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To my parents and Yousuf,

with love

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A. List of abbreviations

2-ME	β-Mercapthoethanol
aa	Amino acid
AGPs	α_1-Acid glycoproteins
AHR	Airway hyperreactivity
ALEX	Allergy and Endotoxin
APC	Antigen presenting cell
APS	Ammonium persulfate
BAL(F)	Bronchoalveolar lavage (fluid)
BCG	*Mycobacterium bovis* BCG
BHR	Bronchohyperresponsiveness
BIRs	Baculovirus inhibitor repeats
BSA	Bovine serum albumin
CARD	Caspases recruitment domain
CLRs	C-type lectine receptors
CoDE	Cow-shed dust extract
DC-SIGN	DC-specific intercellular adhesion molecule-grabbing nonintegrin
DMEM	Dulbecco's minimal essential medium
DTT	Dithiothreitol
e.g.	*Exempli gratia*; for example
EDTA	Ethylendiaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
et al.	*Et alii*; and others
FABPs	Fatty acid-binding proteins
FACS	Fluorescence activated cell sorting
FcεRI	High affinity IgE receptor
FID	Flame ionization detector
FITC	Fluorescein-5-isothiocyanate
FPLC	Fast protein liquid chromatography
Glc	Glucose
GC-MS	Gas chromatography and coupled mass spectrometry
GlcNAc	*N*-Acetyl-glucosamine
GM-CSF	Granulocyte macrophage – colony stimulating factor
GPI	Glycosylphosphatidylinositolo anchored protein
Gram+/-	Gram-positive /Gram-negative

GroCys	*S*-(2,3-dihydroxypropyl)cysteine
h	Hour
HBSS	Hanks buffered salt solution
HEK	Human embryonic kidney
HEPES	*N*-[2-Hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid]
HIC	Hydrophobic interaction chromatography
HPLC	High-performance liquid chromatography
i-	Iso-
i.p.	Intraperitonial
IEF	Isoelectric focussing
IFN-γ	Interferon- γ
Ig	Immunoglobuline
IL-	Interleukin
kDa	KiloDalton
LBP	LPS binding protein
LMW	Low molecular mass standard
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTA	Lipoteichoic acid
LRR	Leucine-rich repeats
M	Molar; mol/L
MALDI-TOF	Matrix-assisted laser-desorption/ionization-time of flight
MAPKs	Mitogen-activated protein kinases
MDP	Muramil dipeptide
meso-DAP	*meso*-Diaminopimelic acid
MHC	Major histocompatibility complex
min	Minute
moDC	Monocyte derived dendritic cell
MS	Mass spectrometry
MurNAc	*N*-Acetylmuramic acid
NDV	Newcastle disease virus
NF-κB	Nuclear transcription factor – κB
NK	Natural killer
NLR	NOD-Like receptor
NOD	Nucleotide-binding oligomerization domain
OD	Optical density
OVA	Ovalbumin
PAGE	Polyacrylamide gel electrophoresis

PAMP	Pathogen-associated molecular pattern
PARSIFAL	Prevention of Allergy Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle
PAS	Periodic acid-Schiff
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PFA	Para-formaldehyde
PGN	Peptidoglycan
PITC	Phenylisothiocyanate
PMF	Peptide mass fingerprint
PMSF	Phenylmethanesulfonylfluoride
poly I:C	Polyinosine-polycytidilic acid
PRR	Pattern-recognition receptor
PYD	Pyrin domain
PVDF	Polyvinylidene difluoride
RIG-I	Retinoic acid-inducible gene I
RLRs	Retinoic acid-inducible gene I (RIG-I)-like receptors
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
ShDE	Sheep-shed dust extract
SNP	Single nucleotide polymorphism
TBS	Tris-buffered saline
TBST	TBS + Tween 20
TEMED	*N*,*N*,*N*',*N*' tetraethylenediamine
TF	Tissue factor
TFG-β	Transforming growth factor-β
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor-α
TNPT	Tris-HCl, NaCl, PMSF, Triton X-100
T_{regs}	Regulatory T-cells
Tris	Tris[hydroxymethyl]amino methane
UV	Ultraviolet
v	Volume
V	Volt
w	Weight
WHO	World health organisation

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1. Introduction

1.1 Allergy, asthma and atopy

Allergy is a generic term to indicate aberrant reactions to common inhaled proteins known as allergens and includes a range of clinical diseases such as asthma, rhinitis, atopic dermatitis and anaphylaxis. Most patients who develop atopic disorders also develop acute immediate hypersensitivity which is a damage to host mediated by pre-existing immunity to self or foreign antigen[(1)]. Rather than being classified as a single disease allergic asthma is the result of systemic inflammatory reactions triggered by an aberrant Th2-type cytokines response to allergens and characterized by three major features: (1) at the physiological level reversible and intermittent airway obstruction leading to chest tightness, cough and wheezing, (2) airway inflammation which involves high secretion of the Th2-type cytokines, namely interleukine-4 (IL-4), IL-5 and IL-13, with increased amounts of total IgE and eosinophils and (3) bronchohyperresponsiveness (BHR) which is defined as a high sensitivity to bronchoconstrictors such as histamine or cholinergic agonist[(2)]. The hypersensitive reactions are usually classified in four groups, according to the type of immunoresponse and to the effector mechanism responsible for the damage to cells and tissues. Type I hypersensitivity is an immediate allergic response caused by exposure to antigens against which the host has pre-existing IgE antibody. IgE in blood serum of healthy people are present at a low concentration and their half life is only 2-3 days, but in sensitized individuals much of IgE is bound to high affinity receptors (FcεRI) on mast cells and basophils, and in this state the half-life reaches about three weeks. The cells are activated by the cross-linking of the FcεRI receptors *via* antigen binding to the bound IgE molecules. This cross-link leads to the degranulation of mast cells with consequent release of primary inflammatory mediators which cause the acute inflammatory reaction. Hay fever, eczema, asthma and allergic rhinitis belong to the type I hypersensitivity. Type II hypersensitivity is mediated by IgM and IgG antibody against surface or extracellular antigens. The process of pathogenicity and tissue damage is determined by recruitment of neutrophils and macrophages by Fc dependent mechanism or by recruiting complement *via* the classical pathway. Autoimmune diseases belong to type II hypersensitivity. Type III hypersensitivity is mediated by immune complexes of IgG antibody bound to soluble antigens. Contrary to Type I hypersensitivity, in this case the IgG are not bound to mast cells so that only preformed complexes can bind to the low affinity FcγRIII. Finally the damage to tissues can be provoked by T lymphocytes

which activate mechanisms of delayed type hypersensitivity (DTH) or lysate directly the cells target. Type IV hypersensitivity is the only class of hypersensitive reactions to be triggered by antigen-specific T-cells. Delayed type hypersensitivity results when an antigen presenting cell, typically a tissue dendritic cell after processing an antigen and displaying peptide fragments bound to class II MHC is contacted by an antigen specific Th1-cell. The resulting activation of the T-cell produces cytokines such as chemokines for macrophages, other T-cells and, to a lesser extent, neutrophils as well as TNF-β and IFN-γ. The consequences are a cellular infiltrate in which mononuclear cells (T-cells and macrophages) tend to predominate.

1.1.1 Epidemiology of allergic diseases

Epidemiological studies are complicated by a missing objective diagnostic tool for atopic disorders. Most epidemiological studies use questionnaires and reports of physician diagnosis of asthma which sometimes may not be accompanied by an objective pulmonary function data[3]. Studies maintaining a constant definition of "asthma" throughout time showed worldwide increases in asthma prevalence since the 1960s[4]. Although asthma and related phenotypes are common throughout the world, differences in their prevalence have been reported related to geographical and ethnic differences ranging from 0.5% to 6%[5-7]. Furthermore, countries are classified according to their asthma rate into low incidence countries (Africa and Asia), medium incidence countries (USA, Canada, European countries and United Kingdom) and high incidence countries (New Zealand and Australia)[8]. Differences in the prevalence of asthma have been also described in rural and urban areas: in Japan asthma prevalence has increased since the population moved away from traditional ventilated houses to western-style buildings[9]. Furthermore von Mutius showed that asthma and atopy were significantly more frequent in children in the former West Germany, thus suggesting that the prevalence of asthma is the result of westernisation and although a lot of environmental and genetic factors have been thought to play key roles in the development of asthma and allergies, the cause and pathophysiology of this syndrome are not completely defined[10].

1.1.2 Genetic and environmental factors: the hygiene hypothesis

The allergic phenotype depends on the interaction between two major components: a genetic predisposition and a gene-environment interaction[11]. Although it has been

recognized that allergic diseases run strongly in families and have a hereditary component, the genome-wide screens for allergy and asthma susceptibility loci is relative young[12]. Genetic studies within the last years have identified several chromosomal regions which may contain genes responsible for asthma and related phenotypes. Linkage for high level of IgE for example has been found on chromosomes 5q, 11q and 12q[13-15], while other chromosomal regions have shown linkage to bronchial hyperresponsiveness[16;17] . Moreover in the last decades asthma genetics detected several susceptibility genes crucial for asthma pathogenesis and defective tolerance induction to allergens. Vercelli D. *et al.* classified asthma susceptibility genes in four main groups: i) genes associated with innate immunity and immunoregulation, ii) genes associated with Th2-cell differentiation, iii) genes associated with epithelial biology and mucosal immunity, and iiii) genes associated with lung function and airway remodelling[18]. However, even among the list of highly replicated genes associated with asthma, the results have not been consistent among all of the populations[19] thus suggesting that those genes are not associated with asthma under every conditions analyzed. A probable explanation is that asthma and atopic disorders are the result of different diseases rather than one pathology, and genes may have role in only subsets of asthma. Genetic factors are important in the regulation of atopic diseases, but the rise in atopy has occurred within too short a time frame to be explained only by a genetic shift in the population, thus pointing to environmental or lifestyle changes[20]. In 1989 Strachan first formulated the so-called “hygiene hypothesis” pointing out an inverse relation between family size and development of atopic disorders and proposed that a lower incidence of infection in early childhood could be cause of the rise in allergic diseases[21] (Figure 1).

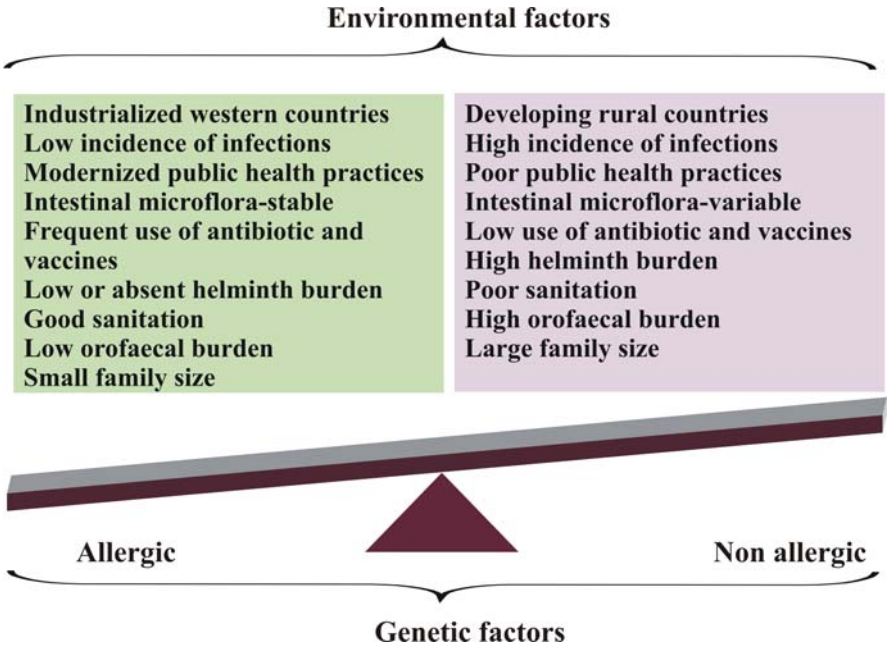

Figure 1: The hygiene hypothesis: genetic and environmental factors.

Genetic and environment factors involved in the development of the allergic disorders. Family size and incidence of infections are the most stressed factors involved in the regulation of the balance between the allergic and non allergic phenotype.

It has been shown that in neonates, the immunological population of T lymphocytes in the umbilical cord is predominantly Th2, the same as in the allergic individuals[22]. It seems that there is a natural predisposition to develop allergic diseases during the childhood, but then the immunological balance is kept by infection diseases, namely viral, bacterial and helminth infections[23]. Consistent with this view the hygiene hypothesis supported the fact that children of affluent countries are less likely to be in contact with microbial compounds due to modernized public health practices, thus leading to a gradual change in the frequency of childhood infection, affected by the introduction of clean water supplies, sterilized and processed foods and the routine use of antibiotics and vaccines[24]. The hygiene hypothesis concept was then put forward by von Mutius supporting the fact that viral infections such as hepatitis A, measles and tuberculosis are negatively associated with the prevalence of allergic disorders[25;26].

1.1.3 Protective effect of farm animal exposure

Numerous studies have shown that the exposure to a farm environment is negatively associated to the prevalence of hay fever, asthma and atopic sensibilization[27-29]. The ALEX

(**AL**lergy and **E**ndoto**X**in) study, a cross-sectional study which included more than 800 children from rural areas in the south of Germany, Switzerland and Austria, showed that the exposition to microbial compounds early in life reduces the risk to develop allergic diseases[30]. The results of those studies were supported by a study in Swedish conscripts[31] and among Finnish university students demonstrating lower prevalence rates of self-reported allergic rhinitis in students who had been raised on a farm as compared with students from a nonagricultural environment[31;32]. Another example of study for the investigation of the relationship between gene and environment in allergic disorders and atopy is the PARSIFAL study (**P**revention of **A**llergy-**R**isk factors for **S**ensitization **I**n children related to **F**arming and **A**nthroposophic **L**ifestyle). It was initiated in 2000 as a cross-sectional study of the role of different lifestyles and environment exposures in farm children, children from Steiner schools and other reference groups[33].

Converging studies have speculated that the contact or exposure to bacterial compounds reduces the incidence of atopic disorders in childhood. The protective effect has been related to the direct contact to livestock and the consumption of non-pasteurized milk[34]. Furthermore the diagnosis of asthma was inversely associated with agriculture, pig farming, frequent stay in the animal sheds and child´s involvement in haying, whereas the presence of sheep was positively associated with wheezing, thus suggesting the importance of the farm exposure[35]. Numerous studies have been attributing the reduced risk of allergic symptoms to the exposure to a farm-related microbial environment, in particular to livestock. Microbes and microbial products can be easily brought inside the farm houses, thus being part of the environment also for newborn children. It seems also that the family lifestyle plays a pivotal role in the risk of atopic disorders as demonstrated in a study in Sweden[36]: lower risk of atopy among children adopting an anthroposophic lifestyle was found and this could be related to differences in diet, exercise level, use of medication and exposure to air pollution[37]. Interestingly, epidemiological studies stressed the importance of the time window in the regulation of allergic disorders. Although little is known about the relevant temporal sequence of the allergic outcome, it seems that the prenatal period exposure is more relevant then exposure while the mother is breast-feeding. Thus, the prenatal period and the early childhood (12-24 months) are considered to be critical for the establishment and maintenance of a normal immune Th1/Th2 balance[38].

1.1.4 "Microflora hypothesis" of allergic diseases

The hygiene hypothesis concept has lead to the theory that a lack of early microbial stimulation results in an aberrant immune response to innocuous antigens late in life[39]. Next to this the "microflora hypothesis" suggests that changes of the gastrointestinal microflora, due for example to the use of antibiotics or dietary differences in the "industrialized" countries, has lead to an alteration of the normal micro biota-mediated mechanism of the immunological tolerance in the mucosa, which is the reason for an increased incidence of allergic airway diseases[40]. Noverr *et al.* demonstrated that mice can develop allergic airway responses to allergens if the endogenous micro biota is altered at the time of the first contact with the allergen[41]. Thus it seems that micro biota play a pivotal role in maintaining mucosal immunologic tolerance and are essential in shaping the development of the immune system[42]. In line with this view, a number of studies provided evidences that a non-invasive contact with microbes influences immune development, homeostasis, and as a consequence, allergic risk[43;44]. In this respect for example, the intestinal flora have been shown to facilitate post-natal immune development and prevent Th2-polarized responses to dietary allergens[45-48].

1.2 The allergic immune response

Recent increases in the prevalence and incidence of allergic and autoimmune diseases indicate a fundamental defect in immune regulation that appears to be related to lifestyle and exposure factors[49].The allergic immune response is induced by increasingly generated Th2-cells, which dominantly secrete IL-4, IL-13 and IL-5 cytokines, trigger IgE antibodies and prolong eosinophilic granulocyte survival. IL-4, IL-5, IL-9 enhance the survival of eosinophils and prime them for activation and chemotaxis[50]. The early phase of allergic inflammation is initiated when the allergen comes into contact with IgE-primed mast cells/basophiles. The cross-link of allergen-specific IgE on the surface of mast cells and basophiles leads to the release of Th2-type cytokines[51]. The expression of IgE receptors on antigen presenting cells allows these cells to capture allergen in a specific way through surface-bound IgE[52]. The late phase of the allergic reaction involves the recruitment of activated $CD4^+$ Th2 T-cells and eosinophils to sites of allergen exposure. Eosinophils are important effector cells responsible of the epithelial cell damage through release of toxic proteins, bronchoconstruction through leukotrienes production and contributing to airway hyperresponsivenes AHR[50].

1.2.1 Molecular mechanism of allergic reaction

The respiratory tract is continuously exposed to a vast array of environmental allergens ranging from harmless protein to potentially harmful pathogens.

The first challenge of the local airway mucosa immune system is to discriminate between proteins and pathogens. In healthy individuals the normal response to innocuous non-self allergen is set to non-reactivity or active tolerance, but this delicate equilibrium can be disrupted following infections or atopic disorders, such as asthma[53-55]. Allergic inflammation is often classified into temporal phases. Early-phase reaction occurs within seconds to minutes of allergen challenge and is mainly regulated by mast cells and their mediators. In sensitized individuals mast cells have already allergen-specific IgE bound to their surface high-affinity IgE receptors (FcεRI)[56;57]. The cross linking of adjacent IgE molecules by allergens leads to aggregation of FcεRI which trigger a complex signalling pathway with activation of pre-synthesized molecules contributing to the acute symptoms associated with early-phase reactions[58]. The synthesis of preformed mediators includes i) histamine that increases the secretion of mucus and induces bronco-constriction, ii) tryptase that is a protease causing local swelling, iii) chemotactic factors of eosinophilic cells that increase the local inflammation, and iiii) kinogenase that increases the vasodilatation[59]. This reaction is followed by an inflammatory response leading to changes in the bronchial structure and airway hyper-responsiveness. The infiltration in the airways of mononuclear cells, mainly CD4+ T-cells leads to production of Th2 cytokines (IL-4 and IL-13) which participate in the production of mast cells and IgE synthesis, as well as IL-5 in the activation of eosinophils[60;61]. After this first pre synthesis, mast cells respond to IgE and allergens also releasing a broad variety of newly synthesized cytokines, chemokines and growth factors in the late phase. The interaction with the antigen and the activation of the mast cells leads to the synthesis of other molecules like leukotrienes namely B4, C4, D4[62] which are potent inflammatory lipid mediators involved in the pathophysiology of asthma due to their chemotactic activity and a physiological action like that of the histamine, but thousand times more potent. Then, prostaglandins are synthesized, some of which are chemotactic while others cause swelling and pain. Late-phase reactions typically occur 2-6 h after allergen exposure. Moreover the exacerbation of the allergic disorder is the result of the reduction in the function of the epithelial barrier caused by allergic inflammation. The inflammatory cascade in the allergic reaction is depicted in Figure 2.

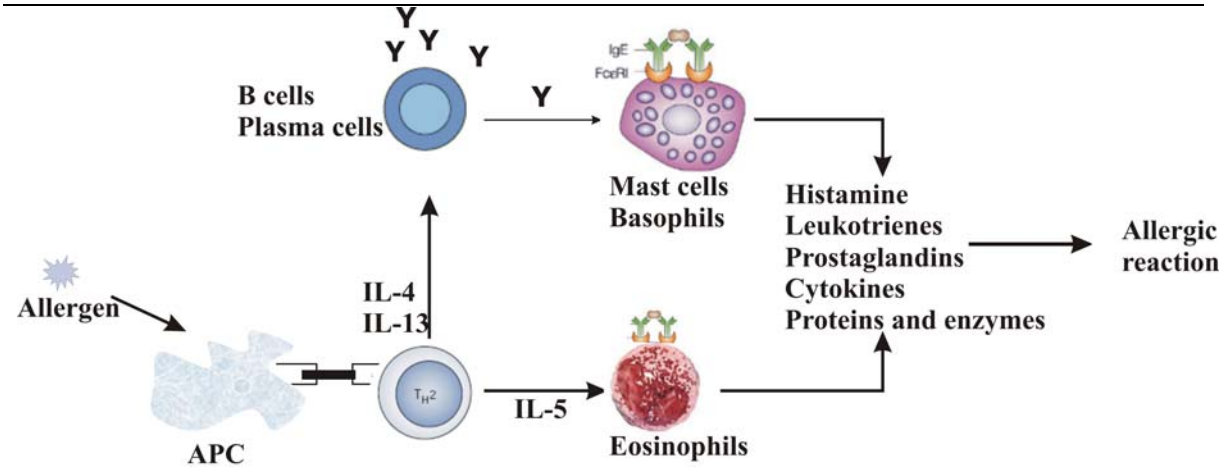

Figure 2: Inflammatory cascade in allergic reaction.

The allergic reaction is boosted by allergens entering the upper airways. The presentation of the antigen by contact to specialized cells leads to activation of Th2-cells, resulting in a release of chemokines and interleukins which selectively stimulate other cell species to produce the allergic reaction at the molecular and histological level. Modified after Bradding *et al.* 2006.

1.2.2 The T_H1/T_H2 paradigm

Conventionally, $CD4^+$ T-cells have been categorized into two subsets (Th1 and Th2) according to the profile of cytokines they produce. Usually, intracellular infections rely on a Th1 type response regulated by IL-12, TNF and IFN-γ which mediate inflammatory responses and promote cell-mediated immunity[63]. In contrast, extracellular parasites are processed by Th2-cells which produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 and are involved in antibody-mediated immunity[64]. Those cytokines stimulate antibody production and promote mast cell and eosinophils proliferation to expel the parasites. Originally the hygiene hypothesis was explained with an imbalance between the Th1/Th2 responses, but more detailed studies demonstrated that the regulation of the immune system does not rely on the symmetrical cross-regulation of Th1 and Th2 cells. It has been shown for example that IFN-γ which is a typical Th1 cytokine is also present in asthma and atopic dermatitis[65]. However, at the same time a defect in the IFN-γ and IL-12 pathway does not lead to an increase of the allergic disorder, thus implying that Th1 is not the regulator of Th2 responses[66]. Th1/Th2 cytokine balance plays an important regulatory role in the immune system, and its de-regulated ratio is responsible for several immunopathological conditions[67]. Th1/Th2 polarization depends on several environmental and genetic factors and particularly, on the local concentration of cytokines, such as IL-12 and IL-4 that induce differentiation of naïve T lymphocytes to the Th1 and Th2 phenotype, respectively[63]

1.2.3 Protective effect of helmints infection

Helminth infections as well as allergic reactions are associated with Th2 type immune reactions activating eosinophils, mast cells and the production of IgE. Despite this similarity on the molecular level the pathological outcomes are clearly different. A study carried out in Gabon demonstrated that individuals affected by helminth infection are less likely to develop allergic disorders[68] and the treatment of the infection leads automatically to an increase of the allergic sensitization[69].

Initially it was believed that Th1 immune response had an antagonistic action to that of Th2 immune response, one inhibiting the other[70]. However this polarized form of classifying the immune response is complicated by induction of regulatory mechanisms which would prevent the exacerbated Th1 and Th2 immune response and would prevent allergic as well as autoimmune disorders[71]. Regulatory T-cells play a pivotal role in this regulation[72;73] maintaining the immunological tolerance by the production of IL-10 and TGF-β so that continues antigen exposure lead to a down-regulation of the immune response.

