INNATE IMMUNITY GENE EXPRESSION IN BRONCHIAL EPITHELIAL CELLS

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Faculty of Life and Environmental Sciences
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2011

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90 ECTS thesis submitted in partial fulfillment of a
Magister Scientiarum degree in biology

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Faculty of Life and Environmental Sciences
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Science does not know its debt to imagination.

-Ralph Waldo Emerson-

Abstract

The airway epithelium forms the interface between the external environment and the interior of the human body. The epithelial cells are constantly challenged by potential pathogens and have the crucial function to provide the first line of defense. The epithelial defense strategy consists of both mechanical and chemical components. The mechanical barrier function maintains the integrity of the epithelium to prevent a bacterial breach. The chemical defense strategy consists of the expression and secretion of factors, such as the antimicrobial peptides (AMPs), to prevent the establishment of bacterial infections. The epithelial cells must be able to recognize microbial threats, resulting in the initiation of appropriate immune responses. Here we further characterize the novel bronchial epithelial cell line, VA10, with regard to its receptor expression. Toll-like receptor (TLR) stimulation alone had no effect on AMP expression, while the stimulation together with 4-Phenylbutyrate (4-PBA) and 1,25-Dihydroxyvitamin D_3 ($1,25D_3$) indicated an interference with signaling pathways mediating AMP expression. Our findings show that the expression of antimicrobial peptides can be augmented by the stimulation with 4-PBA and $1,25D_3$. We additionally found that bacterial clearance can be directly induced through stimulation. Together our findings give further insight into the modulating effects of 4-PBA and $1,25D_3$ on the gene expression in VA10 cells. Both agents aid in the prevention of bacterial infections by augmenting the innate antimicrobial response and acting in anti-inflammatory fashion. These beneficial characteristics further the proposal of 4-PBA and $1,25D_3$ as novel drug candidates to treat bacterial infections.

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List of Abbreviations

4-PBA	Sodium 4-Phenylbutyrate
ALI	Air liquid interface
AMP	Antimicrobial peptide
AP-1	Activator protein 1
ASL	Airway surface liquid
ATP	Adenosine triphosphate
BEGM	Bronchial epithelial cell growth medium (without gentamicin)
bp	Base pair
CAMP	Cathelicidin antimicrobial peptide
cAMP	cyclic adenosine monophosphate
CCR6	C-C chemokine receptor 6
CD36	Cluster of differentiation 36
cDNA	Complementary DNA
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CIITA	Class II, major histocompatibility complex, transactivator
Cl^-	Chloride
CpG	Cytosine-phosphate-guanine
Ct	Threshold cycle
DAMP	Danger-associated molecular pattern
DEFB1	β-defensin 1
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
ds	double-stranded
E. coli	*Escherichia coli*
EGF	Epidermal growth factor
GM	Gentamicin
GPR41	G-protein-coupled receptor 41
hBD-1	Human β-defensin-1
hCAP-18	Human cationic antimicrobial protein 18
HDAC	Histone deacetylase
HT29	Human colon epithelial cell line
IL-1β	Interleukin-1β
INF-α	Interferon-α
IPAF	ICE-protease activating factor
LGP2	Laboratory of genetics and physiology 2
LHC-9	Bronchial epithelial cell groth medium (with gentamicin)
LL-37	Mature human peptide of CAMP
LPS	Lipopolysaccharide
M. tuberculosis	*Mycobacterium tuberculosis*

MAPK	Mitogen-activated protein kinase
MCT1	Monocarboxylate transporter isoform 1
MDA5	Melanoma differentiation-associated gene-5
MOI	Multiplicity of infection
mRNA	Messenger RNA
MyD88	Myeloid differentiation factor 88
n	Number of experiments
Na^+	Sodium
NAIP	NLR family, apoptosis inhibitory protein
NF-κB	Nuclear factor
NLR	NOD-like receptor
NLRP1	NACHT, LRR and PYD domains-containing protein 1
NOD1	Nucleotide-binding oligomerization domain-containing protein 1
NTC	Non template control
P. aeruginosa	*Pseudomonas aeruginosa*
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCL	Periciliary liquid layer
PPARγ	peroxisome proliferator-activated receptor-γ
PRR	Pattern recognition receptor
RIG1	Retinoid-inducable gene 1
RLR	RIG-like receptor
RNA	Ribonucleic acid
RT-PCR	Real time-polymerase chain reaction
RXR	Retinoid X receptor
SCFA	Short-chain fatty acid
SEM	Standard error of the mean
SLC5A3	Solute carrier family 5 member 8
SLPI	Secretory leukoproteinase inhibitor
SP-A	Surfactant protein A
ss	single-stranded
stdev	Standard deviation
THP1	Human monocytic cell line
TIR	Toll-interleukin-1 receptor
TIRAP	TIR-containing adaptor protein
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
TRAM	TRIF-related adaptor molecule
TRIF	TIR-containing adaptor inducing IFN-β
TUBB	β-Tubulin
UVB	Ultraviolet B
VA10	Human bronchial epitheial cell line
VDR	Vitamin D receptor
VDRE	Vitamin D-responsive element
$1,25D_3$	1α,25-Dihydoxyvitamin D_3

