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Microbial spores from farming environments and their role in allergy protection

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The following list contains all abbreviations used in this thesis except SI-units, SI-prefixes as well as one and three letter codes for common α-amino acids.

%C	Percentage of cross-linking bisacrylamide in total acrylamide (w/w)
%T	Percentage of acrylamide in solution (w/v)
°C	Degree Celsius
2-ME	β-Mercapthoethanol
a-	Anteiso-
aa	Amino acid
AHR	Airway hyperreactivity
ALEX	Allergy and Endotoxin
ALI	Air-liquid interface
AMP	Antimicrobial peptide
APC	Antigen presenting cell
APS	Ammonium persulfate
ATCC	American type culture collection
ATZ	Anilinothiazolinone
BAL(F)	Bronchoalveolar lavage (fluid)
BCG	*Mycobacterium bovis* BCG
Blspo	*Bacillus licheniformis* 467 spores
BSA	Bovine serum albumin
CCA	α-Cyano-4-hydroxy cinnamic acid
CD	Cluster of differentiation
cfu	Colony forming unit
DAP	Diaminopimelic acid
DMEM	Dulbecco's minimal essential medium
DPA	Dipicolinic acid
DSM	Difco Sporulation Medium
DSMZ	*Deutsche Sammlung für Mikroorganismen und Zellkulturen*
DTT	Dithiothreitol
e.g.	*Exempli gratia*; for example
EDTA	Ethylendiaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
et al.	*Et alii*; and others
FACS	Fluorescence activated cell sorting
FID	Flame ionization detector
Fig.	Figure
FITC	Fluorescein-5-isothiocyanate
FPLC	Fast protein liquid chromatography
Glc	Glucopyranose, glucose
GC-MS	Gas chromatography and coupled mass spectrometry
GlcNAc	*N*-Acetyl-glucosamine
GM-CSF	Granulocyte macrophage – colony stimulating factor
Gram+/-	Gram-positive /Gram-negative
GroCys	*S*-(2,3-dihydroxypropyl)cysteine

h	Hour
HBSS	Hanks buffered salt solution
HE	Hematoxilin eosine
HEK	Human embryonic kidney
HEPES	*N*-[2-Hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid]
hiBlspo	heat-inactivated spores of *Bacillus licheniformis* 467
HIC	hydrophobic interaction chromatography
HPLC	high-performance liquid chromatography
i-	Iso-
i.p.	Intraperitonial
ieDAP	Isoglutamyldiaminopimelic acid
IEF	Isoelectric focussing
IFN-γ	Interferon-gamma
Ig	Immunoglobuline
IL-	Interleukin
kDa	Kilo Dalton
LB^+	Supplemented Luria-Bertani medium
LMW	Low molecular mass standard
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LSM	Lymphocyte separation medium
LTA	Lipoteichoic acid
M	Molar; $mol \cdot L^{-1}$
MALDI-TOF	Matrix-assisted laser-desorption/ionization-time of flight
MALP-2	Macrophage activating lipoprotein 2 kDa
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
min	Minute
MM	Minimal medium
MNG	Multinucleated giant cells
moDC	Monocyte derived dendritic cell
MOI	Mutiplicity of infection
MS	Mass spectrometry
MurNAc	*N*-Acetyl-muramic acid
NADH	Nicotinamide adenine dinucleotide (reduced form)
NCBI	National center for biotechnology information
NEPHGE	Non-equilibrium-pH-gradient-electrophoresis
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
Pam_3CSK_4	*N*-Palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-[*R*]-cysteinyl-[*S*]-seryl-[*S*]-lysyl-[*S*]-lysyl-[*S*]-lysyl-[*S*]-lysine
PAMP	Pathogen associated molecular pattern

PAS	Periodic acid-Schiff
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PFA	Para-formaldehyde
PG	Peptidoglycan
PITC	Phenylisothiocyanate
PMF	Peptide mass fingerprint
PMSF	Phenylmethanesulfonylfluoride
PRR	Pattern-recognition receptor
PTH	Phenyl thiohydantoin
PVDF	Polyvinylidene difluoride
RE	Raw extract
RPC	Reversed-phase chromatography
sasp	Small acid soluble proteins
SDS	Soduim dodecyl sulfate
SNP	Single nucleotide polymorphism
SpMPF	Membrane protein fraction isolated from *Bacillus licheniformis* 467 spores
spp.	Subspecies
sv.	Serovar
TBS	Tris buffered saline
TBST	TBS + Tween 20
Tc	T-cell
TE	Total extract
TEM	Transmission electron microscopy
TEMED	*N*,*N*,*N*',*N*' tetraethylenediamine
T_H	T-Helper-cells
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNC	Tris-NaCl, complete protease inhibitor
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
vegBl	Vegetative cells of *Bacillus licheniformis* 467
vegMPF	Membrane protein fraction isolated from vegetative *Bacillus licheniformis* 467
w	Weight
WHO	World health organisation

1. Introduction

1.1 Allergy, asthma and atopy

Allergic diseases are defined as excessive immunological reactions to harmless environmental antigens, so called allergens. Clinical symptoms of allergy vary concerning the different ways of allergen uptake and include for example asthma, hay fever (allergic rhinitis), conjunctivitis, dermatitis and food allergies [(1)]. Asthma, the most common chronic disease among children, and a widespread disease among adults, affects about 300 million patients worldwide (WHO). Physiologically, asthma is characterized by airway obstruction, airflow limitations and airway hyperreactivity (AHR). These symptoms are caused by inflammatory airway disorders [(2)]. Asthma can be distinguished between allergic and non-allergic forms. Allergic asthma is characterized by elevated levels of aeroallergen-specific class E immunoglobulin (IgE). Generated by the initial contact with aeroallergens in the sensitization phase, repeated contact to the specific allergen (elicitation phase) induces an excessive release of IgE mediated immediate type allergic responses [(3)]. Cross-linking of allergen-specific IgE antibodies on mast cells induces the release of these immune mediators (e.g. histamine and leukotrienes). The most prevalent aeroallergens able to induce these reactions are derived from house dust mites (Der p 1), plant pollen (Phl p 1) or cat dander (Fel d 1) [(4)]. In allergic asthma these allergens evoke symptoms like chest tightness, wheezing, shortness of breath or bronchial hyperreactivity [(5)].

The presence of allergic and asthmatic individuals has been observed to cluster in families. In the last decades asthma genetics detected several susceptibility genes crucial for pathways involved in asthma pathogenesis and defective tolerance induction to allergens. The propensity to induce exaggerated immune responses associated with these genetic variations [(6)] is called atopy. In epidemiological studies this tendency is tested by skin prick tests to common allergens or by determination of serum IgE levels. Atopy was determined to be present in 30% of the population in developed countries [(7)]. Contrary to this genetic component is the incidence of asthma symptoms in 10-12% of this population [(7)] and the rapid change of epidemiology, doubling the prevalence of allergic disorders in the last 10-15 years. These facts indicate a strong environmental influence [(8;9)].

1.2 Environmental factors and allergic diseases – the hygiene hypothesis

An association between environmental conditions and the prevalence for atopic diseases was initially formulated by Strachan [10] in 1989. In this study the hygiene hypothesis was constructed on the basis of an inverse association between family size and the presence of older siblings on one side and the prevalence of hay fever on the other side. Supporting this hypothesis numerous epidemiological studies have ascertained protective effects as well as risk factors for asthma and allergy.

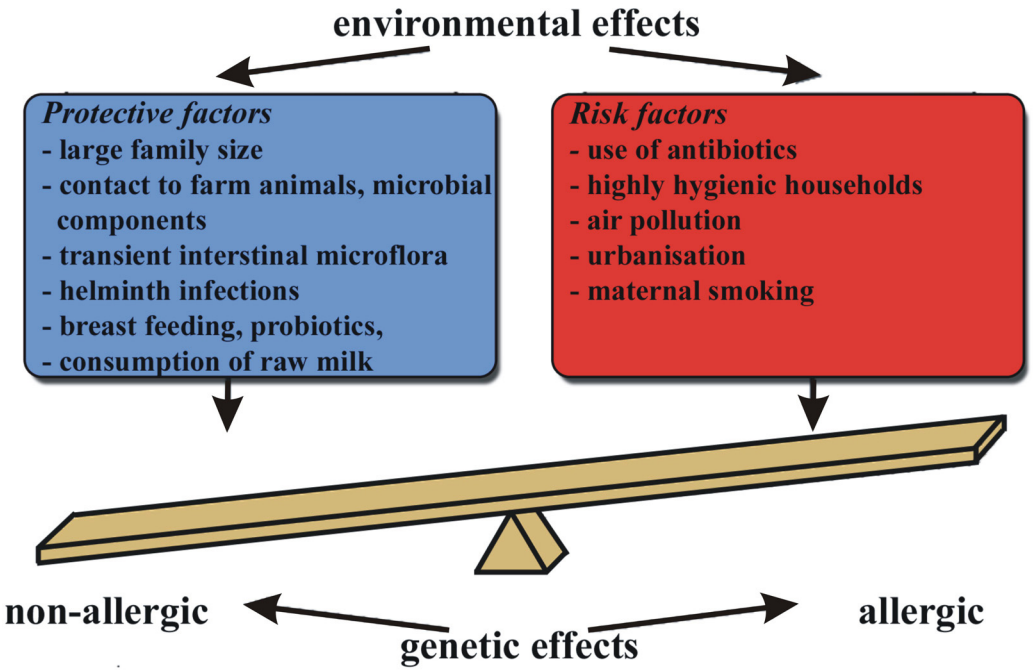

Figure 1.1: Environmental influences on the development of asthma. Besides genetic factors influencing the development and prevention of allergy environmental conditions are important. Numerous epidemiological studies have associated protective factors as well as risk factors for the development of these diseases. A selection of the respective factors is depicted [11].

In recent studies Strachan's initial idea of a protective effect of infections in early childhood on the development of atopic diseases was challenged. Especially respiratory syncytical virus (RSV) and influenza virus infections indicated an effect of certain pathogens on the development rather than the prevention of asthma [12]. In contrast to these results a decrease of allergen-specific IgE has been shown for helminth infections [13]. An influence of changes in intestinal colonization resulted in the same way, suggesting the gut microflora as an important stimulus for the developing immune system in an allergy

protective manner [11]. Furthermore, reductions of infections by the use of antibiotics as well as their influence on the gut flora are associated with an increase of atopy in investigated subjects [14]. However, these factors were mainly identified in studies comparing developing and industrialized countries. Since such studies have to consider many confounding variables the comparison of regional differences in the same countries is of high interest [15]. In this context changes of the gut microflora by e.g. consumption of probiotica and raw farm milk as well as breast feeding decrease allergic symptoms [16-18], whereas maternal smoking [19] and air pollution were elucidated [20] as risk factors.

The multitude of epidemiological studies have revealed a strong inverse correlation between atopy, asthma and growing up on a traditional farm in rural regions of Germany, Switzerland and Austria [21]. Characteristics of these farms are maternal farm work during pregnancy and an early contact of children with the animal shed environment, farm animals and products such as raw milk. Thus, a high exposure to microbial compounds early in life has been linked to the prevention of childhood asthma on these farms [22]. Endotoxin, muramic acid, and bacterial DNA in farm dust were investigated in this context [23;24]. In addition, fungal agents detected in farm children's mattress dust were associated with a protection from atopic wheeze [25]. The proposed beneficial effect of pet contact on the prevention of allergic symptoms was shown to be outbalanced by contact to farm animals [26;27], especially cattle [24;28]. This finding was confirmed by significant decreases in atopic prevalence among farm children in comparison to non-farm children of the same region/village [26;29].

Correlations of these environmental factors with epigenetic variations in the expression of innate immune factors emphasize their importance for the development of asthma and allergy [30]. The mentioned studies described the protective effects as well as the risk factors as particularly important during the development of the immune system *in utero* and in the first years of life. This time is specified as the window of opportunity [31].

1.3 Immunological background of allergy

The molecular basis for pathological excessive reactions to harmless antigens is a defective induction of tolerance to these allergens and an impaired immune reaction of T_{Helper} (T_H)-cells [3].

Immune responses in general are mediated by leukocytes responsible for innate and acquired recognition and clearance of pathogens. Innate immunity is responsible for the early, unspecific immune response and the recognition of invading pathogens by sensing of

pathogen associated molecular pattern (PAMP) [32]. In the lungs pathogens and allergens are taken up by alveolar macrophages, or they are phagocytozed by lung dendritic cells (DCs) of myeloid or plasmacytoid origin [33]. Myeloid DCs play a major role in antigen presentation [34]. The uptake of allergens can be promoted by three different mechanisms. First, allergens can be accompanied by agonists of Toll-like receptors (TLR) implying the immune system to be non-self [35]. As part of the family of pattern recognition receptors (PRR) TLRs recognize PAMPs as unique microbial structures for which reason allergens are phagocytozed like pathogens. Second, enzymatic function of several allergens enables them to cleave protease-activated receptors, inducing proinflammatory mechanisms on epithelial cells [35]. Third, protease activity allows the cleavage of tight junctions, enabling the allergen to pass the epithelial barrier gaining direct access to DCs of the peripheral tissue [4]. Representative antigen fragments are then presented by antigen presenting cells (APC), such as DCs. Peptide fragments are bound to major histocompatibility complex class II (MHC class II) molecules and presented to naïve $CD4^+$ T-cells (CD = cluster of differentiation) in the lymph nodes. Depending on the maturation process of DCs co-receptors are expressed and specific cytokine profiles are provided to polarize naïve T-cells to antigen specific $CD4^+$ T_H-cells [36].

1.3.1 The T_H1/T_H2 paradigm

Mosman et al. initially described the polarization of naïve T-cells to different T_H-cell subsets depending on the signals provided by antigen presenting DCs [37]. In healthy individuals, antigens of intracellular bacteria lead a polarization of T_H-cells to type 1 (T_H1) cells in the presence of Interleukin (IL)-12p70 expressed by DCs. In contrast, helminth antigens are presented by DCs in a T_H2 differentiating cytokine milieu (e.g. IL-4) resulting in the activation of eosinophils, basophiles and mast cells. Fungi and extracellular bacteria are presented under coexpression of IL-23 activating neutrophil effector cells in a T_H17-dependent way [38]. The T_H1- and T_H2-cell subtypes are able to counter-regulate each other by the expression of specific cytokines. Interferon (IFN)-γ a T_H1-cytokine is suppressing the development of T_H2-cells and IL-4 has the opposite effect on T_H1-cells [39].

In fetal and neonatal innate immunity this immune balance is impaired by suppression of T_H1 polarizing cytokines that prevent immune reactions between mother and fetus. Infection induced T_H1 cytokines during this time lead to a pre-term delivery of the fetus [40]. Prenatally and in the first two years of life the immune system matures to a balanced T_H1/T_H2 immunity in healthy individuals by repeated exposure to microbial

components [41]. Missing environmental exposure to microbes can explain the T_H2 biased reaction to harmless allergens in allergic individuals with regard to the hygiene hypothesis [42].

These predisposed, atopic individuals react on initial exposure to allergens by the polarization of naïve T-cells to T_H2-cells, caused by a T_H2 cytokine milieu. This process is known as allergic sensitization. Repeated exposure to the same allergen in the elicitation phase causes increased survival rates and recruitment of effector cells like eosinophils and mast cells. The release of proinflammatory mediators by these cells directs wheezing, sneezing, goblet cell hyperplasia and mucus hypersecretion as well as the induction of bronchial hyperreactivity [4]. The latter leads to contraction of smooth muscle cells and decreased lung function in asthma patients. In addition to the specific effector cells induced by different T_H-cell subsets, their cytokines mediate B-cell activation and antibody class switching. In the case of the T_H2-specific cytokines IL-4 and IL-13, the production of IgE antibodies is induced in response to allergen contact [43]. Release of IgE antibodies by B-cells increases the cross-linking of these antibodies on mast cells multiplying the histamine and leukotriene secretion and therewith the asthmatic symptoms [4;5] (Fig. 1.2).

In contrast, non-atopic individuals fail to induce T-cell proliferation in response to allergens *in vitro* and express a protective T_H1 immune response [44]. This includes the expression of sometimes high amounts of IgA, IgG4 and IgG1 antibodies in the absence of allergen-specific IgE [45]. The frequency of allergen-specific T_H2-cells is decreased and the number of regulatory T-cells (T_{regs}), the major subtype of antigen-specific T-cell suppression, is increased [46]. In this way allergic immune responses are down-regulated.

1.3.2 Antigen tolerance

The mechanism of antigen tolerance is induced by contact to harmless environmental antigens. The higher the exposure to this antigen the stronger is the induction of tolerance in healthy individuals [47]. The underlying mechanisms are based on inefficient T-cell activation leading to clonal depletion or anergy of the allergen-specific T-cell.

An additional mechanism for tolerance induction is the suppression of T-cell maturation by T_{regs} in the presence of IL-10 producing DCs [9;48;49]. Deficiencies in T_{reg} activity are correlated to atopic diseases in humans providing an inappropriate down-regulation of effector T_H2-cells [50]. Evidence for an important role of T_{regs} is more and more discussed and new mechanisms, e.g. the direct T-cell activation by the innate immune receptor TLR2 seem to be important for the prevention of allergic diseases [51].

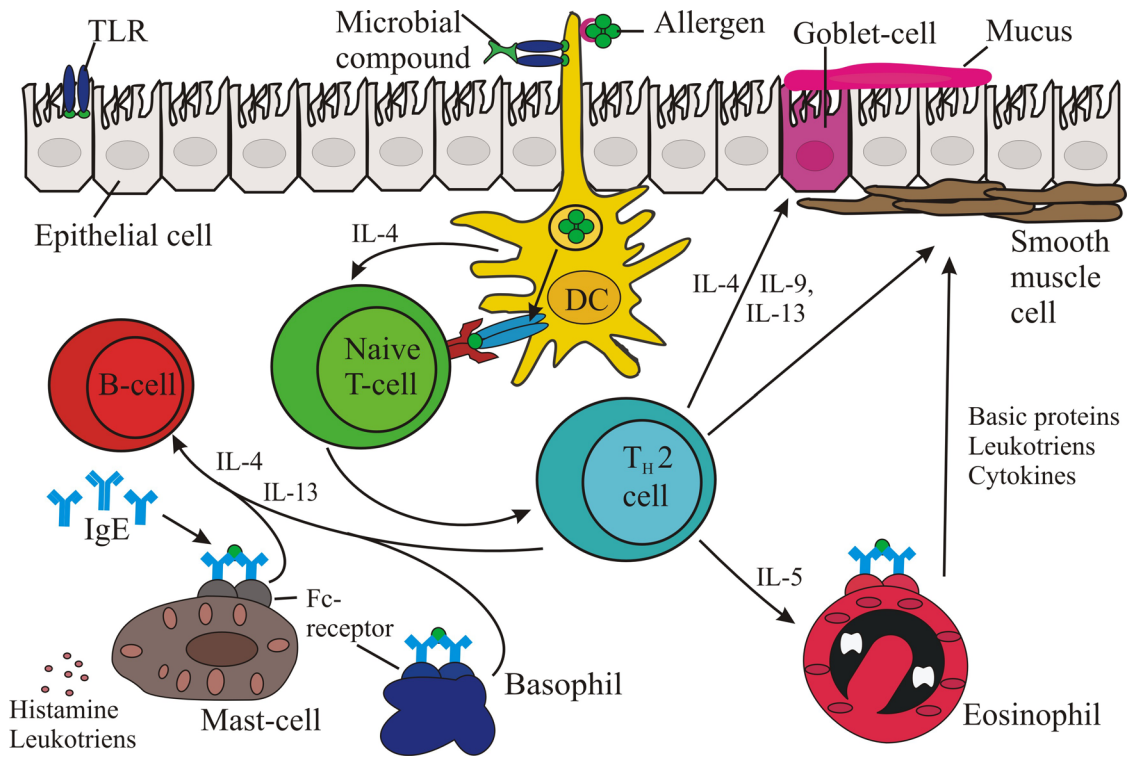

Figure 1.2: Mechanism of allergic reaction. Allergen contact in an atopic person leads to T_H2 polarization of naïve T-cells followed by the activation of effector cells (e.g. eosinophils and B-cells) and the release of immune mediators (e.g. histamine, leukotrienes and cytokines) provoking allergic responses. Among others they promote smooth muscle cell contraction (bronchial hyperreactivity), vascular permeability, and mucus hypersecretion. Increased survival of eosinophils by IL-5 expression leads to late allergic symptoms like tissue damage [4-6].

1.4 T-cell polarization by the innate immune system

As mentioned above the detection of microbial compounds plays a key role in the polarization of T-cells and the initiation of immune reactions in response to infectious agents and allergens [52]. Receptors of the innate immune system recognize highly conserved structures present exclusively on microbes. This system enables the host to recognize a broad range of pathogens and to initiate innate immune responses. In epithelial cells the recognition of pathogens by these receptors leads for example to the expression of anti-microbial molecules like defensins or lysozyme [53]. Among the first cells to come in contact with invading pathogens or inhaled allergens, DCs are important cells to provide T-cell polarization. Expressing a broad range of PRR, DCs sense microbes and undergo maturation processes necessary for T-cell activation. Therefore, they are key players in the decision of allergy induction [54].

The activation of T-cells and the polarization of T_H-cells by DCs depends on three different signals [55]:

1. the presentation of antigen-specific peptides to T-cell receptors via MHC class II molecules
2. the expression of co-stimulatory molecules (e.g. CD 40, CD80, CD83 or CD86) and
3. a soluble or membrane bound polarization factor (e.g. IL12p70 (T_H1) or IL-4 (T_H2))

The decision on one of these signals depends on the type of PRR providing a signal of non-self antigen recognition [55]. Inadequate signal expression provokes allergen tolerance in healthy individuals, whereas T_H2 biased atopic individuals can induce allergic reactions [46].

Experimentally the importance of DCs in the development and the prevention of allergy and asthma is supported by dramatic increases in the number of myeloid DCs (moDCs) in airways of mice with induced asthma [6;56]. Furthermore, allergen-sensitized DCs are able to induce allergic T_H2-cell responses in untreated mice after DC transfer [57].

1.4.1 Pattern recognition receptors

Pattern recognition receptors detect structures unique to microbes allowing the immune system to distinguish between self and non-self antigens [58]. The examples of TLR2 and NOD-like receptors (NLR) associated with allergy and asthma in epidemiological studies are described in the following paragraphs [59;60].

1.4.1.1 Toll-like receptors

Toll-like receptors are members of the PRR family in mammals. Identified due to its homology to the Toll protein in *Drosophila* this evolutionary ancient superfamily of innate immune receptors includes 10 TLRs of different specificities in humans [61]. TLRs are type I transmembrane receptors. The extracellular amino-terminus consists of varying numbers of leucine-rich-repeat (LRR) domains. These LRRs are involved in ligand binding and receptor dimerization [62]. The intracellular *C*-terminus contains a highly conserved Toll/interleukin-1 receptor (TIR) homology domain interacting with downstream signaling molecules such as myeloid differentiation factor 88 (MyD88). Interaction with these

proteins results in nuclear factor (NF)-κB and mitogen activated protein kinase (MAPK) activation as well as cytokine release (e.g. tumor necrosis factor (TNF)-α) [63].

1.4.1.1.1 Toll-like receptor 2 and the recognition of lipoproteins

Toll-like receptor 2 is one of the best characterized members of the TLR family. However, its ligand specificity is still under discussion. The list of proposed ligands recognized by TLR2 includes lipopolysaccharides (LPS) lipoproteins, lipopeptides, lipoarabinomannans and lipoteichoic acids (LTA) [64]. Their common characteristic is a lipid moiety, but they vary in structural details. The broad specificity of TLR2 for these molecules has been explained by heterodimerization with the lipid scavenger receptor molecule CD36 [65]. In addition to these agonists, several studies describe peptidoglycan as a TLR2 agonist. However, highly purified peptidoglycan as well as synthetic muropeptides have shown not to induce TLR2 activity [66;67]. Structural analyses as well as comparison of immunological activation have identified lipoproteins and lipopeptides as TLR2 ligands. The are the only compounds recognized without co-receptor with a sensitivity comparable to binding of LPS to TLR4 [68]. The molecule responsible for this TLR2 activation is the 2 kDa macrophage-activating lipopeptide from *Mycoplasma fermentans* (MALP-2) [69]. Structural differences in lipoproteins, based on their grade of acylation are distinguished by hetero-dimerization with TLR1, or TLR6, respectively [70-72]. The molecular mechanisms of this interaction were enlightened by crystallization of the TLR2-TLR1 heterodimer binding the synthetic triacylated lipopeptide Pam_3CSK_4 [73].

1.4.1.1.2 Bacterial lipoproteins

Lipoproteins are abundant peripheral membrane proteins in bacteria. By covalent binding of a lipid anchor to a conserved *N*-terminal cysteine, even highly hydrophilic proteins can be membrane coupled. The anchoring structure of lipoproteins consists of a conserved *N*-acyl-*S*-diacyl-glyceryl cysteine moiety, first detected as murein-bound lipoprotein in *Escherichia coli* by Braun et al. [74]. Lipoproteins are not only involved in cell wall synthesis, nutrient uptake, degradation, signaling and sporulation of Gram-positive (Gram+) bacteria [75;76], but also function as adhesion and invasion proteins.

The biosynthesis of bacterial lipoproteins is initiated by prelipoprotein expression. Characteristics of prelipoproteins are a positively charged region of 5 to 7 amino acids (aa), following the *N*-terminal methionine [77]. The adjacent hydrophobic region of 7 – 22 uncharged and hydrophobic residues is then followed by the lipobox, carrying the lipobox

consensus sequence [LVI][ASTVI][GAS] C. This sequence occurs within the first 40 aa of the prelipoprotein. The steps of lipoprotein biosynthesis are depicted in Figure 1.3. In a first step diacylglyceryl is transferred from phosphatidylglycerol to the sulfhydryl group of the invariant cysteine by the phosphatidylglycerol-prolipoprotein diacylglyceryl transferase (Lgt). After translocation through the cytoplasmic membrane the lipid anchor is recognized by the lipoprotein signal peptidase (Lsp) II. Cleavage at the *N*-terminus leaves the invariant cysteine to be the first aa of the lipoprotein. In *E. coli* and other bacteria, an *N*-terminal acylation is catalyzed by the *N*-acyl-transferase (Lnt) producing triacylated lipoproteins. In Gram+ bacteria such as *Staphylococcus aureus* and *Bacillus licheniformis* this enzyme is missing [78].

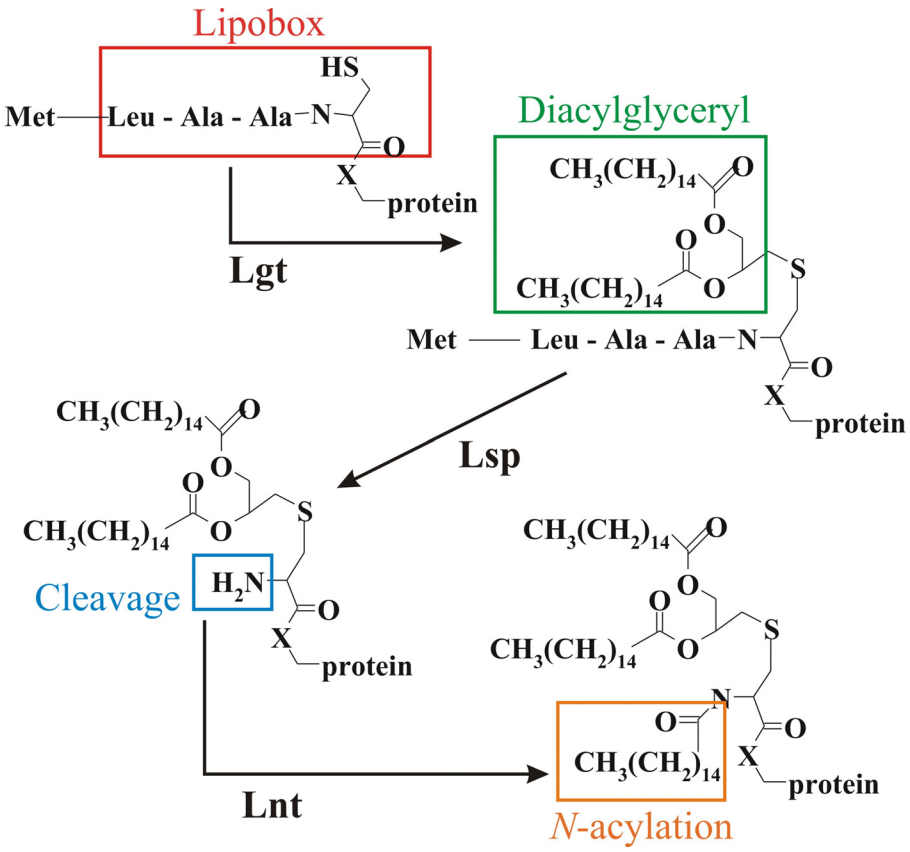

Figure 1.3: Biosynthesis of bacterial lipoproteins. Prelipoproteins are recognized and modified by the phosphatidylglycerol-prolipoprotein diacylglyceryl transferase (Lgt). This enzyme transfers the diacylglyceryl group to the sulfhydryl group of the invariant cysteine in the lipobox. The specific signal peptidase II (Lsp) discerns the diacylglyceryl modification and cleaves the cysteine *N*-terminus. In some bacteria *N*-acylation is catalyzed by *N*-acyl-transferase (Lnt) [78] resulting in triacylated lipoproteins.

The recognition of lipoproteins by their signal sequence is used to predict lipoproteins in genome and protein database analyses. Therefore, the criteria are applied to prediction algorithms to establish a database of bacterial lipoproteins (DOLOP) [77]. Alternative prediction tools are provided by the LipPred algorithm by Taylor et al. [79] and the LipoP algorithm by Juncker et al. [80], respectively. The prosite tool (ExPASy.org) includes an algorithm by Sutcliffe et al. [81] improving lipoprotein prediction for Gram+ bacteria.

1.4.1.1.3 Recognition of fungal cell wall components

The fungal componentsβ-(1→3)-glucan, a component of the fungal cell wall, and zymosan from yeast, have been shown to interact with TLR2 via the Dectin-1 receptor molecule [82]. Dectin-1 is expressed by monocytes, macrophages and neutrophils. Although phagocytosis of detected glycans is mediated by dectin-1 TLR2/6 hetero-dimer formation has been described to be crucial for ongoing signaling [83]. Other studies indicate a role of TLR4 in the recognition of fungal cell wall components [84].

1.4.1.2 Toll-like receptor 2 in allergy and asthma

The paradigm of a general T_H1-promoting signal by triggering IL-12 release in DCs upon general TLR stimulation has been challenged in the last years based on several observations [55]. The example of TLR2 and its role in allergy and asthma clearly shows the broad range of cytokine releases induced by TLR2 ligands depending on the experimental setting. Weigt et al. describe that TLR2 agonists in cooperation with IFN-γ are able to shift an established allergic T_H2 skew to a non-allergic immune response [85]. These results are confirmed by studies using synthetic lipopeptides [86]. In a mouse model the administration of TLR2 and TLR4 agonists during the allergen sensitization phase resulted in decreases of allergic inflammatory responses such as eosinophilia and increased IgE levels [87].

In contrast, the induction of a T_H2 immune response on the co-immunization of Pam_3CSK_4 and the allergen Ovalbumin (OVA) has been observed in experimental asthma [88]. Recently similar findings were presented for TLR4 and its ligand LPS. Ovalbumin sensitization with high amounts of LPS resulted in an increase of T_H1 responses, whereas low doses provoked allergic sensitization [89]. Low doses appear to be essential to induce inflammatory reactions like AHR. These examples emphasize the divergent role of TLRs in asthma and allergy, which can be explained by the involvement of T_{regs}. Toll-like receptor 2 deficient mice are characterized by a lower level of T_{regs} suggesting a direct influence of TLR2 on T_{regs} [90]. Data obtained in mouse models also correlate TLR2

signaling to the expansion of T_{reg} populations, which is important for the induction of tolerance to allergens and suppression of experimental allergic disorders [91;92].

1.4.2 Intracellular NOD receptors

Recently the intracellular nucleotide-binding oligomerization domain molecules (NOD) were identified to detect bacterial peptidoglycan (PG) [93]. Gram+ and Gram-negative (Gram-) PG is distinguished by different members [94;95] of the NOD-like receptor (NLR) family. Besides the NOD domain they contain an *N*-terminal effector domain like the caspase recruitment domain (CARD). *C*-Terminal leucine-rich-repeats (LRR) allow the recognition of PAMPs and let suggest a similarity to TLRs [96].

1.4.2.1 The recognition of peptidoglycan by NOD1 and NOD2

The most prominent members of the NLR family are the PG muropeptide receptors NOD1 and NOD2. Peptidoglycan consists of repeating units of ***N*-**acetyl-glucosamine (GlcNAc) and *N*-acetyl-muramic acid (MurNAc), representing its sugar backbone. Different layers of this backbone are cross-linked by peptide bridges containing specific motifs that allow a discrimination of Gram+ and Gram- bacteria. Their PG differ in thickness, degree of cross-linking and in the third aa of the stem peptide. In Gram+ bacteria this aa is mainly lysine, whereas Gram- bacteria contain diaminopimelic acid (DAP). An important exception to this rule is *Bacillus subtilis*. The minimal motif for the detection by NOD1 is a terminal D-Glu-*meso*-DAP. In comparison, NOD2 is a general PG sensor detecting the muramyl dipeptide MurNAc-L-Ala-D-Glu (Fig. 1.4) [82]. Naturally occurring muropeptides are obtained by cleavage of the β-(1→4)-glycosidic bond between GlcNAc and MurNAc by *N*-acetyl-β-D-muramidases, e.g. lysozymes [97].

1.4.2.2 Peptidoglycan recognition and signaling

Phagocytosis of bacteria and digestion of their peptidoglycan by phagosomes lead to the release of muropeptides and their detection by NOD receptors in the cytosol. The mechanisms of muropeptide transport across membranes is still unclear [96]. Differences in expression of NOD1 and NOD2 were described. Whereas NOD1 is expressed ubiquitously in adult tissues, NOD2 is expressed exclusively in monocytes [98]. By activation of the transcription factor NF-κB NOD signaling induces the expression of pro-inflammatory cytokines, chemokines and the recruitment of neutrophils [99]. Defects in NOD2 recognition can lead to decreased expression of antimicrobial peptides (AMP) and a

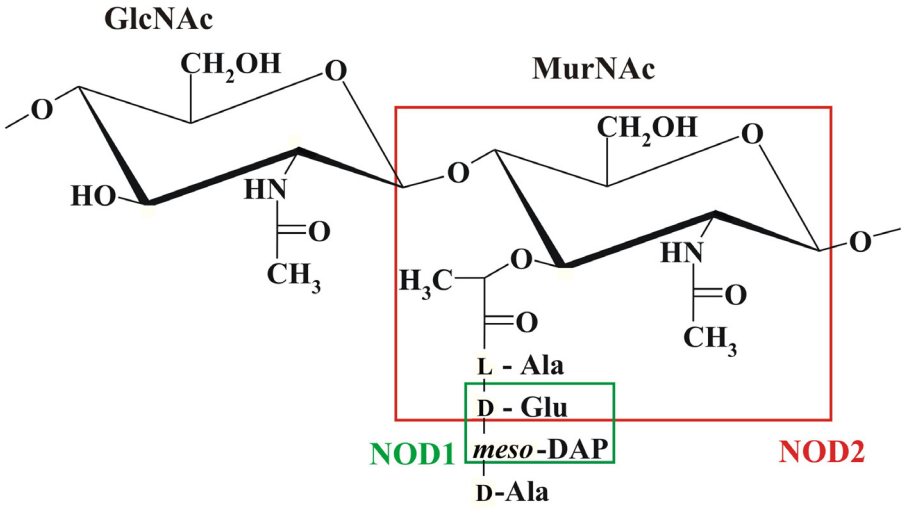

Figure 1.4: Minimal peptidoglycan motifs recognized by the intracellular receptors NOD1 and NOD2. The NOD1 motif contains *meso*-diaminopimelic acid (DAP), mostly present in Gram- organisms. The smallest motif recognized is γ-D-Glu-*meso*-DAP (green box). It is often called ieDAP (isoglutamyldiaminopimilelic acid). In Gram+ bacteria *meso*-DAP is exchanged for L-Lys. The general PG motif muramyl dipeptide (MDP) (red box) is detected by NOD2 [82].

reduced innate immune response by specialized epithelial cells [100]. Furthermore, mutations in NOD2 show a clear correlation to Crohn's disease, where an impaired recognition of muramyl dipeptide (MDP) affects the activation of NF-κB and the expression of pro-inflammatory cytokines [101]. Detailed investigations on the impact of NOD receptors on the immune system remain to be clarified. The only evidence for an involvement in allergic reactions is adapted from the epidemiological observation connecting genetic variations of NOD1 and their connection to allergy protection [59]. This association has to be investigated in detail on a molecular level, to emphasize its molecular effect on the interaction of receptor and ligand.

1.5 Molecular basis of allergy protection

Two mechanisms that explain the increase of allergy on the molecular level have been recently discussed based on the associations of environmental and genetic factors achieved by epidemiological studies (Chapter 1.2). The concept of a missing immune deviation, on the level of T_H1 and T_H2 immunity by a lack of contact to microbial compounds, and the concept of missing immune suppression in case of allergen contact are described [91].

1.5.1 Protection by helminth infections

A paradoxon to the above mentioned protective effect of T_H1 polarization seems to be present in the mechanism of allergy protection by helminth infection. Usually helminth infections as well as allergic reactions are associated with T_H2 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. Chronic helminth infections are characterized by cycles of infection and inflammation followed by anti-inflammatory immune restriction establishing a regulatory network. The immune suppression possibly also involved in the control of allergy is accompanied by high levels of IL-10 [13]. The regulation of this excessive immune reaction is mediated by T_{regs} which are induced by infection related IL-10 release by DCs [102]. Able to inhibit the immune responses of T_H1 and T_H2 polarization, they are suggested to play a key role in the control of immune disorders [103]. This mechanism is supported by the observation of a coeval increase of T_H1-biased diseases (e.g. inflammatory bowel disease) and T_H2-biased allergies. Thus, the idea of missing immune deviation by a lack of microbial contact seems not to be the only cause for increased incidences in asthma and allergy as a consequence of hygienic lifestyles [91].

1.5.2 Molecular evidence of protective farming environment

Epidemiological studies showed a clear relation between the observed protective effect of growing up on a farm and the expression of innate immune receptors like TLRs and their co-receptors in farm children. The Allergen and Endotoxin (ALEX)-study group revealed an increased expression of CD14, TLR2 and TLR4 in peripheral blood leukocytes of farm children [104]. This result was confirmed by investigations of prenatal farm exposure and its relation to the expression of those innate immune receptor molecules. Their expression levels were found to correlate with the number of farm animal species, contacted by the pregnant mother [105].

Moreover, single nucleotide polymorphisms (SNP) in PRR genes were explored to have a modulating effect on the allergy protection after contact to microbes. For example genetic variations in NOD1 recognizing bacterial PG reveal a strong protective effect on allergies by farm contact [59] and variations in TLR2 genes are clearly associated to asthma protection in farm children [60]. Higher expression levels or genetic variations increasing the reactivity of TLR2 and NOD1 can lead to allergy and asthma protection. In case this contact occurred prenatal or in early stages of life they skew the fetal T_H2 bias to a

balanced T_H1/T_H2 immunity [17;105]. These findings emphasize an important association between innate immune receptors and the protection described for early life contact to farms [30], whereas later in life increased levels of the T_H1 cytokine IFN-γ can exacerbate the pathogenic effects in asthma patients [49;105].

The protective effect of farm contact was experimentally proved in a mouse model of allergic asthma. Stable dust extracts used to treat mice intranasally during the whole sensitization process with OVA, inhibited the development of typical phenotypic characteristics of asthma and allergy such as AHR, eosinophilia, and antigen-specific IgE [106].

1.6 Microbial flora of protective stable dusts, a pilot study

As mentioned before recent epidemiological studies show a protective effect on the development of hay fever and allergic asthma, when contact to traditional farms occurred during the first years of life. Bacterial as well as fungal components found in shed and mattress dusts were associated with this effect [22;25]. Based on these studies, experimental evidence has been presented for the protective effect of cowshed dust [106] and isolated cowshed bacteria [107].

To further investigate the microbial variety of animal shed dust a pilot study, including 24 traditional farms in Bavaria, was performed. From these farms dust samples of children's mattresses and animal sheds were collected [108;109]. Aerobic and anaerobic growth of bacterial and fungal species on agar plates identified a great diversity of microbial species, detected by cooperation partners.

For the bacterial species *Bacillus* spp. and the group of coliform bacteria were the most abundant showing the highest colony forming unit (cfu) numbers. Their abundance was specific and could be found in all animal sheds and children's mattresses of the analyzed farms. This led to the question whether transport of animal shed bacteria to children's mattresses occurred indicating a relevant contact to these microbes.

To address this question partial correlation of bacteria in these animal sheds and children's mattresses were calculated. The Pearson's partial correlation coefficients for colony numbers of all microorganisms are depicted in Table 1.1. Moreover the number of children with exposure to the respective microorganisms and their mattresses are denoted. Correlation coefficients for the transport of microorganisms from stable to mattresses were maximal for the genera *Clostridium* and *Enterococcus*. In contrast, the presence of these genera in less than one third of all investigated children's mattresses was low. Contact to

these microorganisms was not as relevant as it was for *Bacillus* spp. The negative correlation for coliform bacteria excluded their transport from animal sheds to children's mattresses. Therefore, they were not suggested to be specific for the protective animal shed effect.

