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Testing novel therapeutics in allergic asthma in mouse model

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Zusammenfassung

Asthma ist eine weit verbreitete Beeinträchtigung der Lungenfunktion, da es zur Kurzatmigkeit, Hypperreaktivität und chronisch obstruktiver Krankheitsverlauf kommen kann.

In dieser Abhandlung meiner Diplomarbeit wird die derzeitig gängige Einsicht untersucht, inwiefern Th2 Helfer T Zellen mit all ihren Auswirkungen betreffend allergischem Asthmas und deren Behandlung durch Auroacetylcystein in Verbindung einer Reduktion durch demselbigen erreichbar scheint. Zur Erreichung eines akuten Asthma Stadiums wurden unterschiedlich, für den jeweiligen Versuch spezifisch, BALB/c female oder C57/B6 female Mäuse verwendet. Im generellen erfolgte die Sensibilisierung der Mäuse, jeweils spezifisch mit OVA Grade V oder Milch Pulver, durch deren Intraperitonealer Injektion von 200 µl am Tage 0 und 21. Beginnend mit Tag 28 bis inklusive Tag 32 wurden jeweils PBS (1x) als positiv Kontrolle und die jeweilige Konzentration Auroacetylcystein (untersuchende Gruppe) intranasal gegeben. Um ein akutes Krankheitsbild allergischen Asthmas zu erhalten, wurde eine „Aerosol challange“ am Tag 31 und 32 für jeweils 1 Stunde, zwei mal am Tage durchgeführt. Am Tag 35 wurden die Mäuse getötet und für die jeweiligen Versuche unterschiedlich Nieren, Milz, Leber, Magen, Dünndarm und für alle gemeinsam Lunge entfernt, welche für weitere Untersuchungen herangezogen wurden. Um Serum für IgG1, IgE und Albumin ELISAs zu erhalten wurde eine Cardiac puncture durchgeführt. Um die zuvor genanten Immunglobuline, Albumin und Differentiell Zell Zählung durchführen zu können wurde ebenfalls Broncheoalveolar lavage gemacht.

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1.) Abbreviations:

Th 2	T helper cell 2
Th1	T helper cell 1
BALB/c	
C57/B6	C57 black 6
OVA	Ovalbumin
PBS	phosphate buffered saline
Ig G1	Immune globulin G1
Ig E	Immune globulin E
Ig A	Immune globulin A
Il- 4, Il-5,Il-10, Il-13	Interleukins 4,5,10 and 13
MHC	Major Histocompatibility Complex
IFN-γ	Interferon- Gamma
NF- κ B	Nuclear factor- kappa B
ICAM	Intracellular adhesion molecule
NAEPP	National Asthma Education and Prevention Program
min	minutes
µl	micro liter
ml	mille liter
µm	micrometer
ng	nanogram
µg	microgram
mg	milligram
g	gram
kg	kilogram
mol	SI unit for the number of an amount of a substance
i.p	Intraperitoneal
i.n	intranasal
BAL	Bronchoealveolar lavage
BSA	Bovine serum albumin
$Na^{+}[AuCl_4]^{-}$	[Tetra chlor- aurum] sodium complex
$H[Au\ Cl_4]$	[Tetra- chloro- aurum] hydrogen complex
$H^{+}[Au\ (Cl)_3\ OH]^{-}$	[tri-(chloro)-hydroxyhydrogen- aurum] hydrogen complex

Au^{+}	Aurum (I) cation
$[Au(CN)_2]^-$ complex	[(di-cyanide) aurum] one minus complex
SH^- group	Sulfur hydrogen- group
Na_2CO_3	Sodium carbonate
HIV	Human Immune deficiency virus
° C	degree Celsius
%	percentage
Wk	week
Rpm	rounds per minute
pH value	the measure of the acidity and alkalinity of a solution
ELISA	Enzyme linked immunosorbent assay
NAC	N-acetylcysteine
AAC	Auroacetylcysteine
H_2O_2	Hydrogen peroxide
ANOVA	analysis of variance
COPD	chronic obstructive pulmonary disease

1.1) General information about allergic asthma

Asthma is a common disease in western civilization. This increase in asthma has been attributed to aspects of Western culture including outdoor and indoor air pollution, childhood immunizations, and cleaner living conditions, but no single cause has been identified. [1] This disease it typified by major difficulty in breathing coupled with wheezing and coughing [2], often in the night. Asthma was general divided into two clearly parts. The extrinsic (allergic) and the intrinsic (non allergic) asthma [4] Today more classification had occurred [3] the allergic, non-allergic/intrinsic, exercise-induced, nocturnal, occupational and steroid- resistant asthma were detected.

Most people who suffered on asthma have the allergic one. This type is triggered by allergens, substances capable of causing an allergic reaction. A subclass is the childhood allergic asthma. This type of asthma occurs more often in young boys than girls and out of all childhood illness accounts for the most missed days in the school. Research showed that continued exposure to cigarette smoking could irritate the respiratory tract and make infants and children particularly vulnerable to allergic asthma. Intrinsic asthma occurs when allergies did not play a part. This type of asthma is typically found in people after the age of 40. The arrival of intrinsic asthma included respiratory

irritants such as perfumes, cleaning agents, fumes, smoke and cold air, upper respiratory infections and gastroesophageal reflux. Intrinsic asthma tended to be less responsive to treatment than allergic asthma. Exercise- induced asthma [5] can affect anyone at any age and may be attributed to the loss of heat and moisture in the lungs that occurred with strenuous exercise. The only symptom in that type of asthma was the frequent coughing during the exercise. Under cold, dry conditions symptoms could be more served. With prophylactic medications it is able to prevent people from exercised induced asthma. Another type of asthma is called nocturnal [6], or sleeping related asthma and affected people when they were sleeping. The term nocturnal means belonging to the night. Symptoms occurred regardless of the time of day a person was sleeping. Often symptoms occurred between midnight and 4 a.m. Nocturnal asthma could be triggered by allergens in bedding or in bedroom, a decrease in room temperature, and gastroesophageal reflux. As a directly result of breathing chemical fumes, wood dust, or other irritants over long period of time occupational asthma occurred. An estimated 15 % of asthmatics have occupational asthma. The case of asthma medications, especially steroids, could lead to the status asthmaticus. In that case a severe asthma did not respond to medication and may required mechanical ventilation to reverse.

1.1.2) The extent of the problem

A lot of people suffer under the inevitable consequences of allergic asthma. Depending on which state the disease is, several problems can occur. The activation of mast cells through secreted IgE leads to contraction of the smooth muscles. These symptoms are associated with over- production of mucus, which hence the coughing, and bronchial edema. The attempted breathe, produced a wheezing sound. An asthma attack could be anything from simple shortness of breathing to profound respiratory distress, which necessitated an emergency treatment in a hospital setting. In special cases this disease can lead to death [7].

The compound we tested was a combination of two strategies, the gold salt that is known as anti inflammatory and cysteine of N-Acetylcysteine, known to cleave the sulfide double bounds and is mycolytic. Based on the findings from Mark R. Holdiness et.al [8] N- acetylcysteine had followed clinical relevance's in pulmonary disease. It is well accepted that N- acetylcysteine had mucolytic actions which could be useful in asthmatic patients. Generally it can serve to persons topically, orally and intravenously. It is also known that Aerosol given N-actetylcysteine or trough instillation it could exert mycolytic, osmotic and other irritative bronchia depending effects on the mucosa which causing mobilization of secretions (Ziment 1986) [3]. P. Bolme and coworkers suggested that the

unknown bioavailability of NAC depended on the greater glutathione levels than the reduced or oxidized NAC levels [9]. They also found that NAC could be quantified after 60 minutes in the plasma levels and further the bioavailability of reduced NAC was 4.0 (2,3-7,2) % and for total NAC 9,1 (4,8-13.1)%.

Pharmacokinetics and Bioavailability of Reduced and Oxidized n- Acetylcysteine

NAC is a derivate of the amino acid Cysteine and include sulfur as naturally compound of meal. The Antidote power of NAC in combination with Paracetamol, an Acetaminophen ambassador gave the first evidence of the importance of NAC [10]. The mycolytic action of NAC was known years ago. Benefits of NAC were the fast and completely resorption by orally given. In the body NAC was found in free or over disulfide connections bound to plasma proteins form. The location was next to liver, kidney, the lungs and bronchial mucus. By passing the duodenum surface NAC will be converted to Cystein by de-acetylating. The major compounds were Cystein and Cysteine, less Taurin and sulfate. Around 30 % were excreted renal; about the mechanism of non renal excretion less was known.

In a deeper view of Pharmacodynamics, NAC as a precursor of the Glutathione synthesis was also important because endogenous Glutathione as effector was one of the most important dose dependent principles of the body to avoid intoxication by the ability to complex toxic reactive, electrophilic metabolites by building of inert complexes. Electrophilic interconnections were inactivated by conjugation and through reductions oxidative interconnections were neutralized. NAC had the power to increase Glutathione concentration and thereby the Antioxidation concentration also increased. Another benefit was that hydroxyl radicals and H_2O_2 radicals absorbed directly [11].

N-acetylcysteine, Cysteine and Glutathione containing sulphur, as a Sulfuric-hydryl-group, as active group. This sulphuric hydryl group, rule the roost for the mycolytic action [12], and the reduction of disulfide boundaries of the mucus by reduction. Through that reduction of the disulfide boundaries the viscosity was reduced and makes the wheezing more easily for asthmatics. In case of Paracetamol poisoning, NAC was given over days. Paracetamol will be metabolized in high dosage through the Cytochrome-P_{450}- system and a toxic metabolite will be produced. Finally a conversion into a mercapto acid conjugate renal excretion through the kidney occurs. It was also notable that in case of high dosage of Paracetamol an alternative metabolism pathway was used and thereby an arylation of cell proteins in the liver cells occur. Dose dependent liver necrosis or liver damage could

happen. NAC could inhibit that pathological process [13]. Further toxic in contamination through m-Acrylnitril, Methyl bromide or Acrylnitril, NAC also was used as an antidote. A benefit in treating of mycoloytic and chronic inflammation of the respiratory tract was also known as a positive effect of NAC [14].

1.1.3) Pathophysiology

Antigen is taken up by antigen presenting cells (e.g. dentritic cells) and presented with MHC to T cells. T cells differentiate into Th1 and Th2 cells. Th1 cells produce IFN-gamma, TNF-alpha and further more. Th2 cells produce Il-4, Il-5, IL10 and Il-13. This Th2 cytokines play a pivotal role in allergic asthma [2]. Th2 cells secrete effector cytokines which induce differentiation of the B cell and activate eosinophils directly. A differentiate B cell, called Plasma cell secrete IgE. This large amount of antigen specific Antibody bind to mast cells with it s Fc receptor and sensitized the mast cells [15]. The second antigen presentation leads to cross-linking and finally to degranulation of the mast cells. This includes the release of histamine and further the maintenance of bronchial spasm.

1.1.4) Th2 memory cells and their maintenance

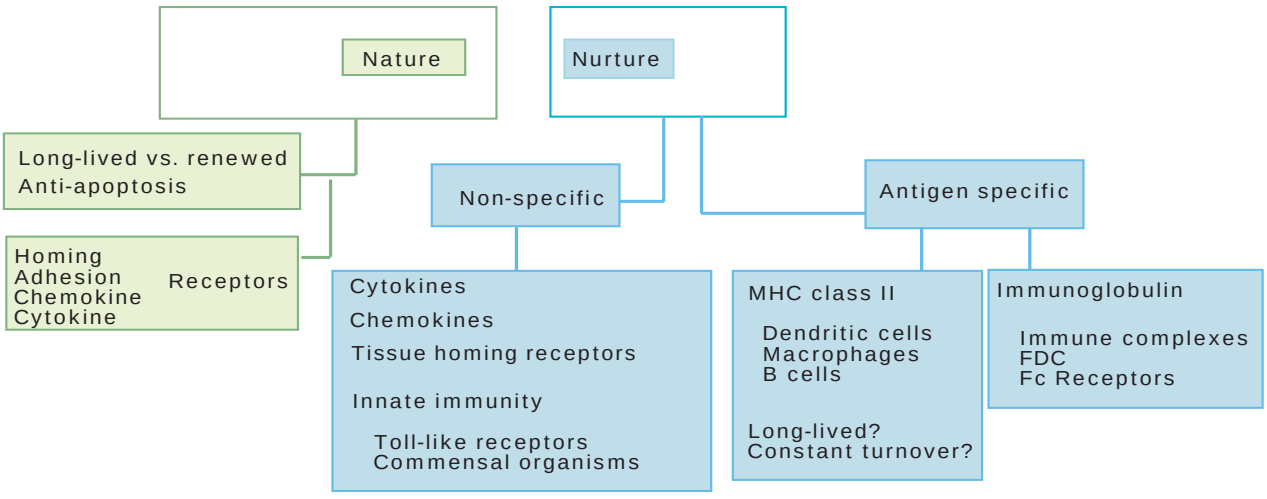

Figure 1: Th2 memory cells and their maintenance (diagram: M. Epstein)

Mossman et. al [16] described for the first time the existence of two T cell subclasses. He also found that Th1 cells mediated through IFN-γ and Th2 cells trough IL-4, IL-5, IL10 and IL-13. It is also

accepted that Th2 memory cells play a pivotal role in establishing and obtaining allergic asthma. The fact that memory T cells didn't undergo apoptosis is at present unclear. The maintenance of Th2 cells belong to two fundamental opinions. Maybe it belongs to the type of cells involved or the lung which mediate Th2 cells. In view of cell interaction, the long lived versus the renewed cells have to be treated. On the other hand Anti – apoptosis could play a role. Further more the receptor function itself could play a role. In homing, adhesion function chemokine and cytokine production. The lung could be differentiated into a nonspecific and antigen specific compartment. In a non- specific view chemokines, cytokines and tissue homing receptors could play a role for the maintenance of Th2 cells. The role of the innate immunity can found in the actions of Toll-like receptors and commensally organisms as bacteria. On the antigen specific side of the lung we could differentiate between MHC class II and Immunglobulines. The role of dentritic cells, macrophages or B cells in context of there long lived and constant turn over rate has to be more studied [2]. Immunglobulines could form immune complexes which lead to decreased apoptosis. Follicular dentritic cells are also acquainted therefore that they stimulate cells in direction to long living. The Fc Receptors which are involved also could play a pivotal role. Taken together the role of cells in the recovery phase in immunological memory is recently poorly understood.

The novel therapeutic allergic asthma drug will be tested in a humoral and cellular view, especially either it has the power to decrease eosinophils as a key player on cellular view in allergic asthma or decrease IgG1 or IgE on a humoral view.

1.2.) Corticosteroids

It is well known that corticosteroids have a powerful action in inhibition in inflammation. They are derived from cortisol which is adrenogenous produced and in asthma used if other medications have to less abatement. The benefit of clucosteroids is that they have the ability to reduce the mucus production of the bronchi and increase other medications. The disadvantages are they have a lot of adverse effects if you took them over a longer period of time. In treating with corticosteroids the dosing and the period of time were the given are essential in preventing side effects. The medication of corticosteroids should be in high dose during the acute phase to reduce the symptoms of asthma as fast as possible and later a slow withdrawal to prevent long time side effects. Orally used corticosteroids are the most powerful once but even if the produced synthetic they have the same effects as its body own hormone Cortisol which affected nearly every organ. To avoid this tremendous side effect, it is always better to administer a relatively low level of oral steroid

combined with a higher level of other drugs with fewer side effects. The best time point in given corticosteroids was early in the morning were the body own cortisol dumping was the highest. This could leads to several side effects, with the body own hormone Aldosteron, including the irradiation of the salt and water metabolism. Therefore, if people suffer on heart diseases and have also problems with asthma a salt diet was very useful.

1.2.1) Corticosteroids as Aerosols

The benefit of aerosols is less side effects of steroids combined with oral given corticosteroids. To attain a high powerful corticosteroid treating agent, this drug is often served in combination with beta-2-adrenergics. For best medication, corticosteroids were given 15 minutes before the beta-2-adrenergika. During a high acute asthma, aerosol treating with aerosols is less useful. The benefits of corticosteroid treatment are given by a reduction of inflammation in the airways that carry air trough bronchial tubes to the lung. Further a mucus decrease by bronchial tubes is visible and so it is easier for asthmatics to breath.

In general there are two ways corticosteroids are administered. The first way is by given it systemically which helps to control narrowing and inflammation in the asthmatic airways of the lung. To achieve a quick relief of asthma during moderate and serve acute asthma attacks and also when a long term treatment of asthma is planed after initial diagnosis corticosteroids were given. Also a benefit of treating asthma with corticosteroids is the fact that they reduce the episode of an attack and prevent re-attacks of asthma episodes. Systemically given Corticosteroids in pill form were given 5 to 7 days and than stopped. In case of serve persistent asthma, corticosteroids were administered by mouth daily or every other day to control their symptoms. It is also notably that there is a difference in medication for adults, teens, children older than the age of 5. Generally the treatment depends on their severity and their long term control. The United States National Asthma Education and Prevention Program (NAEPP) suggested the following approach for treating asthma in adults, teens and children's older than 5 years of age [17].