Allergy is essentially an inflammation triggered by Th2 response, while the normal inflammatory response involves the Th1 subset of cytokines. In individuals infected with helminths, regulatory T-cells promote a state of immunosuppression, leading to inhibition of allergic inflammation[74]. Moreover it has been shown that individuals affected by helminth infection develop mostly anti-inflammatory network, IL-10 and transforming growth factor-β (TGF-β)[75]. Another mechanism explaining the inhibition of allergic inflammation in individuals infected with helminths is that postulated by Yazdanbakhsh and co-authors who demonstrated the first convincing link between worm infection and protection from allergy related to increased production of non-specific IgE in infected patients. According to the authors, the non-specific IgE saturates the IgE receptors on the mast cells (FcεRIs) thus preventing the specific IgE from binding to the allergen. Consequently, since the mast cell degranulation response requires the linkage IgE-FcεRIs to the allergen, it is likely that the production of a redundant IgE blocks the allergic response to allergens[76].

1.2.4 Antigen tolerance

Healthy and allergic immune response to common environmental proteins is characterized by a delicate balance in frequency of allergen specific regulatory cells (Tr1 cells) and allergen-specific Th2 cells[77]. The repeated exposure to the antigens in the airways does not lead always to an hyper reactivity of the immune system, rather to a decrease in the

responsiveness and the development of immunologic tolerance to the antigen[78]. The capacity of DCs to induce stimulatory T-cells or Treg cells depends on the state of the DCs maturation[79]. In the absence of inflammatory stimuli, the immature DCs are not able to induce proliferation of T-cells, thus mediating the tolerance in the lungs since they are the major source of IL-10, a cytokine responsible of the inhibition of inflammatory T-cells response[80]. Two types of immunological tolerance are known[81]: the central tolerance which occurs in the thymus for the T lymphocytes[82] and in the bone marrow for the B cells and the peripheral tolerance that occurs in the peripheral organs[83].

The peripheral tolerance of T-cells can act by 3 different mechanisms: clonal anergy, T-cell death and active suppression by T regulatory cells[84]. When the mature T lymphocytes meet the APC without activation of the co stimulatory molecules, in particular B7-1 (CD80) and B7-2 (CD86) this leads to a non-responsiveness of the T-lymphocytes called anergy[85]. The other possibility is that the T lymphocyte during the contact with the APC uses an inhibitory molecule like cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4)[86]. Another mechanism of anergy is represented by the contact of the T lymphocyte with the co stimulatory molecules, but that presents a distorted peptide in the contact zone with TCR[87;88]. The clonal deletion of mature T lymphocytes is generally the product of the activation-induced cell death (AICD)[89], a kind of apoptosis induced by death signals on the membrane, namely Fas (Fas-L). When Fas-L interacts with Fas, it activates the caspases that are responsible for the apoptosis of the cells. The Fas/Fas-L mediated AICD is dependent from IL-2 which is the responsible factor of T lymphocytes growth[90]. The last mechanism of peripheral tolerance is the deletion which is carried out by regulatory T-cells, once known as lymphocytes T suppressor. The cytokines involved are TGF-β that inhibits T- and B- cells proliferation and IL-10 that inhibits macrophages activation[91]. Regulatory T-cells (Treg) with their anti-inflammatory capabilities are the major mediators of the immunological tolerance[92] and their differentiation is induced by immature DCs in the skin, lungs, blood and spleens for the absence of inflammatory signals[93]. These cells express $CD4^+$ and $CD25^+$ molecules and are also associated with the fork head/winged helix transcription factor (FoxP3)[94]. Immature DCs express low level of MHC class II and co stimulatory molecules, but the expression of these molecules is up-regulated in response to inflammatory stimuli[95;96]. Garza K. M. suggested that the induction of the tolerance *versus* immunity can be explained on the basis of the ratio immature DCs to mature DCs[97]. Treg cells could be induced by immature DCs that in the absence of inflammatory signals, take up protein antigens in the peripheral tissues from apoptotic cell, and in the lymph nodes prime T-cell

precursors to became regulatory rather than effector T-cells[98]. Those primed Treg cells, under inflammatory situations will be reactivated by auto-antigen-presenting DCs to down-regulate the effector T-cells response at the site of inflammation[99]. The influence of immature DCs is restricted to resting/naïve T-cells and the induction of Treg cells is dependent on repetitive stimulation by immature DCs[96]. By contrast, IL-10-modulated DCs induce Ag-specific anergy even after a single contact with the T effector cell[100]. So two DC populations with different functions regulate the T-cell response: from one side the inhibitions of effector T-cells in an antigen-non-specific manner through the activation of Treg cells by immature DCs *versus* the direct-antigen-suppression of effector T-cells by IL-10-modulated DCs[101].

1.3 Dendritic cells and their activation *via* Toll-like receptors

The first time DCs were described in 1868 by Paul Langerhans as a type of cutaneous nerve cells. Two major populations of DCs are present in the skin: Langerhans DCs (LC) typically characterized by the expression of CD1a and the cytoplasmic granules named Birbeck granule (BG)[102] and dermal dendritic cells (DDC). DCs were initially mainly characterized by their high expression of major histocompatibility complex (MHC) class II HLA-DR and their high stimulatory activity toward allogeneic T-cells[103]. Usually DCs are present in all epithelia (skin, mucous and lungs) and are considered the most professional APC because of their ability to induce and coordinate the immune response. DCs are generated in the bone marrow and migrate as precursor cells to the sites of potential entry of pathogens where they up regulate expression of co stimulatory ligands, interact with naïve T lymphocytes and initiate a primary immune response. Dendritic cells are the key players in the presentation of allergens to T-cells in the lungs[104]. They act as sentinel cells that are able to detect the presence of foreign antigenic material and to process it, migrate to lymph nodes and present it to naïve T-cells. Antigens acquired from the extracellular environment are usually processed onto class II MHC, whereas class I MHC molecules bear Ags synthesized in the cytosolic compartment. When present in peripheral blood or in nonlymphoid tissue, DCs are highly specialized for capturing and processing foreign or autologoue antigens[105]. The uptake of high-molecular-weight molecules by DCs may occur through micropinocytosis or more specifically through membrane receptors such as FcγRII and FcεRI loaded with antibodies[106]. In contrast, uptake of low-molecular-weight aptens occurs through binding to glycoproteins and subsequent internalisation.

The presentation of a certain antigen-peptide to the T-cell receptor initiates sensitization and the subsequent immune response to the specific allergen[106]. The nature of this immune response depends on whether other co stimulatory molecules are involved[107]. The efficiency of the T-cells activation depends on the binding of either CD80 or CD86 molecules on the dendritic cell with CD28 on T-cells that leads to sensitization; a lack of those co stimulatory molecules may lead to anergy[108]. DCs are also able to generate IL-12, which is responsible in the maintenance of the balance between Th1 and Th2 response[109]. IL-12 is able to counteract Th2 sensitization, polarizing T-cell differentiation toward a Th1 response[110]. After sensitization, T-cells migrate to the site of antigen presentation under the influence of a chemokine cascade, and also they start to produce a range of cytokines which are mostly expressed on the long arm of chromosome 5, like IL-3, IL-4, Il-5, IL-6, IL9 and IL-13[111]. Th1 T-cells secreting tumour necrosis factor (TNF)α and Interferon (IFN)γ are recruited as the disease becomes more severe[112] and they are responsible for the tissue-damaging and asthma exacerbation[113].

1.3.1 Dendritic cell-mediated T-cell polarization

DCs stimulate the proliferation of naïve T-cells and are involved in the T helper (Th1/Th2) cell polarization through the so-called "three signal" hypothesis[114] depicted in Figure 3. Signal 1 is antigen-specific and requires the presence of MHC II molecules on the cell surface of DCs that recognize T-cell receptors (TCR) on the T-cells[115]. Signal 2 is a co stimulatory one and depends on the interaction between the co stimulatory molecules CD80 or CD86 expressed on DCs after ligation of pattern recognition receptor (PRR), such as TLR that sense infection through recognition of pathogens associated molecular patterns (PAMPs) with the CD28 expressed on the T-cells[116]. The second signal is very strong and in its absence the cell can become anergic, thus losing its ability to react to external stimuli. Signal 3 is needed for polarization of T-cells towards type 1 or type 2 Th-cells ($CD4^+$) and Th1 or Th2 cytotoxic T-cells ($CD8^+$)[117]. IL-12 and IFNγ are the most important members of the type 1 DC-derived cytokines which are secreted after stimulation by various pathogens (bacteria, viruses, protozoa)[118]. DC in their mature stage express CD40 that interact with CD40-ligand (CD40-L) on naïve T-cells and this leads to a higher production of IL-12 that is translated into an activation of Th1 type immune response[119]. From the other side type 2 DC-derived cytokines (IL-4, IL-5, and IL-13) promote and stimulate the activation of Th2-cells.

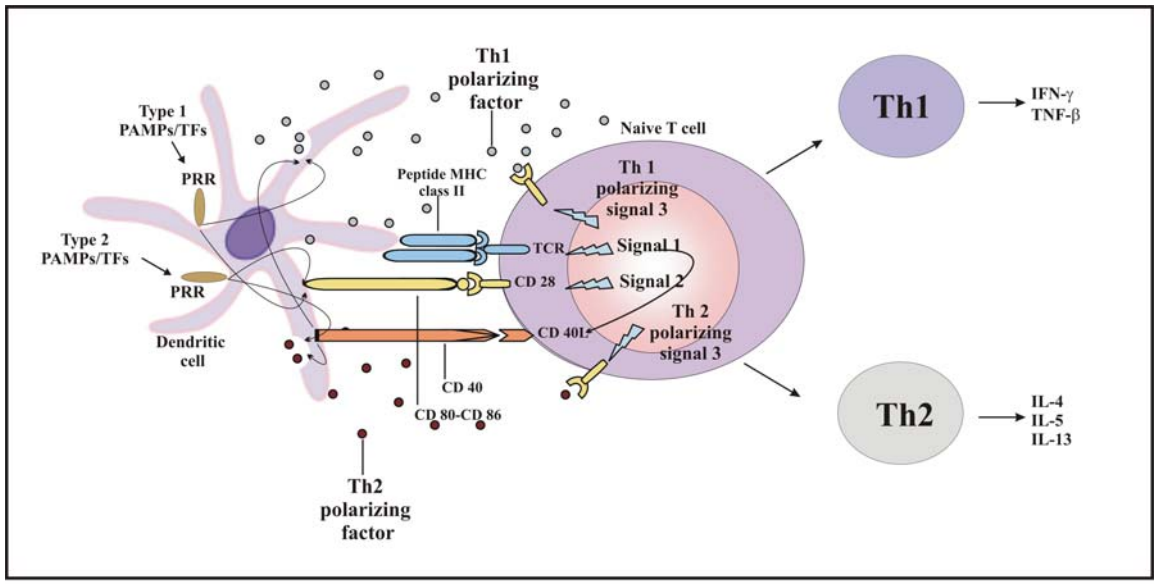

Figure 3: T-cell stimulation *via* "three signals".

Signal 1 is antigen-specific and is mediated by T-cell receptor (TCR) recognising MHC class-II-associated peptides processed from pathogens after internalisation through specialized pattern recognition receptors (PRRs). Signal 2 is the co-stimulatory signal, mainly mediated by triggering of CD28 by CD80 and CD86 that are expressed by dendritic cells (DCs) after ligation of PRRs. Signal 3 is the polarizing signal that is mediated by soluble or membrane-bound factors that promote the development of Th1 or Th2 cells depending on the activation of particular PRRs by PAMPs or inflammatory tissue factors TFs. Type 1 and type 2 PAMPs and TFs selectively prime DCs for the production of high levels of Th1-cell-polarizing or Th2-cell-polarizing factors. Whereas, the profile of T-cell-polarizing factors is primed by recognition of PAMPs, optimal expression of this profile often requires feedback stimulation by CD40 ligand (CD40L) expressed by T-cells after activation by signals 1 and 2. IFN-γ, interferon-γ; TNF-β, tumour-necrosis factor-β, IL, interleukin. Modified after M.L. Kapsenberg 2003.

1.4 Pattern Recognition Receptors

Pattern Recognition Receptors (PRR) recognize highly conserved structures expressed uniquely by microbes of the same class called pathogen-associated molecular pattern (PAMP)[120]. The activation of PRR by endogenous or microbial stimuli leads to the activation of multiple signalling pathways including nuclear factor-kB (NF-KB), mitogen-activated protein kinases (MAPKs) and type I interferon (IFN) which lead to the induction of pro-inflammatory and anti-microbial response[121].

1.4.1 Toll-like receptors

The first class of PRR identified was Toll-like receptors (TLRs). They are localized at the cell surface or within endosomes[122] on leukocytes (TLR1, TLR2), especially peripheral blood monocytes (TLR2, TLR4), macrophages, immature dendritic cells, natural killer cells, and T- and B-cells (TLR7, TLR9, TLR10)[123;124]. Toll was first identified in Drosophila as an important receptor in the early fly development[125] but a homologous family named Toll-like receptor (TLRs) exists also in vertebrates. TLRs are type of PRR that recognize different microbial molecules from bacteria, viruses, fungi and protozoan parasites[126] and they have the important role to distinguish between apoptotic particles generated by the natural tissue turnover and particles that are indicative of infections. TLR are considered the pivotal proteins that link the innate and the acquired immunity and due to the similarity of the cytoplasmic region, they are related to the IL-1 receptors (IL-1Rs). Like those ones, TLRs use the same signalling pathway that includes MyD88, IL-1R-associated protein kinase and tumour necrosis factor receptor-activated factor 6[127]. Thirteen human and 9 mouse TLRs are known so far and they are characterized by an amino-terminal extra cellular domain composed of repeated motifs rich in leucine and known as leucine-rich repeats (LRRs), followed by a single transmembrane domain and a globular cytoplasmic domain called the Toll/interleukin 1 receptor domain, or TIR domain that is also found in IL-1 receptors as well as in adaptors of the TLR signalling pathway[128]. Moreover, heterogeneity in extracellular domains allows for TLR recognition of a wide range of biochemically distinct microbial elements, whereas variability in their intracellular signalling pathways suggests the potential for ligand of different TLRs to induce distinct immunological responses[129]. A schematic view of TLRs and their ligands is depicted in Figure 4.

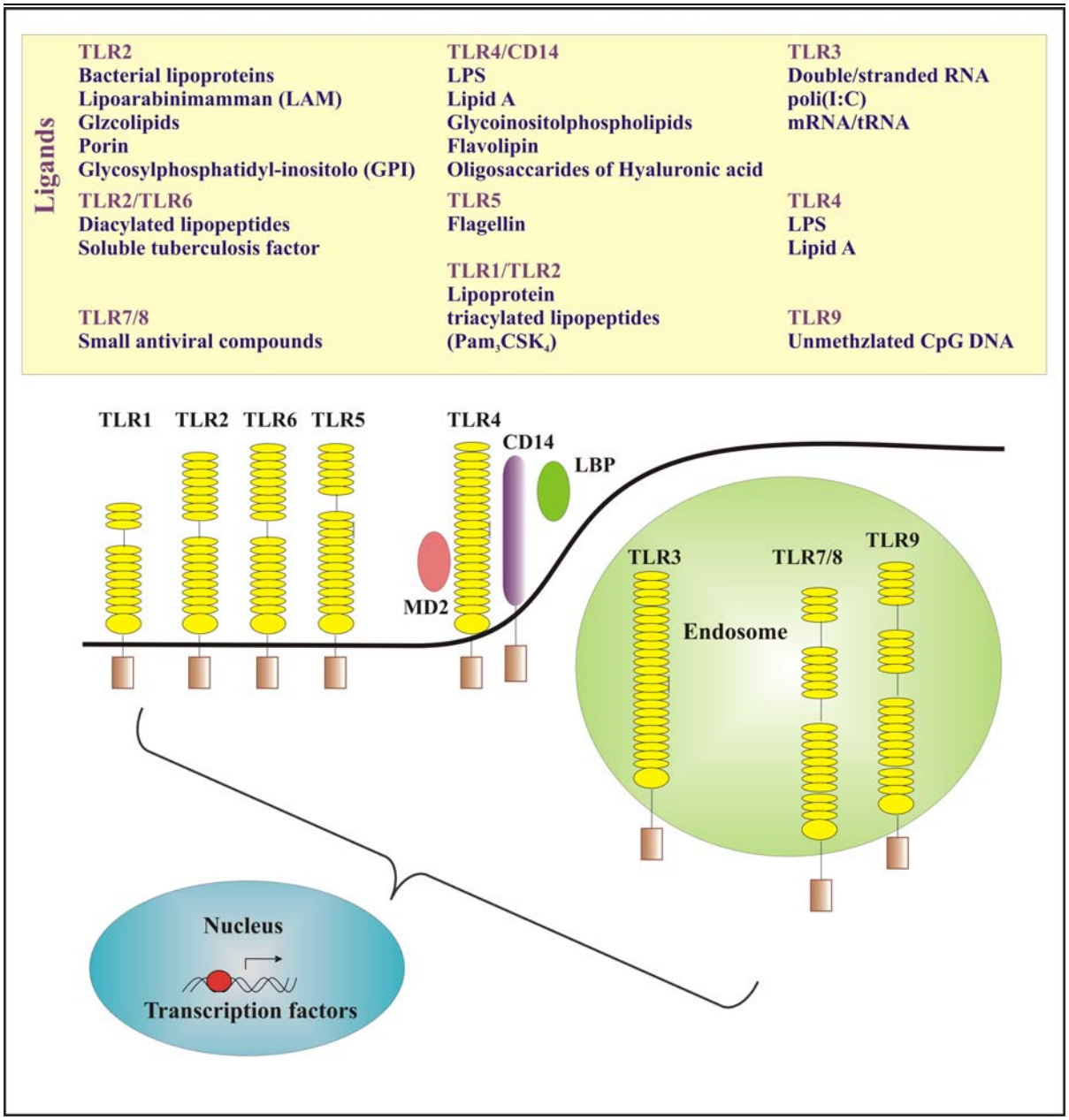

Figure 4: TLRs and their ligands.

TLRs recognize structurally conserved molecules derived from microbes. When activated, TLRs recruit adapter molecules (MyD88, Tirap, Trif and Tram) within the cytoplasm of cells in order to propagate a signal. The adapters activate other molecules within the cell, including certain protein kinases (IRAK1, IRAK4, TBK1 and IKKi) that amplify the signal, and ultimately lead to the induction or suppression of genes that orchestrate the inflammatory response.

Briefly, the lipid portion termed lipid A of the lipopolysaccharide (LPS), which is a compound of the outer bacterial cell membrane of Gram-negative bacteria, is recognized by TLR4 which uses several cofactors like LPS binding protein (LBP), CD14, a glycosylphosphatidylinositolo anchored protein (GPI)[130] and MD-2 anchorless protein.

TLR5 recognizes the D1 domain of flagellin which is a relatively conserved structure of motile species[131]. Amphiphilic negatively charged glycolipid and lipoproteins are recognized by TLR2 and in association with TLR1 or TLR6 can discriminate between triacylated and diacylated lipopeptides respectively[132]. In addition TLR2 modulates also the development of allergic disorders: it was demonstrated that children of farmers who have low probability to develop allergies, have an increased expression of TLR2 mRNA[133]. Moreover polymorphisms identified in the TLR2 gene are important factors to develop allergic diseases[134]. TLR3 is activated by double-stranded (ds) RNA, which is an intermediate of the replication cycle of viral ssRNA or DNA[135]. It can also recognize the synthetic analogue polyinosine-polycytidilic acid (poly I:C) and induce type I IFN[136]. TLR9 recognizes bacterial genomic DNA which contains unmethylated CpG dinucleotides in the so called CpG region[137]. TLR7 and TLR8 recognize single-stranded (ss) RNA rich in guanosine and uridine and ssRNA viruses, like Influenza (VSV) or Newcastle disease virus (NDV)[138-141]. The activation of the TLRs leads to recruitment of adaptor cells involved in the inflammation signalling.

1.4.2 C-Type lectin receptors

In contrast to TLR which are specialized in the induction of intracellular signalling, the C-type lectin receptors (CLRs) are highly specific internalising receptors which play an important role in the recognition of glycosylated antigens. Their activity of recognition and internalisation into DCs leads to the process of antigen presenting cells on MHC class I and II molecules[142]. Contrary to the maturation and activation processes activated by TLRs with consequent activation of the immune response and effectors cells, the uptake of antigen by CLRs does not lead necessarily to the activation of effectors T-cells, but may induce antigen-specific tolerance, thus suppressing the inflammatory response[143]. While TLR rely on the intracellular signalling process to sense and process the pathogen-specific antigens, the CLRs recognize carbohydrates structures even complex and internalise them without activation of DCs. More than 15 CLRs expressed by DCs have been identified so far and it has been found that the specificity of CLRs depends on the glycosylation profile of the protein carrying the carbohydrate moiety, on the length and on the branching of the carbohydrate chain: some CLRs recognize monosaccharides such as fucose, mannose or galactose, while others are more specialized in the recognition of more complex sugar fractions expressed on glycoproteins and glycolipids. CLRs posses a variety of carbohydrate-recognition domain

(CRDs) ranging from a single domain such as DC-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN), Dectin-1, MGL or DCIR up to eight or ten different domain such as mannose receptor (MR) which are differentially expressed on DCs. In vitro and in vivo studies regarding the localization of CLRs in the peripheral tissues demonstrated that their presence on immature DCs play a central role in the maintenance of the tolerance and in the clearance of self-antigen[144].

1.4.3 NOD-like receptors

Different type of PRR are the nucleotide oligomerization domain (Nod)-like receptors (NLRs) and the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) which are helicases recognizing viruses and by contrast to the TLRs are intracellular cytosolic sensors. There are 23 NLR family members in humans and 34 NLR genes in mice known so far. NLR are multidomain proteins containing a variable N-terminal effector region consisting of caspases recruitment domain (CARD), pyrin domain (PYD), acid domain, or baculovirus inhibitor repeats (BIRs) and a C-terminal leucine-rich repeats (LRRs) that senses the PAMPs. There is a big difference between NLRs expressed in plants and the ones expressed in humans: genetic studies demonstrated that LRRs of plant NOD-LRR proteins act as pathogen recognition domains and interact with elicitor produced by pathogens. By contrary Nod1 and Nod2 are able to sense conserved structures shared by many pathogens. Both Nod1 and Nod2 recognize products derived from peptidoglycan (PGN) through their carboxyl-terminal LRRs[145]. PGN is a component of the bacterial cell wall and is composed of glycan chains of repeated *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc)[146]. Muramyl dipeptide (MDP) is the essential structure in the PGN recognized by Nod2[147]. On the other side Nod1 recognizes *meso*-diaminopimelic acid (*meso*-DAP)-related molecules which is an amino acid unique for the PGN structure of all Gram-negative bacteria and some Gram-positive including *Listeria* and *Bacillus*[145].

1.5 The lipocalin protein family

The lipocalin protein family counts a large number of small and hydrophobic proteins which despite a large diversity at molecular level, share a more conserved three dimensional structures. Since its initial identification in 1985[148], the lipocalin family encompasses proteins with diversified structural and functional properties. Currently lipocalin proteins are classified as belonging to two major groups: (1) the kernel lipoproteins which share three

conserved sequence motifs corresponding to the three main structurally conserved regions of the lipocalin fold and (2) the outlier lipocalins characterized by more divergent sequences forming three main groups of proteins: (a) α_1-acid glycoproteins (AGPs), (b) odorant-binding proteins and (c) Von Ebner's – gland proteins[149]. Mostly, lipocalins are identified in vertebrates and few cases reported their presence also in bacteria. They cover large diversified roles in retinol transport, cryptic coloration, olfaction, and pheromone transport; and are also implicated in the regulation of the immune response and cell homeostatic processes. Together with other two families of ligand-binding proteins, the fatty acids-binding proteins (FABPs) and the avidins, the lipocalins are counted in the super family of calycins[150]. Despite their low sequential identity, the overall folding pattern of the lipocalins is highly conserved with a central β-barrel of eight antiparallel β-strands closed back to form a continuously hydrogen-bonded β-barrel that encloses an internal ligand-binding site[149].

1.5.1 The major bovine allergen *Bos d 2*

With the exception of the primary allergen present on cats *Fel d 1*, all major animal-derived allergens causing respiratory diseases identified and cloned so far belong to the lipocalin family. *Bos d 2* is the major cow dander allergen. It is secreted by apocrine sweet glands and then transported to the skin surface as pheromone carrier. It has been shown that *Bos d 2* allergen exists as monomeric non-glycosylated protein[149]. The immunological properties of the lipocalin proteins are poorly understood, but it has been shown for *Bos d 2* that its ability to bind IgE is mainly directed to the carboxy-terminal part[151]. Further studies demonstrated also that a reduction in the internal disulfide bonds corresponded to a significant decrease of the IgE binding property. Virtanen T. *et al.* demonstrated in 1999 that the allergenicity of lipocalins may be associated to the adaptation of the immune system to the presence of endogenous lipocalins. In this case the immune response against *Bos d 2* would manifest itself as a weak reaction with high level of IgE[152]. This kind of reaction was already described for the helminths infection where despite high levels of IgE, allergic reactions are rarely observed in infected individuals[153].

