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Expression of pattern recognition receptors by oral epithelial cell lines

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1. List of abbreviations

µl	micro litre
APC	antigen-presenting cell
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary DNA
CLR	C-type lectin receptor
Conc.	concentration
CRD	carbonate recognition domain
Ct	threshold cycle number
CpG	deoxycytidylate-phosphate-deoxyguanylate
DAMP	danger-associated molecular pattern
DAP	diaminopimelic acid
DC	dendritic cell
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphates
ds/ss	double-stranded/single-stranded
EDTA	ethylenediaminetetraacetic acid
ELISA	enzme-linked immunosorbent assay
EtBr	ethidium bromide
EU	endotoxin unit
FCS	fetal calf serum
FcεRI/II	high-affinity receptor for IgE
g (ng, pg)	gram (nanogram, pictogram)
g	gravity
h	hour
IFN	interferon
Ig	immunoglobulin
IL	interleukin
kDa	kilo Dalton
LAL	Limulus amebocyte lysate
LPS	lipopolysaccharide
LRR	leucine-rich repeats
M (nM, mM)	mol (nanomol, millimol)
MDP	muramyl dipeptide
MHC	major histocompatibility complex
min	minute
ml	millilitre
mRNA	messenger RNA
Neg.	negative
NK-cell	natural killer cell
NLR	NOD-like receptor
nm	nanometer
PAMP	pathogen-associated molecular pattern

PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR (RT-PCR, qPCR)	polymerase chain reaction
Pol.	polymerase
Pos.	positive
PRR	pattern recognition receptor
qPCR	quantitative (real-time) PCR
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase PCR
SD	standard deviation
sec	second
SLIT	sublingual immunotherapy
Ta	annealing temperature
TGF	transforming growth factor
Th	helper T-cell
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptor
Tm	melting temperature
TNF	tumour necrosis factor
UV	ultra violet
w/o	without
w/v	weight per volume

2. Abstract

Sublingual immunotherapy (SLIT) is a safe and effective option for treatment of type I allergy. Still, the immune mechanisms underlying SLIT are not completely understood. In this context, the aim of the master thesis was to analyse the expression of toll-like receptor (TLR) 1-10, C-type lectin receptor DC-SIGN, Dectin-1 and Dectin-2 and NOD-like receptor NOD1 and NOD2 by human epithelial cells. For this purpose, a buccal mucosa cell line, HO-1-N-1, a sublingual epithelial cell line, HO-1-u-1, and two intestinal Caco-2 cell lines were employed. mRNA expression of the different receptors was analysed by reverse-transcription PCR (RT-PCR) and quantitative real-time RT-PCR (qPCR). Moreover, all epithelial cell lines were stimulated with ligands specific for the respective receptors. IL-8 secretion as a readout for the activation of their signalling pathways was determined by ELISA.

HO-1-N-1 cells expressed mRNA for TLR1, TLR2, TLR4, TLR6 as well as NOD1 and NOD2 and responded with increased IL-8 synthesis to ligands specific for these receptors. qPCR also indicated the expression of TLR3, TLR8, TLR10 and Dectin-1 in HO-1-N-1. However, the cells were not activated by ligands specific for these receptors.

Analysing the HO-1-u-1 cell line, mRNA coding for TLR1, TLR3, TLR4, TLR5, TLR6 and NOD1 was detected. qPCR also indicated expression of TLR2, TLR7, TLR8, TLR10 and Dectin-1. However, HO-1-u-1 cells only responded to ligands targeting TLR3, TLR5 and NOD1.

In addition to the epithelial cell lines in the mouth, all experiments were performed with the well-established colorectal epithelial cell lines Caco-2/15 and Caco-2 A9. These cells expressed mRNA for all 10 currently known human TLRs as well as for NOD1. Additionally, mRNA coding for Dectin-2 was detected in Caco-2/15 by qPCR. Analysing the functional response, Caco-2 cells showed IL-8 production upon stimulation with ligands for TLR1/2, TLR2/6 and for TLR5.

In conclusion, we found that sublingual and buccal cells show differences in the expression of pattern recognition receptors and respond to stimulation with microbial ligands in a tissue-specific fashion. The spectrum of ligands activating the buccal cell line HO-1-N-1 indicates that these cells are basically specialised in recognising bacterial compounds. The sublingual epithelial cell line HO-1-u-1 responded to fewer ligands but within a broader spectrum, comprising viral dsRNA, bacterial flagellin and peptidoglycan.

3. Introduction

3.1. The immune system

The human immune system can be divided into the innate or native immunity and the adaptive or specific immunity.

Innate immunity represents the phylogenetically oldest mechanism of the immune system. It provides the first line of defence against microorganisms in order to prevent or eliminate infections of the host, or to stimulate adaptive immune responses. In this context, the epithelial tissue plays an important role as a physical barrier against microbial invasion.

This epithelial barrier consists of cells arranged in continuous layers that cover the body surface or line the body cavity or the lumen of internal organs. They are tightly connected by numerous intercellular junctions. The apical surface of an epithelial cell faces the body surface and may contain cilia and microvilli. The lateral surface faces the adjacent cells on either side, and the basal surface adheres to deeper cell layers or extracellular materials.

The epithelial tissue can be classified into unilaminar epithelium consisting of a single cell layer, stratified epithelium consisting of multiple cell layers, and pseudostratified epithelium, which appears to be multilaminar because not all the cells reach the surface. Furthermore, epithelial cells are classified according to their shape, into squamous, cuboidal and columnar cells, and transitional cells that change their shape.

Epithelial cells have their own nerve supply, but are avascular, and therefore, receive their nutrition by diffusion from neighbouring connective tissue. A high division rate allows the epithelium to constantly renew itself (Standring et al., 2005; Tortora and Derrickson, 2009).

Epithelia function as selective barriers that facilitate or limit the transfer of substances and protect underlying tissues against dehydration and damage. Additionally, as mentioned before, epithelial cells play an important role in the regulation of immune responses, host defence and inflammation (Schleimer et al., 2007).

Further mechanisms of the innate immune system include phagocytic cells like macrophages and natural killer (NK) cells, the complement system and the production of cytokines (Abbas et al., 2007).

Although innate immune responses cannot adapt to particular pathogens during infections, it has been shown that innate immunity is far more specific than previously assumed (Le Bourhis et al., 2007; Akira et al., 2006). Thereby, the innate immune system relies on the

recognition of several structures conserved among microorganisms, known as pathogen-associated molecular patterns (PAMPs), by pattern recognition receptors (PRR) (Takeuchi and Akira, 2010).

Cell-associated PRRs of the innate immune system are located on the surface or in intracellular compartments of various cells, including macrophages, dendritic cells (DC) and epithelial cells. PRR recognise microbial components, which are essential for the survival of the microorganism, as these targets cannot be altered or discarded that easily. Moreover, they detect stressed or injured host cells by danger-associated molecular patterns (DAMPs). PRRs are encoded in the germline DNA of the cell, and are constitutively expressed in order to detect pathogens autonomously of the cell's life-cycle stage. In contrast to the mechanisms of the adaptive immunity, PRRs are nonclonal and do not produce immunological memory (Akira et al., 2006; Abbas et al., 2007).

Several classes of PRRs of the innate immune system have been identified so far, including Toll-like receptors (TLR), C-type lectin receptors (CLR), and NOD-like receptors (NLR).

Each PRR is specific for particular PAMPs. After recognising their ligand, most PRRs upregulate the transcription of genes encoding proinflammatory cytokines, type I interferons, chemokines and other genes involved in inflammatory responses (Takeuchi and Akira, 2010).

The adaptive or specific immunity can be divided into the humoral immune response mediated by antibody-producing B lymphocytes and the cellular immune response mediated by T lymphocytes.

B lymphocytes recognise intact microbes or microbial antigens in their native form and differentiate into antibody-secreting plasma cells. The antibodies circulate in the blood and are important for the neutralization of antigens, opsonisation, activation of the complement system, targeting for phagocytosis and the activation of mast cells. Antibodies/immunoglobulins (Ig) are classified into 5 subtypes, namely IgM, IgD, IgG, IgA and IgE.

T lymphocytes recognise microbial peptides displayed by antigen presenting cells (APC) in combination with a MHC (major histocompatibiliy complex) molecule. Moreover, the T cell needs co-stimulators (f.e. CD80, CD86) for activation. Activated CD8+ cytotoxic T lymphocytes kill infected cells. Activated CD4+ helper T lymphocytes differentiate into

effector cells and secrete cytokines that stimulate different cells of the immune system. Helper T cells can be divided into Th1, Th2 and Th17 cells (Abbas et al., 2007).

3.2. Pattern recognition receptors (PRR) of the innate immune system

3.2.1. Toll-like receptors (TLRs)

TLRs were first described in 1994 by Nomura and coworkers, and are named after the Toll protein in Drosophila (Abbas et al., 2007). They are membrane-bound receptors, namely type I integral membrane glycoproteins, recognising pathogens outside the cell and in intracellular compartments.

TLRs consist of various leucine-rich repeats (LRRs) on the N-terminus, a transmembrane region, and a cytoplamic Toll/IL-1 receptor (TIR) homology domain (Takeuchi and Akira, 2010).

Currently, ten different TLRs have been identified in humans (see *Table 1*), mainly on immune cells such as macrophages, DCs, B- and T-cells, and non-immune cells including fibroblasts and epithelial cells (Akira et al., 2006). However, it is reported that surface epithelial cells of the gastrointestinal tract either lack expression of TLRs or their co-receptors, or express TLRs at rather low levels (Athman and Philpott, 2004).

Table 1: TLRs and their ligands - adapted from "PRRs and their Ligands" (Takeuchi and Akira, 2010).

TLR	**Localization**	**Ligand**	**Origin of the Ligand**
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, fungi, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, fungi, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7, TLR8	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	unknown	unknown

TLR 1, 2, 4, 5 and 6 are expressed on the cell surface, whereas TLR 3, 7, 8, 9 and 10 are expressed in intracellular compartments such as endosomes. The localization of the TLRs is essential, as they are not able to distinguish between self and non-self, nor between pathogenic and commensal microorganisms (Medzhitov and Janeway, 2002).

Each TLR recognises different PAMPs including lipids, nucleic acids and proteins. TLR1, TLR2 and TLR6 sense certain lipoproteins. TLR3 recognises viral double stranded RNA and its synthetic analogue polyinosinic-polycytidylic acid [Poly(I:C)] (Alexopoulou et al., 2001). TLR4 senses lipopolysaccharides in Gram-negative bacteria, but is also involved in the recognition of viruses by binding to viral envelope proteins. TLR5 binds bacterial flagellin. TLR7 and TLR8 bind single stranded RNA, and TLR9 recognizes unmethylated deoxycytidylate-phosphate-deoxyguanylate (CpG) motifs within bacterial DNA (Takeuchi and Akira, 2010).

When TLRs recognise a certain PAMP, they become activated by dimerisation and recruit certain TIR domain-containing adaptor molecules such as myeloid differentiation factor 88 (MyD88), the TIR-domain-containing adaptor inducing IFN-beta (TRIF/TICAM-1), TIR-containing adaptor protein (TIRAP/Mal) or TRIF-related adaptor molecule (TRAM). The activation of protein kinases by these adaptors results in the triggering of downstream signalling cascades, which lead to transcriptional upregulation of genes coding for inflammatory cytokines (e.g. TNF, IL-1 and IL-12), chemokines (e.g. IL-8, MCP-1 and RANTES), endothelial adhesion molecules and other relevant genes of the innate immune system. Thereby, MyD88 is essential for downstream signalling of all TLRs except TLR3, which triggers TRIF-dependent signalling. TLR4 recruits both MyD88 and TRIF, but requires TRAM for TRIF activation (Takeuchi and Akira, 2010; Abbas et al., 2007; Akira et al., 2006).

3.2.2. C-type lectin receptors (CLRs)

C-type lectins are a superfamily of proteins that recognise carbohydrates, mostly on microorganisms, in a calcium-dependent manner (Weis et al., 1998), and stimulate the production of proinflammatory cytokines and other molecules. Moreover, they generate signals that inhibit TLR-mediated immune complexes (Takeuchi and Akira, 2010). CLRs are expressed on the plasma membrane of various cells including macrophages and DCs (Abbas et al., 2007). They are divided into type I and type II receptors according to their carbonate recognition domains (CRD).

