might also modulate DC´s and their function by stimulating maturation. Cederbom et al. 2000 showed that regulatory T cells downregulated the expression of co-stimulatory molecules on DC´s and thereby reflecting a modulating interaction between T regulatory cells and DC´s (Cederbom, Hall et al. 2000). (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Vignali, Collison et al. 2008), copyright 2008)

The CD4+CD25+ thymus-derived naturally occurring Tregs are T cells with immunosuppressive properties acting through cell-cell contact and cytokine secretion (IL-10 and TGF-β), and make up 5-10% of the peripheral circulating CD4+ cells (Sakaguchi 2005). The identification of these cells lies in the determination of CD25+ and their specific transcription factor forkhead box P3 (FOXP3), which is a more specific marker and crucial for the function of these Tregs (Hori, Nomura et al. 2003). Adaptive Tregs or so-called antigen-induced Tregs are mainly distinguished through their secretion profile of cytokines. Tr1 (T regulatory cells of type 1) cells for example produce high amounts of IL-10 and low amounts of IL-5, INF-γ and TGF-β. T helper cells 3 are more different and express high amounts of TGF-β. Interestingly, both cell types have features for inhibiting Th1 and Th2 type of inflammation and are therefore very potent immune regulatory cells, which originate from naïve T cells and are characterized by stimulation of dendritic cells. Naturally occurring Tregs could also be involved in the development and generation of induced Tregs (Cools, Ponsaerts et al. 2007). CD8+ positive T cells with an immunosuppressive effect were also described as T-suppressor cells CD8+CD28- and are also able to inhibit T cell proliferation and adoptive transfer of CD8+CD28- cells into CD8-/- mice inhibited disease progression of EAE (experimental autoimmune encephalomyelitis). CD8-deficient mice were more prone to EAE, reflecting a critical role of T suppressor cells in immune-mediated diseases (Najafian, Chitnis et al. 2003).

Clinical possibilities of using Tregs in treating asthma are interesting as major hints are concerned that Tregs play an essential role in the immunopathology of asthma. Ling et al 2004 showed that CD4+25+ T cells from atopic patients show less abilities to suppress allergen stimulated CD4+ Cd25- T cell proliferation and cytokine production compared to CD4+CD25+ T cells from healthy patients, reflecting a possible deficiency of T regulatory cells from atopic patients to suppress and modulate T cell responses to allergen exposure (Ling, Smith et al. 2004). Matsumoto et al 2004 showed that in a severely unstable group of asthma patients the number of IL-10 producing CD4+CD45Ro+ T cells was decreased compared to a group of mild asthmatics.

Comparison between severely stable and severely unstable patients also showed decreased levels of IL-10 producing T cells, although both groups were treated with the same amount of corticosteroids, reflecting no dependence on corticosteroid therapy (Matsumoto, Inoue et al. 2004). T regulatory cells are one of the major targets of modern immunology research in our time and there are a lot of indications that they also play a critical role in the initiation and development of allergic diseases.

The fact that allergen immunotherapy decreases T cell proliferation and increases IL-10 levels (produced initially by CD4+CD25+ T cells) and the fact that this effect is antagonized by neutralizing IL-10, not only shows the critical role of T cells in initiation of allergy, but also the possibility of using this knowledge for therapy (Akdis, Blesken et al. 1998).

In sum, I have shown that administration of the CRTH2 antagonist CAY has had no significant effect on the cell counts in BAL or whole blood samples, but displayed contrasting data concerning the resistance measurement due to the elevation of resistance in the OVA + Cay treated group. This contrasting data may be the result of a pharmacokinetic process changing the structure of CAY, but no studies have reported this effect yet. Measurement of airway hyperresponsiveness by means of two methods (WBP and direct resistance measurement with flexiVent) has also shown contradistinctive data, reflecting the question of whether the new method of measuring Penh is a real measurement of airway resistance and if correlation between Penh and airway resistance is valid. Furthermore, I have discussed the possible role of CRTH2 antagonism in treatment of allergic diseases. As mentioned, our results have shown no influence of CAY but a high amount of data supports the use of CRTH2 antagonists. CRTH2 antagonists showed in vitro (Royer, Schratl et al. 2007) and in vivo (Uller, Mathiesen et al. 2007; Pettipher 2008) promising results and further investigations in vivo will show if there is a possible use for treatment in human allergic diseases, although Ramatroban (CAY is an analog of Ramatroban) has already been used successfully for treatment of two patients with eosinophilic otitis media. (Wada, Uemaetomari et al. 2006).