2. Aim and rationale for studying the cow and sheep dust extracts

Nowadays there is an increasing interest in the farm environment, in particular in the animal contact because of its potential to influence the development of asthma and allergies[154]. It has been suggested that the "farm effect" can result from the contact or exposure to bacterial compounds, such as moulds, ammonia, faeces and animal proteins[155]. However, recent studies have shown that not all farm environments are always associated to a protective allergic outcome. M. Ege and co-authors found that keeping sheeps is even a risk factor for atopic disorders.

As the determination of the environmental exposure with the greatest impact on the host immunity is difficult, we reasoned that the direct study of the immunological activity of the epidemiological relevant dust extracts (from cow and sheep shed) might be important to shed light on the relation between farm environments and allergic outcome. Since immunomodulatory particulates present within farm environments are concentrated by gravity into settled dust, sterile cow- and sheep-shed dust extracts were chosen for investigations. Moreover Besides the numerous and ever increasing number of data regarding the role of dust in the modulation of the allergic disorder, the dust itself is still poorly characterized. Most of the studies on dust extracts rely on their modulating effect of the allergic response, and on the relation of those dust preparations with the allergic outcome at the epidemiological level. The aim of our work was to isolate allergy-modulating compounds from cow and sheep-shed environment of traditional farms and to investigate their role in the prevention or induction of allergic inflammation. In addition we wanted to confirm whether a real difference between dust preparations from two different farm environments exists at chemical and immune modulating level as suggested by the epidemiological studies. Therefore a biochemical analysis was carried out on two dust samples originating from two different stable environments i.e. cow- and sheep stable with the consequent study of the immune modulating ability of the two dust extract *in vitro* and *in vivo* experiments.

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals and reagents

Reagents listed below were used either in purity grades pro analysis or in HPLC-grade.

PRODUCT	COMPANY
2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris)	BIORAD
Acetic acid	MERCK
Acetic anhydride	FLUKA
Acetone	MERCK
Acetonitrile	MERCK
Acrylamide	BIORAD
Aluminium hydroxide	PIERCE
Ammonia	MERCK
Ammonium acetate	MERCK
Ammonium bicarbonate	MERCK
Ammonium heptamolybdate	MERCK
Ammonium persulfate (APS)	BIORAD
α-Amylase	SIGMA
Aqua bidest.	MILLIPORE
Ascorbic acid	MERCK
Bisacrylamide	BIORAD
Blue dextran	SIGMA
Bovine serum albumine (BSA)	PIERCE, ROTH
Bromophenol blue	MERCK
Calcium chloride	MERCK
Chloroform	MERCK
Concanavalin A, peroxidase labeled from *Canavalia ensiformis*	SIGMA
Coomassie Brilliant Blue R-250	BIORAD

Diff-Quick	DADE DIAGNOSTICS
Dimethyl sulfoxide	SIGMA
Disodium hydrogenphosphate	MERCK
Ethanol	MERCK
Ethylene diamine tetraacetic acid (EDTA)	ROTH
Ficoll	GE-HEALTHCARE
Fluorescein-5-isothiocyanate (FITC)	SIGMA
Formaldehyde	MERCK
Glass beads 0.1 µm	ROTH
Glycerol	MERCK
Glycine	SERVA, BIORAD
Hydrochloric acid	MERCK
Hydrogen peroxide	MERCK
Lithium chloride	MERCK
Low molecular mass standard	BIORAD
Magnesium chloride	MERCK
Manganese chloride	MERCK
Mercaptoethanol	SERVA
Methanol	MERCK
N,*N*-Dimethylformamide	IRIS-BIOTECH
N,*N*,*N*',*N*'-Tetramethylethan-1,2-diamin	BIORAD
Nitroblue tetrazolium-chloride (NBT)	BIOMOL
Naïve T-Cell enrichment column HCD41	R & D System
OVA grade IV	SIGMA
PARA-formaldehyde	SIGMA
Percoll	GE HEALTHCARE
Periodic acid	MERCK
Phenol	MERCK
Potassium chloride	MERCK
Potassium dihydrogen phosphate	MERCK
Pyridine	FLUKA
Rotiphorese®NF-Acrylamide/Bisacrylamide	ROTH
Schiff's reagent	SIGMA

Silver nitrate	BIORAD
Sodium acetate	MERCK
Sodium borhydride	MERCK
Sodium borodeuteride	ALDRICH
Sodium carbonate	FLUKA
Sodium chloride	MERCK
Sodium citrate	MERCK
Sodium dodecylsulfate	BIORAD
Sodium hydrogen carbonate	MERK
Sodium hydrogen phosphate	MERCK
Sodium hydroxide	MERCK
Sodium metabisulfite	SIGMA
Sodium metaperiodate	MERCK
Sodium molybdate tetraoxide	MERCK
Sodium sulfate	MERCK
Sodium thiosulfate pentahydrate	MERCK
Sulfuric acid	MERCK
4-Toluidine salt (5-bromo-4-chloro-3-indolyl-phosphate toluidine) (BCIP)	BIOMOL
Trifluoroacetic acid	MERCK
Trypsin	SIGMA
Tween 20	BIO-RAD
ZIP-tips (C18)	MILLIPORE

3.1.2 Buffers, solutions and media

To avoid contamination with bacterial endotoxin, buffers and solutions were prepared using deionised, sterile filtered water (Milli Q, MILLIPORE) for analytical techniques and sterile, endotoxin-free water (B. BRAUN) for preparative techniques. Unless otherwise indicated, all concentrations listed are final concentrations of dissolved reagents in H_2O. Additionally all glass ware was sterilized at 240°C for 4 h to destroy bacterial endotoxin. All buffers were sterile-filtered and autoclaved subsequently. For the cell cultures the products listed below were used.

PRODUCT	**COMPANY**
Dulbecco's minimal essential medium (DMEM), high glucose, with L-glutamine	GIBCO (Invitrogen)
Fetal calf serum (FCS), inactivated at 56°C for 30 min	GREINER
Hanks buffered salt solution (HBSS)	GIBCO (Invitrogen)
Phosphate buffered saline (PBS)	140 mM NaCl, 2.7 mM KCl, 7.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4
RPMI 1640 with L-glutamine	GIBCO (Invitrogen)
Penicillin/streptomycin	SIGMA
FACS buffer	PBS/1%BSA/2%FCS/1%HS
Recombinant human IL-2:	CHIRON/RED SWAN PHARMA LOGISTICS, PROLEUKIN.
Granulocyte macrophage colony stimulatory factor (GM-CSF)	BIOSOURCE
Interleukin 4	R & D SYSTEM
Tryphan blue	SIGMA
L-glutamine & pyruvate	SIGMA
LPS ultrapure *E. coli* 0111 B4 strain	INVIVOGEN
Saponin	SIGMA
Interferon gamma (IFNγ)	CYTECH
PMA (Phorbol 12 myristate 13 acetate)	SIGMA
Brefeldin A	SIGMA
Ionomycin	SIGMA
Staphylococcus aureus enterotoxin B	SIGMA
Interleukin 2	CETUS
Percoll	GE-HEALTHCARE

Equipments and plastic materials are listed below.

EQUIPMENT	**COMPANY**
2D Electrophoresis WITA vision	WITA
Äkta FPLC system	GE-HEALTHCARE
Amicon-Ultra-15 centrifugal filter units	MILLIPORE
Auto MACS TM	MILTENYI BIOTEC
Autoclave	SYSTEC
Balance	SARTORIUS
Biological safety cabinet	CRYO-TEC
422-Electrolution chamber	BIO-RAD
Cell counter (Thief depth 0.1 mm)	BÜRKER BRIGHT LINE
Centrifuges	BECKMANN COULTER, HEREAUS, HETTICH
CO_2 Water Jacketed Incubator	FORMA SCIENTIFIC
Culture plates (24-, 48-, 96-wells)	COSTAR
Dialysis membranes MWCO 1000	ROTH
Electrophoresis equipment	BIORAD
ELISA reader	BIOTEK INSTRUMENTS
ELISA-plate shaker	EDMUND BÜHLER
FACS Calibur flowcytometer	BECTON DICKINSON
Homogenisator	B. BRAUN
Hi Trap® Q-sepharose 1 and 5 mL column	PHARMACIA BIOTECH
Horizontal rotary shaker	NEW BRUINSWICK SCIENTIFIC
Lyophilizator	ALPHA
MALDI-TOF MS Reflex II	BRUKER DALTONICS
Microcon centrifugal filter devices	MILLIPORE
Mini Protean® 3 Cell	BIORAD
Mini Trans-Blot® Cell Assembly	BIORAD
Multi cytokine ELISA reader	LUMINEX CORPORATION
Multi-well plates	NUNC
Nylon membrane	QIABRANE QIAGEN
Pasteur pipettes	BRAND
Photometer	HELIOS

Pipette tips	SARSTEDT
Procise™ Protein Sequencing System	APPLIED BIOSYSTEMS
Thermomixer	EPPENDORF
Ultracentrifuge: UZ TGA-55	KONTRON

3.2 Methods

3.2.1 Dust extract preparation

3.2.1.1 Dust collection

A total of six cattle stables and five sheep sheds were selected in rural regions of the Alps in the south of Germany. The dust was collected by brushing stables surfaces (windowsill and animal benches) using a common brush and a spatula. Dust was seeped through a common kitchen seep and then pooled down according to the farm origin so that it is referred to cow-stable dust (CoD) and sheep-stable dust (ShD). The sampling of the dust was accompanied by a detailed questionnaire to the family running the farm regarding the dietary component, the number of siblings in the family, the direct contact of children to the animals (for playing or helping in haying) in order to study the farm-related exposure and health outcome.

3.2.1.2 Sodium chloride extraction of the dust samples

Dust samples (10 g) were dissolved in Braun water in the presence of 15 g glass beads (0.4-0.5 mm) until a total volume of 70 mL was reached. In order to obtain a more homogeneous compounds, the dust was homogenized (5 min) in a Braun homogenisator under flux of CO_2 as cooling system[156]. The obtained disrupted samples were transferred to an Erlmeyer flask and filled to a total volume of 1L saline solution [0.9% (w/v) NaCl] in endotoxin-free water and stirred at 22°C for 6 h. The extracted dust was then sedimented (5000 x *g*, 20 min). To remove salts the supernatant was collected, dialyzed against endotoxin-free water and analyzed for its properties to stimulate the immune system in an *in vitro* as well as an *in vivo* model (sections 3.2.2 and 3.2.5 respectively). Additionally after lyophilization the dust extracts were filtered through 0.22 µm Steritip filters to obtain sterile preparations and then lyophilized so that it is referred to cow-shed dust extract (CoDE) and sheep-shed dust extract ShDE.

3.2.2 Cell culture and immunological methods

The cell culture experiments and the immunological study described in this thesis were performed in cooperation with B. A. Pires and B. Everts in the Department of Parasitology at the Leiden University Medical Center, Leiden (The Netherlands) under the supervision of Prof. Dr. Maria Yazdanbakhsh.

3.2.2.1 Isolation of human peripheral blood mononuclear cells

Peripheral blood was drawn into heparinized tubes from healthy donors who had given informed consent. Human peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation using Ficoll[(157)]. Therefore, 10 mL of HBSS medium were added to 15 mL of blood. Subsequently, 13 mL of Ficoll was applied under the cell suspension. After gentle centrifugation at 450 x *g* (22°C, 25 min) the separation of blood plasma (upper phase) and erythrocytes (lower phase) was achieved. The interphase containing PBMCs was collected and washed twice with 1% FCS/HBSS to remove contaminating thrombocytes (4°C, 330 x *g*, 10 min).

3.2.2.2 Isolation of monocytes from PBMCs

A standard Isotone Percoll solution (SIP) was obtained by mixing 2.2 mL of 10 fold concentrated PBS and 19.8 mL of PerColl. The SIP solution was then diluted in 1% FCS/RPMI in order to get a SIP concentration of 47.5% and 34% as shown in the table below. The PBMCs were re-suspended (1 to 5, by volume) in 1% FCS/RPMI and the SIP was subsequently added to reach a final concentration of 60% (6 mL). The cell suspension (2.5 mL) was poured in 15 mL falcon tubes where 5 mL 47.5% SIP and 2 mL 34% SIP had been stratified following centrifugation at 1750 x *g* (22°C, 45 min).

Table 3.1: Preparation of Standard Isotone Percoll solution.

	47.5% SIP	34% SIP
SIP	10.45 mL	3.4 mL
1% FCS/RPMI	11.55 mL	6.6 mL

3.2.2.3 Isolation of monocytes from the upper interface

Cells had been washed twice with 1% FCS/RPMI (228 x *g*, 22°C, 15 min) after which the pellet had been re-suspended in 1% FCS/RPMI (1 to 5, by volume). Cells where counted

and seeded in 24-well culture plates at a density of 5 x 10^5 cells per well and further purified by a 1 h adherence step (37°C, 5% CO_2). Adherent cells were then cultured in RPMI-1640 medium supplemented with 10% FCS, human recombinant IL-4 (250 units/mL) and human recombinant granulocyte-macrophage colony-stimulating factor (500 units/mL). At day 3 the culture medium including the supplements was refreshed. On day 6 or 7 the resulting immature DCs were harvested.

3.2.2.3.1 Differentiation of human monocytes-derived dendritic cells

Monocytes were isolated as described above, concentrated to 10^6 cells per mL and differentiated into immature dendritic cells in the presence of GM-CSF (500 U/mL) and IL-4 (250 U/mL) using RPMI medium containing 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. At day 3 the culture medium including the supplements was refreshed and undifferentiated DCs were harvested after 6-7 days of culture. The maturation of DCs was achieved by addition of $LPS^{(130)}$ (100 ng/mL). In our experiments, immature DCs were matured with (1) LPS alone (100 ng/mL), (2) LPS plus IFN-γ (10^3 units/mL) which stimulates the polarization of naïve T-cells into Th1, and (3) LPS plus Schistosome soluble egg antigen (SEA) (50 µg/mL), a parasite extract which promotes the polarization of naïve T-cells into Th2. In order to test the effect of dust extract samples from cow (CoDE) and sheep-shed dust preparations (ShDE), (4) increasing concentration of CoDE and ShDE were used (20 and 100 µg/mL). Stimulation was followed by (1) analysis of DC supernatant for the presence of IL-10 and IL-12p70 by ELISA (3.2.3.3), (2) analysis of DC surface markers by FACS (3.2.3.2), (3) analysis of cytokines production of DC after co-culture with 2 x 10^4 CD40 ligand-expressing mouse fibroblast (J558 cells) and (4) analysis of DCs ability to skew the T-cells response toward a Th1 or Th2 response in presence of dust extracts stimuli (3.2.2.4.1). Schistosomal egg antigen (SEA) was produced at the Leiden University Medical Centre. It was prepared from schistosomal eggs, collected from trypsin treated liver, homogenate of the *S. mansoni* infected hamsters.

3.2.2.4 Isolation of naïve T-cells

Briefly, naïve T-cells were purified from PBMCs by negative selection with human $CD4^+$ $CD45RO^-$ Naïve T-Cell Enrichment Column kit (R & D Systems Minneapolis, MN).

Highly purified $CD4^+$ $CD45RO^-$ naïve T-cell populations from donors were used for the study of the DC-polarizing effect on T-cells.

3.2.2.4.1 Outgrowth of naïve T-cells in presence of mDCs

Naïve T-cells from an allogeneic donor were co-cultured between 5-7 days in 96-well flat-bottom culture plates with 5 x 10^3 mature DCs in the presence of the superantigen *Staphylococcus aureus* enterotoxin B (SEB) at a final concentration of 100 pg/mL. At day 5, human recombinant IL-2 (10 units/mL) was added and the cultures were expanded. After 10-14 days, the quiescent Th cells were re-stimulated with immobilized CD3 monoclonal antibody (CLB-T3/3, CLB, Amsterdam, the Netherlands) diluted in coating buffer and soluble CD28 monoclonal antibody (CLB-CD28/1, CLB) diluted in 10% FCS/RPMI. IL-10 was measured in 24 h supernatants. The IFN-γ-producing and IL-4-producing T-cell populations were determined by intracellular staining and FACS analysis using phycoerythrin-conjugated anti-human IL-4 (BD Biosciences) and fluorescein isothiocyanate-conjugated anti-human IFN-γ (BD Biosciences) after 5 h of stimulation with phorbol 12-myristate-13-acetate, ionomycin and Brefeldin A. Therefore a solution of 0.5 mg/mL ionomycin/PMA 100 µg/mL in 10% FCS/RPMI was added to the T-cells and incubated for 4 h at 37°C under CO_2 exchange. Additional incubation of 2 h with Brefeldin A was followed. Afterwards the cells were washed in PBS (5 min, 515 x *g*) and then 1 mL of 1:10 (=3.7%) formaldehyde in PBS was slowly added under gentle vortexing. The cells were then incubated for 15 min at 22°C. After 15 h a second washing step in PBS was performed (515 x *g* for 5 min at 4°C) and then the cells were re suspended in 2 mL of PBA [0.5% BSA in PBS]. The following day the cells were washed (10 min 515 x *g* at 4°C) and saponin buffer [0.5% saponin in PBS] containing (1:20) αIL-4-PE and (1:20) αIFNγ-FITC was added to the pellet. After 30 min of incubation at 22°C in the dark, the cells were ready for FACS analysis.

3.2.3 Biochemical methods

3.2.3.1 Flow cytometry

Monocyte derived dendritic cells were stimulated with CoDE and ShDE as described in section (3.2.2.3.1). The maturation level of DCs exposed to different stimuli was assessed by measuring the expression of different surface markers by FACS analysis as explained below.

3.2.3.2 FACS staining and DC surface markers

Between 2500 and 5000 mDC were plated in 96 cone-bottom plates. All the conditions were tested *in duplicate*. Additionally, iDCs and LPS stimulated DC were also stained with CD-1 FITC staining. Cells were washed with 200 µL FACS buffer (330 x *g*, 4°C, 4 min) and re-suspended by gently vortexing and then incubated at 4°C for 30 min with one or more of the following fluorochrome-labeled antibodies (BD Biosciences unless otherwise stated) as indicate in the table 3.2. After staining (30 min at 4°C), the cells were washed once again in FACS buffer (330 x *g*, 4°C, 4 min) and transferred to 1.4 mL FACS tubes placed on ice for the FACS analysis. Cell maturation was analyzed on a FACS Calibur using the CellQuest program. Antibodies of the respective isotype detected unspecific binding in a negative control approach. Data analysis was done using FlowJO 7.2.2 (Treestar, USA). Antibodies used are listed in the table 3.3.

Table 3.2: Fluorochrome-labeled antibodies for FACS analysis.

Mix A	Mix B	Mix C
HLA-DR-FITC, 50*****	**CD-86-FITC,** 50*	**CD-1-FITC,** 50*
CD-80-PE, 50*	**CD-83-PE,** 20*	**7-AAD-Per-CP** 50*
CD-14-Per-CP, 20*		
CD-40-APC, 20*		

**end-dilution*

Table 3.3: Antibodies used in FACS analysis.

Antibodies	Clones	Isotype	Company
CD14 PerCP	MΦP9	mIgG2b k	BD BIOSCIENCE
CD80 PE	L307.4	mIgG1 k	BD BIOSCIENCE
CD83 PE	HB15a	mIgG2b	IMMUNOTECH
CD86 FITC	2331 FUN-1	mIgG1 k	BD BIOSCIENCE
MHC-II HLA-DR FITC	L243	mIgG2a k	BIOLEGEND

3.2.3.3 Sandwich Enzyme-Linked-Immunosorbent-Assay

Enzyme-Linked-Immunosorbent-Assay (ELISA) was performed to determine the concentration of cytokines and chemokines in the culture supernatant of stimulated cells. The first step of the ELISA test was the coating of a 96-well plate with an antibody able to recognize the molecule of interest after incubation for 16 h at 22°C. After unbound antibodies were washed out, uncoated surface was blocked by a blocking solution. After the blocking solution was removed by washing, the samples were added to the plate and incubated at 22°C for 2 h. Removal of unbound sample material by repeated washing steps was followed by incubation with a biotinylated antibody. Its excess was removed by washing, followed by addition of a horse radish peroxides (HRP)-labeled detection antibody. Several washing steps minimized unspecific binding of antibody before addition of tetramethylbenzidine (TMB) substrate. After the HRP-catalyzed substrate conversion occurred the reaction was stopped by addition of sulphuric acid (1.8 M) and the quantification of converted substrate was measured at $\lambda = 450$ nm using an ELISA reader and compared to a standard. PeliKine Compact Human ELISA Kit was used and IL-8, IL-10 and IL12p70 antibody were provided by Sanquin. All the *in vitro* experiments described in this thesis were performed in cooperation with B. Everts and B. A. Pires in the Department of Parasitology at the Leiden University Medical Center, Leiden (The Netherlands), under the supervision of Prof. Dr. Maria Yazdanbakhsh. Experiments attended federal and international guidelines and were approved by the ethical comities.

3.2.4 Serological assay

3.2.4.1 Immunostaining

Immunostaining was performed as follows: the antigen blotted onto a nylon membrane was left to dry 2 h at 37°C. The membrane was then blocked for 1 h in blocking buffer [10% skimmed milk in Dot Blot Buffer] and incubated 2 h at 22°C with the primary antibody diluted 1:200 in Dot blot buffer [200 mM NaCl in 50 mM Tris-HCl pH 7.4]. After repeated washing steps (6 x 5 min each), an AP-labelled secondary antibody diluted 1:1000 in AP-buffer [0.1 mol/L $NaHCO_3$, 1 mmol/L $MgCl_2$ x 6 H_2O PH 9.8] was added to interact with the primary one followed by a second cycle of washing steps (6 x 5 min each) with Dot Blot buffer. The membrane was then incubated with developing solution [10 mL AP buffer + 60 µL 50 mg/mL nitroblue tetrazolium-chloride (NBT) in DMF + 30 µL 50 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate toluidine salt (BCIP) in DMF] 15 min in the darkness and without shaking. Staining produced an insoluble stable purple-coloured deposit at the site of the immunocomplex. The reaction was stopped by the removal of the substrate and the addition of MP-water (30 min at 22°C). The membrane was dried at 37°C or alternatively between Whatman filters paper. If not otherwise indicated, the different steps of the development were made with shaking. As primary antibody blood serum was used which was collected from children living in contact either to sheep or to cattle farms and that were associated to the allergic or non-allergic phenotype as indicated in the table below. Anti human IgE conjugated to alkaline phosphatase was used as Secondary antibody (Zytomed System).

Table 3.4: Primary antibody from blood serum for dot blot test.

Blood serum was collected from children who lived in contact to cattle or sheep farms and then classified according to the positive or negative outcome observed. ID+ and ID- identification numbers are referred to the positively or negatively associated outcome respectively

CONTACT	ALLERGIC OUTCOME	ID + allergic outcome	ID – allergic outcome
Sheep	+/-	2	1
Sheep	+/-	11	21
Sheep	+/-	13	28
Sheep	+/-	27	29
Sheep	+/-	34	30
Cattle	+/-	18	17
Cattle	+/-	25	19
Cattle	+/-	26	20
Cattle	+/-	31	22
Cattle	+/-	32	23

3.2.5 Animal Experiments

The animal experiments described in this thesis were performed in cooperation with PD Dr. Holger Garn in the Department of Clinical Chemistry and Molecular Diagnostics at the Philipps University of Marburg, (Germany) under the supervision of Prof. Dr. Harald Renz. Experiments attended federal and international guidelines and were approved by the regional government (Regierungspräsidium Giessen). Female BALB/c mice aged 6-8 weeks from Harlan Winkelman (Borchen, Germany) were kept under pathogen free housing conditions. Water and OVA-free diet was supplied *ad libidum*.

