universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Cloning, expression and functional characterization of flagellin from *Salmonella typhimurium*

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)

Verfasser:	Stephan Deifl
Matrikel-Nummer:	0204559
Studienrichtung /Studienzweig (lt. Studienblatt):	Biologie - Genetik/Mikrobiologie
Betreuerin / Betreuer:	Barbara Bohle / Thomas Decker

Wien, im März 2009

Acknowledgments

I want to thank Barbara Bohle, my supervisor, who gave me the opportunity to work in her group. She helped out whenever problems occured and took the trouble to improve this diploma thesis.

Special thanks go to Sonja Mutschlechner for her kind advice regarding all questions concerning my work in the lab and her ongoing efforts to bring forward new ideas.
I wish to thank Véronique Schulten for providing assistance with writing this diploma thesis in English.
I want to thank all my colleagues in the lab for the pleasant working atmosphere and the possibility, to discuss any scientific problem with all of them anytime.

Heartfelt thanks go to my parents, not only for supporting me financially, but also for their patience and understanding. Without them I would not have been able to get that far.

Very special thanks go to Angelika, for her patience and her loyalty; for just being there and for her love.

1 Table of contents

2 List of abbreviations

µg	microgram
µL	microliter
Amp	ampicillin
APC	antigen presenting cells
APS	ammonium persulfate
BCA assay	bicinchoninic acid assay
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumine
CIAP	calf intestine alkaline phosphatase
ddH_2O	double distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
FCS	fetal calf serum
FcεRI	high-affinity receptor for IgE
Fig.	Figure
h	hours
HEK cells	human embryonic kidney cells
HRP	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMAC	immobilized metal-ion affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	kilo Dalton
LB medium	lysogeny broth medium
LPS	lipopolysaccharide
LRR	leucin rich repeats
mA	milliampere
mg	milligram
MHC	major histocompatibility complex
min	minute
mL	milliliter
mM	milli Mol
MWCO	molecular weight cut off
nm	nanometer
O/N	over night
OD600	optical density at 600 nm
PAGE	polyacrylamid gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PRR	pattern recognition receptor
rpm	revolutions per minute
RT	room temperature

S. typhimurium	*Salmonella typhimurium*
SDS	sodium dodecyl sulfate
SIT	allergen-specific immunotherapy
SLIT	sublingual allergen-specific immunotherapy
TB medium	terrific broth medium
TBE	tris/borate/EDTA buffer
TBS	tris-buffered saline
TEMED	tetramethylethylenediamine
TGF	transforming growth factor
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TTSS	type three secretion system
UC medium	ultra culture medium
UV	ultra violet

3 Abstract

Toll-like receptor (TLR) ligands may be employed as adjuvants for allergen-specific immunotherapy. For example, CpG motifs recognized by TLR9 were shown to promote the counter regulation of the allergic immune response when added to allergy vaccines. We were interested to investigate whether the bacterial protein flagellin, a TLR5 ligand, excerted immunomodulating effects on cells of allergic patients and to produce flagellin in recombinant form.

As a first step, we stimulated peripheral blood mononuclear cells (PBMC) from birch pollen-allergic patients with commercially available flagellin from *Salmonella typhimurium* and analysed cytokine expression by ELISA. The induction of IFN-γ and IL-10 indicated that flagellin had potential adjuvant activity. Next, we established a read out system to detect TLR5-triggering using human embryonic kidney (HEK) cells stably transfected with TLR5 as well as CaCo-2 cells. In parallel, we assessed the influence of heat-labile serological factors on the ability of flagellin to activate TLR5. Our results indicated that HEK-293/TLR5 cells in the presence of FCS (10%) were best suited for testing TLR5 triggering by flagellin.

Next we tried to clone flagellin from *S. typhimurium* in a suitable vector and express the protein in *E. coli* BL21(DE3)pLysS cells. As different attempts to produce recombinant flagellin failed, we focussed on the expression of the C- and N-terminal fragments of the protein. These conserved regions form the D0 and D1 domain of flagellin responsible for recognition by TLR5. Both protein fragments were successfully expressed in *E. coli* BL21(DE3)pLysS cells and purified by chromatography. However, neither a mixture nor the C- and N-terminal fragments alone bound to TLR5. Therefore, we designed a fusion protein linking both fragments with a hinge region from *E. coli*. This fusion protein was expressed, purified by chromatography and tested on HEK-293/TLR5 cells. Stimulation of the cell line with the fusion protein led to IL-8 synthesis revealing that the N- and C-terminal fragments need to be brought into close proximity to bind and activate TLR5.

In summary, we showed that flagellin may have immunomodulatory effects on cells of allergic patients. Moreover, we established a test system for TLR5 triggering and produced a recombinant fusion protein consisting of the highly conserved N- and C-terminal fragments of flagellin from *S. typhimurium* linked by a hinge region from *E. coli* with the capacity to trigger TLR5.

In the future, it is planned to produce sufficient amounts of the recombinant fusion protein in high purity and free of endotoxins to evaluate its immunomodulatory effects on PBMC of allergic donors.

4 Aims of the study

In this diploma thesis we aimed at

- evaluating whether flagellin from *S. typhimurium* could induce T_H1/T_{reg}-type cytokines and therefore, was a potential adjuvant in allergen-specific immunotherapy.

- establishing a fast read-out system for TLR5 triggering.

- cloning and expressing of flagellin from *S. typhimurium* in *E.* coli cells.

5 Introduction

5.1 Immune system

The human immune system offers various defence mechanisms against infections by pathogens or pathogen-derived products. One can generally differentiate between an evolutionary elder innate immune system and a younger adaptive immune system. The innate or natural immune system constitutes physical and chemical barriers like epithelia, membranes and various cell types such as neutrophils, NK-cells and especially macrophages, which are very important in the first-line defence against pathogens. Typically, the innate immune system recognizes structures shared by many classes of microbes using so-called pattern recognition receptors (PRR). However, the components of the innate immune system are germline encoded and therefore, this system possesses a limited diversity and does not produce immunological memory. In contrast, the adaptive immune system offers a large diversity and the ability to produce immunological memory. The adaptive immune system either acts by humoral components or by lymphocytes. There are two major subclasses of lymphocytes involved in antigen recognition and removal: B- and T-lymphocytes. According to the type of antigen receptor they express, T-lymphocytes are further differentiated into αβ-T-lymphocytes and γδ-T-lymphocytes. The T-cell receptor consists of two transmembrane polypeptide chains named α and β or γ and δ, respectively. The single chains are covalently linked to each other by disulfide bridges and therefore, form the heterodimeric receptor. The αβ-T-lymphocytes are much more frequent. Less than 5% of all T-cells express the γδ-T-cell receptor with variations in different tissues. Mature αβ-T-cells are classified in $CD4^+$ or $CD8^+$ T-cells according to the co-receptor molecules they express. The $CD8^+$ subset is usually called cytotoxic T-lymphocytes (T_C) as they are responsible for direct killing of infected cells after infection with microbes that replicate intracellularly. $CD4^+$ T-cells are generally known as helper T-cells (T_H) as they help in activating phagocytes after ingestion of microbes or mediate B-lymphocyte activation. In addition regulatory $CD4^+$ T-cells exist, that have important functions in the regulation of immune responses and in the maintenance of self-tolerance as they suppress the function of other T-cells. These mechanisms help the immune system to downregulate responses after infections and to keep a balance between pro- and anti-inflammatory immune responses.

B-lymphocytes produce antibodies (immunoglobulins). In humans there exist 5 immunoglobulin isotypes named IgM, IgG, IgA, IgE and IgD.

The main role of the innate and the adaptive immune system is to ensure the defence against all types of different pathogens. Unfortunately, sometimes autoimmune reactions or hypersensitivity reactions occur [1]. According to Coombs and Gell, hypersensitivity reactions of the human immune system are classified into 4 different types. However, today it is discussed whether this classification system summarizes all types of hypersensitivity reactions or whether additional types exist [2, 3]. Type 1 hypersensitivity reactions are based on IgE-mediated degranulation of sensitized basophils and mast-cells. Allergic asthma or rhinoconjunctivitis are examples for Type 1 hypersensitivity reactions. Type 2 hypersensitivity reactions result from the interaction of IgG or IgM antibodies with cell bound antigens. Antigen-antibody binding leads to the local production of anaphylatoxin (C5a) and subsequent tissue injury by the release of hydrolytic neutrophil enzymes [2]. Type 3 hypersensitivity reactions are so-called immune complex reactions. Antigen-Antibody complexes are formed and deposited in the glomerular or pulmonary basement membranes causing tissue injury [2]. Finally, there are cell-mediated reactions or Type 4 hypersensitivity reactions. They are mediated by T-cells, macrophages, natural killer cells or other leukocytes [2].

5.2 Type 1 allergy

The elicitors of Type 1 hypersensitivity reactions are harmless antigens, so-called allergens. Allergens usually are proteins of a molecular weight of 5-100 kDa that can be categorized into different protein families according to their structure [4]. Allergens are frequently glycosylated and easily soluble in body fluids. Some allergens also show protease activity [5]. Common sources of allergens include pollen, mite feces, insect venom or different foods. In industrialized countries the most prominent allergen sources are feces from house-dust mite, pollen from grasses and trees and epithelia from cat [6].

The development of Type 1 allergy is a two step process. At first, sensitization to allergens occurs, which is followed by a reaction phase that can be subdivided into an early and a late phase. At first contact, allergens are taken up by antigen presenting cells (APC) that migrate to the draining lymph nodes. After processing of the allergen by the APC, allergen-derived peptides are bound to major histocompatibility complex (MHC) molecules. Upon contact with the APC, naïve T-cells differentiate into different effector cells depending on key cytokines in their environment. $CD4^+$ T–cells are today subdivided into T_H1 cells, T_H2 cells, T_H17 cells and T_{reg} regulatory cells according to the cytokine pattern produced by these cells. Type 1

allergy is characterized by an overwhelming T_H2 response to allergens. Atopic patients are prone to develop T_H2-like immune reactions whereas non-allergic patients more often show a $T_H2/T_H1/T_{reg}$ balanced immune response to allergens [6]. T_H2 cells secrete cytokines like interleukin-4 (IL-4), IL-5 and IL-13 whereas interferon-γ (IFN-γ) is the major product of T_H1 cells. Under the influence of IL-4 and CD40 ligand, B-cells undergo isotype switching to IgE production. IgE antibodies circulating in the blood are bound to the high-affinity receptor for IgE (FcεRI) expressed on the surface of basophils and mast cells, which renders these effector cells "sensitized". Upon any consecutive allergen encounter, bound IgE antibodies recognize and bind the allergen resulting in cross-linking of IgE molecules and mast cell activation. This reaction phase can be divided into early phase and late phase reactions. Cross-linking of the FcεRI molecules starts a typical signalling cascade resulting in immediate clinical symptoms due to the release of preformed granule contents, synthesis of lipid mediators and also cytokine production. Mediators such as histamine, prostaglandin D_2 or platelet-activating factor cause increased vascular permeability and stimulation of smooth muscle cell contraction as well as vasodilation and bronchoconstriction. Cytokine production and newly synthesized mediators cause the late phase reactions and inflammation [7]. Symptoms of allergic diseases can range from allergic rhinitis to asthma, atopic dermatitis or anaphylactic shock, which is a perilous situation.

Type 1 allergy has become a major threat for public health as up to 20% of adults in industrialized countries develop IgE-mediated or Type 1 hypersensitivity reactions.
The "hygiene hypothesis" tries to provide an explanation for the high occurrence of allergic disorders in industrialized countries [8]. The stimulation of the immune system by microbial compounds in early life may have a protective effect on the development of allergy. Maybe the IL-12 rich environment resulting from permanent stimulation with microbes is important to develop T_H1-like immune responses. Children in industrialized countries often lack the exposure to pathogens because they grow up in high sanitary standards. Therefore, these children often develop T_H2-biased immune responses as the development of the T_H1-like arm is reduced. Studies showing a protective effect on the development of allergic disorders for children that grew up on a farm support this theory [9, 10]. Still, allergic disorders are multifactorial and often have a genetic background. Therefore, family history may be used as a predictor of asthma risk and atopy. Growing up with siblings also seems to decrease chances of developing Type 1 allergies as children who have three or more older siblings develop allergic diseases less frequent as compared to single children [11]. Again, this may be

explained by the exposure to pathogens as elder siblings frequently have contact with other children e.g. in school and therefore, also with a variety of pathogens. However, recent data have excluded a protective effect of the number of siblings and rather support an in-utero programming hypothesis [11, 12].

The influence of environmental factors on the development of allergies is also considerable. Air pollutants were shown to induce an increased release of allergens from pollen and to lead to class switch towards IgE in nasal epithelia [13, 14]. Nevertheless, the development of allergies strongly depends on the genetic background of an individual. Allergic individuals are frequently atopic, i.e. they show a tendency to produce IgE antibodies. IgE levels in plasma of non-allergic patients are normally less than 1µg/mL but can rise up to 1000 µg/mL in atopic patients. In addition several gene loci have been shown to correlate with allergy development such as a polymorphism in the gene for the β-chain of the high-affinity receptor for IgE [15].

5.3 Allergen-specific immunotherapy

The aim of allergen-specific immunotherapy (SIT) is to generate long-term clinical tolerance to natural allergen exposure. To date, SIT is the only causative treatment for Type 1 allergy. Conventional SIT is based on repeated subcutaneous injections of increasing doses of allergen over a long time-period. Other application routes have been tested and sublingual allergen-specific immunotherapy (SLIT) is considered as an alternative because it is safe, non-invasive and more convenient for the patient [16]. Successful SIT/SLIT has been associated with the downregulation of the allergen-specific T_H2-like immune response by inducing IFN-γ-producing T_H1 cells and the shift towards a more physiologic T_H0/T_H1-like response [17]. Additionally, SIT/SLIT induces IL-10- or transforming growth factor β (TGF-β)-producing regulatory T-cells (T_{reg}) that actively suppress allergen-specific T-cell responses [18-20]. IFN-γ and IL-10 also favour the production of allergen-specific IgG antibodies which can function as blocking antibodies and compete with IgE for allergen binding [21, 22]. Consequently, the typical signalling pathways leading to allergic symptoms and immediate hypersensitivity reactions are reduced.

The administration of allergens to the patient bears a risk for IgE-mediated side effects in SIT. Therefore, improvement of allergen-specific immunotherapy is very important. Injection SIT has been improved by the use of recombinant allergens instead of crude allergen extracts [23]. Vaccination with recombinant allergens that were genetically modified to reduce their IgE-binding capacity was shown to prevent progression of allergic disease [24].

Another approach to improve the efficacy of SIT is the use of adjuvants that support the development of T_H0/T_H1-like immune responses to allergens. This can be achieved by targeting the innate immune response, e.g. activation of Toll-like receptors (TLR). Different TLR agonists have been shown to be promising adjuvant candidates in allergy vaccines as they induce regulatory T-cells and T_H1-type immune responses [25]. These approaches were further supported by first positive results in clinical trials and TLR agonists are considered as important in supporting the efficacy of SIT [26].

5.4 Toll-like receptors

TLR are members of the pattern recognition receptor family and are expressed on different cell types but mainly on cells of the innate immune system such as dendritic cells (DC), macrophages and endothelial cells. They are responsible for recognition of pathogen-associated molecular patterns (PAMP). TLR were named after Toll, a gene that is responsible for establishment of dorsal-ventral orientation in *Drosophila melanogaster* and has later been shown to also mediate antifungal responses [27, 28]. To date, the TLR family consists of 11 members in humans and 13 members in mice [29]. All of them are type 1 membrane proteins and have characteristic ectodomains consisting of leucine rich repeats (LRR) by which PAMP of different origin can be recognized. Several TLR including TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are expressed on the cell surface. In contrast, TLR3, TLR7, TLR8 and TLR9 are localized in intracellular compartments. Besides division by cellular localization, TLR can also be categorized according to the type of PAMP they recognize. TLR1, TLR2, TLR4 and TLR6 are required for recognition of lipids. TLR3, TLR7, TLR8 and TLR9 recognize nucleic acids and TLR5 as well as TLR11 are required for recognition of proteins [29]. After recognition, signalling starts via the cytoplasmic domain, which is called Toll/IL-1 receptor (TIR) homology domain. Adapter proteins are recruited leading to activation of protein kinases resulting in activation of transcription factors. Finally, genes are transcribed for expression of inflammatory cytokines such as IL-12 and TNF-α but also for up-regulation of co-stimulatory molecules, e.g. CD80 or CD86. Together, all of these factors support DC maturation and T_H1 differentiation [29]. Apart from that, TLRs also play a role in the induction of immunological tolerance [30].

TLR ligands trigger innate as well as adaptive immune responses by activating the TLR signalling cascades. As already mentioned, some TLR ligands can be considered as potential adjuvants for SIT as they support the shift from a T_H2 type to a T_H0/T_H1 type response in

allergic individuals. For Type 1 allergies, clinical trials with TLR4 and TLR9 ligands have already shown promising results [26, 31].

5.5 Flagellin

Flagellin is a TLR5 ligand and the monomeric subunit of flagella carried by several Gram-positive and Gram-negative bacteria. Flagella are whip-like structures that are up to 15 µm long with a diameter of around 20 nm and composed of many flagellin monomers [32]. Flagella are characterized by a basal body anchored in the inner and outer cell membrane that uses the proton motive force for motor function [33]. Furthermore, there is a torsion hook conferring flexibility to the relatively stiff flagellum, which is a helical filament. Flagella confer bacterial motility either by running, where the flagellar filament rotates counterclockwise, or by tumbling, where motor rotation is quickly changed to a clockwise direction. Flagella can also increase the adhesive potential of pathogenic bacteria, e.g. in colonization of the gut, or they serve as Type 3 secretion systems (TTSS) to secrete virulence factors.

Transcription of the flagellar genes is controlled via the flhDC regulator operon [33].

The flagellar filament is composed of about 30000 flagellin subunits, each consisting of 4 domains (Fig.1). Assembly of the flagellum is achieved by export of the flagellin subunits through the bacterial membrane by a Type 3 secretion like system. After export the flagellin subunits are polymerized, a process that is promoted by a capping structure at the end of the filament (Fig.1). To date more than 200 different flagellin sequences are known and sequence analysis has revealed that flagellin mRNA is highly conserved amongst different bacterial species with regard to the N- and the C-terminus of the protein. In contrast, the central region of the protein varies strongly in different species [33, 34]. The conserved regions are mainly responsible for the formation of the D0 and D1 domains that are characterized by their α-helical structure and are recognized by the innate immune system. The D2 and D3 domains contain β-sheets, formed mainly by the variable central region, and protrude from the compact chain that is formed by the D0 and D1 domains. Therefore, adaptive immune responses like recognition of flagellin by antibodies are mostly directed against the variable regions.

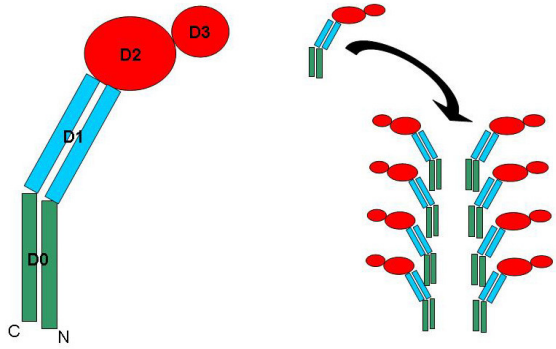

Figure 1: Schematic overview of a flagellin subunit and the flagellar assembly

Recognition of flagellin is accomplished by TLR5 binding to conserved amino acid residues in the D1 domain of flagellin. Binding of flagellin by TLR5 leads to receptor dimerization followed by the recruitment of MyD88 and activation of a typical signalling cascade [35, 36]. Flagellin monomers were demonstrated to be much more potent in eliciting TLR5 activity as compared to filamentous flagella [37].

TLR5 is expressed by monocytes, immature dendritic cells (DC) and also by epithelial cells [35]. TLR5 is also expressed in neutrophils and evidence has been provided that the receptor is recruited to the phagosome upon ligand-driven activation in phagocytic cells [38]. A recent study showed that pancreatic stellate cells expressed TLR5 and responded to flagellin from *Salmonella münchen* [39].

In this diploma thesis we wanted to clone and express the TLR5 ligand flagellin from *Salmonella typhimurium* and then further characterize the recombinant protein with regard to its biological function. *Salmonella* belong to the class of γ-proteobacteria. The family name is enterobacteria and *Salmonella typhimurium* is one serovar of the diverse genus of *Salmonella enterica.* Some *Salmonella* species including *S. typhimurium* are pathogenic for animals or humans and *Salmonella* infections (called salmonellosis) can even be lethal. According to the WHO salmonellosis affects millions of humans every year. This food-bourne disease causes symptoms like diarrhea, abdominal cramps and fever within the first 12 to 72 hours after infection and usually lasts up to 7 days. Salmonellosis is normally not lethal and does not require medical treatment but it may get problematic for immunocomprimised or elder people and children.

6 Materials and methods

6.1 Tissue culture

6.1.1 Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells were isolated from heparinized human blood as follows:

1) Dilute blood 1:2 with 1x DPBS (Gibco®, Invitrogen GmbH, Haus 223, Lofer, Austria).
2) Overlay 15 mL of Ficoll solution with approximately 35 mL of diluted blood.
3) Centrifuge at 22°C at 399x g for 30 minutes (stop without brake).
4) Carefully collect the plasma (upper phase) without harming the interphase. Plasma can be frozen at -20°C.
5) Pipet the PBMC-interphase and residual plasma into a fresh 50 mL tube. Avoid uptake of Ficoll solution and pool 2 interphases per tube. Fill up to 50 mL with 1x DPBS.
6) Centrifuge at 885x g at 22°C for 10 minutes and maximal brake. Discard supernatant directly and resuspend the pellet in 5 mL of 1x DPBS.
7) Pool 2 resuspended pellets in one tube and fill up to 50 mL with 1x DPBS.
8) Centrifuge at 737x g at 22°C for 10 minutes and maximal brake. Decant supernatant and resuspend the pellet in 5 mL of 1x DPBS. Pool resuspended cells in one 50 mL tube and fill up to 50 mL with 1x DPBS.
9) Count cells using a Bürker-Türk counting chamber (LO Laboroptik GmbH, Friedrichsdorf, Germany). Check viability by mixing 10 µL of the cell suspension with 10 µL of Trypane blue solution. Count 100 cells and calculate the percentage of living cells appearing white in the microscope compared to dead cells appearing as blue.

6.1.2 Thawing of frozen human embryonic kidney (HEK) or CaCo-2 cells

One aliquot of cells ($8x10^6$ cells) was taken out of the liquid nitrogen tank and for pressure compensation the cap was slightly opened and closed again immediately. Thereafter, the aliquot was thawed at 37°C in the waterbath. Cells were transferred into a Sterilin tube containing fresh 1x PBS and the cryo-tube was also washed with 1x PBS. Thereafter, the cell suspension was centrifuged for 8 minutes at 737x g at 22°C in a Beckman Coulter Allegra™ X-12R centrifuge (Beckman Coulter GmbH, Krefeld, Germany). The supernatant was discarded. The pellet was resuspended in 5 mL of fresh cell culture medium and this cell

suspension was transferred into a 25 cm² cell culture flask and incubated at 37°C in humidified atmosphere containing 5% CO_2. The next day the used medium was discarded and replaced by fresh, pre-warmed medium. Viability was checked using the microscope.

6.1.3 Culturing of HEK or CaCo-2 cells

Cells were grown to a confluent stage and thereafter, the used medium was discarded. Cells were washed with pre-warmed 1x PBS and Trypsin/EDTA solution was added. Cells were detached by panning the cell culture flask gently. If cells did not detach well the cell culture flask was incubated at 37°C for a few minutes. Detached cells were taken up in 5 mL of pre-warmed medium. The flask was washed once with 5mL of medium. All cells were centrifuged for 8 minutes at 737x g at 22°C in a Beckman Coulter Allegra™ X-12R centrifuge (Beckman Coulter GmbH). The supernatant was discarded and the cell pellet was resuspended in 5 mL of fresh medium. An aliquot of 0.5 mL to 2.5 mL was taken out of the cell suspension and transferred back into the cell culture flask to divide cells by 1:10 or 1:2, respectively. Thereafter, fresh medium was added and the cell culture flask was incubated at 37°C in humidified atmosphere containing 5% CO_2.

6.1.4 Creating frozen stocks of HEK or CaCo-2 cells

To generate frozen stocks of HEK or CaCo-2 cell lines, cells were detached using Trypsin/EDTA as described above and centrifuged for 8 minutes at 737x g at 22°C in a Beckman Coulter Allegra™ X-12R centrifuge (Beckman Coulter GmbH). The supernatant was discarded and cells were resuspended in 10 mL of fresh medium. Cell numbers and viability were determined by counting cells in a Bürker Türk counting chamber (LO Laboroptik GmbH, Friedrichsdorf, Germany). Cells were centrifuged at 737x g at 22°C. All further steps were done on ice. The supernatant was discarded and the cell pellet was resuspended in N_2 medium to reach a final concentration of $8x10^6$ cells per mL. Aliquots of 1 mL of the cell suspension were pipetted into cryo-tubes and immediately frozen at -80°C for at least 12 hours. Thereafter, the cryo-tubes were transferred into a liquid nitrogen tank.