Table 1.1: Correlation of colony numbers in mattress and animal shed dust. Dust was cultivated on different agar plates under aerobic and anaerobic conditions. The listed microorganisms were identified. The presence of these genera correlated with respect to their presence in children's mattress dusts and animal shed dusts.

Microorganism	*N*[§]	*Partial correlation coefficient**	*p-value*
Clostridium spp.	16	0.55	0.0340
Enterococcus spp.	20	0.54	0.0181
Bacillus spp.	61	0.37	0.0039
Cladosporium spp.	16	0.34	0.2212
Wallemia spp.	30	0.31	0.0990
Anaerobic bacteria	57	0.23	0.0841
Mucor spp.	48	0.21	0.1547
Aerobic bacteria	58	0.19	0.1574
Penicillium spp.	14	0.14	0.6389
Lactobacillus spp.	16	0.06	0.8271
Aspergillus spp.	55	-0.02	0.8666
Coliform bacteria	61	-0.17	0.2063

[§] Number of children with exposure to the respective microorganisms in animal sheds.

*Partial correlations are adjusted to family clusters. The log-transformed colony forming units of mattress dust microorganisms were regressed to the family ID and the resulting residues were plotted against the log-transformed colony numbers in animal shed dust into a 95% confidence interval.

(Courtesy of Melanie Korthals and Dr. Markus Ege.)

Detailed analysis of *Bacillus* spp. in all collected dust samples identified *Bacillus licheniformis* to be the most prominent organism, present in 95% of all investigated samples. In descending order *Bacillus pumilus* (31%), *Bacillus subtilis* (28%) and *Bacillus cereus* followed.

Fungal microorganisms showed higher variations in the different dust samples tested. The highest cfu numbers were found for *Aspergillus* and *Mucor* spp. concurrent with small variations. The correlation between mattress and stable dust samples excluded an important role of *Aspergillus* spp. spores with respect to the overall contact of children to these microorganisms. Though the correlations of *Cladosporium* spp. and *Wallemia* spp. were higher (Table 1.1), their presence in mattress dusts was limited to less than one third of the analyzed samples. Based on these results *B. licheniformis* and *Mucor* spp. were isolated from farm dust. The fact that both microorganisms are able to form spores emphasizes the importance of spore forming microorganisms in the protective stable dust.

1.7 Bacterial spores

The presence of high amounts of *Bacillus* spp. in the investigated animal shed and mattress dusts can be explained by their ability to form spores and by their association to farming products such as hay [(110)].

Bacterial spores are dormant forms of bacteria formed as an adoption to environmental stress (e.g. starvation) [(111)]. The process of sporulation enables the bacteria to survive long periods of nutrient limitation. Anaerobic bacteria of the genus *Clostridium* and several aerobic bacteria as e.g. *Bacillus* ssp. are able to initiate the asymmetric cell division initiated by starvation and high cell densities [(112)]. Figure 1.5 demonstrates the process of bacterial sporulation, forming a metabolically dormant spore in a sporulation time of approximately 8 h.

Surrounded by several protective layers the cytoplasm and the DNA are conserved in the spore core. The cytoplasm is dehydrated by accumulation of the spore-specific dipicolinic acid (DPA). Divalent cations form a chelate with DPA reducing the osmotic pressure of the cytosol. This results in an efflux of water, necessary for the heat resistance of the spore and leaves cytoplasm and DNA in a crystalline state [(113;114)]. In addition, the DNA is complexed by small, acid soluble proteins (sasp) [(115)]. The influx of small toxic molecules is warranted by the inner spore membrane. Two different layers of PG are synthesized in between the pre-spore surrounding bilayer membrane. The germ cell wall encases the cytoplasmic core. Peptidoglycan analysis revealed no differences in comparison to the cell

wall of the vegetative bacterium after germination [116]. On top of this a thick layer of spore-specific PG, the cortex, is formed. It contains 50% of the muramic acid in its converted form, as δ-lactam. Every second MurNAc in the repeating structure contains the δ-lactam. The rest is linked to other PG strands by tri- or tetrapeptides [116]. This low degree of cross-linking is essential for the spore dynamics in case of rehydration [117]. It allows an immediate response on the presence of germinants. Furthermore, it functions as a recognition element for the cortex lytic enzymes differentiating the cortex from the germ cell wall [118].

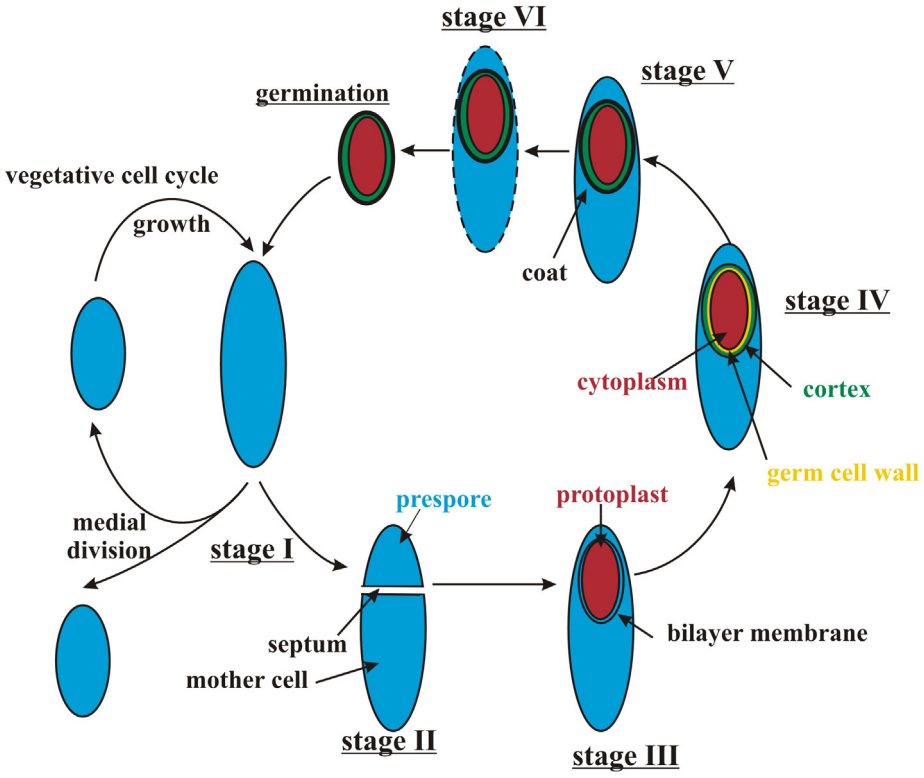

Figure 1.5: Key stages in the sporulation cycle of *Bacillus subtilis*. Sporulation is initiated by e.g. starvation (stage I), leading to an asymmetric cell division and the formation of a prespore and a mother cell (stage II). The prespore is then formed in a bilayer membrane by engulfment (stage III). Germ cell wall and cortex are synthesized between the inner and the outer forespore membrane (stage IV). The final step of spore synthesis is the attachment of the spore coat (stage V). The spore is set free by mother cell lysis (stage IV) and can survive without nutrients in a dormant state or germinate to start the vegetative life cycle in the presence of nutrients [112].

The coat has got a wide range of protective functions, for instance shielding the cortex against lysozyme treatment [119]. However, small germinants such as glucose and amino acids are able to pass the barrier because of its porosity [120]. The coat possesses two layers,

the inner and the outer coat. The assembly of these layers is a complex procedure [121] forming a highly cross-linked coat of 25 proteins, that have been identified by proteomic approaches to date [122]. This complex architecture facilitates enzymatic functions, germination, elasticity and more [123]. The reaction to humidity changes was discovered to be regulated by ridges formed on the spore coat surface allowing a change in spore volume [124]. These shells allow the spore to resist heat, radiation, desiccation, extreme pH values, toxic chemicals and organic solvents [125].

Some species, e.g. *B. anthracis* and the closely related species *B. cereus* and *B. thuringiensis* are enclosed by an additional shell, loosely attached to the spore coat, the exosporium [126]. It consists of proteins, carbohydrates and lipids and contains highly immunogenic molecules. The exosporium has been described to be essential for the interaction of spore and infected host in the pathogenesis of anthrax [127]. Thus, the main difference between pathogenic and apathogenic *Bacillus* spores is the ability to reproduce after phagocytosis by macrophages. In contrast to *B. anthracis*, which is able to germinate, reproduce and colonize the host, *B. subtilis* is killed in the vegetative state after germination [128].

Upon contact to amino acids, sugars or nucleosides, recognized by enzymes of the spore coat, germination is initiated. Releasing the DPA complexes, the core can be hydrated and cortex lytic enzymes hydrolyze the cortex PG. This process of germination is followed by the outgrowth of the spore in return to vegetative growth. The spore is regaining metabolic activity, escaping from the spore coat and synthesizing macromolecules [129].

1.7.1 *Bacillus licheniformis*

Bacillus licheniformis belongs to the *Bacillus subtilis* group of *Bacilli*. *Bacillus subtilis* is the best characterized Gram+ species and the model organism for bacterial sporulation. Genetically *B. subtilis* and *B. licheniformis* are quite distinct from the *cereus/anthracis* group of pathogenic *Bacilli* [130]. *Bacillus subtilis* as well as *B. licheniformis* are neither considered human pathogens nor toxigenic microorganisms [131]. Thermotolerant and halotolerant *B. licheniformis* isolates from extreme environments, were described to produce surface active biosurfactants [132;133]. Rarely cases of food poisoning strains have been isolated [134].

The genomes of two *B. licheniformis* strains have been published so far, ATCC 14580 [135] and DSM13 [130]. Orthology between *B. licheniformis* and *B. subtilis* was appointed to be

80%. Differences include extracellular enzymes, secondary metabolic pathways and spore coat assembly.

1.7.2 Bacterial spores in the farming environment

Bacillus licheniformis can be easily isolated as a spore from soil and plant material all over the world. Therefore, the connection to farming products like hay is obvious. For instance, *B. licheniformis* was detected to be part of the air-borne bacteria found in animal sheds and schools [(110)]. Thus *B. licheniformis* appears to be an interesting object to investigate the relation to protective farming environments. The spore size of about 1.85 µm in length and 0.7 µm of width, reported for *B. subtilis* [(124)], is an important aspect emphasizing their role in inhalational allergies and asthma. Particles of this size are able to intrude into the lower airways of children during their first years of life. Their size also contributes to a slow settling of spores resulting in high spore numbers, especially during litter in animal sheds (unpublished data). Children playing in hay or being present in the stable during their mother's working time may inhale high amounts of bacterial spores [(21)]. *Bacillus clausii* spores applied as a probioticum to allergic children was found to modulate the cytokine milieu in nasal lavage. The treatment decreased the levels of the T_H2 cytokine IL-4 and increased the counter-regulating T_H1 cytokine IFN-γ [(136)]. The usage of bacterial spores as probiotica also suggests an important impact of germination and outgrowth in treated subjects [(137)], emphasizing the importance of comparative analyses between spores and vegetative bacterium.

1.8 Fungal spores

In contrast to bacterial spores fungal spores are part of the reproductive life cycle of these fungi. They are formed as asexual propagules in a broad range of fungi. According to the locus of their formation fungal spores can be divided into sporangiospores, arthrospores and blastospores, by a variety of fungi in complex differentiation processes.

In the context of allergy indoor fungal exposure was associated with IgE mediated hypersensitivity. Today more than 80 fungal species are correlated with this effect. Among those *Mucor* spp. has been found to sensitize to mold allergy [(138)]. Contrary to these results β-(1→3)-D glucan, a component of fungal cell wall, has been described to be non-allergic and associated to the protective farming environment [(25;139)]. Its recognition by the innate immune system can contribute to the allergy protective contact of microbial compounds [(25)].

1.8.1 Sporangiospores of *Mucor* ssp.

Mucor ssp. belong to the group of zygomycetes. Members of this group are able to reproduce sexually, by gametangia fusion or asexually by production of sporangiospores. The most important members of the genus *Mucor* are the species *Mucor racemosus* and *Mucor plumbeus*. Most members of this group are saprobic and can be isolated from decaying grain, fruits and vegetables. The life cycle including sporangiospore formation is depicted in Figure 1.6.

On solid surfaces the fungus is growing as a hypha producing a sporangiophore to form the spores which can be released in the air after maturation. In this way the spore can endure long periods before germination and outgrowth are initiated by appropriate conditions.

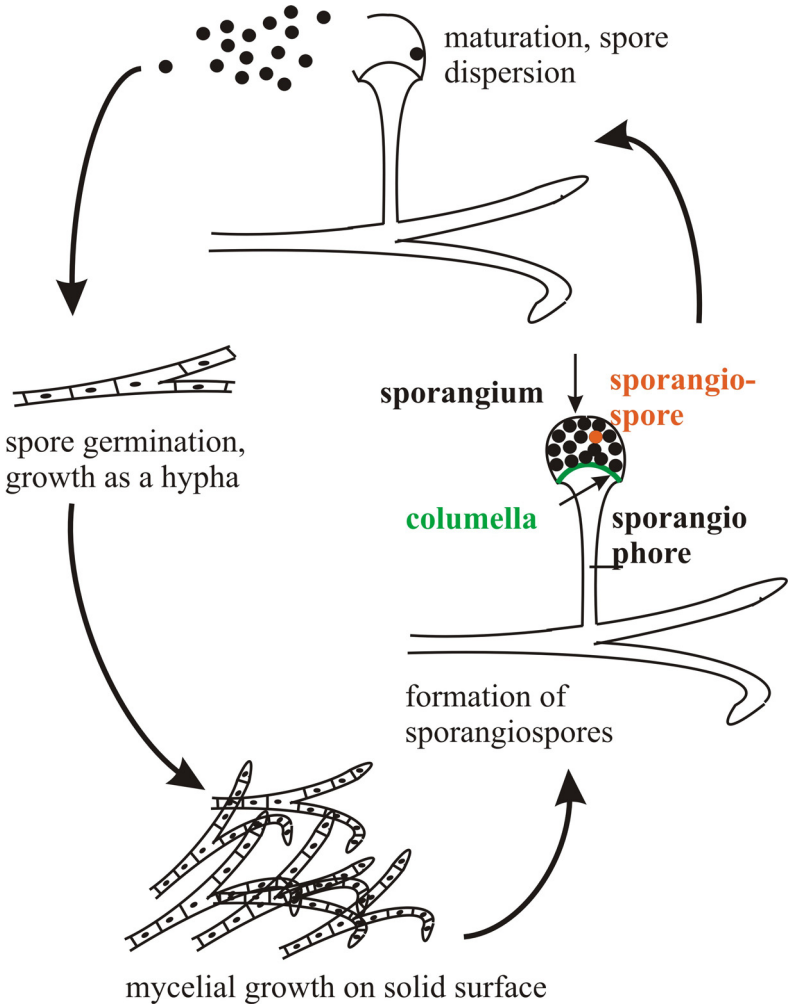

Figure 1.6: Formation of sporangiospores in the asexual life cycle of zygomycetes. *Mucor* spp. grow as mycelium on solid surfaces. They reproduce asexually by forming sporangiospores inside a sporangiophore. Following the complete maturation the sporangium can release the spores, which can be dispersed in the air. Under adequate conditions the spores can grow out and start a new growth cycle [(140)].

2. Aims of the study

Numerous epidemiological investigations showed that a protective effect on the development of asthma, allergy and atopic sensitization is given when growing up on a traditional farm in the alpine regions of Bavaria, Austria and Switzerland (ALEX study). Prenatal and early life contact to farm animals and farming products like hay is associated with an improved prognosis against the development of asthma and allergy. In comparison, children growing up in the same rural area, but without the above mentioned contact, were not decreased in the prevalence of those diseases.
Based on this data dust samples were collected from these farms to investigate the molecular basis of the protective effect of farming environments. The benefits of these dusts extracted with physiological saline were obtained by mouse experiments resulting in a strong asthma protection due to intranasal treatment of mice prior to sensitization combined with allergen provocation.

This knowledge evoked the aim of a detailed analysis of these animal shed dusts and the investigations of their microbial flora. Bacterial and fungal spore forming organisms can be detected in high amounts as outlined in the pilot study, chapter 1.6. Among spore forming microorganisms *Bacillus licheniformis* and *Mucor* spp. were the most common representatives of bacterial and fungal spores. They clearly indicated a relevant contact to farm children by their presence in animal sheds and farm children's mattress dusts. Due to their size and the fact that they occur in a dispersed form in stable air during litter in line with a slow settling their inhalation to the lower airways of newborns and small children is highly probable. Although this should raise the interest for their effect on the lung, studies about their potential to stimulate the immune system by inhalation is rarely found in literature.

Therefore, the aim of this study was to investigate *B. licheniformis* and *Mucor* spp. spores with respect to their contribution to the protective effect of traditional farms in alpine regions. Moreover, components able to stimulate the immune system in relation to allergy protection had to be isolated to carry out and clarify the molecular interactions responsible for this effect.

More specifically the aims of this thesis were formulated as follows:

- grow highly purified bacterial and fungal spores of the above mentioned candidates in adequate amounts,
- investigate their specific abilities to stimulate the human innate immune system, which is important for the development and prevention of allergic diseases in early childhood,
- differentiate the specificity of spore stimulation in comparison to the vegetative form of the respective organism, considering the possibility of spore germination and outgrowth,
- test their influence on the development of asthma and allergy in a mouse model of allergic asthma, and
- isolate potential immune stimulating components responsible for effects shown in former experiments.

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals and reagents

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

Product	Company
1,4-Dithiothreitol (DTT)	ROTH
1-Butanol	SIGMA
1-Propanol	SIGMA
2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris)	BIORAD
2-Propanol	SIGMA
4-Chloro-1-naphthol	SIGMA
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
Antifoam agent SAG 471	BAYER SILICONES
Aqua bidest.	MILLIPORE
Ascorbic acid	MERCK
Bisacrylamide	BIORAD
Blue dextran	SIGMA
Bovine serum albumine	PIERCE, ROTH

Bromophenol blue	MERCK
Calciumchloride	MERCK
Chloroform	MERCK
Cobalt chloride	MERCK
Complete proteinase inhibitor cocktail	ROCHE
Concavalin A, peroxidase labeled from *Canavalia ensiformis*	SIGMA
Coomassie Brilliant Blue R-250	BIORAD
Copper chloride	MERCK
Diff-Quick	DADE DIAGNOSTICS
Dimethyl sulfoxide	SIGMA
Disodium hydrogenphosphate	MERCK
DNAse	GE-HEALTHCARE
Ethanol	MERCK
Ethylene diamine tetraacetic acid (EDTA)	ROTH
Ferrous sulfate	MERCK
Ficoll	PAA LABORATORIES GmbH
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
Lysozyme	ROTH
Magnesium chloride	MERCK
Malachite green	MERCK
Manganese chloride	MERCK
Mercaptoethanol	SERVA
Methanol	MERCK
N,*N*,*N*',*N*'-Tetramethylethan-1,2-diamin	BIORAD

Naïve $CD4^+$ T-Cell Isolation Kit	MYLTENYI BIOTECH
β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate	SIGMA
N-Palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-[*R*]-cysteinyl-[*S*]-seryl-[*S*]-lysyl-[*S*]-lysyl-[*S*]-lysyl-[*S*]-lysine (Pam_3CSK_4)	EMC MICROCOLLECTIONS
Octyl β-D-glucoside	SIGMA
Octyl-sepharose	SIGMA
OVA grade IV	SIGMA
PARA-formaldehyde	SIGMA
Percoll	GE HEALTHCARE
Periodic acid	MERCK
Phenol	MERCK
Phenylmethylsulfonylfluoride (PMSF)	SIGMA
Polyfect	QIAGEN
Potassium chloride	MERCK
Potassium dihydrogen phosphate	MERCK
Propylene oxide	SIGMA
Proteinase K	MERCK
Pyridine	FLUKA
Rotiphorese®NF-Acrylamide/Bisacrylamide solution 30% (29:1)	ROTH
Safranin O	MERCK
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 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
Titriplex I [Nitrilotriacetate, 2-(bis(carboxymethyl) amino)acetic acid]	MERCK
TOTO®-3-iodate	INVITROGEN
Trifluoroacetic acid	MERCK
Triton X-100	SIGMA
Trypsin	SIGMA
Tween 20, Tween 80	RIEDEL-de HAEN
Uranyl acetate	MERCK
Zinc sulfate	MERCK
ZIP-tips (C18)	MILLIPORE

3.1.2 Buffers, solutions and media

Buffers and solutions were prepared using deionized, sterile filtered water (Milli Q, MILLIPORE) for analytical techniques and alternatively sterile, endotoxin-free water (B. BRAUN) for preparative techniques to avoid contamination with bacterial endotoxin. All concentrations listed are final concentrations of dissolved reagents in H_2O unless otherwise indicated.

Additionally all glass ware was sterilized at 240°C for 5 h to remove bacterial endotoxin. All buffers were sterile filtered and autoclaved subsequently.

3.1.2.1 Bacterial preparations

Phosphate buffered saline (PBS)	37.5 mM Na_2HPO_4, 0.137 M NaCl, 12.5 mM KH_2PO_4
LB medium (Lennox)	ROTH
LB sporulation medium (LB^+)	20g·L^{-1} LB Medium, 0.02 g·$L^{-1}$$MnSO_4$ x H_2O, 0.01 g·$L^{-1}$$MgSO_4$ x 7 H_2O

Difco Nutrient Broth	DIFCO
Difco Sporulation Medium (DSM)	8 $g \cdot L^{-1}$Nutrient broth, 3 mM KCl, 1 mM $MgSO_4$ x 7 H_2O, autoclaved and 1 mL of each of the following sterile stock solutions were added: 1 M $Ca(NO_3)_2$, 10 mM $MnCl_2$ x 4 H_2O, 1 mM $FeSO_4$ x 7 H_2O.
Minimal Medium (MM) [141]	10 mM NH_4Cl, 0.5 mM $MgSO_4$ x 7 H_2O, 0.1 mM $CaCl_2$, phosphate buffer: 400 mM Na_2HPO_4 + 400 mM KH_2PO_4, pH 7.5, trace element solution 1mL, glucose (varying concentration), H_2O bidest.
Trace element solution	1.28 g Titriplex I, 2 g $FeSO_4$ x 7 H_2O, 190 mg $CoCl_2$ x 6 H_2O, 100 mg $MnCl_2$ x 4 H_2O, 148 mg $ZnSO_4$ x 7 H2O, 6 mg H_3BO_3, 24 mg $NiCl_2$ x 6 H_2O, 2 mg $CuCl_2$ x 2 H_2O, 36 mg $NaMoO_4$ x 2 H_2O, adjust pH to 6.5 using NaOH, fill up to 1 L H_2O bidest.

3.1.2.2 Cell culture

Dulbecco's minimal essential medium (DMEM), high glucose, with L-glutamine	PAA LABORATORIES
Fetal calf serum (FCS), inactivated at 56°C for 30 min	LINARIS
Hanks buffered salt solution (HBSS)	PAN BIOTECH
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	PAA LABORATORIES
Penicillin/streptomycin 100x	PAA LABORATORIES
Antibiotic/Antimycotic solution	BIO WEST
Rxx10	RPMI 1640, 1% (v/v) penicillin/streptomycin, 10% (v/v) FCS

Lymphocyte separation medium	PAA LABORATORIES
Elutriation medium	1 x HBSS, 2% (v/v) FCS, 3.5% (v/v) sodium hydrogen carbonate in aqua injectabilia
Granulocyte macrophage colony stimulatory factor(GM-CSF)	STRAHTMANN BIOTECH
Interleukin 4	STRAHTMANN BIOTECH

The preparation of special buffers is described in the section of the appropriate method.

3.1.3 Equipment and plastic material

Equipment	**Company**
2D Electrophoresis WITA vision	WITA
Amicon-Ultra-15 centrifugal filter units	MILLIPORE
Auto MACS TM	MILTENYI BIOTEC
Autoclave	SYSTEC
Balance	SARTORIUS
BioFlo110 fermenter/bioreactor	NEW BRUINSWICK SCIENTIFIC
Biological safety cabinet	CRYO-TEC
Bioreactor probes pO_2 & pH	METTLER TOLEDO
Biotrap®/Elutrap® Electrophoresis chamber	MILTENYI BIOTEC
CASY TT cell counter	SCHÄRFE
Centrifuges	BECKMANN COULTER, HEREAUS, HETTICH
CO_2 incubator for cell cultures	HERAEUS
Confocal laser scanning microscope TCS SP	LEICA
Culture plates (24-, 48-, 96-wells)	NUNC
Electrophoresis equipment	BIORAD
ELISA reader	BIOTEK INSTRUMENTS
ELISA-plate shaker	EDMUND BÜHLER
Elutriation chamber JE-6B	BECKMANN
FACS Calibur	BECTON DICKINSON
Homogenisator	B. BRAUN
Horizontal rotary shaker	NEW BRUINSWICK SCIENTIFIC

HPLC	ABIMED GILSON
Lyophilizator, Lyovac GT 2E	OMNILAB
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
Pasteur pipettes	BRAND
Photometer	HELIOS
Pipette tips	SARSTEDT
Procise™ Protein Sequencing System	APPLIED BIOSYSTEMS
Thermomixer	EPPENDORF
Transmission electron microscope	ZEISS
Transmission light microscope	ZEISS
Ultracentrifuge: UZ TGA-55	KONTRON

3.2 Methods

3.2.1 Preparation of bacterial and fungal spores

3.2.1.1 Isolation and strain characterization of *Bacillus licheniformis* strains and *Mucor* spp.

Bacillus licheniformis strain 467 was isolated on agar plates from a mattress of a farm child involved in this study. In comparison to this, strain 830 was isolated from a mattress of a non-farming child. Strain characterization was performed following instructions by Relman et al. [142] with regard to 16S rDNA PCR and sequencing, and to Logan et al. [143] for further biochemical differentiation. Sequencing results of 16S rDNA were aligned to reference strain *B. licheniformis* ATCC 14580 (GenBank entry CP000002) as published by Rey et al. [135]. Apart from some changes also present in other *B. licheniformis* strains characteristic sequence similarities were identified.

Mucor was identified by macroscopic as well as microscopic techniques using sticky tape preparation in combination with lactophenol blue staining.

3.2.1.2 *Bacillus subtilis* DSM 618

Bacillus subtilis DSM 618 (MERCK) was compared to *B. licheniformis* as a closely related reference strain [130]. Spores were used for inoculation of cultures in LB-medium according to the growth conditions for *B. licheniformis*.

3.2.1.3 *Bacillus licheniformis* growth and sporulation for *in vitro* and *in vivo* experiments

Bacillus licheniformis was grown in LB medium (Lennox) at 37 C. Vegetative *B. licheniformis* 467 was harvested after 6 h cultivation on a rotary shaker in the middle of the exponential growth phase. Then the bacteria were washed twice with fresh LB medium to prevent sporulation, and finally lyophilized in aliquots.

Spore preparations were performed by cultivation of *B. licheniformis* 467 in LB medium (Lennox) supplemented with 20 $mg \cdot L^{-1}$ $MgSO_4$ x 7 H_2O and 10 $mg \cdot L^{-1}$ $MnSO_4$ x H_2O on a rotary shaker for 60 h followed by the sporulation for 3 weeks without shaking. Subsequently, *B. licheniformis* was purified by heat inactivation of vegetative cells at 80°C

for 30 min, followed by density gradient centrifugation using 90% Percoll [144] in 0.15 M NaCl. Spores were washed five times with distilled water and purity was controlled by Wirtz-Conklin staining (3.2.4.1). Colony forming units were determined in preparations free of vegetative cells by plating the respective dilution on LB agar plates. Heat killed spores were obtained by steam sterilization (121°C, 200 kPa, 20 min).

3.2.1.4 Upscale of *Bacillus licheniformis* spore production for the preparative isolation of proteins

Within a diploma thesis [145] a high yield method for the production of bacterial spores was established. Based on the above mentioned growth conditions for the sporulation of *B. licheniformis* a 10 L bioreactor (BioFlo 110) was utilized to produce high quantities of spores needed for the isolation of immune stimulating bacterial components such as lipoproteins.

To achieve this different media as well as different growth conditions were tested for their yield in induction of sporulation. Optimal outcomes for the spore yield were going along with a reduction of the fermentation and sporulation time from 3 weeks to 30 hours.

The optimal process for the growth of *B. licheniformis* 467 was initiated by a glycerin culture grown in 30 mL preparatory culture for 7 h and a transfer to a secondary preparatory culture with a final volume of 320 mL. This culture was inoculated to 8 L bioreactor culture after 12 h of incubation.

The batch process was performed under following conditions for 30 hours. The parameters were controlled online within the given variation:

pH-value:	7 ± 0.1 (set by addition of sodium hydroxide and sulfuric acid, respectively)
Temperature:	37°C
Oxygen saturation:	50% (set by increased agitation)
Foam level:	controlled by addition of anti-foam agent SAG-471

After 6 h for the growth of vegetative cells or 30 h of incubation for spore production the bioreactor culture was harvested and bacteria were purified as described before (3.2.1.3) (Fig. 3.1).

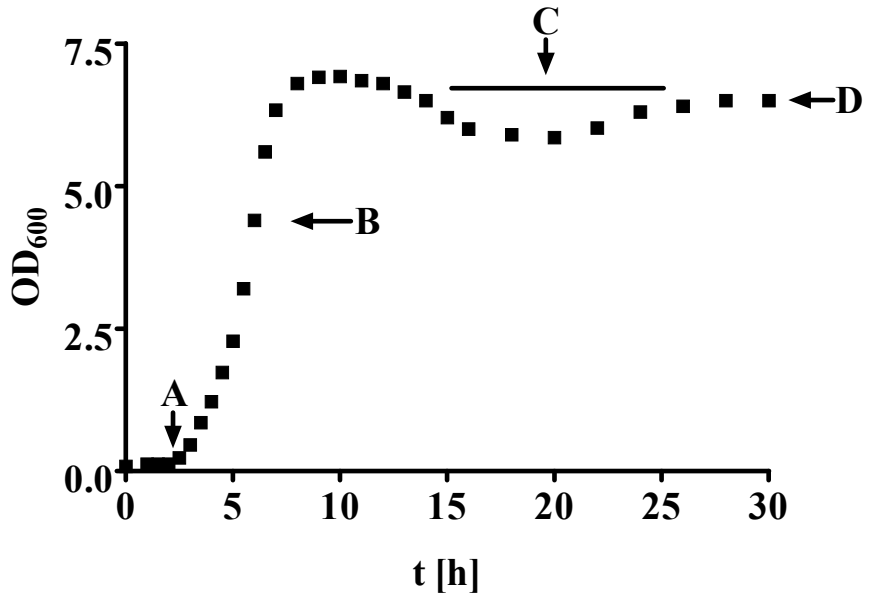

Figure 3.1: Exemplary growth curve for *Bacillus licheniformis* 467 in LB medium. Growth was performed in LB-medium supplemented with divalent cations (LB^+) in an 8 L batch fermentation (BioFlo 110). The end of the lag-phase (**A**) as well as the times for harvesting of vegetative *B. licheniformis* cells (**B**) or spores (**D**) are indicated. Period **C** shows the typical decrease of optical density (OD), aroused by sporulation of bacteria. The same growth parameters were applied to obtain spores and vegetative bacteria.

3.2.1.5 Purity control of *Bacillus licheniformis* spore preparations

Purified preparations of *Bacillus* spores were tested by Wirtz-Conklin staining (3.2.4.1) for the presence of vegetative bacteria and endospores. Furthermore, contaminations of bacteria of other species were investigated by bacterial growth on LB-agar plates and by Gram-staining (3.2.4.2). All investigations mentioned in this thesis were performed with preparations free of vegetative bacteria, endospores and free of contaminations with bacteria of other species.

3.2.1.6 *Mucor* spp. growth and sporulation

Mucor ssp. spores isolated from cowshed dust were grown on agar plates (DG18 and Sabouraud) in cooperation with Dr. Karin Schwaiger (Technical University of Munich). The plates were dashed with 5 mL of sterile water and stirred horizontally. The scraped mycelium was filtered through four layers of sterile gauze. The filtrate was centrifuged at 300 x *g* for 5 min. After that the upper layer was transferred to a new tube and the centrifugation of both tubes was repeated. Supernatants were united and resuspended in H_2O. The same was done for the sediments to repeat centrifugation at 300 x *g* for 2 min till

half of the spores sedimented. Supernatants and sediments were separated and the procedure was repeated till pure spore preparations were achieved. The purity was controlled microscopically.

3.2.2 Cell culture and immunological methods

3.2.2.1 Isolation of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from citrate-buffered blood of healthy donors by gradient centrifugation using Ficoll [(146)]. Therefore, 10 mL of lymphocyte separation medium (LSM) were mixed with 35 mL of diluted blood. The blood was mixed with equal amounts of sodium citrate solution (10% w/v). Gentle centrifugation without disturbing the gradient at 300 x *g* (25°C, 35 min) achieved the separation of blood plasma (upper phase) and erythrocytes (lower phase). The interphase containing PBMCs was collected and washed twice to remove contaminating thrombocytes (4°C, 300 x *g*, 10 min). Cells where counted in a CASY TT cell counter diluted to $1 \cdot 10^6$ cells per mL and plated on culture plates. Stimulation with the respective form of *B. licheniformis* 467, LPS of *Salmonella enteria* sv. Friedenau (kindly provided by Prof. Dr. H. Brade, Research Center Borstel) or Pam_3CSK_4 followed. If required, cells were separated by counterflow elutriation centrifugation to obtain monocytes and lymphocytes. Cells from different healthy donors were isolated for each independent experiment.

Separated PBMCs were resuspended in elutriation medium and isolated by counterflow centrifugation according to Contreras et al. [(147)]. Cells were pumped through the elutriation chamber with a flow of 25 $mL \cdot min^{-1}$ while the rotor was turning with 1720 x *g*. The pump flow was increased in small steps to 50 mL min^{-1}. In this way first lymphocytes and later monocytes were eluted and separated. Purity was controlled by CASY TT or fluorescence activated cell sorting (FACS) (3.2.3.1).

3.2.2.2 Differentiation of human monocyte-derived dendritic cells

Monocytes and lymphocytes were isolated as described above, concentrated to 10^6 cells per mL and differentiated into immature dendritic cells in the presence of GM-CSF and IL-4 (500 $U \cdot mL^{-1}$ each) using RPMI medium containing 10% heat-inactivated fetal calf serum, 100 $U \cdot mL^{-1}$ penicillin, and 100 $\mu g \cdot mL^{-1}$ streptomycin. Every 2-3 days half of the medium was exchanged and undifferentiated DCs were harvested after 5-7 days of culture. Prior to stimulation cells were counted, and plated to a respective culture plate. Two hours later cells were stimulated at indicated multiplicities of infection (MOI) with the respective

bacteria or bacterial stimuli e.g. 10 ng·mL^{-1} LPS of *Salmonella enterica* sv. Friedenau. Stimulation for a denoted time was followed by the analysis of DC supernatants for the presence of TNF-α, IL-10, IL-8 or IL-12p70 by ELISA (3.2.3.2). Alternative DCs were analyzed by FACS for the expression of cell surface markers (3.2.3.1) or for their T_C-polarization in a mixed leukocyte reaction (3.2.2.4). The mentioned methods were performed in cooperation with Dr. Jessica Mittelstädt (Research Center Borstel).

3.2.2.3 Isolation of naïve T-cells from lymphocytes

Naïve T-cells were isolated from enriched lymphocytes after elutriation. Thereto, lymphocytes were separated by the Naïve CD4$^+$ T-Cell Isolation Kit. This Kit allows the removal of any contaminating cells by binding specific surface markers on B-cells, NK cells, CD8$^+$ cells etc. and separating them from CD4$^+$ cells by magnetic absorption. Purities were tested by FACS analyses of surface markers CD4 and CD45RA. Preparations with purities of > 85% were used for mixed leukocyte reactions (MLR).

3.2.2.4 Mixed leukocyte reaction

To analyze the cytokine secretion of human T-cells [148] and differentiation of naïve T-cells, heterologous lymphocytes (2×10^5) were seeded into 96-well round-bottom plates with a total volume of 100 µl per well, 2×10^4, 2×10^3, and 2×10^2 DC stimulated prior to MLR were added to give final ratios of 1:10, 1:100 or 1:1000 DC:lymphocyte. Cocultures were incubated for 5 days. Cell-free culture supernatants were analyzed by Beadlyte Human Multi-Cytokine Detection System 4 (3.2.3.2.2). Lipopolysaccharide of *Salmonella enterica* sv. Friedenau, a strong stimulator of DC maturation [149], as well as *Mycobacterium bovis* BCG, known to induce DC maturation without any T_H-cell polarization [150] were applied as positive controls. There results were compared to the specific forms of *B. licheniformis* 467.

3.2.2.5 Transfection of Human Embryonic Kidney-293 cells

The human embryonic kidney cell line HEK-293 was cultured in DMEM to a confluence of up to 80%. Transfection with the expression plasmids for TLR2 (200 ng·mL^{-1}), TLR4 (200 ng·mL^{-1}) and CD14 (25 ng·mL^{-1}) as well as NOD1 and NOD2 (200·ng·mL^{-1} each) was performed as described before [70;151]. Thereto, 2.5 x 10^4 cells were seeded into 96-well plates. The following day transfection with Polyfect was carried out according to the manufacturer's instructions. After 24 h cells were washed and stimulated for 18 h with

either live or lyophilized vegetative cells of *B. licheniformis* strains 467 or 830, *B. subtilis* or *Mucor* spp. spores. Moreover, heat-inactivated spores and preparations of proteins and peptidoglycan were analyzed. Concentrations of IL-8 in cell-free supernatants were determined by ELISA. Pam_3CSK_4 (TLR2) and LPS of *S. enterica* sv. Friedenau (TLR4) as well as ieDAP (NOD1) (courtesy of Prof. Koichi Fukase (Osaka, Japan)) and MDP (Nod2) (provided by Shoichi Kusomoto (Osaka, Japan)) were applied as positive controls. Toll-like receptor 5 transfected HEK cells were used to investigate samples qualitatively due to the lack of reproducible positive controls. The experiments were performed under technical assistance of Ina Goroncy using the facilities of PD Dr. Holger Heine (Research Center Borstel).

3.2.2.6 Stimulation of epithelial cells

The epithelial cell line A549 was purchased from *Deutsche Sammlung für Mikroorganismen und Zellkulturen* (DSMZ: culture ACC 107). Cells were cultured in DMEM medium supplemented by 10% FCS, 1% L-glutamine and 1% penicillin/streptomycin solution. As soon as a confluence higher than 80% was reached, cells were transferred to 48-well culture plates in a density of 25000 cells per well. Stimulation with the respective bacterium was performed as described in the results section.

Primary epithelial cells were isolated from airway biopsies and were stored in aliquots at a temperature of -80°C. Aliquots were thawed and transferred to two 175 cm^2 culture dishes. Growth was stopped at a confluence of 80% in epithelial cell growth medium. Cells were detached from the plastic material by trypsin-EDTA treatment, centrifuged and resuspended in 12.5 mL medium. They were placed on 24 transwells, 1 mL medium was added and cells were grown till they appeared to be confluent. Medium was exchanged by 650 µL of differentiation medium. Differentiation was completed, when resistance between upper and lower sides of the epithelial cells was >200 mΩ. If this was achieved, a stimulus was added on the air-facing side of the cells in a minimized volume of PBS. These data were obtained in cooperation with Frank Klescz and Prof. Dr. Dr. Robert Bals (Philipps University Marburg).

3.2.2.6.1 Phagocytosis of *Bacillus licheniformis* by dendritic cells

Fluorescence activated cell sorting was performed to investigate phagocytosis of *B. licheniformis* by moDCs. For this purpose cells were stimulated with fluorescein-5-

isothiocyanate (FITC)-labeled vegetative *B. licheniformis* (3.2.4.4) or its spores. The incubated cells were transferred into a FACS tube after different time points of incubation, sedimented (300 x *g*, 5 min) and the supernatant was deployed for ELISA experiments. The cells were conserved in 200 µL azide solution and 200 µL of para-formaldehyde (PFA) solution were added for fixation. Unstimulated cells were applied to set the gate for dendritic cells and about 10000 cells in this gate were counted. Sample analyses followed in the FACS Calibur using WinMDI software. Therefore, auto-fluorescence of untreated DCs was set as a reference and the percentage of cells gaining fluorescence by phagocytosis of FITC-labeled bacteria was calculated. Fluorescence was excited by an argon laser (488 nm) and emission was measured at 515 nm. The experiments were performed in cooperation with Dr. Jennifer Debarry (Research Center Borstel).