3.2.5.1 Allergen sensitization, provocation and dust treatment

Allergic diseases are provoked by a repeated contact to an allergen during the so called sensitization phase. A later contact to the same allergen leads to allergic symptoms. Mice were sensitized to allergens by means of three intraperitoneal (i.p.) injections of 10 µg of OVA grade IV adsorbed to aluminium hydroxide diluted in 200 µl PBS and injected on day 0, 14 and 21. Allergen challenge was performed on 3 consecutive days (day 26, 27 and 28) by application of a 1% OVA (w/v in PBS) aerosol for 20 min in order to provoke a local

allergic reaction in the lungs. This is a standardized treatment that challenges a local allergen induced inflammatory response in the lungs[158]. Dust extracts (1 mg/mL) were given three times a week intranasal (50 µL each) starting 13 days before the first sensitization with OVA allergen. Analysis was performed 24 h (AHR) and 48 h (all other parameters) after the last challenge. A group of 8 mice was sham treated with PBS over the whole process of sensitization and challenge as a negative control. Figure 5 shows the schematic protocol of provocation and treatment.

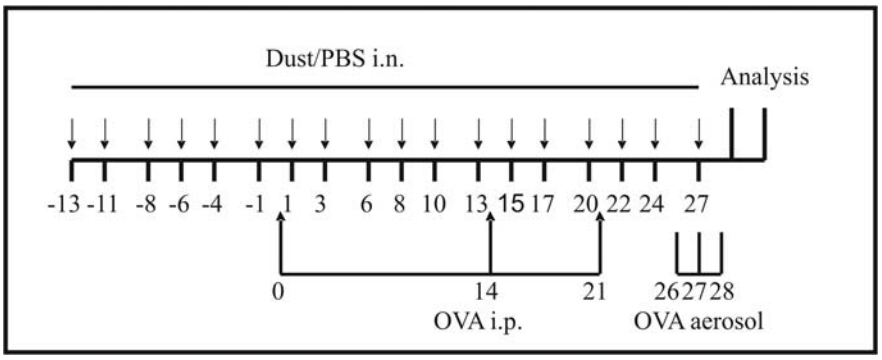

Figure 5: Schematic protocol of mouse experiments.

Female BALB/c mice were sensitized at day 0, 7 and 14 by ovalbumin (OVA) injections (i.p.) followed by OVA aerosol exposure at days 26, 27 and 28. Thirteen days before the first OVA injection and during the whole sensitization period mice received CoDE and ShDE, intranasal every second day. Mice were analyzed 24 h (AHR) and 48 h (all other parameters) after the last challenge.

3.2.5.2 Airway responsiveness to methacholine

Mice were investigated for the hyperresponsiveness of their airways 24 h after the last challenge[159]. To simulate the decrease in tidal volume of asthma patients, mice were analyzed for airway reactivity to stimuli such as β-methacholine directly related to the airway obstruction of the mouse. Therefore, mice were placed into a body plethysmograph and tidal volumes of unaffected mice were measured and set to 100%. Afterwards, mice were exposed to increasing amounts of β-methacholine and the concentrations causing a 50% (EF_{50}) reduction of airflow were compared. High airway obstruction resulted in elevated AHR at low concentrations of β-methacholine.

3.2.5.3 Cell analysis of bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) was performed 48 h after the last allergen challenge as suggested by Neuhaus-Steinmetz *et al.*[160]. The total leukocyte number in BAL fluid (BALF) was determined with a Casy TT cell counter. The principle of this cell counter is a change of a constant resistance in a chamber of measurement. The resistance is shifted by passing cells in the presence of intact cell membranes acting as an isolator. The resistance changes depending on the cell size, which enables the method to determine different cell populations in a mixture. Furthermore cytospins were sedimented at 300 x *g* and cells were stained with Diff Quick. To minimize mistakes 100 cells were counted in a double estimation for every cytospin. The remaining cell free BALF were stored at -20°C for further investigations of cytokines.

3.2.5.4 Detection of cytokines in bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid was obtained as described in section 3.2.5.3 and relevant cytokines for the investigation of asthma and allergy related shifts in immune response were determined using OptEIA ELISA kit. Here we report on TNF-α, IL-6, IL-10, IL-4, IL-5 and IL-13 levels in BALF. The utilized ELISA was performed as a sandwich ELISA as described in section 3.2.3.3.

3.2.5.5 OVA-Specific immunoglobulins

Two days after mice had obtained the last OVA provocation blood samples were taken and used to determine the serum concentrations of OVA-specific immunoglobulins. Therefore multi-well plates were incubated with 20 mg/mL OVA at 4°C for 16 h. Unbound OVA was removed by washing and plates were blocked with BSA. Repeated washing steps were followed by incubation with monoclonal IgG_1, IgG_{2a} or IgE antibodies (4°C, 16 h). After washing, biotinylated IgG_1, IgG_{2a} or IgE antibodies were added and plates were incubated for 2 h. Incubation with peroxidase labelled streptavidin followed at 25°C. Plates were measured in an ELISA reader by comparison to immunoglobulines of known concentrations. Antibodies used are listed in the table below.

Table 3.5: Antibodies used in ELISA analysis.

	Clones	Isotype	Company
Isotype	X56	$mIgG_1$	BD BIOSCIENCE
	R35-72	mIgE	BD BIOSCIENCE
	R19-15	$mIgG_{2a}$	BD BIOSCIENCE

3.2.6 Statistical analysis

All data obtained in animal experiments are shown as median and interquartil range. Significance was analyzed using SAS 9.2 software and the Mann-Whitney-U-test. Significant differences were considered at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, indicating differences between PBS treated mice and CoDE or ShDE treated mice. In this work, the statistical analysis was performed in collaboration with Dr. med. Mascha Rochat of the Department of Pediatric Pneumology at the Ludwig Maximilian University of Munich (Germany) under the supervision of Prof. Dr. Erika von Mutius.

All data obtained in the *in vitro* experiments are shown as means of at least three independent experiments ± SEM. Significance was analyzed using one-sided Student´s t test and the software package GraphPad Prism 4 (GraphPad Software, San Diego, CA) kindly provided by Dr. Norbert Reiling. Significant differences were considered at * $p < 0.05$, ** $p < 0.01$.

3.2.7 Analytical techniques

3.2.7.1 Gel-permeation chromatography

3.2.7.1.1 Sephadex G10

Size exclusion chromatography using Sephadex G10 column was used to purify additionally the dust preparations from salts. CoDE and ShDE samples (50 mg) were purified using a 2.5 cm x 120 cm column (BioRad) of Sephadex G10 superfine (Pharmacia) in MP-water at a flow rate of 150 mL/h connected to a Minipuls 2 Abimed Gilson pump. A differential refractometer (Knauer R100) was used as detection system. Fractions of 2.5 mL were collected.

3.2.7.1.2 TSK 40

To separate the oligo- and polysaccharides fractions from the dust preparations a 2.4 cm x 120 cm column (BioRad) of TSK-40 S (Pharmacia) in water was utilized connected to a Minipuls 2 Abimed Gilson pump and equipped with a differential refractometer (Knauer-R100). Fractions were collected in 6 min intervals.

3.2.7.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Electrophoretic separation of proteins by gel electrophoresis was performed as a discontinuous SDS-PAGE[161]. Gels were prepared in a BIORAD (8 x 10 cm) system. The resolving gel was prepared as shown in the "Gel Formulations" table, cast between glass plates and polymerized for 30 min. The stacking gel (5%) was poured on top of it.

Table 3.6: Gel formulations [10 mL].

Resolving gel (15%)	**Volume (mL)**
Bisacrylamide 30%	4.3
1.5 M Tris-HCl pH 8.8	2.5
H_2O	3.1
SDS 10% (w/v) in H_2O	0.1
APS 10% (w/v) in H_2O	0.05
TEMED	0.005
Stacking gel (5%)	
Bisacrylamide 30%	1.3
0.5 M Tris-HCl pH 6.8	2.5
H_2O	6.1
SDS 10% (w/v) in H_2O	0.1
APS 10% (w/v) in H_2O	0.05
TEMED	0.001

3.2.7.2.1 Sample preparation

Protein extracts were quantified due to their protein content by Bio-Rad protein assay as shown in section 3.2.6.8. One to five µg of protein were mixed with equal amounts of

sample buffer [12.5% (v/v) 0.5 M Tris-HCl pH 6.8, 2% (w/v) SDS, 5% β-mercapto-ethanol, 1% (v/v) glycerol, 0.01% (w/v) bromophenol blue 2 M NaCl in equilibration buffer], centrifuged, heated to 100°C for 5 min, centrifuged again and loaded onto a gel. The gel chamber was filled with 500 mL of running buffer [25 mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3] prior to loading the sample material. Gels were run at 22°C and 200 V for about 1 h. The run was stopped as soon as the bromophenol blue front ran out of the gel.

3.2.7.3 Staining methods for the detection of proteins and carbohydrates following polyacrylamide gel electrophoresis

3.2.7.3.1 Silver staining for the detection of proteins

Silver staining of proteins after SDS-PAGE was performed by successive steps according to *Tsai and Frasch 1982*. Proteins were first fixed for 1 h or alternatively 16 h in fixative solution [40% ethanol (v/v), 5% acetic acid 55% deionized water] under constant shaking. Then the oxidation step was achieved by reaction with periodic acid solution in water [0.07% (w/v) sodium metaperiodate in deionised water]. The gel was then extensively washed 4 times with water for 10 min and the proteins were then stained in an alkaline silver nitrate solution [1 mL of 25% NH_3, 280 µg 5 M NaOH, 2.5 mL of 20% A_gNO_3, 71 mL deionised water] for 10 min under constant shaking. Washing steps as above were followed by incubation with developing solution [85 µL of 37% formaldehyde, 9.45 mg of sodium citrate, 189 mL deionised water] until formation of brown colour. The reaction was stopped by addition of 7% (w/v) acetic acid.

3.2.7.3.2 Coomassie Brilliant blue staining

Gels were stained by Coomassie Brilliant Blue [2 g/L Coomassie Brilliant Blue R-250 in 40% methanol-10 % acetic acid] to detect specifically proteins based on the interaction of the dye with the amino functions of proteins, according to Coomassie *et al*.. Background was minimized with distaining solution [10% (v/v) acetic acid, 10% methanol and 80% deionised water].

3.2.7.3.3 Periodic acid-Schiff staining of carbohydrates

Periodic acid-Schiff staining detects carbohydrates by the reaction of Schiff's base with aldehyde groups of carbohydrates forming a magenta colour imine[162]. Gels were fixed in fixative/distaining solution [10% (v/v) acetic acid, 35% methanol in water] for 1 or 2 h, incubated in periodic acid reagent [0.7% (w/v) periodic acid in acetic acid:water (5:95 v/v)] for 1 h and treated with sodium metabisulfite [0.2% (w/v) sodium metabisulfite in acetic acid:water 5:95 (v/v)]. The gel appeared yellowish and distained in the same solution after ongoing treatment. Subsequently Schiff's reagent (premixed from SIGMA) was added for incubation until the carbohydrate containing bands were stained magenta. Distaining followed to reduce the background colour.

3.2.7.4 Electroelution of proteins

Protein bands were electroeluted in a 422-Electro-Eluter® system of BIO-RAD. The protein of interest was separated by SDS-PAGE and stained partly with Coomassie Blue R-250 (3.2.6.3.2). Bands were excised from the gel and cut into small pieces, prior to application to the 422 Electro-Eluter® devices. Membrane caps were soaked at 60°C in elution buffer [25 mM Tris-HCl, 192 mM glycine, 0.1 % SDS] for at least 1 h prior to use. At the bottom of each glass tube was placed a frit and together they were pushed into the electro-eluter module. Grommet needed to be moist with elution buffer in order to fix the device easier. Pre-wetted membrane caps were placed in the bottom of each silicon adaptor and filled with elution buffer. To avoid air bubbles around the dialysis membrane the buffer was pipetted up and down the adaptor. Tubes were filled with elution buffer and gel slides were placed inside the tubes. After the entire module was placed into the buffer chamber, the lower buffer chamber was filled with 600 mL of buffer and the upper one with 100 mL. Vigorous stirring during the run was preventing bubbles on the dialysis membrane. Elution was carried out at 10 mA/glass tube for 4 h.

3.2.7.5 Electrotransfer of proteins to polyvinylidene fluoride (PVDF) membrane (Western blot)

Proteins were transferred from the resolving gel to a PVDF membrane for the lectin blot analysis using concanavalin A. After SDS-PAGE was finished the gel was equilibrated

in transfer buffer. The same was done with fibre pads and filter papers as well as with the methanol-activated PVDF membrane (soaked for 20 sec).

The transfer stack was assembled by placing the fibre pad, filter paper, gel, membrane, a second filter paper and a second fibre pad on top of each other. All air bubbles were removed, the sandwich was placed into a cassette holder and finally the cassette to a blotting chamber. The blot was cooled by a cooling pack. The buffer was stirred on a magnetic stirrer and proteins were transferred with a voltage of 100 V for 1 h. Later, the membrane was stained by a lectin blot as described in section 3.2.6.5.1. Transfer buffer was prepared using [25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3].

3.2.7.5.1 Lectin blot

Lectin blots were performed to investigate glycoproteins conjugated with mannose or glucose residues. After blotting, the membrane was washed in TBST buffer [20 mM Tris, 150 mM NaCl, adjusted to pH 7.5, 0.1 mM $MnCl_2$, 0.1 mM, $CaCl_2$, 0.1% (v/v) Tween 20] and incubated with 100 µg of HRP-conjugated Concanavalin A under constant shaking for 60 min. The lectin was removed by washing with water and the blot was stained [7 mL 4-chloro-1-naphthol (3mg/mL in methanol), 39 mL H_2O, 2 mL Tris 0.2 M, pH 7.5, 20 µL H_2O_2] in the dark for 15 min. The membrane was washed and documented by scanning.

3.2.7.6 Peptide mass finger print

Separated proteins after gel electrophoresis were identified by peptide mass fingerprinting (PMF)[163] in cooperation with Helga Lüthje and PD Dr. Buko Lindner at the Division of Immunochemistry, Research Center Borstel. Picked protein spots from SDS-PAGE were digested enzymatically *in situ* by Trypsin cleaving [50 mM ammonium bicarbonate in H_2O + 12.5 ng/µL trypsin] the C-termini of the amino acids lysine and arginine. Therefore, picked gel spots were washed and covered with acetonitrile to achieve a shrinking of the gel spot. Drying of gels and re-hydration in wash solution [100 mM ammonium bicarbonate in H_2O/acetonitrile 1:1] was performed to remove Coomassie R 250 completely. Incubation in DTT solution [10 mM dithiothreitol, 100 mM ammonium bicarbonate in H_2O] at 56°C for 45 min was followed by a 30 min alkylation step using iodacetamide solution [55 mM iodaceteamide, 0.1 M ammonium bicarbonate in H_2O] at 25°C under light exclusion. Washing and drying of the samples was repeated and proteins were

digested with trypsin for 16 h. Subsequently, gel spots were covered with 25 mM ammonium bicarbonate as well as an equal volume of acetonitrile. Repeated ultrasonic treatment for 10-15 min effected the extraction of peptides after digestion. The supernatants were collected and concentrated under vacuum. Extracted peptides were re-suspended in 0.1% trifluoroacetic acid and purified by reversed-phase material in Zip-tips. Mass spectrometric analyses were carried out on a MALDI-TOF MS Reflex system. The peptides were embedded in a matrix of α-cyano-4-hydroxy cinnamic acid (CCA) in TA solution [trifluoroacetic acid 0.1% acetonitril 2:1] according to the dried-droplet method. Ionization was achieved by energy transfer of laser light (nitrogen laser, λ = 337 nm). Ions were accelerated by a currency of -20 kV and measured in the positive ion mode. Calibration was achieved by the use of proteins with known masses e.g. angiotensin II, insulin, cytochrome C and BSA. The spectra were recorded by ACQ 4.04 software and monoisotopic masses were inserted to the national centre for biotechnology information (NCBI)-database (www.mascotscience.com).

3.2.7.7 Fast protein liquid chromatography

FPLC separation and analysis was performed in cooperation with Dr. Uwe Mamat at Research Center Borstel. Isolation of proteins from the complex mixture of the dust preparations was performed in anionic conditions using 5 mL Hi Trap Q Sepharose column connected to fast performance liquid chromatography (FPLC Äkta; Amersham Pharmacia, Biotech, Sweden). The flow was set to 1 mL/min, the sample was applied manually and the gradient of 1-100% of 2 M NaCl in 20 mM Tris-HCl, 0.05% tween-20, pH 7.5 was used. The fraction volume was set to 0.5 mL. The elution was monitored with UV lamp at 280 nm using a UPC-900 UV lamp detection system.

3.2.7.7.1 Preparation of the samples for FPLC

After extraction in NaCl solution the two dust preparations (60 mg circa) were risospended in Braun water 10 mL and then dialyzed again against Millipore water using membranes with MWCO 3500. After 3 days of dialysis 0.05% Tween-20 was added to the sample and vigorously vortexed. The so obtained sample was loaded on the column for FPLC separation.

3.2.7.8 Bradford protein detection assay

Proteins were detected and quantified by BioRad protein assay based on the principle of the Bradford test[164]. The absorption maximum of Coomassie Brilliant Blue G 250 is shifted from 465 to 595 nm when binding to a protein. Bovine serum albumin was used as standard in concentrations 0 to 20 µg/mL. The standard and the sample were pipetted to a reaction vial and filled up to 800 µL with water. After 200 µL of premixed BioRad protein assay reagent were added, samples were mixed vigorously and incubated at 22°C for 15 min. Absorption was measured at 595 nm and protein concentration was calculated according to the standard curve.

3.2.7.9 Determination of phosphate

The phosphate measurement[165] was performed to detect the presence of inorganic and total phosphate in our preparations. The samples were tested directly after the extraction. Sample material (50 µL) was transferred into glass tubes and dried in a vacuum cabinet at 40°C for 16 h. Volumes of 0 to 10 µL of disodium hydrogen phosphate solution (5 mM) were used as standard. The dried sample material was digested by addition of 100 µL hydrolyzing solution [6.7 mL aqueous perchloric acid (70% w/v), 30.6 mL sulphuric acid, and fill to 100 mL H_2O], incubated at 100°C for 1 h and then heated to 165°C for additional 2 h. The glass tubes were cooled to 22°C and 1 mL of precooled staining solution [1 mL sodium acetate 1 M, 1 mL ammonium heptamolybdate (2.5% w/v), 1 mL ascorbic acid (10% w/v) and 7 mL H_2O] was added (4°C). The samples were incubated at 37°C for 90 min prior to their photometric quantification at 820 nm. The amount of organic bound phosphate was determined as difference between the total and the inorganic phosphate.

3.2.7.10 Gas liquid chromatography

3.2.7.10.1 Methanolysis

Two kinds of methanolysis have been carried out for the dust extract samples: a weak one using 0.5 M methanolic hydrochloric acid at 85°C for 1 h and a strong one in 2 M methanolic hydrochloric acid for 16 h. After methanolysis the glass tubes were cooled to 22°C, and their content was transferred to glass vials. The mixture was evaporated and peracetylated by the addition of 100 µL water free acetanhydride and 50 µL of pyridine

heated to 85°C for 15 min. Samples were dried under nitrogen, washed 3 times with methanol and dissolved in chloroform. Gas chromatographic separation was achieved on Hewlett Packard series II 5890 chromatograph using a HP-5MS-capillary column. Helium was used as mobile phase with a pressure of 10 psi. Electron ionization was recorded at a currency of 70 eV. Samples were injected at 150°C and the column was heated for 5°C/min to a final temperature of 320°C. Molecules were detected by mass spectrometry.

3.2.7.10.2 Detection and quantification of neutral sugars

The analysis of neutral sugars was performed by gas chromatography and coupled mass spectrometry (GC-MS) of the corresponding alditol acetates[166]. For quantification various sample materials were hydrolyzed at 120°C in 2 M trifluoroacetic acid for 2 h. Allose was added to the evaporated sample and used as internal standard. All volatiles were removed by rotary evaporation and samples were washed twice with a 9:1 mixture of hexane and diethylether to remove fatty acids. One mL of water was added and the pH was adjusted to 8-9 by addition of sodium hydroxide. The carbonyl groups of sugars were reduced by the addition of 150 µL 0.3 M sodium borohydride, for the analysis of neutral sugars, or borodeuteride for amino sugars (22°C in the dark for 16 h). The reaction was stopped by addition of 2 M hydrochloric acid until the mixture stopped foaming which indicated the destruction of excessive borodeuteride. The borate was converted to the corresponding methyl ester by washing three times with glacial acetic acid (5% v/v in methanol]. Subsequently, peracetylation was performed as described before. The dried and peracetylated samples were resolved in 50 µL of chloroform and 1 µL was injected to the gas chromatograph. Alditol acetates were separated on a poly-(5%-diphenyl-95%-dimethyl)-siloxan SPB-5-capillary column (30 m, 0.32 mm). Hydrogen was applied as mobile phase with a pressure of 60 kPa. The gradient started at 150°C followed by heating (3°C/min) to a final temperature of 300°C. Signals were detected by flame ionization. The identification was achieved by comparison with a preinjected mixture of known monosaccharides modified by the same procedure. The quantification was based on comparison of area under the curve values to that of the internal standard allose.

3.2.7.10.3 Total fatty acids

Further investigations of fatty acids were performed according to Wollenweber *et al.*[166]. The samples were hydrolyzed together with 17:0 as internal standard in 4 M hydrochloric acid at 100°C for 4 h. Alkaline hydrolysis followed in 1 mL of sodium hydroxide 5 M at 100°C for 30 min. Released fatty acids were extracted in chloroform:water (1:3 v/v) at pH 3 by vigorous shaking and centrifugation to separate the two phases. The organic phases of repeated extractions were collected, dried over sodium sulphate and concentrated under nitrogen. Diazomethane was added drop wise to 300 µL of organic phase till the solution turned slightly yellow. After gentle shaking the diazomethane was removed under nitrogen until the yellow staining vanished. Methylation was repeated and after removal of diazomethane samples were transferred to 1 mL glass vial by solubilisation in chloroform. For analysis 1 µL sample was injected to perform gas chromatography on a Packard 438A chromatograph and separated on a poly-methylsiloxan column (Ultra-1, 12 m x 0.2mm HEWLETT PACKARD). Hydrogen was used as carrier gas at a pressure of 1.5 bars. The separation was achieved with a temperature gradient of 120°C for 3 min with a linear increase to 260°C (5°C/min).

The qualitative and quantitative determination was performed by gas-liquid chromatography (section 3.2.6.10) using for comparison the following fatty acid methyl esters as external standard to determine their individual response factors: 12:0, 12:0(2-OH), 14:0, 14:0(2-OH), 14:0(3-OH), 16:0, 17:0, 18:1, and 18:0.

3.2.7.10.4 Determination of aminosugars

Samples were hydrolyzed in 4 M hydrochloric acid at 100°C for 4 h to release amino sugars. Repeated evaporation removed the volatiles and the sample was washed with ether/hexane (1:9 v/v) to withdraw fatty acids. Peracetylation was performed as described above (3.2.6.10.2), prior to the reduction of carbonyl groups by sodium borodeuteride treatment. Peracetylation was repeated to achieve acetylation of the free amino groups. The so obtained peracetylated samples were dissolved in 50 µL of chloroform and 1 µL was ingected to gas chromatograph. Analysis was carried out using Hewlett Packard series II 5890 gas chromatograph.

4. Results

4.1 Dust sampling

The sampling of the dust was carried out within a pilot study in rural regions of the Alps in the south of Germany selecting six cow sheds and five sheep sheds. A strong difference in the farm environments was observed: generally the cow sheds were bigger and with a higher percentage of humidity than the sheep ones. Moreover the cow stables resulted more “dirty” and fuller of dust compared to the sheep ones where the collecting process was difficult in terms of amount of dust collected. The sheep sheds usually presented more light points at different sides of the building and mostly the rooms were more aerated and more exposed to meteorological effects e.g. wind and rain compared to the cow sheds.

A difference in the sampling yield was observed with about 95.5 g of dust was obtained pooling the six different cow sheds dust and about 30 g from the five sheep sheds. Figure 6 shows two farm environments: cow and sheep shed where the dust was collected.

Figure 6: Cow and sheep shed environments.

Two representative farm environments are shown: on the upper panels a cow shed dark, humid and with big amount of dust on the windows sills and on the animal benches. On the lower panel a sheep shed environment more aerated, bright and exposed to meteorological effects but with less amount of dust.