6.1.5 Stimulation of PBMC

All stimuli were diluted in UCΦ medium (Lonza Ultra culture medium supplemented with Glutamine, β-mercaptoethanole and Gentamycin) and 100 µL/well were added to flatbottomed 96 well plates (Corning B.V. Life Sciences, Schiphol-Rijk, The Netherlands). If polymyxin treatment was necessary, the stimuli were pre-incubated with polymyxin (Sigma-Aldrich Handels GmbH; 20 µg/mL final concentration) at 37°C for 30 minutes. PBMC (500 000 cells/well) were seeded in 100 µL of UCΦ medium per well. Supernatants were harvested after 48 hours of incubation at 37°C in humidified atmosphere containing 5% CO_2 and cytokine levels were assessed by ELISA.

6.1.6 Stimulation of HEK or CaCo-2 cells

HEK cells stably transfected with TLR5 or CaCo-2 cells (40000 cells/well) were seeded into flatbottomed 96 well plates (Corning B.V. Life Sciences) in a volume of 100 µL/well in the respective culture medium. Plates were incubated at 37°C in humidified atmosphere containing 5% CO_2 for 24 hours to let the cells grow confluently. Medium was removed carefully to avoid loss of any cells and 100 µL/well of fresh medium containing the specific stimuli were added.

Supernatants were collected after 20 h of incubation at 37°C in a humidified atmosphere containing 5% CO_2.

6.1.7 Cytokine ELISA

The levels of different cytokines after stimulation of either HEK or CaCo-2 cells or PBMC were determined by ELISA. Nunc Maxisorb 96 well plates (Thermo Scientific Inc., Rockford, USA) were coated with the respective coating antibodies diluted in carbonate buffer (pH = 9.6) in a final volume of 100 µL/well and incubated O/N at RT. The next day, the coating antibodies were removed and 150 µL of PBS/0.05% (v/v) Tween20/4% (w/v) BSA were added per well and incubated for 60 minutes at RT as blocking solution.

Thereafter, plates were washed 3 times using 200 µL of PBS/0.05% Tween20 (v/v).

Standards and samples were diluted in PBS/0.05% (v/v) Tween20/4% (w/v) BSA, pipetted on the plates in a volume of 50 µL/well and incubated at RT for 60 minutes. They were discarded and the Biotinlyated antibodies diluted in PBS/0.05% (v/v) Tween20/4% (w/v) BSA were added to the plates in a volume of 50 µL/well and incubated at RT for 60 minutes. Plates were washed 3 times using 200 µL of PBS/0.05% (v/v) Tween20. Thereafter, HRP conjugated streptavidin (1.25 mg/mL) was added in a dilution of 1:20000 (diluted in PBS/0.05% (v/v) Tween20/4% (w/v) BSA) and incubated for 30 minutes at RT.

After washing with 200 µL of PBS/0.05% (v/v) Tween20 three times, 100 µL/well of TMB/E solution (Chemicon International Inc, Billerica, USA) were added and incubated for 30 minutes in the dark at RT. Finally, the reaction was stopped by adding 0.18 M H_2SO_4 (100 µL/well) and the absorbance was measured at 450 nm (reading) and 630 nm (reference) using a Spectra max Plus 384 plate reader (Molecular Devices, Sunnyvale, USA) and the SOFTmax Pro 4.8 software.

Antibody	Cytokine ELISA			
	IL-8	IL-10	IFN-γ	TNF-α
Coating	Endogen M801 (2 µg/mL)	Endogen M010 Isotype: Rat IgG_1 (1 µg/mL)	Calbiochem Anti γ-IFN mouse mAb (0.5 µg/mL)	Endogen M303 (2 µg/mL)
Standard	Endogen S-IL8 (500 pg/mL)	Endogen S-IL10 (500 pg/mL)	Endogen S-IFNγ (500 pg/mL)	Endogen S-TNF-α (500 pg/mL)
Biotinylated	Endogen M802B (0.2 µg/mL)	Endogen M011B (1 µg/mL)	Endogen M701B (2 µg/mL)	Endogen M302B (0.9 µg/mL)

6.2 Cloning

6.2.1 Purification of total DNA from Gram negative bacteria

Total DNA from Gram negative bacteria was purified using the QIAGEN DNeasy blood and tissue kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. All centrifugation steps were performed in an Eppendorf 5417C tabletop centrifuge (Eppendorf AG, Hamburg, Germany).

Procedure:

1) Harvest cells (maximum $2x10^9$ cells) in a microcentrifuge tube by centrifuging for 10 min at 7500 rpm. Discard the supernatant.

2) Resuspend pellet in 180 µL of Buffer ATL.

3) Add 20 µL of proteinase K. Mix thoroughly by vortexing and incubate at 56°C until the cells are completely lysed. Vortex occasionally during incubation to disperse the sample.

4) Vortex for 15 s. Add 200 µL of Buffer AL to the sample and mix thoroughly by vortexing. Then add 200 µL of ethanol (96-100%) and mix again thoroughly by vortexing.

5) Pipet the mixture from step 4 (including any precipitate) into the DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge at 8000 rpm for 1 min. Discard flow-through and collection tube.

6) Place the DNeasy Mini spin column in a new 2 mL collection tube, add 500 µL of buffer AW1 and centrifuge for 1 min at 8000 rpm. Discard flow-through and collection tube.

7) Place the DNeasy Mini spin column in a new 2 mL collection tube, add 500 µL of Buffer AW2 and centrifuge for 3 min at 14000 rpm to dry the DNeasy membrane. Discard flow-through and collection tube.

8) Place the DNeasy Mini spin column in a clean 1.5 mL microcentrifuge tube and pipet 200 µL of Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, then centrifuge for 1 min at 8000 rpm to elute the DNA from the column.

9) For maximum DNA yield, repeat elution once as described in step 8.

Step 9 was performed in a fresh 1.5 mL microcentrifuge tube.

Eluted DNA was analysed by agarose gel electrophoresis and stored at 4°C for short time periods or was frozen at -20°C for longer time periods.

6.2.2 Plasmid preparation using the QIAprep Spin Miniprep Kit

The QIAprep Spin Miniprep Kit (QIAGEN) was used for plasmid preparations following the manufacturer's instructions. All centrifugation steps were performed in an Eppendorf 5417C tabletop centrifuge (Eppendorf AG).

Procedure:

1.) After centrifugation at 8000 rpm for 3 minutes resuspend pelleted bacterial cells in 250 µL of Buffer P1 and transfer to a microcentrifuge tube.

2.) Add 250 µL of Buffer P2 and mix thoroughly by inverting the tube 4-6 times.

3.) Add 350 µL of Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.

4.) Centrifuge for 10 min at 13000 rpm in a table-top microcentrifuge.

5.) Transfer the supernatants from step 4 to the QIAprep spin column by decanting or pipetting.

6.) Centrifuge for 30-60 s. Discard the flow-through.

7.) Wash the QIAprep spin column by adding 0.5 mL Buffer PB and centrifuging for 30-60 s. Discard the flow-through.

8.) Wash QIAprep spin column by adding 0.75 mL Buffer PE and centrifuging for 30-60 s.

9.) Discard the flow-through, and centrifuge for an additional 1 min to remove residual wash buffer.

10.) Place the QIAprep column in a clean 1.5 mL microcentrifuge tube. To elute DNA, add 50 µL Buffer EB (10 mM Tris-CL, pH 8.5) or water to the center of each QIAprep spin column, leave it for 1 min, and centrifuge for 1 min.

In order to increase the concentration of the eluted DNA, the final elution step was done using 35 µL of Buffer EB.

DNA concentration was measured by spectrophotometry with a Hitachi U-1800 spectrophotometer (Hitachi High Technologies, Berkshire, UK) at 260 nm and 280 nm and plasmid DNA was stored at -20°C.

6.2.3 Preparation of frozen bacterial stocks

To generate frozen bacterial stocks for long term storage, 800 µL of an overnight culture of bacterial cells were transferred to a cryo-tube and put on ice for 2 to 5 minutes. Thereafter, 70 µL of DMSO were added, mixed by pipetting and frozen at -80°C directly.

6.2.4 Preparation of chemically competent *E.coli* cells

A frozen cryo-culture of competent *E.coli* cells was streaked out on a LB medium plate using a sterile toothpick. Selective medium plates were used for cells carrying the appropriate antibiotic resistance gene. Incubation was performed at 37°C O/N. The following day a single colony was picked and used for inoculation of 5 mL of LB medium and incubated at 37°C O/N on a shaker.

All solutions were put in the refrigerator and chilled down to 4 – 8°C. Tips and centrifugation tubes were stored at -20°C.

The next day, 200 mL of 2x LB medium in a 2 L Erlenmeyer flask were inoculated with 5 mL of the O/N cultures under sterile conditions and incubated at 37°C until the solution reached an OD_{600} of 1.0. All further steps were carried out on ice to keep cells close to 4°C. Cells were transferred into a chilled centrifugation tube and centrifuged at 4°C and 1975x g in a GSA rotor for 15 minutes. For all centrifugation steps a SORVALL RC SC PLUS centrifuge. (Thermo Scientific Inc., Rockford, USA) was used. The supernatant was carefully discarded and 50 mL of icecold 10 mM NaCl were used for resuspending the pellet. The solution was chilled on ice for 10 minutes and then centrifuged again at 4°C and 1975x g. The supernatant was decanted and the pellet was resuspended in 50 mL of icecold 75 mM $CaCl_2$ and incubated on ice for 35 minutes. After a last centrifugation step for 15 minutes at 4°C and 1975x g, the supernatant was again discarded and the pellet was resuspended in 3.0 mL of icecold 75 mM $CaCl_2$. Thereafter, the solution was transferred to a sterile flask with 0.488 mL of autoclaved 100% glycerole. The sample was mixed thoroughly, incubated on ice for 1 hour and then aliquots of 100 or 200 µL were transferred into 1.5 mL Eppendorf tubes kept on ice and shock-frozen in liquid nitrogen. Frozen aliquots were stored at -80°C until use.

6.2.5 Transformation of chemically competent *E.coli* cells

One aliquot (100 µL) of chemically competent *E.coli* cells was thawed on ice. The respective ligation samples (up to 10 µL) were added and cells were kept on ice for 30 minutes. Heat shock was performed at 42°C for 45 seconds. Thereafter, the cells were incubated on ice for 2 minutes. Subsequently 1.0 mL of SOC medium was added. To increase transformation efficiency, cells were incubated at room temperature for 15 minutes prior to incubation at 37°C for 60 minutes on a shaking incubator (300 rpm).

The cells were centrifuged at 4000 rpm for 2 minutes in an Eppendorf 5417C tabletop centrifuge and the pellet was resuspended in 100 to 200 µL of the supernatant, and plated on LB media plates containing the appropriate antibiotic if the cells carried a resistance gene. Plates were incubated at 37°C O/N.

6.2.6 DNA digestion

DNA samples were digested at 37°C for 1-2 h using the respective restriction enzymes and the corresponding buffers in a volume of 20-50 µL with approximately 2 µg of DNA (see below). Digested samples were separated by DNA gel electrophoresis or were used for further processing.

Reagent	Amount	Company
Restriction enzyme	0.5 – 2 µL	Fermentas GmbH
10x Tango buffer	4 - 10 µL	Fermentas GmbH
DNA sample (2 µg)	X µL	
Nuclease free water	Y µL to make up to the final volume of 20 – 50 µL	Fermentas GmbH

6.2.7 Dephosphorylation of digested vectors

Digested vector samples were dephosphorylated with 5 U of Calf Intestine Alkaline Phosphatase (CIAP; 1 U/µL, Fermentas) by incubation at 37°C for 30 minutes followed by incubation at 85°C for 15 minutes to inactivate the enzyme.

6.2.8 DNA ligation

Ligation of digested DNA fragments was performed using T4 DNA ligase and the corresponding T4 DNA ligase buffer at a vector : insert ratio of 1 : 5. The concentration of DNA was determined by spectrophotometry.

Ligation settings were pipetted in volumes from 10 to 25 µL with 2.5 units of T4 DNA ligase (see below). Incubation periods varied from 2 to 3 hours at room temperature.

Reagent	Amount	Company
T4 DNA ligase (5 U/µL)	0.5 µL	Fermentas GmbH
10x T4 DNA ligase buffer	1 – 2.5 µL	Fermentas GmbH
DNA insert		
Restricted vector		
Nuclease free water	To make up the final volume	Fermentas GmbH

6.2.9 Identification of positive clones by PCR

PCR screening was used for fast identification of positive clones carrying the insert of interest. Single colonies grown on selective media after ligation were picked with sterile toothpicks and transferred to a PCR reaction mix (see table).

In parallel, subcultures of each positive clone were prepared on LB medium plates containing selective antibiotics if *E.coli* cells carried the resistance gene. Plates were incubated O/N at 37°C.

Reagent	Amount	Company
Dynazyme™ 2 DNA polymerase (2 U/µL)	0.5 µL	Finnzymes Oy, Espoo, Finland
10x buffer for Dynazyme™ DNA polymerase	2 µL	Finnzymes Oy
dNTPs (10 mM each)	0.5 µL	Applied Biosystems Inc., Foster City, USA
Forward primer (10 pmol/µL)	0.5 µL	Sigma-Aldrich Handels GmbH, Vienna, Austria
Reverse primer (10 pmol/µL)	0.5 µL	Sigma-Aldrich Handels GmbH
Nuclease free water	16 µL	Fermentas GmbH

PCR programme:

95°C → 6 minutes

35 cycles:

95°C → 30 seconds

64°C → 40 seconds

72°C → 1 minute

Final elongation step:

72°C → 8 minutes

4°C → ∞

PCR products were analysed by DNA gel electrophoresis.

6.2.10 Amplification of DNA samples by PCR

To amplify DNA samples, 0.1 to 1.0 µg of DNA template was added to the PCR reaction mix in a final volume of 50 µL (see below). Self-designed primers were purchased from Sigma Aldrich (Sigma-Aldrich Handels GmbH).

Reagent	Amount	Company
Phusion DNA polymerase (2 U/µL)	0.5 µL	Finnzymes Oy
5x HF buffer for Phusion DNA polymerase	10 µL	Finnzymes Oy
dNTPs (10 mM each)	1 µL	Applied Biosystems Inc.
Forward primer (10 pmol/µL)	1.5 µL	Sigma-Aldrich Handels GmbH
Reverse primer (10 pmol/µL)	1.5 µL	Sigma-Aldrich Handels GmbH

PCR programme:

95°C → 5 minutes

35 cycles:

95°C → 30 seconds

64°C → 40 seconds

72°C → 1 minute

final elongation step:

72°C → 8 minutes

4°C → ∞

PCR products were analysed by DNA gel electrophoresis.

6.2.11 DNA gel electrophoresis

DNA samples were separated using Agarose gels containing EtBr. For a 1% (w/v) gel, 0.8 g of agarose were boiled in 80 mL of 1x TBE in a microwave oven until the solution was completely clear. For a 0.6% (w/v) gel, 0.48 g of agarose were boiled in 80 mL of 1x TBE. After cooling to approximately 60°C, 2 drops of a 0.07% EtBr solution (0.7 mg/mL; AppliChem GmbH, Darmstadt, Germany) were added. Gels were poured in the BioRad (Bio-Rad Laboratories Ges.m.b.H., Vienna, Austria) tray and polymerized at RT in a fume hood to avoid inhalation of EtBr.

Before loading, each DNA sample was mixed with the appropriate volume of Fermentas 6x DNA loading dye #R0611 (Fermentas GmbH). Gene Ruler^{TM} 100 bp DNA ladder marker (Fermentas GmbH) was used as a size marker.

Gels were run at 70 to 80 V in the BioRad Sub-Cell GT Agarose Gel Electrophoresis System (Bio-Rad Laboratories Ges.m.b.H.) using 1x TBE as a running buffer.

6.2.12 DNA extraction from agarose gels using the QIAquick Gel Extraction Kit

Extraction of DNA from agarose gels was done using the QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's protocol. All centrifugation steps were performed in an Eppendorf 5417C tabletop centrifuge (Eppendorf AG).

Procedure:

1.) Excise the DNA fragment from the agarose gel with a clean, sharp scalpel

2.) Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg correspond to 100 µL)

3.) Incubate at 50°C for 10 min. To completely dissolve the gel, vortex the tube every 2-3 min during the incubation.

4.) After the gel slice has completely dissolved, the color of the mixture must be yellow (similar to Buffer QG without dissolved agarose).

5.) Add 1 gel volume of isopropanol to the sample and mix.

6.) Place a QIAquick spin column in a provided 2 mL collection tube.

7.) To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.

8.) Discard flow-through and place QIAquick column back in the same collection tube.

9.) Recommended: Add 0.5mL of Buffer QG to QIAquick column and centrifuge for 1 min

10.) To wash, add 0.75 mL of Buffer PE to QIAquick column and centrifuge for 1 min.

11.) Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 13000 rpm.

12.) Place QIAquick column into a clean 1.5 mL microcentrifuge tube.

13.) To elute DNA, add 50 µL of Buffer EB (10 mM Tris-CL, pH 8.5) or water to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µL elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.

The eluted DNA was applied to the column a second time and centrifuged as before to increase the purity of the DNA sample.

DNA concentration was measured by spectrophotometry at 260 nm and 280 nm. DNA was stored at -20°C if not processed directly.

6.2.13 DNA sequencing

DNA samples were sequenced at MWG Eurofins Operon (Eurofins MWG Operon, Ebersberg, Germany). DNA samples (50 – 100 ng/µL) in volumes of 15 – 20 µL were sent in for sequencing.

Standard T7 polymerase primers were used. If internal primers were needed, they were purchased from Sigma Aldrich (Sigma-Aldrich Handels GmbH) and diluted to a working concentration of 2 pmol/µL in a volume of 15 µL according to the guidelines for internal primers from MWG (Eurofins MWG Operon).

6.3 Protein purification

6.3.1 Preparation of SDS-PAGE gels

Gels were prepared using the Biometra Minigel gel electrophoresis system (Biometra biomedizinische Analytik GmbH, Goettingen, Germany)

Reagent	Separating gel*		Stacking gel
	15%	17%	
Polyacrylamid solution	10 mL	11.3 mL	0.7 mL
Lower buffer	5 mL	5 mL	1.1 mL
ddH_2O	5 mL	3.6 mL	2.6 mL
TEMED	10 µL	10 µL	5 µL
10% APS	60 µL	60 µL	30 µL

* amounts to prepare 3 minigels

6.3.2 Protein gel electrophoresis

12 µL of the protein solution were mixed with 4 µL of 4x protein sample buffer, boiled at 95°C for 15 minutes and loaded on the gel. Fermentas Page Ruler™ 8-16% Tris-glycerine SDS-PAGE marker was used as a molecular weight marker. The stacking gel was run at 20 mA. When the marker front reached the separating gel 30 mA were used.

6.3.3 Coomassie staining and destaining of SDS-PAGE gels

Gels were stained with Coomassie staining solution (Coomassie brilliant blue G250) on a shaker at room temperature for 1 hour or O/N incubation. The staining solution was discarded and gels were destained at room temperature.

6.3.4 Silver staining of SDS-PAGE gels

A silver staining kit (Bio-Rad Laboratories Ges.m.b.H., Vienna, Austria) was used for the detection of proteins separated by SDS-PAGE. The staining procedure was carried out according to the following protocol:

1) Incubate the gel for 30 minutes in solution 1 (50% methanol, 12% acetic acid in ddH_2O) on a shaker.

2) Change to solution 2 (10% ethanol, 5% acetic acid in ddH_2O) and incubate the gel for 30 minutes changing to fresh solution 2 every 10 minutes.

3) Wash the gel for 5 minutes in BIORAD OXIDIZER (cat. 161-0444) covered with aluminium foil.
   BIORAD OXIDIZER stock solution is 10x concentrated and has to be diluted 1:10 in ddH_2O.

4) Wash for 4 times (5 minutes each) in ddH_2O.

5) Incubate the gel in BIORAD Silver Nitrate (cat. 161-0445) for 5 minutes covered with foil on a fluorescent box and thereafter, for 25 minutes on a shaker.
   The stock solution is 10 x concentrated and has to be diluted 1:10 in ddH_2O.

6) Discard silver nitrate solution and add BIORAD DEVELOPER (cat. 161-0447; 3.2 g in 100 mL ddH_2O) whilst shaking to avoid precipitates sticking to the gel.
   Do 2 quick changes (2 minutes each) and then a third change until the gel reaches optimum staining.

7) Stop the developing reaction by incubation in 1% (v/v) acetic acid for about 2 minutes.

8) The gel can be stored in ddH_2O.

6.3.5 Immunoblotting

Separated proteins were transferred onto a Whatman® Protran® Nitrocellulose Transfer membrane with 0.2 µm pore size (Whatman GmbH, Dassel, Germany) using an Amersham Biosciences TE22 Tank Transfer Unit (GE Europe GmbH, Munich, Germany) for 1 hour in a blotting chamber at 150 mA.

Scheme:

Negative pole

↓

Cassette for blotting chamber

↓

Sponge

↓

Wet towel

↓

Whatman Paper

↓

Gel

↓

Nitrocellulose membrane

↓

Whatman paper

↓

Wet towel

↓

Sponge

↓

Cassette for blotting chamber

↓

Positive pole

(all equilibrated in transfer buffer)

Thereafter, the membrane was saturated O/N with blocking buffer (2.5 g of milk powder in 50 mL of TBS containing 0.1% (v/v) of Tween 20) at 4°C. The next day, the membrane was washed with 15 mL of TBS/0.1% (v/v) Tween 20 for 10 minutes on a shaker at room temperature, a step that was repeated twice. Then the membrane was incubated with the primary antibody specific for the His-tag of the protein, a mouse Penta-His™ IgG_1 antibody (QIAGEN) diluted 1:4000 in TBS/0.1% (v/v) Tween 20 supplemented with BSA (4% (w/v).
After an incubation period of 2 hours, the antibody solution was discarded and the membrane was washed 3 times as described above.
Thereafter, the secondary antibody, an anti-mouse IgG1 (H&L) HRP-linked antibody (Cell Signaling Technology Inc, Danvers, USA) was added at a dilution range of 1:4000 to 1:10000

in blocking buffer (or TBS/0.1% (v/v) Tween 20/4% BSA, alternatively). The membrane was incubated with the secondary antibody for 1 to 2 hours followed by 3 washing steps as described above.

Finally, the membrane was soaked in the Lumigen™ PS-3 detection reagent (GE Healthcare UK Ltd, Birminghamshire, UK) which was prepared according to the manufacturer's protocol and was used for incubated for 5 minutes. The solution was drained off the membrane and the membrane was covered with a sheet protector and fixed in a Hyperfilm detection box. An Amersham Hyperfilm™ MP (GE Europe GmbH, Munich, Germany) was exposed to the membrane in the darkroom. Exposure times had to be determined empirically. Films were developed in an AGFA CP1000 developing unit (Agfa Graphics Germany GmbH & Co. KG, Düsseldorf, Germany)

6.3.6 Protein test expression

A frozen stock of *E.coli* BL21(DE3)pLysS cells (Invitrogen GmbH, Lofer, Austria) carrying the desired vector was used for inoculation of 5 mL of LB medium supplied with the necessary selective antibiotic. Cells were grown at 37°C in an incubator rotating at 180 rpm for 14 to 16 hours. 1.7 mL of this pre-culture were then used to inoculate 50 mL of fresh LB medium containing the selection antibiotic. This culture was then grown to an OD_{600} of 0.6 to 1.0.

Before protein expression was induced, a 1 mL sample of the suspension was taken and centrifuged at 13000 rpm for 2 minutes in an Eppendorf 5417C tabletop centrifuge (Eppendorf AG). The supernatant was discarded and the pellet was resuspended in 100 µL of 1x protein sample buffer and stored on ice.

Protein expression was induced by the addition of IPTG (final concentration of 0.4 mM). Thereafter, a sample (500 µL) of the cell suspension was collected and centrifuged for 2 minutes at 17900x g every hour. The supernatant was discarded and the pellet was resuspended in 50 µL of 1x protein sample buffer and stored on ice.

After 6 hours of incubation at 37°C and 200 rpm the remaining cells were harvested by centrifugation at 4000x g and 4°C for 30 minutes in a SORVALL RC SC PLUS centrifuge (Thermo Scientific Inc.). The supernatant was carefully decanted and the pellet was resuspended in Lysis buffer (50 mM Tris/HCl pH = 7.5, 0.5 M NaCl, 30 mM imidazole). To lyse the cells the sample was frozen in liquid nitrogen and thawed at 37°C, which was repeated twice. If the cell lysate was very viscous, 5 µg/mL of DNase were added and incubated shaking gently for 30 to 60 minutes at RT.

Thereafter, the cell suspension was centrifuged at 15000x g and 4°C for 20 minutes in a SORVALL RC SC PLUS centrifuge (Thermo Scientific Inc.). The supernatant containing the solubly expressed proteins was stored at 4°C. The pellet was resuspended in 5 mL of 6M urea and centrifuged under the same conditions as before. The supernatant containing the insolubly expressed protein fraction was stored at 4°C.

To monitor protein expression over time, 12 µL of the soluble and insoluble fractions as well as of each sample taken every hour were loaded on SDS-PAGE gels (15 or 17%, page 29).

6.3.7 Protein expression under salt stress

15 mL of TB medium containing the respective selective antibiotic were inoculated with *E.coli* BL21(DE3)pLysS (Invitrogen GmbH) cells containing a vector with the gene of interest using a sterile toothpick. Cells were grown O/N at 30°C shaking at 200 rpm.