Serve persistent asthma is treated by high-dose inhaled corticosteroids and long- acting inhaled beta-2-antagonists and if it is recommended corticosteroid tablets. Notably is that the dose should not be higher than 2 mg/kg/day and not more than 60 mg/day. For moderate persistent asthma low to medium dose inhaled corticosteroids and long- acting inhaled β-2-antagonists is the common treatment. An alternative is given by increasing inhaled corticosteroid concentration with medium-

dose range of inhaled corticosteroids or leukotrine modifier also called leukotrine receptor antagonist or thophylline which is a methylxanthine. Mild persist asthma is treated proffered with low dose corticosteroids or alternative with mast cell stabilizer like cromolyn or nedocromil or leukotrine modifier which sustained release thophylline regulator to a serum concentration of 5 to 15 microgram per ml. If mild intermittent asthma is observably serve episodes of asthma visible. Therefore no daily medication is needed, as quick relief all patients receive short acting bronchodilator. 2-4 puffs of short- acting inhaled β -2-anatgonists are in use. An alternative to corticosteroids mast cell stabilizers as Cromolyn and nedocomil are in use but they not so efficient than corticosteroids. In moderate persistent or severe persistent asthma, using long-acting inhaled $beta_2$-agonists along with inhaled corticosteroids is the best combination of medications to improve lung function and symptoms and to reduce overuse of quick-relief medications.

Another opportunity is to add leukotrine pathway modifier or thophylline to corticosteroids, but they do not improve asthma control as long acting β -2-agonists do. If a children have more than three wheezing episodes lasting more than 1day in the past year and they have risk factors for asthma such as allergic rhinitis or a parent with a history of asthma [18]. People with severe persistent asthma may need to take corticosteroids by mouth daily or every other day to control their symptoms. In children in the age of 5 or younger some problems can occur. The first problem which must be solved is the inhalation. In this time moderate or serve persistent asthma is found till yet and so special treating must performed by nebulizer instead of inhalers. Instead of inhalers, nebulizer have the benefit that they can used with a face mask that ensures that the really inhale the medication. Another opportunity is a metered- dose inhaler with a spacer and a face mask, they as effective as nebulizer but there no studies in this age available till yet. However, the NAEPP guidelines recommended the following approach for threading children's age 5 or younger [18]. For children with serve persistent asthma, high dose inhaled corticosteroids and long acting ß-2-agonists should be administered. If it is recommended corticosteroid tablets or syrup long term medication should be given in a dose 2mg/kg/day and no transgression of 60 mg/day. To get a deeper in view, repeated attempts with tablets or syrup should administer and maintained with control high dose inhaled corticosteroids. Moderate persistent asthma should be treated in children's age 5 years or younger with low dose inhaled corticosteroids and long term beta-2-agonists or medium dose corticosteroids for inhaling. Leukotrine pathway modifier or thophylline medication could be an alternative in treating asthma. In children with recurring serve asthma episodes, medium- dose inhaled corticosteroids and long term acting β-2-agonists recommended. An alternative medium dose inhaled corticosteroids and either leukotrine pathway agonists or thophylline should be given. Mild

persistent asthma in children age 5 years or younger should be treated with low dose inhaled corticosteroid or Cromolyn as a mast cell stabilizer. An opportunity is given by leukotrine pathway modifier. In case of mild intermittent asthma no daily medication is necessary. As a quick relief all patients could use a Bronchodilator which acts by short acting beta-2-agonists or alternative oral beta-2-agoists. In case of viral respiratory infections a bronchodilator should be used 4 to 6 hours but no longer than 24 hours. This depends also on the physician consult in general but indeed no longer usage of 6 weeks is recommended. If β-2-agonist used more than twice a week a long control therapy is indispensable. For young children, abovementioned leukotrine pathway modifier normally in us in oral formulations leads to less efficient Cromolyn and nedocromil mast cell stabilizers also in us instead of corticosteroids [22]. In case of viral respiratory infections it could be necessary treating children with corticosteroids by mouth in case of serve asthma episodes. In moderate persisted or serve persisted episodes of asthma long acting β -2-agonist along with a combination of inhaled corticosteroids reduce overuse of quick medications and improve lung function and symptoms [18]. Another combination with corticosteroids was using a leukotrine pathway modifier as thophylline but they were not as good as long acting β -2- agonists in improves asthma control. Research reported that the administration of orally or inhaled corticosteroids in mild to moderate asthma had equal effects, after acute asthma episode [19].

Instead of corticosteroids, dexamethasone acetate will be an alternative for treating asthma, but they suppress the function of the adrenal glands more than oral corticosteroids do.[3] In treating with dexamethasone acetate for a longer period of time, side effects of the medication like slower growth or stunted growth in children could occur. Further problems with the body's capability to glucose could direct to diabetes mellitus. Osteoporesis or the possibility of bone death (aseptic necrosis of the femur) by changes in blood supply could also be a possibility from changes. Hypertension or repeated infection, bruising, and kind thinning as you find in atrophy was a expression of treating with dexamethasone acetate. Time to time it was less likely if patients had fewer, based that corticosteroids camouflage them. Cataract of the eyes leads to clouding of the lens and therefore it is recommended to minimize or prevent side effects by treating with a low dose of corticosteroids as while asthma control was possible [20]. Steroids by mouth or injection may be given as short-term treatment after an episode or when asthma has not been under control. "Burst" treatment with steroids may be continued for 3 to 14 days. A person who has frequent asthma episodes while being treated with inhaled steroids may need to have the medication increased. In children, research indicates that the most important factor in reducing the severity and length of an acute asthma

episode is giving the oral corticosteroid early in the episode, within one hour of the first signs of symptoms [21]. Pregnant women who use steroids by mouth or injection may have babies with low birth weights. Women who use steroids by mouth or injection and who have gone through menopause need to take extra calcium and vitamin D to prevent bone loss (osteoporosis).

1.3.) Gold

Brief in general, gold has a molecular weight of 196,96 g/mole. Chrysotherapy exerts anti allergic and immunosuppressive properties *in vitro* including inhibition of mast cell histamine and leukotrine release. Further more inhibition of mediator induced smooth muscle contraction and antibody production. It is well accepted that gold had an immunomodulatory effect in the treatment of a variety of inflammatory and autoimmune conditions. It is also believed that this inhibitory effect in the presence of gold decrease the enhancement of eosinophil survival based on IL-5 [22]. Further, B. Merchant et al.[23] Demonstrate that gold salts largely bound to albumin and globulin before carrying out by plasma to nearly every tissue of the body.

1.3.1) General about metallic gold and gold compound

If people think about gold they often connect it with treasures and gifts but it is also a known medication for rheumatics and has its place in dental fillings. Less trace elements have a bigger fascinating to human than gold. In the earth crust gold, from the Latin aurum (Au), with its density of 19,3 gram per cubic centimeter which is one of the ten heaviest elements, affiliate to the ten rare earth elements. In nature you will find them bound to another element called Tellur but mostly dignified as Au 0. In General metallic gold was used for medicine. Plinius the older (23 to 79 after Christus) talks in his *Naturalis Historiae* Libri from a lot of indications. Giovanni dÁrcoli (Arculanus) first talks about dental fillings with platen gold in the year 1448 past Christus. Till today such fillings in use as gold inlays and gold crowns. After the discovery from aqua regia, (Kings water), by Abu Musa Jabir around 1100 to 1160 past Christus also salt connections known called Halogen with oxidation steps +1 and +3. Till yet an essential role of gold is not known. In vestiges you will find them in nourishments and the uptake of gold is less than 7 µg rather it is not used as pigment for decoration of food and drinks (Code E 175). Metallic gold had a less resorption than gold complexes.

1.3.2) Gold connections as therapeutics

At the end of the 19th century, scientists start to use gold as $Na^{+}[AuCl_4]^{-}$ for treatment of syphilis and tuberculosis. In the year 1880 past Christus, Robert Koch first demonstrate the bactericide agency of gold cyanide and during the next forty years an intensive research by tuberculosis. In the year 1929 Jacques Forestier believed that rheumatic arthritis is an infections disease and so he used gold as propanol sulfonate as an agent in 15 patients, with the goal that a part of them was treated successfully. Since that day on gold connections used as disease- modifying antireumathic drugs as basic therapeutics. There is less known about the action of gold in the organism. In fact anionic ligands could be bound and there is a high affinity to sulphur and nearly no to oxygen. This leads to the assumption the biological effect of gold was determined as a sulfur hydrogen inhibitor. In fact this opportunity could be nearly excluded. Metallic gold also known as colloid gold was manufactured in our case by reduction of tetra-chloro-gold acid with basic sodium carbonate as reduction agent, which leads to gold particles of 1- 50 μm in diameter. Those gold particles had a high affinity to macro molecules like albumin or immune globulin and bound tightly by pH of isoelectric point to them. Macrophages, Cupper's star cells or other defense cells of the immune system phagozytice them or take them up by bounding to macro molecules.

Binary gold (I) connections known in combination with high electronegative elements:

This salty halogen and pseudo halogen only stable in combination with reducing ligands as cyanide (CN^{-}) or iodide (I^{-}) and the metal ligands bond was more covalent, and finally leads to water soluble polymer. Gold (III) connections had in water milieu always 4 ligands, e.g. H^{+} $[AuCl_4]^{-}$ or H^{+} $[Au(Cl)_3\ OH]^{-}$. Those circuits are soluble in organic phases which allowed the fusion through lipid membranes and via reduction agents finally to metallic gold or stable Au^{+}. The oxidation power is in acid or neutral milieu higher than those of hydrogen peroxide. For stabilization of Au^{+}, the SH^{-} group of proteins which were essential through binding of two ligands. That contact places of the ligands were exchangeable and so the Au^{+} - ion in the goldthiosufonate comes to the albumin through intravenous passage. Albumin consist of two free SH groups and one by one Au+ exchange the hydrogen of the sulphur hydrogen, that was not understood in detail but in general a second intracellular exchange occurred. The Cyanide ligand from Thiocyanide and the Au^{+} metal ion leads to a coordination and finally an anionic stable $[Au(CN)_2]^{-}$ exists. Cyanide ion connections suspected to be around areas with fluoride inflammation.

In cohesive of gold and the anti inflammatory agents two major ties were analyzed in detail, Auranofin (AF) and Aurothiomalate (ATM). In-vitro- experiments with endothelial cells and neutrophil granulocytes clearly demonstrate that both AF and ATM inhibit the offence of zytotoxic granulocytes against endothelial cells.

AF acts trough blocking transcription factor NF-κ B, which regulates the interaction of ICAM and E-selectin which was important for the zytotoxic reaction and the contact between this different cell types. In macrophages, AF suppressed the appearance of Tumor necrosis factor alpha (TNF-α) which is a potent activator for zytotoxic activity.

Salicylate and the glucocorticoid drug dexamethasone also showed this ability of decreased in cytotoxicity. It is believed that the inactivation of NF-κ B could lead to benefits in treating chronic inflammation reactions. ATM had no ability in this way, it only decrease the expression of E-selectin, on the surface of such cells. Many gold complexes were analyzed about their options as anti-infective, anti- inflammatory and anti- neoplastic chemotherapeutics. Two different classes were analyzed. The class I connections of which had at least on ligand bound, they water soluble and preponderant polymer. For example sodium salts of Aurothiosulfate, Aurothiomalate, Aurothiopropanolsulfonat and Aurothioglucose. Only Aurothiosulfonate had two sulphur ligands which could interact with gold. All the others had a ratio of gold and sulphur as 1:1. Class II-connections were called Aureate, and their characteristic was, they had as immediate neighbor only one thiolate. There were monomer, electric neutral and lipophil. Only one agent, Auranofin (Triethylphosphin (tetra-acetyl) glycosylthioaurat), got the accreditation as medicament. Others were tested against anti- tumor agents, for asthma bronchioles and HIV infections. The radioactive 198 Au were also in use as a combination in beta/gamma emitter for treating local malign tumor and for nuclear medical diagnostic. The best known gold salts and complex had a less bioavailability only Auranofin with 25 percent was known had a good one. In case of intra- muscular administration of class I gold salts as Aurothioglucose or ATM, gold visible first in the blood were 95 percent bound to albumin and with further administration visible in the whole body particularly in the Synovial liquid of the pivot, liver, kidney and adrenal glands.

In the synovial liquid the concentration is ten times higher as in all the other tissues, bones and adipose tissue. The half time of elimination had a variation of seven days up to eighty days with a start concentration of 50 mg. It is longer as a year detectable in the urine and gold were separated as Au+ with a range of 60 to 90 percentages. The rest were separated through faeces.

It is also an interesting fact that the uptake of sulphur hydrogen connections like N-Acetylcystein or Penicillamin increases the gold expulsion in the urine. Auranofin as a member of the class II gold salts had a less concentration in the blood. The accumulation in the tissues was only 25 percentage of the class I. The steady state phase was around 8 up to 12 weeks. The half life is around 80 days and the separation effected trough faeces.

In the year 1980 first reports clearly shown an interaction of contact dermatitis with gold as allergen. In the year 2001 the American society for contact dermatitis nominates gold as number one for contact allergen. 13 percentages of the people in north and 10 percentages of European people suffered under gold induced dermatitis based on batch tests with ATM. In Sweden after nickel, gold was the second important metal allergen when they wear it as adornment, dental crown or pricings of gold. This leads to eczema, lichenin and pityriasis like coetaneous irritations. Finally it was not clearly kwon if these aberrations came from gold or from allow with old. An anaphylaxis was known by uptake of gold including spirituous beverages. Evan arthritis patients which were treated with gold salts develop a contact allergy in 105 of the cases. To test the tolerance of gold, patients got a low starting dose to see what is going on in the body before the recommended dose were given.

Another field of gold in medication was called Chrysotherapy of arthritis, which means a treatment with metallic gold without radioactivity. Indications were detected by inflammatory inflammations like rheumatic and psoriatic arthritis, further the Sjogrens Syndrome which is a non disseminating Lupus erythematodes and a Phemphigus.

The most important field was treating of arthritis, were 15 percentage of the patients exhibit a permanent remission. 60-70 percentages indicated a decrease of symptom and 15-20 percentages had to stop the treatment because of side effects or none responding. In case of side effects the treating should interrupt, that happened after 3 to 6 years in more than 50 percentages of the cases. That effects based, that is the currant meaning, on immunological mechanism and including dermatoid reactions with painful pruritus. An ornery metallic taste in mouth as inflammation of the mouth mucosa or in eye could also lead to damage of the cornea or lens.

One side effect which always present was the sedimentation of gold in the organism which leads to Chrysiasis, a blue- grey colored dermis or in case of the eye the callus. Haematopoiesis after chrysotherapy was also demonstrated in some cases and leads to a lacking developed (aplastic) anemia, further Nephro- and Hepato-toxicity and neuron- and encephalopathy which occurred in class I gold connections.

Based on those findings each patient who suffered on a kind of that side effects, liver and gland function should be monitored as same with eye function and heamogram, after gold therapy. The best evidence of Chryasis was the detection of increased gold levels in body liquids and tissue with Atomic absorption spectrometer (AAC) or Atomic emission spectrometer with inductive coupled Argon plasma (ICP-AES), which allowed detection up to 1 µg/kg

Normal range was 0,002 up to 0,85 µg/ l in blood. Newer day's resonance ionization mass spectrometer (RIMS) the choice with a detection level of 1 ng/ kg. The detection border for gold was 0,1 ng/kg. To give a complete overview neutron activation analyze will be also chosen for less detection levels, but for that I want go deeper in view. It allowed the detection of gold in salivary, traces of blood tissue and faces, further in environment probes like sediments, air and sewage sludge.

It is the affinity to sulphur hydrogen which allowed the treating of chrysotherapy over dosing with dimercaprol [24].

1.4.) Acetylcystein:

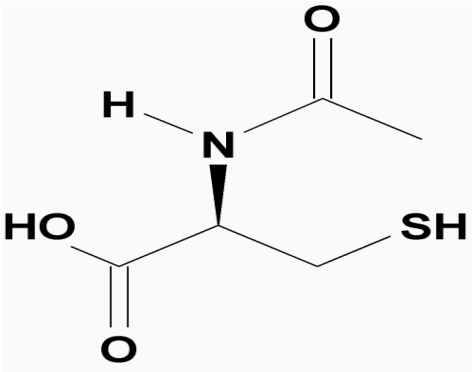

Figure 2: chemical formula of Acetylcystein

The clinical Pharmacokinetics of N-Acetylcysteine (NAC) demonstrates that this agent has benefits in the treatment of chronic bronchitis and other pulmonary diseases as a useful mucolytic agent [2]. The metabolism of N-Acetylcysteine (after DeCaro et al 1989) proposes that NAC interact as labile disulphide complexes with tissue and plasma proteins. Further Cysteine was build and this incorporated into protein chains [25]. NAC were present major in three forms as N acetylcysteine, as N,N- Diacetylcysteine and as Cysteine. After Metabolism Cystic acids, Cysteine, Inorganic sulphates and Gluthathione were secreted. N-Acetylcysteine is useful as a mycolytic agent for

bronchopulmonary disorders complicated by viscid sputum. The mucolytic potency of this drug is possessed by it is free sulfhydryl group that can rupture disulfide chemical bounds.[2] A further benefit of N-Acetylcysteine was shown in the fact that coughing may be stimulated and the mucociliary clearance mechanism may be activated [26]. Side effects of N-Acetylcysteine are mentioned in increased blood pressure, chest pain, hypotension, rectal bleeding, respiratory distress, headache, lethargy, fever and skin allergy (Miller& Rumack 1983).