4.2 Analytical characterization of dust extract

4.2.1 Dust extraction and composition analysis

In order to isolate soluble compounds from the dust samples, the cow shed and the sheep shed dust samples were extracted as described in section 3.2.1. using an aqueous solution of NaCl (0.9%). The total yield of the two extractions was different for the two samples, with CoDE giving a total yield of 10% against only about 5% of ShDE. After the extraction, no microbial growth was observed when CoDE and ShDE aliquots were cultured in blood agar plates, thus indicating that no bacterial contaminations were present in the dust preparations. Since the aim of the work was focused on the analytical characterization and comparison of the two dust extract preparations, the first approach in disentangle their complex and heterogeneous composition was to screen the different chemical species present in the two dust extract samples using GC and GC-MS experiments (Figure 7).

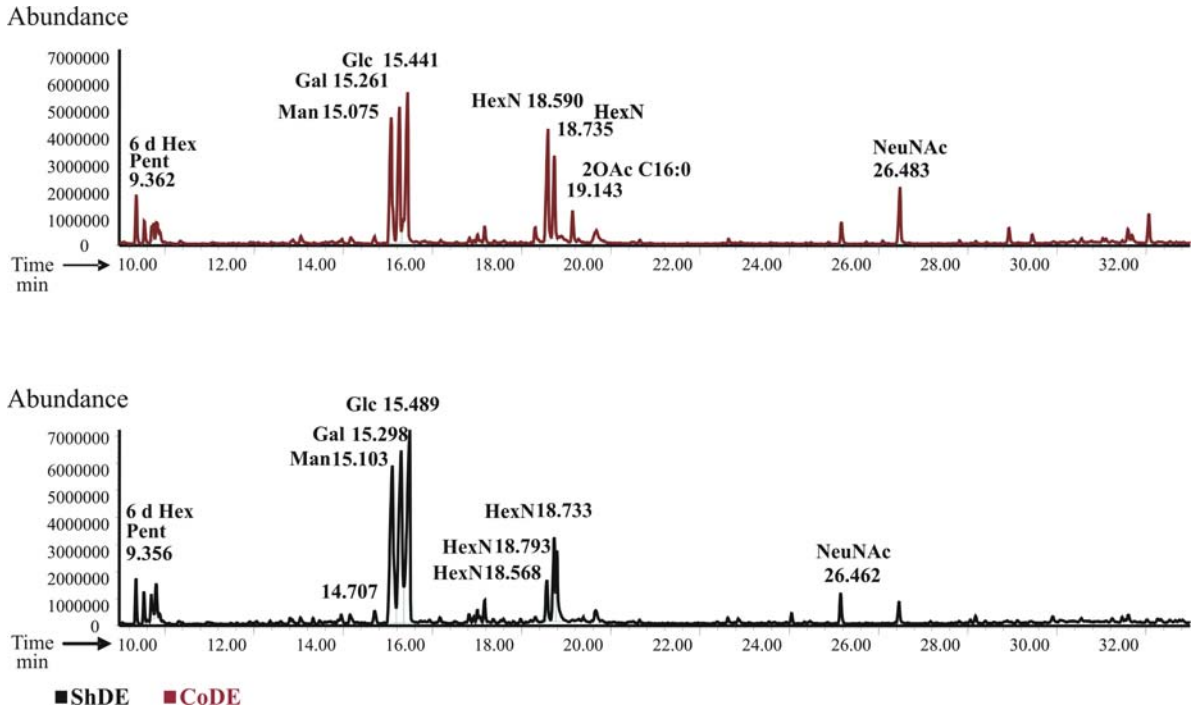

Figure 7: GC-MS analysis of the dust extract samples.

Gas chromatographic separations are depicted. The two dust preparations showed a similar profile with the four main regions almost completely overlapping. In abscissa is depicted the retention time in min., while on the ordinate the abundance. CoDE in red, ShDE in black.

The GC-MS analysis of the two samples was carried out after weak hydrolysis using 0.5 M methanolic hydrochloric acid and peracetylation as described in section 3.2.6.10. The

two dust preparations showed the same profile with four main regions corresponding to four main chemical species: (1) 6-deoxy-hexoses and pentoses, (2) mannose, glucose and galactose as major hexoses, (3) hexosamine and neuraminic acid. In details, an enlarged view of the GC-MS chromatogram showed a complete overlap of the four main regions, thus suggesting that the two dust extracts possess a similar sugar profile. Some variation was observed in the relative amount. The enlarged and overlapped chromatograms are shown in the Figure 8. The strong heterogeneicity of the samples did not allow us to use more sophisticated and precise methods such as NMR experiments.

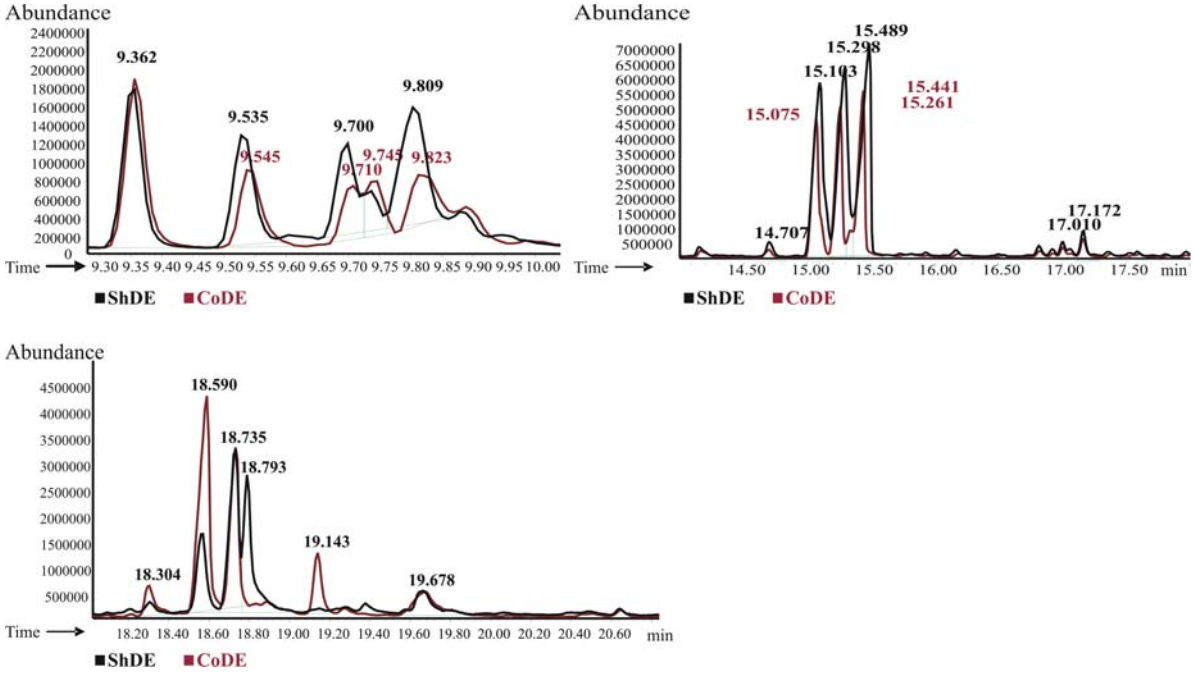

Figure 8: Detailed GC-MS analysis of the dust extract samples.

The three overlapping main regions of the GC-MS chromatogram enlarged are depicted. Gas chromatographic separation was achieved after weak methanolysis using 0.5 M methanolic hydrochloric acid and peracetylation as shown in section 3.2.4.10.1. CoDE in red, ShDE in black.

4.2.2 Sugar analysis

In order to detect and classify in more detail the sugar fractions observed after GC-MS analysis, we studied the neutral and amino sugar profile of the two dust preparations. The detection of the sugar species in the CoDE and ShDE preparations was achieved by gas chromatography as shown in section 3.2.6.10. Table 4.1 summarises the concentration of the different neutral sugars found in the two dust preparations. Interestingly although the same species of sugars were present in both samples, the relative amount was different.

Additionally in none of the dust extract samples were detected heptoses suggesting that no contamination from bacterial compounds was present. In addition, both samples presented glucosamine (GlcN) as unique amino sugar.

Table 4.1: Neutral and amino sugar analysis of the CoDE and ShDE preparations.

Quantification of the sugar fraction by gas chromatography. Sugar concentrations are shown in nmol/mg.

NEUTRAL AND AMINO SUGAR ANALYSIS		
Neutral sugars	**CoDE**(nmol/mg)	**ShDE**(nmol/mg)
Rha	1686	119
Rib	340	38
Ara	1112	114
Xyl	825	89
Man	2108	284
Glc	3034	384
Gal	3253	394
Hep	-	-
Amino sugar	GlcN	GlcN

4.2.3 Fatty acid analysis

Fatty acids detection and quantification was carried out after hydrolysis with HCl and extraction of the lipidic fraction by chloroform-water as described in section 3.2.6.10.3. The two studied dust extract samples (CoDE and ShDE) showed strong differences in the fatty acids composition. The fatty acids found in the two samples were present in traces in the ShDE preparation, but in bigger amount in the CoDE. The analysis revealed the presence of saturated fatty acids such as tetradecanoic acid (14:0), hexadecanoic acid (16:0) and octadecanoic acid (18:0) and monosaturated fatty acids such as oleic acid (18:1) as major fatty acids. In addition CoDE showed also the presence of 2-hydroxytetradecanoic acid [14:0(2-OH)]. The relative amount of fatty acids in the samples was estimated by comparison with an external standard and showed in Table 4.2. In CoDE preparation the most dominating fatty acid was the tetradecanoic acid (14:0) which was found in a concentration of 343.5 nmol/mg.

Table 4.2: Fatty acids analysis by gas chromatography.

Quantification of fatty acids by gas chromatography. Relative amount of fatty acids is shown in nmol/mg.

FATTY ACIDS ANALYSIS		
	CoDE nmol/mg	ShDE nmol/mg
14:0	343.5	7
[14:0(2-OH)]	60.5	-
16:0	130	20.5
18:1	68.5	27
18:0	50.5	14

4.2.4 Analysis of the total and inorganic phosphate

The concentration of total and inorganic phosphate was determined by photometric assay at 820 nm as shown in section 3.2.6.9. The amount of organic-bound phosphate was calculated as difference between the total and the organic phosphate. The analysis revealed the presence of higher amount of organic phosphate in the dust extracted from sheep-shed (ShDE) compared to the one from cow-shed (CoDE).

Table 4.3: Phosphate detection by photometric assay.

The content of inorganic and total phosphate was determined by photometric assay at 820 nm as shown in section 3.2.6.9, while the amount of organic bound phosphate was calculated as difference between the total and the inorganic. The relative amount is shown in nmol/mg.

PHOSPHATE ANALYSIS		
	CoDE nmol/mg	ShDE nmol/mg
Total phosphate	73.4	132
Inorganic phosphate	11.4	67
Bound organic phosphate	62	65

4.3 Analysis of the amino-acidic components

4.3.1 Amino acid profile

The analysis of the aminoacids profile was achieved after hydrolysis and HPLC analysis of the two dust extracts. The pattern of amino acids looked almost identical in both preparations and also the relative amounts were comparable. In descending order, starting

from the most abundant aminoacid we identified glutamic acid (Glu), asparagine (Asn) and glycine (Gly), serine (Ser), alanine (Ala), threonine (Thr), leucine (Leu), proline (Pro) and valine (Val), lysine (Lys) and isoleucine (Ile), arginine (Arg) and phenylalanine (Phe), tyrosine (Tyr), histidine (His), methionine (Met). The relative amount is depicted in Figure 9 as percentage of amino acid compared to a mixture of amino acids used as standard.

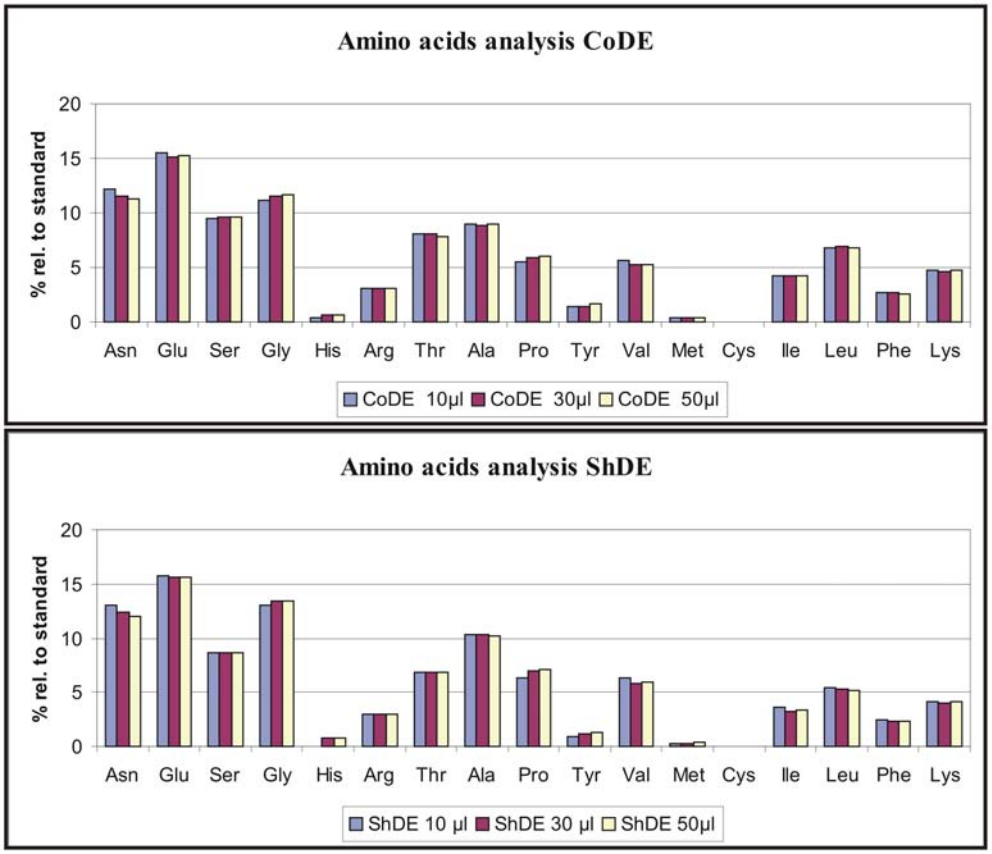

Figure 9: Aminoacid analysis of CoDE and ShDE.

In the upper panel CoDE and in the lower one ShDE preparations are shown. Aminoacid profiles in the two samples were analysed by HPLC after hydrolysis with HCl and the amount was calculated as percentage referred to the standard.

4.3.2 Protein quantification

The total amount of proteins in the two dust preparations was detected and quantified by photometry (BioRad protein assay) as described in section 3.2.6.8. A strong difference between the two dusts preparations was found with 751.8 µg protein/mg of CoDE extract compared to 265.4 µg/mg of ShDE.

4.3.3 Protein pattern analysis

The analysis of the protein profile was carried out by SDS-PAGE and subsequent staining of the gels with silver and Coomassie brilliant blue staining as described in sections 3.2.6.3.1. and 3.2.6.3.2 respectively.

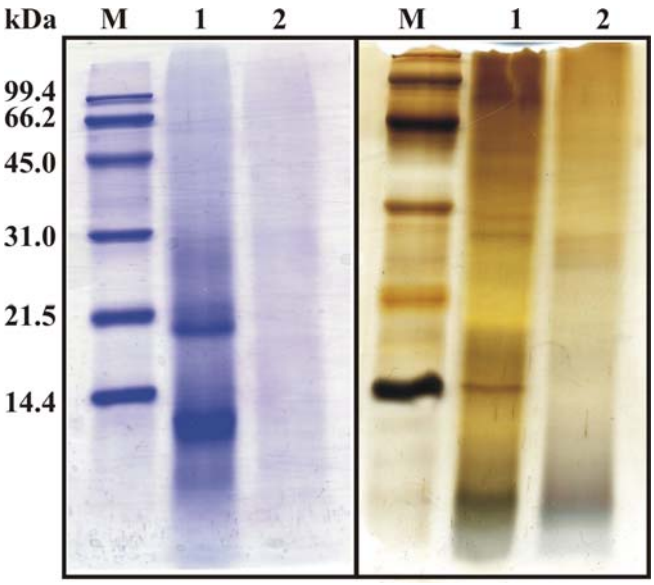

Figure 10: SDS-PAGE of the dust preparations.

SDS-PAGE (15%) of CoDE (1) and ShDE (2). Coomassie Blue and silver staining (100 µg dust extract per lane) are depicted in the left and right panel, respectively. The molecular mass was estimated by comparison to a Low Range Marker standard (M).

As shown in the Figure 10 the Coomassie staining of CoDE (line 1) revealed the presence of several distinct bands mostly at low molecular masses ranging from 10 to 30 kDa which were confirmed also by silver staining. The ShDE (line 2) on the contrary did not show any defined protein pattern, but rather a smear on the gel, thus showing a clear difference in the protein pattern between the two analysed dust samples. Moreover two major proteins from CoDE were detected on SDS-PAGE around 20 and 14 kDa. Those bands were further analysed by MALDI-TOF-MS experiments and peptide mass fingerprint.

4.3.4 Peptide mass fingerprints

Following SDS-PAGE analysis, bioinformatics protein identification was performed on the two major bands isolated from CoDE by gel electrophoresis, namely B1 and B2. The protein bands were directly digested *in situ* with trypsin as described in section 3.2.6.6 and then analyzed by MALDI-TOF-MS experiments. Figure 11 shows the CoDE protein pattern

resolved on SDS-PAGE in different concentrations and stained with Coomassie. Peptide mass fingerprint analyses were performed on the two major bands after trypsin digestion.

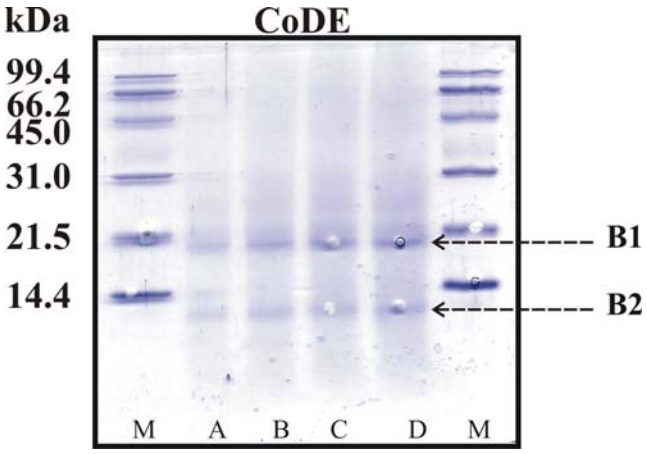

Figure 11: SDS-PAGE separation of CoDE for peptide mass fingerprints.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (15%) of extracted dust sample CoDE. The CoDE is shown after staining with Coomassie blue. Molecular mass was determined by comparison to a standard marker (M). Different concentrations of CoDE are depicted, i.e. A = 30, B = 50, C = 70, D = 100 µg of dust extract. The most promising bands, namely B1 and B2, were digested in situ and then analysed by peptide mass fingerprint.

The band at about 14 kDa (B2) was shown to be only the result of different decomposition products probably due to the ionization strength. In contrary, the band at 20 kDa (B1), the strongest in terms of abundance, was identified by MALDI-TOF MS using the mascot tool on matrixscience.com. Table 4.4 shows the analysis of the major band identified after MALDI-TOF-MS experiments.

Table 4.4: Peptide mass fingerprint analysis on CoDE.

Computational analysis of the two major bands resolved on SDS-PAGE. No identification of B2 was achieved, probably due to decomposition products. B1 was identified by MALDI-TOF MS as major cow dander lipocalin-like protein. Theoretical mass and observed migration in kDa is depicted.

Band no.	UNIProtKB/Swiss-Prot entry accession number	Protein name	Theoretical mass [kDa]	Observed migration [kDa]
B1	Q28133	**ALL2_BOVIN Lipocalin *Bos d 2***	19.833	20

Protein properties as well as the amino acid sequence were then extracted from the UNIProtKB/SwissProt database. The sequence of the protein identified was similar to the major bovine allergen belonging to the lipocalin family and known as *Bos d 2*. Repeated analysis covered up to 41% of the aa sequence (red).

1 MKAVFLTLLF GLVCTAQETP AEIDPSKIPG EWRIIYAAAD NKDKIVEGGP
51 LRNYYRRIEC INDCESLSIT FYLKDQGTCL LLTEVAKRQE GYVYVLEFYG
101 TNTLEVIHVS ENMLVTYVEN YDGERITKMT EGLAKGTSFT PEELEKYQQL
151 NSERGVPNEN IENLIKTDNC PP

4.3.5 Lectin blot

To analyze the glycoprotein pattern in more detail, we transferred the resolved proteins after SDS-PAGE to a PVDF membrane and incubated with Concanavalin A (Con A) from *Canavalia ensiformis*.

Con A belongs to a family of lectins which are carbohydrate-binding proteins and are commonly detected by their ability to precipitate glycoconjugates. In particular Con A has high affinity for terminal α-mannose and α-glucose residues and is used to discriminate and analyze the glycan structures of glycoproteins. Figure 12 shows the lectin blot of the two dust sample preparations.

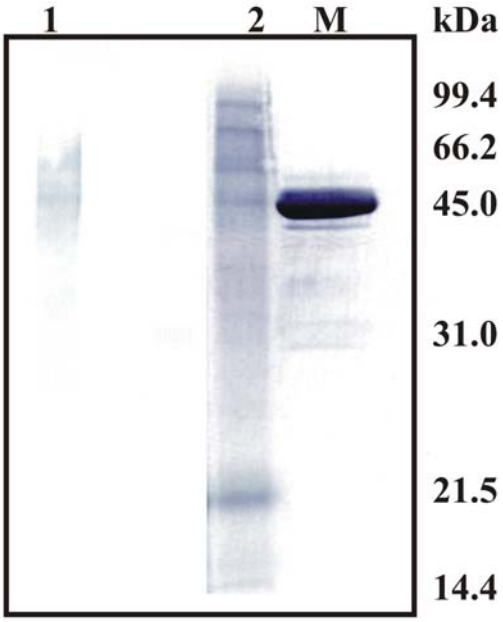

Figure 12: Lectin blot after SDS-PAGE.

Dust preparations were blotted after SDS-PAGE (15%). The ShDE (1) and CoDE (2) are shown (100 µg dust extract per lane). After resolving samples on SDS-PAGE, the gel was transferred to a PVDF membrane and stained with HRP-conjugated Con A as described in section 3.2.6.5.1. Molecular mass was determined by comparison to a standard marker (M).

A pattern of different glycoproteins conjugated with mannose or glucose residues was identified in CoDE, while ShDE did not possess a well defined glycoprotein profile. Further analysis are required to investigate the nature of those glyco-proteins and then eventually to test them as potential immune modulating compounds.

4.3.6 Periodic acid-Schiff staining

In order to detect glycosylated proteins in the dust preparations, we performed PAS staining after SDS-PAGE as described in section 3.2.6.3.3. Thus, after an initial oxidation of carbohydrates by periodic acid the Schiff reagent slightly demonstrated the presence of carbohydrates at high molecular masses in both samples. Moreover any glycosylation was associated with the band (B1) identified by MALDI TOF MS (Figure 13).

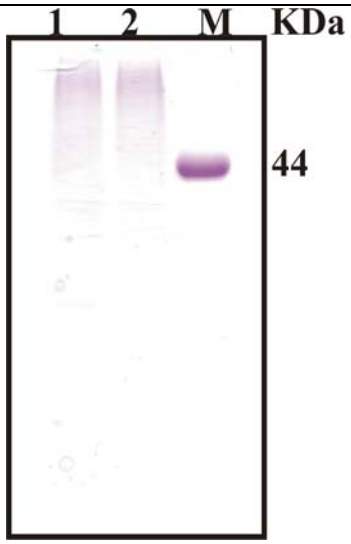

Figure 13: PAS-staining after SDS-PAGE.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (15%) of CoDE (1) and ShDE (2) (100 µg dust extract per lane) is shown. PAS staining allowed the detection of glycoproteins at high molecular mass. Molecular mass was determined by comparison to standard marker (M).

4.3.7 Protein isolation by fast protein liquid chromatography

In order to separate the complex mixture of protein in the dust preparations fast protein liquid chromatography was performed (FPLC).

Standardized procedures use pI values to design the specific eluents for the separation of proteins by FPLC in anionic conditions. In case of CoDE and ShDE none of the standardized procedure appeared to be suitable to the separation of the protein mixture inside the dust preparations. After dialysis of the dust preparations against MP-water for three days, the samples were additionally treated with polysorbate 20 (Tween-20) which was used as solubilising agent. Tween-20 is usually used as detergent; applied to CoDE and ShDE was important to solubilise them additionally in water after the extraction.