Dendritic cell-associated C-type lectin 1 (Dectin-1) is a type II receptor with only a single CRD. It is expressed by DCs but also by macrophages, monocytes, neutrophils and other cell types. Dectin-1 binds β-glucans present in the cell walls of fungi and some bacteria (Hollmig et al., 2009), f.e. β-1,3-glucan (Curdlan), and is able to induce immunity through a Syk and CARD9-dependent pathway (Robinson et al., 2009).

Dendritic cell-associated C-type lectin 2 (Dectin-2) is another member of the type II receptor family. However, the amino acid sequence of Dectin-2 is only 22 % homologous to Dectin-1 (Hollmig et al., 2009). Dectin-2 seems more restricted to DCs than Dectin-1, but is also expressed on macrophages and inflammatory monocytes. It has been shown to bind high-mannose carbohydrates; nevertheless, the PAMPs recognized by Dectin-2 have not yet been fully identified. It seems that Dectin-2 not only recognizes fungal PAMPs, as the receptor might play a role in house dust mite allergy (Robinson et al., 2009).

Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), also known as CD209, is basically expressed on DCs. It has high affinity for intercellular adhesion molecule-3 (ICAM-3) and binds high-mannose glycoproteins like mannan. Thereby, DC-SIGN recognises a variety of microorganisms including certain viruses, bacteria and fungi (Svajger et al., 2010).

3.2.3. NOD-like receptors (NLRs)

Nucleotide-binding oligomerization domain (NOD)-like receptors are a family of cytoplasmatic molecules that recognise bacterial products within the intracellular compartment (Girardin et al., 2003a). NOD1/CARD4 and NOD2/CARD15 are two members of this pattern recognition family, which recognise substructures of bacterial peptidoglycan (Le Bourhis et al., 2007).

NOD1 detects meso-diaminopimelic acid (DAP)-containing peptidoglycan, which is common in Gram-negative bacteria and particular Gram-positive bacteria such as *Bacillus subtilis* and *Listeria monocytogenes.* NOD1 is known to be expressed by spleen cells, macrophages and a variety of epithelial cells (Chamaillard et al., 2003).

NOD2 senses muramyl dipeptide (MDP, MurNAc-L-Ala-D-isoGln), which is found in both Gram-negative and Gram-positive bacteria. However, NOD2 expression seems to be more restricted to certain cell types than NOD1 (Girardin et al., 2003b; Le Bourhis et al., 2007).

Mutations in NOD2 are thought to increase the susceptibility of Crohn's disease, an inflammatory disease of the intestine (Athman and Philpott, 2004).

3.3. Human epithelial cell lines

HO-1-N-1 (Nakata-1; JCRB0831) is a squamous carcinoma cell line isolated from the human buccal mucosa. Although the cell line was established by Moroyama and coworkers in 1986, HO-1-N-1 cells are scarcely characterised with regard to their expression of PRRs.

HO-1-u-1 (Ueda-1; JCRB0828) is a squamous carcinoma cell line isolated from the sublingual mucosa of a 72-year old Japanese male. The cell line was established by Miyauchi and coworkers in 1985. They observed a population doubling time of approximately 23 hours, the hyperdupoidy of the cell's karyotype and the formation of epithelial-like cell layers when grown on cell culture flasks.

In 2007, Uehara and colleagues postulated that HO-1-u-1 expressed the mRNA of TLR2, TLR3, TLR4, TLR7, NOD1 and NOD2, but did not secrete proinflammatory cytokines such as IL-8 in response to bacterial PAMPs.

Moreover, Wang and co-workers (2006, 2008) evaluated HO-1-u-1 cells grown on cell culture inserts as an in vitro model for studying sublingual drug delivery by passive diffusion.

Caco-2/15 and Caco-2 A9 are two heterogeneous human epithelial colorectal adenocarcinoma cell lines. Grown on cell culture inserts, Caco-2 cells are well established as in vitro model of the human intestinal mucosa (Sambuy et al., 2005).

4. Aims of the thesis

The aim of the master thesis was to characterise the expression of PRRs on human oral epithelial cells. For this purpose, we have investigated the expression of TLR 1-10, NLR NOD1 and NOD2, and CLR DC-SIGN, Dectin-1 and Dectin-2 by buccal and sublingual epithelial cell lines.

The presence of PRR mRNA was analysed by reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR (qPCR). Moreover, epithelial cell lines were stimulated with ligands specific for the respective receptors. IL-8 secretion as a readout for the activation of their signalling pathway was determined by ELISA.

In addition to oral epithelial cell lines, all experiments were performed with the well-established colorectal epithelial Caco-2 cell line.

5. Materials and Methods

5.1. Tissue culture

The HO-1-u-1 cell line derived from a sublingual squamous cell carcinoma and the HO-1-N-1 cell line derived from a buccal mucosa squamous cell carcinoma (*Table 2*) were purchased from the Health Science Research Resources Bank (Osaka, Japan).

Table 2: Characteristics of the cell lines HO-1-N-1 and HO-1-u-1.

Cell name	**HO-1-N-1**	**HO-1-u-1**
Cell number	JCRB0831	JCRB0828
Lot number	04092001	10172000
Source (genus/species)	Homo sapiens	Homo sapiens (72-year old male)
Tissue	Buccal mucosa	Sublingual mucosa
Case history	Squamous cell carcinoma	Squamous cell carcinoma
Life span	Infinite	Infinite
Morphology	Epithelial-like	Epithelial-like

5.1.1. Culturing of the cells

HO-1-N-1 and HO-1-u-1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) - Nutrient Mixture F-12 (Ham) + GlutaMAX^{TM} (GIBCO Invitrogen) supplemented with 10 % of fetal calf serum (FCS; PAA).

Caco-2/15 and Caco-2 A9 cells were cultured in DMEM high glucose (PAA) supplemented with 10 % of FCS and additional glutathione.

All cells were cultured at 37˚C in a humidified atmosphere with 5% CO_2.

5.1.2. Isolation of peripheral blood mononuclear cells (PBMC)

15 ml of Ficoll solution were overlaid with approximately 35 ml of heparinised blood, which had been diluted 1:2 with DPBS (GIBCO, Invitrogen), and centrifuged for 30 min at 1310 rpm (22°C, stop without brake). Then, the PBMC-containing interphase was transferred into a new tube, and DPBS was added to a volume of 50 ml. After centrifuging for 10 min at 1950 rpm (22°C, maximal brake), the pellet was vortexed, resuspended in 5 ml of DPBS, filled up

to 50 ml with DPBS and centrifuged for 8 min at 1780 rpm (22°C, maximal brake). The pellet was again vortexed, resuspended in 5 ml of DPBS and filled up to 50 ml with DPBS.

5.1.3. Separation of dendritic cells (DC)

DCs were separated from PBMCs using CD14 MicroBeads (MACS Miltenyi Biotec). The separation was performed according to the manufacturer's protocol.

5.2. Isolation of mRNA and transcription into cDNA

5.2.1. RNA isolation

Quiagen RNeasy kit, performed according to the manufacturer's instructions.

The cells were harvested by trypsinisation and centrifugation for 5 min at 300x g.
350 or 600 µl of RTL buffer containing ß-mercaptoethanol (depending on the amount of cells used for the isolation) were added to the harvested cells, and the lysate was homogenised using a QIAshredder spin column or a syringe.

Then, one volume (350 or 600 µl) of ethanol (70 %) was added to the sample, and up to 700 µl were pipetted on an RNeasy spin column which was spinned for 15 sec before discarding the flow-through. The column was washed with 700 µl of RW1 buffer and twice with 500 µl of RPE buffer (2 min centrifugation step after the second wash). In the end, the column was placed into a new 1.5 ml tube and the mRNA was finally eluted by adding 30 µl nuclease-free dH_2O directly on the membrane followed by a 1 min centrifugation step (max. speed). All pipetting steps were performed using filtered tips.

The final concentration of isolated RNA was measured at a wavelength of 260 nm on Nanodrop.

5.2.2. cDNA transcription

Applied Biosystems High-capacity cDNA reverse transcription kit, performed according to the manufacturer's instructions.

The assay was carried out in volumes of 20 µl, each reaction containing the reagents given in table 3.

Table 3: Reagents for cDNA transcription.

Reagent	**Amount per reaction**
dH_2O (nuclease-free)	3.2 µl
RNase inhibitor	1 µl
RT random primers (10x)	2 µl
dNTP mix	0.8 µl
RT buffer (10x)	2 µl
Reverse transcriptase	1 µl
Isolated RNA	10 µl

The cDNA transcription was performed in a thermocycler (Peqlab 96 Universal Gradient) using the following conditions:

25°C, 10 min
37°C, 120 min
85°C, 5 sec
4°C, indefinitely

Finally, the cDNA was stored at -20°C for further usage.

5.3. Reverse transcription polymerase chain reaction (RT-PCR)

Polymerase chain reaction (PCR) was developed in 1983 by Kary Mullis, and is a sensitive method for amplification and detection of small amounts of a specific target DNA.

Apart from the DNA of interest, specific oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), a reaction buffer optimized for magnesium chloride and a thermostable DNA polymerase are needed in order to perform PCR.

In a denaturation phase, the template DNA separates into single strands and in the annealing phase, the primers anneal to the specific target sequence. During the elongation phase, the DNA polymerase extends the primers by incorporating dNTPs and thereby amplifies the target DNA.

The amount of target DNA increases exponentially in the earlier amplification cycles, but reaches a plateau phase in the later cycles because of the decrease in reaction components. For this reason, RT-PCR is called a semi-quantitative method (Theophilus, 2008).

Primers specific for the different genes of interest (*Table 4*) were designed by Dr. Claudia Kitzmüller using the primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were purchased from Sigma Aldrich.

Table 4: RT-PCR primer.

Gene*	**Primer seqence**	**Length of PCR product**
TLR1a TLR1b	CAC TGA GAG TCT GCA CAT TGT GTG TCT CCA ACT CAG TAA GGT	566 bp
TLR2a TLR2b	TTT ATC GTC TTC CTG CTT CAA GCC C TCT CGC AGT TCC AAA CAT TCC ACG	350 bp
TLR3a TLR3b	GCA AAC CAC AAG CAT TCG GAA TCT G TTG AAG GCT TGG GAC CAA GGC A	713 bp
TLR4a TLR4b	TTT CTG CAA TGG ATC AAG GAC CAG GGA CAC CAC AAC AAT CAC CTT TCG G	440 bp
TLR5a TLR5b	CAG TGA CCA AAA CAG ATT CAA CC AAG AAA CCA GCC AAC ATC CTG	331 bp
TLR6a TLR6b	TCA CCA GAG GTC CAA CCT TAC CAA GTT GTT GCA AAG CTT CCA G	700 bp
TLR7a TLR7b	TCT ACC TGG GCC AAA ACT GTT GGC ACA TGC TGA AGA GAG TTA	388 bp
TLR8a TLR8b	CCG ACT TGG AAG TTC TAG ATC AAT GCT TCA TTT GGG ATG TGC T	316 bp
TLR9a TLR9b	CTT CCT CTA CAA ATG CAT CAC T GTG ACA GAT CCA AGG TGA AGT	488 bp
DC-SIGNa DC-SIGNb	CTC CCA GCG GAA CTG GCA CG GTT TGG GGT GGC AGG GGC TG	385 bp
Dectin-1a Dectin-1b	AGG TGC CAG CCT GGG GAT GT CCA GCC CCA TCC TGT AGG TTT CCA	311 bp
Dectin-2a Dectin-2b	AGG TGC CAG CCT GGG GAT GT CCA GCC CCA TCC TGT AGG TTT CCA	353 bp
NOD1a NOD1b	CAG CCT GTG CTC TGT GCC CC CCC TGC AGG CAC TGG AAC GG	555 bp
NOD2a NOD2b	CCT GTG CCC GCT GGT GTC TG CCA AGG CTT CAG CCA GGG CC	657 bp

* a: forward primer, b: reverse primer

All reagents were kept on ice. With the intention to avoid contamination, filtered tips were used and gloves were changed frequently. The PCR assay was carried out in volumes of 20

µl, each reaction containing the reagents given in table 5. To simplify the pipetting process, master mixtures were prepared.