Azide solution	0.1% (w/v) NaN_3 in PBS
PFA solution	3% para-formaldehyde in PBS

3.2.3 Biochemical methods

3.2.3.1 Flow cytometry

Monocyte derived dendritic cells were stimulated with bacteria (MOI = 1) as described in point 3.2.2.1. To investigate the phenotypic maturation of DCs, expression of different surface antigens was detected by FACS. Following stimulation cells were centrifuged to remove the supernatant. Cells were washed in azide PBS and resuspended in the same solution containing 3% (v/v) human serum. After transfer of cells to FACS tubes primary antibodies were added and the cells were incubated for 20-30 min according to the manufacturer's instructions. If a fluorophor conjugated primary antibody was applied, cells were washed with azide PBS and adjacent addition of the same volume of PFA solution (3% w/v) led to a cross-linking of cells and antibodies. Otherwise incubation with a labeled secondary antibody was performed prior to fixation. 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 WinMDI 2.8 [(148)].

Table 3.1: Antibodies for FACS analyses

Antibodies for FACS	Clone	Isotype	Company
CD14	18D11	mIgG1	DIANOVA
CD80	MAB104	mIgG1	IMMUNOTECH
CD83	HB15e	mIgG1	PHARMINGEN
CD86	FUN-1	mIgG1	PHARMINGEN
Isotype	DAK-GO2	mIgG1	DAKO
	DAK-GO3	mIgG1	DAKO
	DAK-GO5	mIgG2a	DAKO
MHCII	IR-RD1	mIgG2a	BECKMANN COULTER

3.2.3.2 Determination of chemokines and cytokines by Enzyme-Linked-Immunosorbent-Assay

The Enzyme-Linked-Immunosorbent-Assay (ELISA) is based on antigen-antibody interactions and therefore it is specific for the detection of small quantities of molecules. In this thesis the following ELISA approaches were performed to determine the concentration of cytokines and chemokines in the culture supernatant of stimulated cells.

3.2.3.2.1 Sandwich Enzyme-Linked-Immunosorbent-Assay

To perform this kind of ELISA it was necessary to coat a 96-well plate with an antibody able to recognize the molecule of interest by incubation for 16 h. After the unbound antibodies were removed by washing uncoated surface was blocked by bovine serum albumin (BSA). The blocking solution was removed by washing with ELISA buffer. Samples were added to the plate and incubated at 25°C for 2 h. Removal of unbound sample material by repeated washing steps was followed by incubation with 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 sulfuric acid (1 M) and the quantification of converted substrate was measured at $\lambda = 450$ nm using an ELISA reader and compared to a standard.

Table 3.2: Analyzed chemokines and cytokines

Chemokine/ Cytokine	Company
Interleukin-(IL)-8	eBIOSCIENCE
IL-10	eBIOSCIENCE
IL-12	eBIOSCIENCE
TNF-α	eBIOSCIENCE
IL-8	BD BIOSCIENCE
Human ELISA Ready-SET-Go! Kit	NATUTECH
Opt EIA ELISA IFN-γ, IL-5, IL-10	BD BIOSCIENCE

3.2.3.2.2 Multi-cytokine detection

For the detection of T_H-cell polarization by expression of cytokines, the simultaneous detection of several chemokines and cytokines was necessary. The complex cytokine pattern expressed after differentiation of T_H-cells into T_H1- and T_H2-cells was measured by the Beadlyte Human Multi cytokine Detection System 4, which allowed measuring a variety of cytokines and chemokines. The working principle is a mixture of microsphere beads with specific capture antibodies for the different cytokines and chemokines. In contact with the sample the antibodies are able to interact specifically with the cytokine and a biotinylated reporter antibody binds to the cytokine.

After incubation in the dark for 30 min, Beadlyte stop solution was added and results were read out on a Luminex ® 100 instrument. The combination of reporter fluorophore and fluorescent microsphere bead impedes the possibility of unspecific detection. Detailed proceedings of the analysis were performed according to the manufacturer's instructions.

Beadlyte Human Multi-Cytokine Detection System 4	UPSTATE

3.2.3.3 Statistical analysis

All data obtained in *in vitro* experiments are shown as means ± SEM. Significance was analyzed using Graph Prism software and the student's unpaired *t*-test. Significant differences were considered at * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

3.2.4 Microscopic techniques

3.2.4.1 Wirtz-Conklin spore stain

Bacterial suspensions were prepared on an object slide, heat-fixed and covered with 5% aqueous malachite green. The covered slide was heated gently to steaming for 5 min to enhance dye uptake by bacterial spores. Slides were rinsed with water and counterstained for 1 min with 1% aqueous safranin. Rinsing and drying allowed examination by light microscopy [143]. Greenish-blue spheres were malachite green positive spores that passed the whole process of sporulation (dormant spores). They were able to keep the dye by an intact cell wall. Red, safranin positive, spores showed incomplete sporulation (endospores) or initiated germination, in an environment that does not allow complete transition to rod-shaped vegetative bacilli (red) [152].

3.2.4.2 Gram staining

Heat fixed bacteria on an object slide were covered with crystal violet (Gentiana violet solution) for 3-5 min. Washed and dried slides were covered in Lougol's solution for 2-3 min dried and destained with ethanol (96% v/v). Counterstaining with safranin O (0.25% w/v) for 1 min was followed by drying and examination under the light microscope.

3.2.4.3 Confocal microscopy

To control the specificity of bacterial uptake by DCs, the cells were stimulated with FITC-labeled bacteria (3.2.4.4) in µ-slides. At different time points of stimulation (1, 4, 8 and 24 h) cells were centrifuged (300 x *g*, 5 min) and the supernatant was discarded. Cells were washed 3 times with 150 µL of PBS and fixed in 75 µL of 4% PFA solution for 10 min. After washing cells were permeabilized at 25°C by incubation in 75 µL of 0.25% Triton in TBS for 10 min. Triton was removed and an unspecific binding of antibodies was prevented by the blocking with 10% FCS in TBS. First a mouse anti-human TLR2 antibody was diluted 1:20 in 10% FCS/TBS and cells were incubated for 45 min. The unbound antibody was removed while the bound was detected by a secondary goat anti-mouse antibody labeled with the fluorophore Alexa 633 (dilution 1:300). Unbound antibody was rinsed with PBS and nuclei were stained with 75 µL TOTO®-3 solution diluted 1:500 in 10% FCS/TBS. Terminally staining was fixed for 20 min by incubation

with 4% PFA solution, washed with PBS and investigated by confocal laser scanning microscopy in combination with the analyses by TCS NT software.

TBS	50 mM Tris, 150 mM NaCl, pH 7,4
Mouse anti-human TLR2	GENENTECH ATLIMS
Goat anti-mouse Alexa 633	MOLECULAR PROBES

3.2.4.4 Fluorescein-isothiocyanate labeling of bacteria [153]

Bacillus licheniformis spores as well as vegetative bacteria were stained with FITC for investigations in confocal light microscopy (3.2.4.3) and FACS (3.2.3.1). Therefore, lyophilized bacteria were solved in PBS and pelleted at 14000 x *g* for 5 min. The pellet was resuspended in 1 mL FITC solution and incubated at 37°C under constant shaking in the dark for 30 min. To remove excessive FITC bacteria were washed with PBS/Tween20 until no free FITC was visible in the supernatant. Stimulation and examination of bacterial uptake was investigated by confocal laser scanning microscopy and FACS analyses.

FITC solution	10 mg FITC in 10 mL Na_2CO_3 (1M) + 0.05% Tween80
PBS/Tween20	0.05% Tween20 in PBS

3.2.4.5 Transmission electron microscopy

Spores and vegetative bacteria of *B. licheniformis* 467 (3.2.1.3) as well as *Mucor* spp. spores (3.2.1.6) were prepared as explained before. Transmission electron microscopy (TEM) was performed according to Andrä et al. [154]. Spores were washed five times with distilled water whereas vegetative bacteria were washed three times with PBS. The pellets were fixed in 2% osmium tetroxide for 1.5 h and washed three times with distilled water. The post-fixation positive stain was followed by addition of 2% aqueous uranyl-acetate and incubation at 25°C for 1 h. After washing, the pellet was dehydrated in serial ethanol washes. Following the transfer to propylene oxide for 15 min, samples were infiltrated at 4°C for 16 h by a 1:1 mixture of propylene oxide-Epon-resin mix, and polymerization

followed embedding in an Epon-resin at 60°C for 16 h. Ultrathin-sections were cut using a pyramytom, slices were placed on a copper grid, counterstained in lead citrate in a CO_2-free environment and micrographs were taken using an EM Zeiss 910. Electron micrographs were carried out in cooperation with Heike Kühl and PD Dr. Torsten Goldmann (Research Center Borstel).

3.2.5 Animal Experiments

The animal experiments described in this thesis were performed in cooperation with PD Dr. Holger Garn and Dr. Nicole Blümer in the Department of Clinical Chemistry and Molecular Diagnostics at the Philipps University of Marburg, (Germany) under administration 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 libitum*.

3.2.5.1 Allergen sensitization, provocation and bacterial 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. In the experiments of this study mice were sensitized to allergen by three intraperitonial (i.p.) injections of 10 µg OVA grade IV. Ovalbumin was absorbed to 1.5 mg $Al(OH)_3$, dissolved in 200 µl PBS and injected on days 0, 14 and 21. The local allergic reaction in the lung was provoked by inhalation of the same allergen as an aerosol [1% w/v OVA in PBS] on days 19 to 21 once a day for 20 min. This treatment challenged a local allergen induced inflammatory response in the lungs as described [155]. Twelve days before the first OVA injection, mice were anaesthetized to begin intranasal treatment with either 10^8 cfu lyophilized dormant spores of *B. licheniformis* 467, the heat-inactivated spores inhibited in germination or the vegetative cells applicated in fresh LB medium to prevent sporulation. A group of 8 mice was sham treated with PBS over the whole process of sensitization and challenge as a negative control. Figure 3.2 shows the schematic protocol of provocation and treatment.

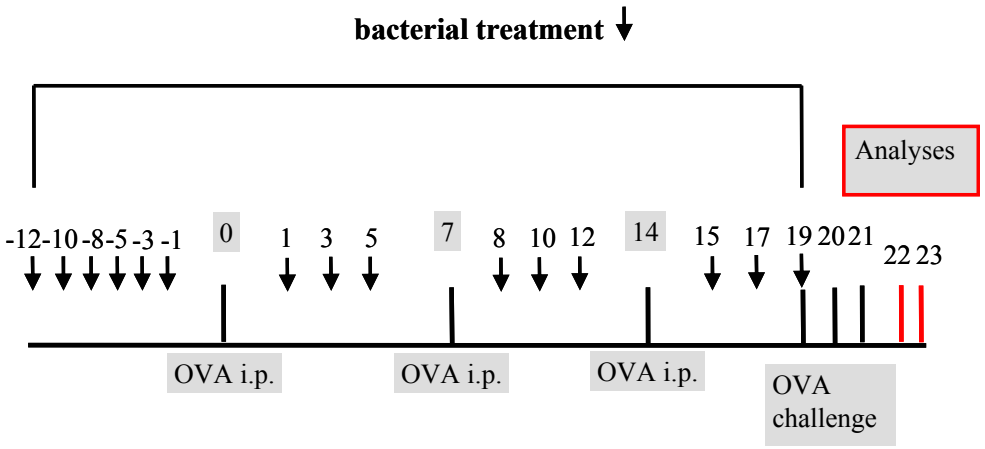

Figure 3.2: Schematic protocol of mouse experiments. Female BALB/c mice were sensitized at day 0, 7 and 14 by ovalbumin (OVA) injections (i.p.) followed by OVA aerosol exposure at days 19, 20 and 21. Twelve days before the first OVA injection and during the whole sensitization period mice received *B. licheniformis* 467 spores, heat-inactivated *B. licheniformis* 467 spores or vegetative *B. licheniformis* 467 intranasally every second day. Mice were analyzed at days 22 and 23 for airway inflammation and lung function.

3.2.5.2 Airway responsiveness to methacholine

At day 22 mice were investigated for the hyperresponsiveness of their airways [156]. 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. Thereto, 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 referring to Neuhaus-Steinmetz et al. [157]. The total leukocyte number in BAL fluid (BALF) was determined by Casy TT cell counter. The principle of this cell counter is a change of a constant resistance in a chamber of measurement. In the presence of intact cell membranes acting as an isolator the resistance is shifted by passing cells. The resistance changes depending on the cell size, what enables the method to determine different cell populations in a mixture. Furthermore cytospins were sedimented at 300 x *g* and cells were differentially stained with Diff Quick. To minimize mistakes 100 cells were counted in a

double estimation for every cytospin. Remaining cell free BALF were stored at -20°C for further investigations of cytokines.

3.2.5.4 Lung histology

Lungs were isolated and fixed *ex situ* by infusion of a 4% (w/v) formaldehyde solution. Sections of 3 µm were cut from paraffin embedded lung tissues. Paraffin was removed by serial incubations in descending concentrations of alcohol. Staining with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS) was performed. Hematoxylin-Eosin-staining resulted in a blue color for acidic molecules, e.g. DNA in the nucleus, and a mangenta one for protein containing tissue material. The PAS staining was conducted to detect mucus production as a response to asthma provocation. Additionally lung slices were analyzed for the detection of inhaled spores by Wirtz-Conklin staining. This staining was done as described before (3.2.4.1) with the modification of an attenuated counterstaining with safranin O (0.5%) for 1 min.

3.2.5.5 Detection of cytokines in bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid was obtained (3.2.5.3) and relevant cytokines for the investigation of asthma and allergy related shifts in immune response were determined by OptEIA ELISA. In this thesis IFN-γ, IL-10 and IL-5 levels in BALF were investigated. The utilized ELISA was performed as a sandwich ELISA (3.2.3.2.1).

3.2.5.6 OVA-specific immunoglobulins

One day after mice had obtained the last OVA-provocation blood samples were retained to determine the serum concentrations of OVA-specific immunoglobulins. Thereto, multiwell plates were incubated with 20 $mg \cdot mL^{-1}$ 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 serum samples or monoclonal IgG1, IgG2a or IgE antibodies (4°C, 16 h). After washing biotinylated IgG1, IgG2a or IgE antibodies were added and plates were incubated for 2 h. Incubation with peroxidase labeled streptavidin followed at 25°C. Plates were measured in an ELISA reader by comparison to immunoglobulins of known concentrations.

3.2.5.7 Statistical analysis

All data obtained in animal experiments are shown as means ± SEM. Significance was analyzed using Graph Prism software and the student's unpaired *t*-test. Significant

differences were considered at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, indicating differences between PBS treated mice and *B. licheniformis* treated mice. Varieties between mice treated with vegetative *B. licheniformis* in medium and the control medium are indicated by $^{\#} p < 0.05$.

3.2.6 Isolation of bacterial components

3.2.6.1 Sodium chloride extraction of *Bacillus licheniformis*

According to the allergy protection obtained by application of stable dust extracted with physiological saline, spores were prepared as described above (3.2.1.3) and washed five times in endotoxin-free water. Following centrifugation 10^9 spores were resuspended in 10 mL physiological saline (0.9% (w/v) NaCl in endotoxin-free water). Spores were disrupted by repeated mixing with glass beads (0.1 mm) and stirred at 22°C for 6 h. Intact spores and cell debris were sedimented (5000 x *g*, 20 min). The supernatant was collected, dialyzed against endotoxin-free water, to remove salts, and investigated due to its properties of stimulation of immune cells *in vitro*.

3.2.6.2 Isolation of peptidoglycan

Peptidoglycan is an essential component of the bacterial cell wall which enables them to resist high osmotic pressure. There are two different kinds of peptidoglycan in bacterial spores. The germ cell wall surrounds the cytoplasmic membrane and is identically to the cell wall of germinated bacteria, and the so called cortex. The cortex is a spore-specific thick peptidoglycan layer responsible for the high resistance of spores to environmental stresses. Depending on the form of the bacteria two modifications of peptidoglycan isolation were carried out.

Prior to cell disruption of bacterial spores the coat, a layer of highly cross-linked proteins conveying resistance to several chemical reagents, was extracted by sodium dodecylsulfate (SDS) and β-mercapthoethanol (3.2.6.4). Sodium dodecyl sulfate was removed by washing the sediment about 15 times with H_2O (60°C) and centrifuging at 5000 x *g* for 10 min.

The following steps of the procedure were performed for both forms in the same way [158]. Approximately 10^{11} cfu of cells or spores were mixed with 30% (w/v) endotoxin-free glass beads (0.1 mm) in disruption buffer. Cells were degraded mechanically by vigorous mixing for five times per 3 min. The supernatant was collected after centrifugation at 5000 x *g* for 15 min and disruption was repeated. Glass beads were removed by intensive washing prior to transfer to ultracentrifugation tubes to collect broken cell walls (45000 x *g*, 25 min). The

pellet was incubated with α-amylase (100 µg·mL^{-1}) at 37°C. DNAse was added 2 h later (10 µg·mL^{-1}) supplemented with 20 mM $MgSO_4$ and cleavage was prolonged 2 h. Afterwards 10 mM $CaCl_2$ was added and the solution was treated with trypsin (100 µg mL^{-1}) for 16 h. In a final concentration of 1% (w/v) SDS was used to inactivate enzymatic digestion in combination with boiling the material for 5 min. To remove SDS and other loosely attached substances cell walls were washed twice with water, once with 8 M LiCl, once with EDTA (100 mM), twice with water and finally twice with acetone prior to lyophilization.

The freeze dried material was stored in aliquots or treated with 48% (v/v) HF at 4°C for 18 h under constant shaking to remove, e.g., covalently bound lipoproteins. Hydrofluoric acid was evaporated under N_2 and the sediment was washed three times with H_2O and utilized for further investigations.

Disruption buffer	100 mM Tris-HCl, pH 7.5, 10 mM EDTA

3.2.6.3 Enzymatic digestion of peptidoglycan

To obtain muropeptides, the building blocks of peptidoglycan, enzymatic digestion by lysozyme was performed. Peptidoglycan was resuspended in phosphate buffer (0.1 M, pH 6.2) in a concentration of 1 mg·mL^{-1}. Lysozyme was added in a concentration of 50 µg·mL^{-1} and incubated under constant shaking at 37°C for 24 h. The reaction was stopped by incubation at 100°C for 15 min. Uncleaved insoluble material was removed by centrifugation at 10000 x *g* for 5 min and the muropeptide containing supernatant was applied to analytical as well as immunological investigations.

3.2.6.4 Spore coat protein extraction

Extraction of the highly cross-linked protein coat of bacterial spores was performed under denaturating conditions according to Henriques et al [159]. Purified spores were sedimented and resuspended in extraction buffer, containing SDS and β-mercapthoethanol. Spores were boiled for 10 min in this buffer, mixed strongly and the extraction was repeated. Spores were centrifuged at 5000 x *g* for 5 min and the supernatant was transferred to centrifugal ultrafiltration devices. Centrifugation at 3000 x *g* for 20 min removed SDS by extensive filtration. The concentrated sample was analyzed by SDS polyacrylamide gel electrophoresis (PAGE) and for immunological activation of cells after estimation of protein content according to Bradford.

3.2.6.5 Isolation of lipoproteins

Bacillus licheniformis was grown in 8 L of LB medium. To gain the biomass the culture was centrifuged and the sediment was washed three times with PBS. Biomass was transferred into plastic tubes and mixed with an equal volume of 0.1 mm glass beads (endotoxin-free). Tubes were mixed intensively three times for 5 min to break the cells, centrifuged and the supernatant was exchanged for new buffer. Breaking of cells was repeated for another three cycles, glass beads were removed completely and centrifugation was performed at 48000 x *g* for 1 h. The sediment was dissolved in PBS and extracted with chloroform:methanol 2:1 (v/v) until the water phase (upper phase) was clear. This phase was freed of organic solvent and lyophilized subsequently. The sample was sonicated in octyl β-D-glucoside buffer and boiled in a water bath for 6 min prior to further purification by a centrifugation step at 27000 x *g* for 30 min. The supernatant was filtered through a 0.45 µm filter and concentrated in an amicon device using an YM-10 membrane. The same device was used to remove salt and detergent by repeated addition of endotoxin-free water. The remaining supernatant was freeze-dried, dissolved in 25 mM octyl β-D-glucoside solution and investigated further (e.g. by proteinase K digestion (3.2.6.6)) or purified continuously by SDS-PAGE (3.2.7.1) and reversed-phase chromatography (3.2.6.8).

Octyl β-D-glucoside buffer	50 mM octyl β-D-glucoside in PBS

3.2.6.6 Proteinase K digestion of lipoproteins

To purify lipopeptides and to test whether the analyzed TLR2 function was due to lipoprotein interaction, proteins were cleaved by proteinase K digestion [160]. Therefore, sample material was dissolved in proteinase K buffer and 10 $\mu g \cdot mL^{-1}$ proteinase K was added. The digestion was performed at 65°C for 90 min. The resulting amino acids were removed by repeated centrifugal ultrafiltration. Because the active center of TLR2-activating lipoproteins is not digested by proteinase K other proteins can be excluded as TLR2 ligands in this way (Fig. 3.3).

Proteinase K buffer	10 mM sodium acetate, 5 mM calcium acetate

3.2.6.7 Mild alkaline hydrolysis and thioether oxidation of lipoproteins

To prove whether isolated proteins were active due to the content of lipoproteins mild alkaline hydrolysis of extracts were performed to cleave the active center of lipoproteins responsible for the interaction with TLR2 [73]. The sample was dissolved in H_2O in a concentration of 5 $\mu g \cdot \mu l^{-1}$. Sodium hydroxide was added to a final concentration of 0.2 M, and hydrolysis was achieved by constant shaking at 37°C for 16 h. Cleavage of fatty acids in Pam_3CSK_4 was applied as a positive control for the reaction (Fig 3.3). Alternative the oxidation of the thioether to the sulfoxide by an incubation at 37°C for 24 h in the presence of 1% hydrogen peroxide (v/v) led to an abolishment of the lipopeptide and lipoprotein interaction with TLR2 [64].

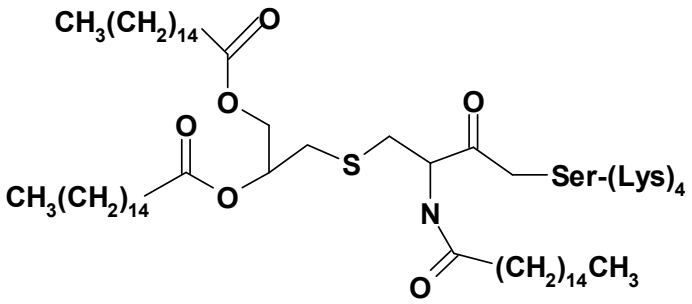

Figure 3.3: Structure of synthetic Pam_3CSK_4. The removal of the fatty acids by mild alkaline hydrolysis as well as the oxidation of the thioether to a sulfoxide are selective methods to abolish the recognition of lipoproteins and lipopeptides by TLR2 [64;71].

3.2.6.8 Reversed-phase chromatography of lipoprotein extracts

Toll-like receptor 2 stimulating fractions obtained by lipoprotein isolation techniques were solved in 25 mM octyl β-D-glucoside in PBS and applied on a Nucleosil® C_{18} reversed-phase column (Nucleosil®, 120-5, 250 x 4.6 mm). In this way the acyl chains of lipoproteins interacted with the hydrophobic stationary phase and were separated from globular, hydrophilic proteins [161;162]. Fractionation was achieved by the following program. The sample was equilibrated and applied in buffer A and kept for 5 min. A gradient was mixed to 100% buffer B up to a time point of 120 min. This condition was retained for 20 min followed by column equilibration in buffer A. The flow rate was set to 0.6 $mL \cdot min^{-1}$. Fractions were collected first every 10 min and then for every 2 min to locate the elution time of the component of interest. Collected fractions were screened for activation of TLR2 in transfected HEK-293 cells and by SDS-PAGE investigation as well as other analytical methods.

Buffer A	5% (v/v) N,N´-dimethylformide, 95% (v/v) H_2O
Buffer B	5% (v/v) N,N´-dimethylformide, 95% (v/v) 2-propanol

3.2.6.9 Isolation of amphiphilic molecules

Bacillus licheniformis was grown in LB medium and was harvested before the end of exponential growth. Approximately 15 g of wet cells were obtained after washing twice in PBS and once in citrate buffer. The cells were dissolved in extraction buffer and were mixed in homogenization bottles with an equal volume of endotoxin-free glass beads (0.1 mm). Disruption of cells was performed by three cycles in a homogenizer (4°C, 5 min). The cells were centrifuged at 5000 x *g* for 15 min and the supernatants of the bottles were combined. Sediments were dissolved in the same volume of extraction buffer and breakage of cells was repeated. Afterwards the cells were washed extensively with buffer to remove glass beads and dissolved in the supernatant of both extractions. An equal amount of n-butanol was added to the collected cell walls and the mixture was stirred at 4°C for 14 h. The extract was transferred to centrifuge tubes and 13000 x *g* were applied for 20 min to separate the organic from the aqueous phase. The aqueous phase was collected and dialyzed against 50 mM ammonium acetate (pH 4.7). By repeated lyophilization the volatile ammonium acetate was removed and freeze dried material was resuspended in chromatography buffer. The insoluble material was centrifuged at 14000 x *g* for 5 min and filtered prior to chromatographic separation on an octyl-sepharose column (3.2.6.10). Fractions of 5 min were collected, the UV absorption at 254 nm was monitored and phosphate was determined (3.2.7.7) to localize the elution of LTA as a reference for the reproduction of several runs. Fractions of interest were collected, lyophilized to remove ammonium acetate and analyzed further by SDS-PAGE or stimulation of immune cells [163].

Citrate buffer	51% (v/v) 0.1 M citric acid, 49% sodium citrate dehydrate 0.1 M, pH 4.6
Extraction buffer	Complete protease inhibitor cocktail dissolved in citrate buffer

3.2.6.10 Separation of amphiphilic molecules on an octyl-sepharose column

For the separation of amphiphilic molecules such as LTA and lipoproteins an octyl-sepharose column was packed [163]. The column was equilibrated with buffer A at a flow rate of 0.5 $mL \cdot min^{-1}$ under low pressure conditions. The same buffer was used for dissolving the extract following the extraction procedure according to Morath et al. [163]. Molecules showing no interaction with the stationary phase were eluted in an 80 min step with buffer A. During 160 min a linear gradient of increased the percentage of buffer B from 0 to 100% and the column was washed for another 80 min with buffer B before equilibration with buffer A was started to prepare the next run. Fractions were collected as described before (3.2.6.9).

Buffer A	0.1 M ammonium acetate, 15% 1-propanol, pH 4.7
Buffer B	0.1 M ammonium acetate, 60% 1-propanol, pH 4.7

3.2.6.11 Isolation of membrane proteins

Bacillus licheniformis was grown in LB^+ medium to the exponential phase either for the isolation of membrane proteins of vegetative bacteria or to sporulate. Afterwards the spores were purified (3.2.1.3). The cells were washed in TN buffer by repeated centrifugation (5000 x *g*, 4°C, 15 min) until the supernatant was clear. Resuspension in TNC buffer (4°C) and mixing with equal amounts of 0.1 mm glass beads were necessary to break the cells by vigorous shaking. Cells were lyzed three times for 5 min per tube, the content of each tube was transferred to a centrifuge bottle and the sediment was obtained after applying 5000 x *g* for 15 min. The supernatants were collected, sediments resuspended and the cell breakage was repeated. Prior to ultracentrifugation the collected supernatants obtained by breaking of spores were cleared by centrifugation at 10000 x *g* for 45 min. Membranes were enriched by centrifugation at 180000 x *g* at 4°C for 1 h and washed three times in buffer [78]. The supernatants were collected and the sediments were dissolved in TNP buffer by sonication and by repeated passages through a fine syringe needle. Membrane preparations were investigated for their hydrogenated nicotinamide adenine dinucleotide (NADH)-oxidase activity to specify the isolations to be membranes (3.2.7.5), analyzed directly by SDS-PAGE or extracted by TNPT buffer. Membrane fractions were resuspended in TNPT buffer in a centrifuge tube and stirred at 4°C for 18 h. Four volumes of ice cold acetone were added and proteins were precipitated for 4-6 h

followed by centrifugation at 5000 x *g* and 4°C for 30 min. The supernatant contains lipids and excess of Triton X-100. Proteins were resuspended in TNC buffer or H_2O containing protease inhibitors.

TN buffer	20 mM Tris-HCl, pH 8, 100 mM NaCl
TNC buffer	TN buffer + complete proteinase inhibitor cocktail
TNP buffer	TN buffer + 1 mM PMSF
TNPT buffer	TNP buffer + 1% (v/v) Triton X-100

3.2.6.12 Purification of membrane preparations by density gradient centrifugation

Ultracentrifugation tubes were filled with the following amounts of sucrose in 10 mM Hepes to assemble a step gradient. The first layer consisted of 5.4 mL of sucrose (2.02 M), overlaid by 18.5 mL of 1.44 M sucrose and 13.1 mL of 0.77 M sucrose. Centrifugation was performed in a swing out rotor at 20000 x *g* and 4°C for 15 h. Purified cytoplasmic membranes were enriched between the upper two levels of sucrose.

3.2.6.13 Triton X-114 phase separation

The membrane protein fraction was resuspended in TNP buffer and 2% (v/v) Triton X-114 was added. Extraction was stirred at 4°C for 16 h followed by incubation at 37°C for 30 min. This temperature change allowed a phase separation between the aqueous and the Triton X-114 phase [(164)]. Both phases were separated by an additional centrifugation step (3200 x *g*, 30 min) and the phases were washed twice with 2% Triton in TNP buffer. The proteins were precipitated with acetone and the pellet was resuspended and dialyzed against endotoxin-free water.

3.2.6.14 Preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis

To isolate and investigate single proteins from a protein extract preparative SDS-PAGE was performed. Thereto, up to 200 µL protein extracts were mixed in equal amounts with sample buffer (3.2.7.1.1), heated at 100°C for 5 min and filled in one pocket of a preparative stacking gel. A low molecular mass marker was added to a second pocket. Samples were separated as described in the analytical part at 50 V for 20 min and at 200 V for an extra hour. To control the run, marker bands and at least a part of the preparative

pocket was stained with Coomassie Brilliant Blue and the band of interest was excised and purified further by electroelution (3.2.6.15).

3.2.6.15 Electroelution of proteins

Protein bands were electroeluted in a Biotrap® system of SCHLEICHER & SCHUELL. The protein of interest was separated by SDS-PAGE and stained at least partly with colloidal Coomassie (3.2.8.4). Stained bands were excised from the gel, destained with Rotiphorese buffer and cut into small pieces, prior to application to the Biotrap® device. The electroelution chamber had to be bordered by BT1 membranes (cut off: 3-5 kDa) as well as by BT2 membranes to fix the gel. Membranes needed to be moist by adjustment of electroelution buffer before the gel is inserted. One to three Biotrap® devices were placed into the electrophoresis chamber and electrophoresis was run at 100 V for 24 h. To detach proteins from the BT1 membrane polarization of the electrophoresis chamber was reverted for 20 s and voltage was increased to 200 V. The electroeluate was transferred immediately to a reaction tube, desalted by ultrafiltration in MICROCON centrifugal filter devices, controlled by SDS-PAGE and applied for further analyses.

Electroelution buffer	25 mM Tris-HCl, 192 mM glycine, pH 8.3

3.2.7 Analytical techniques

3.2.7.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Electrophoretic separation of proteins by gel electrophoresis was performed as a discontinuous SDS-PAGE [165]. Different gel sizes were prepared in a BIORAD (8 x 10 cm) and in a HOEFER system (16 x 18 cm). The concentration of acrylamide was optimized for the separation of membrane isolations to 13% acrylamide monomer (%T) and a percentage of 3.3% bisacrylamide cross-linker (%C). The resolving gel was prepared as follows, cast between glass plates and polymerized for 30 min. The stacking gel (4%T) was poured on top of it.

Gel formulations	
Resolving gel (13%T) (10 mL)	Volume [mL]
Acrylamide/ Bisacrylamide 30%T, 3.3%C	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 (4%T) 10 mL	
Acrylamide/ Bisacrylamide 30%T, 3.3%C	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

3.2.7.1.1 Sample preparation

Protein extracts were quantified due to their protein content by Bio-Rad protein assay. One to five µg of protein were mixed with equal amounts of sample buffer, centrifuged, heated to 100°C for 5 min, centrifuged again and loaded onto a gel.

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

The gel chamber was filled with 500 mL of running buffer prior to loading the sample material. Gels were run at 22°C and 50V for about 20 min to collect proteins at the border between stacking and resolving gel. Once they reached this point the voltage was increased to 200 V and the run was stopped as soon as the bromophenol blue front ran out of the gel.

Running buffer 25 mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3

3.2.7.2 Two-dimensional gel electrophoresis (2D GE)

Two-dimensional gel electrophoresis (2D GE) was performed using WITAvision equipment in cooperation with Nina Grohmann and Dr. Christian Alexander (Research Center Borstel) to separate protein extracts according to charge and molecular mass of the proteins [166]. For the first dimension a non equilibrium pH gradient electrophoresis (NEPHGE) was executed to separate the proteins according to the charge under native conditions. The second dimension was a denaturating SDS-PAGE (3.2.7.1). Isoelectric focusing (IEF) was replaced by NEPHGE to avoid non-satisfying separations caused by basic proteins not being able to penetrate the gel and resulting in non satisfying separations. Using NEPHGE the equilibrium of the pH cannot be obtained and therefore the proteins do not precipitate at their pI, but separate according to their protein charge. All buffers and solutions were purchased from WITA GmbH.

3.2.7.2.1 Sample preparation

Protein extracts were lyophilized in aliquots of 300 µg after acetone precipitation. The dried material was dissolved in 100 µL of ultra-pure water and purified by Ready-Prep™ 2D Cleanup Kit according to the manufacturer's instructions. Samples were freeze dried for storage or dissolved directly in 30 µL AP-HAT buffer at 25°C for 1 h, by mixing every 10 min. By centrifugation at 4°C and 50000 x *g* for 30 min insoluble material was removed and 20 µL of the supernatant were mixed with 80 µL of Agarose-AP-HAT buffer at a temperature of 65°C. The mixture was applied to the 1st dimension.

Ready-Prep™ 2D Cleanup Kit	BIORAD

3.2.7.2.2 First dimension: separation of proteins according to their charge differences (NEPHGE)

First dimension NEPHGE gels were prepared in glass tubes and fixed in a casting chamber. A Sep-gel solution was mixed with 2.5% (v/v) APS solution (0.8% v/v), degassed for 4 min, and filled to the first line of the glass tube. Subsequently, glass tubes were filled in the same way with Cap-gel solution until the second mark was reached. Gels were then polymerized for 30 min. On top of the glass tube 25 µL of water were placed to form a gel pocket. Gel tubes were closed and polymerized for 3 days.

The run was initiated by applying glass tubes to an electrophoresis chamber, the Cap-gel side of the glass tube facing down. The cathode solution was poured into the lower chamber. Sample material was mixed with melted Agarose-AP buffer (1% w/v), applied to the gel pocket, cooled for polymerization and covered with the overlay solution. Afterwards the gel was covered with anode buffer in the upper part of the chamber and electrophoresis was performed according to the following program (Table 3.3).

Table 3.3: Electrophoresis conditions for NEPHGE gels

Voltage [V]	time [min]
100	75
200	75
400	75
600	75
800	10
1000	5

The gel was transferred to equilibration buffer containing 1% (w/v) DTT and incubated under five changes of the buffer for 20 min. Application of the tube gel to the pocket of a 12.5% SDS gel for the 2nd dimension followed. This gel was covered with 1.5 mL of 1% agarose containing bromophenol blue to assign the run.

3.2.7.2.3 Second dimension: SDS-PAGE for the separation of proteins according to their molecular mass

The electrophoresis chamber was filled with running buffer as described under point 3.2.7.1 and the following program was started (Table 3.4):

Table 3.4: Conditions for the 2nd dimension of the 2D electrophoresis

Voltage [V]	time [min]
35	5
55	10
100	15
150	60

Separated proteins were then fixed in the fixative solution of the appropriate silver-staining method modified according to Vorum et al. (3.2.8.3) for further analyses by peptide mass fingerprint, or according to Heukeshoven and Dernik for more sensitive analytical purposes (3.2.8.2).

3.2.7.3 Electrotransfer of proteins to polyvinylidene fluoride (PVDF) membranes (Western blot)

Proteins were transferred from the resolving gel to a PVDF membrane for further analyses by Edman-sequencing and for the lectin blot using concavalin A. After SDS-PAGE was finished the gel was equilibrated in transfer buffer. The same was done with fiber pads and filter papers as well as with the methanol-activated PVDF membrane (soaked for 20 s). The transfer stack was assembled by placing the fiber pad, filter paper, gel, membrane, a second filter paper and a second fiber 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. The membrane was later stained by Coomassie Brilliant Blue R250 for Edman-sequencing of proteins (3.2.7.4) or by a lectin blot (3.2.8.8).

Transfer buffer	25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3

3.2.7.4 Edman *N*-terminal sequencing

Separated proteins were transferred by Western blot onto a PVDF membrane as described in section 3.2.7.3. The blot was stained with Coomassie Brilliant Blue R 250 (3.2.8.4) and protein bands were excised and transferred to a reaction tube. This membrane was applied to a Blot™ cartridge reaction chamber optimized to facilitate the reaction according to Edman in the Procise™ Protein Sequencing System. All reagents and solvents were supplied by APPLIED BIOSYSTEMS.

Protein degradation was performed *N*-terminally. The phenylisothiocyanate (PITC) reagent was vaporized in the reaction chamber to react with the free amino groups under basic conditions. Ethyl acetate and butyl chloride were used to remove by-products prior to cleavage of the modified amino acid by trifluoroacetic acid hydrolysis forming an anilinothiazolinone (ATZ) derivative and leaving a protein with a new free *N*-terminus.

The derivatized amino acids were extracted by 1-chlorobutane and ethyl acetate. The ATZ-amino acids were then converted in the presence of 25% trifluoroacetic acid (v/v) in water at 64°C for 9 min to a more stable phenylthiohydantoin (PTH) derivate. The reagent was evaporated, the dried PTH amino acid was dissolved in aqueous acetonitrile (20% v/v) and the products were transferred to HPLC analysis in a reversed-phase system. The PTH amino acids were identified by comparison to authentic standards. Proteins were identified by the application of sequencing results in the ExPASy database (http://expasy.org/tools/blast/).

3.2.7.5 Nicotinamide adenine dinucleotide oxidase assay

Hydrogenated nicotinamide adenine dinucleotide-oxidase is an enzyme located in the cytoplasmic membrane of bacteria. To prove whether the isolated membranes (3.2.6.11) existed in a native form, NADH in its reduced form was dissolved in a concentration of 12 mM in Tris-HCl (pH 7.5) supplemented with 0.02 mM DTT. Membrane fractions were added to a mixture of 990 µL buffer and 10 µL NADH. The decrease of NADH absorption (λ = 340 nm) was measured. A membrane preparation of *E. coli* was used as positive and the heat-inactivated membrane fraction (100°C, 5 min) as negative control.

3.2.7.6 Bradford protein detection assay

Proteins were detected and quantified by BioRad protein assay based on the principle of the Bradford test [167]. The absorption maximum of Coomassie Brilliant Blue G 250 is shifted from 465 to 595 nm when binding to a protein. Bovine serum albumine was used as standard in concentrations of 0 to 20 $\mu g \cdot mL^{-1}$. The standard and the sample were pipetted to a reaction vial and filled up to 800 µL with water or buffer. After 200 µL of premixed BioRad protein assay reagent were added, samples were mixed vigorously and incubated at 25°C for 15 min. Absorption was measured at 595 nm and protein concentrations were calculated according to the standard curve. Samples of known protein content were analyzed further by gel electrophoresis or were investigated for their potential to stimulate cells of the immune system.

3.2.7.7 Determination of phosphate

The phosphate measurement [168] was performed to control the lipoprotein isolation according to the purification protocol for amphiphilic molecules (3.2.6.9). The fractions collected after separation on octyl-sepharose were analyzed directly in the assay. Sample

material (50µL) 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 (5mM) were compared as standard. The dried sample material was digested by addition of 100 µL hydrolyzing solution, incubated at 100°C for 1 h and then heated to 165°C for additional 2 h. The glass tubes were cooled to 25°C and 1 mL of precooled staining solution was added (4°C). The samples were incubated at 37°C for 90 min prior to photometric quantification at 820 nm.

Hydrolyzing solution	6.7 mL aqueous perchloric acid (70% w/v), 30.6 mL sulfuric acid, fill to 100 mL H_2O
Staining solution	1 mL sodium acetate 1 M, 1 mL ammonium heptamolybdate (2.5% w/v), 1 mL ascorbic acid (10% w/v) cooled to 4°C), 7 mL H_2O

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

3.2.8.1 Silver staining for the detection of proteins

The silver staining method according to Blum et al. [169] was optimized for the sensitive detection of proteins. Gels were fixed in fixative solution for 30 min, washed twice in 30% ethanol and once in water, each time for 20 min. Sensitization by sodium thiosulfate for 1 min, was followed by 3 washing steps with water (each 20 s) and a 20 min incubation in silver nitrate reagent. The staining was developed after removal of excess silver nitrate until bands were visible and clearly differentiable from the background. The reaction was stopped by addition of glycine.