The next day the culture was diluted 1:30 in fresh TB medium supplemented with the selection antibiotic and cells were grown to an OD_{600} of 2.0 at 30°C and 200 rpm. Thereafter, salt stress was induced by the addition of 10 mM betaine, 0.5 M sorbitol and 4% NaCl and cells were incubated at 16°C and 180 rpm for 60 minutes.

One aliquot (1 mL) was collected and centrifuged at 1700x g for 2 minutes in a tabletop centrifuge. The supernatant was discarded and the pellet was resuspended in 100 µL of 1x protein sample buffer. Protein expression was induced by the addition of IPTG (final concentration of 0.4 mM) and cells were cultured at 16°C rotating at 180 rpm. Samples (500 µL) were taken at different timepoints to monitor protein expression. All samples were centrifuged at 1700x g for 2 minutes in a tabletop centrifuge. Supernatants were discarded and pellets were resuspended in 50 µL of 1x protein sample buffer.

Protein expression was induced for 16 hours. Cells were harvested by centrifuging at 5000x g and 4°C for 30 minutes in a SORVALL RC SC PLUS centrifuge (Thermo Scientific Inc.). Supernatants were discarded and the pellet was resuspended in lysis buffer (50 mM Tris/HCl pH = 7.5, 0.5 M NaCl, 30 mM imidazole; one tenth of the original culture volume). Cells were lysed by freezing in liquid nitrogen and thawing in a 37°C waterbath. These steps were repeated twice. Thereafter, 5 µg/mL of DNase were added and incubated at room temperature shaking gently for 30 to 60 minutes.

Thereafter, the cell suspension was centrifuged at 20000x g and 4°C for 30 minutes in a SORVALL RC SC PLUS centrifuge (Thermo Scientific Inc.) and the supernatant containing the solubly expressed proteins was stored at 4°C. The pellet was resuspended in 6 M urea (one tenth of the original culture volume). This suspension was centrifuged under the same conditions as before. The supernatant containing the insolubly expressed protein fraction was stored at 4°C.

To monitor protein expression over time, 12 µL of the soluble and the insoluble fraction as well as of each sample taken at the different time-points were loaded on SDS-PAGE gels (15 or 17%, page 29).

6.3.8 Protein Expression at 16°C

A frozen stock of *E.coli* BL21(DE3)pLysS (Invitrogen GmbH) carrying the desired vector was used for inoculation of 5 mL of LB medium supplemented with the respective selective antibiotic. Cells were grown at 37°C in a shaking incubator at 180 rpm for 14 to 16 hours. 1.7 mL of this culture were used for inoculation of 50 mL of fresh LB medium containing the selective antibiotic and this culture was then grown at 30°C to an OD_{600} of 0.6 to 1.0.

Before induction of protein expression, a 1 mL aliquot of the suspension was collected and centrifuged at 17900x g for 2 minutes in a tabletop centrifuge. The supernatant was discarded and the pellet was resuspended in 100 µL of 1x protein sample buffer and stored on ice.

Protein expression was induced by the addition of IPTG (final concentration of 0.4 mM) and the incubation temperature was reduced to 16°C. Thereafter, several samples (500 µL) were taken at indicated timepoints (Fig. 18) and centrifuged for 2 minutes at 17900x g a tabletop centrifuge. Supernatants were discarded and pellets were resuspended in 50 µL of 1x protein sample buffer. Samples were stored on ice and boiled at 95°C for 15 minutes prior to loading on an SDS-PAGE gel.

After 20 hours of incubation at 16°C and 200 rpm, cells were harvested by centrifugation at 4000x g and 4°C for 30 minutes in a SORVALL RC SC PLUS centrifuge (Thermo Scientific Inc.). The supernatant was decanted carefully and the pellet was resuspended in lysis buffer (50 mM Tris/HCl pH = 7.5, 0.5 M NaCl, 30 mM imidazole). Cells were lysed by three cycles of alternating freezing in liquid nitrogen and thawing at 37°C. If the cell lysate was very viscous, 5 µg/mL of DNase were added and incubated at room temperature shaking gently for 30 to 60 minutes.

Thereafter, the cell suspension was centrifuged at 15000x g and 4°C for 20 minutes in a SORVALL RC SC PLUS centrifuge (Thermo Scientific Inc.) and the supernatant containing the solubly expressed proteins was stored at 4°C. The pellet was resuspended in 6 M urea and centrifuged at the same conditions as before. The supernatant containing the insolubly expressed protein fraction was stored at 4°C.

To monitor protein expression over time, 12 µL of the soluble and the insoluble fraction as well as of each sample taken at the different time-points were loaded on SDS-PAGE gels (15 or 17%, page 29).

6.3.9 Affinity purification of protein samples

Purification of His-tagged proteins was performed using the ÄKTA™ prime protein purification system (GE Europe GmbH, Munich, Germany) according to the manufacturer's instructions.

The His-Trap FF crude 1 mL column (GE Europe GmbH, Munich, Germany) was equilibrated with 5 to 10 column volumes of lysis buffer.

The protein solution was sterile filtered through a MILLEX GV low protein filter unit (0.22 µm; Millipore Corporation, Billerica, USA) and loaded onto the column at a flowrate of 1 mL/min with a backpressure maximum of 0.3 MPa. To elute the protein, a linear gradient starting with 100% of lysis buffer (50 mM Tris/HCl pH = 7.5, 0.5 M NaCl, 30 mM imidazole) and continuously increasing amounts of elution buffer reaching a final concentration of 100% of elution buffer (50 mM Tris/HCl pH = 7.5, 0.5 M NaCl, 500 mM imidazole) was used.

Eluted proteins were collected in fractions of 1.5 mL and analysed by SDS-PAGE (12 to 17% gels) prior to dialysis against PBS.

The His-Trap FF crude column as well as the ÄKTA™ system were washed and equilibrated with ddH_2O followed by ethanol (20% (v/v)).

6.3.10 Dialysis of protein samples

Dialysis membranes with a molecular weight cut off (MWCO) of 6 - 8 kDa (Spectra/Por®, Spectrum Europe B.V., Breda, The Netherlands) were used. Membranes were prepared for 2 hours at 60°C in 2000 mL pre-warmed dialysis preparation buffer with sporadic stirring. This step had to be repeated once. Thereafter, the membranes were incubated for 2 hours at 60°C in pre-warmed ddH_2O. The water was changed several times until the solution was completely clear. Thereafter, the solution was slowly chilled to 4°C. Dialysis membranes were stored in ddH_2O containing chloroform (1 mL/L) at 4°C.

6.3.11 BCA assay

Protein concentrations were determined using the Pierce Endogen BCA (Thermo Scientific Inc., Rockford, USA) assay according to the manufacturer's protocol (microplate procedure).
Preparation of the BCA™ working reagent:
Prepare working reagent by mixing 50 parts of BCA™ Reagent A with 1 part of BCA™ Reagent B. (e.g. 5 mL of Reagent A with 100 µL of Reagent B)

Microplate procedure protocol:

1. Pipette 25 µL of each standard or unknown sample replicate into a microplate well (working range = 20 – 2000 µg/mL).
2. Add 200 µL of the working reagent to each well and mix the plate thoroughly on a plate shaker for 30 seconds.
3. Cover Plate and incubate at 37°C for 30 minutes.
4. Cool plate to RT.
5. Measure the absorbance at 562 nm.

6.4 Buffers and solutions

10x TBE solution:

54 g Tris base

27.5 g Boric acid

20 mL 0.5 M EDTA solution / or: 4.65 g (Na_4EDTA)

Fill up to 500 mL with ddH_2O.

TB Amp medium:

24 g yeast extract

12 g peptone

4 mL 100% glycerol

Fill up to 900 mL with ddH_2O and autoclave.

Meanwhile:

2.31 g KH_2PO_4

12.54 g K_2HPO_4

Fill up to 100 mL with ddH_2O and do sterile filtration.

When autoclaved solution has cooled down add the 100 mL of the other solution and finally add 1 mL of Ampicillin (stock: 100 mg/mL). Store at 4°C.

LB Amp medium:

5 g NaCl

10 g peptone

5 g yeast extract

Fill up to 1000 mL with ddH_2O

Autoclave and when cooled down (handwarm) add 1 mL Amp (stock: 100 mg/mL) under laminar flow/sterile conditions and keep in fridge.

LB medium:

5 g NaCL

10 g peptone

5 g yeast extract

Fill up to 1000 mL with ddH_2O

Autoclave and store at RT.

LB Amp plates:

5 g NaCL

10 g peptone

5 g yeast extract

15 g Agar

Fill up to 100 mL with ddH_2O

Autoclave and when cooled down (handwarm) add 1 mL Amp (stock: 100 mg/mL) and pour plates under laminar flow/sterile conditions.

LB plates:

5 g NaCL

10 g peptone

5 g yeast extract

15 g Agar

Fill up to 100 mL with ddH_2O

Autoclave and pour plates under laminar flow/sterile conditions.

2 M NaCl:

11.69 g NaCl

Fill up to 100 mL with ddH_2O.

50 mL 10 mM NaCl:

29.22 mg of 2 M NaCl

Fill up to 50 mL with ddH_2O

50 mL 75 mM $CaCl_2$:

0.55 g $CaCl_2$

Fill up to 50 mL with ddH_2O.

10x PBS:

80 g NaCl

2 g KCl

14.4 g Na_2HPO_4

2.4 g KH_2PO_4

pH = 7.4 (HCl/NaOH)

Fill up to 1000 mL with ddH_2O.

Coomassie Brilliant Blue:

1 g Coomassie brilliant blue G-250

500 mL methyl alcohol

100 mL acetic acid

400 mL dd H_2O

Dissolve Brilliant Blue in methyl alcohol first O/N and then add the other substances because otherwise the Brilliant Blue won't dissolve properly.

Destaining solution:

100 mL methyl alcohol

100 mL acetic acid

800 mL ddH_2O

Lysis buffer (50 mM Tris/HCl pH = 7.5, 0.5 M NaCl, 30 mM imidazole):

6.057 g Tris

29.22 g NaCl

2.04 g imidazole

Fill up to 1000 mL with ddH_2O pH = 7.5 with HCl

Elution buffer (50 mM Tris/HCl pH = 7.5, 0.5 M NaCl, 500 mM imidazole):

6.057 g Tris

29.22 g NaCl

34.04 g imidazole

Fill up to 1000 mL with ddH_2O pH = 7.5 with HCl

1 mg/mL DNase stock solution:

Buffer: 10 mM Tris/Hcl pH = 7.5, 150 mM NaCl, 1 mM MgCl

For 25 mL of buffer:

0.03 g Tris

0.219 g NaCl

0.00508 g MgCl

Dissolve 2 mg of DNase in 1 mL of buffer and when DNase has dissolved completely add 1 mL of glycerol.

Carbonate buffer (pH = 9.6):

1.965 g Na_2CO_3

2.645 g $NaHCO_3$

Ad 500mL of ddH_2O

Tissue culture media:

HEK-TLR5 medium:

500 mL DMEM (PAA Laboratories GmbH)

10% FCS (PAA Laboratories GmbH) = 50 mL

1 mL Gentamycin (10 g/118 mL)

500 µL Blasticidin (10 mg/mL)

CaCo-2 medium:

500 mL DMEM (PAA Laboratories GmbH)

10% FCS (PAA Laboratories GmbH) = 50 mL

5 mL Glutamin (2.937 g/100 mL)

1 mL Gentamycin (10 g/118 mL)

UCΦ medium

500 mL Ultra Culture medium (Lonza Group Ltd., Basel, Switzerland)

5 mL Glutamin (2.937 g/100 mL)

2.5 mL β-mercaptoethanol (35 µL/50 mL)

1 mL Gentamycin (10 g/118 mL)

N_2 medium

500 mL RPMI 1640 buffered with 25mM Hepes (Gibco®,Invitrogen GmbH)

20% FCS (PAA Laboratories GmbH)

10% DMSO

1 mL Gentamycin (10 g/118 mL)

(Note: Add FCS to RPMI1640 medium and then add DMSO dropwise whilst gently shaking!)

7 Results

Toll-like receptor (TLR) ligands are discussed as possible adjuvants in allergy vaccines. We tested recombinant flagellin, a TLR5 ligand, on PBMC from allergic donors to analyse whether flagellin might be useful for developing new adjuvants for allergy vaccines. Different cell lines carrying TLR5 were used to establish a suited test protocol for the evaluation of TLR5 binding.

7.1 Testing recombinant flagellin from *S. typhimurium* on peripheral blood mononuclear cells (PBMC) from birch pollen-allergic patients

PBMC of birch pollen-allergic patients were isolated and stimulated with recombinant flagellin in the presence or absence of autologous plasma. Cytokine secretion of different key cytokines was determined by ELISA.

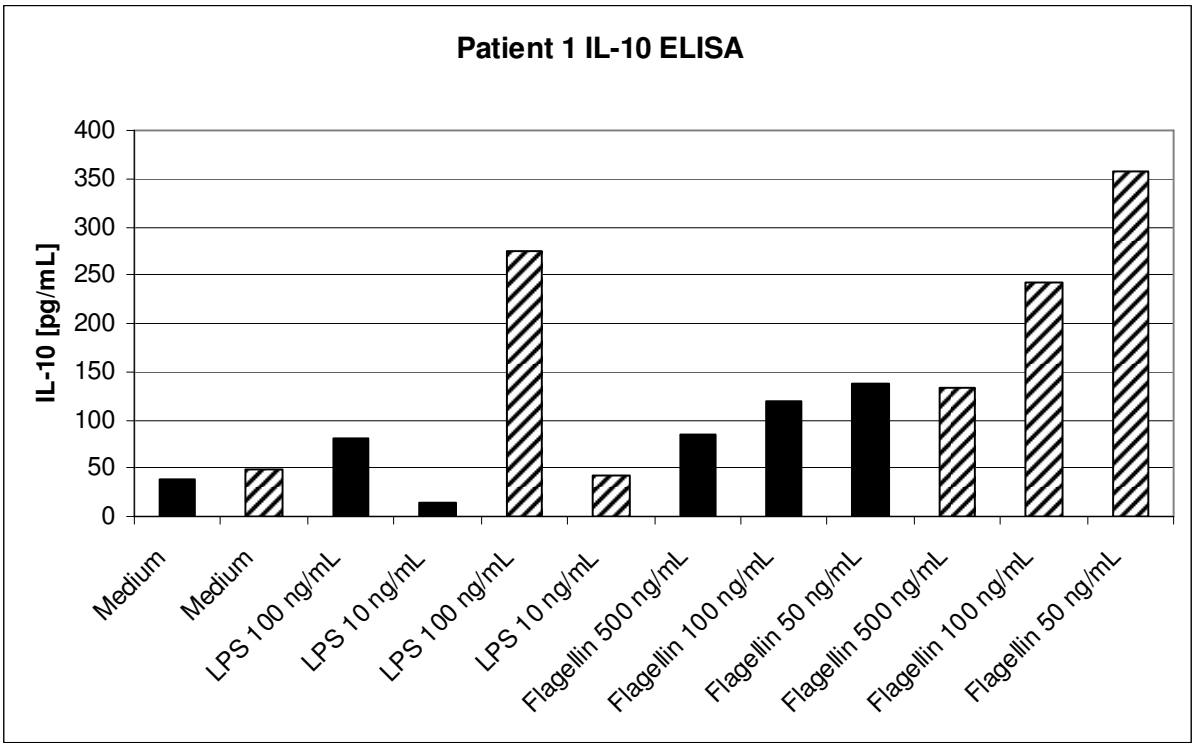

Figure 2: IL-10 production of PBMC from a birch pollen-allergic patient in response to flagellin from *S. typhimurium:* PBMC ($0.5x10^6$ /96 flat-bottomed well) were incubated with either medium alone, different concentrations of lipopolysaccharide (LPS) or different concentrations of flagellin from *Salmonella typhimurium* for 48 hours in medium containing 20 µg/mL polymyxin B either in the presence (hatched bars) or absence (filled bars) of 20% autologous plasma. IL-10 secretion was determined by ELISA.

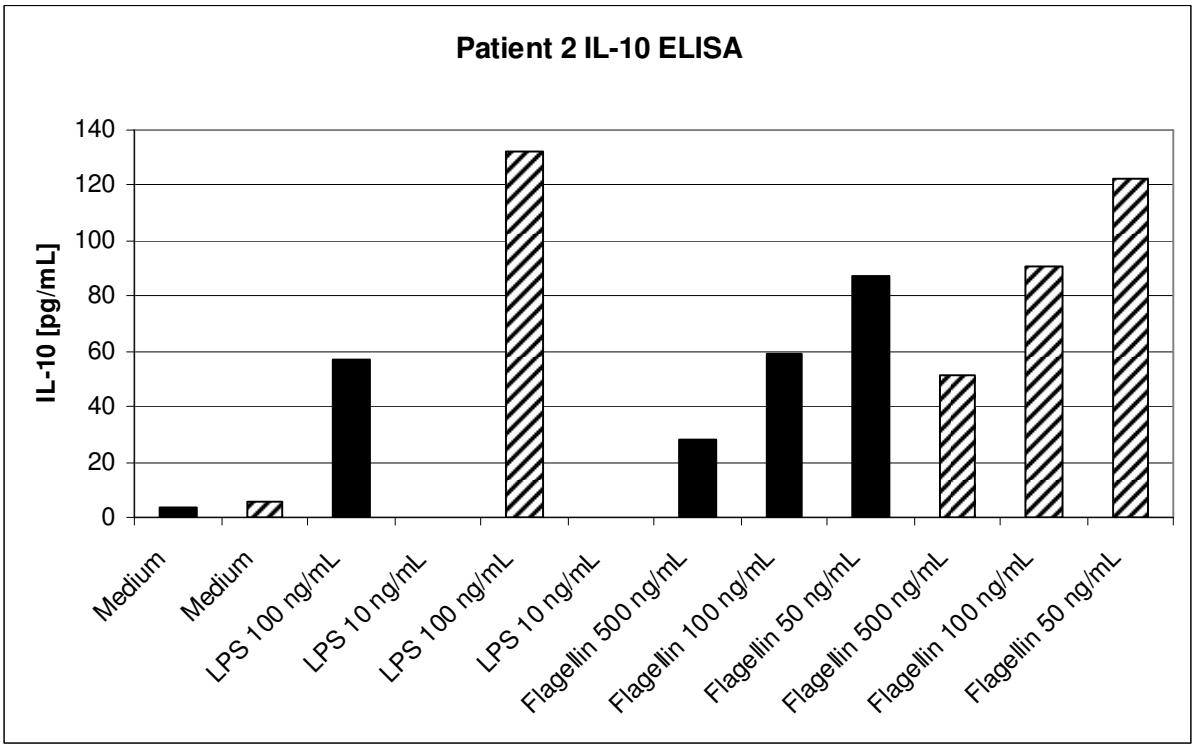

Figure 3: IL-10 production of PBMC from a birch pollen-allergic patient in response to flagellin from *S. typhimurium:* PBMC ($0.5x10^6$ /96 flat-bottomed well) were incubated with either medium alone, different concentrations of lipopolysaccharide (LPS) or different concentrations of flagellin from *Salmonella typhimurium* for 48 hours in medium containing 20 µg/mL polymyxin B either in the presence (hatched bars) or absence (filled bars) of 20% autologous plasma. IL-10 secretion was determined by ELISA.

Figures 2 and 3 show that stimulation of PBMC with flagellin led to increased IL-10 production compared to the medium controls. Different concentrations of flagellin were tested and the concentration of 50 ng/mL triggered the highest IL-10 secretion. Presumably, higher amounts of flagellin were cytotoxic and killed the cells. IL-10 synthesis was increased in the presence of 20% autologous plasma (hatched bars) as compared to stimulation without autologous plasma (filled bars). Stimulation with LPS induced IL-10 secretion in the presence of autologous plasma.

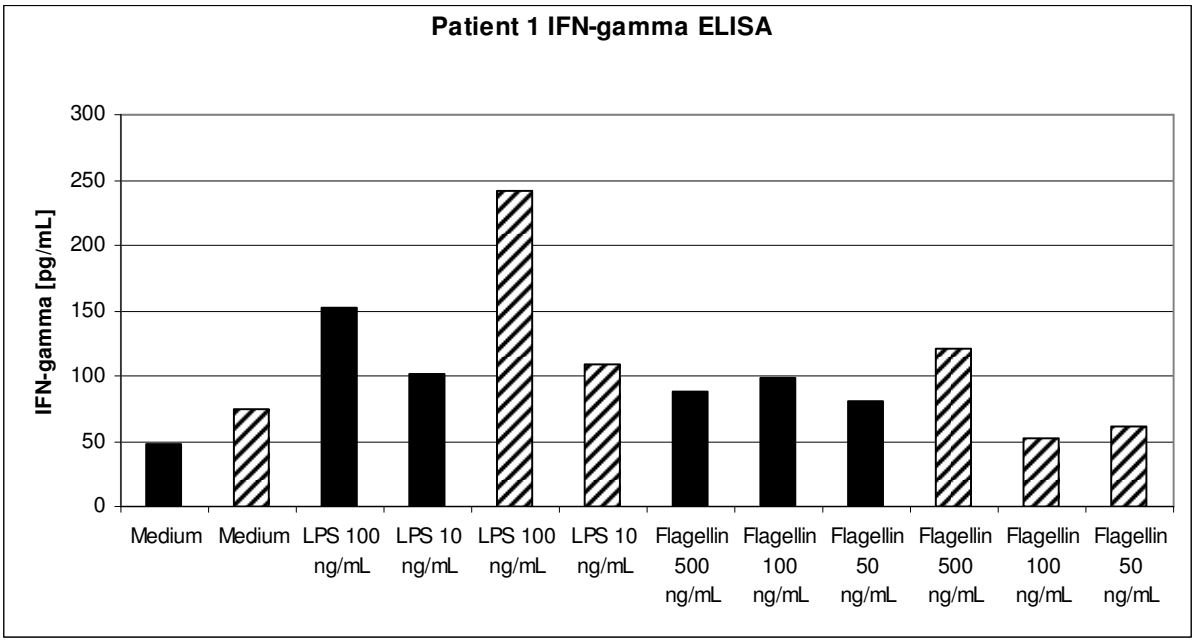

Figure 4: IFN-γ production of PBMC from a birch pollen-allergic patient in response to flagellin from *S. typhimurium:* PBMC ($0.5x10^6$ /96 flat-bottomed well) were incubated with medium alone, different concentrations of lipopolysaccharide (LPS) or different concentrations of flagellin from *Salmonella typhimurium* for 48 hours in medium containing 20 µg/mL polymyxin B either in the presence (hatched bars) or absence (filled bars) of 20% autologous plasma. IFN-γ secretion was determined by ELISA.

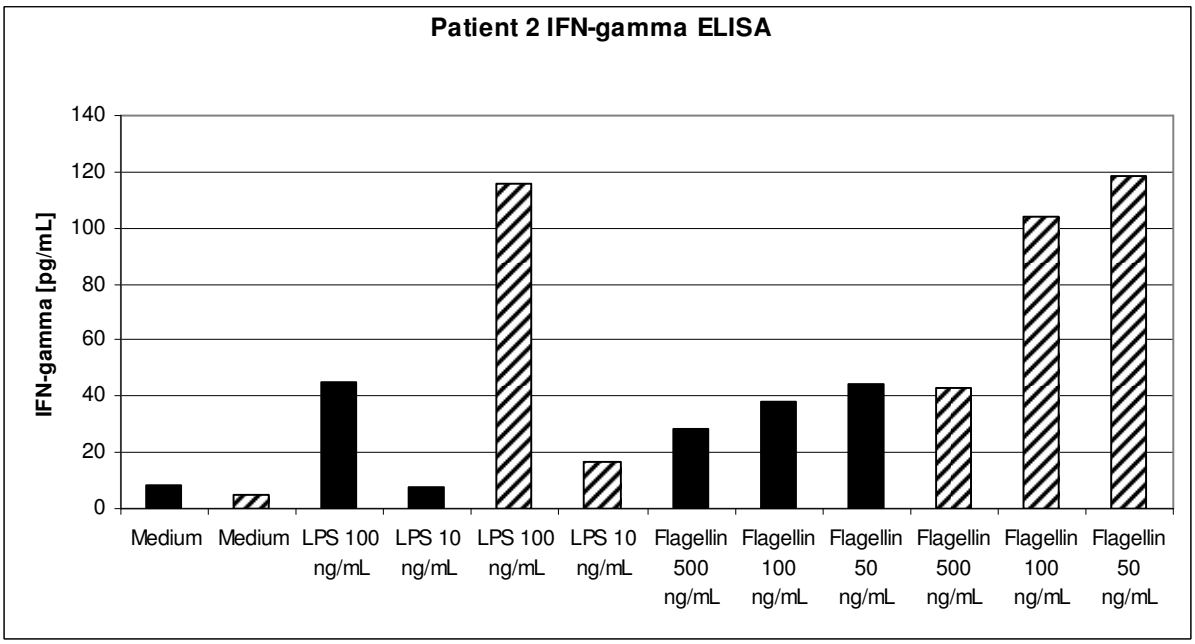

Figure 5: IFN-γ production of PBMC from a birch pollen-allergic patient in response to flagellin from *S. typhimurium:* PBMC ($0.5x10^6$ /96 flat-bottomed well) were incubated with medium alone, different concentrations of lipopolysaccharide (LPS) or different concentrations of flagellin from *Salmonella typhimurium* for 48 hours in medium containing 20 µg/mL polymyxin B either in the presence (hatched bars) or absence (filled bars) of 20% autologous plasma. IFN-γ secretion was determined by ELISA.