1.5.) Milk allergen

To establish a diseased mouse model, mice were treated with 200 µl dissolved milk powder (in PBS 1x). Generally milk as allergen consists of 5 parts of antigen, which called the milk proteins like Alpha-Lactalbumin, ß-lacto globulin, casein, lipoprotein and albumosen-peptosen mixture. They characterized with different physical biological attribute. A more general classification is Casein as the heavy soluble and heat stable milk albumen and heat sensible albumin and globulins. It is also notable that Casein is not cow milk specific and if someone establish an allergy against casein each milk product have to be avoided. Alpha Lactalbumin is mostly involved in milk allergy as antigen. A special treatment makes it possible to inactivate lactalbumin by cooking of the milk. In case of Casein as antigen Melanoidine are the key factor for the allergic reaction which was produced by cleavage of sugary compounds and amino acids. Sterile milk and ultra highly heated milk consists more Melanoidine than normal untreated milk [26].

2.) Aim

My aim is to test Auroacetylcysteine novel therapeutic as an allergic asthma drug.

3.) Experimental Design

3.1) Allergic Asthma disease model

To establish a relapsing acute asthma model we injected 6-12 weeks old C57/B6 mice with 10 µg milk powder in PBS (1x) i.p. on days 0 and 21. Between days 28 and 32 mice were treated with 50 µl PBS (1x) or AAC (100 mg/kg/20 g mouse) i.n. in addition to naïve control mice. The mice were killed on day 35 with i.p. injection of 0,5 ml Rompun Ketanest.

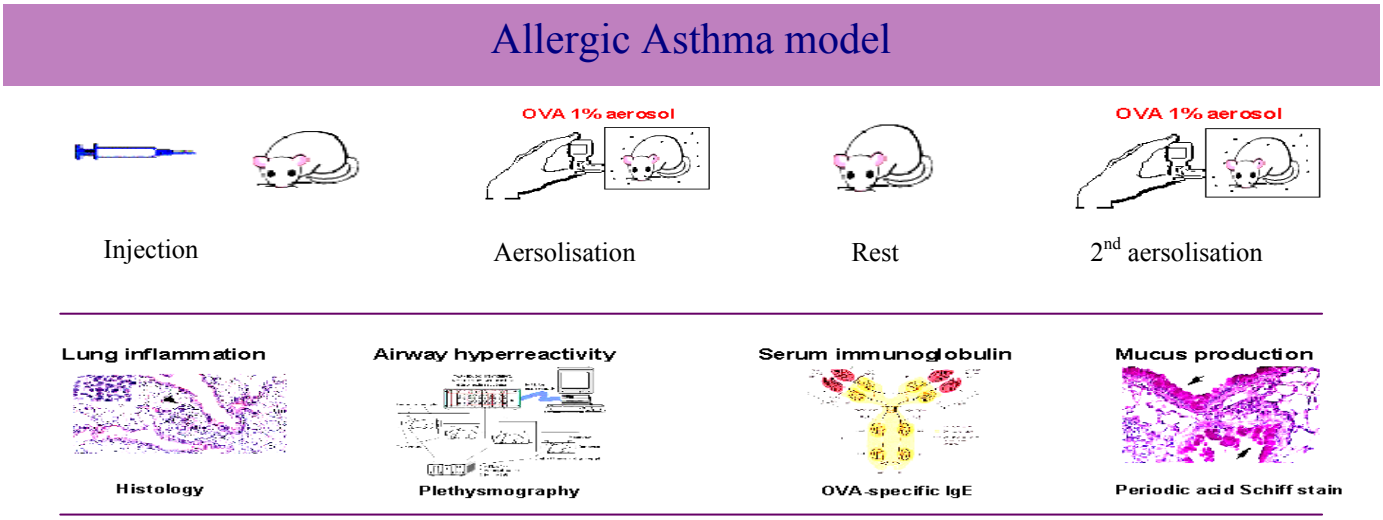

Figure 3: allergic asthma mouse model (adapted from M.Epstein, MD)

Allergic Asthma treatment:

Naive: PBS $_{aerosol}$ + PBS $_{aerosol}$
Recovered: Recovered: milk powder $_{i.p}$ milk powder $_{aerosol}$ PBS $_{aerosol}$
Rechallenged: milk powder $_{i.p}$ milk powder $_{aerosol}$ milk powder $_{aerosol}$

Intraperitoneal: 10µg milk powder/200µl PBS (1x)
Aerosol 1% milk powder or PBS twice a day for 1 hour

After assessment of the mice we took sera for IgG and IgE measuring with ELISA. Further we performed a May Giemsa Grünwald (MGG) staining, to measure several cell types in the BAL. The lung was harvested for PAS (Periodic Acid Schiff) staining to characterize the mucus producing goblet cells and the red colored mucus secretion based on a 5 Grade percentage calculation. Luna staining gave us a deeper insight in the eosinophilia of the treated and untreated mice and were calculated by counting 10 fields by 100_x objective lens and were differentiated into central and peripheral lung tissue. Also including counting's around the airways and the blood vessels. Hämatoxylin and Eosin Staining (H&E staining) will show the infiltration situation of the lung and was calculated on a three graduated system, including parabroncheal and Para vascular areas, based on Mehlop et. al. [29] Albumin ELISA gave us and idea if edema will be occurs or not.

4.) Material and Methods

4.1) Animals

Animals were purchased from the Charles River Laboratories, Germany. Four to eight week old C57/B6 or BALB/c female mice bred and maintained in the animal facility at the Pharmacy Institute were used in these experiments. The University of Vienna, Vienna, Austria, approved all animal experimental protocols. Mice were housed in the Pharmacy facility with OVA-free food and water provided ad libitum.

4.2) Apparatus and instruments

Ultrasonic nebulizer: Aerodyne omega (Kendall/ Tyco Healthcare)
Cooling Zentrifuge (Beckmann)
Freezers –20 °Celsius(Liebherr, Bischofshofen,Austria)
Vortex Genie 2 ^{TM} (Bender&Hobein AG, Zürich, Switzerland)

4.3) Plastic Materials

Centrifuge tubes (Falcon, New Jersey, USA)
Pipettes (Costar, Cambridge, USA)
Sterile tips
Syringes (Braun Medical AG Omnifix R, Emmenbrücke, Switzerland)
Needles (BD Drogheda, 27Gx3/4“ Nr. 20, Ireland)
Cassettes (paraffin) (Simport Histosette ^{R} II, with lid/AVEC, Beloil, Canada)
Cassettes (frozen) (Gyromold r Standard, Sakura Finetek Europe, Zoeterwounde, Netherlands)

4.4) Glassware

Cover slip
Dropper
Glass bottle
Slides

Paraffin sections
Microscope slides 90° ground edges, precleaned, twin frosted end, Marienfeld GmbH& CoKG, Lauda- Königshofen, Germany)

4.5) Reagents

Rompun 2% solution (Bayer AG, Leverkusen, Germany)
Ketanest S (Pfizer Corporation Austria Ges.m.b.H. Vienna, Austria)

PBS
Including:
$Na_2HPO_4x2\ H_2O$ (Merck GmbH, 64271 Darmstadt, Germany)
KCL (Merk GmbH, 64271 Darmstadt, Germany)
KH_2PO_4 (Merk GmbH, 64271 Darmstadt, Germany)
NaCl (Merk GmbH, 64271 Darmstadt, Germany

10 % Giemsa solution (Merck GmbH, Darmstadt, Germany)
May-Grünwald solution (Merck GmbH, Darmstadt, Germany)
Paraformaldehyd, 4% (Merck GmbH, Darmstadt, Germany)

4.5.1) Material and Methods

0,4 % Trypan blue solution (lot no. 44K2394, Sigma Aldrich, Germany)

4.5.1.1) May Grünwald Giemsa staining

Methanol p.a. (lot no. K16453309, Merck, Germany)
May Grünwald Eosin Methylenblaulösung modifiziert (lot no. 0B547524. Merck, Germany)
Giemsas Azur-Eosin- Methylenblaulösung (lot no. 0B540249, Merck, Germany)

4.5.1.2) ELISA

Tween 20 (lot no. 143034A, Bio-Rad laboratories, CA 904547, USA)
BSA (lot no. 120K 1216, Sigma Aldrich, Germany)
PBS (lot no. 13938, Gibco, Invitrogen, Paisley, United Kingdom)
OVA Albumin from chicken white egg (lot no. aA5503-25G, Sigma Aldrich, Germany)
Distilled water

Tips

96 well Plate (Nunc-Immuno^{TM}Plate MaxiSorp^{TM} Surface, Denmark)

Plate sealers (cat no. M30 Quantity 100, Dynatech AG 7306 Denkendorf, West Germany)

Goat anti mouse IgG1 Biotinylated (lot no. G046-T456, Southern Biotechnology) associates, Inc. USA

Streptavadin-HRP (lot no. D126-S786H, Southern Biotechnology associates, Inc. USA)

OPT (lot no.054 K 8216, Sigma Aldrich, Germany)

Buffer with urea H_2O_2 (lot no. P8812, Sigma Aldrich, Germany)

4.5.1.3) PAS Staining

Ethanol absolute (lot no. K30598083, Merck, Germany)

Ethanol 70% vergällt (lot no. F11A027V, AKH Anstaltsapotheke, Vienna)

Ethanol 96% vergällt (lot no. F 21A314A9R, AKH Anstaltsapotheke, Vienna)

Xylenes Isomerengemisch (lot no.456173/1, Fluka Chemika, Buchs, Switzerland)

n-Butylacetate p.a (lot no.K 24240052, Merck, Germany)

Papanicolaou´s solution 1 a Harris hematoxylin solution (lot no. 0B546994, Merck. Germany)

Eukitt ® (O.Kindler GmbH& Co KG, Ziegelhofstraße 214, 79110 Freiburg, Germany)

0.01%Proteinase K

10µl of 10mg/ml stock+990µl of water

2N HCl 16.7 ml of 37%HCl +83.3 ml of H_2O

3% H_2O_2 1ml of stock 30%H_2O_2 make up to 10 ml with water in dark.

Immersion oil n_e^{23} = 1,518 (Wetzler GmbH)

4.5.2) Anesthesia

10 ml of Ketanest S (25mg/ml) was mixed with 2 ml of Rompun 2% solution. The volume was adjusted with autoclaved water to 20 ml end volume.. For killing mice we took 0,5 ml.

Albumin from chicken egg white, Grade V (Sigma-Aldrich Inc. Chemie Ges.m.b.H., Steinheim, Germany)

4.6) Cuttings

4.6.1) Paraffin cuttings

After harvesting, the lungs were stored for 3hours or more in 4 % paraformaldehyd and than transferred into Histocasettes and stored in PBS (1x). All further steps were done in the AKH Wien except till embedding which was performed by us.
Paraffin cutting sections had been carried out with the Microm Heidelberg machine as 4 µm cuts.

5.) Protocol

5.1.) Microscope

All staining pictures are performed with the Nikon Y-IDP microscope. As light camera we used the CCD-VDS 1300 and for the monochromatic light spectroscopy we used the DS- U1. All pictures were made with the magnification 10_{x}, 20_{xs} 40_{xs} 60_{xs} or 100_{xs}
0,4 % Trypan blue solution (lot no. 44K2394, Sigma Aldrich, Germany)
TMB KIT solution (lot no. 512606 KC, BD Pharmingen, 2350 Qume Drive, San Jose, CA 95131-1807, USA)

5.2) Machine

Spectra MAX 2500 Photometer
Microtome Cutting machine, Heidelberg, Germany

5.3) Compound calculation:

Auroacetylcysteine:
Empirical formula

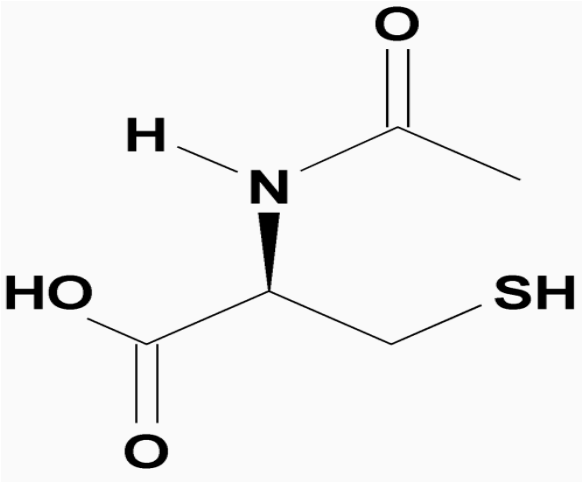

Figure 4: Explanation of NAC

The H of the sulfide group is substituted by an Aurum molecule.
The molecular weights of the used chemicals were AAC= 359,17 g/mol, Au+ = 196,97 g/mol and Na_2CO_3 = 106,00 g/mol. Our final concentration of AAC was either 100 mg/kg/20 g mouse or 10 mg/kg/20 g mouse. Therefore we calculated 0,5 mol Na_2CO_3 plus 1 mol of AAC as our final concentration. This leads us to calculate 359,97 g/mol AAC plus 53 g/mol Na_2CO_3 in one liter and for the preparation of 25 ml final solution 1 gram AAC and 0,147 g Na_2CO_3 in 25 ml distillated water. To avoid irritation of the mucous membrane the pH was set between 7,2 and 7,4 with hydrochloric acid.

6.) Staining

6.1) PAS Staining

Literature for PAS staining method we choose a method based on the description of McManus and Carson [27]. The 4 µm sections of the paraffin block where dried at 60-70 ° Celsius. Followed by the de-paraffinization with a gradient of Xylene for ten min and an ethanol gradient with 100/96/80 % for one minute and finally we wash with aqua distilled as long as no cords observably. We incubated the slides for ten minutes in 0,5 % Periodic acid and washed with aqua dest. three to five minutes. To store the slides a while on tissue should remove the most of the water but notice they should not dry

out. 4°C cold Schiff´sche reagent is used for the incubation of the slides for thirty min in a 60-70 °C oven. Next we washed 3 times with SO_2 water, each chamber two min. We washed with floating tepid hot water for fifteen min and incubated with Papanicolaou´s solution 1a towards Harris for thirty seconds. After this a washing steps performed and the dehydration were done by using 80/96/100 and 100 % Ethanol each ten min. Finally, we fixed the tissue with Eukitt® stable- fast hardening mounting medium. The sections were examined under the light microscope with the magnification 20x.

6.2) H&E staining

To get an in view about the infiltration situation in treated and untreated mice we choose the method of Hämatoxylin and Eosin (H&E staining) staining [28]. 4µm sections of the paraffin block were brought to the AKH where all further steps were done. After preparation the slides were examined under the light microscope with the magnification 10_x, 20_x and 60_x. The grading of inflammatory infiltrate based on Melhop et. al 1997 [29] based on a 4 grading system. Including the differentiation of the3 localization of pathology in parabroncheal and perivascular areas, Grade zero was characterized as normal tissue. Grade one indicate one or two centrally located foci, followed by grad two which extended the localization from center till middle (one divided by three) of the lung. Grade three was indicated by serve dense infiltrate localization from central to visceral pleura. To obtain more powerful meanings of the characterization, the values were translated into percent with grad zero as zero and grade three as 100 percent.

6.3) Luna staining

For further characterization Luna staining was performed to analyze Eosinophils in the tissue [34].
The method in brief, first the 4 µm sections of the paraffin block were dried at 60-70 °C followed by de-deparaffinization with a gradient of Xylene for ten min and an ethanol gradient with 100/96/ 80 percentage for one min and finally we wash with distilled water as long as no cords observably. Following we stained with working Hämatoxylin- bierbrich scarlet solution for seven min and thirty seconds. For differentiation three dips in 1 % acid alcohol were done until desired nuclear detail is achieved. Further we rinse in tap water to remove acid alcohol. Two dips in 0,5 % Lithium Carbonate solution were done until the sections turned into blue and eosinophil granules are bright red. Next we washed for two minutes and dehydrate as described previously. The sections were fixed in Eukitt® fast mounting medium and examined under the light microscope with the magnification

100x. For calculation of Luna staining 10 fields including central and peripheral lung tissue, further around central and peripheral airway and blood vessels were analyzed.