Table 5: Reagents for RT-PCR.

Reagent	**Concentration**	**Amount per reaction**	**Company**
dH_2O (nuclease-free)	-	14.3 µl	-
dNTPs	10 mM	0.5 µl	Fermentas
PCR buffer	10x	2 µl	Finnzymes
forward primer	5 µM	1 µl	Sigma
reverse primer	5 µM	1 µl	Sigma
cDNA*	-	1 µl	-
Dynazyme pol.	-	0.2 µl	Finnzymes

* replaced by nuclease-free dH_2O in neg. controls

5.3.1. Evaluation of optimal PCR conditions for the primer pairs of TLR 1-9

As a rule of thumb, annealing temperature is expected to be 5-10°C below the lower Tm° of the primer pair. The Peqlab 96 Universal Gradient thermocycler is able to establish a temperature gradient, which results in a different temperature for each of the 12 rows (8 samples each) of the cycler. In this experiment, a gradient of 57°C +/- 10°C was used in order to achieve appropriate annealing temperatures for each PCR sample (*Table 6*).

Table 6: Evaluation of the optimal annealing temperature.

TLR	**Tm° primer a**	**Tm° primer b**	**annealing temp. – optimal range**	**annealing temperatures tested**
TLR1	60.6°C	57.3°C	47.3 - 52.3°C	47.0°C, 48.0°C, 49.5°C, 51.4°C
TLR2	70.6°C	72.8°C	60.6 - 65.6°C	60.1°C, 62.3°C, 64.3°C, 65.7°C
TLR3	72.8°C	73.6°C	62.8 - 67.8°C	62.3°C, 64.3°C, 65.7°C, 66.6°C
TLR4	70.0°C	72.8°C	60.0 - 65.0°C	60.1°C, 62.3°C, 64.3°C, 65.7°C
TLR5	64.4°C	65.1°C	54.4 - 59.4°C	55.8°C, 58.0°C, 60.1°C, 62.3°C
TLR6	62.9°C	65.8°C	52.9 - 57.9°C	53.6°C, 55.8°C, 58.0°C, 60.1°C
TLR7	64.5°C	62.0°C	52.0 - 57.0°C	51.4°C, 53.6°C, 55.8°C, 58.0°C
TLR8	59.4°C	66.5°C	49.4 - 54.4°C	48.0°C, 49.5°C, 51.4°C, 53.6°C
TLR9	59.8°C	60.6°C	49.8 - 54.8°C	48.0°C, 49.5°C, 51.4°C, 53.6°C

Expecting the Dynazyme polymerase to synthesize 2000 nucleotides per minute, an elongation time of 1 min was chosen.

- Sample: PBMC cDNA (diluted 1:5 in nuclease-free dH_2O)
- Negative control: nuclease-free dH_2O

PCR was performed in a thermocycler (Peqlab 96 Universal Gradient) using the following conditions:

95°C, 5 min

35 cycles:

Denaturation: 95°C, 30 sec

Annealing: gradient (57°C +/- 10°C) - individual annealing temperature for each primer pair (see table above), 40 sec

Elongation: 72°C, 1 min

72°C, 8 min

4°C, indefinitely

5.3.2. PCR screening of epithelial cell lines for TLR 1-9

- Samples: cDNA derived from the epithelial cell lines (diluted 1:5)
- Positive control: PBMC cDNA (diluted 1:5)
- Negative control: nuclease-free dH_2O

Amplification was performed in a thermocycler (Peqlab 96 Universal Gradient) using different cycle numbers:

95°C, 5 min

35, 30, 28 or 25 cycles:

Denaturation: 95°C, 30 sec

Annealing: gradient (57°C +/- 10°C) - individual annealing temperature for each primer pair (see *table 7*), 40 sec

Elongation: 72°C, 1 min

72°C, 8 min

4°C, indefinitely

Table 7: Individual annealing temperatures for each primer pair (see Results).

Primer pair	Ta°	Primer pair	Ta°	Primer pair	Ta°
TLR1	51.4°C	TLR4	64.3°C	TLR7	55.8°C
TLR2	62.3°C	TLR5	58.0°C	TLR8	48.0°C
TLR3	65.7°C	TLR6	60.1°C	TLR9	49.5°C

5.3.3. PCR screening of epithelial cell lines for DC SIGN, Dectin-1, Dectin-2, NOD1 and NOD2

- Samples: cDNA derived from the epithelial cell lines (diluted 1:5)
- Positive control: cDNA from PBMCs and DCs
- Negative control: nuclease-free dH_2O

PCR was performed in a thermocycler (Peqlab 96 Universal Gradient) using the following conditions:

95°C, 5 min

30 cycles:

Denaturation: 95°C, 30 sec

Annealing: 60°C, 40 sec

Elongation: 72°C, 1 min

72°C, 8 min

4°C, indefinitely

5.3.4. Evaluation of cDNA concentration by β-actin amplification

Human β-actin is a so-called house-keeping gene, and therefore, suitable for comparing the concentration of the different cDNA samples used in PCR screenings.

- Samples: cDNA, diluted 1:5 with nuclease-free dH_2O.
- Negative control: nuclease-free dH_2O

All reagents were kept on ice. With the intention to avoid contamination, filtered tips were used and gloves were changed frequently. The PCR assay was carried out in volumes of 20

µl, each reaction containing the reagents given in table 8. To simplify the pipetting process, master mixtures were prepared.

Table 8: Reagents for β-actin amplification.

Reagent	**Concentration**	**Amount per reaction**	**Company**
dH_2O (nuclease-free)	-	14.3 µl	-
dNTPs	10 mM	0.5 µl	Fermentas
PCR buffer	10x	2 µl	Finnzymes
ß-actin forward primer	5 µM	1 µl	Sigma Aldrich
ß-actin reverse primer	5 µM	1 µl	Sigma Aldrich
cDNA (1:5) *	-	1 µl	-
Dynazyme pol.	-	0.2 µl	Finnzymes

* replaced by nuclease-free dH_2O in neg. controls

β-actin amplification was performed in a thermocycler (Peqlab 96 Universal Gradient) using the following conditions:

95°C, 1 min

94°C, 3 min

25 cycles:

Denaturation: 95°C, 30 sec

Annealing: 55°C, 35 sec

Elongation: 72°C, 45 sec

72°C, 7 min

4°C, indefinitely

5.3.5. DNA gel electrophoresis

PCR products were analysed by DNA gel electrophoresis.

For a 1 % (w/v) gel, 1 g of agarose was added to 100 g of 1 x TBE buffer, and the mixture was heated in the microwave oven in order to solve the agarose. After cooling to approximately 40°C, 2 drops (60 µl) of a 0.07 % (w/v) ethidium bromide solution were added, and the gel was poured in a BioRad electrophoresis tray (BioRad Laboratories Ges.m.b.H.) with two 20-well combs. When the gel has finally polymerised, the combs were

removed and the gel was transferred to the electrophoresis chamber (BioRad Sub-Cell GT Agarose Gel Electrophoresis Systems), filled with 1 x TBE as a running buffer.

Before loading, 3 µl of a 6x DNA loading dye (Fermentas) were added to 15 µl of the samples. Subsequently, 18 µl of each sample and, additionally, 12-18 µl of DNA ladder (e.g. GeneRuler^{TM} 100 bp or FastRuler^{TM} middle range DNA ladder, Fermentas) were loaded on the gel, and electrophoresis was performed for 40 min at 90 volt.

Finally, DNA bands were visualized under UV-light.

5.4. Quantitative Real-time PCR (qPCR)

In real-time PCR, relative quantification of PRR mRNA was performed by measuring increasing fluorescence signals that results from SYBR green binding to double stranded DNA during amplification. The cycle number at which the detected fluorescence reaches a fixed threshold within the exponential phase of the DNA amplification is called threshold cycle number (Ct).
10x QuantiTect Primer Assays for the following PRRs were purchased from Quiagen (*Table 9*).

- Samples: cDNA derived from the epithelial cell lines
- qPCR controls: PBMC cDNA, DC cDNA
- Negative control: nuclease-free dH_2O

Table 9: QuantiTect primer assays.

Assay name	**Gene symbol**	**Cat. No.**	**Lot no.**
Hs_TLR1_3_SG	TLR1	QT01667218	98207961
Hs_TLR2_1_SG	TLR2	QT00236131	98207163
Hs_TLR3_1_SG	TLR3	QT00007714	98207962
Hs_TLR4_2_SG	TLR4	QT01670123	98207164
Hs_TLR5_3_SG	TLR5	QT01682079	98207265
Hs_TLR6_1_SG	TLR6	QT00216272	98207963
Hs_TLR7_1_SG	TLR7	QT00030030	98207266
Hs_TLR8_2_SG	TLR8	QT01666420	98207267

Hs_TLR9_1_SG	TLR9	QT00015183	98207268
Hs_TLR10_1_SG	TLR10	QT00205478	98207269
Hs_CD209_2_SG	CD209 (DC-SIGN)	QT01665447	98207271
Hs_CLEC7A_1_SG	CLEC7A (Dectin-1)	QT00024059	98207964
Hs_CLEC6A_2_SG	CLEC6A (Dectin-2)	QT01342292	98239840
Hs_NOD1_1_SG	NOD1	QT00054082	98207965
Hs_NOD2_1_SG	NOD2	QT00025872	98207270

The lyophilised primers were solved in TE-buffer (pH 8; Ambion) according to the manufacturer's instructions and stored at -20°C.

All reagents were kept on ice and, in order to avoid contamination, filtered tips were used. The PCR assay was carried out in volumes of 20 µl in a 96-well PCR plate. To simplify the pipetting process, master mixtures were prepared (see *table 10*). The amplifications were performed in triplicates, and a negative control without a template was included in each run.

Table 10: Reagents for qPCR.

Reagent	**Conc.**	**Amount/reaction**	**Company**
Power SYBR Green PCR Master Mix	2 x	10 µl	Applied Biosystems
QuantiTect Primer Assay	10 x	2 µl	Quiagen
cDNA template*	5 ng/µl	2 µl	-
Nuclease-free dH_2O	-	6 µl	-

* replaced by nuclease-free dH_2O in neg. controls

Additionally, the house keeping gene EF1 in human was amplified as an internal control gene (see *table 11*). Similar to β-actin, human EF1 is expressed at constant levels in all cell lines tested. The purpose of this internal standard is to normalise the PCR for the amount of DNA used in the reaction (Livak and Schmittgen, 2001).

Table 11: Reagents for EF1 amplification.

Reagent	**Conc.**	**Amount/reaction**	**Company**
Power SYBR Green PCR Master Mix	2 x	10 µl	Applied Biosystems
hEF1 Primer (forward)	500 nM	2 µl	Sigma Aldrich
hEF1 Primer (reverse)	500 nM	2 µl	Sigma Aldrich

cDNA template*	5 ng/µl	2 µl	-
Nuclease-free dH_2O	-	4 µl	-

* replaced by nuclease-free dH_2O in neg. controls

In the end, the wells of the 96-well PCR plate were sealed with a plastic foil, and the plate was centrifuged for 2 min at 1800 rpm to eliminate air bubbles.

Quantitative real-time PCR was performed with an ABI 7900 HT Fast Real-Time PCR System (Applied Biosystems) using the SDS 2.3 software.

Assay: Standard curve, 96-well plate, blank template

Thermal cycling parameters:
Step 1: 50°C, 2 min
Step 2 – enzyme activation: 95°C, 10 min
Step 3 – amplification (40 cycles): 95°C, 15 sec; 60°C, 1 min
Step 4 – dissociation curve: 95°C, 15 sec; 60°C, 15 sec

Data analysis of qPCR was performed using the SDS 2.3 software and Microsoft Office Excel.

The cycle number at which the detected fluorescence reaches a fixed threshold was determined from an exponential plot of the fluorescence vs. cycle number. A threshold cycle number (Ct) above cycle 35 implies negative results.