Figures 4 and 5 show that stimulation with flagellin led to increased IFN-γ production as compared to medium alone. This effect was enhanced by the presence of autologous plasma.

We also assessed the production of TNF-α in response to flagellin. No production of TNF-α was detected.

In summary, we found a weak induction of the T_H1-type cytokine IFN-γ and a stronger induction of the immunosuppressive cytokine IL-10. We considered flagellin as a potential adjuvant for allergy vaccination.

7.2 Establishing a test system for TLR5 triggering

7.2.1 Human embryonic kidney (HEK) 293 cells stably transfected with TLR5

We stimulated HEK-293 cells stably transfected with TLR5 (HEK-293/TLR5) with different concentrations of flagellin (Invitrogen GmbH) and determined IL-8 secretion by ELISA.

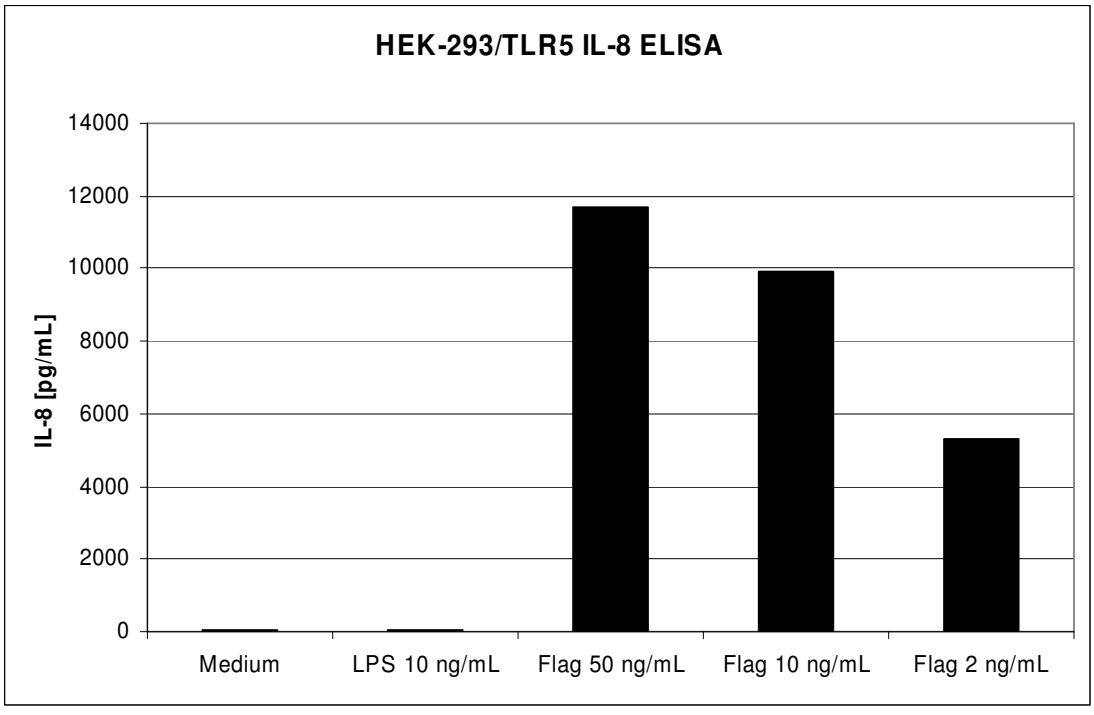

Figure 6: IL-8 production of HEK-293/TLR5 cells in response to flagellin from *S. typhimurium*: HEK-293/TLR5 cells were seeded in a flatbottomed 96 well plate (40000 cells/well) and incubated for 24 hours. Medium was removed and replaced by medium containing the specific stimuli. After 20 hours IL-8 production was determined by ELISA.

Figure 6 shows that stimulation of HEK-293/TLR5 cells with flagellin induced the production of IL-8 (black bars) whereas lipopolysaccharide (LPS), a TLR4 ligand, which served as a negative control did not induce IL-8 synthesis. The synthesis of IL-8 after stimulation with flagellin was dose dependent.

7.2.2 CaCo-2 cells

This immortalized cell-line derived from human epithelial colorectal adenocarcinoma cells shows the ability to differentiate into small intestinal-like cells after confluent growth and it carries TLR5. We were interested in the effects of flagellin stimulation on these cells as they can be used as a model system for intestinal functions.

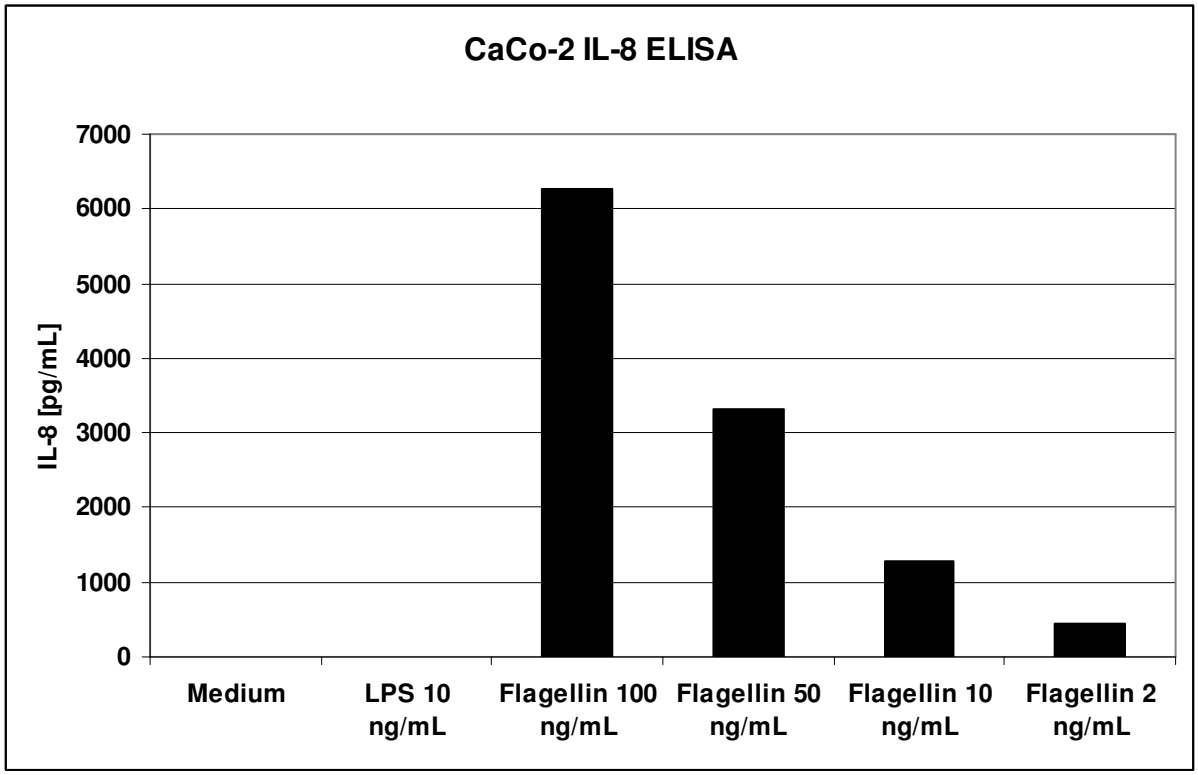

Figure 7: IL-8 production of CaCo-2 cells in response to flagellin from *S. typhimurium*: CaCo-2 cells were seeded in a flatbottomed 96 well plate (40000 cells/well) and incubated for 24 hours. Medium was removed and replaced by medium containing the specific stimuli. After 20 hours IL-8 production was determined by ELISA.

Figure 7 shows that stimulation with flagellin triggered CaCo-2 cells to synthesize IL-8 in a dose dependent manner. LPS was not able to induce IL-8 secretion. HEK-293/TLR5 produced higher levels of IL-8 (Fig.6) as compared to CaCo-2 cells (Fig.7).

Moreover, the HEK-293/TLR5 cell line was more convenient to work with in the tissue culture as cells were easier to detach and showed enhanced growth compared to the CaCo-2

cells. Therefore, we decided to use the HEK-293/TLR5 cell line as our standard cell line to evaluate TLR5 binding.

7.3 Evaluating the influence of heat-labile serological factors on TLR5 triggering

As serological factors might play a role in the signalling cascade of TLR, we examined whether heat-labile serological factors had an influence on the biological capacity of recombinant flagellin to trigger TLR5.

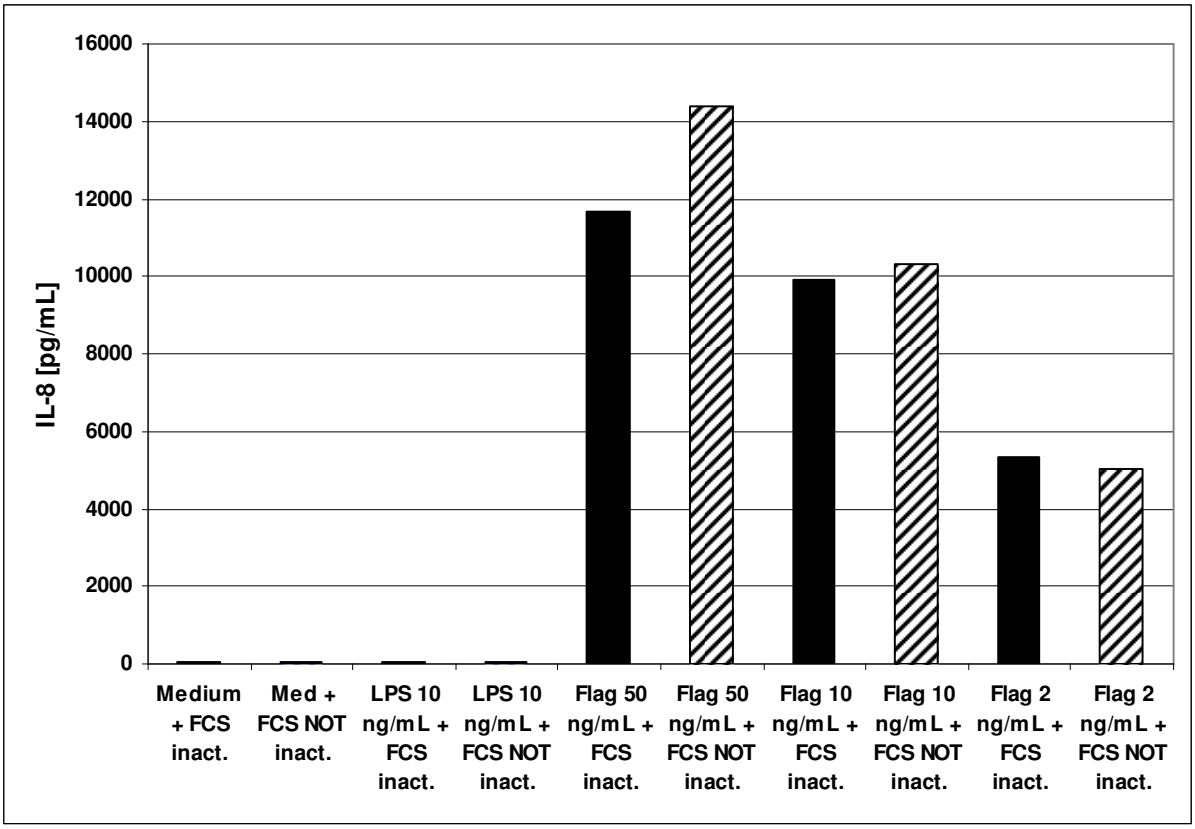

Figure 8: Influence of serological factors on TLR5 stimulation by flagellin: HEK-293/TLR5 cells were seeded in a flatbottomed 96 well plate (40000 cells/well) and incubated for 24 hours. Medium was removed and replaced by medium containing the specific stimuli. Medium either supplemented with inactivated FCS (full bars) or non-inactivated FCS (hatched bars) was used. After 20 hours IL-8 secretion in the cell supernatants was determined by ELISA.

Figure 8 shows the IL-8 production of HEK-TLR5 cells in response to different concentrations of recombinant flagellin from *S. typhimurium* (Invitrogen GmbH) either in the presence of 10% FCS inactivated by incubation at 56°C for 30 minutes (full bars) or non-inactivated FCS (hatched bars). No difference in the ability of flagellin to trigger TLR5 was observed. The medium control and the negative control did not show different results when

non-inactivated FCS was added. We decided to use heat-inactivated FCS in our standard protocols.

To further investigate the possible adjuvant activities of flagellin from *S. typhimurium* we wanted to recombinantly express the protein in bacteria to have sufficient amounts of the protein and save costs.

7.4 Amplification and cloning of DNA encoding flagellin from *S. typhimurium* into pETBlue-2 vector

Prior to isolation of the genomic DNA of *Salmonella typhimurium* using the QIAGEN DNeasy blood and tissue kit, *Salmonella typhimurium* (ATCC® 35987) was cultured in LB medium. DNA samples were separated on a 0.6% (w/v) agarose gel containing EtBr.

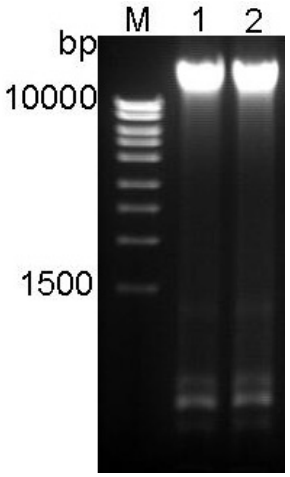

Figure 9: Genomic DNA of *S. typhimurium*: Two DNA samples (Lanes 1&2) were separated on a 0.6% (w/v) agarose gel containing EtBr Lane M: Fermentas DNA High range marker ready to use

Figure 9 shows very intense bands in a size of more than 10 kb resulting from the genomic DNA of *S. typhimurium* in lanes 1 and 2. Weak bands were detected in both lanes resulting from RNA and degraded DNA fragments.

Both samples of genomic DNA were then used as templates for PCR with primers specific for the flagellin protein (Fig. 10).

Forward primer: GAGA**CCATGG**CTCTGGGCACCGCTATCGA

Reverse primer: GAGA**CTCGAG**ACGCAGTAAAGAGAGGACG

Bases matching the DNA sequence of flagellin are underlined. Bases encoding restriction sites for NcoI and XhoI are shown in bold. Primers were designed excluding the signal peptide of flagellin since it is not necessary for the antigenic capacity of the protein.

```
   1 aaggaaaaga tcatggcaca agtcattaat acaaacagcc tgtcgctgtt gacccagaat
  61 aacctgaaca aatcccagtc cgctctgggc accgctatcg agcgtctgtc ttccggtctg
 121 cgtatcaaca gcgcgaaaga cgatgcggca ggtcaggcga ttgctaaccg ttttaccgcg
 181 aacatcaaag gtctgactca ggcttcccgt aacgctaacg acggtatctc cattgcgcag
 241 accactgaag gcgcgctgaa cgaaatcaac aacaacctgc agcgtgtgcg tgaactggcg
 301 gttcagtctg ctaacagcac caactcccag tctgacctcg actccatcca ggctgaaatc
 361 acccagcgtc tgaacgaaat cgaccgtgta aatggccaga ctcagttcag cggcgtgaaa
 421 gtcctggcgc aggacaacac cctgaccatc caggttggtg ccaacgacgg tgaaactatc
 481 gatatcgatc tgaagcagat caactctcag accctgggtc tggatacgct gaatgtgcaa
 541 caaaaatata aggtcagcga tacggctgca actgttacag gatatgccga tactacgatt
 601 gctttagaca atagtacttt taaagcctcg gctactggtc ttggtggtac tgacgagaaa
 661 attgatggcg atttaaaatt tgatgatacg actggaaaat attacgccaa agttaccgtt
 721 acgggggggaa ctggtaaaga tggctattat gaagtttccg ttgataagac gaacggtgag
 781 gtgactcttg ctgcggtcac tcccgctaca gtgactactg cgacagcact gagtggaaaa
 841 atgtacagtg caaatcctga ttctgacata gctaaagccg cattgacagc agcaggtgtt
 901 accggcacag catctgttgt taagatgtct tatactgata ataacggtaa aactattgat
 961 ggtggtttag cagttaaggt aggcgatgat tactattctg caactcaaga taaagatggt
1021 tccataagta ttgatactac gaaatacact gcagataacg gtacatccaa aactgcacta
1081 aacaaactgg gtggcgcaga cggcaaaacc gaagtcgtta ctatcgacgg taaaacctac
1141 aatgccagca aagccgctgg tcatgatttc aaagcagaac cagagctggc ggaacaagcc
1201 gctaaaacca ccgaaaaccc gctgcagaaa attgatgctg ctttggcaca ggttgacacg
1261 ttacgttctg acctgggtgc ggtacagaac cgtttcaact ccgctattac caacctgggc
1321 aacaccgtaa acaacctgtc ttctgcccgt agccgtatcg aagattccga ctacgcgacc
1381 gaagtctcca acatgtctcg cgcgcagatt ctgcagcagg ccggtacctc cgttctggcg
1441 caggcgaacc aggttccgca aaacgtcctc tctttactgc gttaa
```

Figure 10: DNA sequence encoding flagellin from *S. typhimurium* (1485 bp, NCBI Accession No.: M11332,[40]): Primer base pairing sequences are underlined

Thereafter, PCR products were checked on a 1% (w/v) agarose gel containing EtBr and bands of the correct size indicating the expected DNA fragment were excised under UV light and used for DNA extraction by the QIAquick Gel Extraction Kit. One sample was used for further steps. The other sample was stored at -20°C.

Prior to ligation into the pETBlue-2 vector from Novagen (EMD Chemicals Inc., San Diego, USA), the excised DNA sample and the vector were digested with restriction enzymes NcoI and XhoI. The vector was dephosphorylated by CIAP treatment to prevent religation. After ligation of the excised DNA into the vector, the sample was used for transformation of *E.coli* TOP10 cells. Ligation was checked by PCR screening using the grown colonies as templates and the primers mentioned above. PCR products were checked by DNA gel electrophoresis.
We used this vector as it allowed the addition of a histidine-tag to the C-terminus of the protein sequence. This His-tag facilitates the purification of the protein of interest.

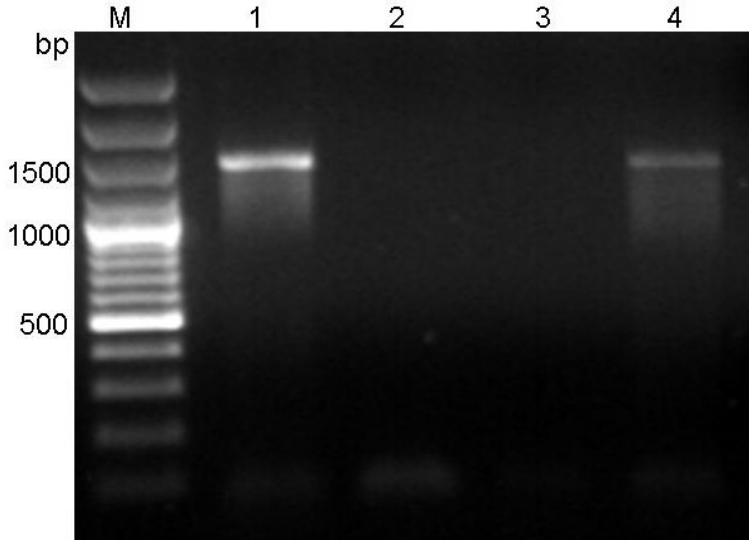

Figure 11: Flagellin in pETBlue-2 vector colony screening: 4 colonies (Lanes 1-4) were checked for successful ligation of the flagellin sequence into the pETBlue-2 vector in a 1% (w/v) agarose gel containing EtBr
Lane M: Fermentas 100 bp DNA ladder marker

Figure 11 shows that PCR of colonies 1 and 4 amplified bands with a size of 1500 bp that correlated with the calculated size of flagellin from *S. typhimurium.* Lanes 2 and 3 showed no bands at the expected size indicating that these clones did not carry the vector containing the correct insert.

As only 2 positive clones were found, more colonies were picked and screened by PCR to identify additional clones containing the correct insert. PCR products were again checked by DNA gel electrophoresis and revealed more clones carrying the vector with the insert.

To check whether those clones giving positive results in the PCR screening indeed carried the insert of interest, a plasmid preparation of an O/N culture of each individual colony was performed. The plasmid DNA was digested with the restriction enzymes NcoI and XhoI and loaded on a 1% (w/v) agarose gel containing EtBr.

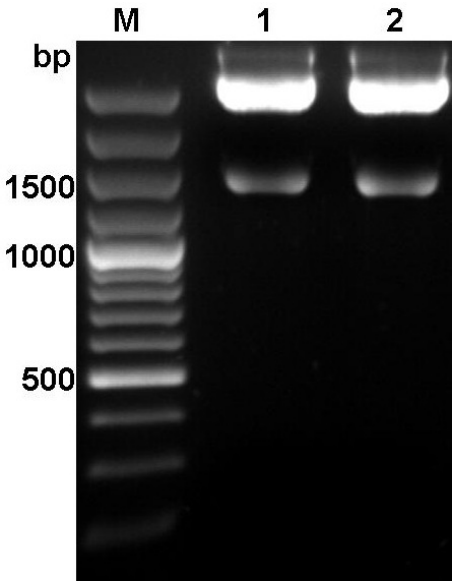

Figure 12: Flagellin in pETBlue-2 vector NcoI/XhoI control digest: Plasmid DNA (Lanes 1 and 2) was digested with restriction enzymes NcoI and XhoI and checked on a 1% (w/v) agarose gel containing EtBr. Lane M: Fermentas 100 bp DNA ladder marker

Figure 12 shows that 2 clones (Lanes 1 and 2) contained a DNA insert in the size of 1500 bp matching the expected size for flagellin from *S. typhimurium.*
Isolated plasmid DNA samples of positive clones were sequenced by MWG (Eurofins MWG Operon, Ebersberg, Germany) using standard T7 primers. Obtained sequences were matched with the flagellin sequence (NCBI Accession No.: M11332, Fig.10) by BLAST analysis (data not shown).

The DNA sequences of all clones contained several mutations in the sequence encoding the N-terminal part of the protein. Therefore, the whole process was started again from the PCR with the genomic DNA sample as template and all steps were performed as described above.

Finally, 3 clones were used for inoculation of 5 mL of LB Amp to perform plasmid preparation and digestion of the DNA samples with NcoI and XhoI. Digested DNA was analysed on a 1% (w/v) agarose gel containing EtBr.

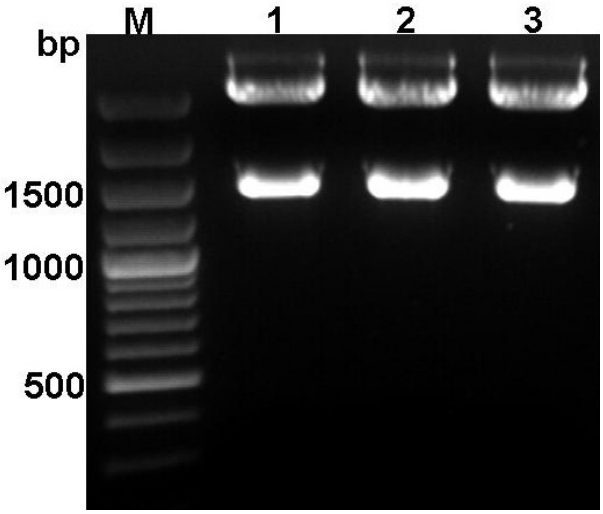

Figure 13: Flagellin in pETBlue-2 vector NcoI/XhoI control digest: Plasmid DNA (Lanes 1-3) was digested with restriction enzymes NcoI and XhoI and checked on a 1% (w/v) agarose gel containing EtBr.Lane M: Fermentas 100 bp DNA ladder marker

Figure 13 shows 3 positive clones (Lanes 1 to 3) carrying an insert with the correct size of approximately 1500 bp. DNA samples of positive clones were sequenced by MWG and matched with the flagellin sequence (NCBI Accession No.: M11332, Fig.10) by BLAST analysis. The sequences obtained after sequencing using the standard T7 primers showed that there were no mutations in the N-terminal part of the protein (data not shown). As the standard primers were not sufficient to obtain full-length sequences, internal primers were designed. The forward primer was designed according to the partial sequence and the pETBlue-2 DOWN primer was used as a reverse primer. The internal primers were then used for further sequencing of the samples.