Fixative solution	40% methanol (v/v), 10% acetic acid, 50% deionized water
Sensitizer	0.02% (w/v) sodium thiosulfate in deionized water
Silver nitrate reagent	0.2% (w/v) silver nitrate, 0.02% (v/v) formaldehyde (37% v/v)
Developer	3% (w/v) sodium carbonate, 0.05% formaldehyde (37% v/v), 2.5 % (v/v) sensitizer
Stop reagent	0.5% (w/v) glycine

3.2.8.2 Silver staining for 2D gels

Two-dimensional gels were stained by the following procedure to detect trace amounts of proteins [170]. Gels were fixed in fixative solution for 30 min and then incubated for 24 h, washed three times for 30 min in water and stained for 1 h in silver nitrate-reagent. The development was followed by incubation for up to 10 min before the stop solution was added. Gels were washed three times with H_2O and incubated in glycine buffer, to complete the reaction.

Fixative solution	30% ethanol (v/v), 10% acetic acid (v/v)
Incubation-reagent	0.2% (w/v) sodium thiosulfate, 0.5% glutaraldehyde (v/v), 0.5 M sodium-acetate, 30% (v/v) ethanol
Silver nitrate reagent	0.1% (w/v) silver nitrate, 0.02% (v/v) formaldehyde (37% v/v)
Developer	2,5% (w/v) sodium carbonate, 0.01% formaldehyde (37% v/v)
Stop-reagent	50 mM EDTA

3.2.8.3 Vorum silver staining for peptide mass fingerprint analyses

This modification of the silver staining was improved for the combination with MALDI-TOF analyses [171]. Gels were fixed for at least 2 h and washed in 35% (v/v) ethanol three times for 20 min. Sensitization with thiosulfate followed for 2 min. Excess was removed by washing three times with water and the gel was stained for 20 min. After removal of free silver nitrate staining was developed and stopped by a pH-shift to pH 3, as soon as proteins were visible. For protein identification the gels were stored in 1% acetic acid, spots were picked and purified for the analyses by matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS).

Fixative solution	50% methanol (v/v), 12% acetic acid, 0.05% (v/v) formaldehyde (37% v/v)
Sensitizer	0.02% (w/v) sodium thiosulfate in deionized water
Silver nitrate reagent	0.2% (w/v) silver nitrate, 0.076% (v/v) formaldehyde (37% v/v)
Developer	6% (w/v) sodium carbonate, 0.05% formaldehyde (37% v/v), 2.5% sensitizer
Stop reagent	50% (v/v) methanol, 12% acetic acid

3.2.8.4 Colloidal Coomassie staining

Gels were stained by colloidal Coomassie Brilliant Blue staining to detect specifically proteins based on the interaction of the dye with the amino functions of proteins. Isopropanol and acetic acid were added to precipitate the proteins during staining. Solutions 1 and 2 were applied for 30 min each and solution 3 for 1 to 24 h. In this way background staining was minimized and destaining with 10% (v/v) acetic acid is hardly necessary.

Staining solution:

1	25% (v/v) 2-propanol, 10% (v/v) acetic acid, 0.03% (w/v) Coomassie Brilliant Blue R 250
2	10% (v/v) 2-propanol, 10% (v/v) acetic acid, 0.03% (w/v) Coomassie Brilliant Blue R 250
3	10% (v/v) acetic acid, 0.03% (w/v) Coomassie Brilliant Blue R 250

3.2.8.5 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 mangenta colored imine [172]. Gels were fixed in fixative/destaining solution for 1-2 h, incubated in periodic acid reagent for 1 h and treated with sodium metabisulfite. The gel appeared yellowish and destained in the same solution after ongoing treatment. Subsequently Schiff's reagent was added for incubation until the carbohydrate containing bands were stained mangenta. Destaining followed to reduce the background color.

Fixative/destaining solution	10% (v/v) acetic acid, 35% methanol in water
Periodic acid reagent	0.7% (w/v) periodic acid in acetic acid:water (5:95 v/v)
Sodium metabisulfite solution	0.2% (w/v) sodium metabisulfite in acetic acid:water (5:95 v/v)
Schiff's reagent	premixed (SIGMA)

3.2.8.6 Coomassie staining of Western blots

Following the transfer of proteins to PVDF membranes (3.2.7.3) the membrane was placed in staining solution for 2 min, washed with ultra-pure water and placed in destain solution I under agitation. To completely remove the background the solution was exchanged with destain solution II for about 10 min.

Staining solution	0.1% (w/v) Coomassie brilliant blue R 250 in 50% (v/v) methanol, 7% (v/v) acetic acid
Destain solution I	50% (v/v) methanol, 7% (v/v) acetic acid
Destain solution II	90% (v/v) methanol, 10% (v/v) acetic acid

3.2.8.7 Reversible Ponceau-S red staining of blot membranes

After Western blot the proteins were stained with Ponceau-S red to control the efficiency of protein transfer. Thereto, dry blots were soaked in methanol and covered with stain under agitation for 1 min. The background was destained by rinsing in ultra-pure water. Afterwards the blot was scanned and the stain was completly removed by washing with 0.1 M sodium hydroxide. Subsequently, the blotting membrane was stained with the lectin concanavalin A (3.2.8.8).

Stain	0.2% (w/v) Ponceau-S red, 3% trichloroacetic acid, 30% (w/v) sulfosalicylic acid) in 1% acetic acid

3.2.8.8 Lectin blot

Lectin blots were performed to investigate glycoproteins conjugated with mannose or glucose residues. After blotting the membrane was washed in TBST buffer and incubated with 100 µg of HRP-conjugated lectin under constant shaking for 60 min. The lectin was removed by washing with water and the blot was stained in the dark for 15 min. The membrane was washed and documented by scanning.

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
Staining solution	7 mL 4-Chloro-1-naphthol (3mg·mL^{-1} in methanol), 39 mL H_2O, 2 mL Tris (0.2 M, pH 7.5), 20 µL H_2O_2

3.2.9 Peptide mass finger print

Separated proteins after 1 or 2 dimensional gel electrophoresis were identified by peptide mass fingerprinting (PMF) [173] in cooperation with Helga Lüthje and PD Dr. Buko Lindner (Research Center Borstel). Picked protein spots from SDS-PAGE were digested enzymatically by Trypsin cleaving the C-termini of the amino acids lysine and arginine. Thereto, picked gel spots were washed and covered with acetonitrile to achieve a shrinking of the gel spot. Drying of gels and rehydration in wash solution was performed to remove Coomassie R 250 completely. Incubation in DTT solution at 56°C for 45 min was followed by a 30 min alkylation step using iodacetamide solution 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 resuspended 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 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 center for biotechnology information (NCBI)-database (www.mascotscience.com).

Wash solution	100 mM ammonium bicarbonate in H_2O / acetonitrile 1:1
DTT solution	10 mM dithiothreitol, 100 mM ammonium bicarbonate in H_2O
Iodacetamide solution	55 mM iodaceteamide, 0.1 M ammonium bicarbonate in H_2O
Trypsin solution	50 mM ammonium bicarbonate in H_2O + 12.5 $ng \cdot \mu L^{-1}$ trypsin
TA solution	Trifluoroacetic acid 0.1%:acetonitril 2:1

3.2.9.1 *O*-Deacylation of lipoproteins prior to peptide mass fingerprint

Due to the hydrophobic interaction of fatty acids and SDS gels the *O*-deacylation of identified lipoprotein spots was performed to increase the yield of peptides eluting from the gel. Therefore, picked spots were incubated in 12% aqueous ammonia at 37°C for 16 h as described for the *O*-deacylation of LPS [174]. The treatment was continued according to the procedure of PMF (3.2.9).

3.2.10 Gas chromatographic investigations

3.2.10.1 Methanolysis

Bacterial cells as well as extracted peptidoglycan material were hydrolyzed in methanolic hydrochloric acid in a sealed glass tube. Two kinds of hydrolysis have to be differentiated. A weak hydrolysis using 0.5 M methanolic hydrochloric acid at 85°C for 1 h and a strong methanolysis in 2 M methanolic hydrochloric acid for 16 h. After hydrolyzation the glass tubes were cooled to 25°C, opened and their content was transferred to glass vials. The mixture was evaporated and peracetylated by the addition of 100 µL water free acetanhydride followed by incubation at 85°C for 15 min. The reagents were dried under nitrogen and 100 µL of pyridine were mixed with 50 µL of acetanhydride in the sample containing vial. For peracetylation of the sample the vial was heated again to 85°C for 30 min. Samples were dried under nitrogen, solubilized in chloroform and transferred to a sample vial, which allowed easy injection of small amounts into the gas chromatograph.
Gas chromatographic separation was achieved on 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^{-1} to a final temperature of 320°C. Molecules were either detected by mass spectrometry or by flame ionization detection (FID).

3.2.10.2 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 altidol acetates [175]. 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 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 methylated monosaccharides (25°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 (3.2.10.1). 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^{-1}) 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 based on comparison of area under curve values to that of the internal standard allose.

3.2.10.3 Total fatty acids

Further investigations of fatty acids were performed according to Wollenweber et al. [176]. The sample material was 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 phases. The organic phases of repeated extractions were collected, dried over sodium sulfate and concentrated under nitrogen. Diazomethane was added dropwise 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 vials by solubilization 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 approved by a temperature gradient of 120°C for 3 min with a linear increase to 260°C (5°C·min^{-1}). Retention times were compared to known standard methyl esters of fatty acids.

3.2.10.4 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 (3.2.10.2), prior to the reduction of carbonyl groups by sodium borodeuteride treatment. Peracetylation was repeated to achieve acetylation of the free amino groups.

4. Results

4.1 *Bacillus* sporulation and purification

Bacillus growth and sporulation has been well described for the model organism *B. subtilis*. Factors initiating sporulation are nutrient limitation and high cell densities under cell culture conditions [112].

Therefore, different culture conditions were tested to find an optimal procedure for the sporulation of *B. licheniformis* isolated from farm children's (strain 467) and non-farm children's mattress dusts (strain 830) with the aim to establish a high yield production of the different microbial spores.

Adopted from Monteiro et al. [177] Difco Sporulation Medium (DSM) was tested first for its ability to induce an efficient sporulation of *B. subtilis*.

Table 4.1: Sporulation efficiency of *Bacillus subtilis* DSM 618 under different culture conditions. Used media contained DSM, 2x DSM, and 2x DSM supplemented with glucose (3.5 $g \cdot L^{-1}$), respectively. Cells were harvested after 72 h of cultivation and counted on agar plates before (total cfu) and after heat inactivation (80°C, 30 min) to inhibit growth of vegetative cells). The ratio of cfu after heat inactivation (spores) and total cfu is shown as sporulation efficiency [%]. The means of 3 experiments ± SD are depicted.

Medium	total cfu·mL^{-1} ± SD	cfu·mL^{-1} after heat inactivation ± SD	Sporulation efficiency [%]
DSM	$1.75 \pm 0.26 \cdot 10^7$	$1.05 \pm 0.36 \cdot 10^7$	60
2xDSM	$2.25 \pm 0.12 \cdot 10^7$	$1.81 \pm 0.42 \cdot 10^7$	81
2xDSM + Glc	$6.70 \pm 0.19 \cdot 10^7$	$5.84 \pm 0.34 \cdot 10^7$	89

The variations of the original DSM resuted in an increase in sporulation efficiency with the highest ratio applying the glucose-containing 2x DSM as described by Monteiro et al. [177].

Since the most important factor for further investigations of the spore-specificity was the spore purity, preparations were purified by density gradient centrifugation using 90% Percoll™ in 0.15 M NaCl [144]. Figure 4.1 depicts the results of spore purification after Wirtz-Conklin staining of the spore preparations shown in Table 4.1.

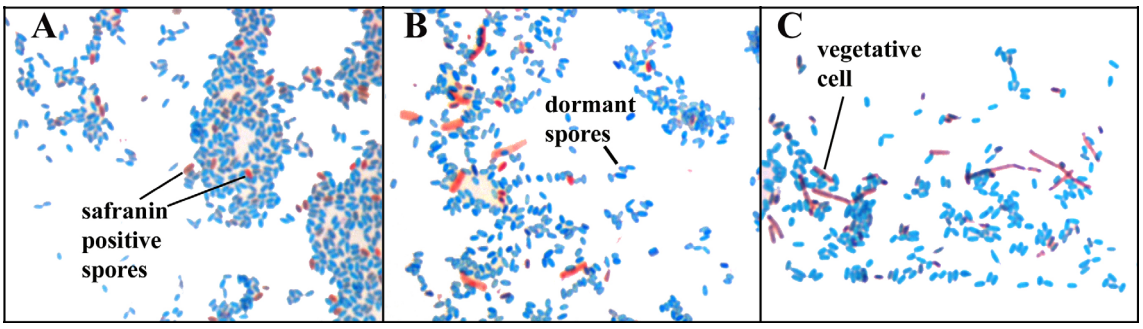

Figure 4.1: Wirtz-Conklin staining of *Bacillus subtilis* spore preparations. Spores were grown in DSM (**A**), 2x DSM (**B**) or 2x DSM + 3.5 g·L^{-1} glucose (**C**) for 72 h. The suspensions were stained on an object slide by malachite green. Dormant spores completing the program of sporulation were malachite green-positve. Spores stained safranin O red are unable to keep the malachite green stain, because of initiated germination or incomplete sporulation [152]. Rod shaped vegetative bacilli can be observed in panels **B** and **C**.

Such a purity control differentiated spores and vegetative cells of *B. subtilis*. Increasing nutrient concentrations (Fig. 4.1 B, C) and the addition of glucose (Fig. 4.1 C) resulted in incomplete purification of cell suspensions. As confirmed by the spore pellet size during the purification, plating cells after heat-inactivation of vegetative *B. subtilis* was an insufficient marker for the determination of pure spore preparations. The increase of nutrient concentrations did not result in higher concentrations of dormant spores, but in higher cell numbers resisting heat-inactivation. Thus, this method was not performed continuously to determine the efficiency of sporulation but was replaced by cfu determination after purification of bacterial suspensions and miscroscopic control.

An elongation of culture time up to three weeks increased the purity of spore preparations cultured in DSM. Apart from this a complete removal of vegetative cells by gradient centrifugation was not achievable after growth in media with increased nutrient concentrations.

Based on these results sporulation of *Bacillus* ssp. was also tested in LB medium (Lennox) and a minimal medium, to analyze whether these nutrient compositions afford pure spore preparations. The medium was supplemented with divalent cations such as Mg^{2+} (20 mg·L^{-1} MgSO4 x 7 H_2O) and Mn^{2+} (10 mg·L^{-1} $MnSO_4$ x H_2O) (LB^{+}) to increase bacterial sporulation [178]. The minimal medium was tested to clarify whether a limeted nutrient supply results in efficient induction of sporulation [141].

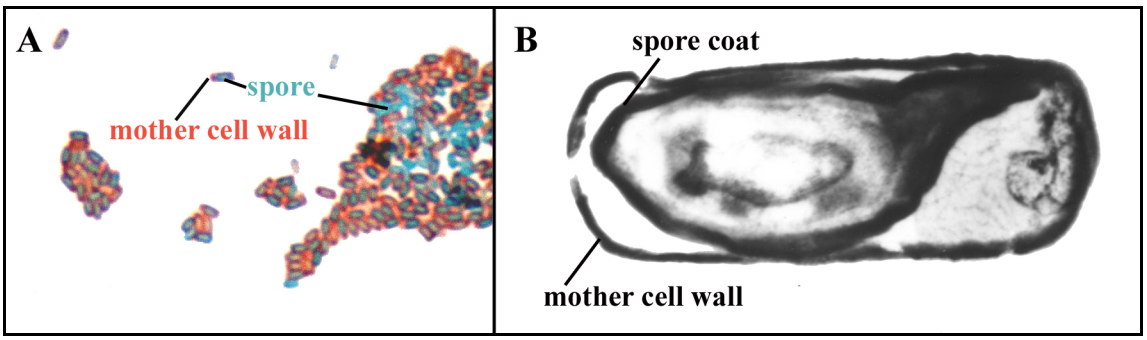

Figure 4.2: *Bacillus licheniformis* 467 preparation of incompletely sporulated cells. The formation of endospores is detectable in panel **A** by the Wirtz-Conklin staining of a *B. licheniformis* 467 spore preparation after growth in minimal medium and subsequent purification. Malachite green spores were surrounded by the safranin O red mother cell (magnification 1000x). Investigations of these endospores by electron microscopy allowed to distinguish the mother cell wall and the electron dense spore coat (**B**) (magnification 25000x).

The presence of incompletely sporulated, safranin-positve *B. subtilis* cells was not affected by any of these culture conditions. Their total depletion by gradient centrifugation was not possible due to marginal density differences to dormant spores. Additionally, in minimal medium the presence of endospores, surrounded by their mother cell wall, occurred. Similar results were obtained for the growth of *B. licheniformis*. An example of such preparations is shown in Figure 4.2 for *B. licheniformis* 467. Furthermore, growth in minimal medium gave the lowest yields of spores. Table 4.2 shows the results of *B. licheniformis* 467 growth and sporulation in the above mentioned media over three weeks.

Based on these data, *Bacillus licheniformis* 467 growth in LB^+ was suitable best to obtain the highest cfu numbers after purification of the spore preparations. Besides this vegetative cells were absent after purification.

Since the outcomes of purification were similar for *B. licheniformis* strains 467 and 830 when grown in LB^+ medium, following spore preparations were performed in LB^+ medium. Growth of *B. subtilis* DSM 618 resulted in higher spore cfu numbers when cultured in DSM. However, to minimize differences affected by medium variations all bacteria were grown in LB^+ medium for subsequent experiments.

Optimization and upscale of the method to an 8 L batch fermentation, gaining higher yields of *B. licheniformis* spores, were approached in a diploma thesis by Björn Alich [145]. Based on the results described above the fermentation process was optimized with regard to a constant pH-value and growth limitations like oxygen saturation. The results for

B. licheniformis 467 showed that an optimal cell growth to high cell densities coexistent with nutrient limitation at late time points of the culture was essential for optimal cfu numbers and spore purity within the shortened fermentation time of 30 h.

Table 4.2: Growth and sporulation of *Bacillus licheniformis* 467 cells in different media. Colony forming units after spore purification by density gradient centrifugation is shown as well as the relative amount of rod shaped vegetative cells after purification and density gradient centrifugation. Culture medium from shaking flasks (250 mL) were purified.

Media	*B. licheniformis* 467 spore cfu·mL^{-1} ± SD following purification	Relative amounts of vegetative cells
DSM	$1.24 \pm 0.26 \cdot 10^8$	—
LB$^+$	$1.99 \pm 0.55 \cdot 10^8$	—
MM	$5.80 \pm 4.35 \cdot 10^6$	10%

4.2 Immune stimulation of *Bacillus licheniformis in vitro*

4.2.1 Specificity of *Bacillus licheniformis* spore stimulation

For specific immunological investigations on the effects of bacterial spores inhaled to the lung it had to be considered that dormant spores may germinate in the lower airways.

In vitro experiments, performed under conditions without any nutrient limitation in the applied media needed to carefully attend germination and outgrowth in order to obtain spore-specific results. To address this question germination of spores was observed in Dulbecco's minimal essential medium as it was applied for *in vitro* experiments containing penicillin and streptomycin. *Bacillus licheniformis* strain 467 was chosen for these experiments. The germination was investigated by staining spores according to Wirtz-Conklin [(152)] after different incubation times at 37°C in the presence or absence of antibiotics. Germination occurred for a small percentage of spores, but their outgrowth to rod shaped vegetative cells was not observed in the presence of antibiotics (data not shown). Moreover spore cell density was not significantly decreased. In contrast, medium without antibiotics induced outgrowth after 3 h of cultivation.

Since the inhaled contact of spores with the epithelial layer appeared to be the first line of host defense [(179)] the epithelial cell line A549 was chosen to analyze whether incubation of these cells lead to a spore-specific cytokine expression profile *in vitro*.

To answer this question A549 cells were stimulated) *in vitro* with *B. licheniformis* 467 spores able to germinate (Blspo), hindered in germination by heat-inactivation (hiBlspo) and vegetative *B. licheniformis* cells (vegBl) (Fig. 4.3). Spores and vegBl differed significantly, whereas Blspo and hiBlspo induced the same level of IL-8 expression.

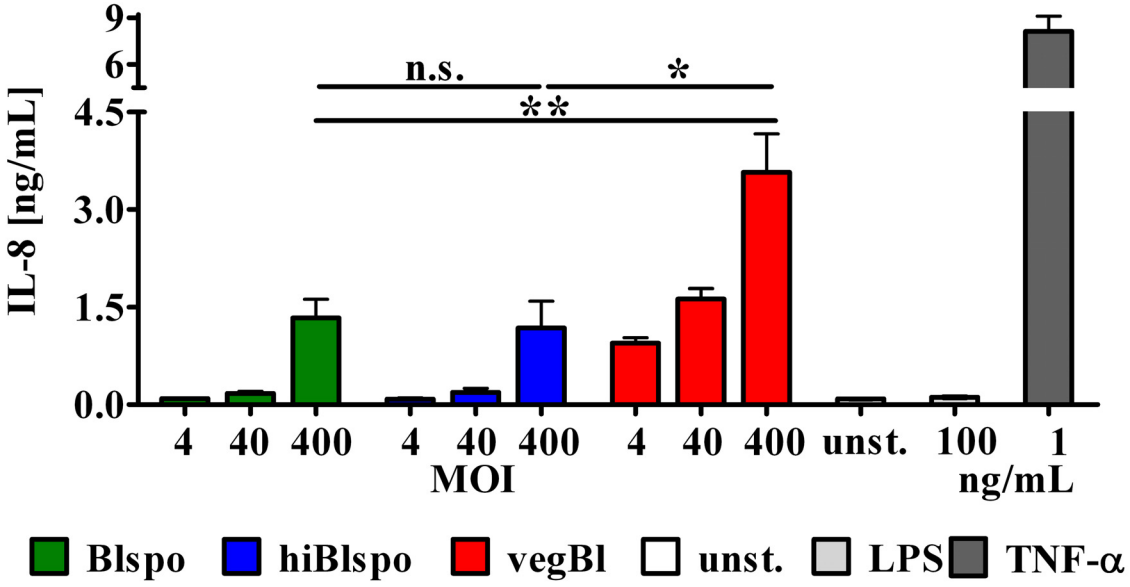

Figure 4.3: Specificity of A549 epithelial cell stimulation by *Bacillus licheniformis* 467. The expression of IL-8 after incubation with vegetative cells (vegBl), spores (Blspo) or heat-inactivated spores (hiBlspo) was measured in a dose dependent manner for 24 h. The ratio was 4, 40 and 400 bacteria per cell (MOI). Results show the means ± SEM of three independent experiments. The significance between stimulations with a MOI of 400 is indicated. Lower doses of stimulation resulted in comparable significances. The cells were unresponsive for the stimulation with LPS. Therefore, TNF-α was applied as a positive control. Unstimulated cells functioned as negative control. *$p < 0.05$, **$p < 0.01$.

To have a closer look on the interaction with epithelial cells, primary airway epithelial cells, isolated from lung biopsies, were cultured in air-liquid interface (ALI) cultures. Stimulation of these cells was performed obtaining similar results but showing higher variations between independent experiments (data not shown).

4.2.2 Activation of the innate immune system

Since the innate immune system is the next line to defend the host against inhaled bacteria, DCs presenting antigens and polarizing T-cells were important research objects in the context of allergic disorders [(180)].

4.2.2.1 Stimulation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were stimulated to investigate their cytokine expression in response to the stimulation with vegBl, Blspo and hiBlspo. These cells include important cells of the immune system e.g. precursors of moDCs and macrophages. They were investigated concerning their specificity for spore stimulation as well as for the optimal stimulation dose in further experiments. The TNF-α release after *B. licheniformis* stimulations at MOI of 0.1, 1 and 10 were measured (Fig. 4.4).

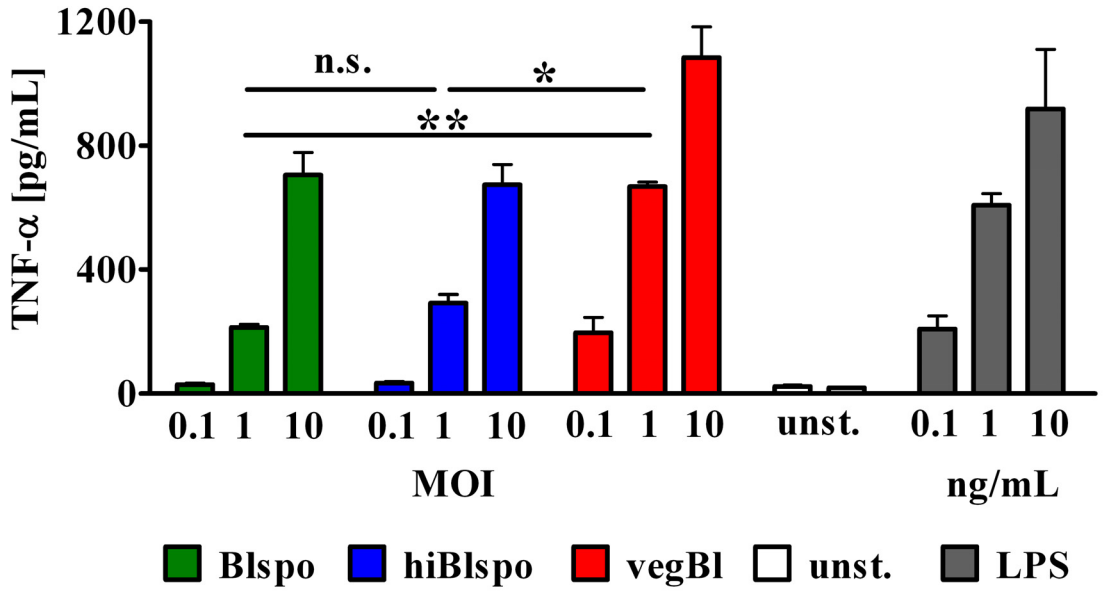

Figure 4.4: Stimulation of PBMCs by *Bacillus licheniformis* 467. Investigation of specific cytokine expression levels by spore stimulation. Blspo, hiBlspo and vegBl were compared. Cells isolated from different healthy donors were stimulated dose dependently ($1x10^6$ cells per well) at a MOI of 0.1, 1 and 10 for 24 h. Means of three independent experiments are depicted ± SEM. * $p < 0.05$, ** $p < 0.01$, unst.: unstimulated, n.s. not significant.

Significant differences between cells incubated for 24 h in the presence of spores or vegetative cells were calculated for values obtained at a MOI of 1. Vegetative bacteria induced a significantly higher level of TNF-α in the culture supernatant of PBMCs. A comparison of dose dependent PBMC and A549 cell stimulations resulted in similar significances for the different forms of *B. licheniformis* 467.

Based on these results further investigations addressing the *in vitro* stimulation of immune cells were performed in the tested medium at a MOI of 1.

4.2.2.2 Recognition of *Bacillus licheniformis* by pattern recognition receptors

The HEK-293 cell line was transiently transfected with the PRRs TLR2, TLR4, NOD1 and NOD2, in order to define the role of these receptors in the recognition of *B. licheniformis* by DCs. The Gram+ bacterium *B. licheniformis* stimulated TLR2 and NOD 2 (Table 4.3). Recognition by NOD1 was significantly lower as described by Girardin et al. [93]. Expression levels of IL-8 were comparable for Blspo and hiBlspo. The cytokine level induced by stimulation of vegBl was significantly higher than those induced by spores, as it was shown for PBMCs and epithelial cells. Moreover, vegBl induced exclusively the expression of IL-8 in TLR5 transfected HEK cells, recognizing bacterial flagellin. This activation was carried out qualitatively. Thus, it is missing in Table 4.3.

Furthermore, TLR4 which detects LPS was used to identify possible contaminations by low amounts of Gram- bacteria or LPS due to its high level induction of DC maturation and cytokine expression.

Table 4.3: Expression of IL-8 by transiently transfected HEK-293 cells. HEK-293 cells, transfected with expression plasmids for TLR2 and NOD2 were stimulated with *B. licheniformis* 467 preparations at a MOI = 1 for 24 h. Vegetative bacteria activated transfected HEK-293 cells stronger than spores did. The means ± SEM of 3 independent experiments are depicted. Pam_3CSK_4 was approached as positive control for stimulation of TLR2 transfected HEK-293 cells [70]. The positive control for the stimulation of NOD2 transfected cells was muramyl dipeptid (MDP) § [95].

HEK-293 cells expressing	HEK-293 cell expression of IL-8 [pg·mL^{-1}]				
	Blspo	hiBlspo	vegBl	unstimulated	positive control §
TLR 2	1245.5 ± 345.1	1253.2 ± 185.47	12365.5 ± 2273.3	8.2 ± 4.7	5234.5 ± 477.2
NOD2	305.0 ± 102.1	193.5 ± 7.5	1015.4 ± 81.9	156.3 ± 31.5	1103.6 ± 57.6

Comparable results for PBMC stimulation and interaction with innate immune receptors were achieved by stimulation with *B. licheniformis* 830 isolated from mattress dust of a child living in a non-farming environment. Similar findings were also obtained for *B. subtilis* strain DSM 618 (data not shown). For this reason further investigations to elucidate differences between Blspo and vegBl concentrated on strain 467. Moreover, the

similarity of Blspo and hiBlspo found in the *in vitro* experiments excluded the requirement of parallel experiments for both forms, since germination was neither observed microscopically nor on the level of cytokine expression.

4.2.2.3 Phagocytosis of *Bacillus licheniformis* by dendritic cells

Due to the significant differences in recognition by TLRs, bacterial phagocytosis was analyzed to explain the significant differences in cytokine expression by PBMCs.

To investigate phagocytosis vegetative bacteria and spores were labeled by incubation in FITC. Fluorescence activated cell sorting was performed in a time course. The cells were either left unstimulated or incubated with FITC-labeled Blspo and vegBl for up to 24 h, respectively. An example of the differences in moDC fluorescence before and after phagocytosis of FITC-labeled bacteria is depicted in Figure 4.5.

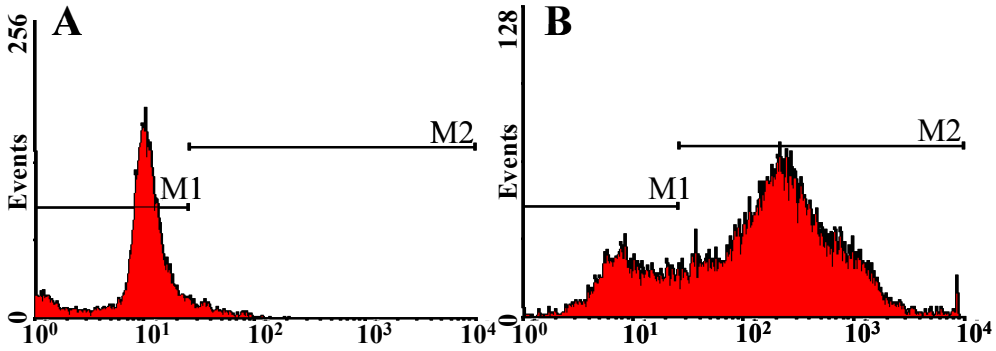

Figure 4.5: Fluorescence shift of moDCs, by phagocytosis of *B. licheniformis*. Part **A** shows the auto-fluorescence of unstimulated moDCs at 515 nm after excitation at 488 nm. Unstimulated cells were analyzed by FACS in order to set markers to quantify the relative fluorescence shift by phagocytosis of FITC-labeled bacteria (M1: auto-fluorescence of DCs, M2 fluorescence after uptake of FITC-labeled bacteria). In comparison part **B** depicts the fluorescence pattern after stimulation of DCs with FITC-labeled vegBl for 21 h.

The relative percentage of FITC-positive cells (marker M2 in Figure 4.5) was used as a measure for bacterial uptake by moDCs (Fig. 4.6 A). The proinflammatory cytokine IL-8 was measured in culture supernatants of the same experiments (Fig. 4.6 B).

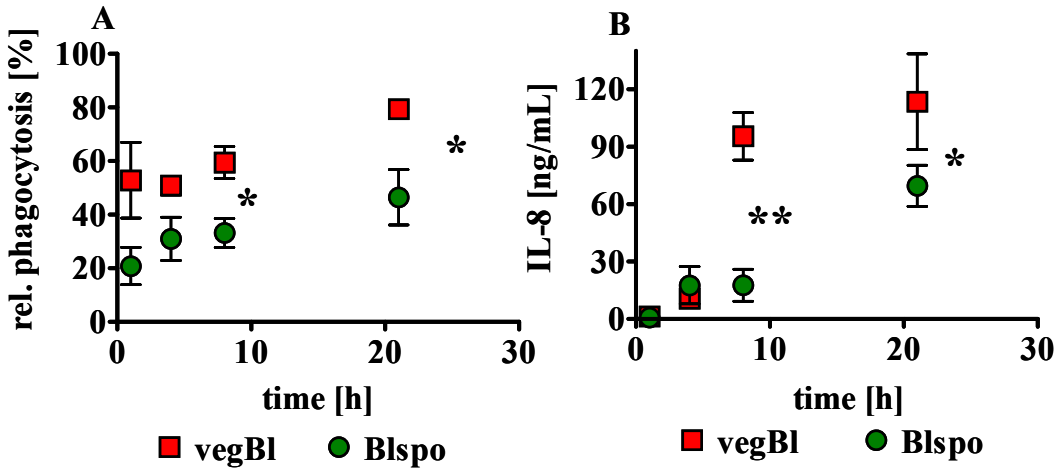

Figure 4.6: Relative phagocytosis of FITC-labeled Blspo and vegBl by moDCs. Cells were cultured for the indicated time in the presence of labeled bacteria. Percentages of FITC-positive DCs in FACS analyses are depicted in panel **A**. The culture supernatants of the same stimulations were analyzed for IL-8 expression by moDCs (**B**). The means determined in three independent experiments are shown ± SEM. $p < 0.05$, ** $p < 0.01$. Phagocytosis was controlled by confocal microscopy (Fig. 4.7).

Statistical differences between phagocytosis of Blspo and vegBl explained the differences in cytokine expression by the cell types stimulated. After 1 h of incubation more than 50% of the cells were detected to be FITC-positive when stimulated with vegBl. The maximum of 80% FITC-positive cells was reached after 21 h. In comparison, 20% of the cells phagocytozed Blspo after 1 h, increasing constantly to a maximum of 46% after 21 h. Panel B in Figure 4.6 shows the significant differences after 8 and 21 h of stimulation. The expression of IL-8 after stimulation with spores was delayed. An increase was observed after 8 h of incubation, whereas stimulation with vegetative bacteria resulted in a strong increase between 4 and 8 h of incubation. This early increase of cytokine expression was based on the high rate of phagocytosis reached after 1 h of stimulation.

To check whether fluorescence of FITC-positive DCs was due to uptake or attachment of bacteria to the cell surface, cells were investigated by confocal microscopy following stimulation with *B. licheniformis*. Thereto, cells were stained intra- and extracellularly with an anti-TLR2 antibody for visualization. The location of FITC-labeled bacteria was analyzed (Fig. 4.7).

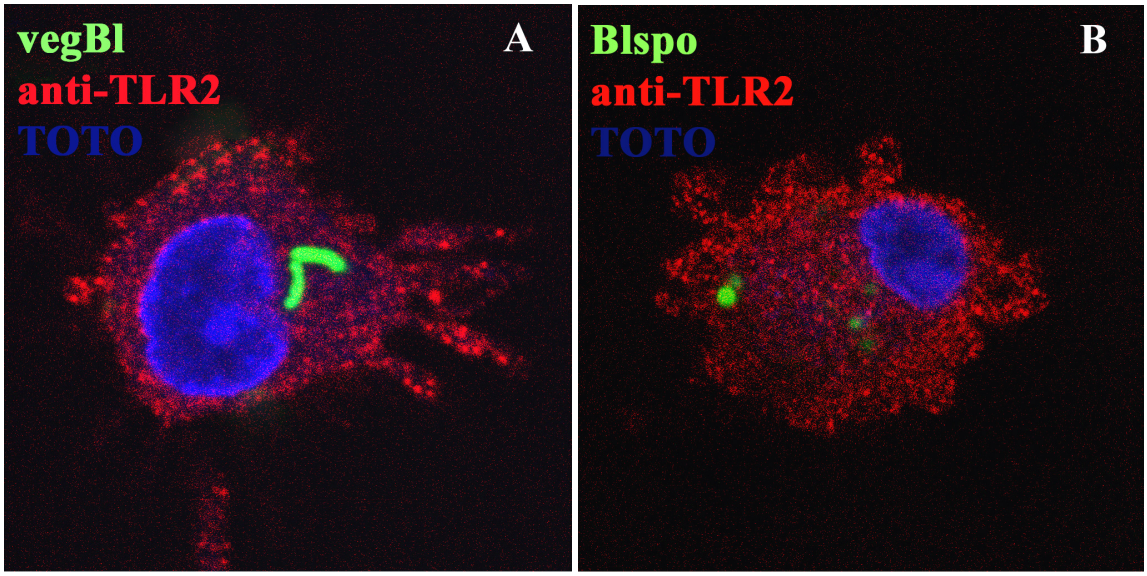

Figure 4.7: Uptake of FITC-labeled *Bacillus licheniformis* 467 by moDCs. Blspo and vegBl were labeled by incubation with FITC solution. Stimulation of moDCs was performed with labeled bacteria at a MOI of 1. Depicted are representative results of moDCs incubated for 24 h in the presence of vegBl (**A**) or Blspo (**B**). Dendritic cells were stained with anti-TLR2 antibody in combination with a secondary Alexa 633 antibody (red) as well as with TOTO®-3 to visualize the nucleus (blue).

The phagocytosis of spores and vegetative cells was proved in this way and an incomplete removal of attached bacteria was excluded.

4.2.2.4 Maturation of dendritic cells

Significant differences in the interaction with innate immune receptors expressed on moDCs as well as the effects on phagocytosis can result in variations during DC maturation. The expression of the co-stimulatory molecules CD86 and CD83 after DC maturation into APCs is depicted (Fig. 4.8). Comparing moDC maturation initiated by vegBl and Blspo clearly showed differences in CD86 expression. Vegetative cells achieved the strongest maturation, whereas Blspo led to lower CD86 expression levels than the *Mycobacterium bovis* BCG (BCG) control. In contrast, expression of CD83 was comparable following stimulation of dendritic cells by vegBl and Blspo.

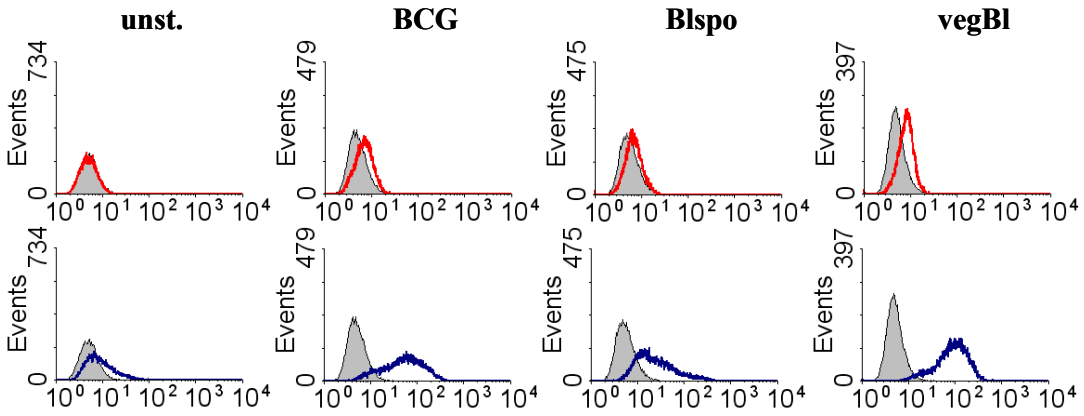

Figure 4.8: Phenotypic maturation of dendritic cells. Dendritic cells isolated from peripheral blood of healthy donors were stimulated in three independent experiments. Representative results of moDCs obtained from one donor are shown. Cells were either left unstimulated (unst.), stimulated with *Mycobacterium bovis* BCG, Blspo or vegBl at a MOI of 1. After 24 h cells were incubated with CD83 antibody (red) or CD86 antibody (blue). Unspecific binding was controlled by isotype antibodies, depicted as shaded histograms.

4.2.3 Polarization of T_{Helper} cells and activation of the adaptive immune system

To examine the effect of DCs on the polarization of T_H-cells, moDCs were isolated from peripheral blood of healthy human donors. Analyses of differential cytokine expression in response to *B. licheniformis* 467 stimulation in form of Blspo and vegBl were investigated for their impact on DC maturation and their activation of naïve T-cell polarization [180].

The stimulatory effect on the expression of the proinflammatory cytokine TNF-α (Fig. 4.9 A), the regulatory cytokine IL-10 (Fig. 4.9 B), and the T_H1-directing cytokine IL-12p70 (Fig. 4.9 C) [181] were analyzed. Expression of TNF-α as well as IL-10 was significantly higher after DC stimulation with vegBl. In contrast, spores induced the expression of IL-12p70, the most important cytokine responsible for T_H1 polarization of naïve T cells. Furthermore, the expression of IL-12p70 was close to the detection limit of 15 $pg \cdot mL^{-1}$ in case incubation was performed in the presence of *B. subtilis* DSM 618 or *B. licheniformis* 830. The stimulation of IL-12p70 release by these strains was similar to the induction by vegetative *B. licheniformis* 467. Therefore, IL-12p70 expression was considered to be specific for *B. licheniformis* 467 spores isolated from a farm children's mattress.