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1 Introduction

The airway epithelium is the largest epithelial surface of the human body that is in direct contact with the external environment and thereby presents a large target for infections. During the course of the day an average human lung inhales approximately 10.000 liters of air [1]. The airway epithelial cells are the first point of contact and therefore constantly challenged by a vast diversity of inhaled microorganisms, including potential pathogens, as well as organic and inorganic particles and other environmental pollutants [2-5]. The moist and warm surface of the lung epithelium presents an ideal growth environment for pathogenic colonization. This interface between the internal and external environment represents a common route of pathogen entry into the human body [6-8]. The fact that respiratory infections are relatively rare in healthy individuals only demonstrates how efficient the host defense mechanisms at the mucosal surface of the upper respiratory epithelia really are. It is evident that the integrity of the respiratory airway critically depends on the tightly regulated host defense mechanisms that inactivate and clear the inhaled pathogens in order to prevent the establishment of infections. Among these vital mechanisms is the innate immune system, which provides an initial broad and rapid protection against invading pathogens [1, 4, 8]. The cells responsible for augmenting innate immune responses are phagocytes, such as neutrophils and macrophages, and the epithelial cells themselves [1, 9]. In addition to providing a physical barrier function and being responsible for mucociliary clearance, the airway epithelium is involved in the recognition and distinction between potentially harmful microorganisms and harmless foreign particles. The epithelial cells must react appropriately in order to prevent the contact to microorganisms from progressing to an infection or avoid unnecessary inflammatory responses [4, 7, 10]. In general, the innate immune response is activated by the recognition of microorganisms through surface receptors. The recognition of microbial patterns usually results in the activation of signaling pathways, which lead to the activation of transcription factors, which in turn mediate the induction of innate immune gene expression, including pro-inflammatory cytokines, chemokines, and host defense peptides, such as defensins and cathelicidins [8, 11] as well as the activation and coordination of adaptive immune responses.

1.1 The human respiratory system

1.1.1 Lung histology

The human lung consists of a complex network of branching airways (Figure 1, see figure references). The conducting airways consist of the trachea and a complex network of branching bronchi [12]. This great number of bronchi eventually branch into bronchioles which terminate in clusters of alveoli, where the gas exchange between the alveolar epithelium and the blood occurs. The bronchioles form the transition between the conducting airways and the exchange epithelium of the lung.

There are striking differences between the cellular composition and the function of the epithelium lining the conducting airways and the alveolar epithelium. Both epithelia are lined by a monolayer of a variety of specialized cells responsible of regulating the lung