Partial sequence of flagellin obtained after sequencing with standard T7 primers:

CCCCTCTAGACTTACAATTTCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGA
TCGGTACGGGCCTCTTCGCTATTACGCCAGCTTGCGAACGGTGGGTGCGCTGCAAGGCGA
TTAAGTTGGGTAACGCCAGGATTCTCCCAGTCACGACGTTGTAAAACGACGGCCAGCGAG
AGATCTTGATTGGCTAGCAGAATAATTTTGTTTAACTTTAAGAAGGAGATATACCATGGC
TCTGGGCACCGCTATCGAGCGTCTGTCTTCCGGTCTGCGTATCAACAGCGCGAAAGACGA
TGCGGCAGGTCAGGCGATTGCTAACCGTTTTACCGCGAACATCAAAGGTCTGACTCAGGC
TTCCCGTAACGCTAACGACGGTATCTCCATTGCGCAGACCACTGAAGGCGCGCTGAACGA
AATCAACAACAACCTGCAGCGTGTGCGTGAACTGGCGGTTCAGTCTGCTAACAGCACCAA
CTCCCAGTCTGACCTCGACTCCATCCAGGCTGAAATCACCCAGCGCCTGAACGAAATCGA
CCGTGTATCCGGCCAGACTCAGTTCAACGGCGTGAAAGTCCTGGCGCAGGACAACACCCT
GACCATCCAGGTTGGTGCCAACGACGGTGAAACTATCGATATCGATCTGAAGCAGATCAA
CTCTCAGACCCTGGGTCTGGATACGCTGAATGTGCAACAAAAATATAAGGTCAGCGATAC
GGCTGCAACTGTTACAGGATATGCCGATACTACGATTGCTTTAGACAATAGTACTTTTAA
AGCCTCGGCTACTGGTCTTGGTGGTACTGACCAGAAAATTGATGGCGATTTAAAATTTGA
TGATACGACTGGAAAATATTACGCCAAAGTTACCGTTACGGGGGGAACTGGTAAAGATGG
CTATTATGAAGTTTCCGTTGATAAGACGAACGGTGAGGTGACTCTTGCTGGCGGTGCGAC
TTCCCCGCTTACAGGTGGACTACCTGCGACAGCAACTGAGGATGTGAAAATGTACAAGTT
GCAAATGCTGATTTGACAGAGCT

Flagellin internal forward primer: GGTACTGACCAGAAAATTGATGGCG

The sequence used for design of the internal forward primer is underlined.

pETBlue-2 DOWN primer (bp 505-525 in the Novagen pETBlue-2 vector): GTTAAATTGCTAACGCAGTC

Flagellin protein sequence (*S. typhimurium*):

ALGTAIERLSSGLRINSAKDDAAGQAIANRFTANIKGLTQASRNANDGISIAQTTEGALN
EINNNLQRVRELAVQSANSTNSQSDLDSIQAEITQRLNEIDRVNGQTQFSGVKVLAQDNT
LTIQVGANDGETIDIDLKQINSQTLGLDTLNVQQKYKVSDTAATVTGYADTTIALDNSTF
KASATGLGGTDEKIDGDLKFDDTTGKYYAKVTVTGGTGKDGYYEVSVDKTNGEVTLAAVT
PATVTTATALSGKMYSANPDSDIAKAALTAAGVTGTASVVKMSYTDNNGKTIDGGLAVKV
GDDYYSATQDKDGSISIDTTKYTADNGTSKTALNKLGGADGKTEVVTIDGKTYNASKAAG
HDFKAEPELAEQAAKTTENPLQKIDAALAQVDTLRSDLGAVQNRFNSAITNLGNTVNNLS
SARSRIEDSDYATEVSNMSRAQILQQAGTSVLAQANQVPQNVLSLLR-

Sequencing resulted in the following translated sequences of each clone:

Clone 1 translated sequence:

MALGTAIERLSSGLRINSAKDDAAGQAIANRFTANIKGLTQASRNANDGISIAQTTEGALNEINNNLQRVRELAV
QSANSTNSQSDLDSIQAEITQRLNEIDRVSGQTQFNGVKVLAQDNTLTIQVGANDGETIDIDLKQINSQTLGLDT
LNVQQKYKVSDTAATVTGYADTTIALDNSTFKASATGLGGTDQKIDGDLKFDDTTGKYYAKVTVTGGTGKDGYYE
VSVDKTNGEVTLAGGATSPLTGGLPATATEDVKNVQVANADLTEAKAALTAAGVTGTASVVKMSYTDNNGKTIDG
GLAVKVGDDYYSATQNKDGSISINTTKYTADDGTSKTALNKLGGADGKTEVVSIGGKTYAASKAEGHNFKAQPDL
AEAAATTTENPLQKIDAALAQVDTLRSDLGAVQNRFNSAITNLGNTVNNLTSARSRIEDSDYATEVSNMSRAQIL
QQAGTSVLAQANQVPQTSSLYCVSSTTTTTTNVN-

Clone 2 translated sequence:

MALGTAIERLSSGLRINSAKDDAAGQAIANRFTANIKGLTQASRNANDGISIAQTTEGALNEINNNLQRVRELAV
QSANSTNSQSDLDSIQAEITQRLNEIDRVSGQTQFNGVKVLAQDNTLTIQVGANDGETIDIDLKQINSQTLGLDT
LNVQQKYKVSDTAATVTGYADTTIALDNSTFKASATGLGGTDQKIDGDLKFDDTTGKYYAKVTVTGGTGKDGYYE
VSVDKTNGEVTLAGGATSPLTGGLPATATEDVKNVQVANADLTEAKAALTAAGVTGTASVVKMSYTDNNGKTIDG
GLAVKVGDDYYSATQNKDGSISINTTKYTADDGTSKTALNKLGGADGKTEVVSIGGKTYAASKAEGHNFKAQPDL
AEAAATTTENPLQKIDAALAQVDTLRSDLGAVQNRFNSAITNLGNTVNNLTSARSRIEDSDYATEVSNMSRAQIL
QQAGTSVLAQANQVPQTSSLTASRAPPPPPLMLIKLGVVIIVIINTPDCVSNLTVINYRIKAIR-

Clone 3 translated sequence:

MALGTAIERLSSGLRINSAKDDAAGQAIANRFTANIKGLTQASRNANDGISIAQTTEGALNEINNNLQRVRELAV
QSANSTNSQSDLDSIQAEITQRLNEIDRVSGQTQFNGVKVLAQDNTLTIQVGANDGETIDIDLKQINSQTLGLDT
LNVQQKYKVSDTAATVTGYADTTIALDNSTFKASATGLGGTDQKIDGDLKFDDTTGKYYAKVTVTGGTGKDGYYE
VSVDKTNGEVTLAGGATSPLTGGLPATATEDVKNVQVANADLTEAKAALTAAGVTGTASVVKMSYTDNNGKTIDG
GLAVKVGDDYYSATQNKDGSISINTTKYTADDGTSKTALNKLGGADGKTEVVSIGGKTYAASKAEGHNFKAQPDL
AEAAATTTENPLQKIDAALAQVDTLRSDLGAVQNRFNSAITNLGNTVNNLTSARSRIEDSDYATEVSNMSRAQIL
QQAGTSVLAQANQVPQNVLSLLRLEHHHHHH-

The amino acid sequence of each clone was compared with the amino acid sequence of flagellin from *S. typhimurium* (NCBI Accession No.: AAA27072;[40] excluding the signal peptide) using the NCBI protein BLAST tool (Fig. 14).

```
 Score =  802 bits (2071),  Expect = 0.0
 Identities = 427/465 (91%), Positives = 440/465 (94%), Gaps = 5/465 (1%)

Query  1    ALGTAIERLSSGLRINSAKDDAAGQAIANRFTANIKGLTQASRNANDGISIAQTTEGALN  60
            ALGTAIERLSSGLRINSAKDDAAGQAIANRFTANIKGLTQASRNANDGISIAQTTEGALN
Sbjct  2    ALGTAIERLSSGLRINSAKDDAAGQAIANRFTANIKGLTQASRNANDGISIAQTTEGALN  61

Query  61   EINNNLQRVRELAVQSANSTNSQSDLDSIQAEITQRLNEIDRVNGQTQFSGVKVLAQDNT  120
            EINNNLQRVRELAVQSANSTNSQSDLDSIQAEITQRLNEIDRV+GQTQF+GVKVLAQDNT
Sbjct  62   EINNNLQRVRELAVQSANSTNSQSDLDSIQAEITQRLNEIDRVSGQTQFNGVKVLAQDNT  121

Query  121  LTIQVGANDGETIDIDLKQINSQTLGLDTLNVQQKYKVSDTAATVTGYADTTIALDNSTF  180
            LTIQVGANDGETIDIDLKQINSQTLGLDTLNVQQKYKVSDTAATVTGYADTTIALDNSTF
Sbjct  122  LTIQVGANDGETIDIDLKQINSQTLGLDTLNVQQKYKVSDTAATVTGYADTTIALDNSTF  181

Query  181  KASATGLGGTDEKIDGDLKFDDTTGKYYAKVTVTGGTGKDGYYEVSVDKTNGEVTLA--A  238
            KASATGLGGTD+KIDGDLKFDDTTGKYYAKVTVTGGTGKDGYYEVSVDKTNGEVTLA  A
Sbjct  182  KASATGLGGTDQKIDGDLKFDDTTGKYYAKVTVTGGTGKDGYYEVSVDKTNGEVTLAGGA  241

Query  239  VTPAT---VTTATALSGKMYSANPDSDIAKAALTAAGVTGTASVVKMSYTDNNGKTIDGG  295
             +P T     TAT     +  AN D   AKAALTAAGVTGTASVVKMSYTDNNGKTIDGG
Sbjct  242  TSPLTGGLPATATEDVKNVQVANADLTEAKAALTAAGVTGTASVVKMSYTDNNGKTIDGG  301

Query  296  LAVKVGDDYYSATQDKDGSISIDTTKYTADNGTSKTALNKLGGADGKTEVVTIDGKTYNA  355
            LAVKVGDDYYSATQ+KDGSISI+TTKYTAD+GTSKTALNKLGGADGKTEVV+I GKTY A
Sbjct  302  LAVKVGDDYYSATQNKDGSISINTTKYTADDGTSKTALNKLGGADGKTEVVSIGGKTYAA  361

Query  356  SKAAGHDFKAEPELAEQAAKTTENPLQKIDAALAQVDTLRSDLGAVQNRFNSAITNLGNT  415
            SKA GH+FKA+P+LAE AA TTENPLQKIDAALAQVDTLRSDLGAVQNRFNSAITNLGNT
Sbjct  362  SKAEGHNFKAQPDLAEAAATTTENPLQKIDAALAQVDTLRSDLGAVQNRFNSAITNLGNT  421

Query  416  VNNLSSARSRIEDSDYATEVSNMSRAQILQQAGTSVLAQANQVPQ  460
            VNNL+SARSRIEDSDYATEVSNMSRAQILQQAGTSVLAQANQVPQ
Sbjct  422  VNNLTSARSRIEDSDYATEVSNMSRAQILQQAGTSVLAQANQVPQ  466
```

Figure 14:Amino acid sequence comparison of the cloned flagellin (Sbjct) and the published protein(NCBI Accession No.: AAA27072;[40];Query) by protein BLAST: One representative example is shown.

All 3 clones contained mutations mainly in the central region of the protein at amino acids 236 to 267 (Fig.14). The N- and the C-terminal regions of the protein contained hardly any mutations. As the same mutations were detected in all 3 clones, we assumed that the model sequence of flagellin was taken from another isotype of *S. typhimurium*. This assumption was further supported by the fact that the central region of flagellin is known to be highly diverse amongst different bacterial species whereas the N- and C-terminal regions are highly conserved. Therefore, we used clone 3 for further experiments and kept frozen bacterial stocks of the other clones.

7.5 Protein expression and purification of recombinant flagellin from *S. typhimurium*

7.5.1 Test expression of flagellin from *S. typhimurium*

The vector containing the correct insert was transformed into *E.coli* BL21(DE3)pLysS cells and protein test expression of flagellin from *S. typhimurium* over a time period of 6 hours was performed. Expression was induced with 0.4 mM IPTG and protein expression over the investigated time course was checked by SDS-PAGE and staining with Coomassie Brilliant Blue.

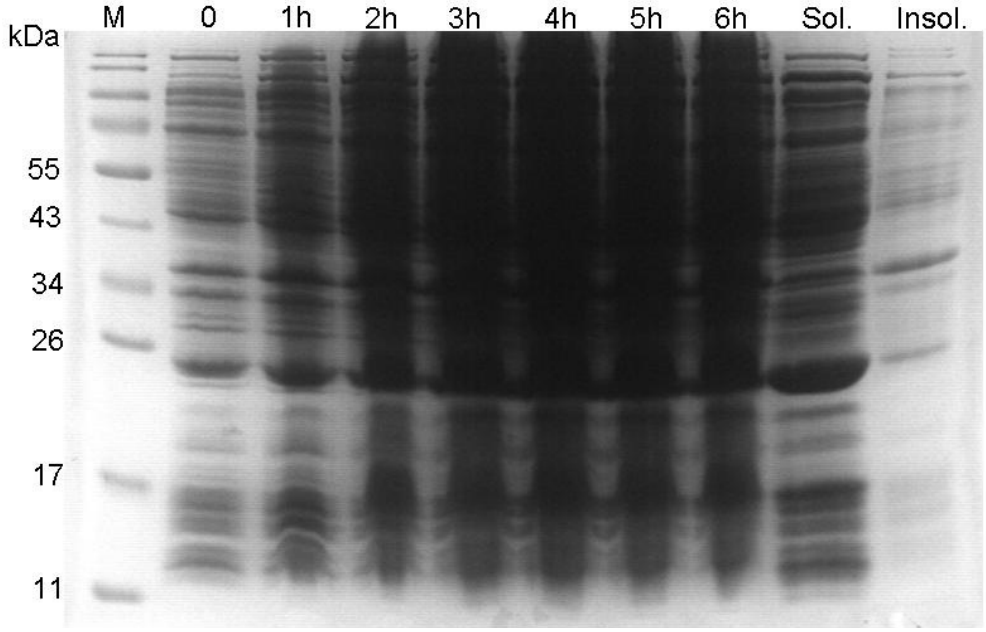

Figure 15: Test expression of flagellin from *S. typhimurium*: Samples were collected at different timepoints (Lanes 0–6h) after induction with IPTG and also samples representing the solubly expressed (Lane Sol.) and the insolubly expressed (Lane Insol.) protein fractions were collected. All samples were separated by SDS-PAGE (15%) and the gel was stained with Coomassie Brilliant Blue to detect proteins. Lane M: Fermentas 8-16% Tris-glycerine SDS-PAGE marker

The calculated size of recombinant flagellin including the His-tag was 49.9 kDa. Figure 8 shows that the protein expression increased over the timecourse of 6 hours (Lanes 1h to 6h) as compared to the uninduced stage (Lane 0). Several proteins of lower as well as of higher molecular weight were detected. Prominent bands appeared indicating proteins of a molecular weight of approximately 25 kDa and 37 kDa. These proteins represented putative degraded products of flagellin. A band in the range of 49 kDa was visible in all lanes except for the insoluble protein fraction (Lane Insol.). This band probably resulted from the recombinant

flagellin protein. The majority of the proteins seemed to be solubly expressed as there were more intense bands in this fraction (Lane Sol.) as compared to the insolubly expressed protein fraction (Lane Insol.).

The expression of His-tagged proteins over the time period of 6 hours was analysed by immunoblotting. Samples separated by SDS-PAGE were transferred onto nitrocellulose. After saturation of non-specific binding sites the sheet was incubated with a mouse anti Penta-His™ antibody. Detection was achieved by a secondary antibody, that was HRP-linked and the Lumigen™ detection reagent.

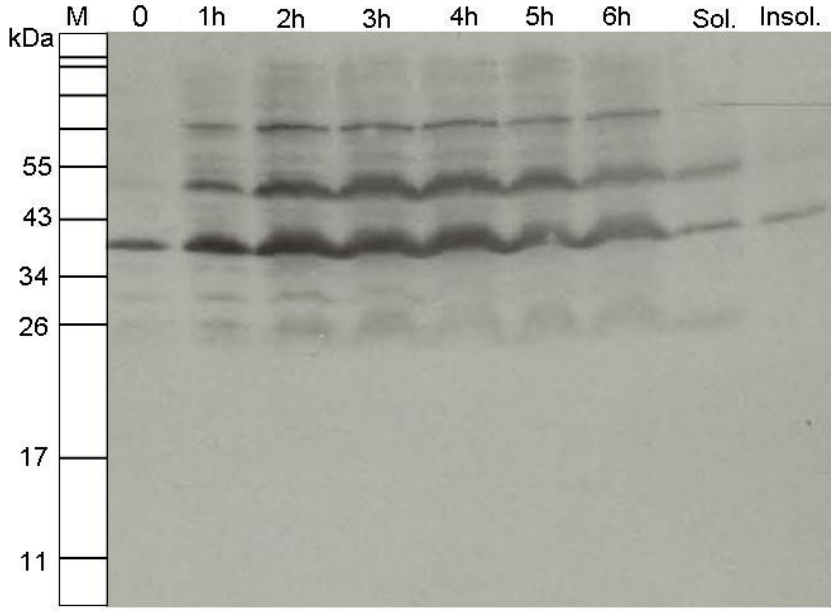

Figure 16: Immunoblot of the recombinant flagellin protein from *S. typhimurium:* The same samples (Lanes 0-Insol.) as described for Fig.8 were separated by SDS-PAGE (15%), blotted onto a nitrocellulose membrane and finally, detected with the Lumigen™ detection reagent and developed on a hyperfilm. Lane M: Fermentas 8-16% Tris-glycerine SDS-PAGE marker

Figure 16 shows that His-tagged proteins of 37, 50 and 72 kDa, respectively, were expressed. Interestingly, optimum expression was found 4 hours (Lane 4h) after induction. Longer induction seemed to lead to degradation of the proteins (Lanes 5h and 6h). The expected protein had a calculated size of 49.9 kDa and we were able to detect a protein at the approximate size of 50 kDa after induction with IPTG (Lanes 1h to 6h). This protein was also present in the soluble protein fraction (Lane Sol.) but not in the insolubly expressed protein fraction (Lane Insol.). Bands appeared in all fractions excluding the insolubly expressed protein fraction at approximately 37 kDa probably resulting from degradations. Another

prominent band was detected at 72 kDa, which can not result from dimers or multimers of the protein as flagellin has a calculated size of 49.9 kDa.

To reduce the expression of other proteins than flagellin or to reduce degradations of the protein, we tried expression under salt stress as well as expression at 16°C.

7.5.2 Test expression of flagellin from *S. typhimurium* under salt stress

Protein expression under salt stress is one approach to ensure that only essential proteins are expressed. As a halophilic environment (mimicked *in vitro* by salt stress) means harsh conditions for *E.coli* cells, these cells will only synthesize essential proteins or proteins that are under the control of strong promoters.

We performed salt stress by addition of 10 mM betaine, 0.5 M sorbitol and 4% NaCl prior to induction with 0.4 mM IPTG. Finally, we checked protein expression over the investigated time course of expression by SDS-PAGE and staining with Coomassie Brilliant Blue.

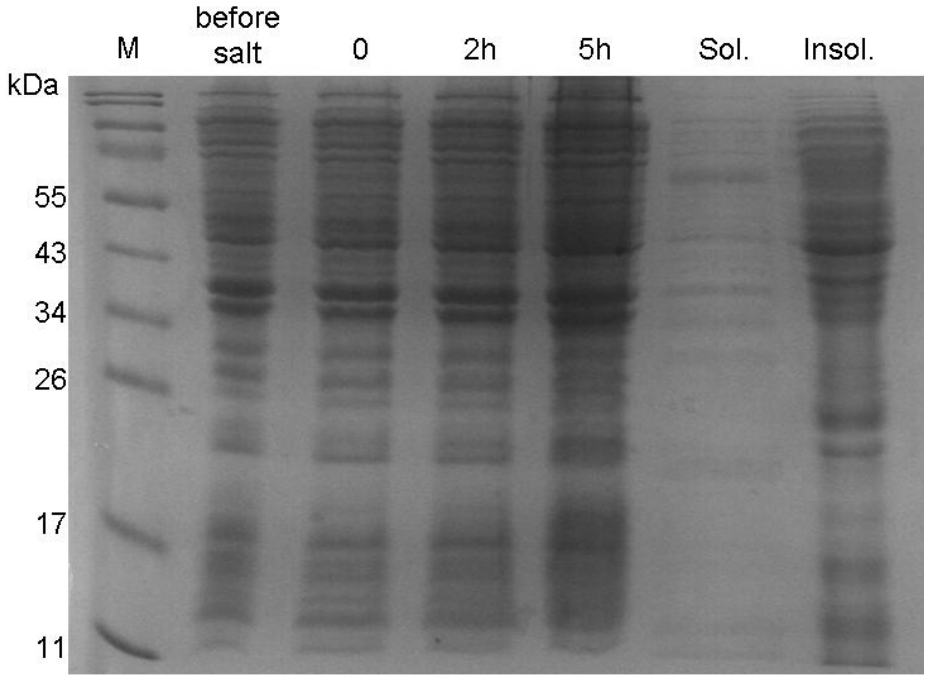

Figure 17: Test expression of recombinant flagellin from *S. typhimurium* under salt stress conditions: Samples were collected at indicated timepoints of expression (Lanes before salt–5h) and also samples representing the solubly expressed (Lane Sol.) and the insolubly expressed (Lane Insol.) protein fractions were collected. All samples were separated by SDS-PAGE (15%) and stained with Coomassie Brilliant Blue. Lane M: Fermentas 8-16% Tris-glycerine SDS-PAGE marker

The results in Figure 10 indicate that protein expression under salt stress did not sufficiently decrease the amount of undesired expressed proteins. Again, prominent bands with approximately 37 kDa were detected over the investigated time period (Lanes before salt – 5h) but neither in the solubly (Lane Sol.) nor in the insolubly (Lane Insol.) expressed protein

fractions. In addition, a protein of the expected size of 49.9 kDa was detected (Lanes before salt – 5h). The expression of this protein was slightly increased after induction with IPTG (Lanes 2h and 5h) compared to the uninduced state (Lane 0). This protein seemed to be expressed in inclusion bodies, as it was only detectable in the insolubly expressed (Lane Insol.) protein fraction.

As compared to expression under standard conditions (Fig.15), expression under salt stress resulted in a decreased expression of proteins with a higher molecular weight.

7.5.3 Test expression of flagellin from *S. typhimurium* at 16°C

Protein expression at lower temperatures decreases the rate of protein expression. This decreased rate of protein synthesis results in an enhanced capacity to fold proteins correctly.

We induced protein expression by addition of 0.4 mM IPTG and incubated the cells at 16°C as *E. coli* cells still grow and express proteins at this temperature. Finally, we checked protein expression over the investigated time course of expression by SDS-PAGE and staining with Coomassie Brilliant Blue.

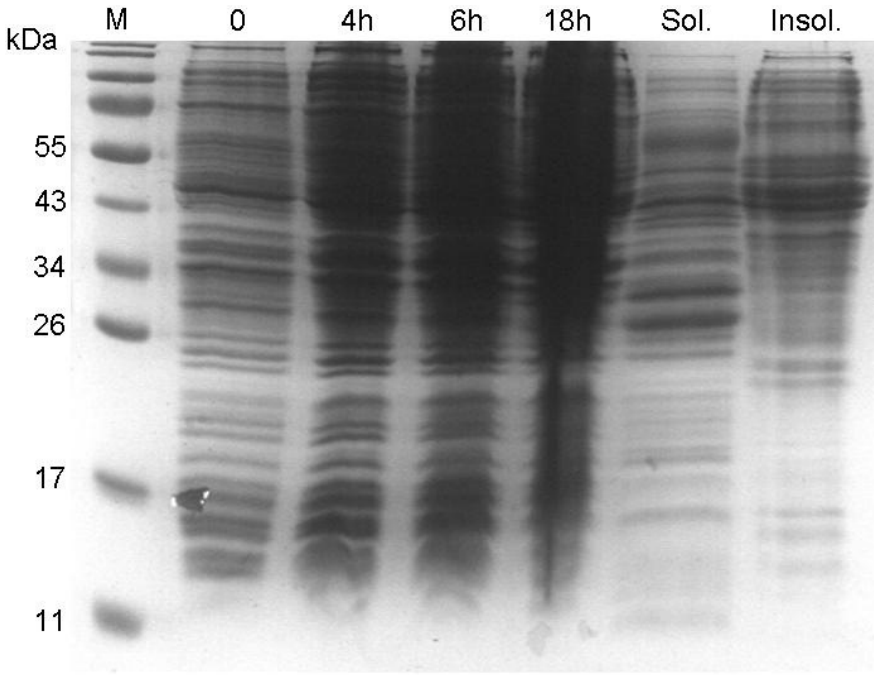

Figure 18: Test expression of recombinant flagellin from *S. typhimurium* at 16°C: Samples were collected before induction and after different timepoints of expression (Lanes Unind.–18h). Samples representing the solubly expressed (Lane Sol.) and the insolubly expressed (Lane Insol.) protein fractions were collected too. All samples were separated by SDS-PAGE (15%) and stained with Coomassie Brilliant Blue. Lane M: Fermentas 8-16% Tris-glycerine SDS-PAGE marker

Figure 18 shows a high number of bands in all lanes. The expression of some proteins only slightly increased over time. A band indicating a protein of a molecular weight of 49.9 kDa

was detected (Lanes Unind. to 18h). However, the expression of this protein (Lanes 4h to 18h) did not increase compared to the uninduced sample (Lane Unind.). The protein was detected in the solubly expressed (Lane Sol.) and in the insolubly expressed (Lane Insol.) protein fraction. The most prominent bands appeared in the soluble protein fraction at a size of 26 and 30 kDa (Lane Sol.).

As neither expression under salt stress nor expression at 16°C led to improved expression of the flagellin protein we decided to skip Immunoblotting and continue with a large scale protein expression under standard conditions to purify the protein.

7.5.4 Large scale expression of flagellin from *S. typhimurium* and protein purification by immobilized metal-ion affinity chromatography

Protein expression was performed under standard conditions in 1000 mL of culture and His-tagged proteins were purified by immobilized metal-ion affinity chromatography (IMAC) which is based on the covalent binding of amino acids to metals. In our case the expressed flagellin carried a histidine-tag. Therefore, we used GE Healthcare His-Trap FF crude 1mL columns where the histidine residues are bound to nickel during the loading step. Thereafter, bound proteins can be eluted by an increasing gradient of elution buffer containing imidazole. Imidazole competes with His-tagged proteins for nickel binding.