In order to compare the relative amounts of a DNA template in different cell lines, Ct values of the internal standard hEF1 was subtracted from Ct values of PRRs tested:

$\Delta Ct = Ct_{PRR} - Ct_{EF1}$

Specificity of the PCR was confirmed by dissociation/melting curve analysis. Hereby, dissociation of the PCR products is expected to result in one peak at a specific melting temperature. Several smaller peaks at different temperatures indicate contaminations, mispriming or primer-dimer artefacts.

5.5. Limulus Amebocyte Lysate (LAL) - Assay

PRR ligands used for stimulation of the cells were tested for bacterial endotoxin using QCL-1000 Endpoint Chromogenic LAL-Assay (Lonza). Hereby, Gram-negative bacterial endotoxin catalyses activation of an enzyme in LAL, which releases p-nitroaniline from a chromogenic substrate. The experiment was performed according to the manufacturer's instructions.

5.5.1. Microplate-Method

50 µl of the standards (*E. coli* Endotoxin: 1.0, 0.5, 0.25, 0.1 and 0.0 EU/ml) and samples were pipetted in the wells of a 96-well-plate at 37°C (heating block) in duplicates. At the time point T = 0 min, 50 µl of LAL were added to each well, and at T = 10 min, 100 µl of pre-warmed chromogenic substrate solution (2 mM) were added. Finally, the reaction was stopped at T = 16 min by addition of 50 µl of 25 % v/v acetic acid per well, and absorbance was measured at 405 nm at the Spectra Max Plus 384 plate reader (Molecular Devices) using SOFTmax Pro 4.8 software.

In order to determine the proper endotoxin concentration of the samples, endotoxin-free materials and water were used in the experiment, and all reagents were pipetted in the same order.

5.6. Stimulation of epithelial cell lines with PRR ligands

HO-1-N-1, HO-1-u-1 and Caco-2 cells (40 000 cells per well in 100 µl) were seeded in a flatbottomed 96-well plate (Corning B.V. Life Sciences) in their appropriate culture medium, and were incubated for 24 h at 37°C in a humidified atmosphere containing 5 % CO_2. Then, medium was removed, and the cells were stimulated with three different concentrations of PRR ligands (*Table 12*) in a final volume of 100 µl in fresh medium. The assay was performed in triplicates, and cells in plain medium without any stimuli were used as negative control.

Table 12: PRR ligands.

PRR ligands (Source)	**Company**	**PRR**	**Conc. 1**	**Conc. 2**	**Conc. 3**
Pam_3CSK_4 (synthetic)	InvivoGen	TLR1, TLR2	10 µg/ml	1 µg/ml	100 ng/ml
Poly(I:C) (synthetic)	InvivoGen	TLR3	100 µg/ml	10 µg/ml	1 µg/ml

LPS (*Escherichia coli*)	-	TLR4	1 µg/ml	100 ng/ml	10 ng/ml
Flagellin (*Bacillus subtilis*)	InvivoGen	TLR5	20 nM	2 nM	200 pM
FSL-1 (*Mycoplasma salivarium*)	InvivoGen	TLR2, TLR6	100 ng/ml	10 ng/ml	1 ng/ml
R848 (synthetic)	InvivoGen	TLR7, TLR8	1 µg/ml	100 ng/ml	10 ng/ml
Unmethylated CpG DNA	-	TLR9	10 µM	1 µM	100 nM
iE-DAP (synthetic)	InvivoGen	NOD1	100 µg/ml	10 µg/ml	1 µg/ml
MDP (synthetic)	InvivoGen	NOD2	100 µg/ml	10 µg/ml	1 µg/ml
Curdlan (*Alcaligenes faecalis*)	Sigma	Dectin-1	100 µg/ml	10 µg/ml	1 µg/ml
Mannan (*Saccharomyces cerevisiae*)	Sigma	DC-SIGN	1 mg/ml	100 µg/ml	10 µg/ml

The cells were incubated for another 24 h at 37°C/5 % CO_2. Finally, supernatants were collected, and the level of IL-8 secretion was determined by cytokine ELISA.

5.7. Thymidine incorporation

Medium containing [3H]-thymidine was added to the stimulated cells after collecting supernatants for ELISA. After 12-18 h of incubation at 37°C/5 % CO_2, the cells were harvested on a filter. The filter was dried in the microwave, and sealed in plastic foil after addition of 4 ml of scintillation fluid (Betaplate scint, Perkin Elmer). Finally, a beta-counter was used for measuring the radioactivity of incorporated [3H]-thymidine, being directly proportional to the number of living cells.

5.8. Determination of IL-8

A Nunc Maxisorp 96 well ELISA plate (Thermo Scientific Inc.) was coated with 100 µl of an anti-human IL-8 coating antibody (SIL-8, Lot IJ107699; Thermo Fisher Scientific), diluted to a concentration of 2 µg/ml in carbonate buffer (pH 9.6), and incubated overnight at room temperature (RT).

On the next day, the coating-antibody solution was discarded, and unspecific binding sites were blocked with 150 µl/well of PBS containing 0.05 % Tween 20 and 4 % (w/v) of bovine serum albumin (PBS/T/BSA) for 1-2 h at RT.

Afterwards, the plate was washed three times with PBS/0.05 % Tween (PBS/T), and 50 µl of the collected supernatants (see "*Stimulation of the epithelial cell lines with PRR ligands"*) were transferred to the plate. Due to the high level of IL-8 secretion observed, supernatants of HO-1-N-1 cells were diluted 1:100 in PBS/T/BSA while supernatants of HO-1-u-1 cells were diluted 1:250 in PBS/T/BSA. Additionally, IL-8 was applied as a standard in several concentrations in the range of 500-0 pg/ml in PBS/T/BSA.

After 1 h, the standards/samples were discarded, and adsorbed IL-8 was bound by a biotinylated anti-IL-8 antibody in a concentration of 0.2 µg/ml, diluted in PBS/T/BSA (1 h, RT). Then, the plate was washed three times with PBS/T, and Biotin was detected using horseradish peroxidise conjugated streptavidin in a dilution of 1:20.000.

Finally, the plate was washed once more, and the wells were incubated with 100 µl of the chromogenic substrate 3,3',5,5'-Tetramethylbenzidine (TMB; Chemicon International Inc) for 30 min in the dark. The colour reaction was stopped by addition of 0.18 M H_2SO_4 (100 µl/well), and extinction was measured at a wavelength of 450 nm (reference wavelength: 630 nm) at the Spectra Max Plus 384 plate reader (Molecular Devices) using SOFTmax Pro 4.8 software. Detected IL-8 was quantified by means of extinction values measured for IL-8 standards.

5.9. Buffers and solutions

10 x phosphate buffered saline (PBS):

80 g NaCl

2 g KCl

14.4 g Na_2HPO_4

2.4 g KH_2PO_4

Fill to 1000 ml with H_2O bidest., pH 7.4

10 x Tris/Borate/EDTA (TBE) buffer:

54 g Tris

27.5 g boric acid

4.65 g Na_4EDTA

Fill to 1000 ml with H_2O bidest.

Carbonate buffer:

37 mM Na_2CO_3

63 mM $NaHCO_3$

Solve in H_2O bidest.

6. Results

6.1. Qualitative detection of PRR mRNA by RT-PCR

In order to perform PCR experiments, mRNA was isolated from epithelial cell lines and transcribed into cDNA. Quality and quantity of the resulting cDNA was confirmed by amplification of β-actin.

6.1.1. Evaluation of optimal PCR conditions for the primer pairs of TLR 1-9

First, optimal annealing temperatures for the different primer pairs used in RT-PCR to detect TLR were evaluated. For this purpose, cDNA isolated from PBMCs was used, as these cells express all TLRs. Each primer pair was tested at four different annealing temperatures (Ta) within the optimal temperature range of the primers. Resulting PCR products were visualised by gel electrophoresis using EtBr-staining. Bands resulting from unspecific amplification indicated that the Ta was too low. A reduced yield of the correct product indicated that the Ta was too high. Figure 1A and 1B depict the PCR products amplified under different PCR conditions. Deduced from these results, the optimal Ta were defined as summarised in table 13.

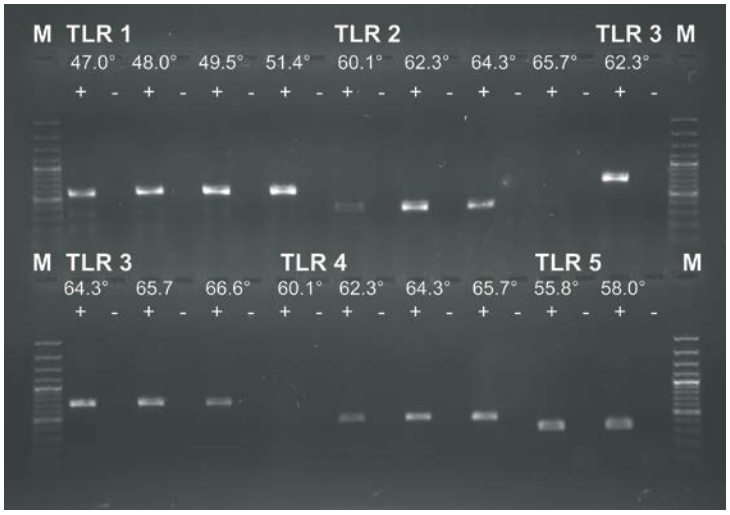

Fig. 1A

Fig. 1A/B: **RT-PCR of different TLR-primers using different annealing temperatures for each primer-pair (35 amplification cycles).**
M = marker (GeneRuler™ 100 bp DNA ladder, Fermentas), + = cDNA, - = neg. control w/o cDNA

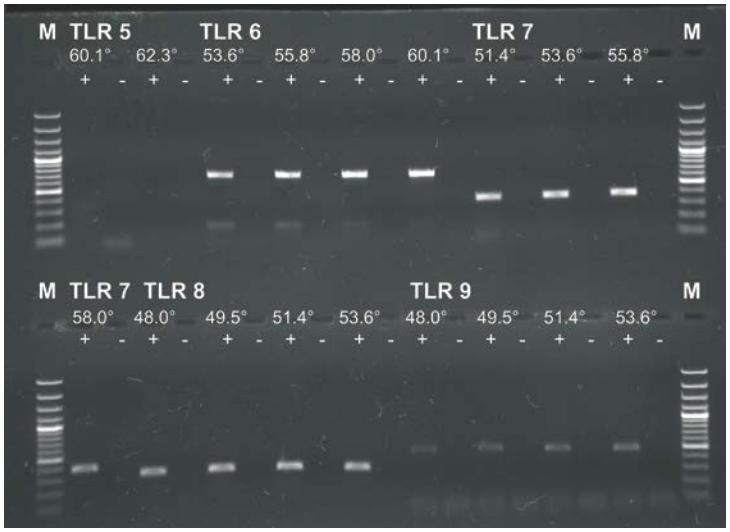

Fig. 1B

Table 13: Optimal Ta for each primer pair used in RT-PCR to detect TLR

Primer pair	Ta°	Primer pair	Ta°	Primer pair	Ta°
TLR1	51.4°C	TLR4	64.3°C	TLR7	55.8°C
TLR2	62.3°C	TLR5	58.0°C	TLR8	48.0°C
TLR3	65.7°C	TLR6	60.1°C	TLR9	49.5°C

PCR conditions evaluated in this experiment were used in all following RT-PCR experiments.

6.1.2. PCR screening of buccal and sublingual cell lines for TLR 1-9 expression

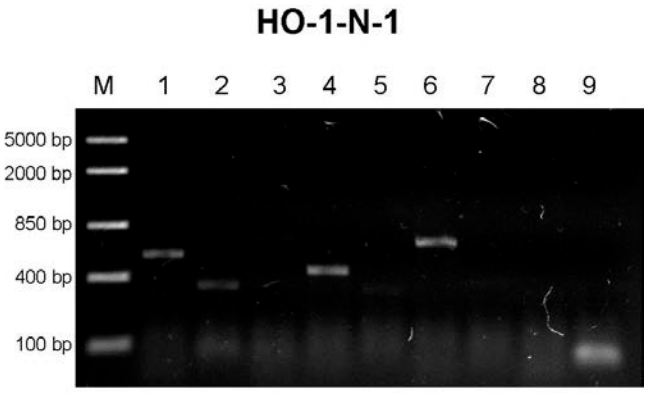

Fig. 2: **PCR screening of HO-1-N-1 cells for TLR 1-9 using 28 amplification cycles.** M = marker (FastRuler^{TM} middle range DNA ladder, Fermentas). The results presented are representative of two independent experiments demonstrating similar results.