6.4) May Grünwald Giemsa staining

For differential Cell counting we performed a May Grünwald Giemsa staining. In brief BAL Cytospin was kept at room temperature for ten minutes. After fixing the tissue in methanol for five minutes we keep them for fifteen min in May Grünwald solution, after clearance with water, slides where incubated for twenty min in 10 % Giemsa solution. Thereafter, we washed with dejonised water till water became clear and the air dry slides were examined under the light microscope with the magnification 100_x for Quantification.

6.5) Albumin ELISA

To get an idea how the whole body system was, we performed an albumin ELISA from sera and BAL samples. It is well known that the colloidal pressure in a body is determined through globular proteins such as albumin and globulin. The Human Plasma contains 60 % of albumins and 40 % globulins. The method in brief, after coating the plate for one hour with coating Buffer (pH 9,6), we washed the plate three times with washing buffer (pH 8,0). Further we block with blocking solution including 1,21 g Tris, 1,64 g NaCl and 2 g BSA in 200 ml dest aqua with a final pH 8.

After blocking for 30 minutes the sample were added with 50 µl of each PBS (1x) or 100 mg/kg/20 g AAC. Followed by a three times washing step the samples were added as first Antibody for sixty minutes. Next a three times washing step were done and the second antibody was added for 30 minutes. Another three times washing step were performed and finally incubated with TMB solution for 30 minutes in the dark. Finally a 2 N Hydro sulfuric stop solution were added and measured with the spectra Max Photometer with the wave length 450 nm.

7.) Generation of allergic asthma in wild-type BALB/c female mice

Our allergic asthma model allowed us to differentiate between acute disease, a recovery period and a rechallenged phase. We choose two different models to establish our acute disease mouse model. In general we sensitized with 10 µg OVA or with 10 µg milk powder on day zero and twenty one.
Toxicity test
For toxicity test we sensitized 6 (7-9 wk old) BALB/c female mice as described before and aerosol challenge them on day 28 and day 29 with 1 % OVA bid for 1 hour. Before delivering the two different AAC concentrations (10 mg/kg/20 g mouse and 100 mg/kg/20 g mouse) on five consecutive days starting with day 22, we anesthetized all 6 BALB/ c female mice with 100 µl Rompun Ketanest. Three mice got either 50 µl AAC (10 mg/kg/20g mouse) or 50 µl AAC (100mg/kg/20 g mouse) intranasal. Mice were killed by 0,5 ml i. p. injection of Rompun Ketanest

Mice were scarified on day 30 and cardiac puncture were performed to get sera for testing AAC levels in blood (will be done by Oliver Scheiber, Member of Noe´s lab, Pharmacy Institute, Althanstraße, Vienna). Further Liver, Kidney, Spleen, Duodenum and Stomach were harvested for paraffin sections.
Before delivering the two different AAC concentrations on five consecutive days starting with day 22 we anesthetized all 6 BALB/ c female mice with 100 µl Rompun Ketanest. Three mice got either 50 µl AAC (10 mg/kg/20g mouse) or 50 µl AAC (100mg/kg/20 g mouse) intranasal. Mice were killed by 0,5 ml i. p. injection of Rompun Ketanest.

8.) Collecting of the sera

Through cardiac puncture blood were collected and stored at 4 °C over night. Next day a centrifugation step for 10 min at 4 °C with 13200 rpm were performed to collect the sera for further analysis of IgE, IgG 1 and albumin levels. Clot were stored at minus twenty degree.

9.) Results

9.1) Meanderings of this experiment

One mouse getting the high does die before scarifying. And all mice had extended stomachs. Thereby we suggest the combination with Anesthesia and compound will be to strong for the mice and further experiments were done without anesthetize the mice.
To examine whether the Anesthesia or the compound was the reason for the extended stomachs we treated six BALB/ c female (7-9 wk old) mice as described before.

Our findings demonstrate that all stomachs were bigger as normal but in Anesthesia treated BALB/c female mice was it more than in AAC treated. So the combination of both could be cause problems, and leads as not expected to death of one mouse. In brief, three BALB/ c female mice (7-9 wk old) received 50 µl AAC (10 mg/kg/20 g mouse) i.n the other three administrated 50 µl AAC (100 mg/kg/20 g mouse) i.n. Cardiac puncture was done as *described above* and BAL was performed by flushing the lungs with four times 0,5 ml PBS 1_x. After ten minutes centrifugation at 1200 rpm supernatant was collected and stored at –20 °C for further use. The pellet was resuspended in 1 ml PBS 1x and counted with Neubauers chamber, using 50µl solution and 50 µl 0,4 % Trypan blue. For Cytospin $1*10^5$ cells were obtained and centrifuged for ten min @ 80 rpm further May Grünwald Giemsa staining were and differential cell counting were done.

May Grünwald Giemsa staining on Cytospin BAL slides were performed as described before.. Based on this experiment we found no infiltrates in the lung tissue but an increased mucus production which leads us to the findings that eventually hyperplasia or hypertrophy occurs. To demonstrate that this is not ostentatious and AAC has inhibition power of allergic asthma further experiments were done.

9.1.1) Pathology of the lung

9.1.2) Grading of Inflammatory infiltrate

Our Grading system depends on the staining we used.

9.1.3) H&E Grading

For graduation of the lung infiltrates we used the method by Mehlop et al., 1997 [30]. Therefore we analyzed the lung sections in view of peribroncheal and perivascular infiltrates and there localization of pathology. Grad zero showed no infiltrates and determines normal tissue. Grade one examines one or two centrally located foci of infiltrates. Grade two is characterized by dense peribronchela and perivascular infiltration and extended from center till the middle 1/3 of the lung. Grade three was marked through dense infiltration from central to visceral pleura infiltration. The examination was done calculating Grade three as 100 % and a graduation of the lung sections in between.

9.1.4) PAS Grading

This grading includes six Grades, from Grade zero to Grade five. We concerned goblet cells and mucus and finally we calculated the results into % with Grade five as 100 %.

9.1.5) Luna Grading

For the graduation of the Luna slides we choose a method including counting ten different fields of the lung by 100x objective lens. We examined fields of central and peripheral tissue, airway sections, including central and peripheral passages and blood vessels of the central and peripheral area.

9.1.6) Albumin Calculation

For the calculation of the Albumin levels itself a sandwich ELISA were performed [31,32].
The sigmoid curve of this ELISA was achieved by a semi logarithmic diagram, including the concentration on the x-axis as normal logarithmic and the Extinction on the y-axis.

To calculate the linear regression it was important to linerize the sigmoid curve. Therefore the y-axis values were translated into Logit values.
Finally you should observe a straight line in the double logarithmic diagram, and it is called Logit-log- plot.
To achieve Logit values from Extinction values we have to normalize (n) the extinction values (w) to get numbers from 0 to 1.

With the lower (u) and upper (o) asymptote of the sigmoid curve it was possible to normalize them easily.

$$n = \frac{w - u}{o - u}$$

Reverse Function

$$w = u + n \cdot (o - u)$$

This normalized values were used fort he Logit- equation (L).

$$L = \ln\left(\frac{n}{1 - n}\right)$$

Reverse Function

$$n = \frac{e^L}{1 + e^L}$$

This pair of variants from the Logit-log-Plot with normal logarithmic from the concentration and Logit values from the extinction calculation will be used to calculate the linear regression. This gave us the high (a) and the acclivity (b) from the linear equation.

$$y = a + b \cdot x$$

Reverse Function

$$x = \frac{y - a}{b}$$

For the interpolation from unknown values you will need the reverse functions of the linear equation from the calibration curve. The best accuracy was given in the middle of the sigmoid curve because the acclivity where here the highest. The best results were achieved in the area with n= 0,5 and L = 0. It makes no matter either you use the decadic or logarithmic calculation, important is only to normalize the values before the Logit calculation will be done.

10.) Treatment

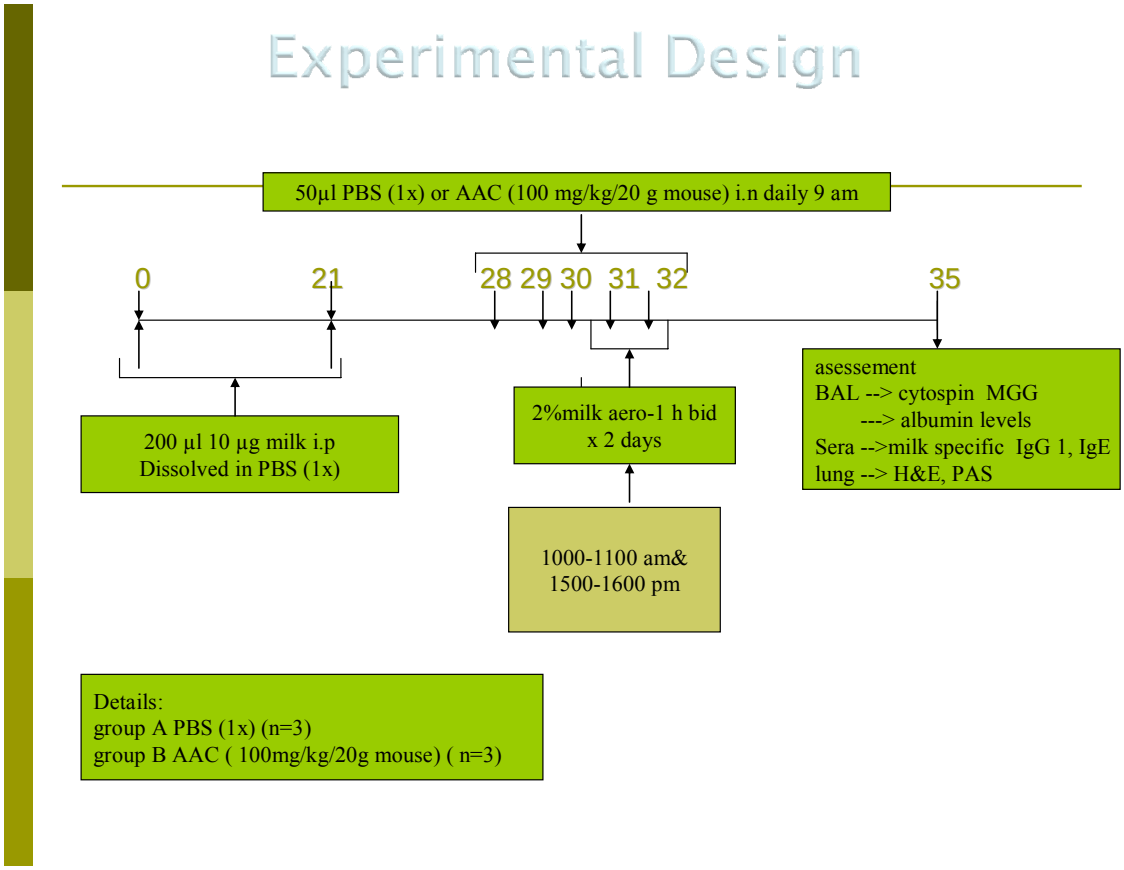

Figure 5: Treating scheme for C57/B6 mice with AAC (100 mg/kg/20 g mouse)

As mention in figure 5 200 µl of 10 µg milk powder dissolved in sterile PBS (1x) were given i.p. on day zero and twenty – one. during days 28 and thirty-two aerosol challenge were performed twice a day for one hour between ten and eleven am and fifteen and sixteen pm. Assessment of the mice on day 35 were done to get BAL for Cytospin and further for May Grünwald Giemsa staining and for measuring albumin levels in BAL.

Further sera were collected through cardiac puncture to measure IgG1 and IgE levels in blood.

Finally paraffin section of the lung was made to do H&E staining for infiltrates and PAS for mucus production of the lung in mice.

11.) IgG1 diagram

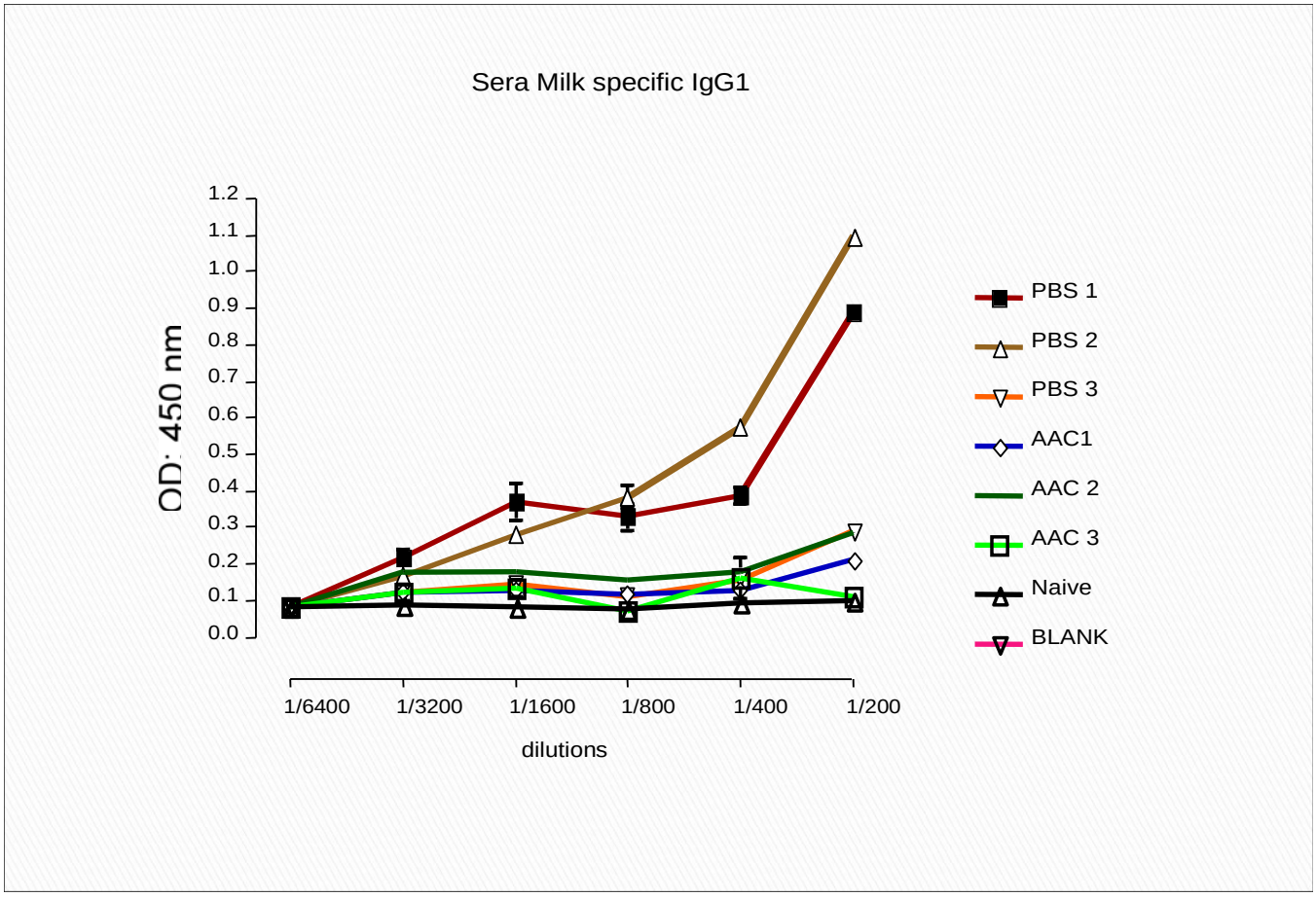

Figure 6: IgG1 levels of C57/B6 female mice

After performing an IgG1 ELISA values were examined with the ELISA Plate reader. To achieve reproducible results dilutions of 1/200 to 1/6400 were done and after thirty min the OD were measured at 450 nm with the SpectraMax M5 from Molecular Devices. Our Data suggest two important things. The first thing as we suspected was that the PBS (1x) positive group showed much higher level of IgG1 as the AAC treated. Thereby, we found that PBS 3 was however a no responder. This could happen from time to time notwithstanding the treatment were done as usual. The treated AAC group had a four to five time decreased IgG 1 levels compared to the PBS group which demonstrates the powerful action of AAC. Interestingly the AAC group was nearly treated to naïve which was highly interestingly because that indicates an interaction of IgG1 and allergic asthma in combination with B cell interaction. The common view was that allergic asthma is a Th2 depended [33]. This T cell subtype produces cytokines which activates B cells. After class switch to IgG, IgA and IgE, IgE activate Mast cells which produce Histamines, which causes allergic asthma reaction**.** Based on our findings AAC inhibits the B cell activation completely.

Based on the Prism Graph pad 2005 software, the one-way analysis of variance is significant for IgE and IgG1 in the PBS (1x) group. We also found a significant increase of neutrophiles of PBS (1x) and AAC. As Borderline of significance we choose P as lower than 0,05. We demonstrate a significant decrease of IgG1 level in the AAC treated group.