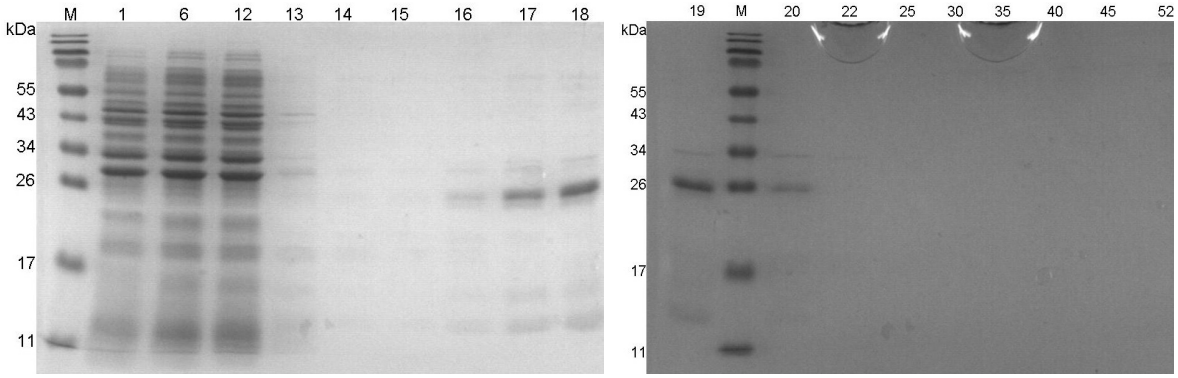

Figure 19: Purification of the recombinant flagellin protein by IMAC: Samples (Lanes 1-18 and Lanes 19-52) were separated by SDS-PAGE (15%) and stained with Coomassie Brilliant Blue Lanes 1-12: flow-through Lane M: Fermentas 8-16% SDS marker

Figure 19 shows that protein purification by IMAC did not lead to purification of a protein of the expected size of approximately 50 kDa. Instead, one prominent band at a size of around 26 kDa was detected in the fractions 17, 18, 19 and 20. These fractions also contained smaller proteins that might result from degradation of the protein. Bands around 50 kDa were found in fractions 1, 6 and 12 indicating that the sample contained a protein of the expected size but

either in too small amounts to be detectable after purification or the protein did not bind to the column and could therefore not be purified.

7.6 Testing the ability of *E. coli* lysates to activate TLR5

Although the purification of recombinant flagellin from *E.coli* BL21(DE3)pLysS cells did not work, we tested crude bacterial lysates of cells that had been transformed with the vector containing the flagellin insert for their ability to activate HEK-293/TLR5 cells. HEK-293/TLR5 cells respond to stimulation with flagellin by production of IL-8. The cytokine concentration was determined by ELISA.

Standard protein expression was performed as described. The solubly expressed protein fraction was used for stimulation of HEK-293/TLR5 cells. "Empty" *E.coli* BL21(DE3)pLysS cells transformed with a pETBlue-2 vector without insert served as a control. Purchased flagellin (Invitrogen GmbH) served as a positive control and LPS was used as a negative control.

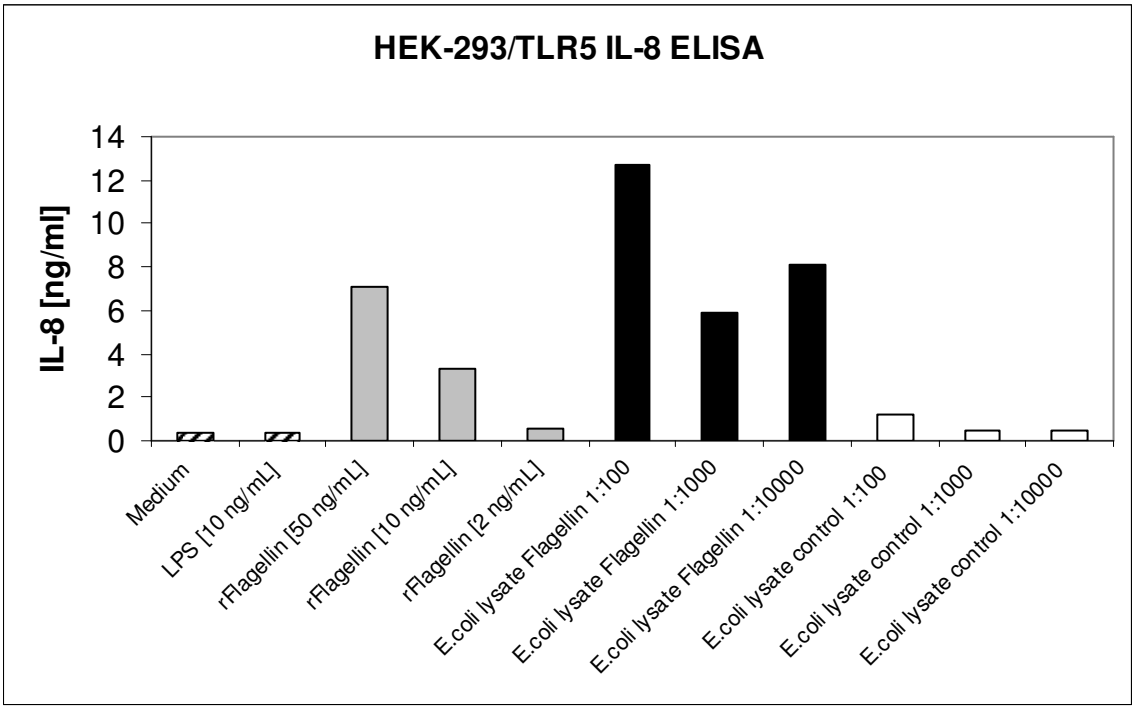

Figure 20: IL-8 production of HEK-293/TLR5 cells in response to *E.coli* lysates: HEK-293/TLR5 cells were seeded in a flatbottomed 96 well plate (40000 cells/well) and incubated for 24 hours. Medium was removed and replaced by medium containing specific stimuli (medium, LPS: hatched bars; purchased flagellin: grey bars; different concentrations of lysate of bacteria transformed with vector containing flagellin: black bars; control lysates of bacteria transformed with the vector alone: white bars). After 20 hours IL-8 levels in the supernatants were determined by ELISA.

Figure 20 shows that neither medium alone nor stimulation with LPS, a TLR4 ligand (hatched bars) induced IL-8 production of HEK-293/TLR5 cells. Stimulation with different concentrations of recombinant flagellin (grey bars) resulted in IL-8 synthesis in a dose dependent manner. The lysate of bacteria containing the vector with the flagellin insert (filled bars) induced strong IL-8 production after stimulation of HEK-293/TLR5 cells whereas the control lysate from bacteria containing an empty vector (empty bars) did not. We concluded that flagellin was expressed and had the capacity to trigger TLR5 stimulation. The levels of the expressed protein were possibly too low to allow purification of the protein by IMAC.

7.7 Amplification of DNA encoding the C- or N-terminal fragments of flagellin from *S. typhimurium* and cloning into pHis parallel 2 vector

As our attempts did not lead to successful high level expression of the full-length flagellin protein we decided to individually express the conserved regions of the protein. For this purpose we cloned the flagellin C-terminus as well as the flagellin N-terminus into the pHis parallel 2 vector and separately expressed both proteins in *E.coli* BL21(DE3)pLysS cells.
The DNA coding for the N- or the C-terminal fragment of the flagellin protein, which was synthetically produced, was used as a template for amplification by PCR.
Primers were designed including restriction sites for NcoI and XhoI:

Primers:

Flag C forward: TATA**CCATGG**GAACCGAAAACCCGCTGCAGAAA

Flag C reverse: TATA**CTCGAG**TTAACGCAGTAAAGAGAGGACG

Sequences homologous to the C-terminal fragment of the flagellin protein DNA sequence are underlined and sites for enzymatic digest with restriction enzymes are indicated in bold.

Flag N forward: TATA**CCATGG**CTCTGGGCACCGCTATCGA

Flag N reverse: TATA**CTCGAG**TTATTGCACATTCAGCGTATCCAGACC

Sequences homologous to the N-terminal fragment of the flagellin protein DNA sequence are underlined and sites for enzymatic digestion with restriction enzymes are indicated in bold.

The C-terminal sequence (277bp; bp 1209 – 1485 in the full-length sequence of flagellin, Accession No.: M11332) of the flagellin protein from *S.typhimurium*:

gaccgaaaa cccgctgcag aaaattgatg ctgctttggc acaggttgac
acgttacgtt ctgacctggg tgcggtacag aaccgtttca actccgctat taccaacctg
ggcaacaccg taaacaacct gacttctgcc cgtagccgta tcgaagattc cgactacgcg
accgaagttt ccaacatgtc tcgcgcgcag attctgcagc aggccggtac ctccgttctg
gcgcaggcga accaggttcc gcaaaacgtc ctctctttac tgcgttaa

The N-terminal Sequence (458bp; bp 83 – 540 in the full-length sequence of flagellin, Accession No.: M11332) of the flagellin protein from *S.typhimurium*:

ctctgggcac cgctatcgag cgtctgtctt ccggtctgcg tatcaacagc
gcgaaagacg atgcggcagg tcaggcgatt gctaaccgtt ttaccgcgaa catcaaaggt
ctgactcagg cttcccgtaa cgctaacgac ggtatctcca ttgcgcagac cactgaaggc
gcgctgaacg aaatcaacaa caacctgcag cgtgtgcgtg aactggcggt tcagtctgct
aacagcacca actcccagtc tgacctcgac tccatccagg ctgaaatcac ccagcgcctg
aacgaaatcg accgtgtatc cggccagact cagttcaacg gcgtgaaagt cctggcgcag
gacaacaccc tgaccatcca ggttggtgcc aacgacggtg aaactatcga tatcgatctg
aagcagatca actctcagac cctgggtctg gatacgctga atgtgcaa

PCR-amplified products were checked on a 1% (w/v) agarose gel containing EtBr and bands indicating the DNA fragment of the correct size were excised under UV light. Bands were excised from the gel and digested with the restriction enzymes NcoI and XhoI. In parallel, the pHis parallel 2 vector was digested with NcoI and XhoI and dephosphorylated with CIAP prior to ligation. The vector containing the insert was then transformed in *E.coli* TOP10 cells. Successful ligation was checked by PCR screening of grown colonies. PCR products were checked by DNA gel electrophoresis.

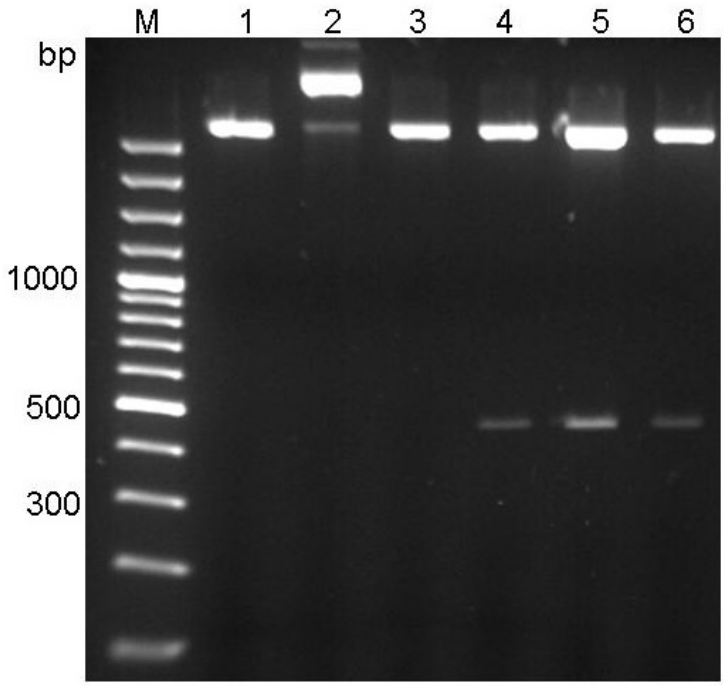

Figure 21: Test digestion of vectors containing the C- and N-terminal fragments of flagellin from *S. typhimurium*: The C-terminal (Lanes 1–3) and the N-terminal (Lanes 4–6) DNA samples were separated on a 1% (w/v) agarose gel containing EtBr. Lane M: gene ruler 100 bp DNA ladder marker

Figure 21 shows that three colonies transformed with the vector containing the C-terminal insert (Lanes 1 – 3) were negative as no bands appeared in the expected size of approximately 300 bp. Lane 4 – 6 show bands of approximately 500 bp indicating that the N-terminal fragment of flagellin was ligated successfully into the pHis parallel 2 vector.

As no amplified products were found for the C-terminal fragment the colony screening was repeated with additional colonies.

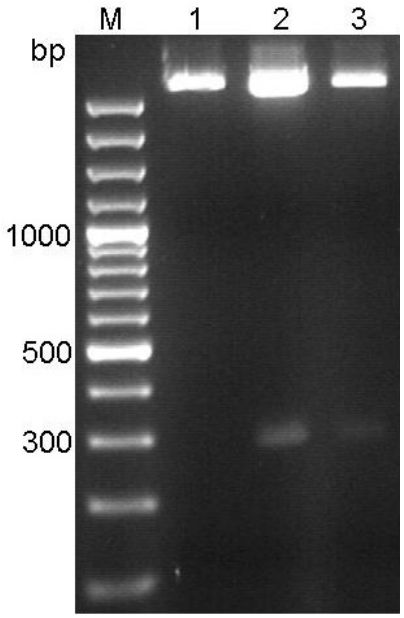

Figure 22: Test digestion of the vector containing the C-terminal fragment of flagellin from *S. typhimurium*: Samples (Lanes 1-3) were separated on a 1% (w/v) agarose gel containing EtBr Lane M: gene ruler 100 bp DNA ladder marker

Figure 22 shows that two out of three screened colonies carried a vector containing an insert of approximately 300 bp (Lanes 2 and 3) indicating that the C-terminal fragment of flagellin was successfully ligated into the vector and transformation into *E.coli* TOP10 cells had worked. Sample 1 did not show any bands at the expected size.

All positive clones for the C- and N-terminal fragments of flagellin were sequenced and compared to the original sequences by BLAST analysis. Sequence comparison showed that the inserts displayed no mutations (data not shown). Subsequently, we continued with the expression of the C- and N-terminal fragments of flagellin as proteins.

7.8 Protein expression and purification of the C- and N-terminal fragments of flagellin from *S. typhimurium*

As a first step, *E.coli* BL21(DE3)pLysS cells were transformed with the vectors carrying the C- or the N-terminal fragment of flagellin, respectively. After transformation, a protein test expression was performed.

7.8.1 Test expression of the C-terminal fragment of flagellin from *S. typhimurium*

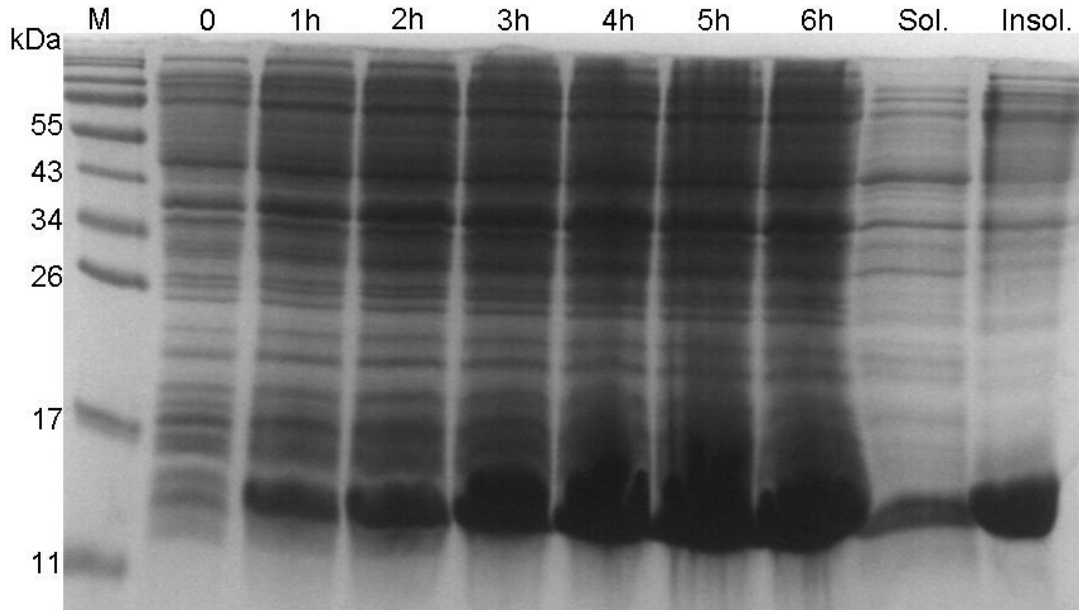

Figure 23: Test expression of the C-terminal fragment of flagellin from *S. typhimurium*: Samples collected over the timecourse of expression (Lanes 0-6h) and samples containing the solubly expressed protein fraction (Lane Sol.) and the insolubly expressed protein fraction (Lane Insol.) were separated by SDS-PAGE (15%) and stained with Coomassie Brilliant Blue Lane M: Fermentas 8-16% Tris-glycerine SDS-PAGE marker

Figure 23 shows the expression of a protein in the range of 11 - 17 kDa over the studied time period of 6 hours (Lanes 1h to 6h). This expression product perfectly matched a calculated size of 13.1 kDa of the C-terminus of flagellin including the His-tag. The amount of the expressed protein increased over time. Both, the soluble and the insoluble protein fraction contained the protein, even though the major part of the protein seemed to be insolubly expressed (Lane Insol.).

In addition, several bands indicating proteins of higher molecular weight in all lanes were visible. However, expression of these proteins did not increase after induction with IPTG.

An Immunoblot was performed using a detection system specific for the His-tag to assess, whether the massively expressed protein detectable in the Coomassie stained gel indeed was the protein of interest.

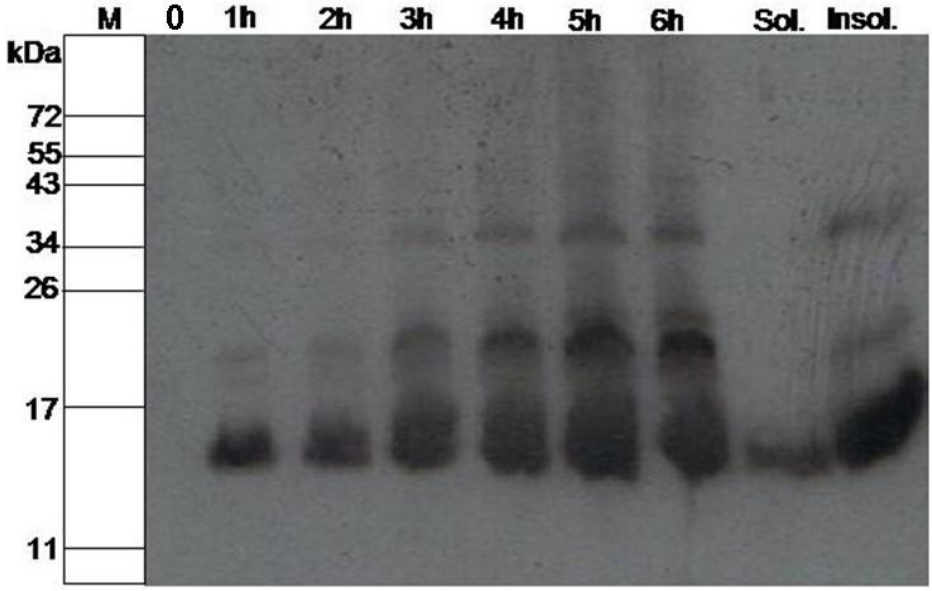

Figure 24: Immunoblot of the C-terminal fragment of flagellin from *S. typhimurium*: The same samples (Lanes 0–Insol.) as described for Fig.17 were separated by SDS-PAGE (15%) and transferred onto a nitrocellulose membrane. After saturation and washing, a Penta-His™ antibody was added. A HRP-linked antibody served as a secondary antibody and the Lumigen™ detection reagent was used in combination with a hyperfilm to detect proteins carrying the His-tag. Lane M: Fermentas 8-16% Tris-glycerine SDS-PAGE marker

Figure 24 shows His-tagged proteins of around 13.1 kDa in all lanes except for Lane 0 (uninduced sample). This observation indicated that the C-terminal fragment was not expressed in high amounts as long as the lac repressor remained active. Upon induction with IPTG the protein was strongly expressed. Immunoblotting confirmed the result obtained by Coomassie staining of the test expression (Fig. 23). However, His-tagged proteins of a molecular weight of 23 kDa and 38 kDa (Lanes 3h to 6h and Lane Insol.) were also detected. These bands cannot represent dimers or trimers of the C-terminal fragment of flagellin. The solubly expressed protein fraction (Lane Sol.) exclusively contained the protein of the expected size of 13.1 kDa.

As optimum protein expression was observed after 5 hours (Fig. 23 & Fig.24) a period of 5 hours was chosen for protein expression of the C-terminal fragment of flagellin.

7.8.2 Test expression of the N-terminal fragment of flagellin from *S. typhimurium*

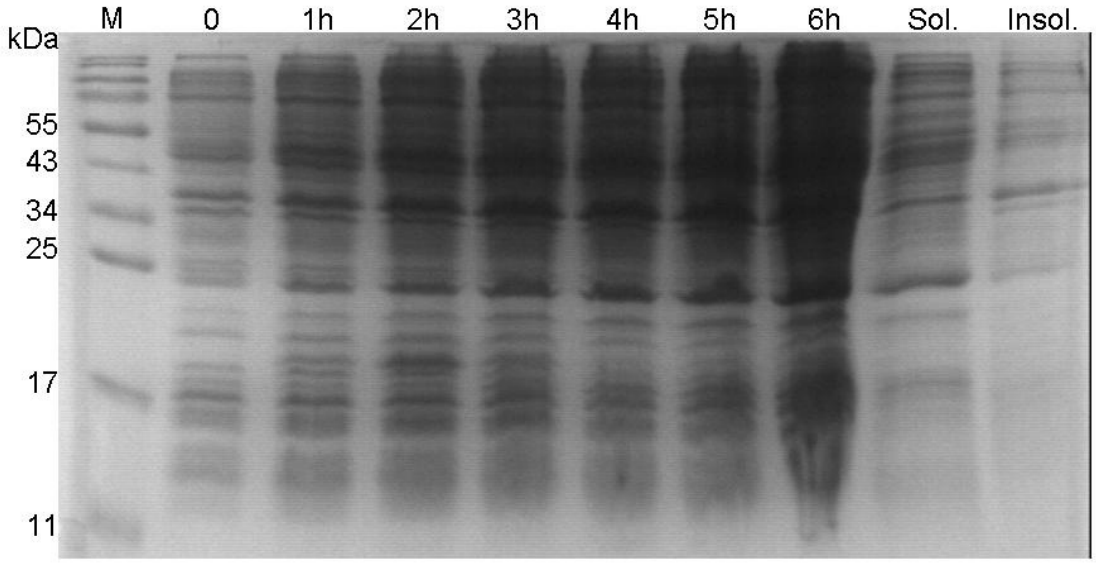

Figure 25: Test expression of the N-terminal fragment of flagellin from *S. typhimurium*: Samples were collected over a time period of 6 hours (Lanes 0-6h) and separated by SDS-PAGE (15%) together with samples containing the solubly expressed protein fraction (Lane Sol.) and the insolubly expressed protein fraction (Lane Insol.). The gel was stained with Coomassie Brilliant Blue Lane M: Fermentas 8-16% Tris-glycerine SDS-PAGE marker

The calculated size for the N-terminal fragment including the His-tag was 18.8 kDa.
Figure 18 shows a weak band in the range of 20 kDa after 1 to 3h of expression (Lanes 1h to 3h) which disappeared after longer expression periods (Lanes 4h to 6h) and was also not detectable in the soluble (Lane sol.) and insoluble protein fraction (Lane insol.). Additional bands around 25 kDa and approximately 40 kDa were visible and seemed to increase in their intensity over the timecourse of expression. These bands were also detected in the soluble and the insoluble protein fraction (Lane Sol. & Lane Insol.).

To analyse whether the weak bands of approximately 20 kDa represented the protein of interest we performed an Immunoblot to specifically detect His-tagged proteins.