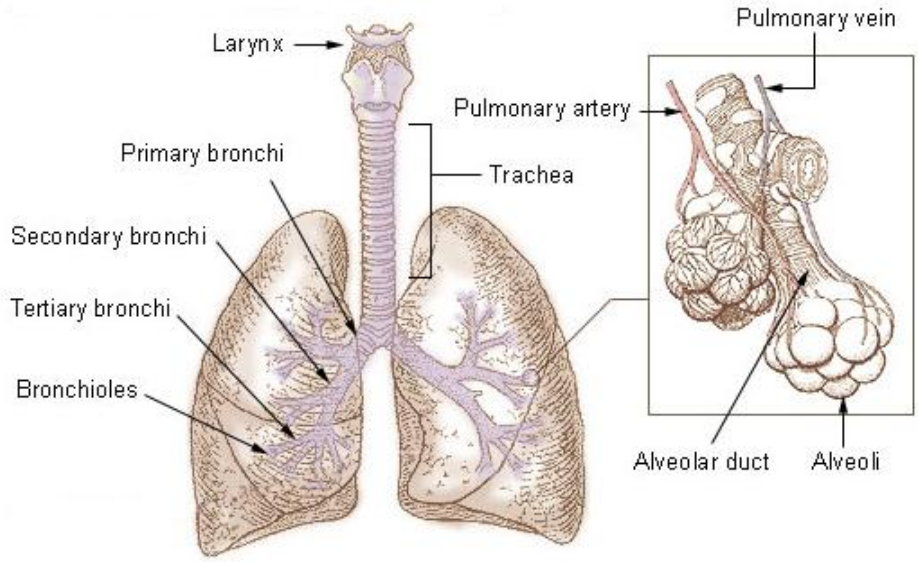

Figure 1*: Schematic representation of the human lung anatomy.*

fluid, the clearance of inhaled agents as well as the secretion of mediators and thereby the attraction and activation of inflammatory cells in response to injury or infection [13]. The proximal respiratory epithelium of the lung is lined by a pseudostratified columnar epithelium consisting mainly of ciliated cells, basal cells and secretory cells, including the goblet cells and the cells of the submucosal glands (Figure 2) [7, 13]. These cells are underlined by basal cells, which are responsible for the attachment of the epithelial cells to the basement membrane. The epithelium of the conducting airways is supported by cartilage rings to prevent a collapse. The distal respiratory epithelium or the alveolar epithelium is composed of a single layer of type I and type II respiratory alveolar cells. While the gas diffuses through the very thin type I alveolar cells, the type II alveolar cells are responsible for the synthesis and secretion of surfactant and the regulations of the amount of fluid present on the alveolar surface [14]. In addition to the structural respiratory epithelial cells there are other cells present, including the mast cells, dendritic cells, and alveolar macrophages [5, 7, 13]. These immune cells either reside within the epithelium or transit through the lumen to be recruited to the site of inflammation or epithelial damage [13].

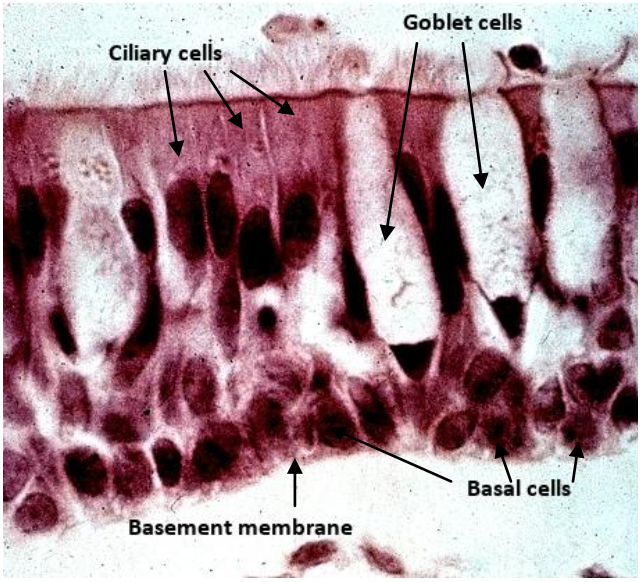

Figure 2: *Microscopic image of the human bronchial epithelium. This pseudostratified columnar epithelium consists of ciliated cells, basal cells and secretory Goblet cells.*

The bronchial epithelial cells are joined by cell-to-cell junctions, including tight junctions, adherence junctions, gap junctions and desmosomes [7, 15]. The tight

junctions are crucial to prevent microbial penetration of the epithelium [10] by establishing a tight link between adjacent cells at the apical and basolateral interface. However, this physical barrier to microbial entry still allows the paracellular diffusion of fluids, electrolytes and other essential molecules [7, 13, 15]. The adherence junctions and desmosomes play a similar important role in the cell-cell adhesion, while the gap junctions aid the rapid intercellular exchange of molecules and ions between adjacent epithelial cells.

1.1.2 Mucociliary clearance

The primary function of the ciliated cells of the conducting airways is the directional transport and clearance of mucus from the lower airways to the throat, thereby facilitating the constant clearance of inhaled agents that are entrapped in it [5, 6, 12, 13].