One-way analysis of variance	value
P value	0,0337
P value summary	*
Are means significant different? (P < 0.05)	yes
Number of groups	10
F	2,180
R squared	0,2189

Table1: milk IgG1 level C57/B6 female mice 450 nm AAC (100 mg/kg/20 g mouse) vs. PBS (1x)

11.1) IgE diagram

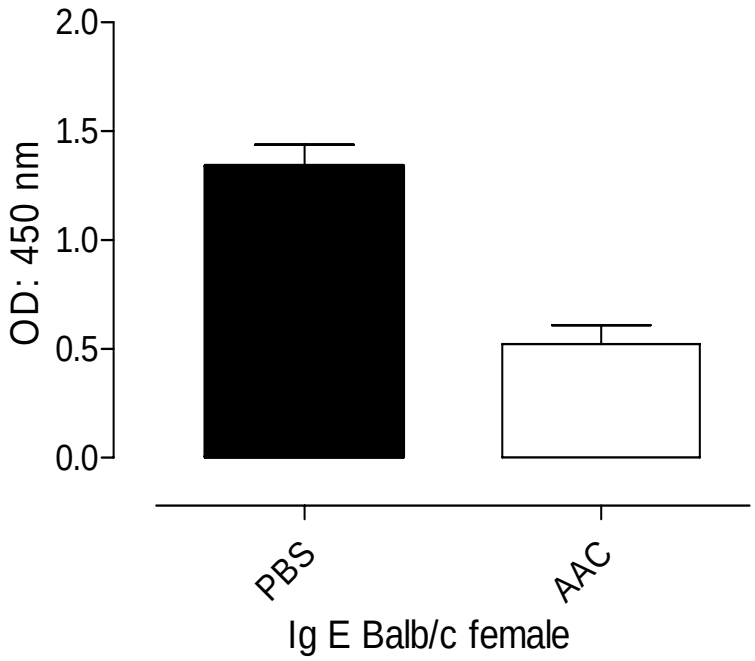

Figure 7 IgE level of PBS (1x) vs. AAC 100mg/kg/20 g mouse n= 3

To get an idea if the values are in a significant relationship we analyzed our summarized data with the Prism graph pad 2005 version number 5 software. Our analysis based on the one paired nonparametric ANOVA test of variances. Our final goal was to demonstrate if our data significant differs from the treated AAC group compared to the untreated PBS (1x) group. As borderline for significant we choose P < 0,05. Our data clearly demonstrate a significant differ of the means by one-way analysis of variance. For calculation we used 6 groups with R squared of 0, 8207 and a F of 21,98.

Table Analyzed	IgE
One-way analysis of variance	
P value	P>0,001
P value summary	***
Are means significant different? (P < 0.05)	Yes
Number of groups	6
F	21,98
R squared	0,8207

Table 2: Ig E C57/B6 female mice AAC (100 mg/kg/20 g mouse) vs. PBS (1x)

11.2) Macrophages diagram

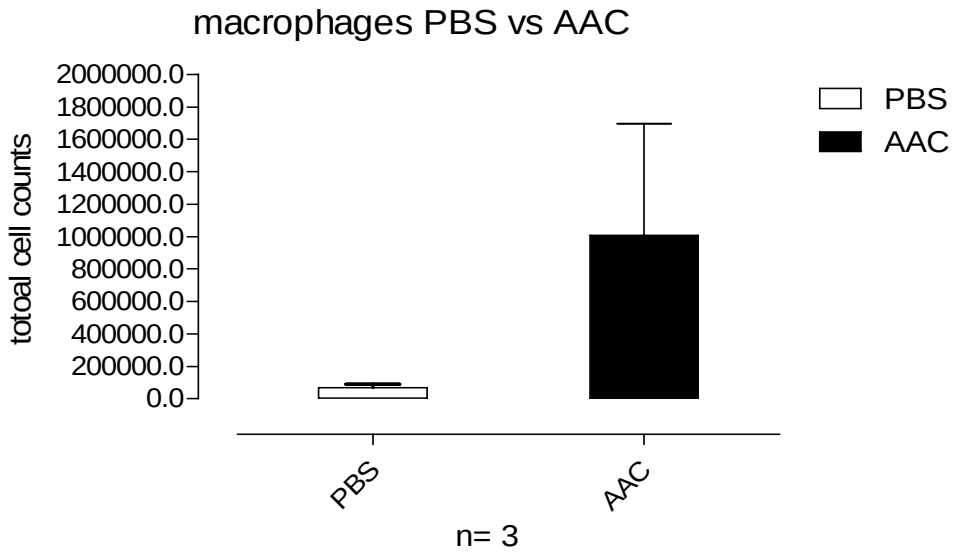

Figure 8: MacrophagesC57/b6 female mouse PBS (1x) vs. AAC (100 mg/kg/20 g mouse)

11.3) Eosinophil diagram

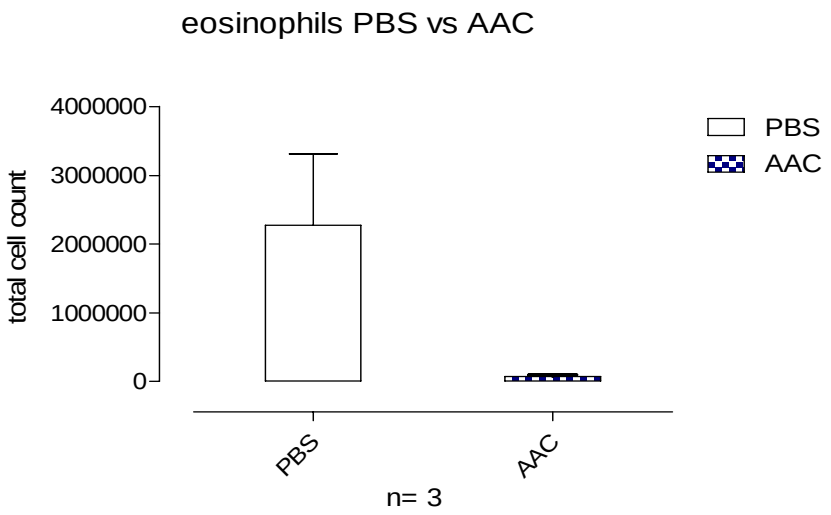

Figure 9 Eosinophils PBS vs. AAC

The diagram is explained in table two.

11.4) Lymphocytes diagram

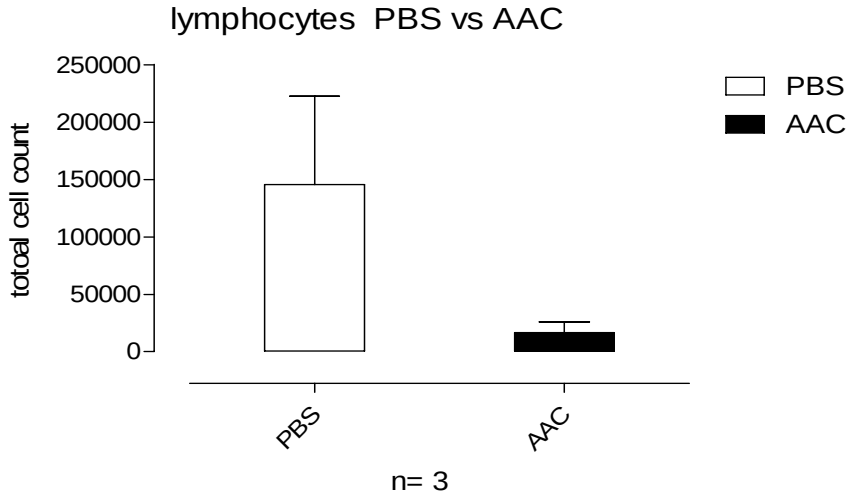

Figure 10: Lymphocytes PBS vs. AAC

11.5) Neutrophiles diagram

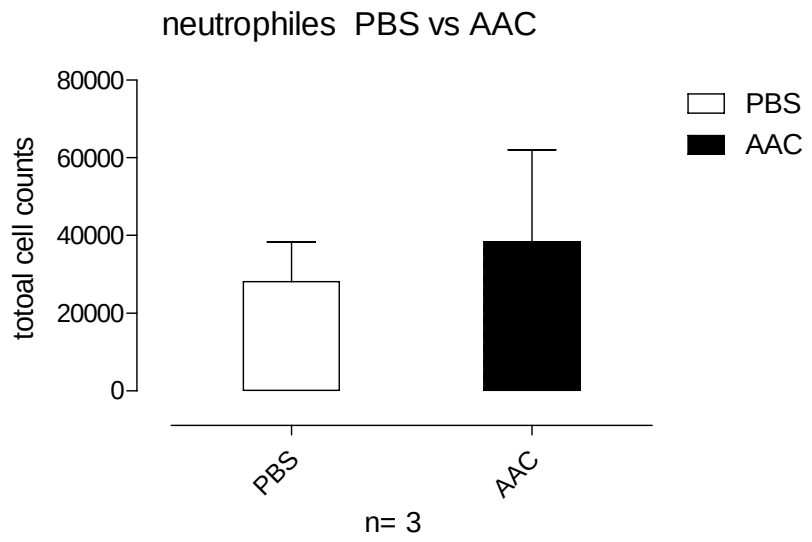

Figure 11: Neutrophiles PBS vs. AAC

Table analyzed	**PBS (1x) vs. AAC (100 mg/kg/20 g mouse)**	
Analysis	**Two-way ANOVA**	
Source of Variation	**% of total Variation**	**P value**
Interaction	34,31	0,0181
Time	3,11	0,2859
Row Factor	21,81	0,0701
Source of Variation	**P value summary**	**Significant?**
Interaction	*	Yes
Time	ns	No
Row Factor	ns	No

Table 3: PBS (1x) vs. AAC (100 mg/kg/20 g mouse) C57/B6 mouse neutrophiles

11.6) Summary Experiments diagram

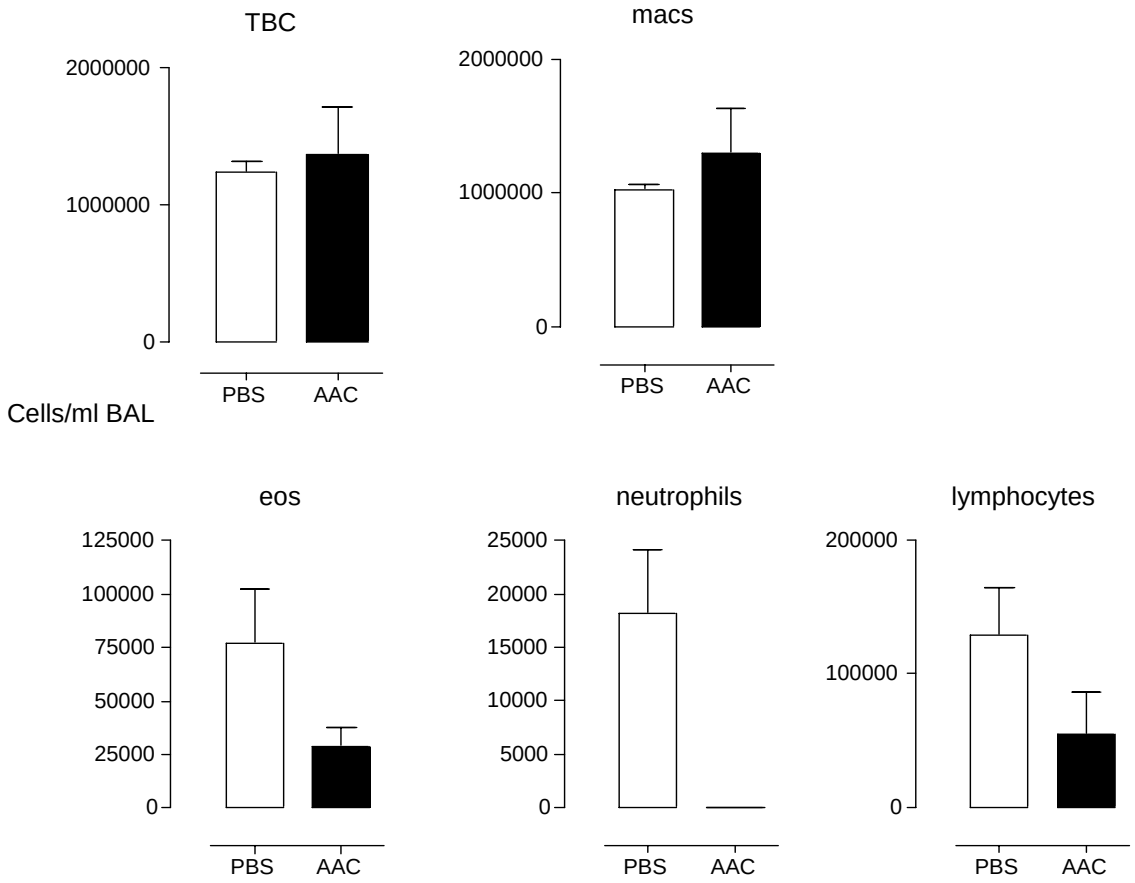

Figure 12: summary TBC, macrophages, eosinophils, neutrophiles, lymphocytes

11.6.1) General information

To get a deeper in view about the situation of the mouse, body differential cell count were performed. The graph on top left show the total BAL count in cells per ml. for calculation of the cells depending macrophages (macs), eosinophils (eos), neutrophiles and lymphocytes, a total of 300 cells were counted with the light microscope under the magnification 100 x. Therefore the slide was first scanned to get an overview about the situation itself on the slide and than continued with starting from one side and going downward by a criss cross movement and counting with the counting machine which is in Principe nothing else than a metric machine with fife wheels which allowed the different counting of the different cell subtypes. The fifth wheel counted the total number of the cells.

After flushing the lungs with a total 1,5 ml PBS (1x) cells were stored on ice after a centrifugation step including 1200 rpm for 10 min. The supernatant were collected and stored on -20 °C. The cells

were resuspended in 1 ml PBS (1x) three times and than counted with the cell counting chamber based on Neubauer. In Brief the chamber contains a reticule of 4 squares. Each of them contains sixteen squares per square of the four. For correct counting two of the four squares where counted and the middle value was taken. Based on this counting an aliquot of the resuspended cell volume were taken to achieve a final cell concentration of 1 multiply by the power of ten to the fifth. 300 µl of this vortex mixture were used for differential cell counting. Therefore we centrifuged the solution 3 min at 80 g. After counting the BAL cells total Ball Count (A) macrophages (B) eosinophils (C) neutrophiles (E) and lymphocytes were shown in the graph. After counting of 300 cells per BAL slide total statistics were done with the Excel and Prism software. In brief, we found that there is no difference in the mean of total BAL counts. We found an increased but not significant difference in TBC. One possibility to decrease the symptoms of asthma was the reduction of the eosinophils which was demonstrated in a reduction of more than 50 % in mean Neutrophils were absent in the AAC treated group which was demonstrated in figure E. The reduction of lymphocytes in AAC treated mice hallmark the powerful action of the drug

11.6.2) albumin level

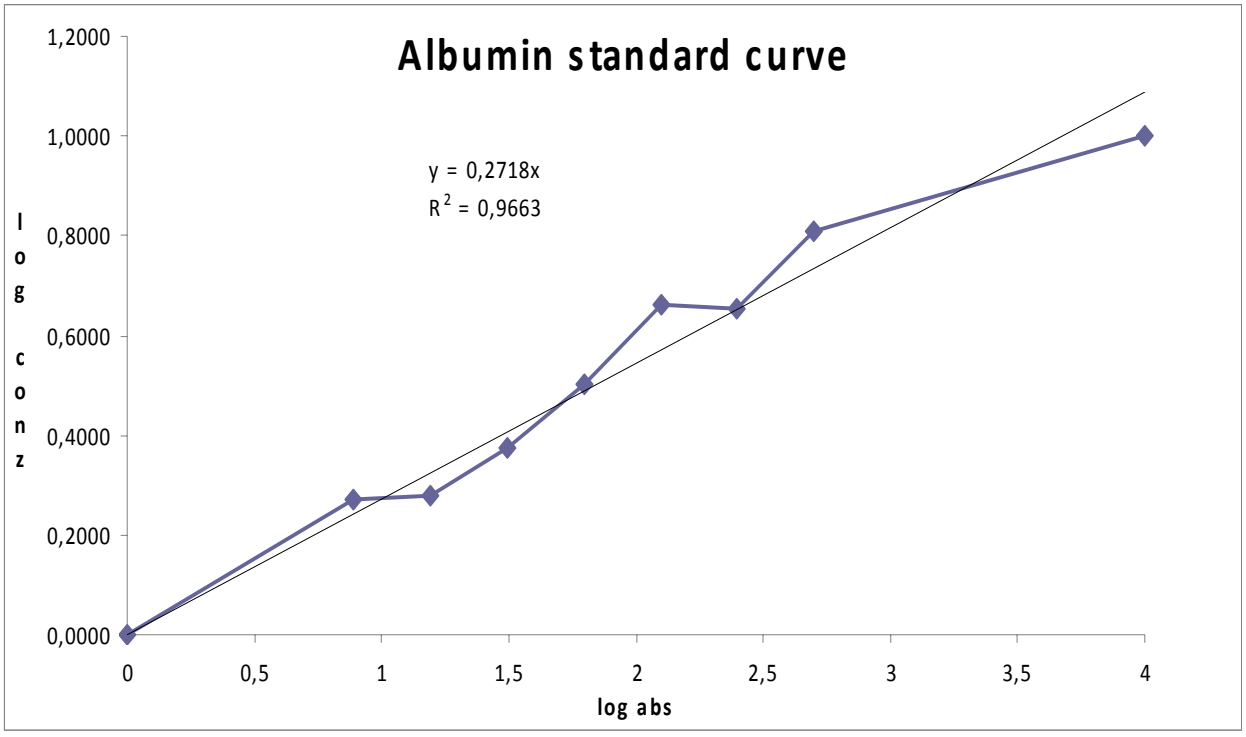

Figure 13: graph albumin levels in C57/B6 female mice

			average	norm values	
nv BAL	0,3892	0,4211	0,4052	0,1888	-0,63313
nv sera	0,5876	0,5682	0,5779	0,2881	-0,39288
PB	0,5099	0,4817	0,4958	0,2409	-0,49846
AB	0,5561	0,5893	0,5727	0,2851	-0,39925
Ps	0,8725	0,8348	0,8537	0,4468	-0,09277
AS	0,8104	0,8203	**0,8154**	0,4247	-0,13181
	500,0000	2,699	0,8089		
	250,0000	2,398	0,6526		
	125,0000	2,097	0,6615		
	62,5000	1,796	0,5030		
	31,2500	1,495	0,3744		
	15,6250	1,194	0,2800		
	7,8000	0,892	0,2700		

0,0000 0,000 0,0000

Table 4: Albumin C57/B6 BAL and sera values

Explanation of the albumin graph

PBS (1x) BAL	***21,78 mg/ml***
AAC BAL	***29,24 mg/ml***
Naïve BAL	***73,48 mg/ml***
PBS (1x) sera	***107,74 mg/ml***
AAC sera	***103,61 mg/ml***
naïve sera	***48,88 mg/ml***

Table 5: Values of Albumin in C57/B6 female mice

12.) Pathology of the treated and untreated BALB/c female and CB57/B6 female mice

To get a deeper in view of the situation of the mouse after treating with 100 mg/kg/ 20 g AAC different organs were analyzed.