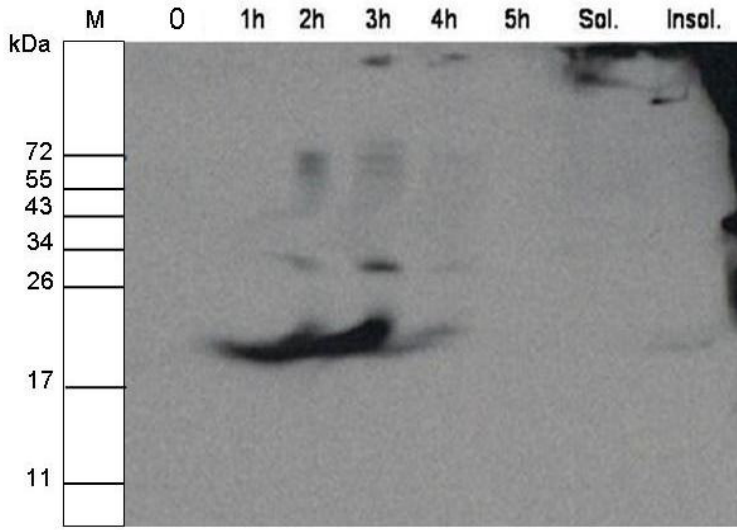

Figure 26: Immunoblot of the N-terminal fragment of flagellin from *S. typhimurium*: The same samples (Lanes 0–Insol.) as described in Fig.17 except for the 6h sample were separated by SDS-PAGE (15%) and transferred onto a nitrocellulose membrane. After saturation and washing a Penta-His™ antibody was added. A HRP-linked antibody served as a secondary antibody and the Lumigen™ detection reagent was used in combination with a hyperfilm to detect proteins carrying the His-Tag. Lane M: Fermentas 8-16% Tris-glycerine SDS-PAGE marker

Figure 26 shows that a protein of approximately 19 kDa (Lanes 1h to 3h) appeared and was totally degraded after 3 hours of induction and therefore could not be detected by immunoblotting either at later time points (Lanes 4h and 5h) or in the soluble protein fraction (Lane Sol.). The insoluble fraction (Lane Insol.) shows a very weak band which is much weaker compared to the intense bands in Lanes 2h and 3h. Lanes 2h and 3h also show bands of weaker intensity of approximately 30 kDa and also of 72 kDa.

Western Blotting confirmed the results obtained by Coomassie staining. A time period of 2 hours was chosen to express the N-terminal fragment of flagellin from *S. typhimurium*.

Thereafter, a large scale protein expression was performed and proteins were purified by immobilized metal ion affinity chromatography as described before.

7.8.3 Large scale expression of the C- and the N-terminal fragments of flagellin from *S. typhimurium* and protein purification by immobilized metal-ion affinity chromatography

7.8.3.1 Protein purification of the C-terminal fragment of flagellin by IMAC

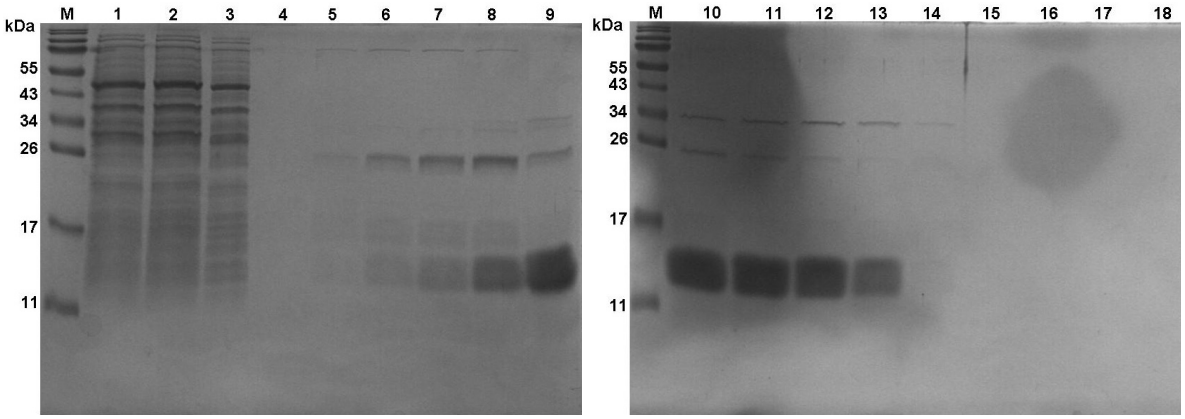

Figure 27: Purification of the C-terminal fragment of flagellin by IMAC: Samples (Lanes 1-9 and Lanes 10-18) were separated by SDS-PAGE (15%) and stained with Coomassie Brilliant Blue Lanes 1-3: flow-through Lane M: Fermentas 8-16% Tris-glycerine SDS-PAGE marker

Figure 27 shows strong protein expression in different fractions (Lanes 9 to 13) indicating that the C-terminal fragment of flagellin with a calculated molecular weight of 13.1 kDa was purified by chromatography. The fractions showing the flow-through (Lanes 1 to 3) contained no bands at the expected size of 13.1 kDa. This confirms that the protein containing the His-tag had bound efficiently to the column. The band at 26 kDa (Lanes 5 to 12) presumably resulted from a dimer of the protein. Lanes 10 to 13 contained bands at a size of approximately 36 kDa that might result from a trimer of the protein. Fractions 14 to 18 contained no bands which proved that the proteins bound to the column were washed out at lower concentrations of imidazole.

Fractions 9 to 13 were pooled for dialysis of the protein.

7.8.3.2 Protein purification of the N-terminal fragment of flagellin by IMAC

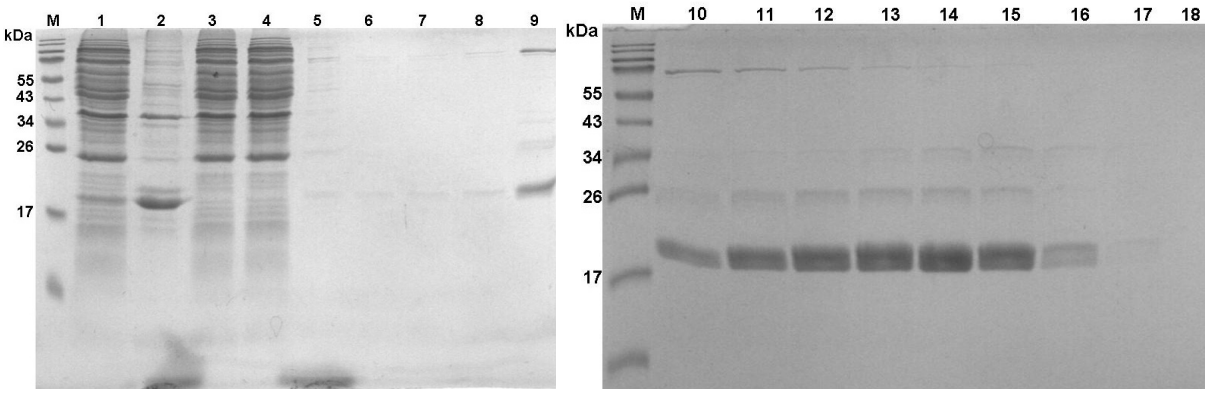

Figure 28: Purification of the N-terminal fragment of flagellin by IMAC: Samples (Lanes 1–9) were separated by SDS-PAGE (15%) and stained with Coomassie Brilliant Blue Lanes 1-4: flow-through Lane M: Fermentas 8-16% Tris-glycerine SDS-PAGE marker

Figure 28 shows that the N-terminal fragment of flagellin from *S. typhimurium* was purified by IMAC as the Coomassie Brilliant Blue stained SDS-PAGE gels showed intense bands at the calculated size of 18.8 kDa in Lanes 9 to 16. Lanes 1 and 2 also contained bands around this size possibly resulting from a protein, that had not bound to the column and was directly washed out. Some weak bands were detected indicating proteins of a higher molecular weight (Lanes 10 to 15) that might result from multimers of the N-terminal fragment of flagellin. Lanes 5 to 8 showed only very weak bands indicating that the protein was obviously eluted at higher concentrations of imidazole.

Fractions 10 to 16 were pooled for dialysis.

Pooled fractions were dialysed against 1x PBS and then analysed for purity by SDS-PAGE.

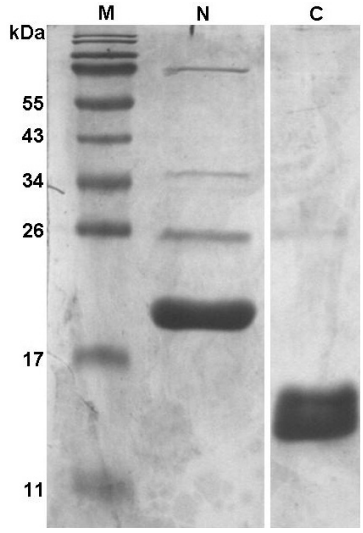

Figure 29: Coomassie staining of the dialysed N- and C-terminal fragments of flagellin from *S. typhimurium*: Fractions were pooled after IMAC and dialysed against 1x PBS. Samples (Lanes N and C) were separated by SDS-PAGE (15%) and stained with Coomassie Brilliant Blue Lane M: Fermentas 8-16% Tris-glycerine SDS-PAGE marker

Figure 29 shows that the protein sample for the C-terminal fragment of flagellin was pure as only one very intense band at the expected size of approximately 13 kDa (Lane C) was detected. The sample for the N-terminal fragment showed a very intense band at the expected size of approximately 19 kDa but also several weak bands indicating proteins of a higher molecular weight (Lane N).

To confirm whether the intense bands really reflected the N- and C-terminal flagellin proteins we performed an immunoblot to detect His-tagged proteins.

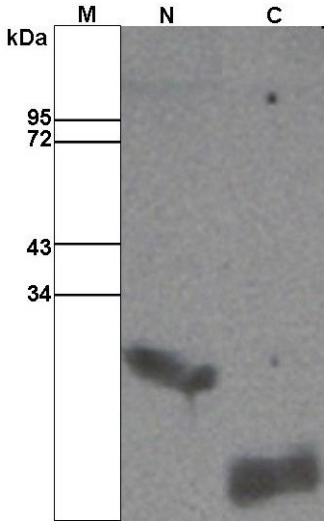

Figure 30: Immunoblot of the dialysed N- and C-terminal fragments of flagellin from *S. typhimurium*: Fractions were pooled after IMAC and dialysed against 1x PBS. Samples (Lanes N and C) were separated by SDS-PAGE (15%) and transferred onto a nitrocellulose membrane. After saturation and washing, a Penta-His™ antibody was added. A HRP-linked antibody served as a secondary antibody and the Lumigen™ detection reagent was used in combination with a hyperfilm to detect proteins carrying the His-tag. Lane M: Fermentas 8-16% Tris-glycerine SDS-PAGE marker

Figure 30 shows that the pooled fractions contained the proteins of interest after dialysis. Lane N shows one single band at the expected size of approximately 19 kDa and Lane C shows the expected band at the size of approximately 13 kDa. As Lane N does not show any other bands, the slight bands in the Coomassie stained gel must have resulted from proteins different than the His-tagged N-terminal fragment.

7.9 Testing the ability of the C- and N-terminal fragments of flagellin from *S. typhimurium* to activate TLR5

7.9.1 Testing the ability to activate TLR5 on HEK-293/TLR5 cells

The purified proteins of the C- and N-terminal fragments of flagellin from *S. typhimurium* were tested for their potential to activate TLR5 using HEK-293/TLR5 cells as described before.

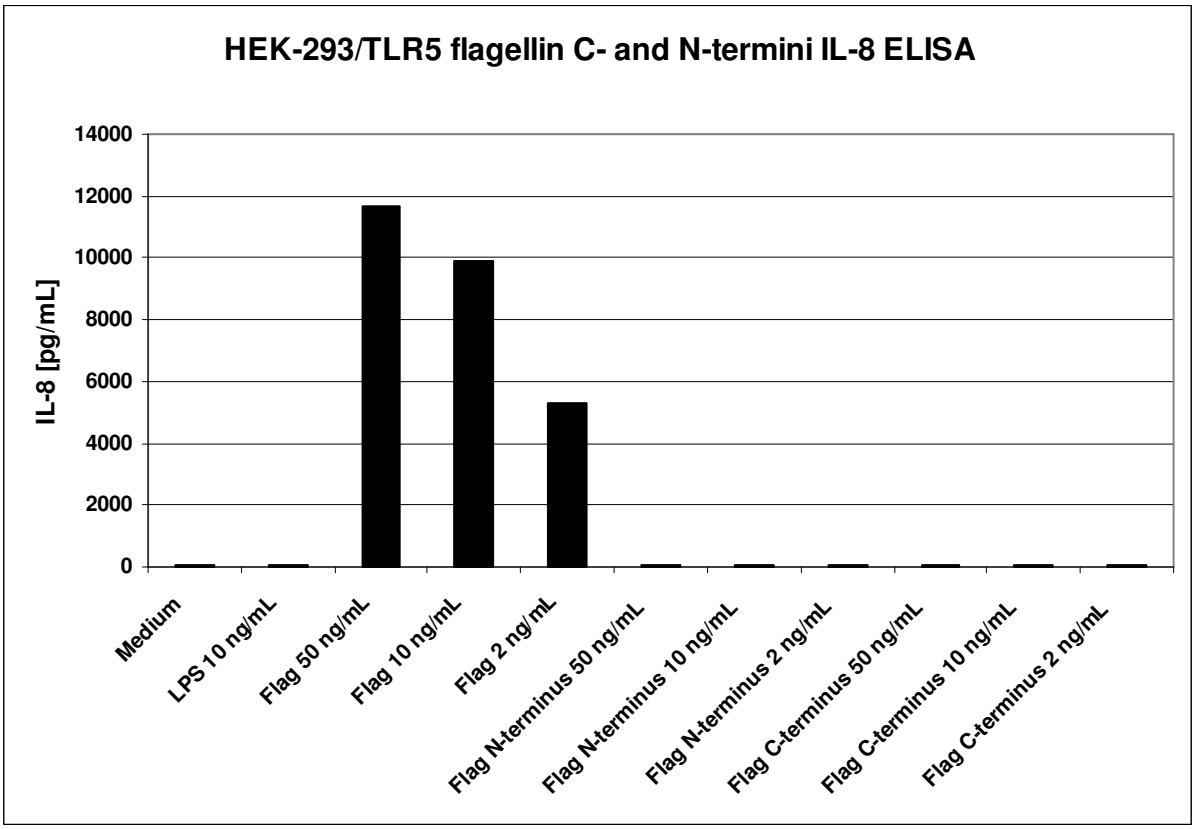

Figure 31: TLR5 activation of the C- and N-terminal fragments of flagellin: HEK-293/TLR5 cells were seeded in a flatbottomed 96 well plate (40000 cells/well) and incubated for 24 hours. Medium was removed and replaced by medium containing the specific stimuli. After 20 hours IL-8 production was determined by ELISA.

Figure 31 shows that HEK-293/TLR5 cells responded to stimulation with recombinant full-length flagellin from *S. typhimurium* in a dose-dependent manner. Neither equimolar amounts of the N-terminal fragment and the full-length protein nor the C-terminal fragment and the full-length protein resulted in IL-8 synthesis. We conclude that the conserved regions of flagellin alone are biologically inactive concerning activation of TLR5. As expected, LPS had no effect on IL-8 production of HEK-293/TLR5 cells.

7.9.2 Testing the ability to activate TLR5 on CaCo-2 cells

To test the effects of flagellin on different cell types we employed CaCo-2 cells. This immortalized cell-line derived from human epithelial colorectal adenocarcinoma cells shows the ability to differentiate into small intestinal-like cells after confluent growth. We were interested in the effects of flagellin stimulation of these cells as they could function as a model system for intestinal functions.

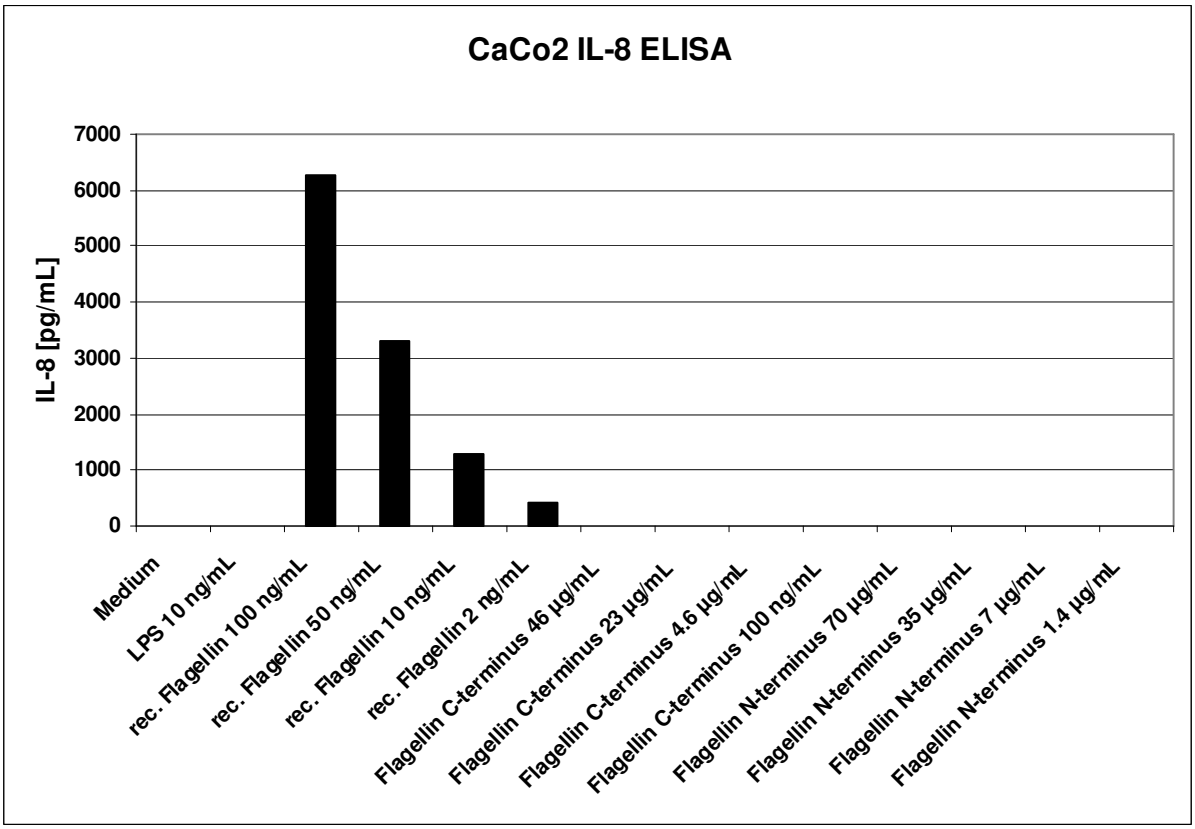

Figure 32: Stimulation of CaCo-2 cells with the C- and N-terminal fragments of flagellin: CaCo-2 cells were seeded in a flatbottomed 96 well plate (40000 cells/well) and incubated for 24 hours. Medium was removed and replaced by medium containing the specific stimuli. After 22 hours IL-8 production was determined by ELISA.

Figure 32 shows that stimulation of CaCo-2 cells with recombinant flagellin of *S. typhimurium* resulted in the secretion of IL-8 in a dose-dependent manner whereas neither the C-terminal nor the N-terminal fragment alone triggered IL-8 secretion. CaCo-2 cells did not respond to stimulation with LPS.

These results showed that HEK-293 cells stably transfected with TLR5 as well as CaCo-2 cells responded to stimulation with recombinant flagellin of *S. typhimurium* with IL-8 secretion. HEK-293/TLR5 cells produced higher amounts of IL-8 compared to CaCo-2 cells.

7.10 Amplification and cloning of DNA encoding a fusion protein of the N- and C-terminal fragments of flagellin from *S. typhimurium* linked by the hinge-region of the *E.coli* MukB protein into pHis parallel 2 vector

As neither the N- nor the C-terminal fragment of flagellin separately bound to TLR5 and induced IL-8 secretion, we linked the N- and C-termini with a hinge region of *E.coli* that has previously been shown to bring amino acid carboxyl protein regions into hairpin stem structures [41]. For this purpose, new primers were designed to clone the hinge region in between the N- and the C-terminal fragments of flagellin (Fig. 33).

***E.coli* MukB hinge region(bp 1996 – 2349 of NCBI Accession No.:NC_009801)**

```
ccagg tggttctgaa gatcagcgtc tgaacgcgct ggcggagcgt

tttggtggtg   tgctgctgtc   agaaatttat   gacgacgtta   gcctggaaga   tgcgccgtac
ttctcagcac   tgtatggccc   gtcacgccac   gccatcgtgg   tgccagatct   gtcacaggta
accgaacacc   tggaaggctt   gaccgattgc   ccggaagatc   tctatttgat   cgaaggggat
ccgcagtcat   tcgatgacag   cgtgttcagc   gttgatgagc   tggaaaaagc   ggtggtggtg
aaaatcgccg atcgtcagtg gcgttattca cgtttcccgg aagtgccgct gtttggtcgt
gctgcgcgt
```

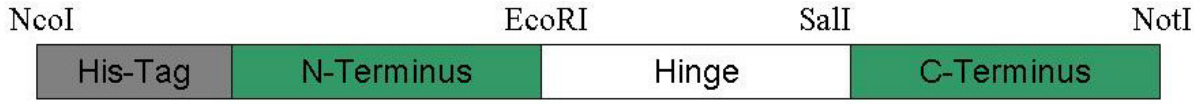

Figure 33: Cloning scheme for the hinge region of *E.coli* MukB in between the N- and C-terminal fragments of flagellin from *S. typhimurium*

Primers:

Flag N forward NcoI: TATA**CCATGG**CTCTGGGCACCGCTATCGA

Flag N reverse EcoR1: TATA**GAATTC**CTTGCACATTCAGCGTATCCAGAC

Flag C forward Sal1: TATA**GTCGAC**CCGAAAACCCGCTGCAGAAAA

Flag C reverse Not1: TATA**GCGGCCG**CTTAACGCAGTAAAGAGAGGACG

Hinge forward EcoR1: TATA**GAATTC**CAGGTGGTTCTGAAGATCAGC

Hinge reverse Sal1: TATA**GTCGAC**GTACGCGCAGCACGACCAAACAG

Sequences homologous to the DNA sequence are underlined and sites for enzymatic digest with restriction enzymes are indicated in bold.

The cloning procedure was performed as described for the N- and C-terminal fragment of flagellin. As a first step the N-terminal fragment of flagellin was cloned into the pHis parallel 2 vector. Next, the hinge-region was cloned into the vector. Finally, the C terminal fragment was cloned into the vector already containing the other two fragments. The complete construct was used for transformation of *E.coli* BL21(DE3)pLysS cells.

7.11 Protein expression and purification of the fusion construct of the N- and C-terminal fragments of flagellin from *S. typhimurium* linked with the hinge-region of *E.coli* MukB protein

7.11.1 Test expression of the fusion protein

Protein expression was performed under standard conditions as described before.

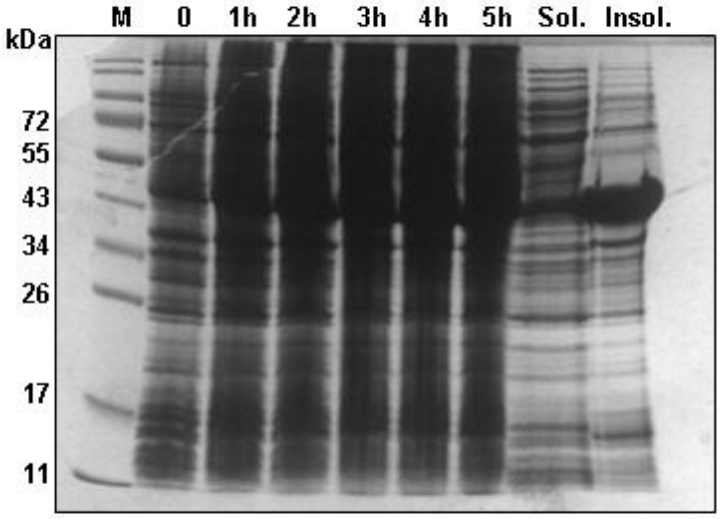

Figure 34: Test expression of the N-terminal fragment – hinge region – C-terminal fragment construct: Samples were collected over the timecourse of expression (Lanes 0–6h) and separated by SDS-PAGE (15%) together with samples containing the solubly expressed protein fraction (Lane Sol.) and the insolubly expressed protein fraction (Lane Insol.).The gel was stained with Coomassie Brilliant Blue Lane M: Fermentas 8-16% Tris-glycerine SDS-PAGE marker

Figure 28 shows increased expression of a protein with a molecular weight of approximately 45 kDa over time upon induction with IPTG (Lanes 1h-5h). This protein was found mainly in the insolubly expressed protein fraction (Lane Insol.) but was also detected in the solubly expressed protein fraction (Lane Sol.). The calculated size for the construct is 42.79 kDa corresponding to the visible bands of around 45 kDa. A slight increase in expression of a protein of approximately 72 kDa (Lanes 1h-5h) was also observed.

As a next step we performed an Immunoblot of the solubly and the insolubly expressed protein fractions to check whether these fractions indeed contained the His-tagged protein of interest.

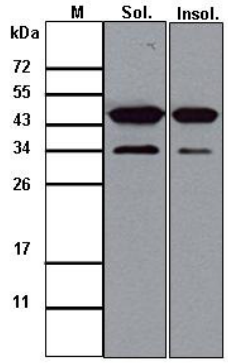

Figure 35: Immunoblot of the expression of the N-terminal fragment – hinge region – C-terminal fragment construct: Samples of the solubly (Lane Sol.) and the insolubly (Lane Insol.) expressed protein fractions were separated by SDS-PAGE (15%) and transferred onto a nitrocellulose membrane. After saturation and washing, a Penta-His™ antibody was added. A HRP-linked antibody served as a secondary antibody and the Lumigen™ detection reagent was used in combination with a hyperfilm to detect proteins carrying the His-tag. Lane M: Fermentas 8-16% Tris-glycerine SDS-PAGE marker

Figure 35 shows that a protein of approximately 45 kDa was expressed in both, the soluble and the insoluble protein fraction (Lanes Sol.&Insol.). Both lanes also contained a His-tagged protein with a molecular weight of 34 kDa that might result from degradation.
Next, a large scale protein expression of the soluble protein fraction was performed and the protein was purified by IMAC.