The addition of Tween-20 to increase the solubility of the dust extracts resulted in a clearer and heterogeneous compound with reduction of insoluble materials. After FPLC separation of CoDE, from the sixty fractions eluted we could not observe any apparent separation on the chromatogram, but only a smear through the column. The only three peaks observed were in the flow through region (Figure 14). The intense brown colour of the dust extract suggested that the big smear and the high adsorption (3500 mAU) could be probably due to chromophores present in the preparation which adsorb at the same wave length of the proteins, thus hiding the proteins themselves. Thus, we spotted on SDS-PAGE almost all the fractions obtained after FPLC. Surprisingly, after Coomassie staining the fractions eluted at

the beginning of the gradient showed the presence of distinct band in particular the fraction number 7 was pure enough to appear as a unique band. Both, the use of the Tween-20 and the use of water instead of Tris-HCl as dissolving agent for the sample, appeared to be essential for the separation and elution of a single protein within the 2M NaCl gradient. Figure 15 shows the SDS-PAGE after CoDE separation through FPLC.

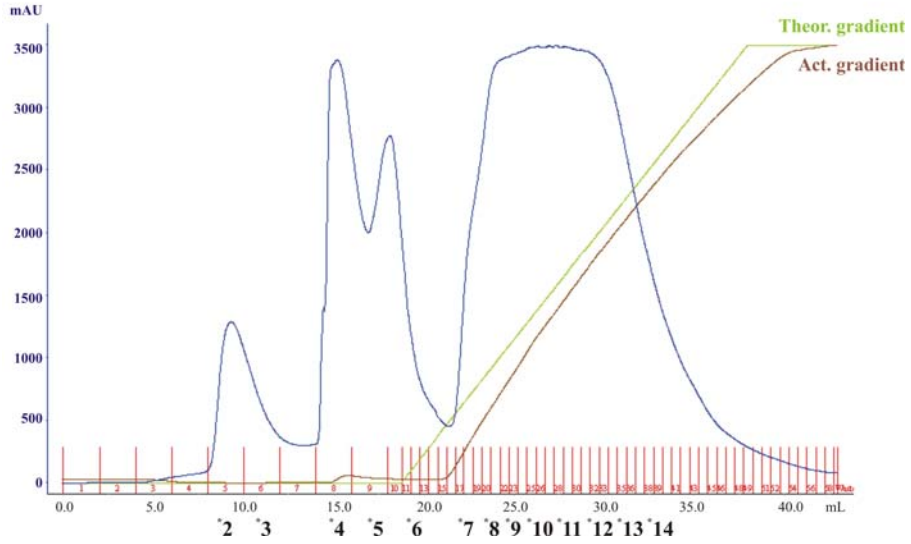

Figure 14: FPLC separation of CoDE.

FPLC experiment of CoDE sample (50 mg) in MP-water after dialysis and filtration with 0.22 µm filters. The isolated and analysed fractions were labelled with *2-14. The protein content was measured at 280 nm and the result is showed in mili arbitrary units (mAU). Theor. – Theoretical gradient; Act. – Actual gradient.

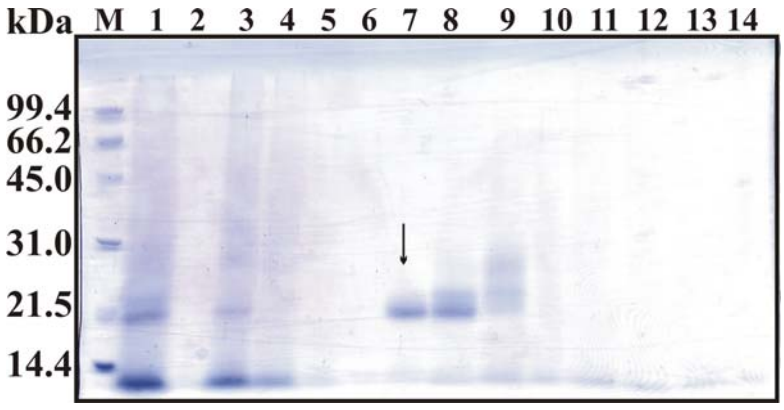

Figure 15: SDS-PAGE after FPLC separation of CoDE.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (12%) of CoDE sample after FPLC. Column 1 shows CoDE (100 µg of dust extract) before FPLC separation. Columns 2-14 show the thirteen fractions selected after FPLC (10 µl). Coomassie staining of the SDS-PAGE is depicted. Molecular mass was determined by comparison to standard marker (M).

A different situation was observed in case of ShDE (Figure 16). The chromatogram profile was similar to the one from CoDE but no protein separation was achieved with this method and the SDS-PAGE separation of the different fractions also did not show any distinct band. The "smearing" behaviour of the SDS-PAGE separation (Figure 17) was completely reflected on FPLC and then again on electrophoresis gel of the different fractions.

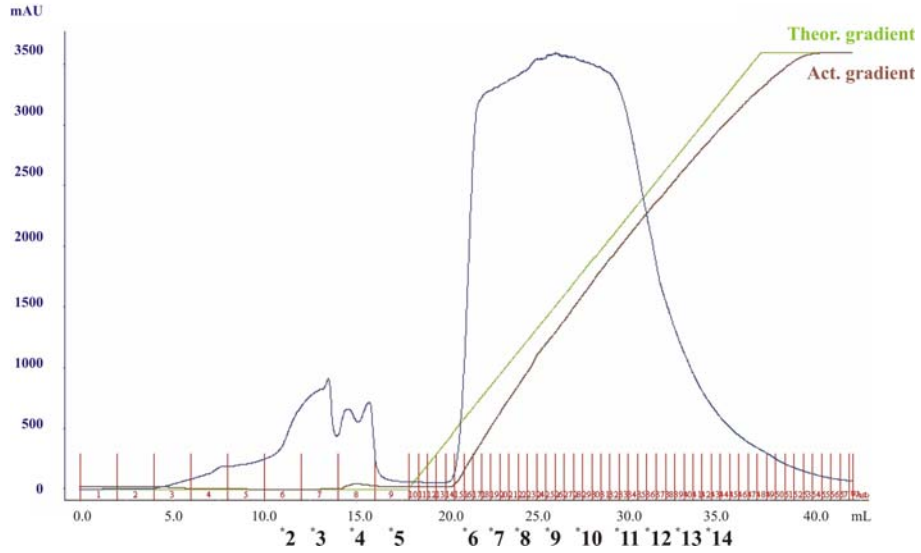

Figure 16: FPLC separation of ShDE.

After FPLC the isolated and analysed fractions were labelled with * 2-14. The protein content was measured at 280 nm and the result is showed in mili arbitrary units (mAU).

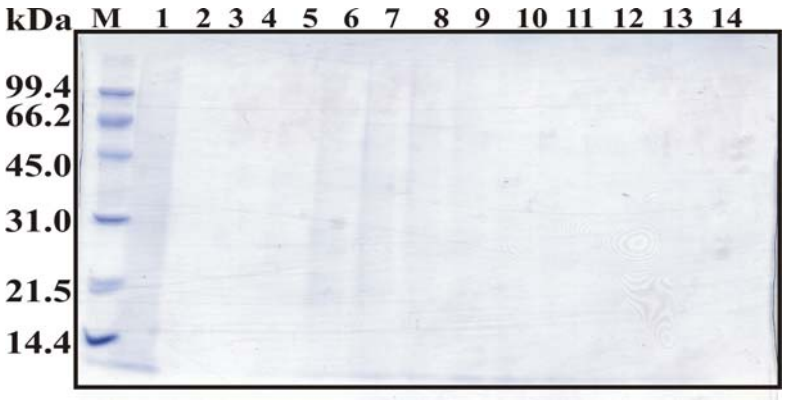

Figure 17: SDS-PAGE after FPLC separation of ShDE.

Column 1 shows ShDE (100 µg of dust extract) before FPLC separation. Columns 2-14 show different fraction after FPLC (10 µl). Coomassie staining of the SDS-PAGE (12%) is depicted. Molecular mass was determined by comparison to standard marker (M).

4.4 Immunostaining (dot blot assay)

Initial experiments allowed us to set up the optimal conditions to perform dot blot assays. One microgram of the dust preparations was used and 1 to 200 diluted blood sera from patients with and without allergic reactions was applied as mixture of primary antibodies. A 1 to 1000 diluted secondary antibody conjugated with alkaline phosphatase were used to detect the immunocomplex. In the dot blot assays using IgE as secondary antibody neither CoDE nor ShDE preparations were able to be recognized by the antibodies in the sera of the patients with and without allergy. This result suggests that the four groups of blood sera (Sheep – allergy, Sheep + allergy, Cattle – allergy and Cattle + allergy) did not reveal a positive reaction to the CoDE and ShDE preparations (Figure 18).

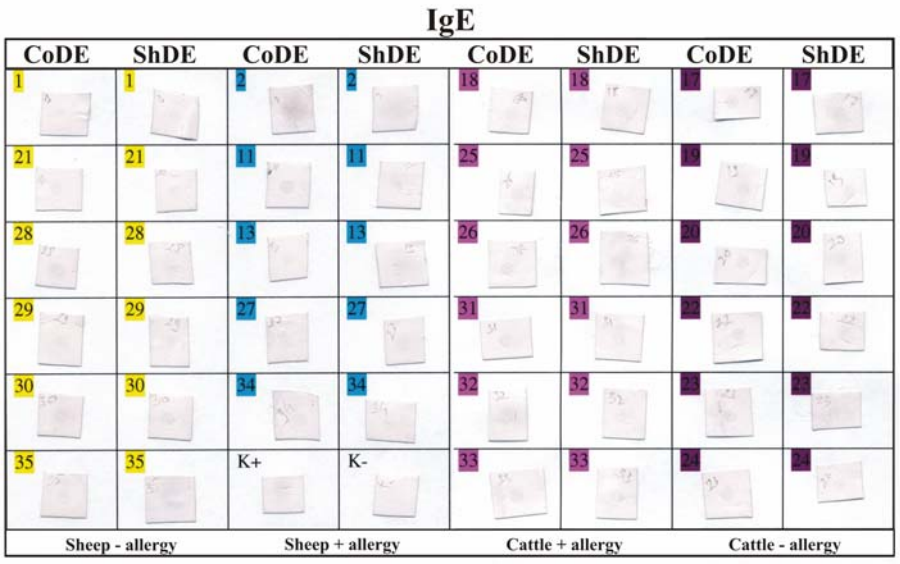

Figure 18: Dot blot assay.

CoDE and ShDE were used as antigen mixture. Dot blot assay was performed using individual sera from patients with or without allergies. All the sera samples were tested in duplicate indicated by numbering. Four different groups of sera were collected and classified according to the farm contact and to the pathological outcome (positive or negative allergic reaction). Sera were added to a dilution of 1:200, dust preparations at a concentration of 1 µg/well and the secondary antibody conjugated to alkaline phosphatase at a dilution of 1:1000. None of the dust preparations induced formation of the immunocomplex.

4.5 Dust modulation of human moDC *in vitro*

To investigate whether CoDE and ShDE preparations were able to stimulate the innate immune system for Th-polarization, the stimulatory capacity of the two dust extracts on

cytokine production by PBMC-derived DCs was analysed. Therefore different concentrations of CoDE and ShDE were used to stimulate dendritic cells after their maturation process. The dust samples were tested with and without LPS as maturation factor to study whether LPS on its own had a significant effect on the two dust preparations. As the dust preparations themselves gave a strong DCs maturation, the data shown in this work are without LPS, unless otherwise stated. Results were compared to LPS alone used as a control condition.

4.5.1 Dendritic cell surface markers

Based on the expression of surface markers and co-stimulatory molecules the maturation status of the dendritic cells after CoDE and ShDE stimuli was determined and subsequently compared to the maturation level induced by LPS which was used as control (Figure 19).

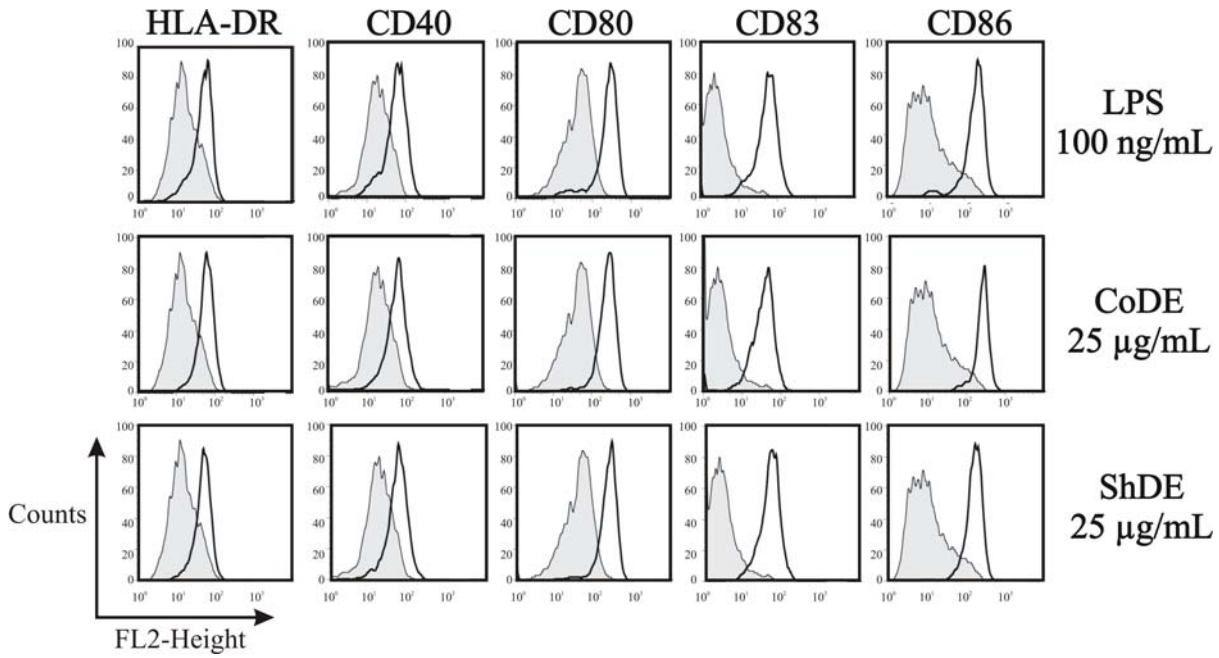

Figure 19: DC maturation by dust extracts.

Analysis of HLA-DR (MHC class II molecule), CD40, CD80, CD83 and CD86 in human DCs after treatment with indicated stimuli is shown. Grey histogram shows the isotype control and black open histogram shows the stimuli. The event counts can be seen on the ordinate; the frequency on the abscissa.

We found that in every condition expression of maturation markers showed the tendency to be higher than levels measured on immature DCs and more similar to the levels induced by LPS alone. The tendency toward a strong maturation of DCs was achieved after treatment with both dust preparations as shown from the up-regulation of the CD40, CD83,

CD80 and CD86 expression level. Moreover DCs stimulated with CoDE and ShDE preparations showed an up-regulation trend of the antigen presenting MHC class II molecule HLA-DR.

4.5.2 Pro- and anti-inflammatory cytokines production by DCs

After stimulation dendritic cells are known to produce a stimulus-dependent pattern of cytokines which is responsible for addressing alternatively the Th1 or Th2 immune responses. IL-12 is a dimeric pro-inflammatory cytokine of two subunits: p35 and p40 and the most important Th1 driving factor, whereas IL-4, IL-13 and IL-10 are known to inhibit Th1 response and to promote Th2 cell type differentiation or regulation. After stimulation with the dust extracts, the IL-10 and IL-12p70 profile was studied directly from the supernatant of the DCs at 24 h and after co-culture with CD40 ligand (CD40-L). CD40-L is a molecule expressed on T-cells that binds CD40 and activates DCs, thus promoting the production of IL-12.

4.5.2.1 IL-10 and IL-12p70 from dendritic cells

A significant up-regulation in the production of IL-10 by DCs in presence of CoDE (25 µg/mL) compared to the level of IL-10 produced after stimulation with LPS alone was observed (Figure 20 A). As previously described[167] SEA-exposed DCs always showed the tendency to produce higher amounts of IL-10 compared with those produced by LPS-exposed DCs alone, whereas IFN-γ exposed DCs showed a down-regulation trend in IL-10 production. No significant difference in the IL-10 cytokine production was observed after ShDE stimulating DCs compared to the "neutral" maturation factor LPS control. Looking at the IL-12p70 profile after stimulation of DCs it was observed that in presence of IFN-γ the cytokine expression was up-regulated compared to DCs maturated with LPS alone whereas SEA-exposed DCs produced a down-regulation trend in the production of IL-12p70 compared to the control as expected from previous studies[168]. The ShDE and CoDE preparations were associated with IL-12p70 levels comparable to LPS (Figure 20 B) and none of the dust extract treatments was able to induce a significant difference compared with LPS control. The IL-10/IL-12 ratio showed a positive trend toward a higher production of IL-10 from CoDE exposed DCs although no significant difference could be demonstrated (Figure 21).

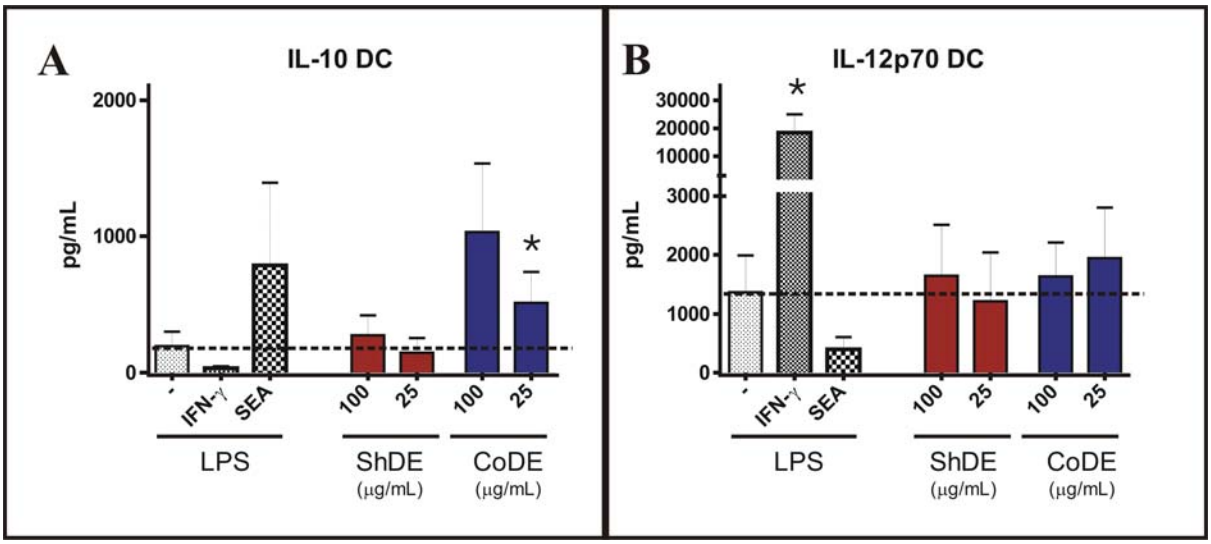

Figure 20: IL-10 and IL-12p70 cytokine production by DCs.

Human monocytes-derived DCs were stimulated as indicated. IL-10 and IL-12p70 were measured directly from the supernatant at 24 h using ELISA test as described in section 3.2.3.3. LPS, IFN-γ and SEA were used as controls. Results shown are means of three independent experiments ± SEM. Significant differences between control LPS and dust extract-pulsed DCs are indicated by *P < .05.CoDE stimulated DCs show significant up-regulation of IL-10 compared to LPS stimulated DCs (dotted lines).

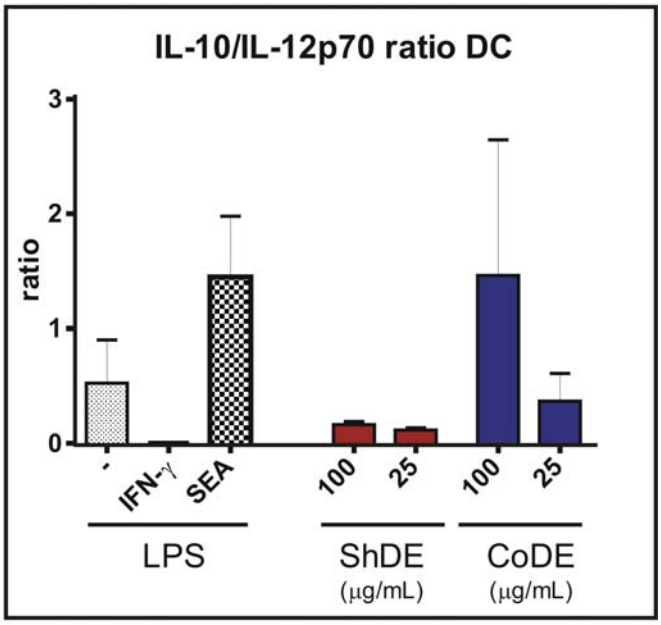

Figure 21: IL-10/IL12 ratio from DCs.

The IL-10/IL-12 ratio showed positive trend toward higher IL-10 production from DCs after CoDE stimulation. Results shown are means of three independent experiments ± SEM.

4.5.2.2 Cytokine profile from DCs after co-culture with CD40-L

Interaction of CD40 with CD40-L on dendritic cells provides a strong release of IL-12. IL-12 is a heterodimeric cytokine composed by two subunits p35 and p40 and produced by antigen presenting cells (APC) and other phagocytic cells. It is a pro-inflammatory cytokine and has a central role in the Th1 polarization by stimulating the production of IFN-γ[169-171] in a T-cell dependent pathway resulting from the interaction between CD40-L with CD40. CD40-L is indeed necessary for T-cell dependent IL-12 production by DC[172-174] thus, we used this system to study the skewing of naïve T-cells after interaction with maturated dendritic cells. Upon stimulation with CD40-L we could show that ShDE and CoDE display the tendency to condition DCs to produce low level of IL-12p70 compared with the level of IL-12p70 produced by DCs in the presence of LPS alone but no significant differences could be demonstrated (Figure 22 B).

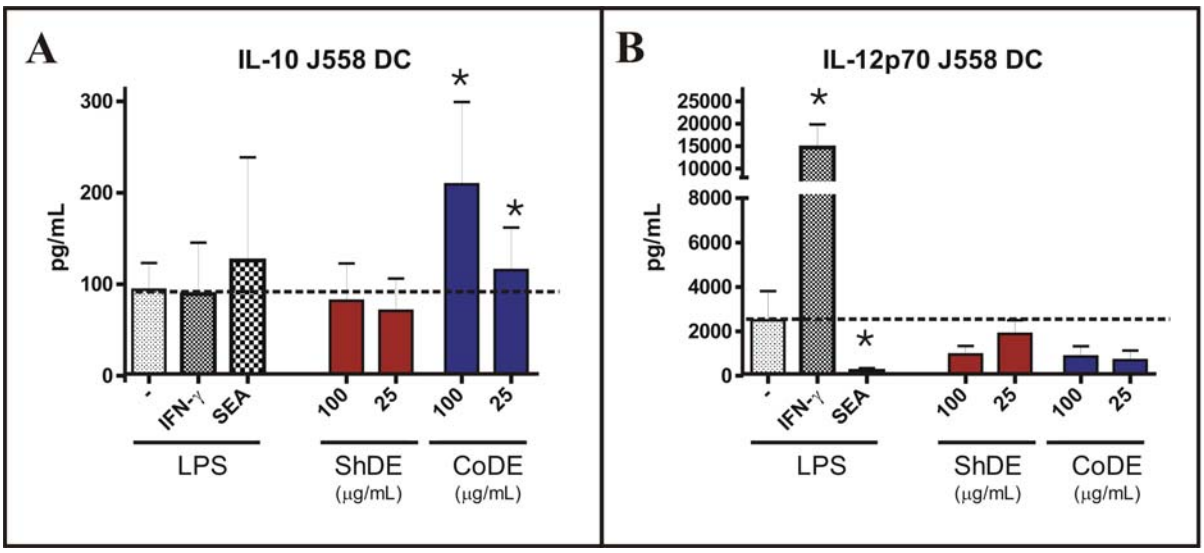

Figure 22: IL-10 and IL-12 production by DCs following activation *via* CD40 ligation.