First of all we looked at the situation of the lung and we found a huge infiltration area in the centre of the lung and less infiltrates in the periphery.

12.1) Lung

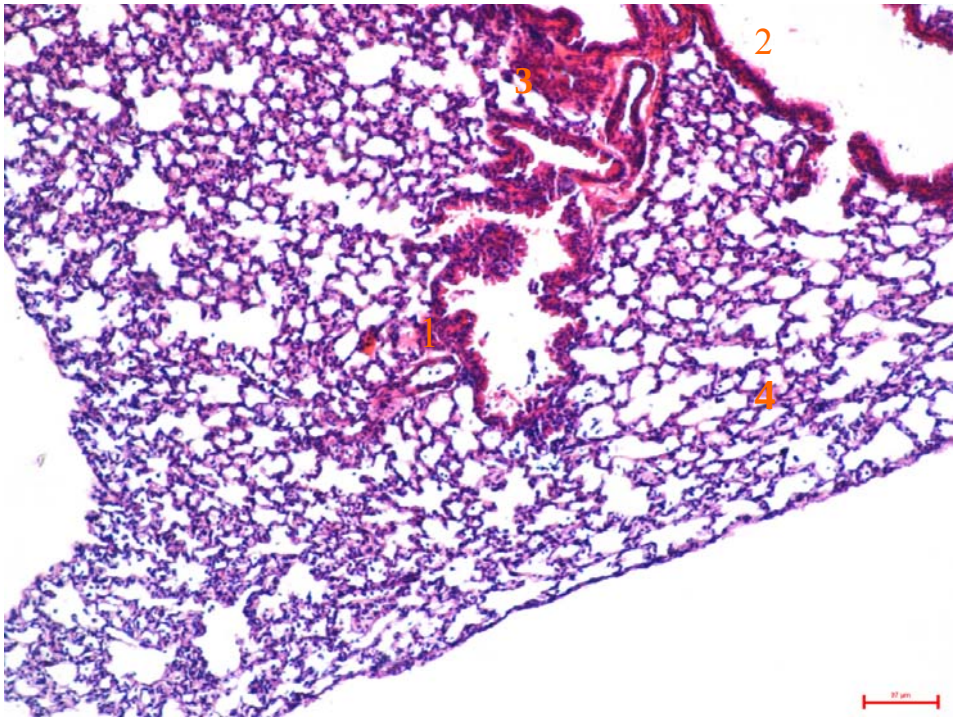

Figure 14: H&E staining AAC (10 mg/kg/20 g mouse)

To analyze the effect of AAC (10 mg/kg/20 g mouse) paraffin lung section of 4 µm were performed with the microtome cutting machine and analyzed under light microscope with the magnification 20 x. The lung is structured in general in the lung entering primary bronchus (2) which is the airway entering site of the lung. Followed of the proximal and distal midlevel airway areas (1) and bronchiole were the first second and third branches accordingly. In general as proximal parabroncheal and proximal midlevel areas respectively the proximal zone and distal midlevel areas and bronchioles were known as distal areas. The cutting demonstrates a typical cutting in our treated mice. As we found in all our sections, infiltrates were visible in the centre of the lung near the Para bronchioles and proximal midlevel areas (3) but not conspicuously in distal areas.

12.2) Duodenum

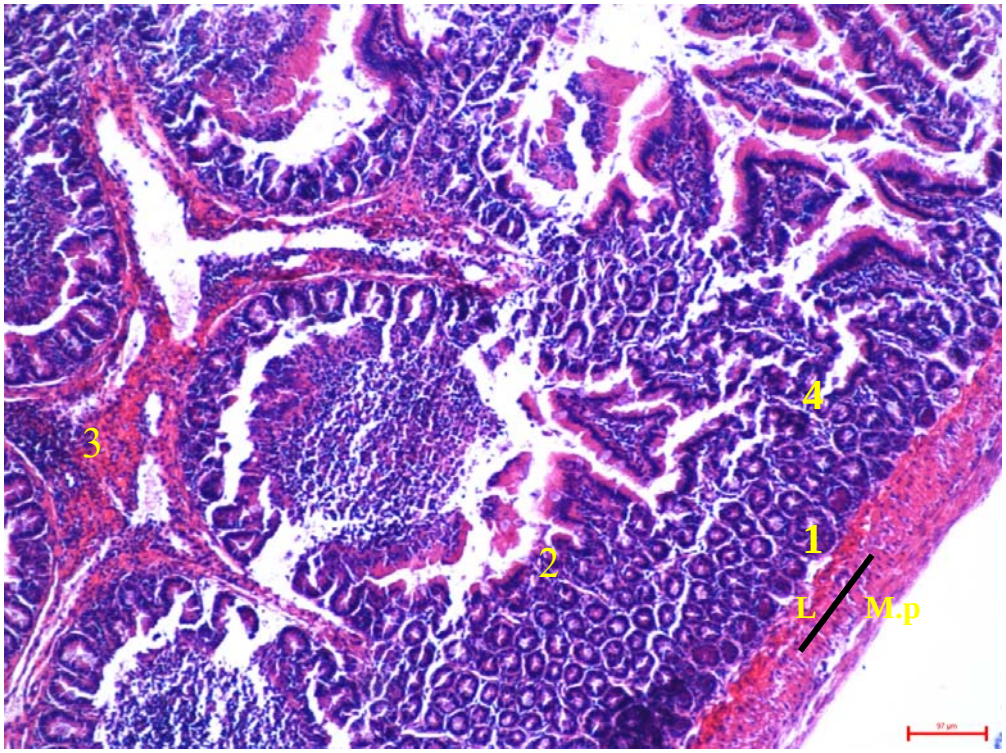

Figure 15: duodenum (100 mg/kg/20 g mouse AAC) BALB/c female

The duodenum is a part of the small intestine. It is retroperitoneal quire which fixes them and allows no movement. The cardinal features of the duodenum were uptake food in portions from the stomach and transport them into the duodenum and further through the small intestine. Further ingress of the liver, gallbladder and pancreas secrets was added in the compartment of the duodenum. This part of the small intestine is the only with influx of secrets in the sub mucosa via Brunner adenoids.

Explanation of the picture:

In general the architecture of the duodenum include from posterior to anterior the tunica muscularis propria (1) which was separated into longitudinal muscle (L) and ring muscle of the muscularis propria (M.p). Further we can differentiate sub mucosa (2) into Brunner adenoids and flanked by a small layer of Tunica muscularis mucosa (3). An accomplishment of a Brunner adenoid was shown in (4).

12.3) Kidney

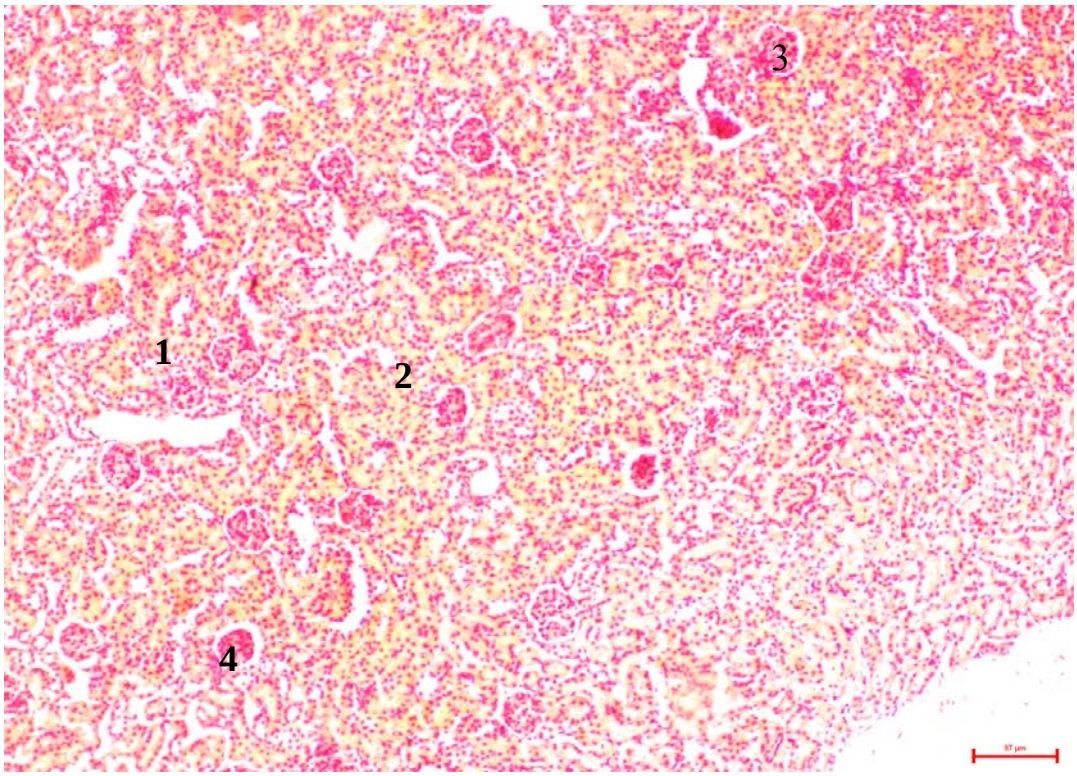

Figure 16: kidney (100 mg/kg/20 g mouse) AAC

The kidney controls the uptake of water and minerals and further produces hormones like Rennin which regulates the blood pressure and erythropoietin for blood regeneration. Also for Vitamin D production, kidney plays a pivotal role. The kidney is structured in cortex and medulla (not shown). Further we had March beams which starts at the Medulla and go forward into the Cortex. The cortex labyrinth (2) hallmarks the inter-medium of two glamorous (4). The functional unit of the kidney is called Nephron (1) and consists of the kidney corpuscle (3), which includes the glamorous and Bowman capsule and kidney tube, which on your part includes the proximal tube, the Henle bow and the distal tube [35].

12.4) Spleen

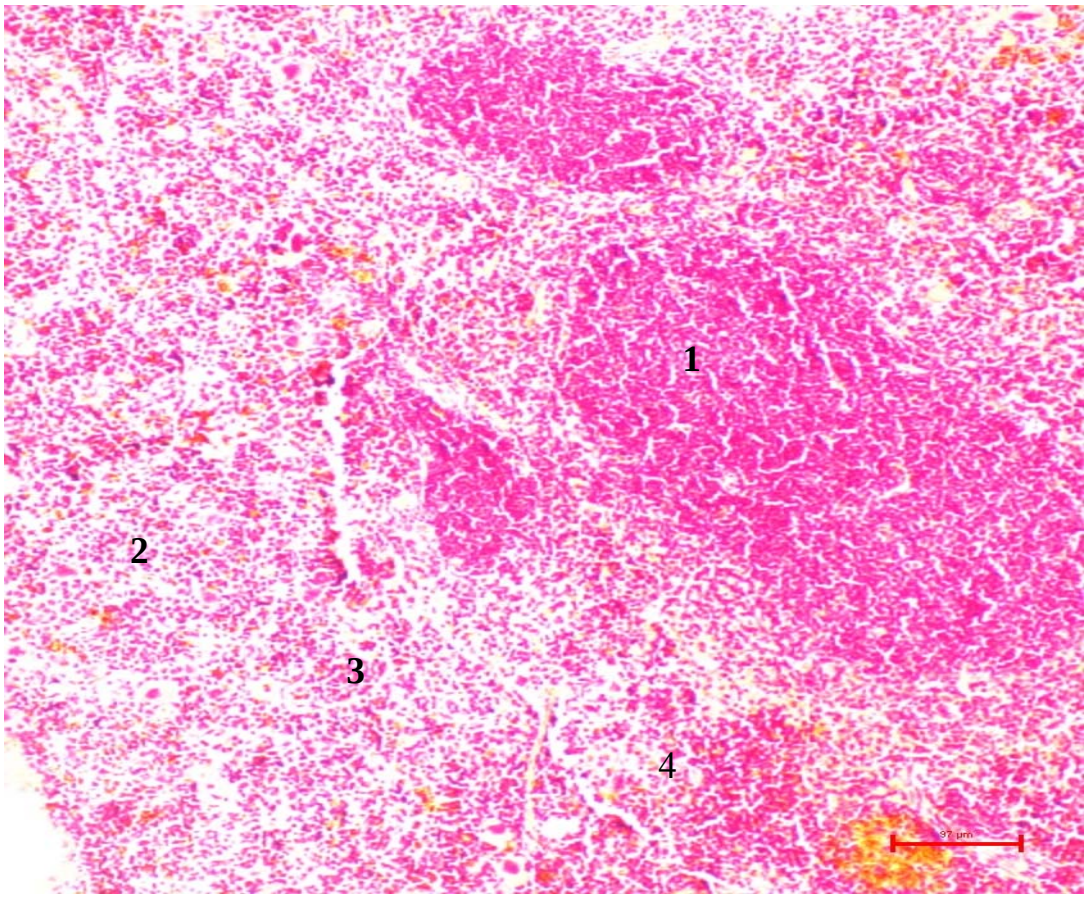

Figure 17: Spleen (10 mg / kg/20 g mouse AAC) BALB/c female

The spleen is a lymph reticular organ which is embedded in a connective-mucosal capsule. it is part of the lymphatic and blood circulation. It is a major part of the secondary immunological system which filtrate antigens throughout the blood. The spleen parenchyma is characterized by the white pulp which includes the whole lymphoretricular arteries parting, called T cell area and the red pulp. This red pulp consists with a reticular network with organized sinus which filtrate the blood. In this area you can found a lot of macrophages which major part is to eliminate all the old erythrocytes and all their end products (Hem, iron and so on) and recirculation to the bone marrow and further to the blood regeneration. The dark zone determines the follicle area (1), which is flanked by the marginal zone (2), (3) Indicate the red pulpa area. A further area in the spleen is called periateriell lymphocyte borderline (4).