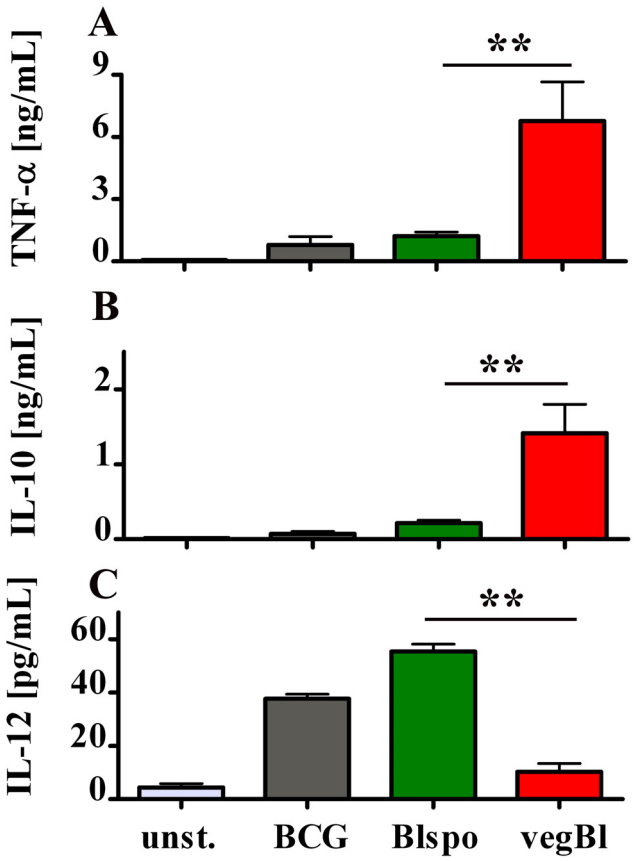

Figure 4.9:. Stimulation of moDCs by *Bacillus licheniformis* 467. Cells were isolated from healthy blood donors and incubated in the presence of Blspo and vegBl for 24 hours (MOI=1). The proinflammatory cytokine TNF-α (**A**) as well as the regulatory cytokine IL-10 (**B**) and the T_H1-directing cytokine IL-12p70 (IL-12, **C**) were measured by ELISA. Results are denoted as means of cytokine measurements in these experiments (± SEM). Statistical significance is indicated by **p < 0.01 unst.: unstimulated, BCG: *Mycobacterium bovis* BCG.

These results revealed a spore-specific cytokine profile following DC stimulation with Blspo. Stimulation prior to a mixed leukocyte reaction for 24 h was affiliated [182]. Characteristic cytokines expressed by T_H-cells after differentiation into T_H1-cells (IFN-γ) or T_H2-cells (IL-5, IL-13) were measured by ELISA.

The strongest expression of IFN-γ was achieved by stimulation of DCs with Blspo. The resulted maturation and expression of DC cytokines (as shown before) caused a significant increase in the expression of IFN-γ, whereas the expression levels of IL-5 and IL-13 remained on the level of unstimulated DCs. In contrast, IL-5 and significantly IL-13 expression was increased by incubation with vegBl.

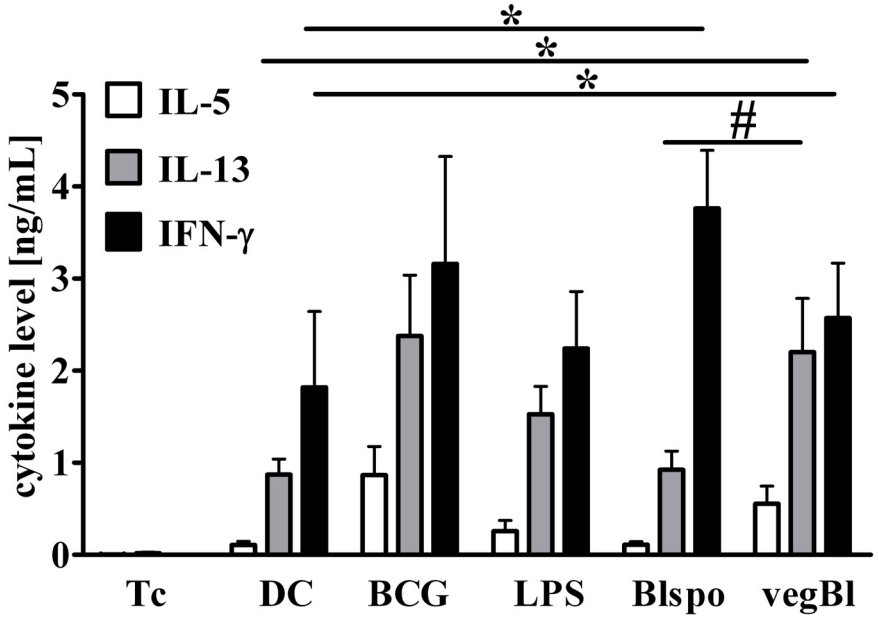

Figure 4.10: Mixed leukocyte reaction. Stimulation of moDCs by *Bacillus licheniformis* 467 (MOI = 1). Vegetative cells and spores of *B. licheniformis* were used to stimulate cells for 24 h (see Figure 4.9), prior to a coculture with lymphocytes in a ratio of 1 APC: 10 lymphocytes. Cells were cocultured for 5 days [(148)] and the results are depicted as means of cytokine measurements in these experiments ± SEM. The cells were isolated from blood of healthy donors in three independent experiments. * $p < 0.05$, depict significant differences between cocultures with unstimulated DCs and *B. licheniformis* 467 stimulated DCs. # $p < 0.05$, depicts difference between Blspo and vegBl. TC: leukocytes alone, DC: unstimulated DCs plus leukocytes, BCG: *Mycobacterium bovis* BCG, LPS 10 ng·mL^{-1}.

These results clearly differ in the expression of typical T_H1- and T_H2-cell cytokines induced by Blspo and vegBl and emphasized the importance of *in vivo* experiments.

4.3 Recognition of *Mucor* spp. spores by the innate immune system

Experiments, as they were described before, for the immune recognition of bacterial spores were repeated for the fungal spores of *Mucor* spp. isolated from a farming environment. Purified *Mucor* spp. spores were provided by Dr. Karin Schwaiger (Chair of Animal Hygiene, Technical University Munich).

Stimulation of primary epithelial cells in a MOI of 4, 40 and 400 were performed to compare *B. licheniformis* 467 spores to *Mucor* spp. A weak induction of IL-8 expression by *Mucor* spores was observed (Fig. 4.11).

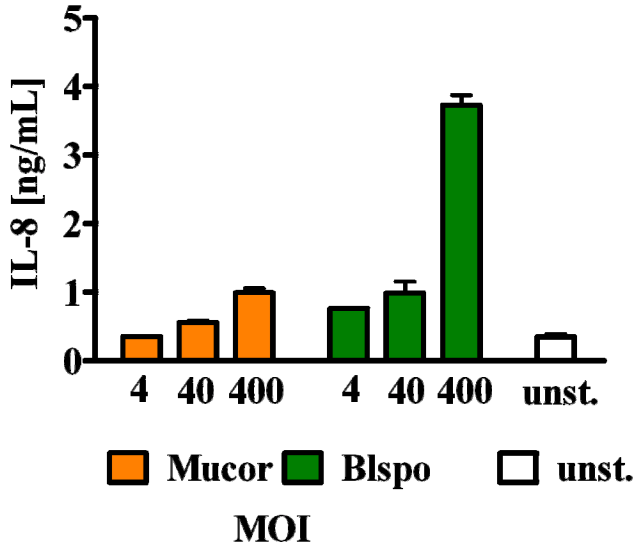

Figure 4.11: Comparison of spore stimulations on airway epithelial cells. Spores of *Mucor* spp. and *B. licheniformis* 467 (Blspo) were applied at a MOI of 4, 40 and 400 to measure a concentration-dependent release of IL-8 by primary airway epithelial cells, isolated from biopsies and grown in an air liquid interface culture. Shown are the means ± SEM of three different experiments with primary cells isolated from different biopsies. The cytokine was measured in duplicate for each experiment. Cells were left unstimulated as negative control.

Investigations on the level of innate immune stimulation revealed no stimulatory effect (data not shown). PBMCs were stimulated at the same MOI as *B. licheniformis*, but no concentration dependent stimulation was observed (data not shown).
Lacking these basic ways of immune activation by *Mucor* spp., subsequent experiments focused on the *in vivo* effects of *B. licheniformis* spore inhalation alone.

4.4 Comparison of *Bacillus licheniformis* spores and vegetative bacteria by transmission electron microscopy

Ahead of *in vivo* experiments the ability to intrude the airways was checked by comparing size and shape of vegBl and Blspo. These experiments were performed by transmission electron microscopy. A comparison of spore and vegetative cell showed the clear decrease in size resulting from sporulation of *B. licheniformis* 467. The length of the spore was less than half of the vegetative cell. The width, in comparison, was almost identical. Its shape and the shell-like protection layers emphasize an easy inhalation of the spore.

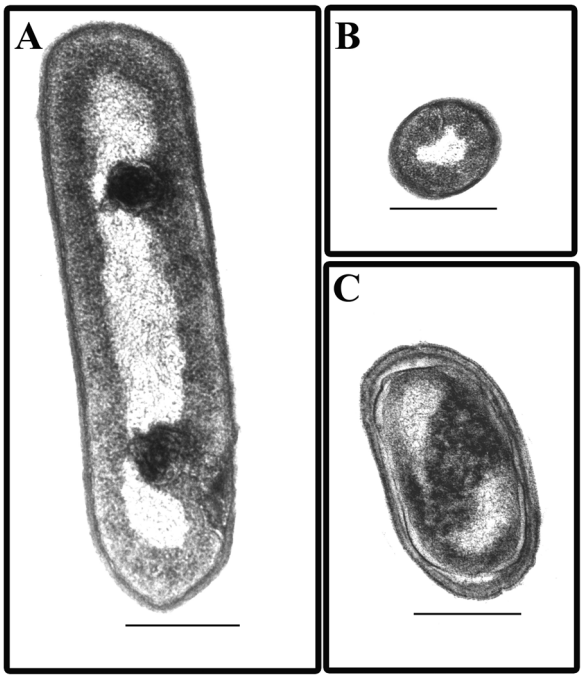

Figure 4.12: Comparison of *Bacillus licheniformis* 467 by transmission electron microscopy. The vegetative bacterium obtained in the log-phase of growth in LB^+ medium is depicted in longitudinal (**A**) and cross (**B**) sections. In comparison, panel **C** depicts the condensed dormant spore with the core protected by several layers of highly cross-linked proteins and peptidoglycan. Bars indicate 0.5 µm.

4.5 Attenuation of allergic asthma induction in mice by *Bacillus licheniformis* 467 pretreatment

Due to the spore-specific results obtained for the immune stimulation by *B. licheniformis* 467 spores *in vitro*, further on an *in vivo* mouse model of allergic asthma was employed. In this model the effect of *B. licheniformis* spore inhalation prior to allergen sensitization and provocation was analyzed. *Bacillus licheniformis* spores and hiBlspo with vegBl were investigated for spore-specificity *in vivo*. Vegetative cells were lyophilized in fresh LB medium to prevent sporulation.

Pretreatment of mice with the respective form of *B. licheniformis* was started 12 days before the first OVA dose was injected i.p. to obtain sensibilization. Every second day 10^8 cfu were applied during the whole process of sensitization (Fig. 3.2) by intranasal application. Local allergen challenge was achieved by OVA inhalation on days 19-21.

4.5.1 Analyses of bronchoalveolar lavage cells

Bronchoalveolar lavage was performed 48 h after the last allergen challenge. The presence of eosinophils (T_H2 effector cells), neutrophils (T_H1 effector cells), lymphocytes and macrophages was detected by differential staining. Results are depicted in Figure 4.13.

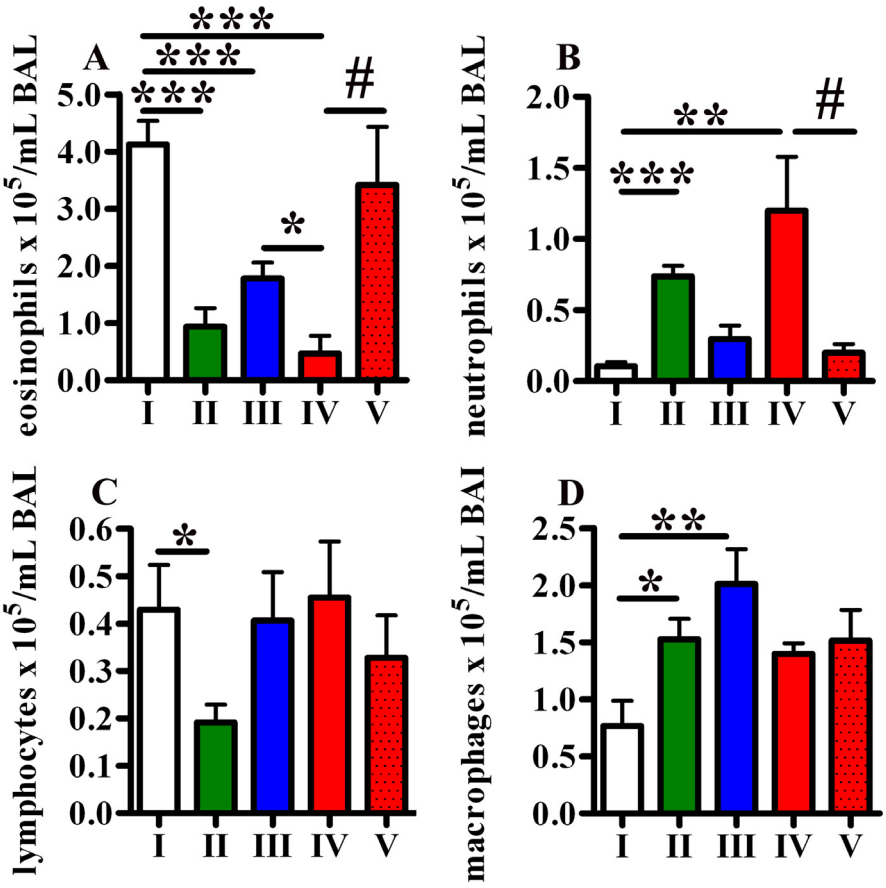

Figure 4.13: Bronchoalveolar lavage cells of *Bacillus licheniformis* 467 treated and OVA allergen provoked mice. Mice (n = 8) were treated with Blspo (II), hiBlspo (III), vegBl in medium (IV) and a medium control (V) prior to and during the induction of experimental asthma by OVA provocation. The control group was sham treated with PBS (I). Mice BALF were sedimented in cytospins and stained differential for the detection of eosinophils (**A**), neutrophils (**B**), lymphocytes (**C**) and macrophages (**D**). Depicted are the means ± SEM. Significances between the vegBl treated group and the control medium are indicated by # $p < 0.05$. Differences to sham treated mice are depicted by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Eosinophil counts were significantly decreased by *B. licheniformis* 467 treatment in contrast to the sham treated group. Comparing the different *B. licheniformis* forms resulted in a reduction of eosinophils to a lesser degree than vegBl showing the strongest decrease (Fig. 4.13 A). Contrary, significantly increased numbers of BAL neutrophils were confined

to vegBl and Blspo treatment (Fig.4.13 B). In both cases the numbers of eosinophils and neutrophils of Blspo treated mice were between those of vegBl and hiBlspo. The numbers of lymphocytes were decreased exclusively in the Blspo treated group (Fig 4.13 C). Treatment with hiBlspo and vegBl did not affect the level of lymphocytes in comparison to sham treated mice, provoked by OVA sensitization and challenge.

The macrophage numbers in the Blspo treated group were significantly increased in comparison to the PBS treated group. This revealed macrophage recruitment as a response to spore treatment but not as a response to OVA treatment. Similar results were obtained for the increase of neutrophils described before. Significance between PBS and vegBl treated groups were not calculated, due to the increase of macrophage numbers in mice lungs treated with the control medium.

4.5.2 Analyses of lung histology

4.5.2.1 Periodic acid-Schiff staining of lung tissue

To observe the effects of allergen provocation with regard to the hypersecretion of mucus and the peribronchial wall thickening [183], lung slices of mice were stained according to the protocol of PAS staining (Fig. 4.14).

Allergen provocation evoked a clear increase of peribronchial inflammation in comparison to untreated mice [107]. The decrease of this bronchial wall thickening (double headed arrow) revealed by *B. licheniformis* treatment was independent of the form of *B. licheniformis*. The number of mucus producing goblet cells was almost completely reduced by *B. licheniformis* treatment. Thus prevention of goblet cell hyperplasia was also independent of the form of *B. licheniformis*. However, the strongest reduction was achieved by vegBl.

4.5.2.2 Formation of multinucleated giant cells

As indicated by the differential cell counts of Figure 4.13, neutrophils and macrophages infiltrated the BALF as a response to *B. licheniformis*. The surrounding tissue was analyzed for evidences of inflammation by histological investigations of bronchial airways. Tissue inflammation by neutrophil and macrophage influx was supported by the presence of multinucleated giant cells in the lung tissue (Fig. 4.15).

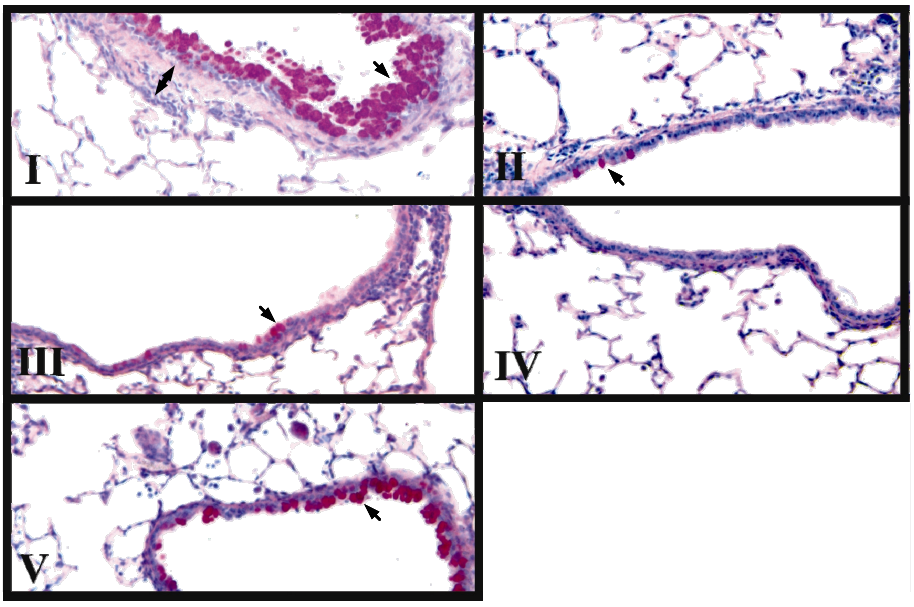

Figure 4.14: Periodic acid-Schiff staining of lung tissue. Thin sections of mouse lungs obtained after allergen provocation and PBS treatment (**I**) in comparison to *B. licheniformis* treated mice (**II**: Blspo, **III**: hiBlspo, **IV**: vegBl, **V**: control medium). Slices of bronchial airways were stained by PAS staining to detect mucus producing goblet cells (arrows). Acidic molecules like DNA were stained blue, detecting nuclei of infiltrating cells. Representative micrographs for the different mice groups are shown. A peribronchial wall thickening is indicated in panel I (double headed arrow).

Although the recruitment of macrophages resulted in the highest cell numbers in the hiBlspo treated groups the formation of giant cells, by fusion of mononuclear cells such as macrophages, was not restricted to lung tissues of spore treated mice. It was either detected in the other treated mice groups.

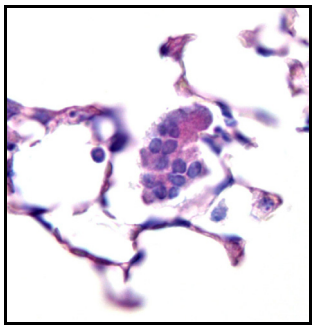

Figure 4.15: Formation of multinucleated giant cells. These cells were detected in lung tissue at a magnification of 400 fold. One representative cell fusion forming a giant cell including 11 nuclei is presented. The micrograph was taken from a mouse treated with control medium. However, similar results were detected in all treated mice groups.

Further steps in granuloma formation such as the organization of epitheloid-like macrophages and the formation of Langhans-giant cells were not detected.

4.5.3 Improvement of airway hyperreactivity

Improvements of AHR in asthma are important for the prevention of asthma attacks. Therefore, the reactivity to β-metacholine (MCH) was measured in mice following the different *B. licheniformis* 467 treatments. Metacholine concentrations causing a 50% reduction of airflow are reported in Figure 4.16.

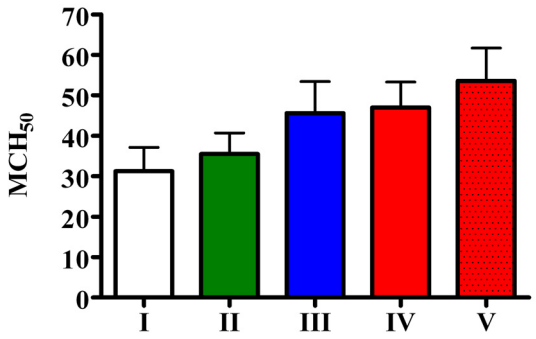

Figure 4.16: Analyses of lung function by head-out body plethysmography. Mice were sensitized and challenged to OVA during and after bacterial treatment with different forms of *B. licheniformis* 467. Metacholine concentrations that caused a 50% reduction in baseline midexpiratory airflow (MCH_{50}) are displayed in comparison to the sham treated mouse group. Treatment with I: PBS, II: Blspo, III: hiBlspo, IV: vegBl, V: control medium was perfored intranasally.

No significant decrease of AHR to MCH and therefore significant increased levels of MCH_{50} were achieved by *B. licheniformis* treatment. Moreover treatment with control medium resulted in a higher resistance to metacholine exposure than it was observed in all groups treated with *B. licheniformis* 467.

4.5.4 Bronchoalveolar lavage cytokines

Cytokine levels in BALF of mice were measured to clarify the state of inflammation in the lung at the time of BAL execution. Measurements were performed 48 h after the last allergen challenge. The IFN-γ, IL-10 and IL-5 levels measured by ELISA are shown in Figure 4.17. Treatment of mice with Blspo led to an increase of IFN-γ levels to about 50 $pg \cdot mL^{-1}$ BALF. In contrast, IFN-γ was slightly increased after treatment with hiBlspo as well as after vegBl treatment in comparison to the PBS treated control group. A similar induction was observed for the BAL-level of IL-10 after Blspo treatment. In comparison to sham treated mice this cytokine was increased more than three fold, whereas no cytokines were detectable after vegBl and hiBlspo treatment. In comparison to the T_H1-cytokine

IFN-γ and the regulatory cytokine IL-10, the T_H2-related cytokine IL-5 changed not significantly.

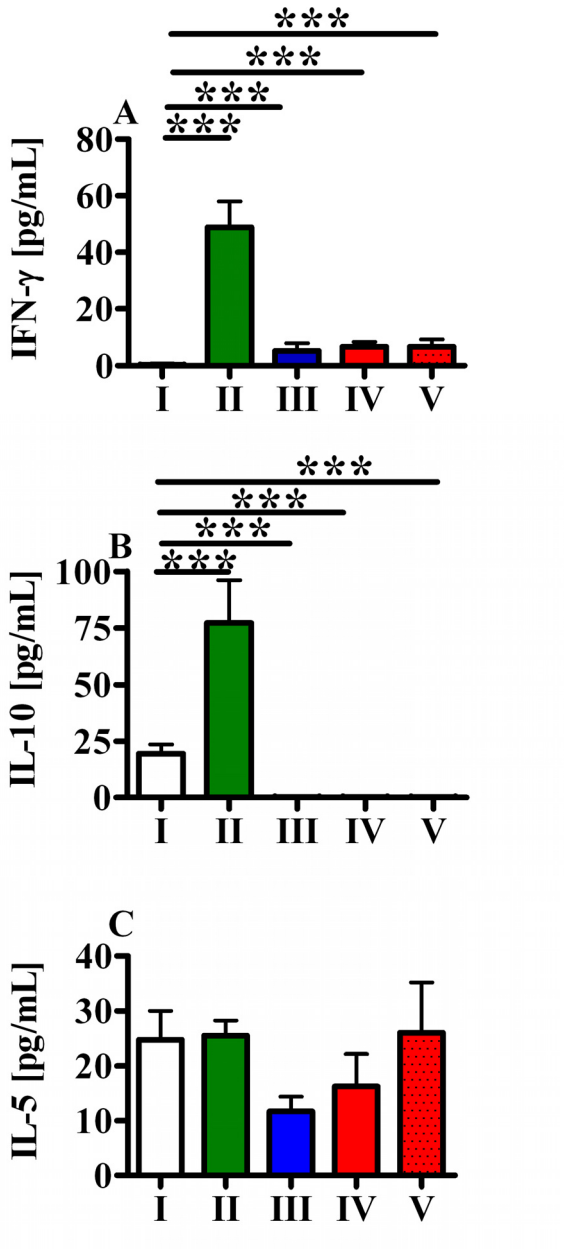

Figure 4.17: Cytokines in bronchoalveolar lavage fluid. Bronchoalveolar lavage was performed and cytokines were measured by ELISA, following OVA sensitization and challenge as well as treatment of mice with PBS or *B. licheniformis*. Depicted are the means ± SEM of cytokine measurements by ELISA for each treated group (n = 8). Significant differences between sensitized PBS- and bacteria treated mice are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The treatment was performed by intranasal application of I: PBS, II: Blspo, III: hiBlspo, IV: vegBl, V: control medium.

4.5.5 Persistence of *Bacillus licheniformis* 467 in the lung

Due to the elevated levels of IL-10 and IFN-γ in BALF Wirtz-Conklin staining was accomplished to examine the presence of *B. licheniformis* in lung tissue. The staining of *B. licheniformis* spores was performed in a modified manner to reduce the counterstaining of lung tissue (Fig. 4.18).

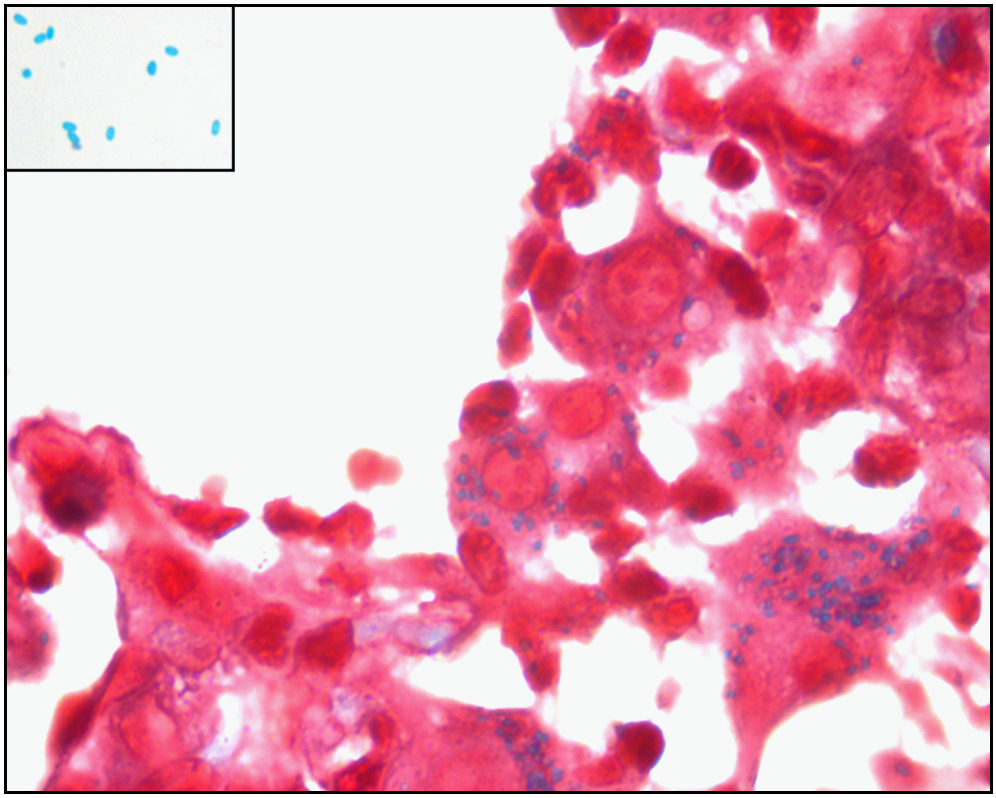

Figure 4.18: Lung tissue of mice treated with Blspo stained by Wirtz-Conklin staining. Fixation of lung tissue was carried out 4 days after the last treatment. Bacterial spores stained greenish on an object slide were used as a positive control (upper left corner of the figure). Spores were phagocytozed by alveolar macrophages. Similar results were obtained by staining lung cytospins of BAL. Spores were detected after treatment of mice with Blspo and hiBlspo, respectively. One representative microphagh of a Blspo treated mouse lung is depicted. Magnification 1000x.

Bacillus licheniformis spores detected in lung tissue were phagocytozed by lung cells. The surrounding tissue was free of spores. The presence of spores, independent of heat inactivation, was confirmed by staining BAL cytospins, showing an accumulation of spores in BAL cells. At this time four days after the last treatment with vegBl, Blspo or hiBlspo no rod shaped vegetative cells were detected. Neither by Gram-staining nor by Grocott or Ziehl-Nielsen staining vegBl were detected in vegBl or Blspo treated mice. These findings revealed a continuous immune activation to inhaled spores even four days after the last treatment with Blspo and hiBlspo. The strong deviations between hiBlspo and Blspo on the level of BAL cells as well as BAL cytokines showed a difference in the immune reaction caused by heat-inactivation of Blspo, although both forms were present in the lung. The persistence of immune activation was exclusively observed in Blspo treated

mice, able to germinate in the lungs. They activated a delayed or long lasting immune response and cytokines could be detected at the time point analyzed.

4.5.6 Detection of OVA-specific antibodies

The impact of *B. licheniformis* treatment on the systemic prevention of allergy was observed in contrast to the local effects on asthma regarding OVA-specific antibodies in the blood of mice. In response to the release of T_H1 or T_H2 cytokines B-cells produce IgG2a and IgE, respectively. IgG2a antibodies are secreted in response to INF-γ and TNF-α, whereas IgE is released in response to T_H2 cytokines IL-4, IL-5 and IL-13 [(184)].

As indicated in Figure 4.19 OVA-specific IgE concentrations were not changed significantly by any treatment. This antibody is produced as a result of a B-cell shift following a contact to allergens. These marginally changes in the levels of IgE antibodies in comparison to the sham treated groups showed a missing response to *B. licheniformis* treatment for a systemic, allergic immune response. The decrease of the T_H1 related IgG2a antibody was observed exclusively as a response to vegetative cells. It points at differences between spores and vegetative cells on the level of antibody release and allergy development. *Ex vivo* controls of B-cells isolated from spleen cells and restimulated with OVA, detected no changes of antibody release comparing PBS treated and *B. licheniformis* 467 treated mice cells (data not shown).

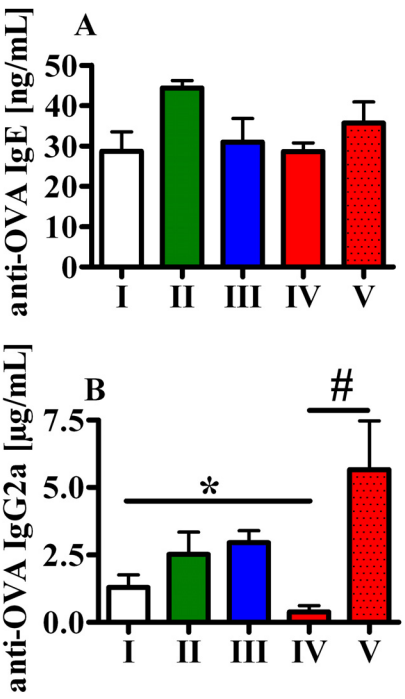

Figure 4.19: Investigation of immunoglobulin titers in the blood of OVA sensitized mice (n = 8). Mice blood was examined for the presence of IgE (**A**) and IgG2a (**B**) titers by ELISA. The means ± SEM are depicted and significant differences between the groups are indicated. The difference between the PBS treated group and *B. licheniformis* treated groups is indicated as * $p < 0.05$. The variety between vegBl treated group and its control medium is indicated by # $p < 0.05$. The mice were treated with I: PBS, II: Blspo, III: hiBlspo, IV: vegBl, V: control medium.

4.6 Isolation of bacterial components

The specificity of T_H1 polarization by spores *in vitro* and their difference to the obtained *in vivo* results emphasized the importance of investigations using isolated bacterial components for possible applications in asthma treatment. Based on the allergy protective properties observed using an physiological saline extraction of stable dust the same method was tested for its ability to extract spore components of interest [106]. Immunological tests on PBMCs and transfected HEK-293 cells showed a stimulation of these cells by the obtained extracts in response to recognition by TLR2 and NOD2 (data not shown), as it was described for the whole cells. Since the yields of these extractions were below 0.01% further experiments, especially the purification of immune stimulatory factors was not possible.

Against the background of these results alternative extraction procedures for the purification of peptidoglycan and lipoproteins were performed, to identify their role in cytokine induction *in vitro* and *in vivo*. The spore-specific IL-12p70 expression of moDCs, a mediator of T_H1 polarization, and the expression of the immune regulatory cytokine IL-10 were of interest for allergy protection.

4.6.1 Isolation of peptidoglycan from *Bacillus licheniformis* spores

The first component isolated from spores for further investigations was peptidoglycan. Its immune stimulating properties, based on the recognition by the intracellular NOD2 receptor were found in vegetative cells as well as in spores. As described in chapter 1.7 the peptidoglycan of spores differs from the PG found in vegetative cells due to their relative contribution to the dry cell weight as well as in their structures [185]. Known to be specific for spores and vegetative bacteria by studies in *B. subtilis* PG was an interesting target to explain the differences obtained in animal experiments by vegBl and Blspo treatment. Vegetative bacteria and spores were prepared to compare the IL-12p70 release induced by spores with the vegetative cells lacking IL-12p70 induction, but providing TNF-α and IL-10 induction in moDCs. Prior to disruption the spore coat was removed to prevent its strong protective effects on mechanical and chemical treatment (chapter 1.7). Following disruption with glass beads peptidoglycan was extracted according to the procedure of de Jonge et al. [158]. The purity of isolated peptidoglycan was controlled by methanolysis of the different preparations. The major components detected in this way, were identified to be MurNAc, hexosamine (HexN), diaminopimelic acid (Dpm) as well as several aa (Fig. 4.20). By amino sugar analyses HexN was identified to be GlcNAc. Similar results were

obtained by methanolysis of PG from vegBl (vegBl-PG) (data not shown). Minor differences were detectable at early elution times overlaid by the aa detection. Thus no further discrimination by GC-MS was viable.

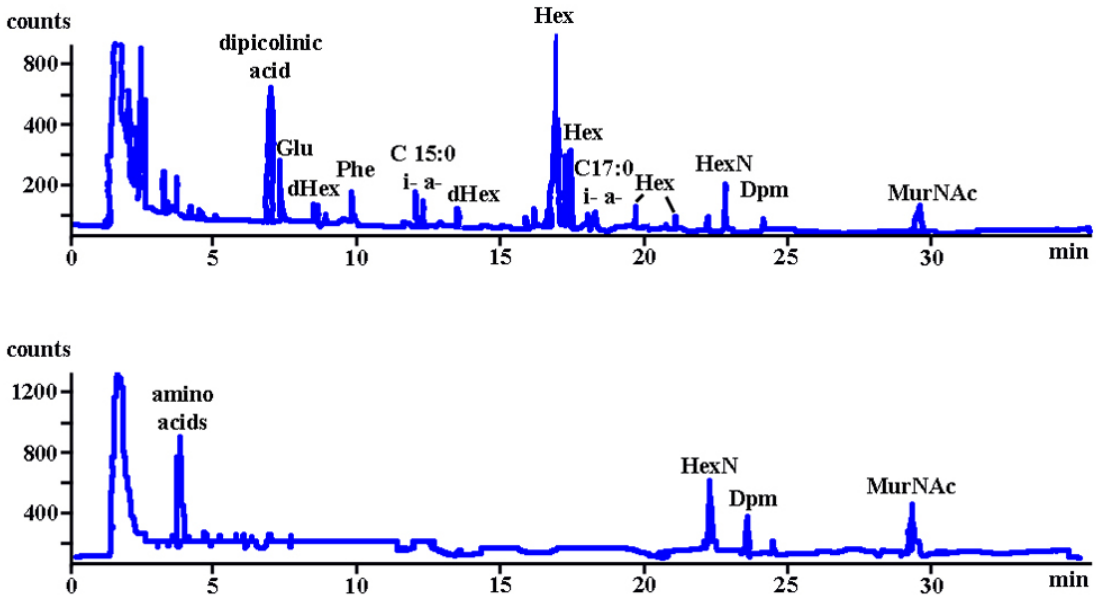

Figure 4.20: Methanolysis of *Bacillus licheniformis* 467 spores (upper panel) and spore peptidoglycan (lower panel). For each analysis 100 µg of dry mass were dissolved in 2 M methanolic hydrochloric acid, and peracetylation was performed following methanolysis at 85°C for 16 h. Components were separated by GC and identified by mass spectrometry. The detected components in the lower panel are specific for peptidoglycan. Glu = Glutamic acid, dHex = deoxyhexose, Phe = phenylalanine, C15:0 i-, a- = iso (i) and anteiso (a) forms of unsaturated pentadecanoic acid, Hex = hexose, C17:0 i-, a- = iso (i) and anteiso (a) form of unsaturated heptadecanoic acid, HexN = aminohexose, Dpm = diaminopimelic acid.

Furthermore, samples were checked for their purity of NOD2 ligation in immunological investigations on transfected HEK cells. Spore peptidoglycan was recognized exclusively by NOD2 whereas preparations of peptidoglycan from vegBl were activating TLR2 as well. This activation was due to lipoprotein contamination, as confirmed by oxidation of the sulfur residue, which abolished the TLR2 recognition (data not shown). Its interaction with TLR2 occurred despite a repeated treatment with HF to break phosphate bridges described to remove covalently attached lipoproteins and lipopeptides [(74)]. Although differences in purity were detected the response on immune cell recognition was similar (Fig. 4.21). Both preparations were compared before and after treatment with lysozyme. Prior to lysozyme (an *N*-acetyl-β-D-muramidase) cleavage both peptidoglycan preparations

were insoluble. In contrast to spore-PG enzymatic hydrolysis increased the solubility of vegBl-PG. The stimulation of HEK-293 cells and their release of IL-8 was also significantly increased. On the other hand, PG isolated from Blspo induced higher IL-8 releases before cleavage. Degradation and removal of insoluble material by centrifugation decreased this capacity dramatically. Therefore, cleavage of spore-PG by lysozyme was observed to be inefficient.

Although an efficient digestion of PG from Blspo (Blspo-PG) was not achieved these isolates were applied to moDCs to test their allergy protective properties as indicated before.

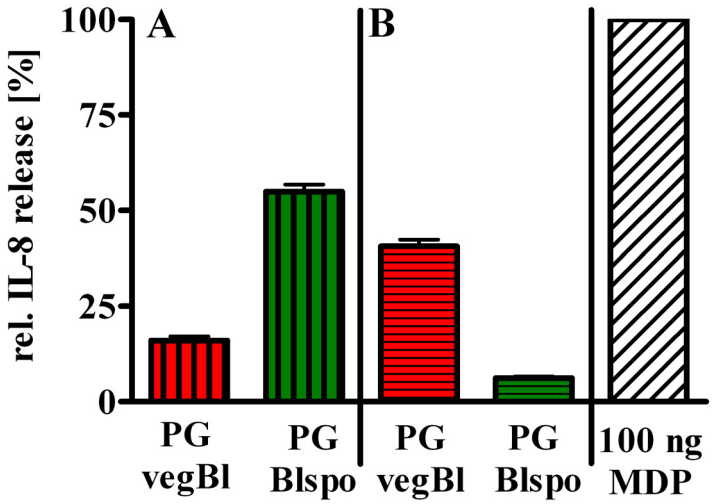

Figure 4.21: Relative IL-8 release of PG by stimulation of NOD2 transfected HEK-293 cells. Peptidoglycan was tested after isolation from Blspo and vegBl in the insoluble form (**A**) and the muropeptides after lysozyme treatment for 16 h and removal of insoluble material (**B**) in a concentration of 100 ng·mL^{-1}. The weight was determined before cleavage. Interleukin-8 expression induced by MDP stimulation (100 ng·mL^{-1}), the minimal motif for NOD2, was set to 100% to compare different experiments. The means ± SEM of three independent experiments are presented.

The expression of TNF-α was exclusively found in vegBl-PG stimulated DCs, coexisting with the ability of this PG to stimulate TLR2 transfected HEK 293 cells. Hydrolyzed vegBl-PG did neither stimulate TLR2 transfected HEK cells nor TNF-α and IL-10 expression of moDCs. Cell activation and cytokine expression was not detectable after stimulation with hydrolyzed or untreated Blspo-PG at concentrations of 0.1 and 1 µg·ml^{-1} (data not shown). Due to the lack of cytokine expression in comparison to the whole spore,

an impact on T_H1 polarization or immune suppression of isolated peptidoglycan structures was excluded. In contrast, the TNF-α expression of vegBl was associated to the expression of TLR2 ligands.