In order to prevent microbial infections of the distal airway epithelia, the lungs utilize the strategy of directed air-flow to induce impaction and sedimentation of inhaled microorganism and particles onto the mucus of the branching conducting airways [4, 10]. The airway epithelia are responsible for regulating crucial aspects of host defense by facilitating the balanced transepithelial transport of electrolytes, thereby regulating both the volume and the proper composition of the airway surface liquid (ASL) [7, 12]. The ASL consists of two layers, the thin periciliary liquid layer (PCL) and the thick mucus layer that covers the PCL [12]. The PCL is approximately 5 µm thick and has two crucial functions by providing a low-viscosity solution, in which the cilia can beat rapidly, and by shielding the epithelial call surface from the overlaying mucus layer (Figure 3) [7]. There are approximately 50-200 cilia [2] on the surface of each ciliated cell that coordinately beat at a frequency of about 8-15 Hz and thereby effectively clear the mucus layer and the entrapped particles out of the lung. The layer of mucus consists of high-molecular weight, heavily glycosylated macromolecules forming a tangled network of polymers [2]. These so-called mucins, such as mucin-5AC and mucin-5B, are secreted by goblet cells and the mucus cells of the submucosal glands of the airway epithelium [4, 7, 10, 16]. In addition, this ASL is known to contain antimicrobial factors, including lactoferrin, lysozyme, collectins and antimicrobial peptides [2, 6, 16] which are produced and secreted by both the submucosal gland epithelia and the surface epithelial cells [5]. This mucociliary clearance, together with the immobilization of the pathogens by the mucus and the direct killing of pathogens by the antimicrobial proteins, can be seen as a primary innate airway defense mechanism [2, 4, 12].

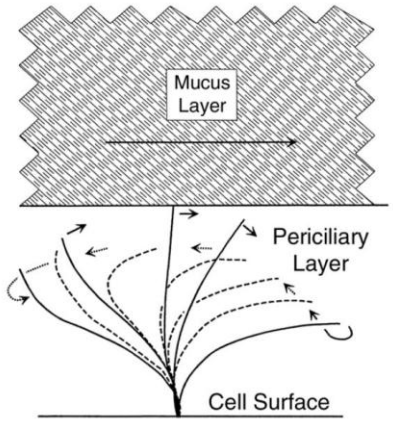

***Figure 3**: Schematic representation of the mucociliary clearance of the ASL from the surface of the bronchial epithelium.*

1.2 Innate immunity

The innate immune system of the human airways consists of the epithelium itself as well as resident phagocytic cells and other migrating immune effector cells, such as dendritic cells and neutrophils [1, 7, 10]. The epithelial cells of the respiratory tract form the interface between the internal and external environments and thereby are the first cells that come in contact with inhaled microorganisms and particles [6]. The epithelial cells play a key role in local immunity by mediating the first line of defense against invading pathogens. They are crucial components of innate immunity by providing a physical barrier to microbial entry, but also by actively contributing to the innate immune system by expressing and releasing inflammatory mediators and chemotactic mediators, to recruit leukocytes to the site of microbial entry, which ingest and kill the microorganisms, and the direct killing of pathogens by inducing the expression and secretion of antimicrobial peptides (Figure 4) [1, 4, 10]. All these innate immune defense mechanisms are known to be highly inducible upon sensing microbial products as well as host products and are crucial for the health of the host, as failure of the local host defense mechanisms often results in microbial colonization and subsequent infection of the airway epithelia [1, 10]. The adaptive immune system provides an addition to the overall immune strategy by eliminating pathogens in the later phase of infection as well as the generating specific immunological memory [9].

The innate immune system is involved in events that occur immediately after exposure to microorganisms, while a few hours post exposure, the immune response shifts to early adaptive immune responses, which are antigen-specific and require cellular relocation of receptors in addition to the expression of immunoglobulin genes to provide the antigen specificity [4]. In contrast to the adaptive immune system, innate immunity has several distinct characteristics that are related to the processes of inflammation. First of all, there is the rapid activation of effector mechanisms, which are constantly active at low background levels, but are immediately amplified in reaction to microbial exposure and inflammatory stimuli without requiring previous encounters or immunologic memory for their activity [17, 18]. The microbial recognition occurs through receptors on the surface of epithelial and immune cells [1, 18]. The innate immune system has traditionally been seen as the first line of defense responsible for discrimination between "self" from "nonself". This