12.5) Liver

Basic function of the liver

The most important features of the liver were the complex structure, the metabolism and filter of the toxins. Therefore the conversion from meal including sugars especially glucose into glycogen as a storage product and further into fat, once if the glucose storage space is full, is one of the most important pathway of the body. This process is possible in two ways. From glucose to glycogen and backwards if it is required. Another important feature of the liver is the production of mostly blood proteins like clotting factors, albumin and hormones like cholesterol. Bile acids which were important for digestion of fat and vitamin absorption also produced in the liver. The degradation of hemoglobin, cholesterol and proteins all occurred partly in the liver. Via deamination amino acids were produced from digested proteins in the liver. The filter action of chemical toxins which were not water soluble was also part of the liver filtration system through chemical modifying. Water soluble toxins and waste products were secreted via kidneys by urine secretion. To get a deeper in view of the action of AAC and PBS in mouse liver, we also looked in cut liver tissue about aberrations of the liver. The initial response of the liver to disease is inflammation and fatty change. Inflammation can be caused by contact with toxic substances such as bufotoxin, so care should be taken when handling/disposing of a creature like the Cane Toad. Heavy drinking will cause alcoholic hepatitis. Prolonged or severe inflammation leads to cirrhosis (scarring of the liver). When a section of liver is removed the liver will regenerate, but scarring in cirrhosis stops this regeneration and so the damage is irreversible. The most obvious sign of liver disease is jaundice, where the skin and whites of the eyes are yellow. Neonatal jaundice is very reversible: the current treatment is increased fluids and UV light therapy. Other symptoms include constant, extreme tiredness and lack of energy; itching; nausea; pain to the right shoulder; and pain in the right upper area of the abdomen. The complications of liver cirrhosis include: lack of albumin causing swollen legs and abdomen; lack of clotting factors causing easy bruising and internal hemorrhages (haematomas); impaired mental state from low-blood sugar and toxins (acting like morphine and sleeping tablets) normally removed by the liver; and cancer of the liver (hepatoma). Another malfunction of the liver is the inability to breakdown LDL (low density lipoprotein) cholesterol, and statins5 need to be taken to keep LDL at a safe level to prevent arterial disease, known as atherosclerosis. If a clot forms and blocks a narrowed artery, it can cause a heart attack or stroke. LDL (Low-density-lipoprotein) cholesterol is the harmful (bad) cholesterol, as opposed to the HDL (high-density lipoprotein) cholesterol which is absorbed and used as energy etcetera. The only current treatment for liver failure is transplantation. Death usually occurs from infection of a suppressed immune system, or vomiting blood from abnormal vessels in the esophagus (varices). With drugs, the liver combines cocaine and alcohol and creates a toxic third substance, coca ethylene, which magnifies cocaine's euphoric effects. The mixture of

cocaine and alcohol is the most common two-drug combination which results in drug-related death, the second being paracetamol and alcohol (this is usually an attempt at suicide and if it fails the patient will almost certainly have long-term liver damage).

Diagnosis

Liver disease is often first detected because of abnormal liver blood tests, often called liver function tests (LFTs), although they are not actually a measure of liver function. Abnormal albumin and blood clotting tests, demonstrate abnormal liver function. The pattern of the abnormalities of LFTs points to whether the problem is the liver, a hemolytic anemia, or the biliary system. Blockage of the biliary system6 and hemolytic anemia cause jaundice as well.

Blood tests can diagnose viral hepatitis and autoimmune liver diseases. Iron and copper levels in blood tests check for iron overload (haemachromatosis) and copper overload (Wilson's disease), both inherited diseases that can cause cirrhosis. An ultrasound scan will show blockage of the bile duct, fatty liver, cirrhosis and liver tumors. A liver biopsy may be necessary to examine the liver under the microscope, look for iron or copper, or determine the amount of virus present.

Treatment

The main aim of treatment of liver disease is to prevent cirrhosis. Obviously alcohol consumption must stop. Both Hepatitis B and C can be treated with antiviral drugs. Where the patient's immune system is attacking the liver, powerful suppressants of the immune system are given. N-acetylcysteine is given to prevent damage from paracetamol overdoses. Iron or copper overload can be treated with chelating agents that help the body to eliminate the excess heavy metals.

Treatment of cirrhosis involves preventing complications (e.g. drugs to prevent bleeding from varices); ensuring good nutrition, and monitoring for liver cancer. Water tablets (chemical diuretics) for the treatment of fluid retention in the legs (peripheral edema) or abdomen (ascites) should not be taken without medical consultation, particularly if the patient is already taking other medication. The current prescription drug for water retention, Bendroflumethiazide, lowers blood-pressure; and lists as potential side effects like mineral changes in body salts; feeling physically sick; loss of appetite; feeling dizzy or light-headed on standing up; feeling weak, tired, drowsy or sleepy; confusion; and muscle cramps. It can cause gout, impotence, skin rash, itchy skin and allergic reactions.

Laxatives (usually a syrupy solution called lactulose) need to be administered to prevent constipation and to reduce the chances of the poisonous substances from the bowel bypassing the liver and reaching the brain, causing drowsiness, confusion and coma (hepatic encephalopathy).

For someone recovering from any form of liver disease, it is very important to minimize the amount of fat in the diet: eating bread without any form of butter or margarine; frying eggs in a non-stick pan without any fat, etc.

Vitamin A

Although liver and liver products, such as pâté and liver sausage, are good sources of iron, they can also contain very high concentrations of vitamin A8. If taken in excess, this vitamin can build up in the liver and cause serious harm to a growing fetus. As a result, the Department of Health advises all pregnant women to avoid eating liver and liver products.

Liver structure:

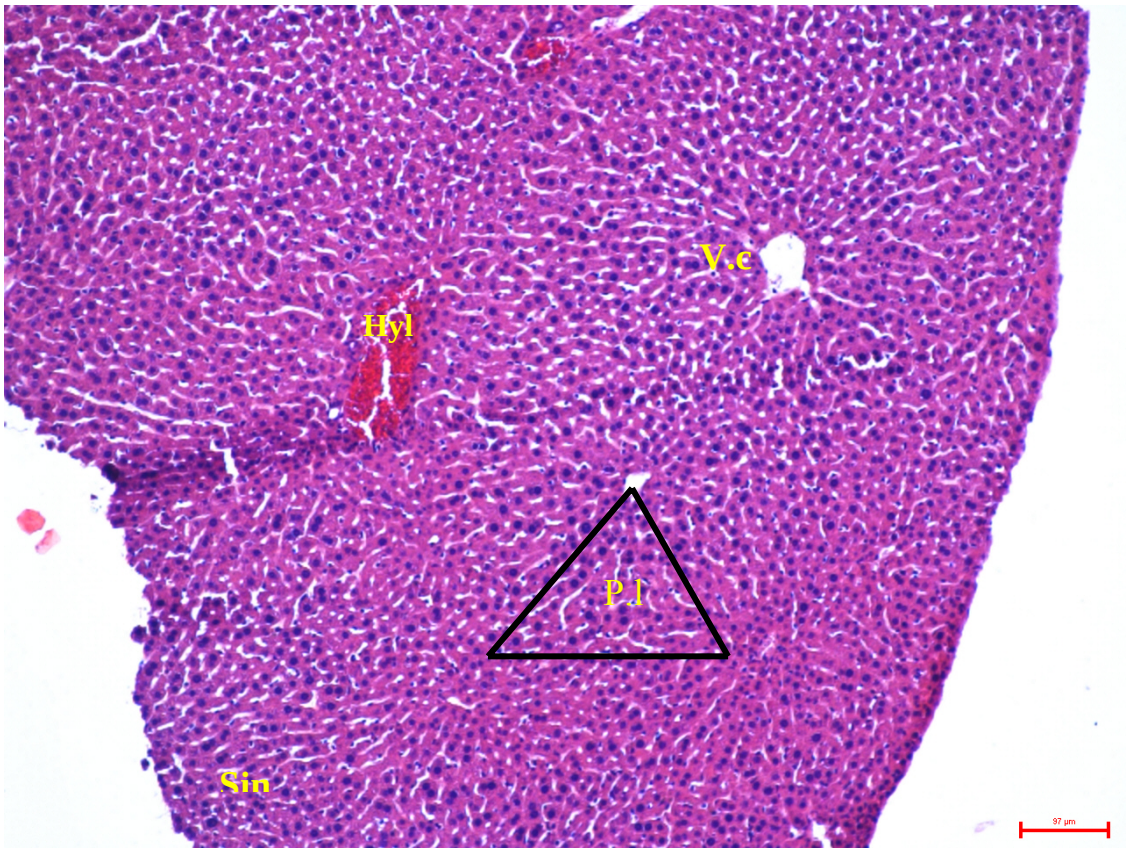

Figure 18: liver (100 mg/kg/20 g mouse AAC) BALB/c female mouse

The functional unit of the liver is called liver lobe. In general there where three possibilities of such liver lobes, the classical with Vena centralis (V.c) is in the middle of such hexagonal liver field and on each vertex you will find a Vena portalis. The second version is called Portal lobe with a trigonic Vena centralis (black lines) field in the middle of three hexagonal portal lobes (P.l). And the third liver lobe is called liver acinuses with two of the three Vena centralis in the middle of the two hexagonal portal lobes. A further characteristic, shown in this cutting are the sinusoids (Sin) which includes three types of cells, the Sinus endothelia, the Cupper star-cells and the lipocytes or Ito-cells. Further we found Hyalin (Hyl) in the liver which is an accretion of the metabolism and found sometimes in mice

12.6) Luna staining

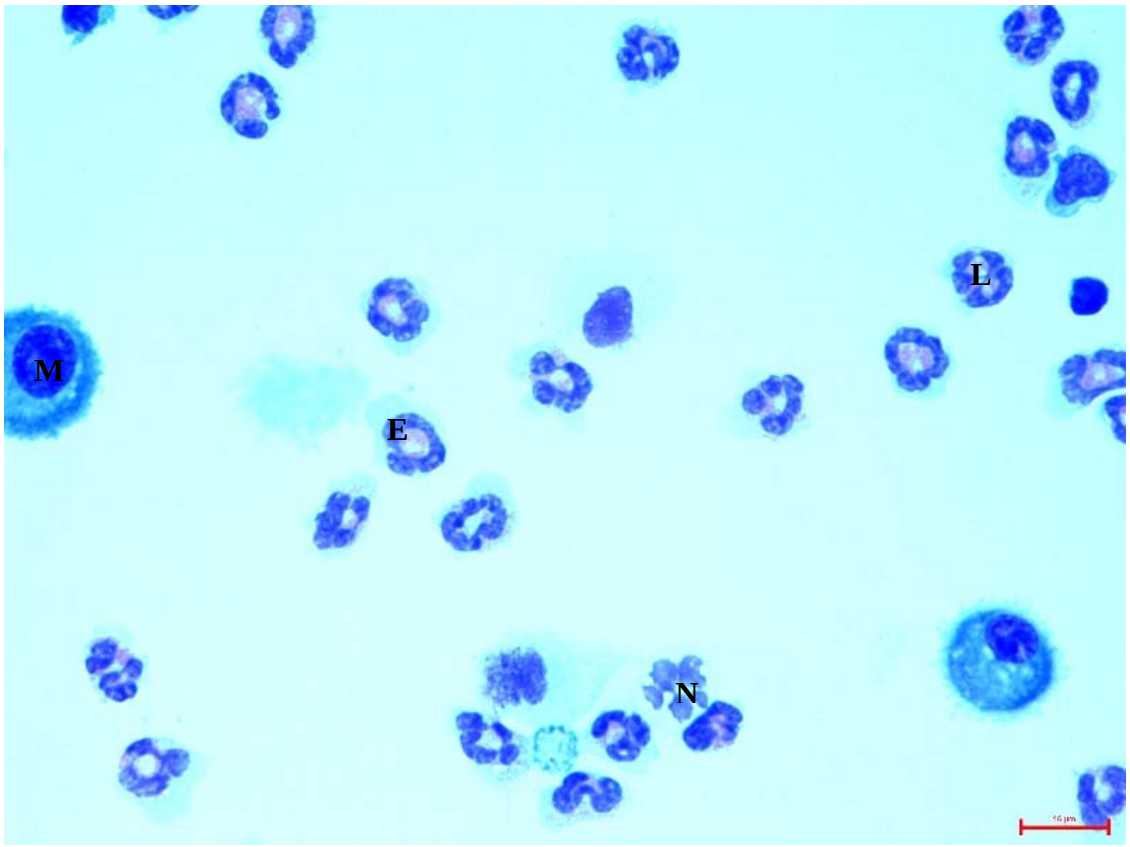

Figure 19: Luna staining

The Luna staining allowed us to get a more detailed characterization about the BAL involved cell types. Macrophages (M) are member of free connective tissue and have a size of 20 µm. Their high Phagocytes activity is the consequence of tentacle Pseudopodia and hook shaped cytoplasm protuberance. Its characteristic is a less developed endoplasmatic reticulum and a good established Golgi apparatus. A further characteristic is the availability of osmiophilic granules. Eosinophils (E) are a member of free connective tissue. One to four percentages of leukocytes are eosinophilic granulocytes in blood. The cross section dimension of an eosinophil granulocyte is 12 µm, and therefore less than macrophages. The nucleolus is characterized by bilobularity segmentation. A special characteristic of eosinophils is their pink colored granules. The oval discus shaped granules including a central long shaped crystallographic body which is called Internum. The Internum is enclosed from the Exertum or matrix. This acidophilic granules are modified lysosoms which including several acid hydrolase's like acid Phosphatases, Kathepsin, Peroxidases, Arylsulfatases and ribonucleases. The cytoplasm has less organelle. There major mechanism is including phagozytosis of antigen antibody complexes and the have the availability of amoeboid movement.

Neutrophils (N) are also a member of granulocytes and member of the white blood cell and have a size of 9 to 14 µm. The name Neutrophil based on the fact that either amphocyte or eosinophil dyestuff gives a colored granular as they do in eosinophils. Another name of Neutrophils is polymorph meaty, based on the types of the nucleolus which has several types. Times ago it was believed that neutrophils have more than one nucleolus. Bulks off White blood cells are neutrophils. If there was an infection it is part of the neutrophils to leave the blood circulation and opsonized the virus bacteria. In sanies you will find a lot of neutrophils.

In general there are 5 different types of neutrophils visible. Most of them are segment meaty. This means the have two or more segments in the nucleolus. Another expression of neutrophils is called rod meaty neutrophil granulocytes. Commonly in the range of 4 percentages young neutrophil granulocytes in the blood circulation but in case of infection rod neutrophils are increased.

Another part of neutrophiles are called hyper segmented neutrophils. The nucleolus is segmented in more than five compartments. Vitamin B12 or folic acid deficiency you will find an increase of this type of neutrophiles. In this context left- shifting means the increase development of immature preliminary stage of neutrophil granulocytes. Infection often determines a left-shifting and an increase of rod meaty, metamyelozyts, myeolocytes or Promyeleocytes. In the blood circulation 45 to 75 percentages of leukocytes are Neutrophils. This is equal to 1900 to 8000 cells per micro liter or absolute 1.9 to 8.0 multiply with ten to the power of nine cells per micro liter. It is also notable that the increase of neutrophiles is sensible. Evan stress can lead to an increase of the value [36]

12.7) Luna staining of the lung

Luna staining was used as a special staining for eosinophils in several tissues as lung tissue. The benefit of such staining was the fine differentiation between eosinophils, neutrophiles, lymphocytes and macrophages. We decided to use this staining to get a deeper in view about the eosinophilic situation of the mouse in lung tissue.

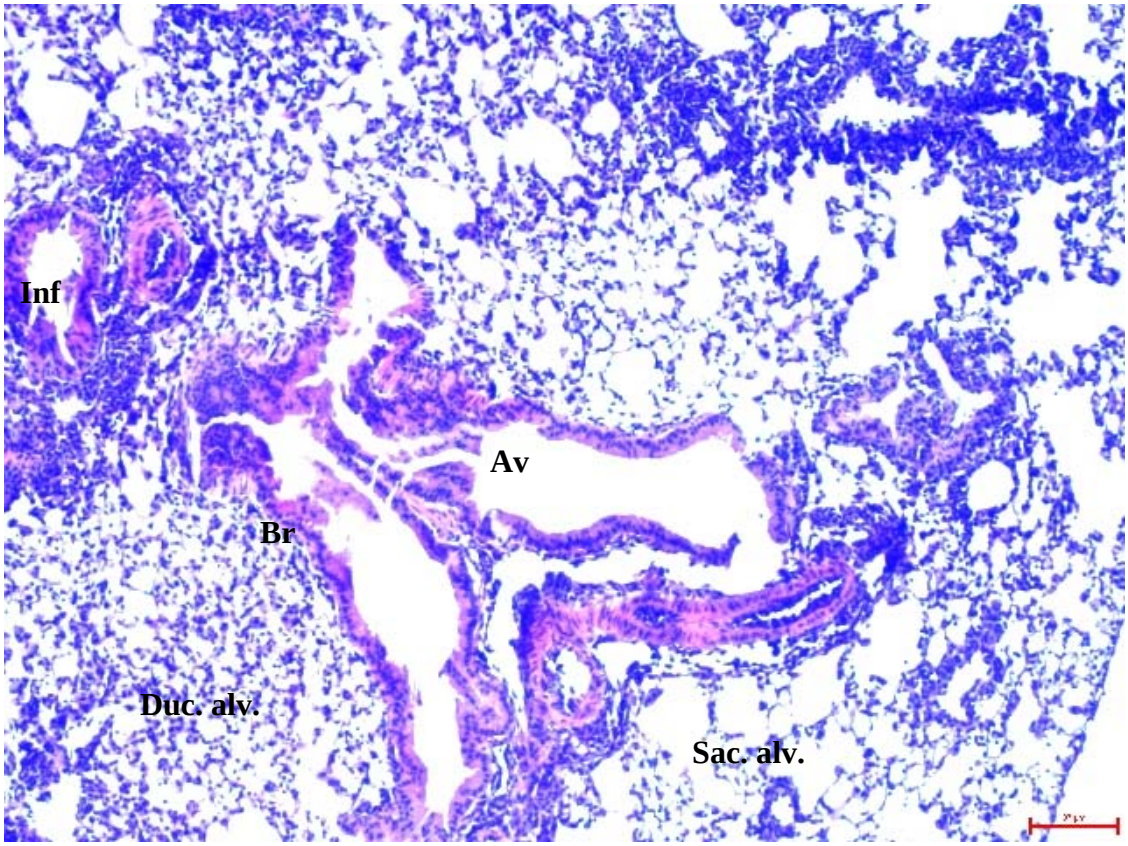

Figure 20: Lung H&E staining in PBS (1x) diseased mouse model

The architecture of the lung showed Bronchia's, Alveolus (Av) and the bronchioles. Further we investigate that the Infiltrates visible in the center, near the bronchioles (Br) and the periphery. An overview through the lung tissue clearly showed the ductus alveolaris (Duc. Alv.) and the alveolus. The diameter of the alveolus had a size of 50 to 250 µm and where enclosed from 6 to 12 capillary mashes. The wand of two neighboring alveoleous called Septum interalveolare. Two barriers of epithelial surfaces were needed for air exchange. First the Alveolar ephitelial cells also called Pneumocytes type I and a thin endothelium called capillary. In between these two epithelia laminates a Basal lamina.