The bridge between the pharmacological approach of using chemical antagonists and the discussed role of the “hygiene hypothesis” is that investigations and findings of infection-induced suppression of allergic diseases, especially asthma, are of exceptional relevance due to their holistic character. Wilson et al. 2005 demonstrated in their study that helminth infection inhibits airway inflammation in creating a change in the whole inflammation response: reduced airway infiltration with eosinophils and neutrophils, reduced number of goblet cells, inhibition of mast cell degranulation, reduced IL-5 and eotaxin levels, increased IL-10 (only in one strain), elevated TGF-β, and elevated T regulatory cells (Wilson, Taylor et al. 2005). (CD4+CD25+Foxp3+).

Major points of the characteristics of allergic airway inflammation were influenced by the helminth infection, reflecting a change in the inflammatory response in different areas with just one therapeutic intervention. Also, Dittrich et al. 2008 described complex changes in response as a whole to allergen challenge after infection with helminthes: reduced airway reactivity to inhaled metacholine, reduced antigen-specific Ig production and pulmonary eosinophilia (Dittrich, Erbacher et al. 2008). I have compared thus the typical approach with chemical antagonism in allergic disease with the etiology-based approach due to the fact that the ratio behind the intervention is different. The antagonist model uses a mechanistic approach of investigating biological systems by searching for inhibitable pathways like cytokine-inhibition (IL-5 antagonists) and receptor-inhibition (CRTH2). The other model uses epidemiologic data in comparison with hard facts from immunological research to imply observations about incidence and prevalence into immunology. The most fascinating fact about this approach is the eventual chance of intervening in the development of allergic inflammation and thereby influencing not just one characteristic point of asthma but targeting the origin of allergic inflammation. A combination of these two approaches will perhaps lead to better treatment of asthma and allergic diseases.

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# Appendix

Curriculum Vitae

Adrian M. Moser

Born 01.09.1982 in Graz/Austria

Address: 8046 Graz, Im Hoffeld 24, Austria

Mother Marianne Moser Mag.phil., born Rannberg Gothenburg/SE

Father Anton Moser Dipl.Ing. Dr. techn. Univ.Prof. born Graz/AUT

| | |
|---|---|
| 1989-1993: | Primary school St.Veit/Graz |
| 1993-2001: | High school Graduation 23.06.2001 |
| 01.07.2000-31.7.2000: | Voluntary medical work at Dr. med. M. Wendler |
| 03.09.2001-02.05.2002: | Military Service at ABC Corps (Nuklear/Biological/Chemical) |
| 2002-2003: | Voluntary work Austrian Red Cross |
| 01.10.2002 -2008: | Study of Medicine at MedUNI Graz |
| 01.10.2003-2006: | Member of ARGE Alpinmedizin (interdisciplinary workgroup) |
| 01.03.2004-2006: | Assistant at the Department of Systemphysiology at MedUNI Graz, Student education in Respiratory Physiology |

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| Sept. 2004: | 4 weeks rotation Department of Medicine LKH Bad Aussee, Styria |
| July 2005: | 2 weeks rotation ICU MedUNI Graz |
| February 2006: | 2 weeks rotation Department of Neurology/ MedUNI Graz |
| July 2006: | 4 weeks rotation at Dr. Michael Wendler, Graz |
| September 2006: | 4 weeks rotation Department of Medicine/ BHB Graz, Head: Prof. Skrabal |
| October 2006-2008 | Research fellow at Department of Pharmacology, MedUNI Graz "Prostaglandin and its receptors DP1 and CRTH2 in eosinophil recruitment" in vivo experiments under guidance of Prof. Irmgard Lippe, |
| January –June 2008 | Erasmus study stay in Tromsö/ Norway |