4.6.2 Spore coat protein

Since the positive effect of peptidoglycan from Blspo on the polarization to T_H1-cells was excluded, further investigations were focused on the effect of spore proteins. The isolation of lipoproteins and lipopeptides as TLR2 ligands and their influence on the obtained differences in DC cytokine expression induced by stimulation with Blspo and vegBl was of particular interest. Their importance was underlined by testing the described dust extracts for TLR2 recognition. These experiments had a high yield of lipoprotein extraction by physiological sodium chloride (data not shown). To establish the protein extraction *B. subtilis*, the model organism for spore coat protein analyses, was utilized.

The spore coat is a spore-specific layer of highly cross-linked proteins, protecting the spore from e.g. UV-light, organic chemicals and toxic molecules such as lysozyme [117;159;186;187]. This layer was first examined for the expression of specific immune stimulating proteins. Different procedures were tested to extract this surface layer, surrounding the spores of *B. subtilis* and *B. licheniformis*. Due to the fact, that the extraction buffers described in literature differ in their composition, the extraction efficiency was compared by pellet weight before and after extraction (Table 4.4) as well as by SDS-PAGE analyses (Fig. 4.23).

Table 4.4: Comparison of extraction procedures for spore coat protein extraction. The dry biomass of *B. subtilis* spores (1 mg each) was extracted by the procedures described below. Pellet weight differences before extraction were compared with the washed pellet after extraction.

	Extraction buffer	Conditions	Pellet weight loss
Henriques et al. (159)	125 mM Tris, 4% SDS, 10% 2-ME	100°C, 15 min	54%
Sylvestre et al. (186)	2% 2-ME (v/v), in 50mM Tris-HCl, pH 10, 8 M urea	85°C, 15 min	9%
Tarasenko et al. (187)	2% 2-ME (v/v), in 1mM carbonate/bicarbonate buffer, pH 10	37°C, 120 min	16%

2-ME = β-mercapthoethanol

Comparing these extracts by SDS-PAGE, visualized by Coomassie as well as silver staining, showed detectable amounts of proteins exclusively after spore extraction according to Henriques et al [159]. High temperatures as well as the presence of SDS were necessary to extract spore proteins of *B. subtilis* DSM 618. The high weight loss of the spore pellet (Table 4.5) indicated an efficient extraction but its selectivity had to be investigated. Figure 4.22 compares *B. subtilis* spores before (A) and after protein extraction (B) according to Henriques et al.

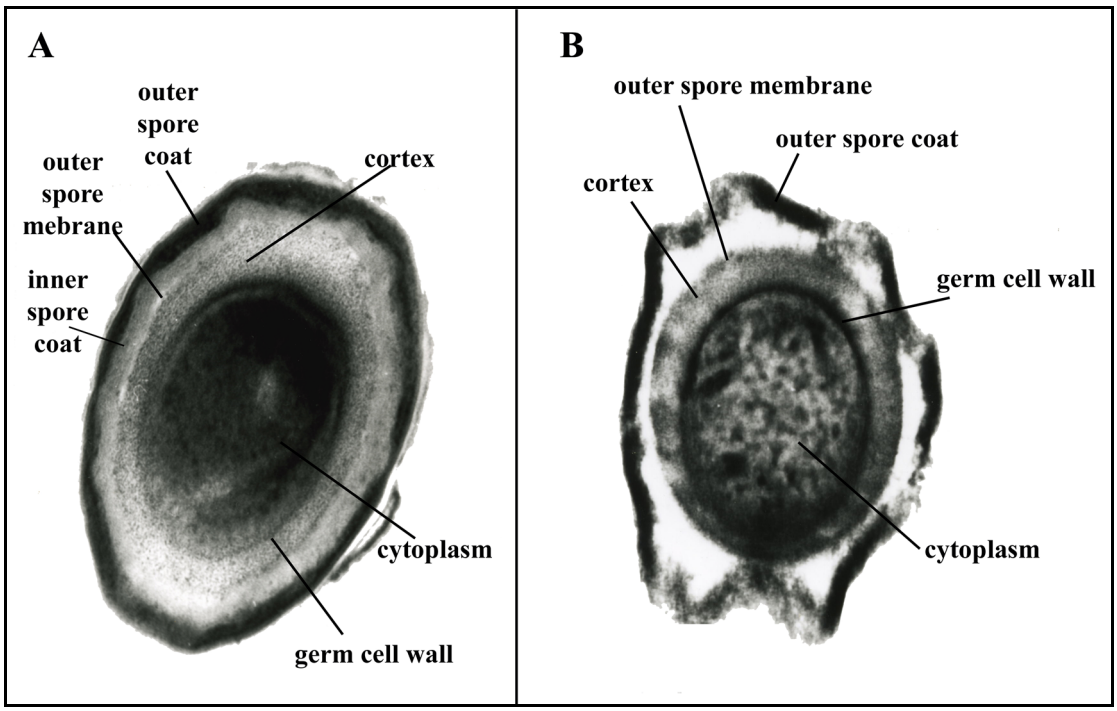

Figure 4.22: Comparison of *Bacillus subtilis* DSM 618 spores before (A) and after protein extraction (B). Transmission electron miscroscopy was performed. Structural elements of spores are indicated. In panel **A** a clear and distinct electron dense outer spore coat surrounding the spore is visible. After protein extraction according to Henriques [159] this layer was partly dissolved. The tight attachment of the outer coat to the spore cortex was removed. The inner coat, visible as electron bright layer between outer coat and outer spore membrane in panel **A** cannot be detected after protein extraction. Magnification 30000x.

The results of transmission electron microscopy showed a partially dissolved outer spore coat when treated with SDS and 2-ME containing extraction buffer. The presence of the inner coat was not detectable. The tight attachment of coat and cortex, as it was present prior to treatment, was detached. The inner part of the spore was affected in its electron density, but surrounded by an intact cortex. Longer extraction times did not increase the extraction yield. Due to these results *B. licheniformis* and *B. subtilis* proteins were extracted as described above.

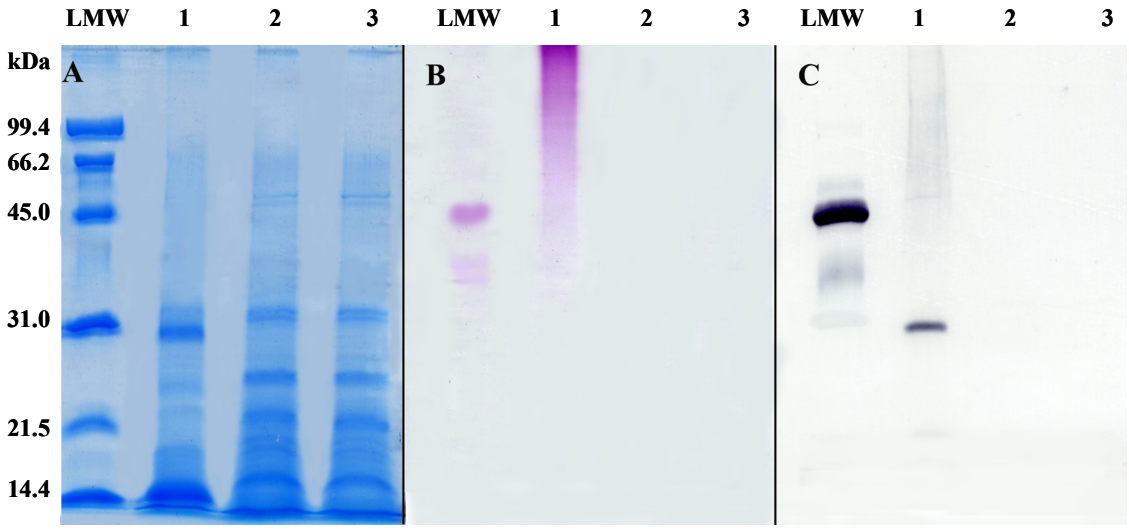

Figure 4.23: SDS-PAGE of spore coat protein extractions. Extractions were performed for *B. subtilis* DSM 618 (**1**), *B. licheniformis* 467 (**2**) and *B. licheniformis* 830 (**3**) according to Henriques et al. [159]. Equal volumes of extraction eluate after dialysis (1% v/v) were mixed with sample buffer. Samples were separated on a 12.5% SDS-PAGE gel. Gels were stained with Coomassie brilliant blue (**A**), PAS staining for the detection of carbohydrates (**B**) or by a Western blot, detecting terminal α-mannose and α-glucose by Concavalin A (**C**). Molecular mass was determined by comparison with low molecular mass standard (**LMW**). Ovalbumin with a molecular weight of 45.0 kDa functioned as a positive control for a glycosylated protein in panels (**B**) and (**C**).

Differences in protein patterns were visible following Coomassie staining. Particularly the detection of carbohydrates and glycosylated proteins, important immunogenic epitops [188], revealed differences between *B. subtilis* and *B. licheniformis*, but no differences within the *B. licheniformis* strains. The high molecular mass region of *B. subtilis* protein extract was identified to contain carbohydrates, reacting with the Schiff reagent in the PAS-staining. Further investigations of these extracts by lectin blot with Concavalin A showed a low affinity of the high molecular mass region. A distinct band identified by this staining method was found at a molecular mass of about 30 kDa. The lanes containing extracts of *B. licheniformis* spores presented no carbohydrate containing molecules, neither in PAS staining, nor by reaction with Concavalin A. The lack of glycoproteins and carbohydrates in the *B. licheniformis* extracts excluded in this way an impact of carbohydrate-containing molecules on the specific T_H1 inducing principle of Blspo.

The active principle after spore extraction of *B. licheniformis* 467 was localized on moDCs (Fig 4.24).

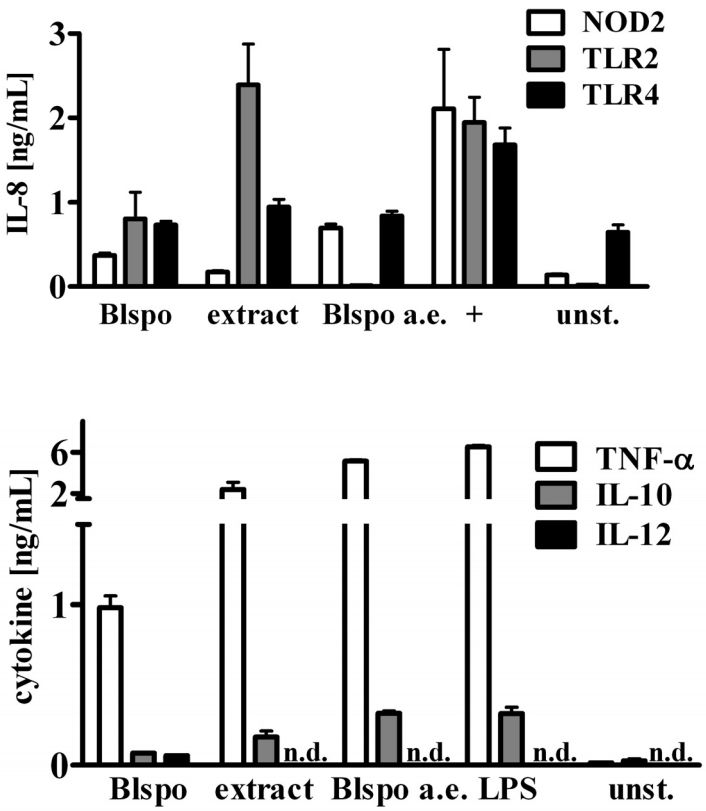

Figure 4.24: Stimulatory capacity of *Bacillus licheniformis* 467 spore coat extract. Spores were extracted according to Henriques et al.[159] and the different fractions were applied to stimulate transfected HEK-293 cells (**upper panel**) (1 µg·mL^{-1}) and moDCs (**lower panel**) (10·µg·mL^{-1}). Sodium dodecylsulfate and 2-ME were removed by dialysis. The fractions were depicted as untreated Blspo (Blspo), spore coat extract (extract) and spores after extraction (Blspo a.e.). Unstimulated cells were used as negative control and LPS as positive control for moDC (10 ng·mL-1) and TLR4 (1 ng·mL^{-1}), Pam_3CSK_4 (100 ng·mL^{-1}) for TLR2, and MDP (100 ng·mL^{-1}) for NOD2. n.d. = not detectable.

The moDC stimulation showed a parallel increase in induction of TNF-α and IL-10 for the protein extract as well as for Blspo after extraction. This parallel induction was similar to the results obtained for vegBl (Fig. 4.9). However, the weak induction of IL-12p70 (~60 pg·mL^{-1}) by Blspo was not detectable after treatment.

The expression levels of TNF-α and IL-10 were doubled in the extract in comparison of extract and dormant spores. The same cytokines were released by the spores after extraction, correlating to the destruction of the spore coat (Fig. 4.22.). Stimulation of HEK-293 cells with the same extracts was performed to exclude LPS contamination. Although the cytokine expression profiles in moDCs were similar for Blspo and LPS, expression levels in TLR4 transfected HEK cells were comparable with the negative

control. The cytokine induction in moDCs treated with the coat extract, was characterized by a strong effect of TLR2 related to TNF-α and IL-10 induction. This TLR2 stimulation was absent in Blspo after extraction, but the spores were able to induce the same cytokine expression in moDCs. The results of the spore coat protein extraction showed a decrease of IL-12p70 expression in moDCs by denaturation during the extraction. This down-regulation of IL-12p70 induction was confirmed by testing heat-inactivated Blspo. After heat-inactivation the expression of IL-12p70 was similar to the expression induced by vegetative cells and close to the detection limit presented in Figure 4.9 (data not shown).

4.6.3 Isolation of lipoproteins

The differences between vegBl, Blspo and hiBlspo in the mouse model of allergic asthma, combined with the cytokine expression profiles in moDCs and BAL cells showed the importance of procedures suitable for extracting lipoproteins without heat-denaturation. Toll-like receptor 2 stimulation was proved to be related to the induction of TNF-α and IL-10 in spore coat protein extracts whereas heat-inactivation decreased the potential of Blspo to induce IL-12p70 expression which is essential for T_H1 polarization.

Since the mentioned differences were found between Blspo and vegBl, lipoprotein extraction procedures were established in order to isolate responsible components on the level of lipoprotein expression and TLR2 activation without heat-denaturation of the protein part.

4.6.3.1 Isolation of lipoproteins/lipopeptides by reversed-phase chromatography

Mühlradt et al. used the amphiphilic properties of lipoproteins and lipopeptides caused by their fatty acid moieties to isolate the macrophage stimulating lipopeptide MALP-2 by reversed-phase chromatography [161]. The octyl β-D-glucoside solubilized extract of vegBl was purified by C_{18} RP-chromatography on an analytical 4.5 x 250 mm column. The fractions were tested for TLR2 activity in a HEK cell assay and compared to the unspecific absorption at $UV_{280\,nm}$ by aromatic amino acids (Fig. 4.25).

The eluate containing the highest concentration of proteins showed no TLR2 stimulation whereas fraction 6 (50-60 min) induced a relative release of about 55% of that obtained by the synthetic lipoprotein Pam_3CSK_4. The TLR2 stimulating component eluted in the middle of the gradient spread over a wide range of 20 min elution time. Collected fractions were freed of propanol, freeze dried and analyzed by methanolysis. Lipoproteins,

lipopeptides as well as *S*-(2,3-dihydroxypropyl)cysteine (GroCys) a motif characteristic for lipoproteins/lipopeptides [69] were neither detectable in the TLR2 active faction 6 nor in any other fraction.

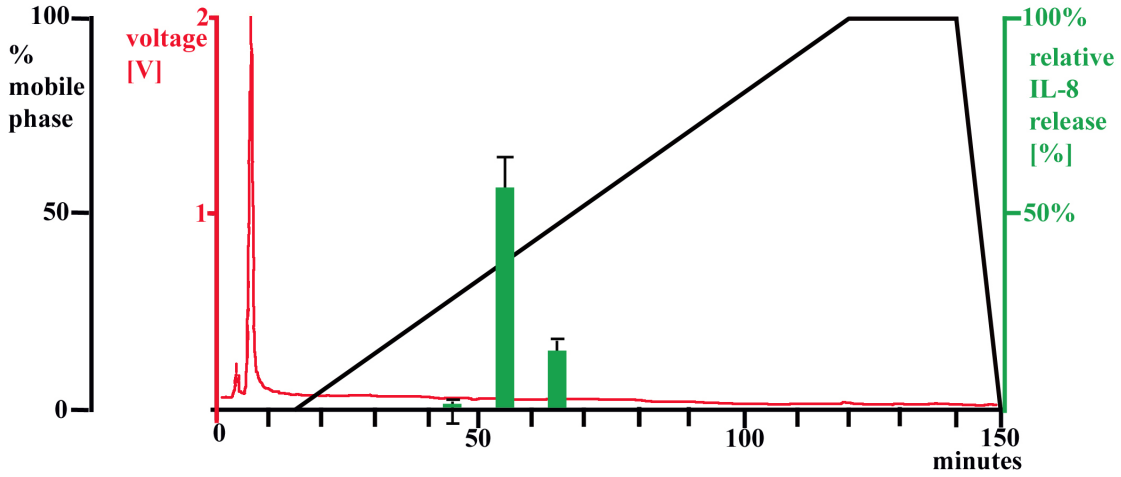

Figure 4.25: Chromatogram of reversed-phase lipopeptide separation. Depicted is one representative chromatogram of a C_{18}-RP chromatographic separation of the lipoprotein extract according to Mühlradt et al. [161] (Nucleosil® 120-5, 250 x 4.6mm). Aromatic aa are detected by UV-absorption at 280 nm (red line). The relative TLR2 activity represented by IL-8 release of TLR2 transfected HEK-293 cells was measured after lyophilization of fractions, collected for 10 min each. The relative releases induced by stimulation with 100 ng material are depicted as green columns. Expression was standardized to the stimulation induced by Pam_3CSK_4 (100 ng). The gradient of 2-propanol is shown as black line.

Analyses of the extract as well as analyses of fraction 6, showing the highest TLR2 activity, by SDS-PAGE are displayed in Figure 4.26. Silver staining of proteins detected several distinct bands in the total extract, but not after HPLC. The TLR2 active fraction 6 of RP-HPLC contained exclusively one high molecular mass band hardly migrating in the gel. This band was stained by silver nitrate, but not by PAS. To clarify this discrepancy proteinase K treatment was performed. Treated total extract revealed identical results in silver and PAS-staining as they were obtained in the analysis of HPLC fraction 6 of the total extract. Extraction of components from preparative SDS-PAGE confirmed the absence of TLR2 ligands, namely lipoproteins or lipopeptides, in this region. The highest activity in HEK cell stimulation was obtained below 15 kDa. In this region proteins were not detected. Oxidation of extracts as well as mild alkaline hydrolyses decreased the activity characterizing the presence of lipoproteins and lipopeptides, respectively [160;189].

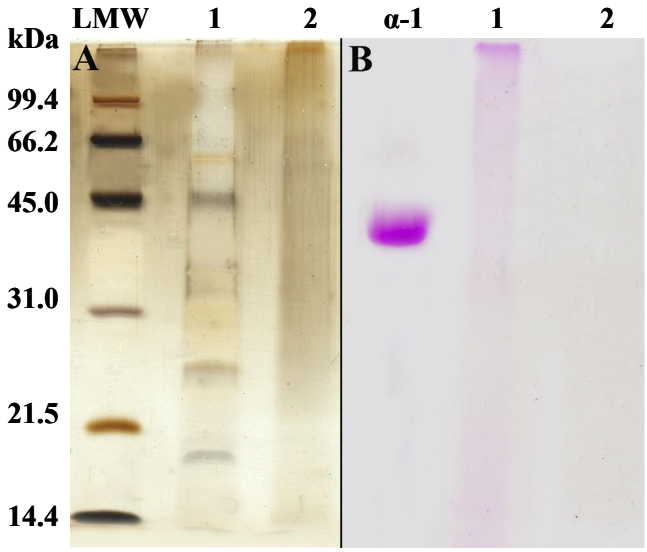

Figure 4.26: Separation of the lipoprotein extract according to Mühlradt et al. [161] by SDS-PAGE. Five µg protein of the total extract (**1**) as well as 50 µg dry mass of the TLR2 activating fraction 6 (**2**) (Fig. 4.25), were applied and visualized by silver (**A**) and PAS staining (**B**). The **LMW** was separated to determine the molecular mass. Alpha-1 acidic glycoprotein (**α-1**) (42 kDa) was used as a positive control for PAS staining.

The use of a preparative HPLC column (RP_{18} Nucleosil® 120-5, 250 x 10 mm) to increase the amount of material resulted in a complete change of the elution pattern with great variations of elution time dependent on the run. Variations of run conditions did not improve the chromatography. Because of this, alternative methods were checked to investigate the effect of components recognized by TLR2.

4.6.3.2 Isolation of amphiphilic molecules on octyl-sepharose

The enrichment of TLR2 agonists by RP-chromatography on a C_{18} column led to unspecific retention for the elution of TLR2 stimulating components in a preparative scale. Alternatively octyl-sepharose was utilized as stationary phase in a low pressure chromatography providing lower binding affinities for amphiphilic molecules [163]. Separation of the extract according to Mühlradt et al. resulted in reproducible chromatograms, but the collected fractions showed low stimulatory capacity on TLR2 transfected HEK 293 cells (data not shown).

According to the method of Morath et al. [163] for the isolation of amphiphilic molecules such as LTA, vegBl was disrupted by vigorous homogenization with glass beads. Molecules were separated between a butanol and an aqueous phase. The aqueous phase was dialyzed and separated on octyl-sepharose, purified in a gradient of 1-propanol

containing 15% ammonium acetate. Chromatograms of this hydrophobic interaction chromatography (HIC) were recorded at a wavelength of 254 nm. The relative IL-8 release by TLR2 transfected HEK 293 cells, induced by the lyophilized fractions, was analyzed to determine the elution of lipoproteins (Fig. 4.27).

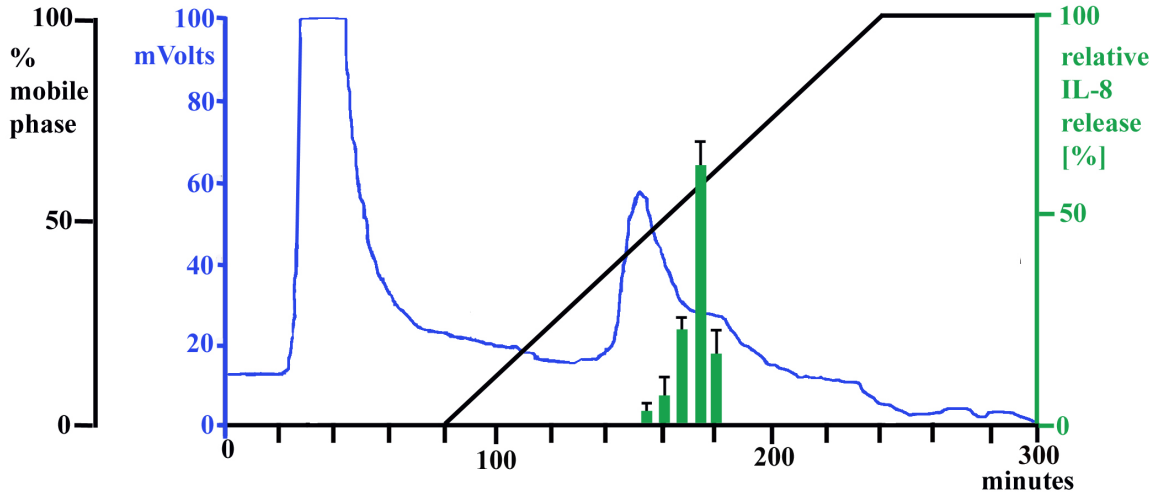

Figure 4.27: Chromatogram of hydrophobic interaction chromatography on octyl-sepharose. Octyl-sepharose was packed to a column for fast protein liquid chromatography (FPLC). Two mg of lyophilized aqueous phase material were solubilized in 15% aqueous 1-propanol and separated in an increasing gradient of 1-propanol in the presence of 0.1 mM ammonium chloride. The blue line represents the recorded absorption at a wavelength of 254 nm. The green columns represent the relative IL-8 release of fractions 30-34, collected for 5.5 min each. These were the only fractions stimulating cytokine release. The relative IL-8 release, measured after stimulation of TLR2 transfected HEK-293 cells with 100 ng of dried material was standardized concerning the release induced by 100 ng Pam_3CSK_4.

Additionally, phosphate was determined in the fractions as an indicator for the presence of LTA. The increase of UV-absorption co-eluted with a maximum of phosphate detection between 140 and 170 min. The results of phosphate measurement gave a maximum at an elution time of 155 min. Phosphate positive fractions and fractions containing TLR2 stimulating components were separated. Despite this, phosphate measurement was performed to check the reproducibility of different chromatographic runs, because the TLR2 activating fraction was not visible in the UV signal.

The fractions with TLR2 stimulating capacity in reproducible chromatographic separations were analyzed by SDS-PAGE to detect proteins of interest. Thereto, comparable amounts of lyophilized material were separated in comparison to the whole extract. As depicted in Figure 4.27 the capacity of TLR2 activation increased from fraction 30 to 33. Although

these fractions were eluted in the middle of the depicted gradient several proteins could be detected. Except for the detection of the Coomassie-positive band at a molecular mass of about 26 kDa, no differences between silver and Coomassie staining were observed in the total extract.

To identify the protein bands visible in the SDS gel they were blotted to a PVDF membrane and identified by *N*-terminal sequencing (Table 4.5).

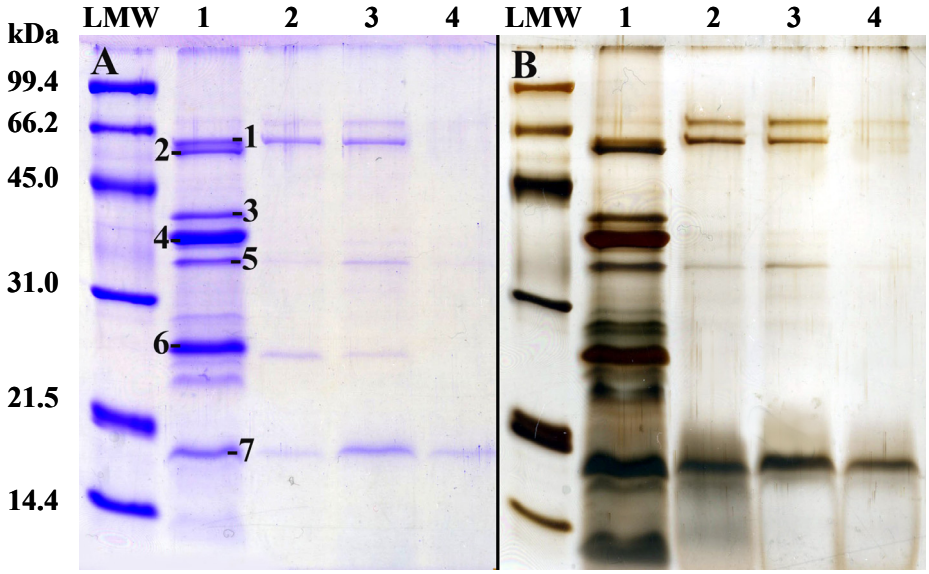

Figure 4.28: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (12.5%) of octyl-sepharose fractions. Fractions 33-31 (**2-4**) were separated in comparison to the total extract (**1**) and stained with Coomassie (**A**) and silver (**B**). Molecular mass determination was achieved by comparison to low molecular weight standard (**LMW**). For protein identification total extract was blotted to a PVDF-membrane and analyzed by *N*-terminal sequencing. The protein labeling refers to table 4.5.

With the exception of protein no. 5 all visible bands of the SDS-PAGE in Figure 4.28 were identified. Sequencing results for protein no. 5 did not allow the identification of this protein. Peptide mass finger print analyses of this protein provided no identification either. Therefore, it was left unidentified.

Amino acid sequences of the identified proteins were applied to compare those proteins to the predicted lipoproteins from the genome of strain ATCC 14580. This was achieved in the database of bacterial lipoproteins (DOLOP). None of the detected proteins was identified to belong to the 73 lipoproteins predicted. The results were confirmed by analyzing protein aa sequence by other prediction tools, using a less conservative aa sequence to predict the lipobox (see chapter 1.4.1.1.2.).

Table 4.5: Protein identification after octyl-sepharose separation. The first 10-14 amino acids were sequenced *N*-terminally after blotting the extract (lane 1, Fig. 4.28). Sequences were identified in the blast tool provided by expasy.org. All proteins were identified according to reference strain *B. licheniformis* ATCC 14580.

Band no.	UNIProt/TrEMBL entry accession number	Protein name	Predicted Lipoprotein[§]	Theoretical mass [kDa]	Observed migration [kDa]
1	Q65DD8	Catalase	No	54.835	58
2	Q65NN2	P5C dehydrogenase	No	56.655	56
3	Q65HN9	NADPH dehydrogenase	No	37.405	39
4	Q65G89	Malate dehydrogenase	No	33.597	37
5	n.i.				35
6	Q65K82	Alkyl hyper. reductase	No	20.403	27
7	Q65F57	MrgA (DNA-binding regul.)	No	17.920	18
8	Q65GE9	Thioredoxin	No	11.424	12

§Lipoprotein prediction was carried out by LipPred [(79)], Prosite [(81)], LipoP [(80)] and by affiliation of the identified aa sequence to the list of predicted lipoproteins from the genome of *B. licheniformis* ATCC 14580. n.i. not identified.

Further on, membrane association was tested by the transmembrane helix prediction tool (TMHMM) provided by CBS Prediction Servers. By checking the presence of hydrophobic transmembrane domains a measure for success of hydrophobic interaction between protein and column material was investigated. Proteins no. 1, 3, 4, and 6 showed the presence of hydrophobic domains. Out of these proteins, with predicted hydrophobic domains, only proteins no. 1 and 6 were enriched by HIC. Additionally protein no. 5 was enriched in TLR2 active fractions.

4.6.3.2.1 HEK-293 cell stimulation by isolated proteins

Despite the fact that the proteins eluted in the middle of the gradient in HIC were not predicted to be lipoproteins, their TLR2 stimulating activity was tested. Due to their enrichment by HIC, bands no. 1, 5 and 6 were chosen for electroelution. Proteins were separated in a preparative SDS-PAGE and excised bands were eluted. The success of elution was controlled by SDS-PAGE. Comparable amounts after SDS-PAGE were used to stimulate HEK-293 cells. Protein quantification was not possible due to low efficiency of electroelution. The results of HEK-293 cell stimulation by eluted proteins no. 1, 5 and 6 are presented in Figure 4.29.

The total extract (TE) activated all three transfected receptors with a clear difference in specificity. The highest IL-8 expression by transfected HEK cells was found for TLR2 transfected cells, followed by NOD2 transfected cells. In those cells expressing TLR4 the IL-8 expression was lowest.

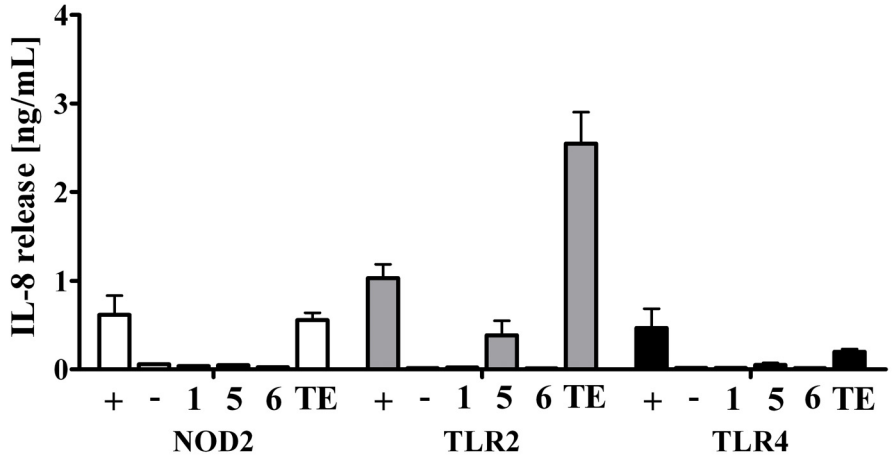

Figure 4.29: Stimulatory capacity of proteins after electroelution following HIC. The total extract (TE, 0.1 µg protein per mL) was tested in comparison to the eluted proteins. Protein bands were tested qualitatively after electroelution and controlled by silver staining. The numbering occurs according to the band labeling in figure 4.28. Human embryonic kidney cells were either transfected with TLR2, NOD2 or TLR4 expressing plasmids. Pam_3CSK_4 (TLR2, 100 ng·mL^{-1}), MDP (NOD2, 100 ng·mL^{-1}) and LPS (TLR4, 100 ng·mL^{-1}) were applied as positive controls (+). The IL-8 release of unstimulated cells is the negative control (-). Shown are means ± SEM of three different electroelutions, tested in duplicate.

Additional to the TE only the unidentified protein no. 5 stimulated TLR2 transfected HEK cells. Protein no. 5 was neither identified by *N*-terminal sequencing nor by peptide mass fingerprint. Therefore, its classification as bacterial lipoprotein was not confirmed by

prediction tools. Since quantification was not viable the stimulation of TLR2 by protein no. 5 was not comparable with the stimulation by the total extract.

4.6.3.2.2 Stimulation of dendritic cells by eluted TLR2 ligands

Qualitative analyses of moDC stimulation by eluted TLR2 ligand protein no. 5 as well as the TE and the positive control Pam_3CSK_4 were performed. Using the same concentrations as for the HEK-293 cell assay no relevant induction of TNF-α expression was observed. Due to these results comparison of Blspo and vegBl on the level of TLR2 ligation was carried out in a different approach.

4.6.3.3 Isolation of membrane protein extracts

Regarding the experiences in purification of lipoproteins, utilizing the hydrophobicity of their fatty acids, chromatographic approaches were not tested for Blspo. To compare vegBl and Blspo lipoprotein expression intact membranes were isolated according to Stoll et al. [78] and tested for NADH-activity in order to refer to their native state of isolation. In this way unspecific interaction of fatty acids and hydrophobic surfaces, as found in the above mentioned chromatography, was minimized.

Vegetative cells and spores were disrupted, the debris was removed by centrifugation and membrane fractions were enriched from the supernatant by ultracentrifugation. Following ultracentrifugation equal amounts of proteins, determined by a Bradford protein assay, were analyzed. Furthermore, the purity of the membrane preparations was tested by density gradient centrifugation in a sucrose gradient according to Kaplow et al. [190]. In this way the turbidity of spore membrane protein extracts was reduced by an additional centrifugation step. Both membrane preparations were opalescent, a characteristic appearance of micelles. Comparing the supernatants after membrane enrichment by ultracentrifugation revealed no differences between Blspo and vegBl (data not shown). Enriched membranes were extracted with Triton X-100 and precipitated proteins were resuspended in TNC buffer. These fractions were named spore membrane protein fraction (SpMPF) and vegetative membrane protein fraction (vegMPF), respectively.

The MPF was solubilized in SDS-PAGE sample buffer and the protein pattern of the membrane preparations was analyzed by differential staining of membrane-associated proteins. By comparing Coomassie and silver staining these proteins were distinguished from glycolipids and other membrane associated molecules. Figure 4.30 depicts an example of the reproducible extraction of membranes and membrane proteins from spores

and vegetative cells. Solubilized in SDS-PAGE sample buffer the MPF was compared to the soluble supernatant of MPF resuspended in aqueous TNC buffer. The total membrane preparations separated before extraction with Triton X-100 showed protein bands identical to MPF. Thus, Triton X-100 extraction was considered complete.

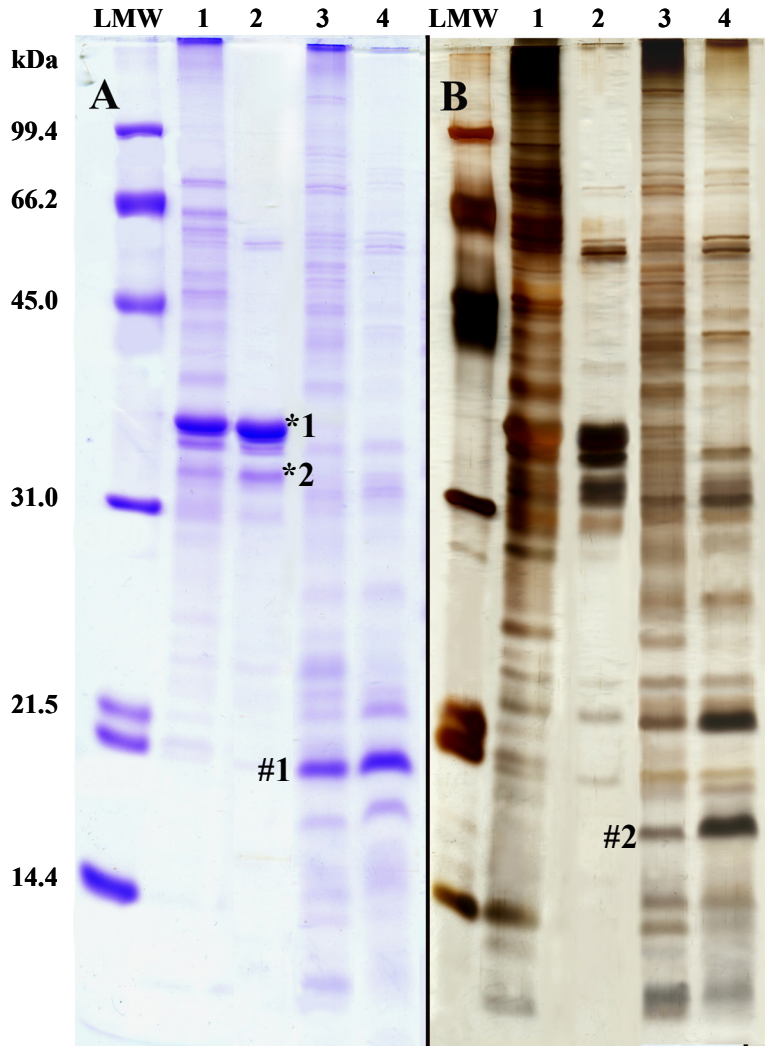

Figure 4.30: Sodium dodecyl sulfate polyacrylamide gel electrophoresis (12.5%) of extracted membrane protein fractions (MPF). The MPF of vegetative *B. licheniformis* 467 cells (vegMPF) as well as the spore MPF (SpMPF) after Triton X-100 treatment of membrane preparations are shown [(78)]. VegMPF (**1**) and SpMPF (**3**) were resuspended in TNC buffer and dissolved in SDS sample buffer. Components soluble in TNC after centrifugation were separated in lane **2** (vegMPF) and lane **4** (SpMPF). The comparison of Coomassie (**A**, 2.5 µg protein per lane) and silver staining (**B**, 1 µg protein per lane) allowed the differentiation of proteins and glycolipids or other components. Major spore-specific bands were marked with # and major bands specific for vegBl preparations with *. Molecular mass was determined by comparison to **LMW** standard.

Specific bands were detected in vegMPF as well as in SpMPF. A Coomassie-positive band of about 37 kDa (*1) was specific for vegBl preparations. Moreover, differences between Blspo and vegBl were detected at a molecular mass of about 34 kDa (*2) in vegMPF extractions. In SpMPF fractions specific bands were detected in Coomassie stained gels (~ 20 kDa, #1) and in silver stained gels (~ 16 kDa, #2). These bands were present in the corresponding TNC soluble fractions. Differences in silver staining between samples soluble in SDS-containing sample buffer and TNC buffer showed strong purification for preparations obtained from vegBl, whereas Blspo preparations differed slightly.
The TLR2 activity was localized to be present in the TNC buffer soluble fraction of the MPF (Fig. 4.31). Centrifugation of the extracts marginally decreased the IL-8 induction of TLR2 transfected HEK cells proving the soluble part to be responsible for their activation.

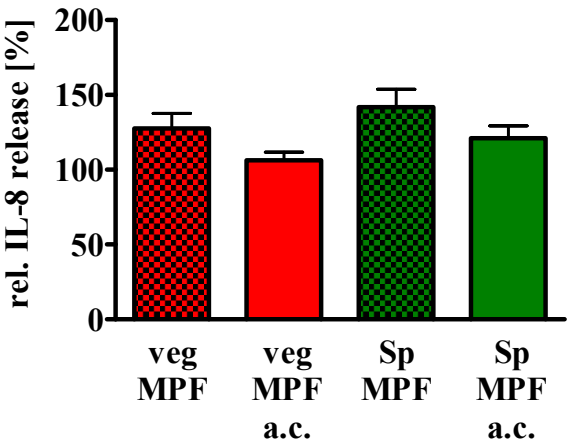

Figure 4.31: Localization of TLR2 stimulating activity in MPF. Membrane proteins of vegBl resuspended in TNC were applied before (vegMPF) and after centrifugation (vegMPF a.c.). The same was done for membrane protein fractions isolated from *B. licheniformis* 467 spores (SpMPF and SpMPF a.c.). Interleukin-8 release induced by 100 ng of protein was standardized on the induction by 100 ng100 ng mL^{-1} Pam_3CSK_4.