7.11.2 Purification of the fusion protein by IMAC

Protein purification was done as described above.

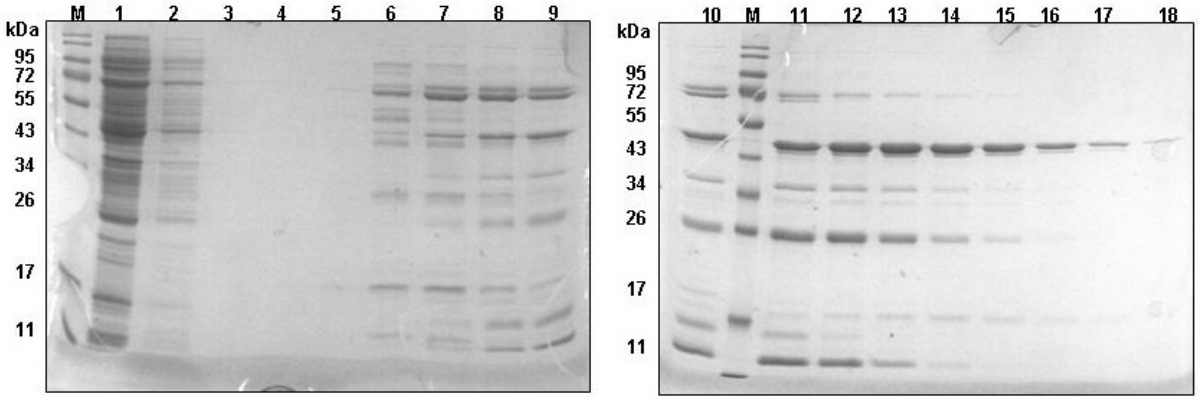

Figure 36: Purification of the N-terminal fragment - hinge region - C-terminal fragment fusion protein by IMAC: Samples (Lanes 1-9 and Lanes 10-18) were separated by SDS-PAGE (15%) and stained with Coomassie Brilliant Blue Lane M: Fermentas 8-16% Tris-glycerine SDS-PAGE marker

Figure 36 shows lots of bands in the flow-through fractions (Lanes 1&2) but both fractions did not show very intense bands at the size of approximately 45 kDa corresponding to the

His-tagged protein. Fractions 3 to 5 did not show any bands indicating that the amount of imidazole was too low to elute bound proteins from the column. Fractions 6 to 10 showed several bands including a band of 45 kDa indicating that the eluted His-tagged protein was rather impure as several bands were visible in these fractions. The smaller bands might result from degradation of the fusion protein, but there were also bands of 72 kDa that could not result from dimers or trimers of the protein. Fractions 11 to 15 showed more intense bands of 45 kDa indicating that the protein of interest was mainly contained in these fractions. However, there were also several smaller bands presumably resulting from degradation and also one band at 72 kDa. The protein could not be purely eluted. Fractions 16 to 18 showed decreased concentrations of the protein at 45 kDa and no additional bands indicating that all bound sample was already eluted at lower concentrations of imidazole.

Fractions 11 to 16 were pooled and dialysed against 1x PBS. The dialysed protein was used to assess, whether the N-terminal fragment – hinge region – C-terminal fragment fusion protein could activate TLR5.

7.12 Testing the ability of the N-terminal fragment – hinge region – C-terminal fragment fusion protein to activate TLR5

To test the biological activity of the fusion protein, HEK cells stably transfected with TLR5 were stimulated and IL-8 secretion was determined by ELISA as described above.

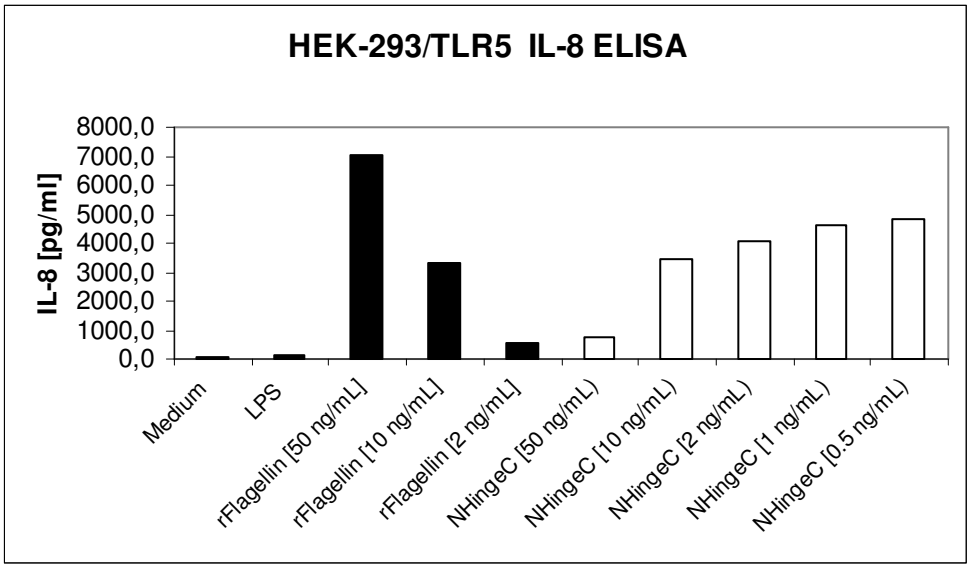

Figure 37: TLR5 activation of the N-terminal fragment – hinge region – C-terminal fragment fusion protein: HEK-293/TLR5 cells were seeded in a flatbottomed 96 well plate (40000 cells/well) and incubated for 24 hours. Medium was removed and replaced by medium containing the specific stimuli (medium: dotted bar; LPS: hatched bar; different concentrations of recombinant flagellin: full bars; different concentrations of the fusion protein: empty bars). After 20 hours IL-8 production was determined by ELISA.

Figure 37 shows that recombinant flagellin (full bars) induced IL-8 secretion in a dose dependent manner after stimulation of HEK-293/TLR5 cells whereas LPS stimulation (hatched bar) did not. Stimulating cells with the N-terminal fragment – hinge region – C-terminal fragment construct (empty bars) also induced IL-8 synthesis by HEK-293/TLR5 cells indicating that the protein was biologically active concerning TLR5 activation.

8 Discussion

TLR ligands are becoming more important in the field of allergy because they may be potential adjuvants for allergy vaccines. Clinical trials with TLR4 and TLR9 ligands have already shown promising results [26, 31]. The TLR2 ligand Pam3Csk4 was shown to induce T_H1-type T-cell responses and regulatory T-cells [26]. A monophosphoryl lipid A-adjuvanted vaccine was shown to reduce seasonal allergen-specific IgE boosts and led to the induction of allergen-specific IgG blocking antibodies [31]. To find out whether flagellin, a TLR5 ligand, could also be a potential adjuvant candidate for allergy vaccines flagellin-induced production of key cytokines in PBMC from allergic patients was analysed. Stimulation of PBMC from birch-pollen allergic patients with commercially available flagellin from *S. typhimurium* induced the immunosuppressive cytokine IL-10 (Fig. 2, Fig. 3) and a weak production of the T_H1-type cytokine IFN-γ (Fig. 4, Fig. 5) but no TNF-α (data not shown). These preliminary results indicated that flagellin might be a potential adjuvant for allergy vaccination. The major aim of this diploma thesis was to produce high amounts of recombinant flagellin in order to confirm this hypothesis.

First, we established a read-out system for TLR5 triggering. For this purpose, HEK-293 cells stably transfected with TLR5 (HEK-293/TLR5 cells) and CaCo-2 cells were stimulated with commercially available recombinant flagellin from *S. typhimurium*. HEK-293/TLR5 responded with IL-8 synthesis in a dose-dependent manner (Fig. 6). LPS stimulation did not lead to IL-8 production indicating that contamination of the flagellin protein with endotoxins did not affect the results (Fig. 6). Stimulation of CaCo-2 cells with flagellin resulted in lower IL-8 synthesis as compared to HEK-293/TLR5 cells (Fig. 7). In parallel, we investigated whether heat-labile serological factors were important for the ability of flagellin to trigger TLR5 responses. We usually supplemented our cell-culture media with FCS that was heat-inactivated, which is not a physiologic condition. With regard to possible allergy vaccines we wanted to ensure that heat-labile serological factors could not influence the biological activity. For this purpose, HEK-293/TLR5 cells were stimulated with recombinant flagellin in the presence of FCS that was either heat-inactivated or not. IL-8 secretion did not differ between both groups (Fig. 8). There was slightly increased IL-8 production in the presence of heat-labile serological factors when stimulated with high doses of flagellin but this effect was diminished when lower flagellin concentrations were applied (Fig. 8). This observation indicated that heat-labile serological factors had no influence on the effects of TLR5 activation.

Based on these findings, we decided to use HEK-293/TLR5 as a read-out system for the ability of a TLR5 ligand to trigger TLR5 responses. Moreover, HEK-293/TLR5 cells could be detached easier compared to CaCo-2 cells and CaCo-2 cells appeared to be more sensitive to stress as they showed a decreased growth rate after splitting of cells.

We amplified the DNA coding for flagellin and transformed bacterial cells with a vector containing the gene of interest. Next, we tried to express the full length flagellin under different conditions including salt stress or low-temperature (Fig.15, Fig.17 and Fig. 18). Unfortunately, none of these approaches resulted in a satisfactory expression of flagellin. Nevertheless, we tried protein purification by IMAC but were not able to purify detectable amounts of the protein (Fig. 19). Interestingly, stimulation of HEK-293/TLR5 cells with a cell lysate of *E. coli* cells containing the vector with the flagellin insert resulted in IL-8 synthesis (Fig. 20). Therefore, we conclude that the flagellin protein was expressed in a biological active form. However, we could not purify it by IMAC nor clearly detect it either by Coomassie Brilliant Blue staining or by Immunoblotting applying a detection system specific for His-tagged proteins. Probably the *E.coli* BL21(DE3)pLysS strain was not able to express sufficient amounts of the protein for detection or the protein was degraded. It could also be possible that the His-tag of the protein was not accessible for purification by IMAC and detection with His-tag-specific antibodies.

Recognition by TLR5 is accomplished by binding of certain conserved amino acids in the D1 region (Fig. 1) [35, 36]. The D0 and the D1 region are mainly formed by structures encoded in the N- and C-terminal parts of the flagellin protein. Since we had problems with expression of the full length protein, the next step was to express the N- and C-terminal fragments of flagellin as individual proteins. Both fragments could be cloned and expressed in *E.coli* cells (Fig. 23 - 26) and purified by IMAC (Fig.27 - 30). The successful expression of both, the N- and C-terminal fragments indicated that the *E.coli* BL21(DE3)pLysS strain could not correctly express the D2 and D3 regions of the flagellin protein, that are formed by structures encoded in the central region of the protein. Testing both flagellin fragments for TLR5 triggering using HEK-293/TLR5 cells revealed that neither the N- or C-terminal fragments alone nor a mixture of both fragments induced the synthesis of IL-8 (Fig. 31). We also tested the two fragments on CaCo-2 cells for binding and activating TLR5. Again, no detectable IL-8 synthesis was observed (Fig. 32). We concluded that the individual N- and C-terminal fragments encoded by the conserved DNA sequences of the flagellin protein were not capable

of activating TLR5. This might be due to incorrect folding of the proteins so that TLR5 is not able to recognize the structure. We speculated that the N- and C-terminal fragments of flagellin have to be brought into close proximity in order to trigger TLR5.

It has previously been reported that the hinge region of the MukB protein of *E.coli* could be used to bring the N- and the C-terminal fragments of flagellin into close proximity [42]. Therefore, we engineered a fusion protein consisting of the N- and C-terminal fragments of flagellin and this hinge region. The resulting fusion protein had a calculated molecular weight of approximately 43 kDa and therefore, was slightly smaller than the full-length flagellin protein with a calculated molecular weight of 49.9 kDa. We were able to express and purify the fusion protein indicating that the *E.coli* BL21(DE3)pLysS strain was able to express sufficient amounts of this artificial construct whereas the full-length *S. typhimurium* protein could not be expressed. Maybe the slight differences in the molecular weight of the proteins were critical in their expression or species related factors were of importance.
Stimulation of HEK-293/TLR5 cells with the fusion protein resulted in the synthesis of IL-8 demonstrating that linking the N- and C-terminal fragments with the hinge region of the MukB protein from *E. coli* resulted in a fusion protein recognized by TLR5. The hinge region has previously been shown to induce hairpin stem structures in amino acid carboxyl protein regions [41]. Maybe this function was important to express the protein in the *E.coli* BL21(DE3)pLysS strain and to recover the biological activity of the fragments.

In this diploma thesis we established a model read-out system for TLR5 activation and attempted to express the TLR5 ligand flagellin from *S. typhimurium* in *E. coli* cells. We were not able to express the full-length protein but the N- and C-terminal fragments of flagellin, which form the conserved parts of the protein. As the individual fragments did not trigger TLR5 we created a fusion construct including a hinge region of the MukB protein from *E. coli.* This approach restored the ability to bind and activate TLR5. We are now able to produce large amounts of this fusion protein to assess, if such a protein may serve as an adjuvant in allergy vaccines.

9 Appendix

9.1 References

1. Abbas, A.K., Lichtman, A.H., Pillai, S, *Cellular and Molecular Immunology*. 6th edition ed. 2007, Philadelphia: Saunders.
2. Rajan, T.V., *The Gell-Coombs classification of hypersensitivity reactions: a re-interpretation.* Trends Immunol, 2003. **24**(7): p. 376-9.
3. Descotes, J. and G. Choquet-Kastylevsky, *Gell and Coombs's classification: is it still valid?* Toxicology, 2001. **158**(1-2): p. 43-9.
4. Ivanciuc, O., et al., *Characteristic motifs for families of allergenic proteins.* Mol Immunol, 2008.
5. Shakib, F., A.M. Ghaemmaghami, and H.F. Sewell, *The molecular basis of allergenicity.* Trends Immunol, 2008. **29**(12): p. 633-42.
6. Kay, A.B., *Allergy and allergic diseases. First of two parts.* N Engl J Med, 2001. **344**(1): p. 30-7.
7. Galli, S.J., M. Tsai, and A.M. Piliponsky, *The development of allergic inflammation.* Nature, 2008. **454**(7203): p. 445-54.
8. Strachan, D.P., *Hay fever, hygiene, and household size.* Bmj, 1989. **299**(6710): p. 1259-60.
9. Leynaert, B., et al., *Does living on a farm during childhood protect against asthma, allergic rhinitis, and atopy in adulthood?* Am J Respir Crit Care Med, 2001. **164**(10 Pt 1): p. 1829-34.
10. Braun-Fahrlander, C., *The role of the farm environment and animal contact for the development of asthma and allergies.* Clin Exp Allergy, 2001. **31**(12): p. 1799-803.
11. Openshaw, P.J. and C. Hewitt, *Protective and harmful effects of viral infections in childhood on wheezing disorders and asthma.* Am J Respir Crit Care Med, 2000. **162**(2 Pt 2): p. S40-3.
12. Ohfuji, S., et al., *Sibship size and prevalence of allergic disorders in Japan: The Ryukyus Child Health Study.* Pediatr Allergy Immunol, 2008.
13. Behrendt, H., et al., *Air pollution and allergy: experimental studies on modulation of allergen release from pollen by air pollutants.* Int Arch Allergy Immunol, 1997. **113**(1-3): p. 69-74.
14. Fujieda, S., D. Diaz-Sanchez, and A. Saxon, *Combined nasal challenge with diesel exhaust particles and allergen induces In vivo IgE isotype switching.* Am J Respir Cell Mol Biol, 1998. **19**(3): p. 507-12.
15. Hill, M.R. and W.O. Cookson, *A new variant of the beta subunit of the high-affinity receptor for immunoglobulin E (Fc epsilon RI-beta E237G): associations with measures of atopy and bronchial hyper-responsiveness.* Hum Mol Genet, 1996. **5**(7): p. 959-62.
16. Frew, A.J., *Sublingual immunotherapy.* N Engl J Med, 2008. **358**(21): p. 2259-64.
17. Ebner, C., et al., *Immunological changes during specific immunotherapy of grass pollen allergy: reduced lymphoproliferative responses to allergen and shift from TH2 to TH1 in T-cell clones specific for Phl p 1, a major grass pollen allergen.* Clin Exp Allergy, 1997. **27**(9): p. 1007-15.
18. Jutel, M., et al., *IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy.* Eur J Immunol, 2003. **33**(5): p. 1205-14.
19. Francis, J.N., S.J. Till, and S.R. Durham, *Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy.* J Allergy Clin Immunol, 2003. **111**(6): p. 1255-61.

20. Bohle, B., et al., *Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation.* J Allergy Clin Immunol, 2007. **120**(3): p. 707-13.
21. van Neerven, R.J., et al., *Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4+ T cells by inhibiting serum-IgE-facilitated allergen presentation.* J Immunol, 1999. **163**(5): p. 2944-52.
22. Nouri-Aria, K.T., et al., *Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity.* J Immunol, 2004. **172**(5): p. 3252-9.
23. Pauli, G., et al., *Efficacy of recombinant birch pollen vaccine for the treatment of birch-allergic rhinoconjunctivitis.* J Allergy Clin Immunol, 2008. **122**(5): p. 951-60.
24. Niederberger, V., et al., *Vaccination with genetically engineered allergens prevents progression of allergic disease.* Proc Natl Acad Sci U S A, 2004. **101 Suppl 2**: p. 14677-82.
25. Lombardi, V., et al., *Toll-like receptor 2 agonist Pam3CSK4 enhances the induction of antigen-specific tolerance via the sublingual route.* Clin Exp Allergy, 2008.
26. Creticos, P.S., et al., *Immunotherapy with a ragweed-toll-like receptor 9 agonist vaccine for allergic rhinitis.* N Engl J Med, 2006. **355**(14): p. 1445-55.
27. Lemaitre, B., et al., *The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults.* Cell, 1996. **86**(6): p. 973-83.
28. Anderson, K.V., L. Bokla, and C. Nusslein-Volhard, *Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product.* Cell, 1985. **42**(3): p. 791-8.
29. Kawai, T. and S. Akira, *TLR signaling.* Semin Immunol, 2007. **19**(1): p. 24-32.
30. Dillon, S., et al., *Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance.* J Clin Invest, 2006. **116**(4): p. 916-28.
31. Mothes, N., et al., *Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies.* Clin Exp Allergy, 2003. **33**(9): p. 1198-208.
32. Yonekura, K., S. Maki-Yonekura, and K. Namba, *Growth mechanism of the bacterial flagellar filament.* Res Microbiol, 2002. **153**(4): p. 191-7.
33. Ramos, H.C., M. Rumbo, and J.C. Sirard, *Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa.* Trends Microbiol, 2004. **12**(11): p. 509-17.
34. Beatson, S.A., T. Minamino, and M.J. Pallen, *Variation in bacterial flagellins: from sequence to structure.* Trends Microbiol, 2006. **14**(4): p. 151-5.
35. Hayashi, F., et al., *The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5.* Nature, 2001. **410**(6832): p. 1099-103.
36. Andersen-Nissen, E., et al., *A conserved surface on Toll-like receptor 5 recognizes bacterial flagellin.* J Exp Med, 2007. **204**(2): p. 393-403.
37. Smith, K.D., et al., *Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility.* Nat Immunol, 2003. **4**(12): p. 1247-53.
38. Parker, L.C., et al., *The expression and roles of Toll-like receptors in the biology of the human neutrophil.* J Leukoc Biol, 2005. **77**(6): p. 886-92.
39. Masamune, A., et al., *Pancreatic stellate cells express Toll-like receptors.* J Gastroenterol, 2008. **43**(5): p. 352-62.

40. Joys, T.M., *The covalent structure of the phase-1 flagellar filament protein of Salmonella typhimurium and its comparison with other flagellins.* J Biol Chem, 1985. **260**(29): p. 15758-61.
41. Melby, T.E., et al., *The symmetrical structure of structural maintenance of chromosomes (SMC) and MukB proteins: long, antiparallel coiled coils, folded at a flexible hinge.* J Cell Biol, 1998. **142**(6): p. 1595-604.
42. Eaves-Pyles, T.D., et al., *Salmonella flagellin-dependent proinflammatory responses are localized to the conserved amino and carboxyl regions of the protein.* J Immunol, 2001. **167**(12): p. 7009-16.

9.2 List of figures

9.3 Zusammenfassung

Liganden für Toll-like Rezeptoren (TLR) werden als mögliche Adjuvantien in Impfstoffen für die allergen-spezifische Immuntherapie diskutiert. So konnte beispielsweise gezeigt werden, dass die Beimengung von DNA, die CpG-Motive enthält und von TLR9 erkannt wird, zu Allergieimpfstoffen die allergische Immunantwort gegen-regulieren kann. In dieser Diplomarbeit wurde untersucht, ob das bakterielle Protein Flagellin, welches von TLR5 erkannt wird, ebenfalls immunmodulierende Eigenschaften aufweist. In weiterer Folge sollte Flagellin von *Salmonella typhimurium* als rekombinantes Protein hergestellt werden.

Zunächst wurden periphere mononukleäre Blutzellen (PBMC) von Birkenpollenallergikern mit Flagellin von *S. typhimurium* stimuliert. Die darauf folgende Zytokinantwort wurde mittels ELISA analysiert. Es wurde die Produktion von IFN-γ sowie IL-10 festgestellt, was darauf hindeutete, dass Flagellin als Adjuvans wirksam sein könnte. Als nächsten Schritt etablierten wir ein Testmodell, um eine Aktivierung über TLR5 rasch nachzuweisen. Dieses Modell basierte einerseits auf der Verwendung von humanen embryonalen Nierenzellen (human embryonic kidney cells, HEK cells), die mit TLR5 stabil transfiziert worden waren und andererseits auf der Stimulation von CaCo-2 Zellen. Parallel dazu wurde die Wichtigkeit der Präsenz von hitzelabilen Serumfaktoren für die Aktivierung über TLR5 untersucht. Die Resultate bei Verwendung von kommerziell erhältlichem Flagellin von *S. typhimurium* zeigten, dass HEK-293/TLR5 Zellen bei Beimengung von fötalem Kälberserum (10%) zum Nährmedium bestens geeignet waren, die Aktivierung über TLR5 nachzuweisen.

Wir versuchten, Flagellin von *S. typhimurium* in einen geeigneten Vektor zu klonieren und das Protein in *E. coli* BL21(DE3)pLysS Zellen zu exprimieren. Da mehrere Versuche, das Gesamtprotein rekombinant herzustellen, scheiterten, haben wir uns auf die Herstellung der C- und N-terminalen Fragmente des Proteins konzentriert. Diese konservierten Bereiche sind wesentlich an der Bildung der D0 und D1 Domänen von Flagellin beteiligt, die von TLR5 gebunden werden. Wir konnten sowohl das C- als auch das N-terminale Protein in *E. coli* BL21(DE3)pLysS Zellen exprimieren und mittels chromatographischer Methoden reinigen, jedoch konnten weder die einzelnen Fragmente, noch eine Mischung beider Proteine, TLR5 aktivieren. Daraufhin wurde ein Fusionsprotein, in dem die C- und N-terminalen Fragmente von Flagellin aus *S. typhimurium* mit einer Gelenksregion aus einem *E. coli* Protein verknüpft wurden, hergestellt. Dieses Fusionsprotein konnte exprimiert und gereinigt werden und erste Tests auf HEK-293/TLR5 Zellen führten zu IL-8 Produktion. Dies zeigte, dass die C- und N-terminalen Fragmente verknüpft und in räumliche Nähe gebracht werden müssen, um TLR5 zu aktivieren.

Zusammenfassend weisen mehrere Ergebnisse, aus Versuchen mit Zellen von Allergikern, darauf hin, dass Flagellin immunmodulatorische Eigenschaften aufweist. Es wurde ein Testmodell für den Nachweis einer Aktivierung von TLR5 etabliert und ein rekombinantes Fusionsprotein hergestellt, das aus den hochkonservierten N- und C-terminalen Fragmenten von Flagellin aus *S. typhimurium* sowie einer Gelenksregion aus *E. coli* besteht. Dieses Fusionsprotein konnte TLR5 aktivieren.

In nächster Zukunft ist geplant, dieses Fusionsprotein in ausreichender Menge endotoxinfrei herzustellen und hinsichtlich seiner immunmodulatorischen Effekte auf PBMC von Allergikern zu testen.

9.4 Curriculum vitae

Personal data:

Name: Stephan Deifl

Date of birth: 20. November 1982

Place of birth: Krems an der Donau

Citizenship: Austrian

Education:

Sept. 1989 - July 1993 Elementary school at the Volksschule Hadersdorf am Kamp, Austria

Sept. 1993 - May 2001 Secondary school at the Bundesrealgymnasium Krems, Austria

Oct. 2001 - Oct. 2002 Civil service at the Red Cross in Langenlois, Austria

Oct. 2002 – Apr. 2009 Studies of Biology/Genetics at the University of Vienna, Austria

Congresses:

6th EAACI - GA²LEN "Davos" Immunology Winterschool, Pichl, Austria;

Jan. 31st – Feb. 3rd 2008

Member of the local organizing committee

Joint Annual Meeting of Immunology of the Austrian and German Societies, Vienna, Austria;

Sept. 3rd – Sept. 6th 2008

S. Deifl, S. Mutschlechner, A. Radakovics, B. Bohle. Flagellin from *Salmonella typhimurium* – a possible adjuvant in specific immunotherapy of Type 1 allergies?

Poster presentation