Human monocytes-derived DCs were stimulated as indicated. IL-10 and IL-12p70 production by DCs were measured using ELISA as described in section 3.2.3.3 after co-culture with J558 mouse fibroblast cell line transfected with CD40-L. LPS, IFN-γ and SEA were used as controls. Means of three independent experiments are depicted ± SEM. Significant differences between control LPS and dust extract-pulsed DCs are indicated by *P < .05. CoDE-pulsed DCs in co-culture with CD40 ligation show significant up-regulation of IL-10 compared to LPS stimulated DCs (dotted lines).

In contrast, IL-10 production by DCs in the presence of CoDE sample was significantly increased compared with the IL-10 production after stimulation with LPS alone. This was not the case of ShDE where the IL-10 production by DCs after co-culture with

CD40-L did not significantly change compared to the LPS control (Figure 22 A). Figure 22 shows the production of IL-10 and IL-12p70 from CoDE and ShDE preparations after co-culture with CD40-L.

4.5.3 Polarization of naïve T-cells by DCs in the presence of dust preparations

To assess the T-cell polarizing capacity of DCs exposed to CoDE and ShDE, stimulated human monocytes-derived DCs were co-cultured for two weeks with allogenic naïve $CD4^+$ T-cells and IL-4 as well as IFN-γ production was determined by intracellular staining upon T-cell restimulation. DCs were stimulated with the two dust extracts in the presence of LPS, to ensure equal maturation and to rule out potential effects on polarization due to differences in maturation status of DCs. It has previously been reported that DCs matured in the presence of SEA or IFN-γ skew T-cells towards Th1 or Th2 immune response respectively[169]. When CoDE-exposed DCs were used to stimulate naïve T-cells there was a significant increase in IL-4 production and reduction of IFN-γ almost to the level of SEA as compared to control LPS. On the other side ShDE-exposed DCs showed the tendency to up-regulate the production of IL-4 but without statistical significance (Figure 23). Those results suggest that CoDE instructed DCs toward the production of IL-4, thus driving a Th2 skewed immune-response.

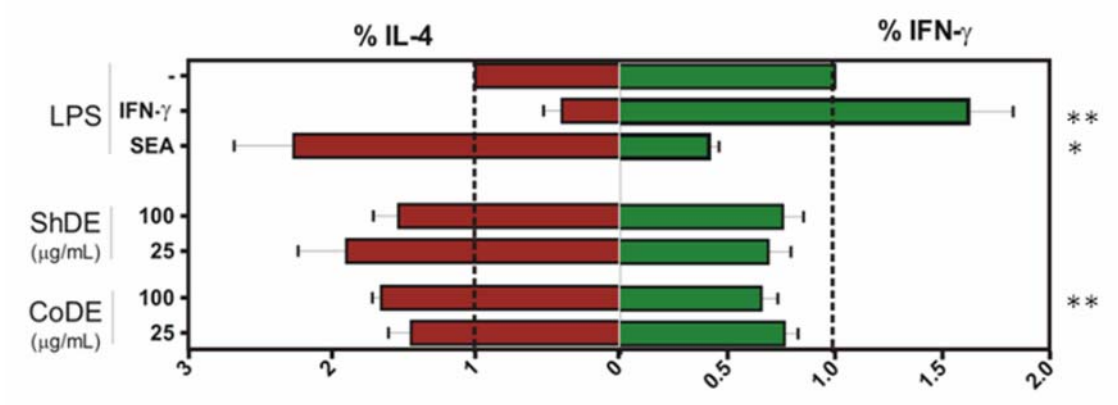

Figure 23: Polarization of naïve T-cells by dust extracts-pulses DCs.

T-cell polarization was determined by FACS analysis by measuring the percentage of cells with intracellular IFN-γ and IL-4 production. Means of three independent experiments are depicted ± SEM. Significant differences between control LPS and dust extract-pulsed DCs are indicated by *P < .05, **P < .01. DCs in co-culture with T-cells after treatment with CoDE show up-regulation in IL-4 production compared to LPS control (dotted lines).

4.6 Effect of dust extracts on acute allergic airway inflammation

4.6.1 Effect of dust preparations on airway hyperresponsivenes

The allergic reaction is characterized by inflammation with recruitment of Th2-cells, eosinophils and basophils mostly associated with elevated production of IgE. Studies of an *in vivo* asthma model are crucial to understand the multiple aspects that orchestrate the allergic response. To determine the effects of dust extracts on the development of the allergic outcome, the immunomodulatory influence of airway dust extracts exposure on allergen-induced AHR was assessed. Experiments were carried out according to the schedule outlined in Figure 24. Therefore mice were first Th2 sensitised to OVA by intraperitoneal injections. Allergen challenge was performed at three consecutive days by OVA aerosol and dust extracts were given 3 times per week by intranasal application starting 13 days before the first sensitisation.

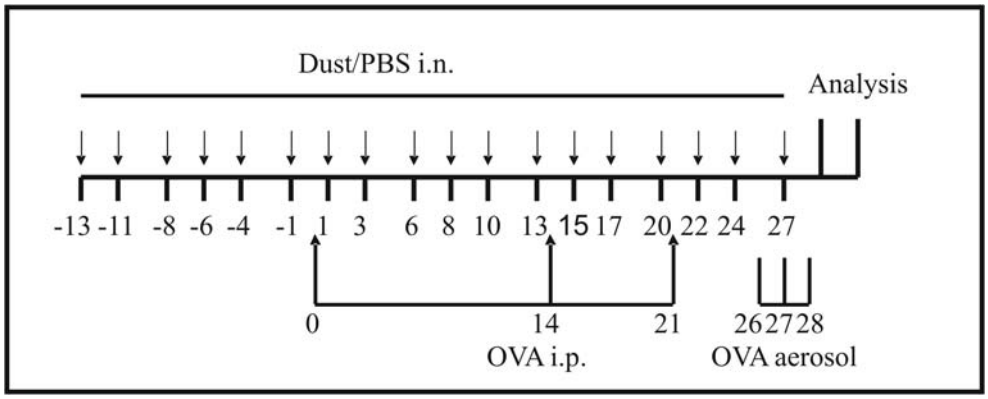

Figure 24: Schematic protocol of allergen sensitization and challenge *in vivo*.

Mice were sensitized to OVA by means of 3 intraperitoneal injections of 10 µg of OVA adsorbed to aluminium hydroxide diluted in 200 µl PBS on day 0; 14 and 21. Allergen challenge was performed on 3 consecutive days (26, 27 and 28) by application of 1% OVA aerosol for 20 min. Dust extracts (1 mg/ml) were given 3 times per week intranasal (50 µl each) starting 13 days before the first sensitization. Analysis was performed at 24 (AHR) and 48 h (all other parameters) after the last challenge.

A methacholine challenge test was performed to analyse the effect on the mice broncho-constriction after treatment with CoDE and ShDE. After sensitisation and challenge, mice were ventilated and AHR to inhaled β-methacholine (determined by alteration in resistance) measured as described in section 3.2.5.2. Assessment of lung function after CoDE and ShDE treatment showed the tendency of nearly normalized airway responsiveness to

methacholine as compared with mice of the OVA group. Responses to methacholine were expressed as the percentage change over saline control (baseline) (Figure 25).

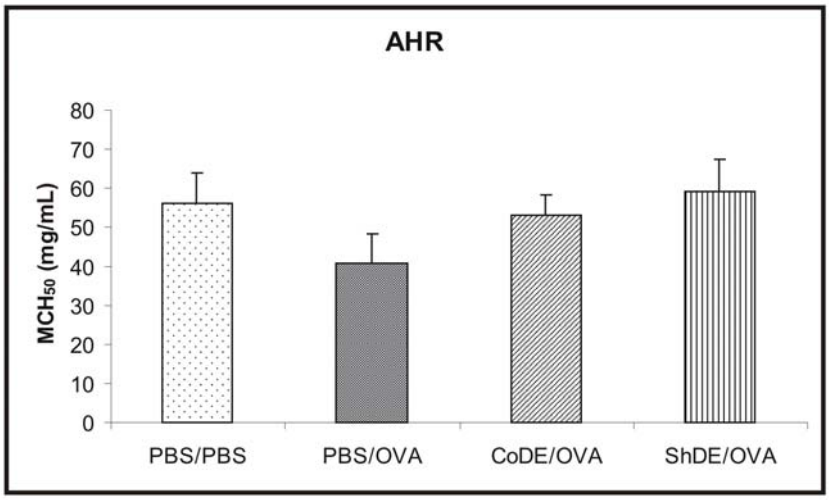

Figure 25: Analyses of lung function by head-out body plethysmography.

Methacholine concentrations that provoke 50% reduction in baseline midexpiratory airflow (MCH_{50}) are shown in comparison to the sham treated mice. None of the treatments was able to achieve statistically significant increase in airway hyperreactivity. Means ± SEM are depicted. Each group of mice consisted of n = 8.

4.6.2 Effect of dust extract on bronchoalveolar lavage cells

Allergic asthma is also characterized by inflammation of the airways with infiltration of eosinophils and other cell types. To assess the local inflammatory response in the airways, we studied the bronchoalveolar lavage fluids (BALF) in order to determine the different cellular population. Two days after the last airway OVA challenge the total number of BALF cells strongly increased, compared to the group treated only with saline solution (Figure 26). In particular mice receiving CoDE during the airway allergen challenge period had mean reduction of ~ 34 % in their BALF total cells (Figure 26). A trend in the reduction of BALF cells was also observed after treatment with ShDE, but in this case the effect was not significant.

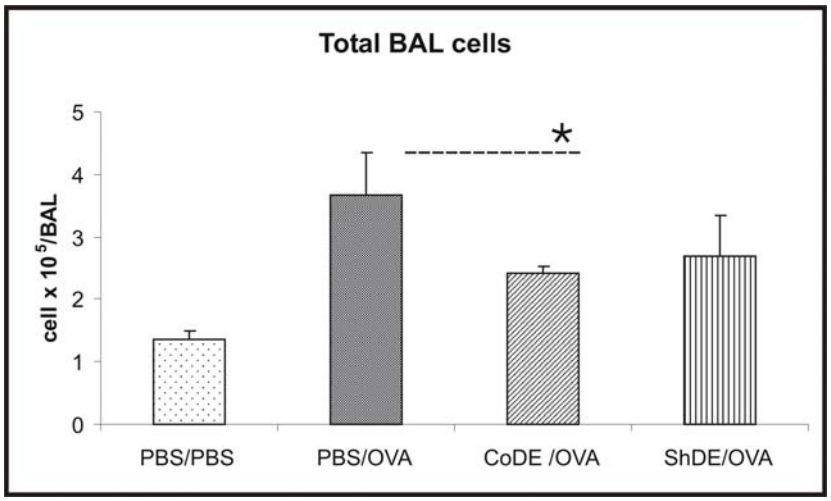

Figure 26: Effect of dust extracts on allergen airway inflammation.

Total number of BALF cells was measured after treatment with dust extracts or in sham mice treated with phosphate buffer saline (PBS) during sensitization. Means ± SEM are depicted. Significant differences between control mice and dust extract-treated mice are indicated by *P < .05. Each group of mice consisted of n = 8.

Both dust extracts were able to modify the number of BALF cells.

An average reduction of 95% and 85% in the eosinophil counts was observed after treatment with CoDE and ShDE respectively (Figure 27 A). Mice treated with CoDE showed additionally the tendency of reducing the lymphocytes count, but this was not the case of ShDE where the number of lymphocytes showed a trend to be increased. Those results showed a strong difference between the two dust samples in the inflammatory process: the repeated local treatment with CoDE and ShDE in both cases reduced the eosinophils recruitment, but contrary to CoDE which induced a weak inflammatory response, ShDE was associated to high level of lymphocytes, thus suggesting that ShDE was not able to shut down completely the inflammatory response (Figure 27 B). Moreover an average increase of 54% and 97% in BALF macrophages and neutrophils was observed respectively after treatment with CoDE (Figure 27 C). On the contrary, ShDE showed level of macrophages comparable to CoDE, but the number of neutrophils after ShDE was not different from the OVA group (Figure 27 D). Comparing the effect of the two dust extract samples we could see a significant reduction in the production of neutrophils and macrophages after ShDE exposure compared to the CoDE treated mice. Interestingly, a trend towards a reduction of lymphocytes production after CoDE exposure was demonstrated. All together those results suggest that there is a tendency from CoDE and ShDE to down-regulate the allergic response

as indicated by the reduction in the eosinophils count, but the treatment with CoDE showed in addition a tendency to reduce the lymphocytes number as well as increase in the macrophage production, thus suggesting a process of clearance of the eosinophils resulting in a non-allergic airway inflammation.

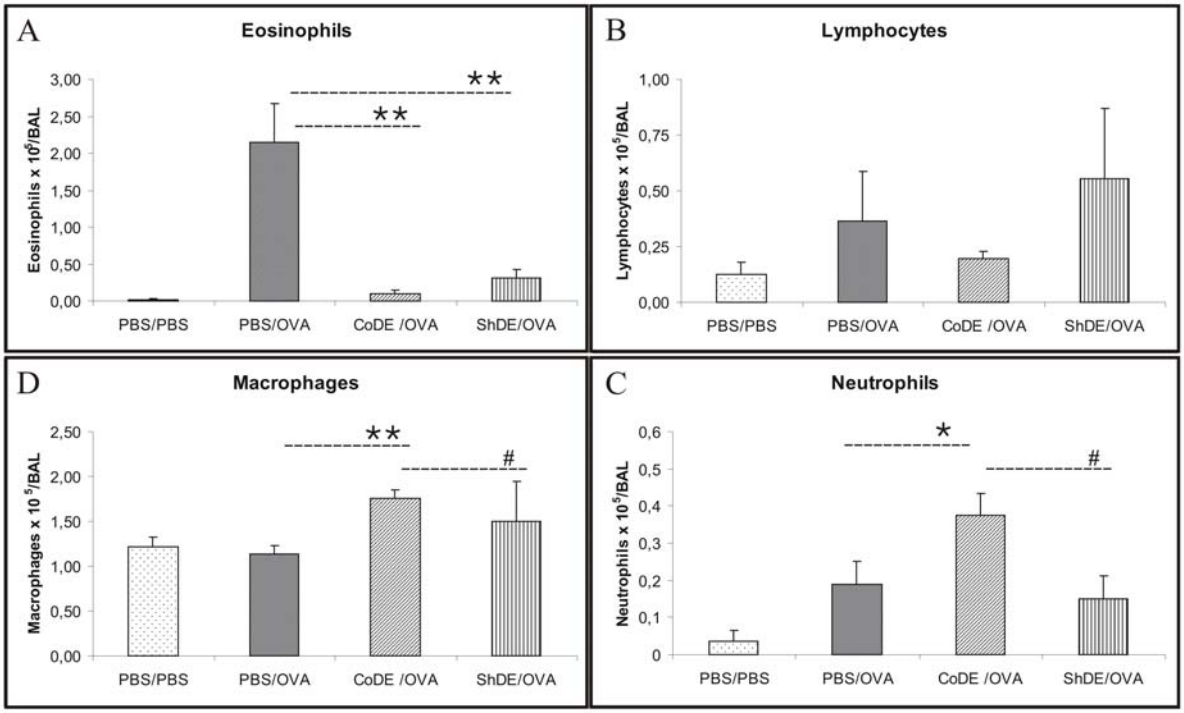

Figure 27: Effect of CoDE and ShDE on allergen induced airway inflammation.

Total number of eosinophils, lymphocytes, macrophages and neutrophils in BAL fluid after treatment of mice with dust extracts or control mice are counted and differentiate. Significant differences between sham-treated (PBS) mice and dust extracts treated mice are indicated by *P < .05; **P < .01. Means ± SEM are depicted as well as well as significant differences ($^{\#}$P < .05) between the two dust extract samples. Each group of mice consisted of n = 8.

4.6.3 Cytokines in re-stimulated lymph node supernatants

Numerous studies have shown that T-cells from the lungs of asthmatic patients express a Th2 pattern of cytokines. In order to analyse the inflammation status in the lungs we studied the cytokine pattern in the re-stimulated lymph node supernatant at 48 h after the last challenge. Because Th2 cytokines are required for the development of airway eosinophilia and IgE production, we analysed the production of those cytokines as marker for the allergic phenotype. Sensitisation and challenge with OVA increased Th1 and Th2 cytokines level. *Ex vivo* CoDE stimulation of draining lymph node cells reduced the concentration of the pro-asthmatic cytokines, IL-4, IL-5 and IL-13 by 55%, 90% and 85%

respectively. Also the production of Th1-depending IL-6 and IL-10 cytokines was significantly reduced by 61% and 88% after CoDE stimulation and the production of TNF-α showed the tendency to be down-regulated after both dust treatments. Those results suggested that CoDE can inhibit allergic airway reactions through inhibiting the recruitment of Th2 cells into the airway rather than enhancing the Th1 response. Interestingly also ShDE-stimulated lymph node induced a reduction in IL-4, IL-5 and IL-13 secretion compared to the OVA group followed by a reduction also in the Th1 type cytokines. In both cases no significant difference between the two dust extract samples was achieved and the two dust extract treatments failed to increase the levels of Th1 cytokines (Figure 28). Further studies are required to address the question whether the reduction of eosinophils observed is an IL-10-dependent process.

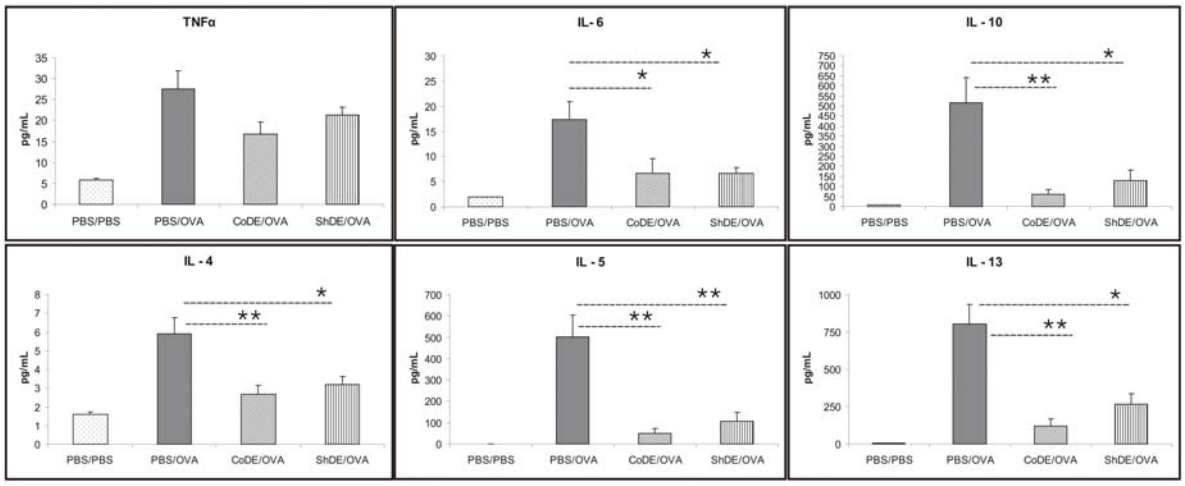

Figure 28: Effect of dust extracts inhalation on cytokines production.

In vitro production of Th1 and Th2 cytokines was measured by ELISA after OVA-restimulation of the lymph nodes. Results are depicted as mean ± SEM. Significant differences between sham-treated (PBS) mice and dust extract treated mice are indicated by *P < .05; **P < .01. Each group of mice consisted of n = 8. IL, interleukin, TNF-α, tumour necrosis factor- α.

4.6.4 OVA-specific immunoglobulines

The release of Th1 or Th2 cytokines is followed by the production of IgG_{2a} and IgE from B cells respectively. In particular IgG_{2a} is secreted from B cells following signal from IFN-γ and TNF-α- producing Th1 cells. From the other side Th2 cells express IL-4, IL-5 and IL-13 and promote the production of IgG_1 and IgE antibodies. The increased production of IgE in response to common allergen is a marker of atopic diseases. IgE binds to the mast cells through the high affinity receptors (FcεRI) thus activating them with following synthesis and

release of a variety of pro-inflammatory mediators and cytokines. To assess the immune-stimulatory capacity of dust extracts, we exposed mice airways to OVA with and without dust extracts. Serum levels of total IgG_1, IgG_{2a} and IgE were determined. After sensitization the serum levels of OVA specific IgE, IgG_1 and IgG_{2a} increased, as shown in Figure 29. The treatment with CoDE and ShDE did not modify the concentration of IgE and IgG_{2a} isotype; CoDE induced a significant increase only in the production of the IgG_1 thus indicating the induction of the Th2 response without enhancing the OVA-specific Th1 response.

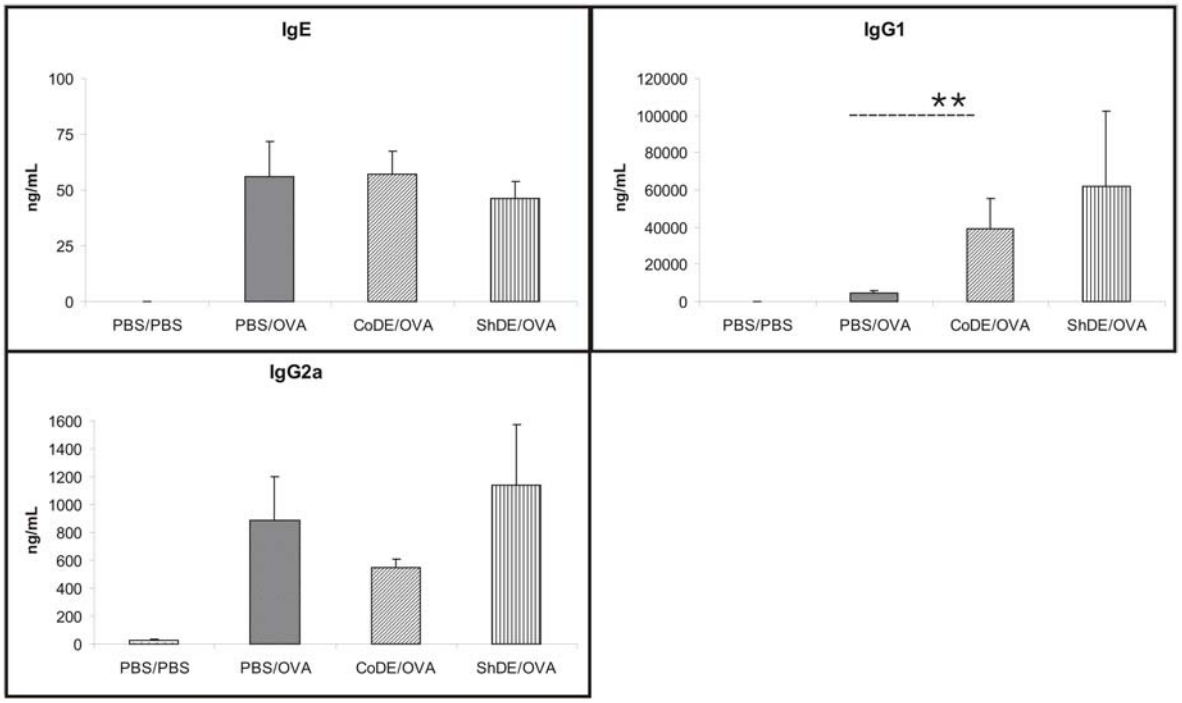

Figure 29: Effect of dust extracts inhalation on allergen-specific immunoglobulines.
Concentrations of OVA-specific IgE, IgG_1 and IgG_{2a} were measured by ELISA. Means ± SEM are depicted and significant differences between sham-treated mice (PBS) and dust extracts-treated mice are indicated by **P < .01. Each group of mice consisted of n = 8.

Despite reproducible and significant differences in cytokine production, at sacrifice, the OVA specific serum IgG_{2a} and IgE levels of mice from the experimental and the control groups did not differ significantly. The only significant effect was that observed in the increase of IgG_1 isotype concentration after treating mice with CoDE: mice receiving dust extract from cow-shed within the time of the airway allergen challenge showed an increase in anti OVA IgG_1 thus promoting the development of Th2-type humoral immune response to

OVA. The treatment with ShDE during sensitisation from the other side did not suppress the production of OVA-specific antibodies in any of the Ig isotypes analysed.

5. Discussion

5.1 Farm environment and allergic outcome

Results from epidemiological investigations have shown the beneficial effect of growing up on farm and living under condition which are not as "aseptic" as found in modern cities. In the recent years big interest has aroused in the "farm effect" because of its potential protective effect in preventing allergic disorders. It has been stressed that the variety of microbial factors found in the dust extracts collected in different farm environment plays a pivotal role in this protection. In recent years studies on the analysis of different farm environments pointed out a divergent effect of two farm exposures to the allergic outcome: being considered the cow-shed a protective environmental factor for the development of allergic disorder whereas sheep shed exposure is considered a risk factor.