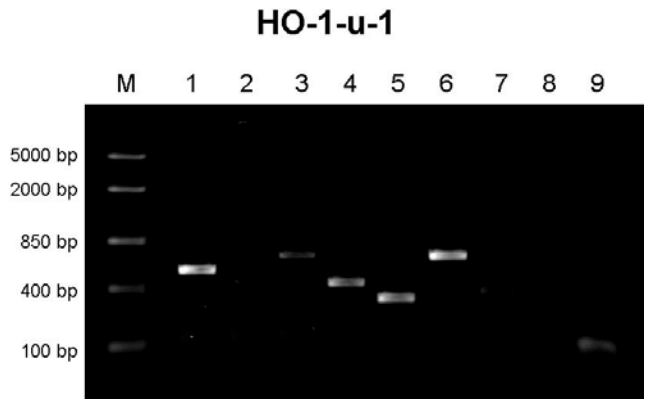

Fig. 3: **PCR screening of HO-1-u-1 cells for TLR 1-9 using 30 amplification cycles.** M = marker (FastRuler™ middle range DNA ladder, Fermentas). The results presented are representative of two independent experiments demonstrating similar results.

Analysing the expression of TLR 1-9 in the buccal cell line HO-1-N-1, best results were achieved after 28 amplification cycles. As seen in figure 2, mRNA coding for TLR1, TLR2, TLR4 and TLR6 was detected.

Screening the sublingual cell line HO-1-u-1 for TLR 1-9, significant results were achieved after 30 amplification cycles. It was found that HO-1-u-1 expressed the genetic information for TLR1, TLR3, TLR4, TLR5 and TLR6 (*Fig. 3*).

6.1.3. PCR screening of intestinal epithelial cell lines for TLR 1-9 expression

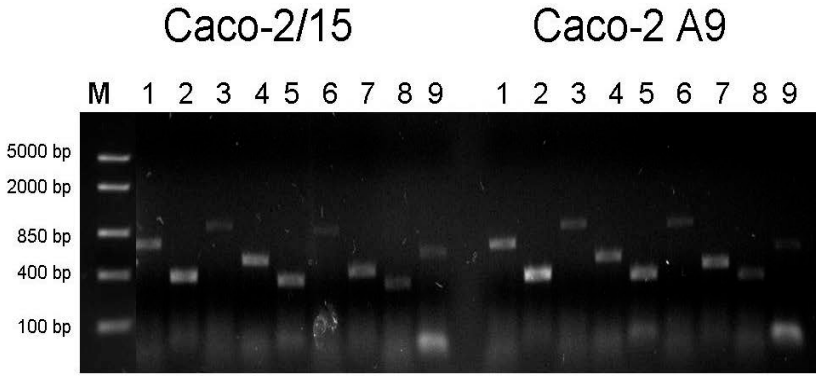

Fig. 4: **PCR screening of Caco-2/15 and Caco-2 A9 cells for TLRs 1-9 using 30 amplification cycles.** M = marker (FastRuler™ middle range DNA ladder, Fermentas). The results presented are representative of two independent experiments demonstrating similar results.

The colorectal epithelial cell lines Caco-2/15 and Caco-2 A9 were found to express mRNA coding for TLR 1-9. Nonetheless, bands representing TLR3, TLR6, TLR8 and TLR9 were of low intensity and barely visible in the EtBr-stained agarose gel (*Fig. 4*).

TLR10 was not tested in any of the RT-PCR experiments.

6.1.4. PCR screening for DC-SIGN, Dectin-1, Dectin-2, NOD1 and NOD2 expression

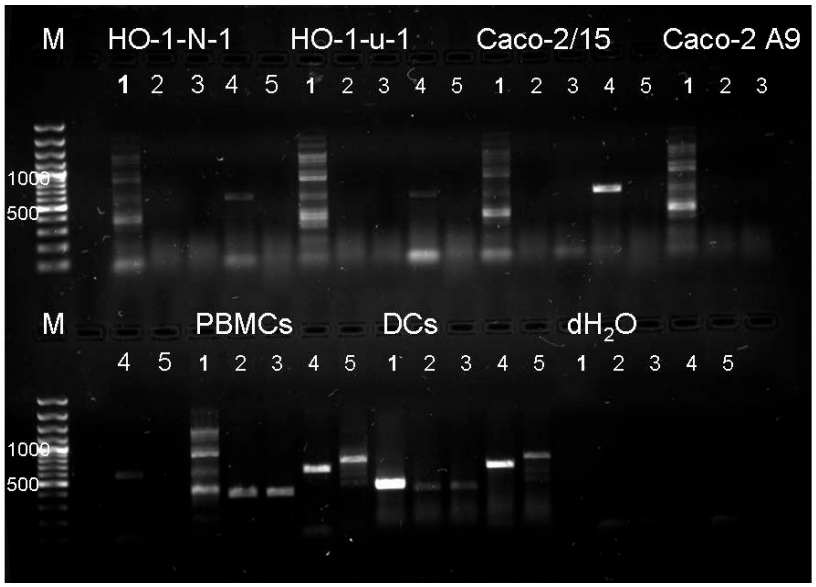

Fig. 5: **PCR screening for C-type lectins and NLRs using 30 amplification cycles.**
M = marker (GeneRuler™ 100 bp DNA ladder, Fermentas), pos. control = PBMC and DC cDNA, neg. control = dH2O instead of cDNA. The results presented are representative of two independent experiments demonstrating similar results.

1… DC-SIGN
2… Dectin-1
3… Dectin-2
4… NOD1
5… NOD2

Analogue to the TLR experiments, RT-PCR screening was performed for the C-type lectins DC-SIGN, Dectin-1 and Dectin-2 and NLRs NOD1 and NOD2.

Epithelial cell lines tested in the experiment were only positive for DC-SIGN and NOD1 (*Fig. 5*). Dectin-1, Dectin-2 as well as NOD2 could not be detected by RT-PCR.

PBMCs and DCs express RNA of all PRRs tested, namely DC-SIGN, Dectin-1, Dectin-2, NOD1 and NOD2, and were therefore used as positive controls.

Interestingly, amplifying DC-SIGN cDNA of epithelial cell lines and PBMCs, seven transcripts could be detected on the gel. In contrast, when analysing the cDNA of the DCs, only one DC-SIGN transcript was amplified.

6.1.5. Limitations of RT-PCR screening

Considering the difference of the bands visible on the agarose gel in terms of intensity, it was supposed that some PRRs are expressed to a lower extent than others. However, RT-PCR is only a semi-quantitative method, and is not suitable for comparing the total amount of mRNA coding for a single PRR.

Traditional RT-PCR detects the amount of replicated cDNA at the end-point of the PCR when the reaction has reached the plateau phase. However, this end-point is variable from sample to sample, which results in different quantities of amplicons at the end of the PCR even when

the same amount of cDNA was used. Moreover, agarose gel resolution is very poor, and EtBr-staining has a relatively low sensitivity.

In qPCR, amplicated cDNA is measured at the exponential growth phase of the reaction, and the number of amplicons is directly proportional to an increase in reporter dye fluorescence. Consequently, qPCR is more sensitive than end-point PCR, and has a higher objectivity as the results are expressed in numbers.

Therefore, qPCR was performed to confirm the results of RT-PCR and to quantify the relative amount of specific PRR mRNA expressed by the different cell lines.

6.2. Detection of PRR mRNA by quantitative real-time RT-PCR (qPCR)

In order to compare the relative amounts of PRR mRNA expressed by a certain cell line, Δ Ct was determined, which is equal to the difference in the Ct values between the PRRs and the internal standard EF1 (see Materials and Methods): $\Delta Ct = Ct_{PRR} - Ct_{EF1}$

As SYBR green binds to any double stranded DNA, specificity of the amplified PCR products was confirmed by melting curve analysis.

Table 14: Δ Ct values for TLR 1-10

Δ Ct	**HO-1-N-1**	**HO-1-u-1**	**Caco-2/15**	**Caco-2 A9**	**PBMC**	**DC**
TLR1	8,9	11	b	15,3	8,4	6,8
TLR2	9	10,4	9,3	7,9	7	5,7
TLR3	8,9	9,2	13,7	11,4	11,7	8,5
TLR4	9,6	11,7	14	13,1	7,3	6
TLR5	a	11,7	13,3	12,9	11,8	9,6
TLR6	6	8	12,7	11,3	7,8	5,5
TLR7	b	15,6	13,6	13,1	9,1	9,3
TLR8	14,7	14,4	14,6	13,1	7,7	4,8
TLR9	b	b	b	b	15,1	b
TLR10	10,7	10,3	12	11,5	8,2	10,3

a…no specific cDNA amplified within 40 cycles; b…Ct > 35

Δ Ct values evaluated for HO-1-N-1, HO-1-u-1, Caco-2/15, Caco-2 A9, PBMCs and DCs for TLR 1-10 are given in table 14. Hence, quantitative real-time PCR indicated that the buccal cell line HO-1-N-1 express TLR 1-4 and TLR6 as well as, to a lesser extent, TLR8 and TLR10. TLR7 and TLR9 were considered negative, as their Ct value exceeded cycle number 35. In case of TLR5, no specific mRNA could be detected within the 40 amplification cycles performed.

In the sublingual HO-1-u-1 cells, all TLRs except of TLR9 could be detected. Nevertheless, TLR3 and TLR6 passed the threshold at lower cycle numbers than the other receptors indicating a higher amount of mRNA expressed for this receptor.

The intestinal Caco-2 cell lines seem to express mainly TLR2 mRNA. However, marginal amounts of mRNA were found for all TLRs except of TLR1 (in case of Caco-2/15) and

TLR9. Nevertheless, compared to the other cell lines, colorectal cells express lower levels of TLR mRNA.

As expected, mRNA coding for TLR 1-10 was detected in PBMCs. Nevertheless, the highest amounts of mRNA coding for TLR 1-8 were found in DCs. They also express TLR10 but to a lower extent.

Table 15: Δ Ct values for CLRs and NLRs

Δ Ct	**HO-1-N-1**	**HO-1-u-1**	**Caco-2/15**	**Caco-2 A9**	**PBMC**	**DC**
DC-SIGN	a	a	a	a	14,6	3,4
Dectin-1	9,7	7,5	b	b	7,4	2,7
Dectin-2	a	a	18,5	b	10,8	10,4
NOD1	10,6	10,6	10,9	10,4	9,8	7
NOD2	14,2	a	a	b	9,5	7,9

a…no specific cDNA amplified within 40 cycles; b…Ct > 35

Δ Ct values evaluated for HO-1-N-1, HO-1-u-1, Caco-2/15, Caco-2 A9, PBMCs and DCs for DC-SIGN, Dectin-1, Dectin-2, NOD1 and NOD2 are given in table 15. qPCR indicted that HO-1-N-1 cells were positive for Dectin-1, NOD1 and NOD2. HO-1-u-1 cells were only found positive for Dectin-1 and NOD1. In the Caco-2 cell lines, NOD1 mRNA could be detected, and Caco-2/15 additionally showed traces of Dectin-2 expression. PBMCs expressed Dectin-1, Dectin-2, NOD1 and NOD2 as well as DC-SIGN. DCs were equally positive for these PRR, but seem to express mainly DC-SIGN and Dectin-1 mRNA.

6.3. Stimulation of epithelial cell lines with PRR ligands

To study the functional response of the cells to PRR ligands, epithelial cell lines were stimulated with PAMPs specific for the respective receptors. Recognition of PAMPs by PRRs result in activation of multiple intracellular signalling events and secretion of proinflammatory cytokines. The level of IL-8 production as a readout for the activation of their signalling pathway was determined by cytokine ELISA. In order to avoid false positive and false negative results, all ligands were tested for endotoxin contamination, and the viability of the activated cells was tested by [3H]-thymidine incorporation (data not shown). Notably, excessive basal levels of IL-8 production were observed in the oral epithelial cell lines HO-1-N-1 and HO-1-u-1 cells. In comparison, colorectal Caco-2 cells produced rather small amounts of IL-8 without any stimulus added to the culture medium.