Since the TLR2 activity was detected in the supernatant of MPF during extraction of vegetative cells as well as in spore preparations, further investigations were performed with these soluble fractions.

4.6.3.3.1 Identification and characterization of isolated MPF proteins

The specific proteins *1, *2 and #1 (Fig. 4.30) stained by Coomassie were identified by *N*-terminal sequencing and peptide mass fingerprints of the SDS-PAGE bands after vegMPF and SpMPF separation. Band *2, specific for vegMPF was enriched by Triton X-114 phase separation, to purify amphiphilic molecules [164]. This purification was confirmed by an increase of stimulation in TLR2 transfected HEK-293 cells (data not shown).

Such increase in TLR2 recognition was going along with an enriched detection of proteins no. 6 and 7 by SDS-PAGE of the Triton X-114 phase extract (Fig. 4.32). Proteins were separated by SDS-PAGE and identified by peptide mass fingerprint (Fig. 4.32, Tab. 4.6).

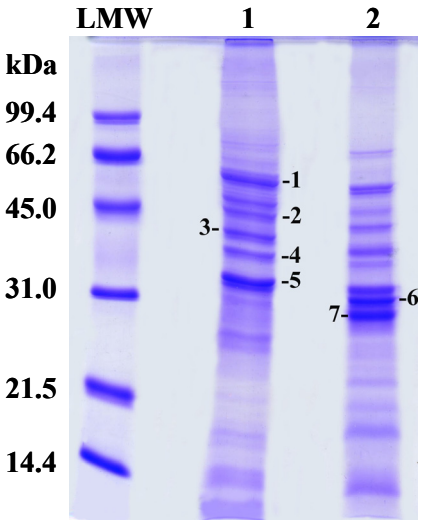

Figure 4.32: Separation of vegMPF before and after Triton X-114 extraction by SDS-PAGE (12.5%). VegMPF (lane **1**, 5 µg protein) and the Triton enriched phase of vegMPF (lane **2**, 5 µg protein) are compared. Proteins no. 6 and 7 were enriched by phase separation of Triton X114 (2% v/v) and H_2O. Results of the protein identification are denoted in Table 4.6. The molecular mass was determined by comparison to low molecular mass standard (**LMW**).

Protein band no. 5, identified to contain the flagellin protein Hag, corresponded to the vegMPF-specific band *1 in Figure 4.30. By Triton X-114 extraction band no. 6 was enriched and identified to be the manganese transporter system substrate binding protein (MntA).

Bioinformatic lipoprotein prediction for the identified proteins was contradictory. The MntA protein was not homolog to any predicted lipoprotein in the DOLOP database for *B. licheniformis* ATCC 14580. In contrast, the LipPred algorithm by Taylor et al. [79] (www.jenner.ac.uk/LipPred/) as well as the LipoP [80] and the ExPASy prosite tool predicted them to be lipoproteins. The later adapted an algorithm established for lipoprotein identification in Gram+ bacterial genomes [81].

Table 4.6: Protein identification of SDS-PAGE bands in vegMPF by peptide mass fingerprint (Fig. 4.32). Peptide mass fingerprint analyses identified proteins using the mascot tool on matrixscience.com. Protein properties as well as the complete aa sequence were then extracted from the UNIProt/TrEMBL database. Lipoprotein prediction[§] was carried out by LipPred [(79)], Prosite [(81)], LipoP [(80)] and by affiliation of identified aa sequence to the list of predicted lipoproteins from the genome of *B. licheniformis* ATCC 14580.

Band no.	UNIProt/TrEMBL entry accession number	Protein name	predicted Lipoprotein[§]	Theoretical mass [kDa]	Observed migration [kDa]
1	Q65K42	Pdh~C	No	46.357	45
2	Q65PA9	EF-Tu*	No	43.653	44
3	Q65K44	Pdh~A	No	41.712	42
4	Q65K43	Pdh~B	No	35.504	37
5	Q65EB8	Hag[+]	No	33.221	34
6	Q65EZ1	MntA[$]	Yes	34.344	33
7	Q65EH0	OPF0042	No	33.637	32

[~] pyruvate dehydrogenase, * elongation factor Tu, [+] flagellin protein Hag, [$] manganese transporter system substrate binding protein.

To determine whether MntA represents a lipoprotein, MALDI-TOF experiments were performed. Repeated analyses covered up to 70% of the aa sequence (Fig. 4.33).

1 MKWKQTLAIA AALILILAAG CSSKSSSEEN GKLKVVTTYS ILYDIVKQVG
51 GDHIDLYSIV PVGTDPHEYD PLPKDVQKTT DADVVFYNGL NLETGNGWFN
101 KLLETAEKDG DDAPVYKLSK GVKPKHLTAK GKETEEDPHA WLDIENGIQY
151 AKNARDALIK NDPDHKEDYE KNAEAYIGKL QKLHDEAVNR FKDIPKERRV
201 LVTSEGAFKY FASAYGVDAQ YIWEINTENE GTPGQMKKIV DTVKKKDVPA
251 LFVETSVDPR SMESLSAETG VPIKAKVFTD SIGKPGEAGD SYYKMMKENL
301 DRIHQGLAE

Figure 4.33: Sequence coverage of MntA. The protein band was identified by MALDI-TOF MS and obtained masses were transferred to MASCOT peptide mass fingerprint. Sequence coverage was up to 70% (red) missing the first 47 aa of the protein. According to lipoprotein prediction the possible lipobox (green) is cleaved and the invariant cysteine is modified. This modification prohibited the identification of this peptide by MASCOT search.

Despite this the first 47 aa of the predicted prelipoprotein including the possible modification of a lipid anchor were missing in every single experiment. Additionally peptide masses were screened for modified peptides starting at the predicted *N*-terminal cysteine. This cysteine is modified by attachment of the lipid anchor catalyzed by the diacylglyceryl transferase (Lgt) at position 21 of the prelipoprotein. Masses were screened including the dipalmitoylated lipid anchor [78], but none of them was identified to be part of the predicted lipoprotein.

4.6.3.3.2 Characterization of spore membrane extract proteins

Protein identification was performed in the same way for SpMPF. Following SDS-PAGE analysis spore proteins were identified by *N*-terminal sequencing as well as by peptide mass fingerprint (Fig. 4.34, Table 4.7).

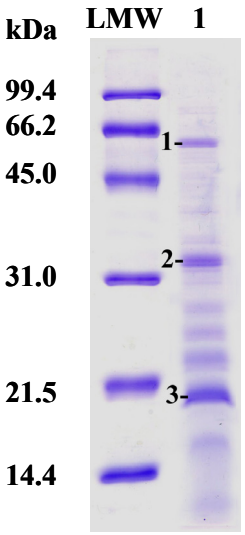

Figure 4.34: Separation of the spore membrane protein fraction supernatant. The SpMPF extract after solubilization and centrifugation (SpMPF a.c., lane 1) was separated by SDS-PAGE (12.5%) and proteins were detected by Coomasssie staining. Bands labeled with no. 1, 2 and 3 were analyzed by MALDI-TOF MS. The low molecular mass standard (**LMW**) was separated to estimate the protein size.

Bands no. 1 and 2 were neither identified by peptide mass fingerprint nor by *N*-terminal sequencing. Band no. 3 was identified to be the putative uncharacterized protein YhcN (spore-specific protein #1 Fig. 4.30). The YhcN protein was described by Bagyan et al. [191] to be a spore protein found in *B. subtilis*. Membrane protein isolations located this protein in the inner forespore membrane. Additionally to the presence in isolated spore membranes a sequence similarity to the lipoprotein consensus sequence was suggested. Sequences of both YhcN proteins from *B. licheniformis* and *B. subtilis* were aligned in BioEdit using the PAM250 matrix. An aa identity of 43.3% as well as a similarity of 62.9% of aa was detected.

Table 4.7: Protein identification of SDS-PAGE bands in SpMPF by peptide mass fingerprint (Fig. 4.34). Peptide mass fingerprint analysis was performed and proteins were identified using the MASCOT tool on matrixscience.com. Protein properties as well as the complete aa sequence were then extracted from the UNIProt/TrEMBL database. All proteins were identified according to reference strain *B. licheniformis* ATCC 14580.

Band no.	UNIProt/TrEMBL entry accession number	Protein Name	predicted Lipoprotein[§]	Theoretical mass [kDa]	Observed migration [kDa]
1	n.i.				
2	n.i.				
3	Q65M28	YhcN	Yes	20660	20

[§]Lipoprotein prediction was carried out by LipPred [(79)], Prosite [(81)], LipoP [(80)] and by affiliation of identified aa sequence to the list of predicted lipoproteins in the DOLOP database of bacterial lipoproteins from the genome of *B. licheniformis* ATCC 14580. n.i. = not identified

The bioinformatic lipoprotein prediction was contradictory. Both YhcN sequences were not predicted to be lipoproteins by the DOLOP database algorithm as well as they were missing in the list of predicted lipoproteins from genome analyses. The other tools for lipoprotein prediction, mentioned above, predicted them to be lipoproteins. False positive results were excluded by prediction of the *N*-terminal membrane spanning domains. The lipoproteins carry an *N*-terminal membrane domain not extending the cysteine in position one of the lipoprotein. Both criteria were used to control bioinformatic lipoprotein analysis in literature [(81)].

Peptide mass fingerprint analyses for YhcN were similar to those results presented for MntA analysis. Sequence coverage of repeated experiments was up to 76%, but the first peptide fragments containing a possible modification were not detected. Removal of ester bound fatty acids by incubation in 12% aqueous ammonia was performed to detect the *N*-terminal peptide, with an additional mass of 74 Da for the attached glycerol. The corresponding fragments were not detected, although an improvement of peptide elution from the gel was expected. In this way similar results were obtained as they were described for the MASCOT analysis of MntA (Fig. 4.33).

4.6.3.3.3 Specificity of HEK-293 cell stimulation of eluted proteins

To investigate whether the predicted lipoproteins function as specific TLR2 ligands extracts as well as eluted proteins were tested on transfected as well as untransfected HEK cells. In addition to the specific stimulation of TLR2 transfected HEK cells by vegMPF and SpMPF, unspecific stimulation of untransfected HEK cells was detected after stimulation with vegMPF (Fig. 4.35, upper panel).

Single protein bands were isolated and tested for their specificity of stimulation. The vegMPF-specific proteins Hag and MntA were isolated. The protein MntA was predicted to be a lipoprotein, whereas Hag, the full-length flagellin protein of *B. licheniformis* [(192)], was a candidate for TLR5 recognition of flagellin [(193;194)]. Additionally, PdhC was isolated to control the purity of the electroelution. Since the amounts eluted from SDS-PAGE were not enough to perform these experiments quantitatively, their effect was tested after electroelution and controlled by SDS-PAGE and silver staining. Comparable amounts were used to stimulate transfected and untransfected HEK-293 cells qualitatively (Fig. 4.35 lower panel).

The obtained results indicate that vegMPF as well as the isolated Hag protein and MntA were able to stimulate HEK cells in an unspecific way. All transfected as well as untransfected cells were stimulated to release IL-8. For PdhC this unspecific stimulation was very low in comparison to the other vegBl-proteins. The specific recognition of flagellin protein Hag by TLR5 was not confirmed in this way.

Two-dimensional gel electrophoresis of the TNC soluble vegMPF was performed, to check whether the protein bands for Hag also contained other proteins. By this technique the major spots were identified to be modifications of the Hag protein. However, the co-migration of other proteins present in significantly lower amounts cannot be excluded (Fig. 4.36).

In contrast to MntA and Hag, specific for the vegetative extract, YhcN extracted from SpMPF specifically activated TLR2. Following this purity control the effect of YhcN as an exclusive ligand for TLR2 was investigated on moDCs.

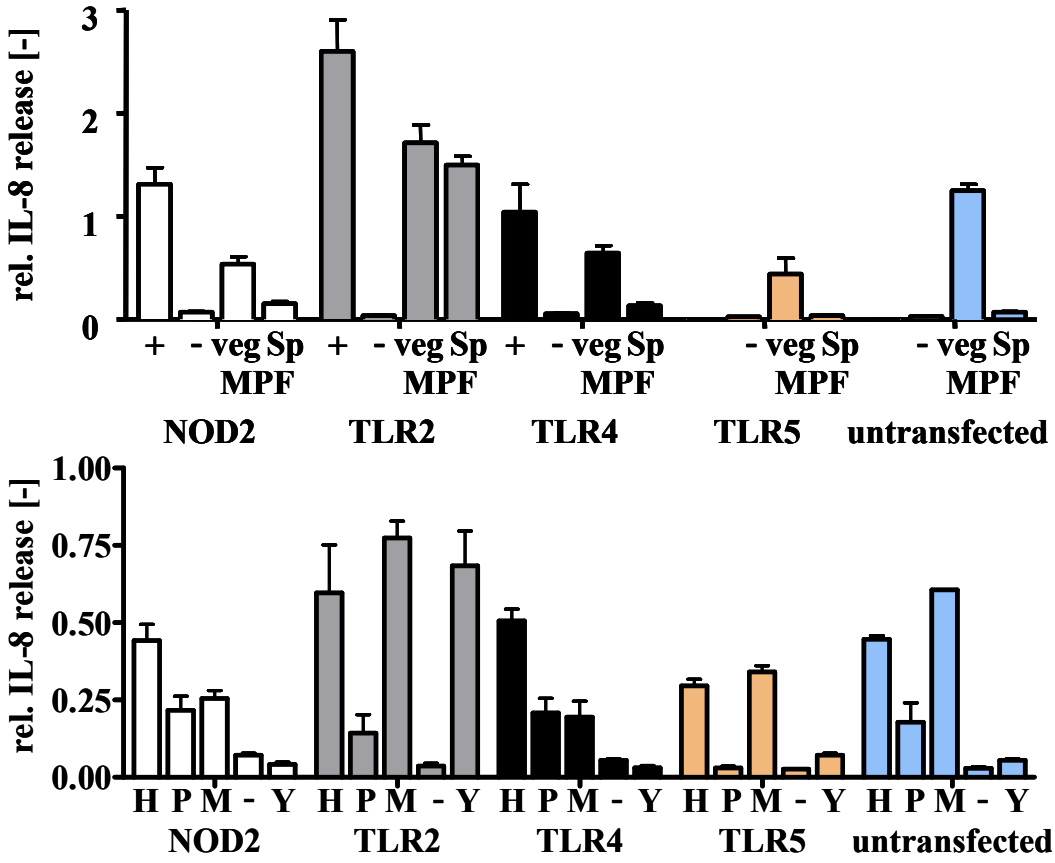

Figure 4.35: Specific stimulation of HEK-293 cells by MPF extracts. Analyzed cells were either transiently transfected with expression plasmids containing NOD2, TLR2, TLR4 and TLR5 or were left untransfected. The **upper panel** shows the relative IL-8 release after stimulation by membrane protein fractions vegMPF and SpMPF. Interleukin-8 expression is depicted relative to the stimulation with 1 ng TNF-α. Pam_3CSK_4 (TLR2, 100 ng·mL^{-1}), MDP (NOD2, 100 ng·mL^{-1}) and LPS (TLR4, 100 ng·mL^{-1}) were applied as positive controls (+). The viability of TLR5 transfected HEK cells and untransfected cells was tested by stimulation with TNF-α. As negative control (-) cells were left unstimulated. Shown are the means ± SEM of two different extractions, tested in 3 independent experiments each.

The **lower panel** shows the relative IL-8 release of eluted protein bands standardized to the release induced by TNF-α. Vegetative proteins Hag (H), PdhC (P) and MntA (M), were eluted by excision of their respective band in SDS gels of vegMPF. Eluted proteins were tested for success of elution by SDS-PAGE and silver staining. Comparable amounts were used to stimulate HEK cells. Depicted are results of two independent electroelutions measured twice in the HEK cell assay. Negative controls are represented by unstimulated cells (-). In the same way YhcN (Y) was eluted following SDS-PAGE separation of SpMPF.

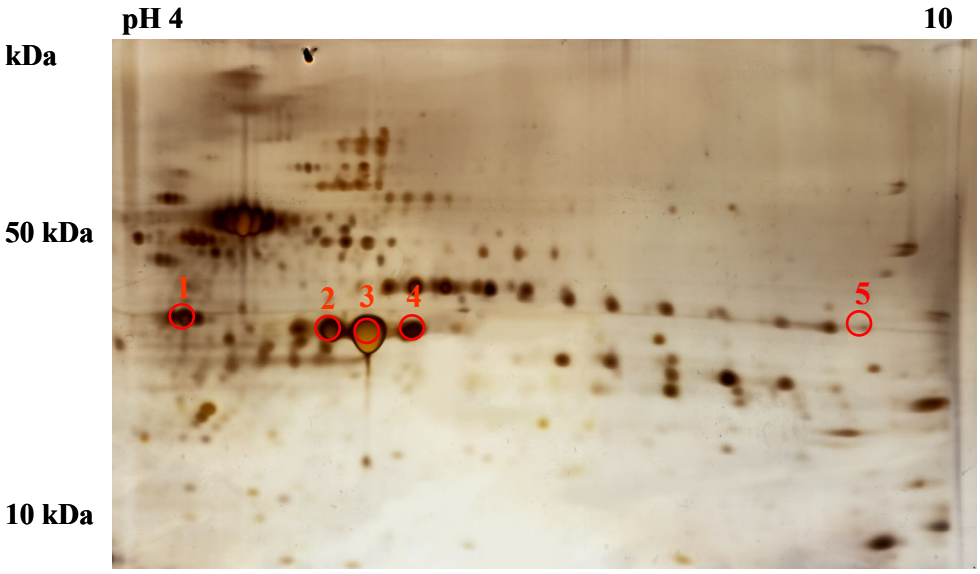

Figure 4.36: Two dimensional gel electrophoresis of the soluble supernatant of MPF extraction. Proteins were extracted and purified by Ready Prep 2D clean up kit. The lyophilized material was solubilized in rehydration buffer. Twenty five micrograms of protein were separated in the first dimension by NEPHGE. The second dimension was a 12.5% SDS-PAGE. Marked spots in the molecular weight region of the vegMPF protein Hag were identified by peptide mass fingerprint as follows: **1**: pyruvate dehydrogenase β-subunit (PdhB), **2**, **3**, **4**: flagellin protein Hag, **5**: Cytosine/ adenine deaminase. Depicted is one representative experiment out of four. Molecular mass and pH were determined by parallel separation of a protein standard.

4.6.3.4 Effect of membrane protein extracts on cytokine expression of moDCs

The question of whether the MPF of Blspo and vegBl cells can explain the differences in moDC stimulation observed for the intact cells, the different fractions obtained during extraction of membrane proteins were tested with respect to their cytokine expression. Results of TNF-α expression are depicted in Figure 4.37. Corresponding fractions of membrane protein extractions from vegBl and Blspo were compared. By application of equal amounts of protein no significant differences between the fractions of Blspo and vegBl were detected.

As presented before TNF-α expression is an appropriate marker to reveal differences between spores and vegetative cells (Fig. 4.9). TNF-α expression induced by different fractions of membrane preparations showed no differences between corresponding extracts of Blspo and vegBl preparations. In contrast, differences resulting in moDC stimulation with intact vegBl and Blspo cells were highly significant. The TNF-α release was above 6 ng·mL^{-1} following stimulation with vegBl at a MOI of 1 and below 2 ng·mL^{-1} for Blspo. To exclude that these differences between whole cells and extracts were due to

concentration effects, higher amounts were tested to stimulate moDCs. Amounts up to 10 µg·mL^{-1} led to a decrease in TNF-α expression (data not shown).

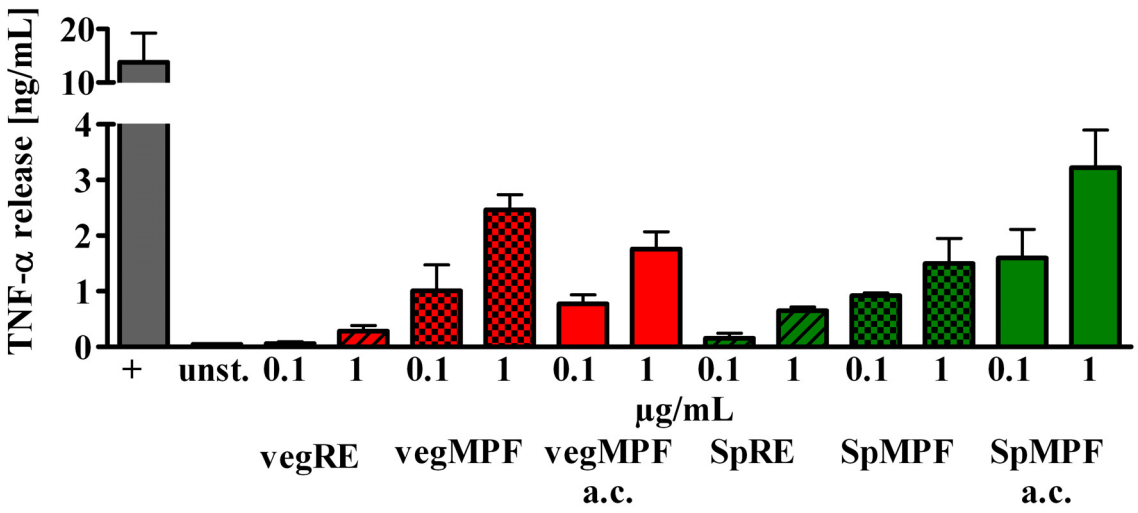

Figure 4.37: Stimulation of TNF-α expression by membrane protein preparations. Different fractions of MPF isolation were tested. Supernatants received after cell disruption of Blspo and vegBl followed by ultracentrifugation of membranes are depicted as raw extract (RE). This membrane protein free fraction was compared to the enriched membrane protein fractions (MPF) prepared as described above. For each extract 0.1 or 1 µg protein were used to stimulate 10^6 moDCs per mL. Lipopolysaccharide of *Salmonella enterica* sv. Friedenau (10 ng·mL^{-1}) was applied as positive control (+) and unstimulated cells were negative controls (-). Depicted are the means ± SEM of two independent extraction procedures tested in a concentration dependent manner on moDCs. Cells were isolated from peripheral blood of 3 different patients in independent experiments. Statistically significant differences were not detected.

The expression profiles of TNF-α release, induced by the different extracts, were similar to the IL-8 release of TLR2 transfected HEK-293 cells (Fig. 4.21). The release of IL-8 by transfected HEK cells was higher after induction with MPF before centrifugation. In case of moDC stimulation the spore extract doubled TNF-α expression after removal of insoluble material. As Figure 4.30 depicts the most abundant protein present in soluble supernatants of SpMPF is YhcN. Since this protein was isolated as a specific TLR2 ligand investigations of cytokine induction by single proteins were performed. The same preparations of Hag, MntA, PdhC and YhcN tested on transfected HEK cells (Fig. 4.35) were applied to stimulate moDCs. As described for the elution of HIC-isolated proteins no relevant stimulation of TNF-α release was observed.

5. Discussion

In this thesis the protective effect of early life contact to microbial spores and their compounds on the development of childhood asthma and allergy was analyzed. The investigations based on epidemiological evidence for prenatal and early life contact to farm animals and farm milk as well as sheltering in stables. These factors were evidently associated with a decrease in atopic sensitization of farm children. The suggested effect was recently proved in a mouse model of allergic asthma. Intranasal treatment with stable dust extract prior to sensitization avoided the development of asthmatic phenotypes. To gain further information about possible organisms and components responsible for this beneficial effect the microbial flora of stable dust was investigated. In a pilot study dusts were collected from farms involved in the mentioned epidemiological studies and spore forming bacteria and fungi were detected to be the most prominent. Among those *B. licheniformis* and *Mucor* ssp. were identified to be the most relevant microorganisms farm children are in contact with. They were detected to be present in stable dust and clearly correlated to transport from stable to farm children's mattresses. Therefore, the organisms were isolated from these mattresses.

This work deals with the specific ability of spores to stimulate components of the innate immune system. By comparison to vegetative cells their specific impact on the development of allergies and their association to the prevention of asthma provocation was analyzed in a mouse model of allergic asthma. *Bacillus licheniformis* peptidoglycan and lipoproteins were isolated to determine the effect of the major components of bacterial cell wall and membrane modulating innate immune stimulation.

5.1 Preparation of pure spores, excluding germination and outgrowth

Sporulation of *B. subtilis*, the model organism for bacterial sporulation, was described to be initiated by nutrient limitation and high cell densities [112]. The conditions for sporulation of *B. licheniformis* 467 isolated from farm children's mattress dust and *B. licheniformis* 830 from a non-farm children's mattress of the same region had to be optimized. Different growth media were compared with yields for the model organism *B. subtilis*. In contrast to the optimized conditions found for *B. subtilis* (in Difco Sporulation Medium, by Monteiro et al. [177]), *B. licheniformis* was grown to optimal cell densities in LB medium, supplemented with divalent cations. The highest yields of

B. licheniformis spores were found after a three weeks process. This process was divided into a growth phase up to high cell densities and an adjacent sporulation phase. In this way optimal yields in line with high purity of spores after density gradient centrifugation were obtained. Interestingly, literature mentions different procedures for the induction of sporulation, but the degree of spore purification and their control is often unattended or investigated by processes insufficient for highly purified preparations [137;177;191;195].

In a diploma thesis Björn Alich scaled the sporulation process up to an 8 L batch with a fermentation time of 30 h. Different conditions, e.g. pH values and oxygen concentrations were tested. The results confirmed an optimal growth as basis for high cell densities being essential for efficient sporulation. In case nutrients like glucose were added, complete sporulation was prevented due to the germination triggering effects of remaining glucose [129]. In contrast, nutrient limitation directed the formation of endospores, surrounded by their mother cell wall in minimal medium and under oxygen limitation, indicating an incomplete sporulation cycle [112]. Both forms were not removable by density gradient centrifugation, excluding these nutrient limitations as effective inducers of sporulation efficiency.

A limitation for optimized conditions in supplemented LB medium was the varying presence of spores stained red by safranin O. This was a sign of germination without the ability of outgrowth to a vegetative cell. Since the thick spore cell wall is responsible for the inhibition of malachite green release in spores, changes like cortex hydrolyses must have been performed in these cells [152]. Variations in the occurrence of these red stained spores were not related to any tested variation of growth. Thus, preparations with maximal yields of malachite green dormant spores in LB^+ medium were used.

An additional observation with respect to germination was the inhibition of outgrowth in the cell culture media for immunological experiments, containing antibiotics. Germination occurred in a low percentage when spores were incubated for 24 h in cell culture media. However, outgrowth seemed to be prevented by sensing the spore surrounding environment for the content of the antibiotics streptomycin and penicillin, possible inhibitors for spore nutrient receptors [196]. The detailed mechanisms of how spores sense their environment for toxic molecules is not yet described in literature. Without detailed investigations of germination mechanisms the spore purification was optimized to obtain preparations free of vegetative cells, with an optimal content of malachite green positive spores, to obtain results specific for dormant spores.

5.2 *Bacillus licheniformis* spore stimulation of the innate immune system leads to T_H1 polarization *in vitro*

According to the concepts of missing immune deviation or missing immune suppression on the level of T-cell polarization, immunological experiments were performed to investigate the effect of *B. licheniformis* and *Mucor* spp. spores.

Investigations of spores in immunological experiments had to consider their adoption to the surrounding environment. Within the scope of spore inhalation and its effect on allergy protection by innate immune stimulation environmental conditions can trigger spore germination, following inhalation to the lung. To clarify this germinable and heat-inactivated spores (constricted in germination) were compared to the vegetative form.

In contrast to *Mucor* spp. spores *Bacillus* spores led to a relevant concentration dependent stimulation of epithelial cells and PBMCs. Stimulating these important components of the innate immune system, *B. licheniformis* spores were possible candidates for the polarization of T-cells. Significant differences of cytokine expression and maturation of moDCs were detectable for spores and vegetative cells. A possible reason for those differences is a significantly lower phogocytosis of spores. *Bacillus licheniformis* 467 provoked a spore-specific expression of moDC cytokines, activated by recognition of innate immune receptors such as TLR2 and NOD2. Spores induced higher expression levels of the important T_H1-directing cytokine IL-12p70 [55] than vegetative cells. Converse results were detected for the cytokines IL-10 and TNF-α. Furthermore, the recognition of *B. licheniformis* by the innate immune receptors TLR2 and NOD2, revealed by the induction of IL-8 release in transfected HEK 293 cells, was significantly higher when stimulated with vegetative cells. As proposed, the effect on T_H-cell polarization by the induced moDC cytokines resulted in significant differences. *In vitro*, moDC stimulation with *B. licheniformis* 467 spores, prior to mixed leukocyte reaction induced the up-regulation of IFN-γ. This cytokine, produced by T_H1-cells, is able to down-regulate the expression of T_H2 cytokines [32;39]. In contrast, vegetative cells induced the expression of IL-5 and IL-13, typical cytokines expressed by T_H2-cells [5].

These results suggest the hypothesis of a spore-specific T_H1 cytokine induction in the airways by spore inhalation evoking an anti-allergic signal. It should skew the T_H2 biased immune response in allergic individuals to a non-allergic response and prevent allergy and asthma development [39;136]. Despite spore-specific induction of IL-12p70 the amounts expressed are rather low in comparison to the induction by *Acinetobacter lwoffii* and

Lactocuccus lactis stimulation, as described recently [107]. A mouse model of OVA sensitization and provocation was employed to clarify these effects *in vivo*.

5.3 *Mucor* spp. spores and the missing innate immune recognition

The recognition of fungal components by TLRs is controversially discussed [197]. A possible allergy protection is opposite to the induction of asthma and allergy in response to indoor mould exposure [138;139;198]. *Mucor* ssp. spores which did not stimulate strong cytokine induction in epithelial cells as well as moDCs *in vitro* were excluded from further *in vivo* experiments. Essential for T-cell polarization the stimulation of moDCs was considered as a crucial step for further experiments based on investigations with the above mentioned bacteria isolated from farm cowsheds [107;199]. Against the background of a possible immune suppression by contact to dust extracts the results obtained with *Mucor* spp. spores refer more to a possible immune suppression and can be a subject for future studies.

5.4 The T_H1 specificity of Blspo stimulation does not occur *in vivo*

Intranasal treatment of BALB/c mice during the whole process of allergen sensitization and provocation led to a mitigation of phenotypic characteristics. The allergic airway infiltration of eosinophils was decreased and the establishment of goblet cell hyperplasia and bronchial wall thickening was prevented. The described changes were general effects of *B. licheniformis* 467 treatment. However, the potency of the effect in comparison to PBS-treated mice depends on the form of *B. licheniformis* application. On the level of BAL cells these differences were obvious.

Prevention of eosinophil influx reached a maximum in mice treated with vegBl and a minimum in hiBlspo treated mice. The opposite was found for the influx of neutrophils. Significant increases were observed after vegBl and Blspo treatment. Interestingly, in both cases the effects of Blspo were between those of vegBl and hiBlspo. Spore germination in the airways could be an explanation for this. For macrophages the influx is maximal after hiBlspo treatment and minimal for vegBl.

An explanation for this effect was indicated by lung histology. Malachite green stained Blspo and hiBlspo were detected in alveolar macrophages, even four days after the last treatment. On the other hand vegetative bacteria were neither observed in vegBl treated nor

in Blspo treated mice, although different staining procedures were tested. The detection of spores in spore treated groups was confirmed for BAL cytokine expression, measured four days after the last treatment. Continuous immune activation by germinable spores allowed the detection of increased IFN-γ and IL-10 concentrations. Levels of INF-γ were increased fivefold in Blspo treated mice in comparison to hiBlspo and vegBl treated groups. The BAL level of IL-10, a consequence of Blspo treatment, increased more than threefold. In the other groups the cytokine was not detectable at this time. Concluding these differences, two mechanisms for the clearance of spores and vegetative cells can be suggested. On the one hand an efficient clearance of vegBl mediated by neutrophils and on the other hand a clearance of spores by macrophages. The latter is an ongoing process at the time of analyses [(128)]. Differences in the clearance of spores were described for the pathology of *B. anthracis*. While inhalation anthrax is highly lethal, because spores are phagocytozed and cleared inefficiently by macrophages, cutaneous infections remain local due to the effective clearance by neutrophils [(200)]. *Bacillus subtilis* the closest relative of *B. licheniformis* was cleared by macrophages *in vitro*. This process depends on the germination of the spores, allowing the macrophage to digest the vegetative cell [(201)]. The spore, as an robust bio-particle, can resist the conditions inside the phagolysosome [(128)]. The mechanism proposed for germination and outgrowth of spores inside macrophages is a chemical mimicry of conditions initiating spore germination [(202)]. Differences to hiBlspo are an additional hint to this, because they cannot be cleared via germination. To confirm this suggestion a kinetic approach has to be performed in future studies.

The expression of cytokines has to be either related to the germination of spores or to the denaturation of spore proteins by heat-inactivation, since the detection was not possible in hiBlspo treated mice. The T_H1 subtype of T_H-cells use IFN-γ to recruit macrophages and neutrophils [(203)], but other cells can be responsible for IFN-γ release. Among these $CD8^+$ T-cells, natural killer cells or interferon-producing killer dendritic cells are described recently [(204)]. Outside macrophages no spores were detected, after the repeated removal by BAL. Due to further processing of the BAL, it was not possible to prove the incidence of spores in lung tissue, being able to affect other immune cells. To clarify this discrepancy, repetition of mice experiments is necessary. In these experiments Blspo and vegBl can be detected in a time dependent manner by an immuno-histological approach using specific antibodies.

The cowshed isolates of *A. lwoffii* and *L. lactis*, applied according to the same protocol, down-regulate eosinophilic infiltrations without the up-regulation of neutrophils and

macrophages, respectively [107]. Different mechanisms induced by these bacteria seem to be responsible for the prevented influx of eosinophils. The increase of neutrophils and macrophages by *B. licheniformis* 467 treatment seem to down-regulate eosinophilic infiltration. Therefore, T_H2 effector cells were counter-regulated by T_H1 and T_H17 responses, recruiting macrophages and neutrophils, respectively [38]. The cytokines related to these effects cannot be observed, because a down-regulation of the T_H2 cytokine IL-5 did not occur, although IFN-γ was increased dramatically after spore treatment. Interestingly, the detection of IL-4, the major counter-regulating cytokine of IFN-γ [205], was not possible in any mouse group (data not shown).

Debarry et al. describe a strong induction of IL-12p70 release in moDCs, by *A. lwoffii* and *L. lactis*. This cytokine leads to the induction of T_H1 polarization *in vitro*, but neither a recruitment of T_H1-cells (e.g. macrophages) was observed nor the induction of IFN-γ was measured in the BAL [38]. The authors explain this with a possible induction of tolerance to the bacteria, although *A. lwoffii* causes a significant increase of lymphocytes in the BAL. The relation to the presence of cytokines has not been mentioned in this study.

Their findings stay in contrast to the exclusive effect of Blspo attenuating the lymphocyte increase aroused by OVA sensitization and challenge. Heat-inactivated spores did not affect the concentration of antigen-specific lymphocytes. This can be explained by the destruction of heat-labile antigens on the surface of spores. Interestingly the decrease in lymphocytes as well as the influx of macrophages was also described for the treatment of mice with stable dust extracts [106]. The protective effects on the establishment of lung-allergy in this study were associated to a suppression of moDC generation and the presence of unresponsive T-cells. This alleviated the expression of T_H2 cytokines after *in vitro* restimulation of spleen lymphocytes. The authors do not present data of moDC cytokine expression, thus a further correlation between the mouse model and its relations to human DC cytokines cannot be suggested and needs further investigation. However, murine and human immune responses differ for example in T_H1 and T_H2 cytokine expression, initially observed in mice. Thus, the relations between mouse and human have to be interpreted carefully [206] with respect to possible applications in asthma treatment.

The described effects of *B. licheniformis* treatment showed parallels to the protective effects of dust extract rather than similarities to the effects of the isolated cowshed bacteria *A. lwoffii* and *L. lactis*. Presumably the protective effects of *B. licheniformis* spores, acting locally in the airways, are due to the induction of IL-10 in DCs. This cytokine induces IL-10 producing T_{regs} [5;102] responsible for immune suppressive effects which are

observable also in the allergy protective effect of helminth infections [13]. Regulatory T-cells are able to suppress both T_H1 and T_H2 immune responses and can be activated via DCs or by direct TLR ligation.

Another beneficial effect of *B. licheniformis* treatment was the prophylaxis of goblet cell hyperplasia. Independent whether the treatment was preformed with spores or vegetative cells, the prevention was almost complete. Despite the inhibition of increased goblet cell density associated with the expression of the T_H2 cytokine IL-13 [207], airway hyperreactivity was not improved. According to Kuperman et al. [208] IL-13 has a direct influence on both, the mucus hypersecretion and the hyperreactivity of airways to unspecific stimuli. However, in case of *B. licheniformis* treatment these characteristics of asthma were affected independent from each other.

Recently, the effects of systemic TLR3 and TLR7 ligand treatment were studied [209] in a mouse model. The improvement of AHR and the production of IL-5 were mediated by IL-12p70 produced by APCs [210]. In contrast, eosinophilia was not affected in IL-12 deficient mice. The use of IL-10 antibodies elucidated the importance of this anti-inflammatory cytokine for the suppression of T_H2-cell activation and the prevention of eosinophilic infiltration [5]. Our data partially approved these results. The induction of IL-10 by *in vitro* stimulation of human moDCs with vegBl and the detection of IL-10 in BAL of Blspo treated mice can be a reason for the impeded establishment of eosinophilic airway inflammation.

In contrast, the spore-specific IL-12p70 induction observed *in vitro* cannot be related to improvements in AHR *in vivo*. This can be due to low expression levels in comparison to above mentioned bacteria. Alternatively, the incidence for germination and outgrowth of spores prevented the accumulation of IL-12p70 induced by spores. Taking into account that *B. licheniformis* treatment improves the IL-10 dependent features of allergic asthma, but lacks to affect the IL-12p70 dependent, an influence of regulatory T-cells expressing IL-10 and IFN-γ is very likely [211].

The immunoglobulin E titer, the humoral component of allergic reactions, was not changed by *B. licheniformis* treatment. Barnes et al. described an increase in the titer of T_H1 related IgG2a antibodies by intranasal co-administration of antigen and *B. subtilis* spores correlating with a decrease of T_H2 related IgG1 [212]. They discuss the relation to a strong IFN-γ induction as a result of spore stimulation and IL-12p70 expression in DCs induced by the applied *B. subtilis* strain. Therefore their results indicated a clear T_H1 polarization *in vitro* and *in vivo*, but the source of DC, murine or human, was not mentioned [212] and their

method of stimulation was not published. In the study at hand *B. licheniformis* treatment instead showed only marginal effects on the up-regulation of IgG2a. Moreover a comparison to this study is interesting, since heat-inactivated spores were used lacking any induction of IL-12p70 in case of *B. licheniformis* 467 experiments. In contrast, vegetative bacteria diminished the expression of IgG2a. Surprisingly, IgG1 was not detected in the investigated mice, although it generally appears to be the most abundant immunoglobulin [(213)]. In this way the systemic influence on the allergic response was not confirmed by a change of the IgG1 and IgG2a ratio. In line with other studies, intranasal application protected locally in the lung, but systemic influences are low. In this point a limitation of the mouse model is obvious. Mice are sensitized to OVA allergen by a systemic i.p. injection, which is not physiological, but the intranasal application leads to tolerance induction to allergens in the setting applied [(214)]. The different ways of sensitization and treatment can be an explanation of the low effects on systemic allergy treatment, but the results by Barnes et al. [(212)] for intranasal application of spores are an interesting controversy to the results obtained in the asthma model.

5.4.1 Inflammation in the lung - Formation of multinucleated giant cells

The induction of macrophage recruitment and the formation of MNG are indicators for a possible formation of granuloma as they are common in chronic infections such as tuberculosis or as a consequence of foreign body interaction [(215)]. The presence of IFN-γ in spore treated mice and the detection of spores in phagocytozed lung cells (e.g. alveolar macrophages) after several days confirm this hypothesis. Interestingly, the fusion of cells to MNG was observed in all treated groups. It can be a result of the repeated treatment itself. The formation of these cells questions the way of treatment and the dose and frequency of *B. licheniformis* application. In consecutive studies this is a feature which has to be observed with regard to the physiology of the treatment. Here 10^8 bacteria were applied according to the protocol by Debarry et al. [(107)]. The authors give no clues about the formation of MNGs, since they observed no accumulation of neutrophils as well as macrophages. Physiologic concentrations, inhaled by farm children, are hard to conclude from the obtained cfu numbers in animal shed dust. The amount of airborne bacterial spores depends on the way of fodder, storage of hay, presence of children in the stable and fluctuations between summer and winter time [(216;217)].

5.5 Which components are responsible for the differences between vegBl and Blspo?

According to the protective effect of stable dust extract inhalation observed in mouse experiments, Blspo were extracted by the same method, to investigate compounds which can contribute to the asthma protective effects of dusts and spores.

The same innate immune receptors TLR2 and NOD2 responded on the contact with both the intact spores as well as the Blspo extract. The low yield of this extraction prevented further purification steps, but the extraction supported the relevance of these two receptors.