12.8) PAS staining mouse model diseased

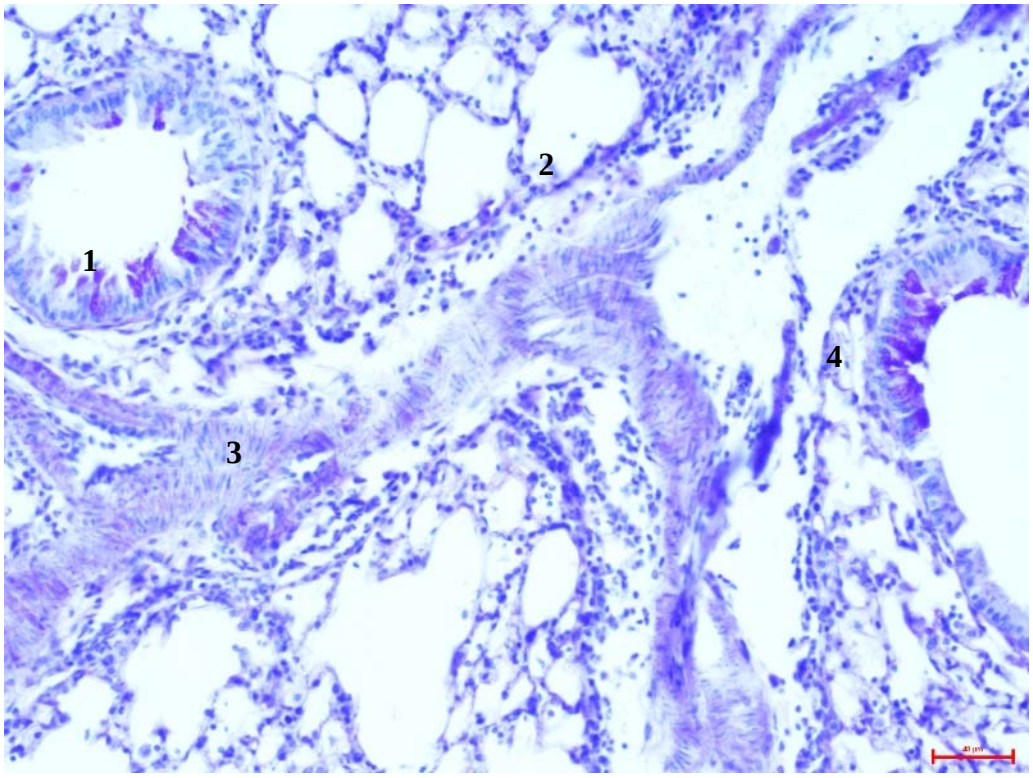

Figure 21: PAS staining AAC (100 mg/kg/20 g mouse) Magnification 25x

The general PAS staining gave us an idea about the situation of mucus production by administer of AAC (100 mg/kg/20 g mouse). Therefore a 4 µm section of paraffin lung tissue were taken and stained with PAS. We found a huge mucus mucous production in the centre of the lung (1) while mucous was absent in the periphery. This fact was a different found to PBS (1x) treated mice which have mucous production in the centre the same way as the AAC treated group has but further more mucous production in the periphery was found. Further differentiate was shown in figure 22.

12.9) PAS staining detail

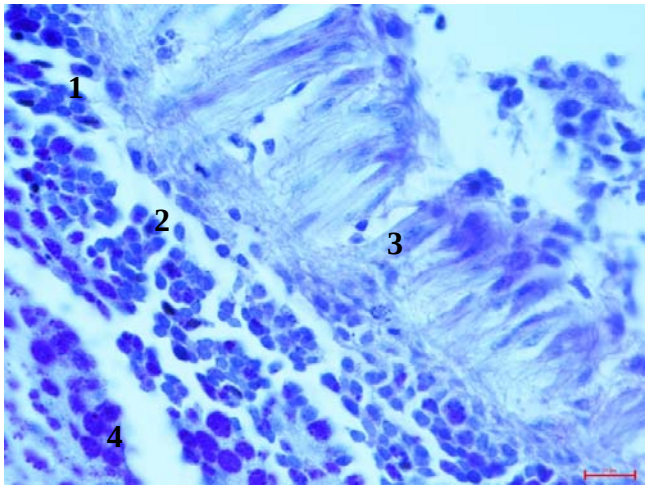

Figure 22: detail PAS staining AAC (100 mg/kg/20 g mouse) magnification 60 x

Goblet cells are one of the common intraepithelial adenocytes (4), which were found often in the duodenum stomach and in lung epithelial. They producing mucus called muzine via the merorine gland as an extrusion mode directly to the surface. The structure of goblet cells include often a basal bellied or cup-shaped (1) enhanced, apical in turn on corded (2), further the interaction between basal membrane and extraction tiller is characterized by cannikin shaft (3). The figure(figure 22) showed also the lamia propria mucosa (4).

13) Discussion

13.1) Asthma model

Our acute Asthma model allowed us to differentiate between acute, recovery and rechallenged asthma.

In my diploma work we want to figure out how and why Auroacetylcysteine (AAC) has effects on milk powder sensitized and either one or two percentage aerosol challenged BALB/c female or C57/B6 mice in allergic asthma. To get a deeper in view, we analyzed the body system in general with our albumin ELISA. To get an idea about the humoral immune response we looked at the IgG1 and IgE levels of several different treated mice. It was also a goal to look at the cellular immune response of the treated mice and therefore we used the method of differential cell counting. Based on our findings, the combination with high dose AAC (100mg/kg/20g mouse) and Rompun Ketanest as

aesthesia agent leads to extended stomachs and finally to death of one of the treated mice. That guides us to do all our experiments without Anesthesia, Rompun Ketanest.

It is well accepted that allergic asthma is related to Th2 T helper cells and therefore we analyzed the relationship between IgG1, which is a Th2 related Immunoglobulin. Further, we analyzed IgE which plays a pivotal role as key mediator in allergic asthma. PBS (1x) as positive control and AAC as our treated group were tested to naïve as background to analyze the effect based to the key features of allergic asthma in mouse model.

13.1.1) Toxicology

13.1.1.1) Lung

Based on the findings we found that infiltrates appear in the centre of the lung in areas called bronchioles. We also can clearly distinguish between the PBS (1x) and the AAC treated group which hallmark the positive treated group, in which the AAC treated group showed only in the centre infiltrates, whereas the PBS (1x) treated group show infiltrates in the centre and also in the periphery. Based on our findings we could suggest an optional benefit of AAC in treated mice in either BALB/c female mice or C57/B6 mice. Further more we looked about the mucous production of the mice in lung. It was no huge surprise that we found a decrease mucous production in AAC treated group whereas a huge mucous production was found in PBS (1x) group. Taken together our novel therapeutic had a benefit of the body situation of the mouse. This is also hallmarked by a decrease of IgG1 level to nearly naïve and a reduction of IgE levels in AAC (100 mg/kg/20 g mouse) treated BALB/c or C57/B6 mice. Further information will be needed about the gold flow during the body. Therefore information about the gold level in clot, sera and maybe urea will be needed in further studies. This might be interest, because the anti inflammation action of gold as benefit could be alleviated by anti inflammation actions during the route AAC goes through the body, e.g. the nose and trachea. The reason AAC was up taken through the nose and aerosol challenge was through the nose could also be an effect of reduction of infiltrates and mucous production in the lung by a decrease of allergen uptake during this route. This might also be an explanation of this huge reduction. The difference in albumin levels could lead to edema but nothing like this was found in our mouse system.

13.1.1.2) Liver

The shape of the lung is determined by the cavity available in the upper right part of the abdomen, between the rib margin and the diaphragm. As a major part of the metabolism it makes and breaks down proteins, fats, sugars, stored nutrients from the intestine. And removes toxins from the blood.. A further characteristic is the fact that the liver had two blood supplies, the hepatic arteries (20%) and the portal vein (80 %) which carried blood from the intestine. Therefore the liver can also call the powerhouse with recycling and disposal qualities and without life it is not possible. Diseases of the liver often occurs in connection with overdose alcohol, viral hepatitis 2 e.g. B,C3 and the glandular fewer virus Epstein Bar Virus 4, further auto-immune diseases and overdosed drugs or poisoning and indeed inherited disorders. This poisoning effect included also the effect of AAC in treated mice and we looked further to analyzed the toxicology and moreover the Pathophysiology of the liver. Our findings shown no abnormality compared to normal lungs. Only Hyalin sediments occurred but this happened from time to time and means nothing about the body situation of the mouse.

13.1.1.3) Spleen

Based on our explanation with H&E staining we found no aberrations of the spleen. All described compartments seem to be normal. This suggest no aberrations or damage by N-acetylcysteine or gold.

13.1.1.4) Stomach

In combination with Rompun Ketanest and AAC (10 or 100 mg/kg/20 g C57/B6 or BALB/c female mouse), we found extended stomachs, twice big as normal. That fact gave us the apprehension that AAC could have tremendous effects to the whole body of the mouse. To figure out if AAC or either Rompun Ketanest was responsible for the extended stomachs we performed two different series. One was only treated with Rompun Ketanest and the other only with AAC (100 mg/kg/20 g mouse). Interestingly in both, Rompun Ketanest either in the AAC treated group, extended stomachs were visible. To be sure all further experiments were done without to administer an anesthesia. The extension of the stomachs without anesthesia was nearly normal.

13.1.1.5) Duodenum

After performing 4 µm paraffin cuttings with the Microtome cutting machine, H&E and PAS staining were done to get a deeper insight in the situation of the mouse duodenum.

We suspected in case of the extended stomachs that even the duodenum could suffer under the administration of AAC to the mice. Astonishingly we found either in the H&E or in the PAS staining abnormalities compared to a normal tissue [37]. In this case it should be mentioned that we can not exclude at this time point that the pancreas could suffer under the treatment of AAC.

13.1.1.6) Kidney

Another goal of our study in C57/B6 mice was the effect of AAC in analogy to the kidney of healthy mice. As we suspected no difference were found, this could be and the shortness of the time period of the treatment with AAC or the converting via the glutathione pathway was that high that AAC did not pass the kidney. Anyway no aberration of the tissue was found. Pharmacology

13.1.1.7) IgG1

One of the key features in our study of the effects of AAC in allergic asthmatic mice was the decrease of IgG 1 levels in treated C57/B6 female mice. We could clearly distinguish between the PBS (1x) positive treated group and the 100 mg/kg/20 g mouse treated group. The huge reduction to nearly naïve leads us to suspect one possibility in avoiding allergic asthma. Moreover we suspected that this reduction plays a role in the development of B cell class switch into IgE and IgG1. IgE as the key mediator of allergic asthma demonstrate the most powerful indication of inhibition in allergic asthma by inhibiting mast cell degradation and histamine production. Our data leads us to the suspecting that AAC had a pivotal action as drug against allergic asthma by a reduction of the IgE level significantly.

13.1.1.8) Albumin

For testing the albumin levels we performed a sandwich ELISA. As described before in this type of ELISA sera or BAL as first Antibody and a albumin conjugated second antibody were used for detection with TMB as substrate. After measuring with the SpectraMax Photometer we found in the BAL an amount of 21,78 mg/ml and an increase of 26 51 % in the AAC group. In the sera we found compared to the PBS group an decrease of 3,99 % in the AAC group. Interestingly both PBS (1x) and AAC were decreased compared to Naïve albumin in the sera PBS (1x) 2,2 fold more and in the AAC group a 2,11 fold higher albumin level were found. Compared to the naïve BAL we found an increase of 29,64 % in PBS (1x) and a 39,79 % reduction in the AAC group. According to these findings, we can hazard a guess that PBS (1x) as nearly the same AAC have effect of the healthy

mouse system. It is well known that a disharmony in the albumin level could lead to edema but nothing toward an edema was found in our system.

13.1.1.9) Staining

All staining were done as described in the literature or established in our lab as the MGG (May Grünwald Giemsa) staining.

Mucus

To examine the mucus production we performed the PAS staining which hallmarks the mucus in a pink color. We can clearly distinguish between the PBS (1x) positive lung tissue which demonstrate the positive group in our system with a huge mucus production around the centre of the lung and also in the periphery. In contrast to PBS (1x) in the AAC group we could only found mucus in the centre around the bronchioles but it was completely absent in the periphery. This indicates the pivotal action of AAC in allergic asthma and COPD.

13.1.1.10) H&E

To examine the cellular situation in the mouse lung H&E staining were performed. Our findings demonstrated a reduction in the infiltration state in the AAC group. As same as in the mucus production we found infiltrates in the centre of the lung near the bronchioles but nothing in the periphery. A different situation was visible in the PBS (1x) group. Here we found infiltrates similar to the AAC group near the bronchioles but also in the periphery of the lung.

13.1.1.11) Luna

To get a better insight of the eosinophilic situation in the lung, Luna staining was performed. This indicated the Eosinophils in the lung more clearly than the H&E staining. It was no surprise that the PBS (1x) group had Eosinophils in the centre and in the periphery similar to the infiltrates of the lung.

In the AAC group also eosinophils were found but less than in the PBS (1x) group and only in the centre of the lung near the bronchioles. Taken together we can assert that the AAC inhibits the building of mucus, which is important in case of asthma, because a cardinal feature of asthma was wheezing and coughing, which will be inhibited through AAC as drug. There is evidence that the less infiltrates promote the inhibition of allergic asthma in our mouse model.

Compared to the findings of Jean Bousquet et. al,[38] T lymphocytes, eosinophils and mast cells and much more cell types play a role in developing allergic asthma. Chest tightness, current episodes of

wheezing, further coughing was major features of this disease. Since 1959 several definitions of allergic asthma have been proposed. The basic alterations of asthma were considered to be bronchospam, edema, and hypersecretion. Our data suggest a decrease in bronchospam and hypersecretion. Edema was not visible but the ratio between naïve and PBS (1x) or AAC could not definitively exclude this opportunity. Tissue eosinophilia is a characteristic of asthma but it is not necessarily specific to asthma but there is coherence between bronchioles eosinophils and asthma. It is also notable that tissue eosinophilia is greater in fatal asthma than in patients with chronic asthma. Our data demonstrate a decrease of asthma in AAC treated C57/B6 mice compared to the PBS (1x) group.

Further a decrease of eosinophils suggests that there is a reduction in the cardinal features of asthma in our mouse model. The reduction of mucus production also indicates the positive effect of AAC in our model. The reduction of IgG1 demonstrated the tremendous action of AAC. An Explanation to that powerful reduction could lead either in the high dose of AAC or in the action of N-acetylcysteine and gold combination but no advice was found for this or better was not tested in this study. Based on the findings, we can affirm the AAC had absolutely a reduction action of the symptoms of allergic asthma in mouse model. Further we clearly showed that AAC plays a pivotal role in the reduction of asthma.

14.) Curriculum vitae

Curriculum vitae
Roman Ferdinand Kreindl

Home address: 4272 Weitersfelden, Stumberg 7, Upper Austria

Date of birth: 08/11/1979
Gender: Male
Nationality: Austrian

Education

- 1986 - 1990 primary school in Weitersfelden, Austria
- 1990 - 1994 secondary school in St. Oswald/Fr., Austria
- 1994 - 1995 vocational school for agriculture in Freistadt, Austria
- 1995 - 2000 The institutes for higher education for chemical industrial engineering, Wels, Austria. Received general qualification for university entrance on the
- Oct. 2001 - University of Vienna, biology
- Diploma: September 2008

Work experience

- Sept. 2006 – Diploma work in Immunology. Supervisor: Dr. Michelle Epstein

Army experience

- Oct. 2000 - May 2001 Military in the Schwarzenbergkaserne in Wals-Sitzenheim, Salzburg.

Skills

- fundamental basics of statistics
- REFA certificate for logistic part A

Interests

- Immunology and Vaccination development
- Chess
- Philosophy

15.) Literature

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