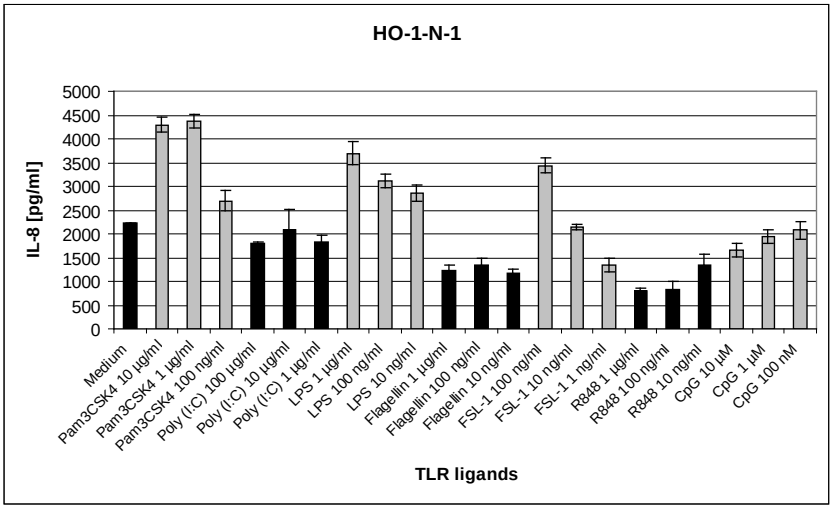

Fig. 6: **IL-8 production of the buccal cell line HO-1-N-1 in response to treatment with different concentrations of TLR ligands.** Values represent the means +/- SD of the results of triplicate samples, and are representative of three independent experiments demonstrating similar results.

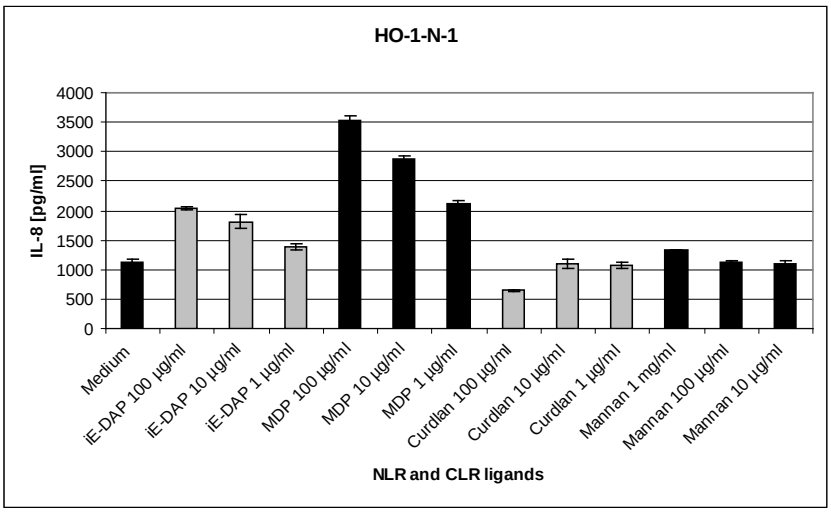

Fig. 7: **IL-8 production of the buccal cell line HO-1-N-1 in response to treatment with different concentrations of NLR and CLR ligands.** Values represent the means +/- SD of the results of triplicate samples, and are representative of three independent experiments demonstrating similar results.

When challenged with different TLR stimuli, buccal HO-1-N-1 cells up-regulated IL-8 secretion in response to Pam_3CSK_4, a TLR1/2 ligand, bacterial LPS, a TLR4 ligand, and to the TLR2/6 heterodimer agonist FSL-1 (*Fig. 6*). Furthermore, the NOD1 and NOD2 ligands iE-DAP and MDP induced increased IL-8 production in a dose-dependent manner (*Fig. 7*). No effects were observed for Poly(I:C), bacterial flagellin and R848 as well as for curdlan and mannan. Moreover, as already reported by Fitzner and coworkers in 2008, unmethylated CpG DNA had a suppressive effect on IL-8 production.

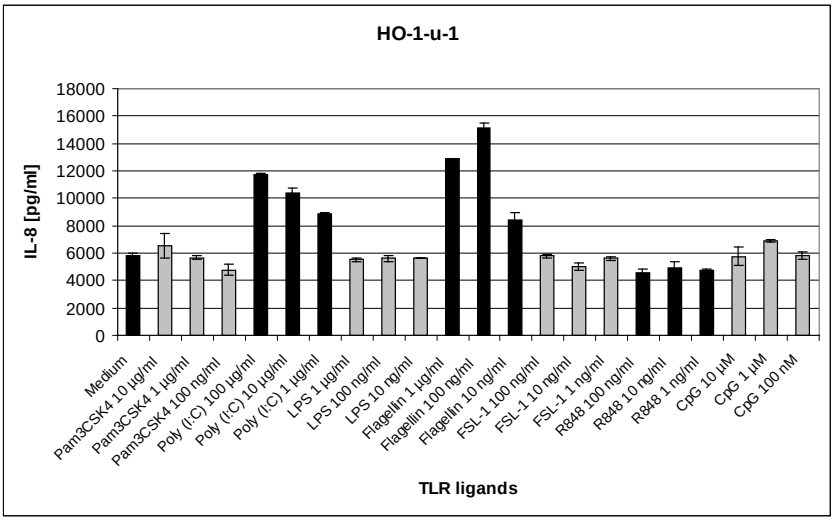

Fig. 8: **IL-8 production of the sublingual cell line HO-1-u-1 in response to treatment with different concentrations of TLR ligands.** Values represent the means +/- SD of the results of triplicate samples, and are representative of three independent experiments demonstrating similar results.

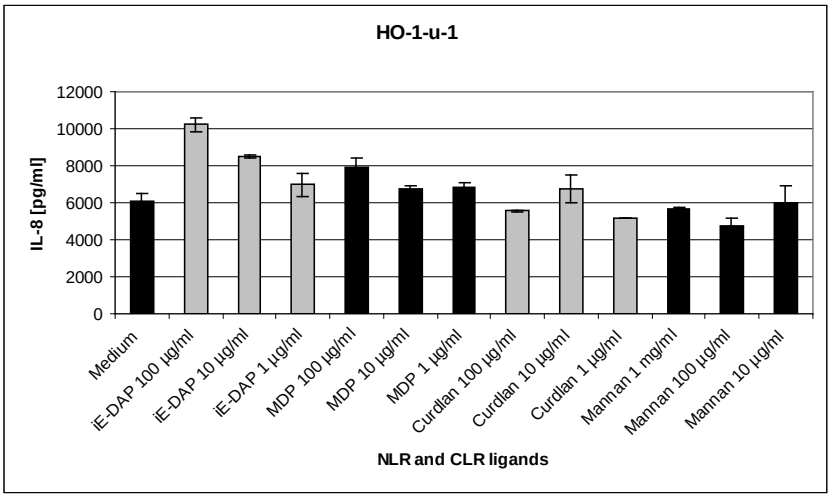

Fig. 9: **IL-8 production of the sublingual cell line HO-1-u-1 in response to treatment with different concentrations of NLR and CLR ligands.** Values represent the means +/- SD of the results of triplicate samples, and are representative of three independent experiments demonstrating similar results.

In the sublingual cell line HO-1-u-1, increased IL-8 secretion was observed upon stimulation with the TLR3 ligand Poly(I:C), the TLR5 ligand flagellin (*Fig. 8*), and the NOD1 ligand iE-DAP (*Fig. 9*). The other PRR ligands used in the experiment did not modify IL-8 release.

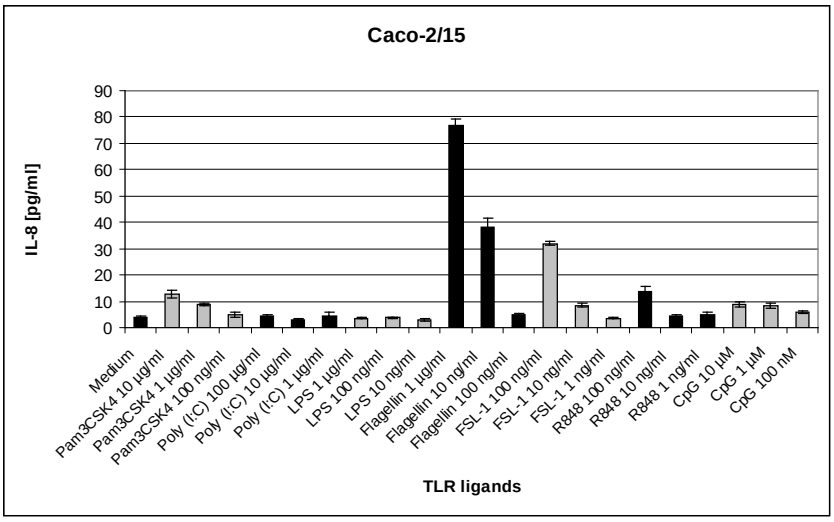

Fig. 10: **IL-8 production of Caco-2/15 cells upon stimulation with different concentrations of TLR ligands.** Values represent the means +/- SD of the results of triplicate samples, and are representative of three independent experiments demonstrating similar results.

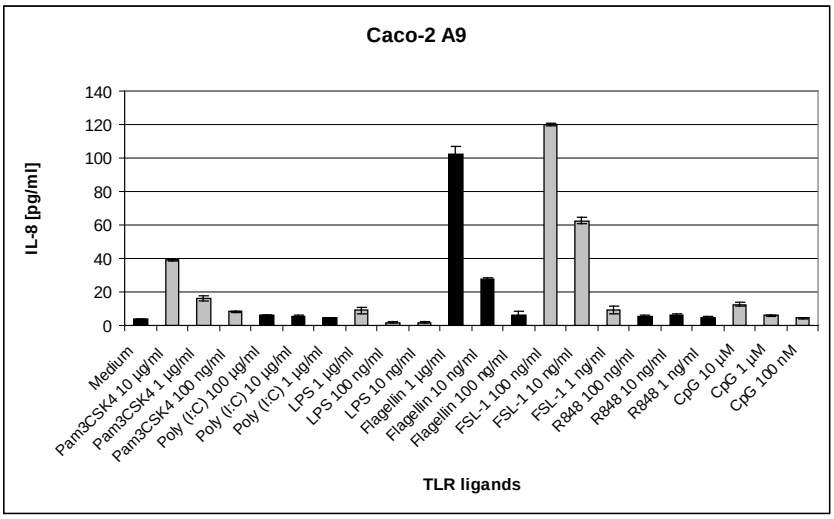

Fig. 11: **IL-8 production of Caco-2 A9 cells upon stimulation with different concentrations of TLR ligands.** Values represent the means +/- SD of the results of triplicate samples, and are representative of three independent experiments demonstrating similar results.

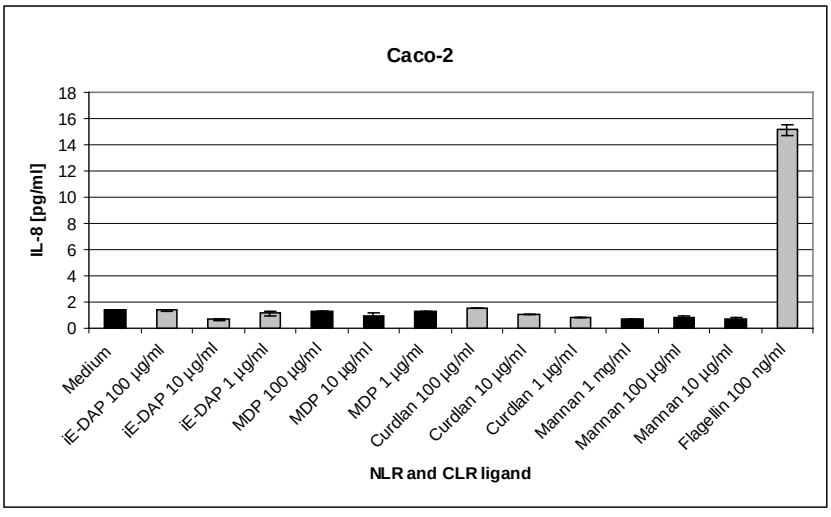

Fig. 12: **IL-8 production of Caco-2 cells upon stimulation with different concentrations of NLR and CLR ligands.** Values represent the means +/- SD of the results of triplicate samples, and are representative of three independent experiments demonstrating similar results. Flagellin was used as a positive control.