5.5.1 Isolation of peptidoglycan

Peptidoglycan was isolated due to the fact that Blspo and vegBl stimulated the intracellular receptor NOD2. The purity of isolated PG structures was tested by HEK-293 cell stimulation and by methanolysis. Besides small differences in the detection of aa, considerable differences were shown for TNF-α release in moDCs following incubation with isolated PG from Blspo and vegBl. The stimulation of TNF-α release correlated with TLR2 activation in transfected HEK-293 cells and was exclusively found in PG preparations of vegBl. Lipoprotein oxidation was able to identify the activity as a lipoprotein contamination [64]. Interestingly, the TLR2 activation was removed by lysozyme degradation and subsequent centrifugation, but not by HF treatment, described to remove lipoproteins covalently linked to PG [74;158]. This TLR2 susceptibility was responsible for the induction of TNF-α expression by moDCs. After hydrolysis the insoluble part was separated. The muropeptide containing supernatant did not induce TNF-α. Interleukin-12p70 expression was neither detected before nor after hydrolysis. The limitation was an incomplete spore-PG degradation. However, the high degree of lysozyme impenetrable material was surprising since the spore lysozyme resistance is a function of the coat that had been removed as a first step of PG-extraction [117].

Spore-specific δ-lactam rings are present in approximately 50% of the disaccharides [113]. To cleave the δ-lactam containing muropeptides specific cortex hydrolases would be necessary recognizing these rings and differentiating cortex and germ cell wall peptidoglycan [97;113]. Since these enzymes are expressed in spores and the destruction of spores in phagolysosomes of APCs seems to depend on the germination of spores [202], a specific stimulation of NOD receptors by spore muropeptides would need an indirect approval. The release of spore-specific muropeptides after spore cleavage in

phagolysosomes of APCs cannot be simulated in a direct approach. A permeabilization of epithelial cells to achieve intracellular NOD signaling, as it was performed by Girardin et al. [94], was restricted to the recognition of Gram- bacterial PG. To mimic the physiological stimulation it would be necessary to provide signal factors for muropeptide transport to the phagolysosome. The intracellular membrane-transport necessary for the translocation from the phagolysosome to the cytosolic receptors are not yet identified [96]. A possible recruitment of neutrophils, as it is reviewed by Matsuzaki et al. [218], for the stimulation of T_H17-cells, has to be investigated in further studies to explain neutrophil recruitment in vegBl treated mice.

5.5.2 Spore coat proteins – the outer layer of spores

The spore coat is the outermost protective layer of spores. Therefore, it is likely that the recognition of spores by the innate immune system is mediated by this layer of highly cross-linked proteins with a variety of protective functions in the spore [219]. The high degree of cross-linking impedes an easy extraction of these proteins. Different methods were compared and only a highly denaturating extraction procedure using SDS and 2-ME, established for the analysis of *B. subtilis* spore coat, was efficient in protein isolation. However, the spore coat was not selectively extracted. Effects on the outer spore membrane and the core were visible by transmission electron microscopy. The comparison of the spore coat of *B. subtilis* DSM 618, a model for spore coat analyses [122;159;220-222], and *B. licheniformis* spore coat showed differences in the protein patterns and the carbohydrate contents. These results confirmed genetic predictions of missing genes for spore coat carbohydrate synthesis in *B. licheniformis* [135]. Describing the differences between these two organisms a list of 16 coat proteins was recently published which are present in *B. subtilis* but not in *B. licheniformis* [219]. These observations are interesting on the background of controversy results described by Barnes et al [212], with T_H1 polarizing effects of *B. subtilis*, and the results of this study, without IL-12p70 induction by *B. subtilis* DSM 618. Moreover, these results excluded the presence of immunogenic glycoprotein epitopes [188] and T-cell stimulating zwitterionic polysaccharides [223].

The immunological activity of Blspo before and after extraction was compared with the extract. A significant stimulation of TNF-α and IL-10 induction in response to moDC treatment with spores and extract, respectively, was observed. For the spore coat extract this activity was clearly associated with the recognition of TLR2 ligands, tested in transfected HEK-293 cells. Interestingly, a similar cytokine expression was obtained by

stimulation of moDCs with spores after extraction, although they were lacking any recognition of TLR2 ligands. This can be explained by the destructive effects of the extraction procedure, which enables an improved degradation of spores by lysozyme as a result of spore coat removal [119]. In this way intracellular TLR ligands like oligonucleotides, possibly not detected in whole cells because of their complexation with sasp [115], can be responsible for immune stimulation [224] as well as the parallel induction of IL-10 and TNF-α.

An additional effect of the extraction procedure was the inactivation of spore-specific IL-12p70 expression. After extraction as well as heat-inactivation of spores the ability to induce this cytokine was lost. In comparison to the release of proinflammatory TNF-α and regulatory IL-10, the induction of IL-12p70 seemed not to be triggered by an exclusive effect of TLR2. Toll-like receptor ligands are heat-stable [226]; with the exception of TLR5 ligand flagellin (a protein) all PAMPs resist heat-inactivation. Napolitani et al. showed the synergistical effect of TLR ligands on the increase of IL-12 release [225], as well as a further amplification in response to CD40 ligation and IFN-γ application. The results are supported by skews of allergic T_H2-cells by MALP-2 and IFN-γ [85]. This dependency on a co-stimulatory protein can explain the heat lability of IL-12p70 induction by Blspo. Further purifications of lipoproteins to identify the impact of TLR2 stimulation on allergy and asthma protective effects in mice considered these effects.

5.5.3 Lipoprotein isolation

5.5.3.1 Reversed-phase chromatography of lipopeptides

The 2 kDa macrophage activating lipopeptide isolated from *Mycoplasma fermentans* is the best characterized lipopeptide and TLR2 ligand [161] so far. For this reason an extraction procedure according to the publication was established based on the hydrophobicity of lipopeptides in comparison to proteins without lipid modifications. The fractions of C_{18} reversed-phase chromatography (RPC) in an HPLC approach were tested on TLR2 transfected HEK-293 cells. Fractions in the middle of a 2-propanol gradient were identified to have a stimulatory activity of about 50% as to the activity of the synthetic lipoprotein Pam_3CSK_4. Anyhow, the active molecule of this fraction was neither identified by SDS-PAGE nor by gas-chromatographic screen for the characteristic GroCys molecule of the lipid anchor [160]. Upscaling of the chromatographic procedure to a preparative HPLC column was necessary for further analysis of the enriched TLR2 ligand. Irreducible

variations in elution times in this scale impeded the identification of the molecule responsible for the TLR2 activation. These variations indicated an unspecific interaction of lipoproteins or lipopeptides with the hydrophobic matrix in RPC. In *B. licheniformis* ATCC 14580 the number of predicted lipoproteins and lipopeptides was 73 with the strictest prediction tool in the DOLOP database [77]. This variety of lipoproteins is recognized by TLR2 via its lipid anchor [73]. Against this background it is very likely, that unspecific isolation of proteins by the described method led to elution of a protein mixture. The usage of this mixture can result in an activation of TLR2 in picomolar concentrations [68], without being detected by the approved methods. The possibility that molecules can be recognized in such low concentration by TLR2 could be a reason for numerous contaminations [64]. HPLC procedures are not optimal for the separation of large proteins. Due to pore size and the high pressure the separation of small molecules with defined structural similarities is preferred and large molecules like proteins can enhance unspecific interactions or even block the system.

5.5.3.2 Lipoprotein isolation by hydrophobic interaction chromatography

Considering the unspecific interaction with highly hydrophobic surfaces like C_{18} RPC a second approach for the isolation of lipoproteins was tested using hydrophobic interaction chromatography, a method mainly used for protein purification [226]. The applied procedure was established for the isolation of *Staphylococcus aureus* LTA, a molecule with similar amphiphilic properties [163]. An increasing evidence for TLR2 activation by lipoprotein provides a basis for lipoprotein isolation by this method. Furthermore, the co-elution of immune stimulating fractions was described for this procedure [227-230]. Water soluble lipoproteins were separated on octyl-sepharose in a gradient of 1-propanol containing ammonium acetate. This technique allows to purify amphiphilic molecules according to their hydrophobicity. However, a strong interaction suggested to be a problem in RPC is circumvented by a low pressure approach. To separate the lipoprotein fraction from the LTA-containing fraction two tests were carried out. Phosphate measurement was performed to detect LTA on the one hand, and on the other hand a HEK cell assay determined lipoprotein activity. The identified TLR2 positive fractions were separated by SDS-PAGE. The result of this purification step was the identification of four protein bands eluting in the middle of the HIC-gradient and interacting with the octyl-sepharose material. Except one protein, they were identified and analyzed by bioinformatic tools screening the protein sequences for characteristics of lipoproteins. Although different prediction tools

were adapted, none of the proteins showed the characteristic properties of lipoproteins, especially the presence of a lipobox within the first aa of the protein [77;79;80]. The interaction of the protein with the hydrophobic column material was further tested by bioinformatic prediction of hydrophobic domains. The results did not prove their specific interaction. Proteins selected by HIC were eluted from SDS gels and tested for their ability to stimulate transfected HEK cells and moDCs. The unidentified protein was exclusively TLR2 active. However, subsequent analyses of moDC cytokines did not result in DC activation, neither by the total extract before purification nor by the eluted protein. Since the eluted protein was applied in an amount according to the control of positive electroelution, determined by SDS-PAGE and silver staining, they were not quantified. The parallel stimulation by the total extract indicates that a relatively high amount of TLR2 ligand seem to be necessary to induce cytokine expression. In comparison to the TLR4 ligand LPS, the TLR2 ligand Pam_3CSK_4 is a relatively weak inducer of DC cytokines [231]. To induce the release of the same amount of TNF-α via TLR2 the amount of ligand had to be increased 100-fold (data not shown).

Combined with the weak staining of SDS gels for eluted proteins it is doubtful whether this method is selective in purification and allows the enrichment of this protein by the performed chromatography. Considering this, the method seemed not to be selective enough for the isolation and comparison to spore lipoproteins. The proportion of spore lipoproteins containing functions in metabolism, like substrate binding [76], is not expressed in metabolic dormant spores and will reduce the likelihood of lipoprotein isolation.

5.5.3.3 Membrane protein extracts

To isolate lipoproteins in a native approach membrane fractions can be enriched [78]. To check the native state of isolation NADH conversion was tested for both preparations. The dormant metabolism in spores prevented the successful application of this method, but activity was found in vegetative cell membrane fractions isolated in parallel. These membranes were treated with Triton X-100 and proteins were precipitated with acetone. By SDS-PAGE the specific MPFs were compared with each other resulting in Blspo and vegBl specific proteins soluble in aqueous buffers without detergents.

The spore lipoprotein YhcN was identified by bioinformatic prediction as well as by similarity search to the *B. subtilis* protein described by Bagyan et al. [191]. The authors located this protein in the inner forespore membrane, but the detected differences between spore and vegetative bacterium were barely visible, although 10 µg of protein were

separated in comparison to 2.5 µg applied for *B. licheniformis* in this study. These differences can be explained by methodical variations in the induction of sporulation and the isolation of proteins.

The lipoprotein MntA, identified by aqueous Triton X-114 phase separation to be a manganese transporter substrate binding protein, was specific for the vegetative cell. The MALDI-TOF analyses of these lipoproteins were not able to prove the presence of a lipoprotein anchor. The *N*-terminus of the protein was not detected in both cases, although the sequence coverage was about 70%. Variations of the peptide mass fingerprint procedure were performed, because it was likely that the *N*-terminal peptide did not elute from the gel after trypsin digestion. The hydrophobic interaction between acrylamide gel and lipid anchor can be a reason for a dramatically reduced efficiency of peptide elution as it was indicated by low blotting efficiencies for these proteins (data not shown). Deacylation of the lipid anchor removed fatty acids by incubation of the picked gel slice in 12% ammonia [(174)]. By this method ester-bound fatty acids of LPS molecules are removed. However, even after this treatment the respective *N*-terminal peptides, modified by the attachment of glycerol were not detectable. This is a possible explanation for the lack of experimental prove for lipoprotein isolation in literature [(64)].

Both lipoproteins were not predicted by sequence analysis using the DOLOP prediction tool. The conserved lipobox domain implemented in the algorithm is [LVI][ASTVI][GAS]C. The identified sequences referring to a database screen for *B. licheniformis* reference strain ATCC 14580 contained the lipobox TAGC for YhcN and AAGC for MntA. This variation in the first aa of the lipobox was included in the prediction algorithms LipoP, LipPred and the prosite tool. Babu et al. described the difference between DOLOP and LipoP to be 73 and 103 lipoproteins, respectively [(77)].

Considering the results of *B. licheniformis* the DOLOP algorithm seemed not to be an appropriate tool to predict lipoproteins in this organism.

Immunological investigations of extracts and isolated protein bands revealed a TLR2-specific induction of IL-8 following stimulation of transfected HEK 293 cells by YhcN. In contrast, the predicted lipoprotein MntA led to an unspecific induction of HEK cells. Both transfected cells, expressing TLR2, TLR4, TLR5 and NOD2, respectively, as well as untransfected cells were examined. Similar unspecific stimulation was obtained by vegBl and the vegetative MPF, as well as the flagellin protein Hag. These results indicate the recognition by TLR5. Although transfection of HEK 293 cells with TLR5 expression

plasmids was a common method [232;233] a recent publication described the constitutive expression in HEK-293 cells [234]. The presence of this protein or its recognized peptide sequence seems to be responsible for the unspecific activation of all TLR transfected HEK cells by samples prepared from vegetative bacteria [192;194]. Even the PdhC protein isolated to control the purity of electroelution, activated unstimulated HEK cells. However, a lower level of cytokine was induced in comparison to the other proteins. Interestingly, the susceptibility to equal amounts of sample material was reduced in TLR5 transfected HEK-293 cells, expected to induce stronger cytokine release as a result of TLR5 overexpression.

The possibility of additional proteins eluted with the excised protein band was investigated by 2D gel electrophoresis of the vegMPF extract. Results of this technique were not able to exclude a co-elution of Hag with other proteins. However, comparison of expression levels shows a probability for an unspecific interaction resulting from TLR5 activation. A preparative isoelectric focusing can circumvent this interaction in subsequent purifications [235].

With respect to the contribution of TLR5 and TLR2 and their effects on moDCs, stimulation was performed with both the extracts and the isolated proteins of MPF from spores and vegetative cells. The expression of TNF-α was the most sensitive marker of cytokine expression by moDCs in response to stimulation by TLRs [236]. The concentration dependent stimulation with MPF of spores and vegetative cells, either before or after centrifugation, did not reveal significant differences. In fact they were comparable to the IL-8 release as a result of TLR2 recognition in HEK cells. The only difference detected was an increase in TNF-α induction by the soluble supernatant of SpMPF. Comparing with the SDS-PAGE a relation to the solubility of the TLR2 ligand YhcN can be assumed. For vegMPF this relation depends on the presence of the water soluble flagellin protein Hag as well as the presence of TLR2 ligands present in soluble and insoluble phases (data not shown). The influence of both components can be distinguished in future studies by TLR2 and TLR5 antibodies on the receptor side and Hag antibodies on the ligand side. Both receptors signal via NF-κB activation inducing proinflammatory cytokines IL-6, IL-8 and TNF-α [237]. The IL-8 inducing principle is described to stimulate neutrophil and macrophage influx into host tissues. For this flagellin was initially reported to stimulate IFN-γ, while other studies provide evidence for IL-4 induction developing T_H2 responses and down-regulating IFN-γ and therefore T_H1 responses [238]. The inconsistency of these results is suggested to be due to the difficulty to prepare flagellin preparations without LPS

and lipoprotein impurities [238]. Diedierlaunt et al. investigated the impact of flagellin stimulation *in vitro* on murine DCs and in an *in vivo* mouse model using BALB/c mice [239]. They mention a T_H2 response for both systems characterized by the up-regulation of IL-4 and IL-13.

The expression of a flagellum is one of the main differences between vegetative cells and spores. It can have an important impact on the stimulation of the innate immune system on the level of DC stimulation and T-cell polarization. The influence of at least two TLR ligands, flagellin and lipoprotein, can act synergistically or antagonistically on DCs [225]. Didierlaunt et al. [239] reported an expression of TRL5 in mouse DCs independent of the genetic background of BALB/c or C57BL/6 mice. Certainly, Means et al. [240] showed for the latter no expression of TLR5 in murine splenic DCs, but described the expression of TLR5 in human moDCs. These results indicate the controversy about TLR5 expression in literature and strongly recommend further experiments to allow a better correlation of *in vitro* and *in vivo* data obtained in this study. The genetic differences between mouse and human have to be considered [241] as well as the differences between *in vivo* data of different mouse strains [242] and tissue specific receptor expression [243]. A recent study provides interesting data for the controversial *in vivo* and *in vitro* differences presented here. T-cell polarization to T_H2-cells is observed in response to vegBl stimulation of human moDCs, but the down-regulation of T_H2 related inflammation *in vivo* is definite. Vicente-Suarez et al. investigated the expression of pro- and anti-inflammatory cytokines in response to TLR5 ligation [244] on macrophages. They described an inhibition of IL-10 expression and the induction of IL-12p70 release by peritoneal macrophages as a consequence of flagellin stimulation. In contrast, the macrophage cell line RAW-264.7 did not express TLR5 and therefore did not express IL-12p70 in response to flagellin stimulation.

In further experiments the purified ligands of this study can be used to clarify the role of *B. licheniformis* flagellin and lipoproteins in the prevention of asthma in the mentioned mouse model. The correlations to the human *in vitro* model, important for possible applications in asthma preventive therapy have to consider alveolar macrophages and lung DCs (belonging to the myeloid or plasmacytoid subset) as important targets for consecutive analyses. These cells are difficult to generate *in vitro* but their role in uptake of inhaled allergens is still unclear [33;245]. For TLR2 and TLR5 ligands identified and isolated in this study a direct interaction with T_{regs} has been reported and should be examined in more detail in subsequent studies [51;238]. Furthermore, the influence of TLR2 ligands

administered to the airways, has been described to induce neutrophil influx by T_H17-cell polarization [246]. This can be an explanation for the accumulation of neutrophils after vegBl treatment and is an important interaction which needs to be investigated to elucidate the involved mechanisms. *In vitro* studies of T_{regs} and T_H17 polarization by IL-23 expression are further projects against this background [38;51].

5.6 Conclusion and perspectives

The epidemiological evidence of a protective farming effect has been shown in numerous studies, but the immunological basis of this protective effect is not yet understood. Two mechanisms are discussed in the context of the hygiene hypothesis. On one side a missing immune balance regarding the T_H2 bias in newborns and on the other side a lack of immune suppression.

Nowadays experimental evidence explaining the epidemiological findings is rare. Peters et al. describe an immunosuppressive effect by inhalation of stable dust extract, prohibiting asthma development in mice. This effect is correlated with the suppressed generation of DCs in the presence of stable dust extract [106]. Contrary to this effect Debarry et al. report a strong T_H1 polarization *in vitro* by DC stimulation with the cowshed bacteria *A. lwoffii* and *L. lactis*. These data encourage the idea of a missing immune balance of the skewed T_H2 immune response in allergic individuals by T_H1 polarizing microbial compounds.

The aim of this study was to provide further insights into the protective effect of exposure to microbial spores on traditional farms in the alpine region. The most common spore forming bacterium *B. licheniformis* is associated to farming products like hay and has the ability to intrude the lower airways due to its size.

Bacillus licheniformis spores have provided a spore-specific T_H1 polarization signal *in vitro*, by the induction of IL-12p70 release in DCs and the increase of IFN-γ production by T-cells. In an *in vivo* mouse model this spore-specificity could not be confirmed. The comparison of dormant germinable spores, with those inactivated for germination as well as with vegetative cells strongly indicated a germination of Blspo after intranasal treatment. The only spore-specific effect detected *in vivo* was the suppression of an allergy-specific lymphocyte accumulation in BALF, which seems to be mediated by a heat-labile component. The identification and isolation of this component is a challenging task for further investigations and should include spore purification by lysozyme treatment [247;248].

In general *B. licheniformis* treatment led to a decrease of allergic T_H2 effector cells, but also to an increase of T_H1 inflammation. The results of these observations let suggest an accumulation of neutrophils and macrophages in the lung being responsible for the clearance of spores and vegetative cells, respectively. However, the formation of multinucleated giant cells was not specific for spores, although they could be detected even four days after the last treatment.

On the molecular level the results of this study contribute to the impact of TLR ligands on the prevention of asthma and allergy. Lipoproteins as well as the flagellin protein Hag have been isolated from *B. licheniformis*, but the data obtained with the isolated TLR2 and TLR5 ligands are difficult to interpret. Variations in mice experiments between described studies contribute to this as well as different *in vitro* systems. *In vivo* the time and way of application varies between the studies as well as the amount and the adjuvant co-administered. For the example of LPS it is shown that contact during the sensitization phase is allergy protective, while at a later time, asthma exaggeration is supported. Moreover, this depends on the concentration of the applied TLR-ligand and the age of the animals [(249)]. Epidemiological studies suggest a window of opportunity for protection in the prenatal and early life phases, the time of immune maturation [(30;40)]. Due to experimental procedures, most studies have been performed with adult mice. This has to be optimized to imitate the physiological situation on farms. Now, first studies investigate a maternal exposure to microbial components, by inhalation and by probiotic application [(250)].

Essential *in vitro* and *in vivo* experiments as basis for clinical application of spore-specific components were provided by this thesis. The use of germinable spores in allergy and asthma prevention was excluded, due to its induction of local lung inflammation. However, interesting results support subsequent purification and analyses of spore-components. To address the window of opportunity also *in vitro* the limitation of healthy, adult blood donors for the isolation of moDCs and T-cells should be improved. The window of opportunity in the developing immune system as a target for the protective farming influence needs blood donors of the respective age. The availability of these samples on the other hand would prevent large studies, necessary to include the strong genetic influence on the development of asthma and allergy.

However, the components at hand, isolated in this study, will improve the possibility to investigate the protective farming effect in more detail.

6. Summary

Numerous epidemiological studies associate a prevention of allergy and allergic asthma in children with growing up on a farm and contact to farm animals in the alpine regions of Germany, Austria and Switzerland.

Recently, evidence for this protective effect was approved in a mouse model by treatment with stable dust extract or cowshed bacteria during allergen sensitization and provocation. Against the background of the hygiene hypothesis these results have been discussed in the concept of a missing immune deviation in early childhood caused by a down-regulation of the prenatal T_H2 bias. Moreover, the concept of a missing immune suppression by a lack of contact to microbial components is described.

Except these studies little is known about the microbial variety in farm stables. Therefore, the microbial flora of stable and mattress dusts was investigated and *Bacillus* (more precisely *B. licheniformis*) and *Mucor* spp. were identified to represent the most relevant contact to farm children. Additional to the high prevalence in the farming environment, these spore forming microbes are of high interest for inhalational exposure. Especially bacterial spores are able to intrude into the lower airways of children and provoke a local stimulation of the immune system.

The isolated bacterial and fungal spores were grown and tested for their ability to polarize the human T_H-cell response to a non-allergic T_H1 type. In contrast to *B. licheniformis, Mucor* spp. spores were not able to stimulate relevant cytokine expression in monocyte derived dendritic cells (moDCs), a crucial step for the polarization of T-cells. Therefore, further experiments focused on the effects of *B. licheniformis* spores. To investigate bacterial spores it was important to consider their ability to germinate under appropriate conditions. Thus, the spore-specificity *in vitro* and *in vivo* was analyzed by comparing *B. licheniformis* spores (Blspo), heat-inactivated Blspo (hiBlspo) and vegetative cells (vegBl).

In vitro Blspo stimulation results in a specific cytokine profile dominated by the expression of IL-12p70. In contrast, the profile expressed by vegBl showed a predominant expression of the proinflammatory cytokine TNF-α and the regulatory cytokine IL-10. As a consequence the stimulation of DCs by spores led to a T_H1 polarization of naïve T-cells in mixed leukocyte reactions. In case vegetative cells were applied for stimulation the expression of T_H2 related cytokines was observed. These differences were related to lower levels of maturation, phagocytosis and recognition by innate immune receptors such as

TLR2 and NOD2 on DCs, when stimulated by Blspo. Furthermore, the differences raised the interest of immune stimulating effects *in vivo*.

Intranasal application of the three different forms of *B. licheniformis* 467 prior to sensitization and provocation of OVA allergy in mice, did not confirm the spore-specific results *in vivo*. As an exception a spore-specific decrease of OVA-related lymphocytes was observed. Instead, the results of BAL cell determination of macrophages, neutrophils and eosinophils obtained for the spore treated group were between those of hiBlspo and vegBl treated mice. This strongly indicated an involvement of two different mechanisms, the clearance of vegBl by neutrophils and the phagocytosis of spores by macrophages. In case of terminable spores an inflammation was detectable, even four days after the last treatment, on the level of BAL cytokines. Moreover, the delayed clearance of spores was observed by spore staining in alveolar macrophages of lung tissue. Unfortunately the origin of IFN-γ and IL-10 release highly elevated in BAL of Blspo treated mice was not determined.

The general protective effect of *B. licheniformis* treatment, independent of its form, included the prevention of eosinophil influx and bronchial wall thickening as well as the development of goblet cell hyperplasia and mucus hypersecretion. In literature these characteristics are related to the expression of IL-10 as a regulatory, immune suppressive cytokine. In contrast, no improvement of airway hyperreactivity was observed, described to be IL-12 dependent.

The effects of *B. licheniformis* treatment on the systemic prevention of allergy-related IgE were marginal. Also the increase of T_H1 related IgG2a by spore treatment and the down-regulation of this antibody by vegBl was a weak but interesting observation. The differences revealed for spores and vegBl *in vitro* and *in vivo* underlined the importance of a screening for spore-specific components responsible for the activation of the immune system in a specific manner. Initial investigations with physiological saline approved the involvement of NOD2 and TLR2 ligands. The analysis of isolated peptidoglycan as a NOD2 ligand identified this component not to be responsible for the differences in IL-12 induction. However, the stimulation of moDCs by the isolated PG revealed an involvement of PG contaminating lipopeptides recognized by TLR2 and the expression of TNF-α by moDCs when stimulation was performed with PG of vegBl.

The correlation of TNF-α expression and TLR2 recognition was confirmed by spore coat protein extraction. The material was extracted and the spore coat showed a stimulation of TNF-α and IL-10 expression in moDCs, clearly associated with the expression of TLR2.

These experiments pointed at a heat-labile component in spores, inducing IL-12p70 expression. Based on these findings subsequent processes focused on the isolation of TLR2 ligands.

Isolation of lipoproteins and lipopeptides, respectively, was carried out by chromatographic HIC and RP-approaches. By means of RP-chromatography the enrichment of TLR2 activation was possible, but not their identification. Most likely this was due to an unspecific elution of TLR2 ligands interacting with the stationary C_{18}-phase. Performance of HIC on octyl-sepharose improved this unspecific interaction. Nevertheless, the selectivity of lipoprotein enrichment was questionable. One TLR2 ligand was isolated in this way, but identification and characterization was not achieved. Due to this experience with chromatographic techniques Blspo and vegBl were compared by enriched membrane fractions. In this way, membrane anchored proteins specific for spores and vegetative cells were identified. The vegBl-specific flagellin protein Hag as well as the lipoprotein MntA were isolated and identified to be TLR5 and TLR2 ligands, respectively. For Blspo the membrane anchored YhcN was identified by homology search based on *B. subtilis* proteins and shown to be a TLR2 ligand in HEK-293 cell assays. Especially the presence of the TLR5 ligand Hag was an interesting aspect with regard to the differences of spores and vegetative cells. Recently, TLR5 ligands are described to induce T_H2 responses, but current literature is highly controversy. This is supported by the differences in TLR5 expression in mouse and human cells and needs further investigations to clarify the mechanisms of allergy prevention and induction of neutrophil and macrophage recruitment by *B. licheniformis*. The description of direct TLR2 and TLR5 ligand interactions with T_{regs} by other groups provide interesting evidence for the importance of these ligands in immune and allergy suppression.

The whole extracts of membrane enrichment obtained no difference for TNF-α expression by moDC. Subsequent studies can now optimize the extraction and purification. This is necessary to clarify the effects of specific molecules on the level of human *in vitro* assays and murine *in vivo* experiments.

In the context of potential asthma prevention the study provided important insight to the contribution of spore inhalation into the lower airways. *Bacillus licheniformis* 467 spores reduce eosinophilic infiltrations and goblet cells hyperplasia, but do not improve airway hyperreactivity. Furthermore, spores provoke neutrophil and macrophage infiltration by germination, clearing vegetative cells and spores, respectively. Though, the *in vitro* spore-

specific effects directing a T_H1 polarization depend on IL-12p70 expression, but were not verified *in vivo*.

Techniques for the isolation of the innate immune receptor ligands for TLR2 and TLR5 were established and subsequent studies can clarify the protective effect of these components in the described model system, to clarify the mechanisms induced in immune reaction to inhaled spores and to investigate prospective microbial components for preventive applications.

7. Zusammenfassung

Zahlreiche epidemiologische Studien der letzten Jahre haben eine eindeutige Assoziation zwischen der Vorbeugung von allergischen und asthmatischen Erkrankungen bei Kindern und dem Aufwachsen auf einem traditionell geführten Alpenbauernhof in Deutschland, Österreich bzw. der Schweiz, gezeigt.

Diese Studien wurden durch Mausexperimente bekräftigt. Die intranasale Applikation von Stallstaubextrakt während der allergischen Sensibilisierung und Provokation verhinderte die Ausbildung phänotypischer Charakteristika von Asthma. Bestätigt wurden diese Ergebnisse durch Experimente mit isolierten Stallbakterien. In Bezug auf die Hygienehypothese wurden diese Ergebnisse auf der Ebene einer fehlenden Immunbalance in früher Kindheit diskutiert. Kontakt zu mikrobiellen Bestandteilen soll demnach für einen Ausgleich der T_H2 geprägten, pränatalen Immunantwort sorgen bzw. diese supprimieren.

Mit Ausnahme dieser Studien ist der Wissensstand auf dem Gebiet der mikrobiellen Vielfalt auf den genannten Bauernhöfen und damit die mögliche Allergieprotektion durch bakterielle Bestandteile sehr gering. In einer Pilotstudie wurden deshalb die im Stall- und Matratzenstaub enthaltenen Mikroorganismen untersucht. Dabei wurden *Bacillus* und *Mucor* spp. als diejenigen Spezies identifiziert, welche den häufigsten Kontakt zu Kindern hatten, die auf diesen Bauernhöfen aufwachsen. Diese sporenbildenden Mikroorganismen sind darüber hinaus interessant in Bezug auf die Inhalation und ihre lokale Wirkung auf die Lunge. Speziell für bakterielle Sporen, wie sie von *Bacillus licheniformis* gebildet werden, ist die Inhalation in die kleinen Atemwege aufgrund ihrer geringen Größe möglich.

Isolate der Bakterien- und Pilzsporen wurden aus diesem Grund kultiviert und aufgereinigt, um ihre Fähigkeit zur Polarisation von humanen T_H-Zellen in Richtung einer nicht-allergischen T_H1 Antwort zu untersuchen. Der initiale Schritt bei dieser Polarisation, die Stimulation von dendritischen Zellen (DCs) wurde von *B. licheniformis*, nicht aber von *Mucor* spp. angeregt. Anschließende Experimente konzentrierten sich aus diesem Grund auf die Wirkung von *B. licheniformis* Sporen. Bei der Untersuchung von Sporen-spezifischen Effekten auf das Immunsystem ist es essentiell eine mögliche Auskeimung von Sporen zu beachten. Daher wurden keimfähige *B. licheniformis* Sporen (Blspo) sowohl mit Sporen verglichen, die durch Hitze-Inaktivierung in ihrer Keimfähigkeit unterdrückt worden sind (hiBlspo) als auch mit vegetativen Zellen (vegBl).

In vitro zeigte die Stimulation von DCs mit Blspo eine sporenspezifische Expression von IL-12p70, wohingegen die Expression von TNF-α und IL-10 das Zytokinprofil nach

Stimulation von DCs mit vegBl dominierten. Naive T-Zellen wurden in Gegenwart von Sporen-stimulierten DCs zur T_H1 Polarisierung angeregt. Die Stimulation mit vegBl hingegen erhöhte die Expression von T_H2-assoziierten Zytokinen. Diese Unterschiede konnten unter anderem mit einem geringeren Grad an DC-Reifung und Phagozytose in Verbindung gebracht werden. Dies wiederum kann mit einer geringeren Interaktion von Sporen und Rezeptoren des angeborenen Immunsystems, wie TLR2 und NOD2, in Zusammenhang stehen. Die sporen-spezifischen Signale weckten ein großes Interesse an der Aufklärung dieser Immunstimulation *in vivo*.

Die intranasale Applikation der drei Formen von *B. licheniformis* 467 vor und während der Ovalbumin-(OVA)-Allergen Sensibilisierung und Provokation beschränkte die Sporen-spezifischen Effekte auf eine Unterdrückung des Lymphozyten-Anstiegs, hervorgerufen durch OVA-Behandlung. Die BAL-Zell-Analyse von Makrophagen, Neutrophilen und Eosinophilen lag hingegen für die keimfähigen Sporen jeweils zwischen den Ergebnissen der hiBlspo- und vegBl-behandelten Mäuse. Diese Daten deuteten einerseits auf eine Beteiligung von zwei verschiedenen Mechanismen bei der Immunabwehr gegen Spore und vegBl hin. Andererseits verweisen die Ergebnisse auf eine Auskeimung der Sporen. Die Untersuchung von BAL-Zytokinen zeigen eine Immunabwehr der Lunge, die noch vier Tage nach der letzten Blspo-Behandlung anhielt. Als Grund anhaltend erhöhter Zytokin-Messungen konnte die Präsenz der Sporen durch ihre Färbung in Lungengewebe ermittelt werden. Die Quelle der Zytokine IFN-γ und IL-10, welche in Blspo-behandelten Mäusen stark erhöht waren, konnte nicht aufgeklärt werden. In einem alternativen Mechanismus scheinen Neutrophile an der Entfernung von inhalierten vegBl beteiligt zu sein.

Der allergieprotektive Effekt der beschriebenen *B. licheniformis* Behandlung beinhaltete die Unterdrückung der Rekrutierung von Eosinophilen, die verminderte Verdickung der Bronchienwand und die Verhinderung einer verstärkten Schleimbildung. Diese Charakteristika von allergischem Asthma werden in der Literatur als IL-10 abhängige Vorgänge beschrieben. Im Unterschied zu diesem regulatorischen, immunsuppressiven Zytokin wurde das T_H1 Zytokin IL-12p70 als wichtig für die Unterdrückung der Atemwegshyperreaktivität beschrieben. Dieser Effekt wurde durch *B. licheniformis* nicht beeinflußt.

Der Einfluss der Bakterien auf die allergische IgE Immunantwort war mäßig. Andererseits war die Erhöhung des T_H1 Antikörpers IgG2a durch die Sporenbehandlung und die Verringerung durch die Behandlung mit vegBl ein interessanter Unterschied zwischen Spore und vegetativer Zelle. Die Unterschiede, welche *in vitro* und *in vivo* beobachtet

wurden, erhöhten das Interesse an der Isolierung Sporen-spezifischer Komponenten. Die Erkennung der intakten Zellen und die Extraktion mit physiologischer NaCl-Lösung zeigte die Bedeutung der NOD2 und TLR2 Rezeptor-Erkennung. Aufgereingtes Peptidoglycan (PG), welches durch den NOD2-Rezeptor erkannt wird, war nicht verantwortlich für die Induktion einer IL-12p70 Expression. Allerdings lieferten diese Experimente wichtige Erkenntnisse über kontaminierende Lipoproteine. Lediglich das PG, welches aus vegetativen Zellen isoliert worden ist, war mit TLR2 Liganden kontaminiert und führte zur Expression von TNF-α in myoloiden DCs.

Diese Korrelation zwischen TLR2-Erkennung und TNF-α sowie IL-10 Expression wurde durch die Extraktion Sporen-spezifischer Hüllproteine bestätigt. Diese Experimente zeigten weiterhin, dass ein Hitze-empfindlicher Bestandteil der Sporen für die IL-12 Expression verantwortlich ist. Aus diesem Grund wurde in nachfolgenden Analysen versucht TLR2 aktivierende Lipoproteine möglichst nativ zu isolieren.

Versuche der Isolation von Lipoproteinen mittels hydrophober Interaktions- (HI)- oder Umkehrphasen- (*reversed-phase*, RP)-Chromatographie ermöglichten die Anreicherung, nicht aber die Identifikation von Substanzen, die an TLR2 binden. In der RP-Chromatographie könnte diese Beobachtung auf eine unspezifische Interaktion von TLR2-Liganden mit der stationären Phase zurückzuführen sein. Die Verwendung der HIC verbesserte dies, aber die Selektivität der Isolation blieb fraglich. Ein TLR2-aktivierendes Protein, welches mit der Octyl-Sepharose in Wechselwirkung trat, konnte isoliert werden. Die Identifizierung mittels MALDI-TOF MS und *N*-terminaler Sequenzierung war jedoch nicht möglich. Basierend auf diesen Ergebnissen der chromatographischen Techniken wurden weitere Aufreinigungen von Lipoproteinen durch eine Anreicherung von Membranfraktionen durchgeführt. Sporen und vegetative Zellen von *B. licheniformis* wurden auf diese Weise verglichen und spezifische Proteine für die verschiedenen Formen identifiziert. In vegBl konnten das membran-assoziierte Flagellin-Protein Hag und das Lipoprotein MntA als TLR5- bzw. TLR2-Liganden identifiziert werden. Das sporen-spezifische Protein YhcN wurde durch seine Homologie zum *B. subtilis* Protein zugeordnet. Die TLR2 Spezifität wurde im HEK-293 Zell-Ansatz überprüft. Insbesondere die Aktivierung der Zellen durch den TLR5-Liganden Hag ist ein interessanter Aspekt zur Erklärung der verschiedenen Expressionsmuster *in vitro*. Die Verbindung zwischen der Stimulation von TLR5 und einer T_H2 Polarisierung wurde kürzlich beschrieben. Die Literatur zu diesem Thema ist allerdings sehr kontrovers. Dazu tragen auch die Expressionsunterschiede von TLR5 auf DCs in Maus und Mensch bei. In dieser Studie

konnte das Flagellin-Protein Hag angereichert und isoliert werden, aber der Einfluss auf Allergieprotektion und die Rekrutierung von Neutrophilen bzw. Makrophagen durch *B. licheniformis* muss in weiteren Studien untersucht werden. Darüber hinaus sind neueste Erkenntnisse über die direkte Aktivierung von T_{regs} durch TLR2 und TLR5 von Bedeutung für die Rolle der isolierten Liganden bei der Prävention von Asthma. Bei der Stimulation von DCs durch die angereicherten Membranprotein-Fraktionen konnten allerdings keine Unterschiede herausgearbeitet werden. In nachfolgenden Studien müssen nun die Prozesse der Identifikation und Isolation optimiert werden und die spezifischen Effekte einzelner Liganden untersucht werden, um die molekularen Ursachen der *in vivo* Experimente im Mausmodell aufzuklären.

Auf dem Hintergrund einer möglichen Asthma-Prävention konnten durch diese Arbeit wichtige Erkenntnisse im Hinblick auf die Inhalation von mikrobiellen Sporen gesammelt werden. Die Rekrutierung von Eosinophilen und die Hyperplasie von Mukus-produzierenden Becherzellen werden durch Blspo reduziert. Sie führen aber nicht zur Verbesserung der Atemwegshyperreaktivität. Die Auskeimung der inhalierten Sporen bewirkt weiterhin eine Akkumulation von Neutrophilen und Makrophagen, welche für die Immunreaktion gegen diese zuständig sind. Die spezifische Expression von IL-12p70, wie sie für die Sporen-stimulation *in vitro* beobachtet werden konnte, erfüllte die Erwartungen *in vivo* nicht.

Weiterhin wurden in dieser Arbeit Methoden zur Aufreinigung von Liganden des angeborenen Immunsystems etabliert und Kandidaten identifiziert. Diese können in nachfolgenden Studien zur genaueren Aufklärung der Immunreaktion gegen inhalierte Sporen verwendet werden und wichtige Erkenntnisse über Möglichkeiten der Asthma Prävention liefern.

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D: List of own publications contributing to this study

Printed publications:

Vogel K, Blümer N, Korthals M, Mittelstädt J, Garn H, Ege M, von Mutius E, Gatermann S, Bufe A, Goldmann T, Schwaiger K, Renz H, Brandau S, Bauer J, Heine H, Holst O. Animal shed *Bacillus licheniformis* spores possess allergy-protective as well as inflammatory properties. Journal of Allergy and Clinical Immunology 2008; 122: 307-12. Highlighted as Editors' Choice

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Poster presentation:

Vogel K, Goldmann T, Bauer J, Heine H, Holst O.
Allergy protective factors of bacterial and fungal spores in farming environments
Summer course Glycosciences; 9th European Course on Carbohydrates, June 6th – 9th, 2006

Oral Presentations:

Vogel K, Korthals M, Schwaiger M, Bauer J, Heine H, Holst O.
Role of carbohydrates from bacterial and fungal spores in allergy protection
2nd Baltic Meeting on Microbial Carbohydrates, October 4th-8th, 2006, Rostock

Vogel K, Blümer N, Korthals M, Mittelstädt J, Garn H, Ege M, von Mutius E, Gatermann S, Bufe A, Goldmann T, Schwaiger K, Renz H, Brandau S, Bauer J, Heine H, Holst O.
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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 09.07.2008

Kay Vogel