Although ever increasing investigations are focused on the preventive role of allergic outcome of the dust extract in the *in vivo* and *in vitro* models, the extract itself is not yet fully characterized and to our knowledge do not exist in the literature works concerning the analytical characterization of such a dust extract. The rational of our work has its origin in numerous epidemiological studies where the authors demonstrated a clear protective effect of some farm environments such as cow stable compared to the risk factor described for sheep stable in the allergic outcomes[175]. In this frame our investigations were directed towards the biochemical analysis of the dust extracts in order to investigate differences and similarities responsible of the protective or risk factors observed by the epidemiological studies.

5.2 CoDE and ShDE possess different chemical properties

In a pilot study started in the south of Germany, stable dust from different farm environments had been collected in the rural areas of the Alps and then pooled down according to the farm origin. During the breathing process air is inhaled together with a mixture of particles that reach the airways and are recognized as own or foreign by the mucosal epithelium. In order to mimic the physiological conditions in the lungs during the breathing process, the dust samples were extracted using an aqueous solution of sodium chloride and then analysed for their chemical content and their ability to modulate the immune system in an *in vitro* and *in vivo* model.

The strong difference between the cow and the sheep environments observed at the epidemiological level was reflected also in the chemical composition of the two dust extracts. Contrary to CoDE, the analysis of the protein profile of ShDE did not show a defined protein pattern. The CoDE preparation showed the presence of a protein which was not present in the sheep-shed one and which was identified by peptide mass fingerprint as a lipocalin-like protein. Usually lipocalin proteins family count a high number of allergens mostly derived from mammalians with a variety of functions such as olfactory and gustatory, coloration and pheromones transport[176]. The protein which we identified revealed a sequence coverage of 41% with the major bovine lipocalin *Bos d 2*. The separation of the protein was achieved only after modification of the separation conditions, so that water was used instead of the buffer solution to dissolve the sample. We noticed that when the sample was dissolved in 20 mM Tris-HCL no separation was achieved within the 2 M NaCl gradient. This was not influenced by the pH as could be expected. After repeated trials the use of water as solvent was necessary to achieve a separation. Our explanation of this phenomenon which still needs experimental confirmation, could be the fact that the suggested Tris HCl concentration resulted to be too high so that was acting at the same time as eluent and solvent, hypothesing also the low protein affinity (with low molecular mass) for the stationary face of the column. In this way the use of water as solvent allowed the bound of the protein to the mobile face and its consequent elution. Further studies are needed to confirm our hypothesis. Additionally, a very strong difference was observed at the sugar and fatty acid level: the same sugar and fatty acid species were found in both dust preparations but a significant difference was observed in their relative amount.

5.3 Stimulation of innate immune system by dust extracts leads to Th2 polarization *in vitro*

DCs are known to play a pivotal role in the initiation and polarization of T-cell responses and as such are also thought to be important players in the development of allergic disorders[177-179]. To investigate the capacity of the dust extracts derived from cow- (CoDE) and sheep-shed (ShDE) to modulate immune polarization through conditioning of DCs, the extracts were tested in a well-established co-culture system of human monocytes-derived DCs and naïve $CD4^+$ T-cells, which is generally thought to mimic *in vivo* DCs mediated T helper cell polarization[180]. Stimulation of the DCs with CoDE and ShDE showed a trend to

induced increase in the expression of maturation markers, to a similar extend as induced by TLR4 ligand *E. coli* LPS, a non-polarizing maturation factor for human DCs. LPS is commonly used to ensure an equal maturation and it has been shown that only in combination with immune stimulating factors it promotes a Th1 or Th2 response, but on its own does not confer any polarizing effect[167;168;168], thus being considered a "neutral" maturation factor. From the literature is known that dendritic cells develop a mature phenotype due to microbial or cytokines stimulation with consequent reduction in the antigen uptake property[181]. Interestingly however, analysis of the cytokine production by the DCs in response to the dust extracts, revealed a different profile when compared to the LPS conditioned DCs. DCs pulsed with CoDE, but not with ShDE, were found to induce a positive trend toward the production of the anti-inflammatory cytokine IL-10 compared to the LPS control, while levels of IL-12, a classical Th1 polarizing cytokine, were not different from LPS-pulsed DCs. Our data indicate the tendency of DCs to display a more anti-inflammatory innate cytokine profile in response to CoDE rather than induction of Th1 response. Moreover a recent study on the effect of cow shed dust extract on dendritic cell biology using cow stable dust extract-pulsed bone marrow-derived dendritic cells showed that the contact with cow stable dust extract affect the allergy-inducing capacity of dendritic cells which afterwards lose their capacity to prime mice for allergic immune response[182].

For the initiation of T-cell responses by DCs antigen presentation and co stimulatory signals are crucial. In addition, the kind of cytokines produced by DCs during the interaction with T-cells is thought to determine for a large extend the direction into which the T-cells will be polarized. In this respect, secretions of high levels of IL-12 are associated with the induction of Th1 responses, while the opposite is a prerequisite for Th2 polarization. To assess the cytokine profile of DCs conditioned with the dust extracts during T-cell contact, the pulsed DCs were co-cultured with CD40-L expressing cells to mimic the interaction with T-cells. It has been previously shown *in vitro* the dominant effect of CD40-L on the Th1 differentiation, recruiting even IL-4 and IL-13 for a further enhance of IL-12 production[183;184]. Analysis of the supernatants revealed that conditioning of DCs with both dust extracts resulted in a tendency towards suppression of IL-12 secretion, although not as potent as induced by SEA, which was used as control for Th2 polarization.

IL-10 on the other hand has a pleiotropic effect on immunoregulation and inflammation. Its activity leads more to reduce T-cells activation and inhibiting the synthesis of Th1 and Th2 cells cytokines[185;186]. In contrast to IL12, IL-10 production by CoDE–pulsed DCs following CD40 ligation tended to be higher than the LPS control. This was not the case

of ShDE-conditioned DCs where the IL-10 production by DCs after co-culture with CD40-L was not different from the LPS control. Interaction of CD40-L with CD40 on dendritic cells provides a strong signal for the release of IL-12 thus we used this system to study the skewing of naïve T-cells after interaction with activated dendritic cells.

To study the T-cells polarizing capacity of the DCs following exposure to the two dust preparations, naïve T helper cells were co-cultured with conditioned DC and subsequently analysed for their cytokine profile. DCs matured in presence of SEA or IFN-γ were taken along as controls for the induction of Th2 or Th1 immune responses, respectively. When ShDE- and CoDE-exposed DCs were used to stimulate naïve T-cells there was a positive trend toward production of IL-4 as compared to control LPS, in agreement with the low IL-12 production observed after CD40 ligation. Taken together, these data show that although both dust extracts condition DCs to favour a Th2 polarized response, only DCs exposed to CoDE harbour a more anti-inflammatory phenotype as evidenced by higher secretion of IL-10. These findings together indicate that the dust extracts induce DC maturation with a similar potency as LPS, but that especially CoDE triggers a more anti-inflammatory innate cytokine profile in DCs than LPS does. The mechanism through which CoDE may drive innate IL-10 production in DCs remains to be established, but signalling *via* C-type lectins provides an interesting possibility as dust extracts are known to contain C-type binding carbohydrates while ligation of these receptors have been shown to promote IL-10 in other systems (*Geijtenbeek Nat. Immun.*).

In addition to the Th2 polarization effect after CoDE stimulus, the presence of the lipocalin-like protein could probably explain the strong difference described at the epidemiological level between the two dust preparations. It has previously been described that *Bos d2* and other proteins related to the lipocalin family are able to induce a Th2 response though weak which afterwards leads to the tolerance instead of an immune reaction[187]. As triggers of the immediate allergic immune response, the lipocalins are known to bind IgE on the carboxy terminal region of the molecule[188], but very few information are available regarding human T-cell response to lipocalin allergens so far. Many immunological data demonstrated that although a Th2-biased immune response to lipocalin is evident, it never reaches strong effects probably because peripheral tolerance is activated so that also at the molecular level the response to the exogenous allergens mimicking the self allergen is also weak[189]. Further studies are required to address the question whether the lipocalin like protein that we identified as major component of the CoDE has an effect on its own on the modulation of the immune response *in vitro*.

5.4 Th2 immune polarization does not occur *in vivo*

The allergic reaction is the result of a very complex immune response and is characterized by inflammation in the lungs with recruitment of Th2 lymphocytes, eosinophils and basophiles[190]. We further asked whether the shift toward Th2 response due to a specific allergen that we observed in the *in vitro* experiments is also able to modulate the allergic inflammatory immune response in a mouse model of experimental allergic asthma *in vivo*. The first experimental approach confirming the epidemiological studies that exposure to cow-shed of traditional farms reduces the risk of allergic disorders was carried out by Peters *et al*[156]. In this study the authors proved that allergy-protective compounds are present in stable dust extract which provoke allergy protection in mice. In line with this study, inhalation of dust extract from cow sheds (CoDE) was also able to switch off the airway inflammation as demonstrated by the reduction in the eosinophils and lymphocytes number[191;192]. Moreover this effect was accompanied by an increase of the number of macrophages and neutrophils. Those results suggested that a process of clearance of eosinophils induced by macrophages and neutrophils probably occurs after CoDE inhalation and that despite the repeated treatment with CoDE, only a weak inflammatory response was induced as shown by infiltration of quite low numbers of neutrophils. Surprisingly also ShDE reduced the afflux of eosinophils but this was not associated to a reduction in the lymphocytes number. In addition the clearance process was only associated to the macrophage increase, thus suggesting the failure of ShDE to completely neutralize the allergic inflammation. Interestingly both dust extracts that we analysed, profoundly inhibited the allergic reactions that we could show with a strong and significant reduction in the eosinophils recruitment into the airways and in addition a tendency to improve the airway hyperresponsiveness to methacholine. Those results confirmed the hypothesis of the presence of biological active compounds in the cow- and sheep- shed dust extracts able to differently modulate the allergic immune response that in case of CoDE and contrary to ShDE result in turning off the inflammatory process.

It has already been shown that improvement in the airway hyperresponsiveness is the result of IL-12 dependent process, while the reduction of the eosinophils infiltration is IL-10 dependent[193]. In our study, we have observed that the administration of CoDE and ShDE resulted in the down-regulation of the Th2 cytokines, IL-4 and IL-5, in BAL fluid, but contrary to previous reports[156], without any significant increase in the levels of Th1 cytokines after CoDE and ShDE pre-treatment. The production of IL-4, IL-5, IL-10 and IL-13 was always significantly down-regulated. A significant down-regulation of the allergic

phenotype induced by the dust extract preparations was demonstrated but this effect was not associated to an increase of the Th1 type cytokines. Increase production of IgE in response to common allergen has been previously described as one of the major marker of atopic diseases[194]. Interestingly, in our study despite reproducible and significant differences in cytokine production, the OVA specific serum IgG_{2a} and IgE levels of mice from the experimental and the control groups did not differ significantly. Despites a significant reduction in the IL-4 production was observed, the expression of IgE was not reduced and at the humoral level the only significant effect was the one that we observed after treating mice with CoDE that increased the production of IgG_1 isotype. Mice receiving dust extract from cow stable within the time of the airway allergen challenge showed an increase in anti OVA IgG_1 thus promoting the development of Th2-type humoral immune response to OVA in line with the *in vitro* results. In general, the intranasal treatment of the BALB/c mice within the entire process of sensitization and challenge, showed the tendency to reduce the allergic airway inflammation despite the fact that the systemic allergic sensitization was only partially influenced as shown by OVA-specific IgE levels.

Those observations suggested that the dust extracts probably act locally in the airways inhibiting the effect of the allergen challenge, more than on the humoral component of allergic disorders. Probably a limitation of the mouse model is also evident since the processes of sensitization and challenge occur through two different systems: the intraperitoneal injection and the aerosol challenge respectively[195;196].

Another strong protective effect of the dust samples was demonstrated by the reduction of the allergic eosinophilic influx in the BAL which was profoundly inhibited after treatment with both dust preparations. An explanation of this effect in case of the CoDE-treated mice could be indicated by the massive increase in the macrophages and neutrophils count probably associated to a process of clearance related also to the tendency of CoDE to reduce the lymphocytes count, thus inhibiting the inflammation reaction.

Two recent works focused on the isolation and characterization of bacterial species found in the cowshed dust demonstrated a decrease in the inflammatory response after treatment with isolated bacteria. Debarry *et al.* explaining the minimal influx of neutrophils and lymphocytes as induction of tolerance toward innocuous microorganisms such as *Acinetobacter lwoffi* and *Lactococcus lactis*[197]. On the other side, Vogel *et al.* observed a down-regulation in the eosinophils infiltrate with consequent increase in the neutrophils and macrophages number, thus stressing the fact that the protective effect of *Bacillus licheniformis* spores induced activation of the T_{reg} cells *via* production of IL-10 and this

would be the reason of an immune suppressive effect, rather than a missing immune deviation[155].

The observation that IL-4 and IL-5 production was inhibited after treatment with our two dust preparations validated our hypothesis of a regulatory effect of the two dust preparations on the allergic phenotype. All our *in vivo* results showed inhibition of the Th2 response, thus suggesting that CoDE and ShDE inhibit allergic airway reactions through inhibiting the recruitment of Th2 cells into the airway rather than enhancing the Th1 response but further studies are required to address the question whether the inhibition of Th2 biased inflammatory response occurs without counter augmenting the Th1 polarizing effect.

Further studies are required to provide possible explanation for this apparent discrepancy between the *in vitro* and *in vivo* studies. A possible explanation could be that different DCs subsets in lungs and derived from monocytes act with different functions. In addition an independent regulatory effect of DCs could be stressed.

As asthma and allergic diseases are disorders with a predominant Th2 immune response, diverting the immune response towards a Th1 phenotype or by suppressing the Th2 immune response by infection with intracellular pathogens may be an important tool to reduce the development of atopic allergy. All together our studies are an important tool to shed light on the mechanism of the allergic immune response. Moreover, this study provides evidence that the dust extract from cow-stable may offer a new therapeutic approach to allergic airway diseases by creating a milieu which prevents or suppresses the development of Th2 type cells. As the dust extract showed a strong heterogeneicity in the chemical composition, we believe that the protective effect of the dust preparation is the result of synergic factors probably acting together and regulated by different mechanisms.

5.5 Conclusion and perspectives

The epidemiological studies so far have clearly shown the protective effect of the dust extract with farm origin in the modulation of the immune response. At the molecular level the mechanism of such a protection is not completely understood. The hygiene hypothesis proposed by Strachan and then revisited in the frame of an immune equilibrium, provided two different mechanisms responsible for the regulation of such as balance: from one side the idea of a missing immune response and from the other side the lack of an immune suppression. At the moment the immune experimental prove of the epidemiological evidence is still poor.

The comparison of the two dust preparations that we analysed showed clearly that the different behaviour observed at the epidemiological level is related to a different chemical composition of the dust extracts. More detailed studies are needed to screen the different chemical species observed so far and to analyse those components which are able to induce the modulation of the immune system *in vitro* and *in vivo*. A more detailed study of the chemical composition of the dust extract would allow us to find out the specific antigen responsible for the immune protection observed after treating mice with the dust preparations. The high heterogeneity of the dust extracts was a limiting factor to our analysis, but more detailed studies, directed to the proteic component identified so far, would allow us to understand the specific epitop or ligand involved in the immune recognition process. Furthermore at the molecular level we could demonstrate, that the dust extract that we studied, in particular the one from the cow-stables (CoDE), posses biological active compounds able to interact with DCs and to modulate their response in relation to the polarization of naïve T-cells. A further study of the mechanism by which the dust extract is recognised, internalised and processed by DCs is the subject of next studies. In addition further studies are needed to understand the mechanism through which CoDE drives innate IL-10 in DCs thus leading to an anti-inflammatory response. Probably a signalling via C-type lectin can be hypothesed since those ligands are known to induce production of IL-10 and TGF-β. Our pioneer work of investigation the different chemical species forming the heterogeneous dust mixture allowed us to screen the major constituent of the dust extract, but new studies are needed to investigate different fractions from carbohydrate, proteins or fatty acids which could play a specific role in this modulation.

6. Summary

In the last years epidemiological studies have focused on the protective effect of farm environments in the atopic outcome, including allergic asthma. Numerous factors have been considered responsible for the rising in atopy in the industrialized countries in the last decades and it has been suggested that the contact with bacterial compounds plays an essential role in the prevention of atopic disorders.

Despite the ever increasing epidemiological studies focused on the relation between different exposures with the allergic outcome phenotype, the real mechanism leading to protection or induction of atopy is still poorly understood. In this work we have demonstrated the immune modulating effect of two dust samples collected in two different farm environments in the Alp region. Although no chemical structure could be identified so far as unique compounds responsible for the immuneregulation of the dust extracts, the chemical analysis of the two preparations clearly showed a peculiar difference concerning the chemical species present in the two preparations. We found the presence of a protein in the cow-stable dust extract (CoDE) which could be responsible of the protective phenotype seen in the *in vivo* allergic mouse model. On the *in vitro* studies we could demonstrate that the two dust preparations possess strong maturating properties as demonstrated by the up-regulation of the surface markers CD40, CD83, CD86 and CD80 on DCs comparable to the maturation level of LPS. In addition however, the study of the cytokine profile in response to CoDE and ShDE showed different profile when compared to LPS control. The CoDE-stimulated DCs produced high level of anti-inflammatory IL-10, while the level of IL-12p70 did not differ from LPS control. This was not the case of ShDE where the down-regulation in IL-12p70 production was not related to an increase in IL-10. Furthermore we have shown that the ability of CoDE and ShDE-pulsed DCs to influence naïve T-cells leads to the activation of the Th2 immune response. All together those data suggest that although both dust extracts induce a positive trend toward the activation of Th2 immune reponse, only CoDE is able to harbor a regulatory effect as result of the IL-10 production. The analysis of the two dust preparations in the allergic mouse model *in vivo* did not confirm the activation of the Th2 cell type shown in the DCs study. On the contrary the allergic phenotype was drastically down-regulated. Both dust preparations had shown the ability to down-regulate the afflux of eosinophils, and Th-2 cytokines such as IL-4, IL-5 and IL-13. At the humoral level the immunoglobulin activity was only partially influenced. No strong difference was observed

between the two dust preparations, but a clear prevention of the allergic phenotype was demonstrated. Further studies are required to isolate and study the specific molecules which act as ligands and which are recognized by the immune system as boosting factor for the allergic immune modulation.

7. Zusammenfassung

In den letzten Jahren wurde in epidemiologischen Studien die protektive Wirkung von Tierstall-Umgebungen in Bezug auf Atopie einschließlich des allergischen Asthmas gezeigt. Zahlreiche Faktoren sind für den Anstieg der Atopie in den Industrieländern in den letzten Jahrzehnten verantwortlich gemacht worden, wobei der Kontakt mit bakteriellen Bestandteilen eine wesentliche Rolle in der Verhinderung von atopischen Erkrankungen spielen soll. Trotz vieler epidemiologischer Studien, die auf die Beziehung zwischen verschiedenen Belastungen und dem entsprechenden allergischen Phänotyp eingehen, ist der wirkliche Mechanismus von Protektion bzw. Induktion der Atopie noch wenig verstanden.

In dieser Arbeit wurde der immunomodulierende Effekt von zwei Staubproben untersucht, die aus zwei verschiedenen Stall-Umgebungen der Alpenregion gewonnen wurden. Obwohl bisher keine Komponente identifiziert werden konnte, die für die Immunregulierung durch Staubextrakte verantwortlich ist, zeigen die chemischen Analyses der zwei Präparationen einen ungewöhnlichen Unterschied in Bezug auf die chemischen Spezien dieser Präparationen. Wir konnten aus dem Kuhstallstaubextrakt (CoDE) ein Protein identifizieren, das im Mausmodell verantwortlich für die protektive Wirkung sein könnte.

In *in vitro* Studien konnten wir zeigen, dass die beiden Staubpräparate starke Zellreifungseigenschaften vergleichbar mit LPS besitzen, die durch die Hochregulierung der Oberflächenmarker CD40, CD83, CD86 und CD80 auf DC demonstriert wurden. Weiterhin konnten wir zeigen, dass CoDE- und ShDE-stimulierte DCs naïve T-Zellen in der Art beeinflussen, dass die Th2-Antwort aktiviert wurde, wobei ein stärkerer Effekt in der IL-4-Ausschüttung durch CoDE als ShDE zu erkennen war. Die Analyse der beiden Staubpräparate *in vivo* im allergischen Mausmodell bestätigte die Th2-Aktivierung der DC-Studie nicht. Im Gegenteil dazu war der allergische Phänotyp drastisch nach unten reguliert, wobei beide Staubpräparate den Anstieg der Eosinophilen sowie der Th2-Zytokine IL-4, IL-5 und IL-13 reduzierten. Bei der Stimulation mit CD40-Ligand-exprimierenden Maus-Fibroblasten (J558-Zellen) konnte CoDE mit einer IL-10 Erhöhung assoziert werden, während die IL-10 Menge nach ShDE-Behandlung mit der LPS-induzierter Fibroblasten vergleichbar war. Die Untersuchung des Zytokin-Profils der mit Staub behandelten DC zeigte deutlich, dass die Stimulierung mit CoDE einen regulatorischen Effekt erzielte (erhöhte Produktion von IL-10), während die Th1-polarisierte Antwort inhibiert wurde (Herunterregulierung der IL-12p70-Produktion). Auf humoraler Ebene wurde die

Immunglobulin-Aktivität nur teilweise beeinflusst. Zwischen den beiden Staubproben wurden keine starken Unterschiede beobachtet, jedoch wurde eine klare Prävention des allergischen Phänotyps erlangt. Es sind weitere Studien sind notwendig, um spezifische Moleküle zu isolieren, die als Liganden agieren und vom Immunsystem als Faktoren zur Steigerung der allergischen Immunmodulation erkannt werden.

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List of own publications

Printed publications

P. Passacantilli, C. Centore, E. Ciliberti, G. Piancatelli and F. Leonelli. Neighboring-group Participation in Nitrile-forming Beckmann Fragmentation: Synthesis of Enantiopure (E)-2, 3-di-O-substituted-5-methoxy-pent-4-enenitriles and their conversion into pyranosylamines. *Eur. J. Org. Chem*., 2004, 5083-5091.

P. Passacantilli, C. Centore, E. Ciliberti, G. Piancatelli and F. Leonelli. A highly efficient and stereocontrolled synthesis of 2-Deoxy-1, 5- thioanhydro-L-hexitols from D-glycals in a tandem nucleophilic displacement reaction. *Eur. J. Org. Chem.*, 2006, 3097-3104

E. Ciliberti, R. Galvani, F. Gramazio, S. Haddas, F. Leonelli, P. Passacantilli and G. Piancatelli. Glycals in Organic Synthesis: A Systematic Strategy for the Preparation of Uncommon Piperidine 1, 2-Dideoxy-L-azasugars and 2-Deoxy-1,5-anhydro L-hexitols. *Eur. J. Org. Chem.* 2007, 1463–1473

Selected oral presentations

E. Ciliberti, O. Holst and E. von Mutius
Analysis of dust from cowsheds: presence of allergy protective components? Baltic Meeting on Microbial Carbohydrates. Rostock - Germany October 2006.

Selected poster presentations

E. Ciliberti, O. Holst and E. von Mutius. Farm and allergy. Marie Curie meeting and Esof open forum 2008 Barcelona July 2008

E. Ciliberti, B. Everts, R. Pires, M. Rochat, H. Garn, M. Yazdanbahkh, E. von Mutius, H Renz and O. Holst Farm environment and allergy modulation. Evaluation meeting of the Research Center Borstel - Germany April 2009

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Curriculum vitae

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Erklärung

Die vorliegende Arbeit wurde von Juli 2006 bis Juli 2009 unter der Betreuung von Herrn Prof. Dr. Otto Holst am Forschungszentrum Borstel in der Laborgruppe Strukturbiochemie angefertigt.

Ich versichere, dass ich die vorliegende Dissertation ohne fremde Hilfe angefertigt und keine anderen als die angegebenen Hilfsmittel verwendet habe. Weder vor noch gleichzeitig habe ich andernorts einen Zulassungsantrag gestellt oder diese Dissertation vorgelegt. Ich habe mich bisher noch keinem Promotionsverfahren unterzogen.

Lübeck, den 17.06.2009