IL-8 production in response to different TLR ligands of Caco-2/15 cells was comparable to that of Caco-2 A9 cells. Both intestinal cell lines showed significant up-regulation of IL-8 secretion in response to flagellin (TLR5) and FSL-1 (TLR2/6) [*Fig. 10, 11*]. Besides, Caco-2 A9 cells significantly increased IL-8 secretion in response to stimulation with Pam_3CSK_4

(TLR1/2). No significant responses were noted upon stimulation with NLR and CLR ligands (*Fig. 12*).

6.4. Summary

Table 16: Summary of PRR expression by epithelial cell lines.

cell type	**HO-1-N-1**			**HO-1-u-1**			**Caco-2/15**			**Caco-2 A9**		
method	PCR	qPCR	IL-8	PCR	qPCR	IL-8	PCR	qPCR	IL-8	PCR	qPCR	IL-8
TLR1	++	++	+	++	+	-	+	-	-	+	(+)	++
TLR2	+	++	+	-	+	-	++	++	+	++	++	+++
TLR3	-	++	-	+	++	++	+	+	-	+	+	-
TLR4	++	++	+	++	+	-	++	+	-	++	+	-
TLR5	-	-	-	++	+	+++	++	+	+++	++	+	+++
TLR6	++	++	+	++	++	-	+	+	+	+	+	+++
TLR7	-	-	-	-	(+)	-	++	+	-	++	+	-
TLR8	-	+	-	-	+	-	+	+	-	+	+	-
TLR9	-	-	-	-	-	-	+	-	-	+	-	-
TLR10	ND	+	ND	ND	+	ND	ND	+	ND	ND	+	ND
DC-SIGN	(+)	-	-	(+)	-	-	(+)	-	-	(+)	-	-
Dectin-1	-	++	-	-	++	-	-	-	-	-	-	-
Dectin-2	-	-	ND	-	-	ND	-	(+)	ND	-	-	ND
NOD1	+	+	+	+	+	+	++	+	-	+	+	-
NOD2	-	+	+++	-	-	-	-	-	-	-	-	-

PCR:

RT-PCR: +++ strong band, ++ clear band, + weak band, (+) unspecific bands, - no band

qPCR: +++ Δ Ct $\leq$ 5, ++ Δ Ct $\leq$ 10, + Δ Ct $\leq$ 15, (+) Δ Ct $\leq$ 20, - Δ Ct > 35

IL-8 ELISA:

HO-1-N-1, HO-1-u-1: +++ >2.5x, ++ 2.5–2.0x, + 2.0–1.5x, - <1.5x more IL-8 secretion than unstimulated cells

Caco-2/15, Caco-2 A9: +++ >15x, ++ 15–10x, + 10–5x, - <5x more IL-8 secretion than unstimulated cells

ND: not determined

In summary (see *table 16*), we found that human buccal cells express and respond to stimulation of TLR1, TLR2, TLR4 and TLR6 as well as NOD1 and NOD2. Human sublingual cells were found to express and respond to stimulation of TLR3 and TLR5 as well as NOD1. Considering the Caco-2 cell lines, we observed mRNA expression and response to stimulation of TLR2, TLR5 and TLR6, and additionally, TLR1 in case of Caco-2 A9 cells. Several other PRRs were detected by PCR. However, stimulation with the respective ligands did not induce activation of these cells.

7. Discussion

Epithelial cells lining the oral cavity play an important role as a physical barrier against microbial invasion in the mouth. Besides, several immunological elements are integrated into this barrier, which allow the recognition of antigens (Novak et al., 2011). During sublingual immunotherapy (SLIT), where allergen extracts are administered under the tongue of allergic patients, epithelial cells may also play an important role. However, so far the immune-reactivity of human oral epithelia has not been well defined. Therefore, we determined PRRs expressed by epithelial cells of the oral cavity and evaluated their role in PAMP recognition. For this purpose, the expression of toll-like receptor (TLR) 1-10, C-type lectin receptors DC-SIGN, Dectin-1 and Dectin-2 and NOD-like receptors NOD1 and NOD2 was analysed in both a buccal mucosa cell line, HO-1-N-1, and a sublingual epithelial cell line, HO-1-u-1. A more detailed knowledge of the oral mucosa will help to reveal the immunological mechanisms underlying SLIT of type I allergy, and consequently, to increase its efficacy.

The term “allergy” was first introduced in 1906 by Clemens von Pirquet and is defined as a hypersensitivity reaction against otherwise non-infectious environmental substances called allergens (Galli et al., 2008). Most allergens are proteins of 5-80 kDa (Valenta, 2002), and some of them have enzymatic properties. However, the reason why some proteins, lipids or carbohydrates function as allergens is still relatively unclear.

Type I allergy is the most common allergic/atopic disorder and is characterized by antigen-specific IgE and a Th2-mediated immune response (Kay, 2001). During sensitization phase, when an atopic person first encounters a certain allergen, the allergen is taken up by APCs, e.g. DCs, which present peptides derived from processed allergens in combination with a MHC class II molecule to naïve T cells. CD4+ T cells differentiate into Th2 cells and produce particular cytokines such as IL-4 and IL-13. These cytokines are responsible for IgE production by promoting Ig class switching of specific B cells, which have also captured the allergen. Sensitisation leads to the establishment of allergen-specific memory B and T cells. Moreover, circulating IgE antibodies bind to the high-affinity IgE receptor FcεRI on mast cells, basophils or other APCs. Cross-linking of IgE-FcεRI complexes on mast cells and basophils by the multiple binding sites of an allergen leads to degranulation and subsequent release of biogenic amines (e.g. histamine), lipid mediators and cytokines. Binding of, for example, histamine to the epithelium increases vascular permeability and stimulates cell contraction.

Late-phase reactions develop 2-6 h after immediate reactions and peak 6-9 h after allergen exposure. They reflect the activation of allergen-specific T cells, releasing proinflammatory cytokines, activation of eosinophils and other mechanisms (Galli et al., 2008; Larché et al., 2006, Kay, 2001; Valenta, 2002).

SLIT is a non-invasive treatment to reduce clinical reactivity to allergens by repeated administration of high doses of the sensitising allergen under the tongue of allergic patients (Larché et al., 2006; Frew, 2008). The epithelium of the sublingual mucosa is thinner than that of other oral mucosal sites, and therefore, was chosen for allergen application (Novak et at., 2011). Although the underlying immunological mechanisms of SLIT are not completely understood, several studies have documented systemic immunological changes such as an enhancement of IL-10 or TGFβ-producing regulatory T cells (Bohle et al., 2007) and production of allergen-specific IgG, mainly the subclass IgG4 (Fanta et al., 1999; Scadding et al., 2010), which competes with allergen-specific IgE for allergen binding sites.

A promising novel approach in SLIT is to apply the allergen in combination with a PAMP to promote allergen-specific regulatory T cells and a shift towards Th1 responses (Novak et al., 2011). In order to use the right PAMPs, knowledge of the functional expression of PRRs by the oral epithelium is required.

The present work shows that the buccal cell line HO-1-N-1 expressed mRNA for TLR1, TLR2, TLR4 and TLR6 as well as NOD1 and NOD2 and increased IL-8 synthesis in response to ligands specific for these receptors. TLR2 senses various components from bacteria, fungi or viruses. However, forming a heterodimer with TLR1 or TLR6, TLR2 recognises triacylated or diacylated bacterial lipopeptides, respectively. TLR4 is mainly known for recognition of LPS of Gram-negative bacteria (Takeuchi and Akira, 2010), and the NLRs NOD1 and NOD2 recognise substructures of bacterial peptidoglycan (Le Bourhis et al., 2007). qPCR also indicated the expression of TLR3, TLR8, TLR10 and Dectin-1 in HO-1-N-1, but the cells did not respond to stimulation with ligands specific for these receptors.

Analysing the sublingual HO-1-u-1 cell line, mRNA coding for TLR1, TLR3, TLR4, TLR5, TLR6 and NOD1 was detected by end-point RT-PCR. The higher sensitivity of qPCR resulted in the additional detection of TLR2, TLR7, TLR8 and TLR10 and Dectin-1. However, HO-1-u-1 cells only responded to TLR3-, TLR5- and NOD1-specific ligands with increased IL-8 secretion. TLR3 recognises viral double-stranded RNA, whereas TLR5 binds bacterial flagellin (Takeuchi and Akira, 2010). NOD1 detects DAP-containing peptidoglycan

common in Gram-negative and particular Gram-positive bacteria (Chamaillard et al., 2003). Notably, Uehara and coworkers postulated in 2007 that HO-1-u-1 additionally expressed NOD2 mRNA, but the cell line did not produce any IL-8 upon PAMP stimulation. However, these findings are contrary to our results, as we could not detect NOD2 mRNA expression but observed high levels of IL-8 secretion.

As a reference, the human epithelial colorectal adenocarcinoma cell lines Caco-2/15 and Caco-2 A9 were screened for the same PRRs. The results of the PCR analysis revealed that the colorectal epithelial cells expressed mRNA for all 10 currently known human TLRs as well as for NOD1. Additionally, mRNA coding for Dectin-2 was detected in Caco-2/15 by qPCR. Analysing the functional response of the PRRs, Caco-2 cells showed IL-8 production upon stimulation with ligands for TLR1/2, TLR2/6 and for TLR5. As mentioned before, TLR2 forms heterodimers with either TLR1 or TLR6 and thereby recognises bacterial lipopeptides, whereas TLR5 acts as flagellin-receptor. Hence, although these cells are continually exposed to commensal bacteria, they secrete proinflammatory cytokines in response to bacterial PAMPs. However, compared with oral epithelial cell lines, colorectal cells expressed lower levels of PRR mRNA and produced marginal amounts of IL-8.

In addition, the screening revealed that the majority of PRRs were not functionally active. These PRRs may not be expressed on the protein level or they require other PRRs or cofactors for receptor function (Melmed et al., 2003). Furthermore, it was observed that the quantified level of PRR mRNA expression did not correlate with the responsiveness to their ligands. Some PRRs with a low level of mRNA expression produced relatively high amounts of IL-8 upon stimulation (e.g. NOD2 in HO-1-N-1) and vice versa.

In conclusion, it was observed that sublingual and buccal cells show differences in PRR expression and respond to stimulation with microbial ligands in a tissue-specific fashion. The spectrum of ligands, which the buccal epithelial cell line HO-1-N-1 responded to, indicates that these cells are basically specialised in recognising bacterial compounds. The sublingual epithelial cell line HO-1-u-1 responded to fewer ligands but within a broader spectrum, recognising viral dsRNA, bacterial flagellin and peptidoglycan.

7.1. Outlines

Buccal and sublingual cell lines grown on cell culture inserts could serve as in vitro model for native oral epithelial cells. Therefore, the properties of HO-1-N-1 and HO-1-u-1 cells will be compared with those of primary epithelial cells isolated from the human oral mucosa. The introduction of an appropriate in vitro model would be particularly useful for further development of SLIT.

Future research will also focus on the potential of using different PRR ligands as immunomodulators to increase the efficacy of SLIT.

8. References

Abbas AK, Lichtman AH and Pillai S (2007). Cellular and Molecular Immunology, 6th Edition. Saunders W.B.

Akira S, Uematsu S and Takeuchi O (2006). Pathogen recognition and innate immunity. Cell 124: 783-801.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Walter P (2008). Molecular biology of the cell. Fifth edition. Garland Science.

Alexopoulou L, Holt AC, Medzhitov R and Flavell RA (2001). Recognition of double stranded RNA and activation of NF-κB by Toll-like receptor 3. Nature 413: 732-738.

Athman R and Philpott D (2004). Innate immunity via Toll-like receptors and Nod proteins. Curr Opin Microbiol. 7: 25-32.

Bohle B, Kinaciyan T, Gerstmayr M, Radakovics A, Jahn-Schmid B and Ebner C (2007). Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. J Allergy Clin Immunol 120: 707-713.

Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L, Ogura Y, Kawasaki A, Fukase K, Kusumoto S, Valvano MA, Foster SJ, Mak TW, Nunez G and Inohara N (2003). An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nature immunology 4: 702-707.

Fanta C, Bohle B, Hirt W, Siemann U, Horak F, Kraft D, Ebner H and Ebner C (1999). Systemic immunological changes induced by administration of grass pollen allergens via the oral mucosa during sublingual immunotherapy. Int Arch Allergy Immunol 120: 218-224.

Frew AJ (2008). Sublingual immunotherapy. N Engl J Med 358: 2259-2264.

Fitzner N, Clauberg S, Essmann F, Liebmann J and Kolb-Bachofen V (2008). Human skin endothelial cells can express all 10 TLR genes and respond to respective ligands. Clinical and vaccine immunology 15: 138-146.

Galli SJ, Tsai M and Piliponsky AM (2008). The development of allergic inflammation. Nature 454: 445-454.

Girardin SE, Boneca IG, Carneiro LA, Antignac A, Jéhanno M, Viala J, Tedin K, Taha MK, Labigne A, Zähringer U, Coyle AJ, DiStefano PS, Bertin J, Sansonetti PJ and Philpott DJ (2003a). Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. Science 300: 1584-1586.

Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, Philpott DJ and Sansonetti PJ (2003b). Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem. 278: 8869-8872.

Hollmig ST, Ariizumi K and Cruz PD (2009). Recognition of non-self-polysaccharides by C-type lectin receptors dectin-1 and dectin-2. Glycobiology 19: 568-575.

Kay AB (2001). Allergy and allergic diseases. First of two parts. N Engl J Med 344: 30-37.

Larché M, Akdis CA and Valenta R (2006). Immunological mechanisms of allergen-specific immunotherapy. Nature Reviews Immunology 6: 761-771.

Le Bourhis L, Benko S and Girardin SE (2007). Nod1 and Nod2 in innate immunity and human inflammatory disorders. Biochem Soc Trans. 35: 1479-1484.

Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25: 402-408.

Medzhitov R and Janeway CA (2002). Decoding the patterns of self and nonself by the innate immune system. Science 296: 298-300.

Melmed G, Thomas LS, Lee N, Tesfay SY, Lukasek K, Michelsen KS, Zhou Y, Hu B, Arditi M and Abreu MT (2003). Human intestinal epithelial cells are broadly unresponsive to toll-like receptor 2-dependent bacterial ligands: Implications for host-microbial interactions in the gut. The Journal of Immunology 170: 1406-1415.

Miyauchi S, Moroyama T, Sakamoto T, Okamoto T and Takada K (1985). Establishment of human tumor cell line (Ueda-1) derived from squamous cell carcinoma of the floor of the mouth. Jpn J Oral. Maxillofac. Surg. 31: 1347-1351. [in Japanese; not available]

Moroyama T, Miyauchi S, Akemi T, Sugata T and Takada K (1986). Establishment and characterization of a human tumor cell line (Nakata-1) derived from squamous carcinoma of the buccal mucosa. Proc. Jpn. Can. Assoc. 45: 242. [not available]

Novak N, Bieber T and Allam J-P (2011). Immunological mechanisms of sublingual allergen-specific immunotherapy. Allergy 66: 733-739.

Robinson MJ, Osorio F, Rosas M, Freitas RP, Schweighoffer E, Gross O, Verbeek JS, Ruland J, Tybulewicz V, Brown GD, Moita LF, Taylor PR, Reis e Sousa C (2009). Dectin-2 is a Syk-couples pattern recognition receptor crucial for Th17 response to fungal infection. J Exp Med 206: 2037-51.

Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A and Zucco E (2005). The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biol Toxicol. 21: 1-26.

Scadding GW, Shamji MH, Jacobson MR, Lee DI, Wilson D, Lima MT, Pitkin L, Pilette C, Nouri-Aria K and Durham SR (2010). Sublingual grass pollen immunotherapy is associated with increases in sublingual Foxp3-expressing cells and elevated allergen-specific immunoglobulin G4, immunoglobulin A and serum inhibitory activity for immunoglobulin E-facilitated allergen binding to B cells. Clinical & Experimental Allergy 40: 598-606.

Schleimer R, Kato A, Kern R, Kuperman D and Avila P (2007). Epithelium: at the interface of innate and adaptive immune responses. J Allergy Clin Immunol 120: 1279-1284.

Standring S (Editor-in-Chief) (2005). Gray's Anatomy: The anatomical basics of clinical practice, 39th edition. Elsevier.

Svajger U, Anderluh M, Jeras M and Obermajer N (2010). C-type lectin DC-SIGN: an adhesion, signalling and antigen-uptake molecule that guides dendritic cells in immunity. Cell Signal. 22: 1397-1405.

Takeuchi O and Akira S (2010). Pattern recognition receptors and inflammation. Cell 140: 805-820.

Theophilus B (2008). Priniciples and medical applications of the polymerase chain reaction. In: Molecular biomethods handbook, edited by Walker JM and Rapley R. Humana Press.

Tortora GJ and Derrickson BH (2009). Principles of anatomy and physiology, 12th edition. Wiley.

Uehara A, Fujimoto Y, Fukase K and Takada H (2007). Various human epithelial cells express functional Toll-like receptors, NOD1 and NOD2 to produce anti-microbial peptides, but not proinflammatory cytokines. Molecular Immunology 44: 3100-3111.

Valenta R (2002). The future of antigen-specific immunotherapy of allergy. Nature Reviews Immunology 2: 446-453.

Wang Y, Zuo Z and Chow M (2008). HO-1-u-1 model for screening sublingual drug delivery – Influence of pH, osmolarity and permeation enhancer. International Journal of Pharmaceutics 370: 68-74.

Wang Y, Zuo Z, Lee K and Chow M (2006). Evaluation of HO-1-u-1 cell line as an in vitro model for sublingual drug delivery involving passive diffusion – initial validation studies. International Journal of Pharmaceutics 334: 27-34.

Weis WI, Taylor ME, Drickamer K (1998). The C-type lectin superfamily in the immune system. Immunol Rev 163:19-34.

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11. Zusammenfassung

Sublinguale Immuntherapie (SLIT) ist eine sichere und effektive Möglichkeit der Behandlung von Typ I Allergien. Die dieser Behandlung zugrunde liegenden Immunmechanismen sind derzeit noch nicht gänzlich aufgeklärt. Detailliertes Wissen über mögliche Immunreaktionen in der Mundhöhle trägt zur Aufklärung der Immunmechanismen von SLIT bei. Ziel der vorliegenden Masterarbeit war daher die Analyse der Expression von diversen Pathogen-recognition Rezeptoren (PRR) auf bukkalen und sublingualen Epithelzellen. Hierfür wurden zwei humane Zelllinien (HO-1-N-1 bzw. HO-1-u-1) eingesetzt. Zusätzlich wurden sämtliche Experimente mit den gängigen intestinalen Epithelzelllinien Caco-2/15 und Caco-2 A9 durchgeführt.

Mittels semiquantitativer Polymerase-Kettenreaktion (polymerase chain reaction, PCR) und quantitativer „real-time“ PCR (qPCR) wurde die mRNA Expression der Toll-like Rezeptoren (TLR) 1-10, C-type Lectin Rezeptoren DC-SIGN, Dectin-1 und Dectin-2 sowie der NOD-like Rezeptoren NOD1 bzw. NOD2 bestimmt. Des Weiteren wurden die Zellen mit Rezeptor-spezifischen Liganden stimuliert, und die im Falle einer Aktivierung der Signaltransduktionswege erhöhte Menge an ausgeschiedenem IL-8 mittels Enyzme-linked immunosorbent Assay (ELISA) bestimmt.

Die bukkale Zelllinie HO-1-N-1 exprimierte mRNA für TLR1, TLR2, TLR4 und TLR6 sowie für NOD1 und NOD2 und reagierte auf Stimulation dieser Rezeptoren mit erhöhter IL-8 Sekretion. Die Resultate der qPCR ließen weiters die Expression von TLR3, TLR8, TLR10 und Dectin-1 vermuten, doch die HO-1-N-1 Zellen reagierten nicht auf die entsprechenden Liganden.

Die sublinguale Zelllinie HO-1-u-1 exprimierte mRNA für TLR1, TLR3, TLR4, TLR5 und TLR6 sowie für NOD1. Die Resultate der qPCR ließen weiters auf Expression von TLR2, TLR7, TLR8, TLR10 und Dectin-1 schließen. Erhöhte IL-8 Ausschüttung konnte jedoch nur nach Stimulation mit TLR3-, TLR5- und NOD1-spezifischen Liganden beobachtet werden.

Die intestinalen Caco-2/15 und Caco-2 A9 Zelllinien exprimierten mRNA für alle 10 bekannten humanen TLRs sowie für NOD1. Zusätzlich konnte bei der Caco-2/15 Zelllinie Dectin-2 mRNA nachgewiesen werden. Erhöhte IL-8 Produktion wurde nach Stimulation mit TLR1/2-, TLR2/6- und TLR5-spezifischen Liganden gemessen.

Zusammenfassend konnte beobachtet werden, dass bukkale und sublinguale Epithelzellen unterschiedliche Mustererkennungsrezeptoren exprimieren und daher auf unterschiedliche

Liganden reagieren. Das Liganden-Spektrum der Zelllinie HO-1-N-1 deutet darauf hin, dass bukkale Epithelzellen hauptsächlich auf die Erkennung von Bakterien spezialisiert sind. Sublinguale Epithelzellen reagieren hingegen auf eine geringere Anzahl an Liganden, dafür waren diese sowohl bakteriellen als auch viralen Ursprungs.

12. Curriculum vitae

Personal data

Name: Martina Schuschnig
Title: Bakkalaurea technica (Bakk. techn.)
Date of birth: 16-Apr-1986
Place of Birth: Vienna, Austria
Citizenship: Austrian

Education

1992-1998: Elementary School at the Volksschule der Piaristen, Vienna

1998-2004: Secondary School at the Bundesgymnasium und Bundesrealgymnasium GRG5 Rainergasse, Vienna

Specialised paper in English (Fachbereichsarbeit): "Cosmic angst and artful comedy – the whimsical world of Woody Allen"

2004-2009: Bachelor studies "Food- and Biotechnology" at the University of Natural Resources and Life Sciences, Vienna

Bachelor thesis: "Nachweis von Allergenen in Lebensmitteln" (Detection of allergens in food)

Since 2009: Master studies „Molecular Microbiology and Immunobiology" at the University of Vienna, Vienna

Advanced course in immunology at the Medical University of Vienna – CD Laboratory for Immunomodulation: "Glutathione S-transferase in birch pollen"

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Attendance at scientific congresses

December 2010: Annual meeting of the Austrian society for allergology and immunology (ÖGAI), Vienna, Austria

September 2011: Annual meeting of the Austrian society for allergology and immunology (ÖGAI), Graz, Austria

Poster presentation: M. Schuschnig, C. Kitzmüller, B. Bohle. Expression of pattern recognition receptors by oral epithelial cell lines.

September 2011: Retreat of the Centre for Pathophysiology, Infectiology and Immunology, Vienna, Austria

Poster presentation: M. Schuschnig, C. Kitzmüller, B. Bohle. Expression of pattern recognition receptors by oral epithelial cell lines.

Working experience

August 2004: bakery shop assistant at Ströck Brot GmbH, Vienna

August 2005: Sea turtle project, Grenzenlos, Costa Rica

September 2005: work placement at Octapharma Pharmazeutikaproduktionsges.m.b.H, Technology transfer unit, Vienna

2005/2006: freelance work for Unique, Journal of the Austrian national student unit (ÖH) at the University of Vienna, Vienna

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2007/2008: CliniClowns donation collecting, Street promotion, Vienna

Since 2008: part-time workforce at Octapharma Pharmazeutikaproduktionsges.m.b.H, Central drug safety unit, Vienna

Language skills

German: native

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French: moderate

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