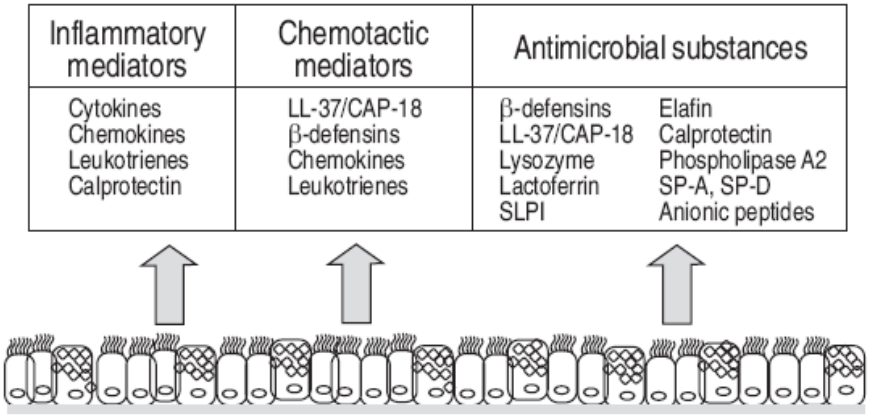

Figure 4: *Overview of immunomodulatory factors expressed and secreted by airway epithelial cells during innate immune responses.*

distinction between host proteins and microbial-derived proteins is achieved by directly recognizing the microorganisms but also, for example, sensing normally present things which are suddenly diminished or absent during the course of infection [19, 20]. Only just recently the "danger" model has been proposed to explain the immune responses to diverse danger signals from injured cells that are exposed to pathogens, their toxins and/or mechanical damage. Such danger signals include host-derived DNA, RNA, heat shock proteins, interferon α (INF-α), and interleukin 1β (IL-1β) [20, 21]. And finally, the limited specificity of innate immunity has the advantage over the adaptive immune response, that it does not depend on an antigen-specific recognition, but recognizes a broad spectrum of molecular and structural motifs associated with microorganisms [18]. However, for an effective host defense both the innate and the adaptive immunity are required [22]. The innate immune system has a critical role in activating and coordinating the adaptive immune system, which relies upon antigen receptors expressed by T and B lymphocytes for highly refined recognition. Macrophages and dendritic cells in the airway epithelium process microbial antigens and present them in association to class I and class II molecules to responding lymphocytes [4, 10].

1.3 Epithelial recognition of pathogens

The airway epithelial cells are able to sense and respond to microbial exposure by increasing their defenses. Such a response includes an increase in the release of antimicrobial peptides to apical or basolateral surfaces of the airway epithelium as well as the release of chemokines and cytokines for the initiation of proinflammatory reactions [1]. These inflammatory reactions result in the recruitment and activation of innate immune cells [6], like macrophages, which ingest and remove microorganisms that are not cleared by the epithelium itself, in addition to dendritic cells and lymphocytes to aid the induction of an adaptive immune response [1, 7].

The mechanisms by which the airway epithelial cells recognize pathogens are crucial to activate an appropriate innate immune response. The epithelial cells are known to express receptors belonging to the class of pattern recognition receptors (PRRs). These receptors recognize conserved microbial patterns derived from a broad range of bacteria, viruses, fungi, and protozoans [1, 6, 7, 9, 23]. Such highly conserved microbial structures are known as pathogen-associated molecular patterns (PAMPs). While these microbial structures enable the host cells to distinguish between self and non-self, the recognition of PAMPs do not allow for discrimination between pathogenic and non-pathogenic microorganisms [6, 10]. Thus, the host cells are unable to distinguish between virulent, pathogenic, potentially dangerous opportunistic microorganisms and the harmless bacteria and will respond to microbial challenges by activating inflammatory responses [6]. In addition to the PAMPs, PRRs are able to identify host molecules that are expressed in response to infection or host molecules that have been modified during the course of infections. Such host-derived molecules are also known as danger-associated molecular patterns (DAMPs) [10, 20, 21].

Although, single PRRs are able recognize only a certain microbial motif, they are thought to cooperate to provide a combinatorial mechanism to cope with the vast diversity of microbial ligands. This is supported by the observation that simultaneous activation of different PPRs results in unique signals that are characteristic, for both the cells and microorganisms involved. The recognition of PAMPs and DAMPs by the PRRs activates

intracellular signaling cascades resulting in the activation of the NF-κB and AP-1 transcription factors, which leads to the expression of effector molecules, such as proinflammatory cytokines and chemokines that are involved in mediation of microbial defense, inflammation, and modulation of adaptive immunity [1, 4, 10, 20, 23].

1.3.1 Pattern-recognition receptors

The innate immune recognition of microbial components is crucial for host defense against infection. Results indicate that airway epithelial cells themselves are able to sense microbial presence through its PRRs.

Toll-like receptors

The Toll-like receptors (TLRs) are intensely studied and today the best understood germline-encoded PRRs that are expressed in epithelial cells and immune cells, including the airway epithelial cells, macrophages, monocytes, dendritic cells, neutrophils, as well as cells of the adaptive immune system. The activation of TLRs has been shown to be

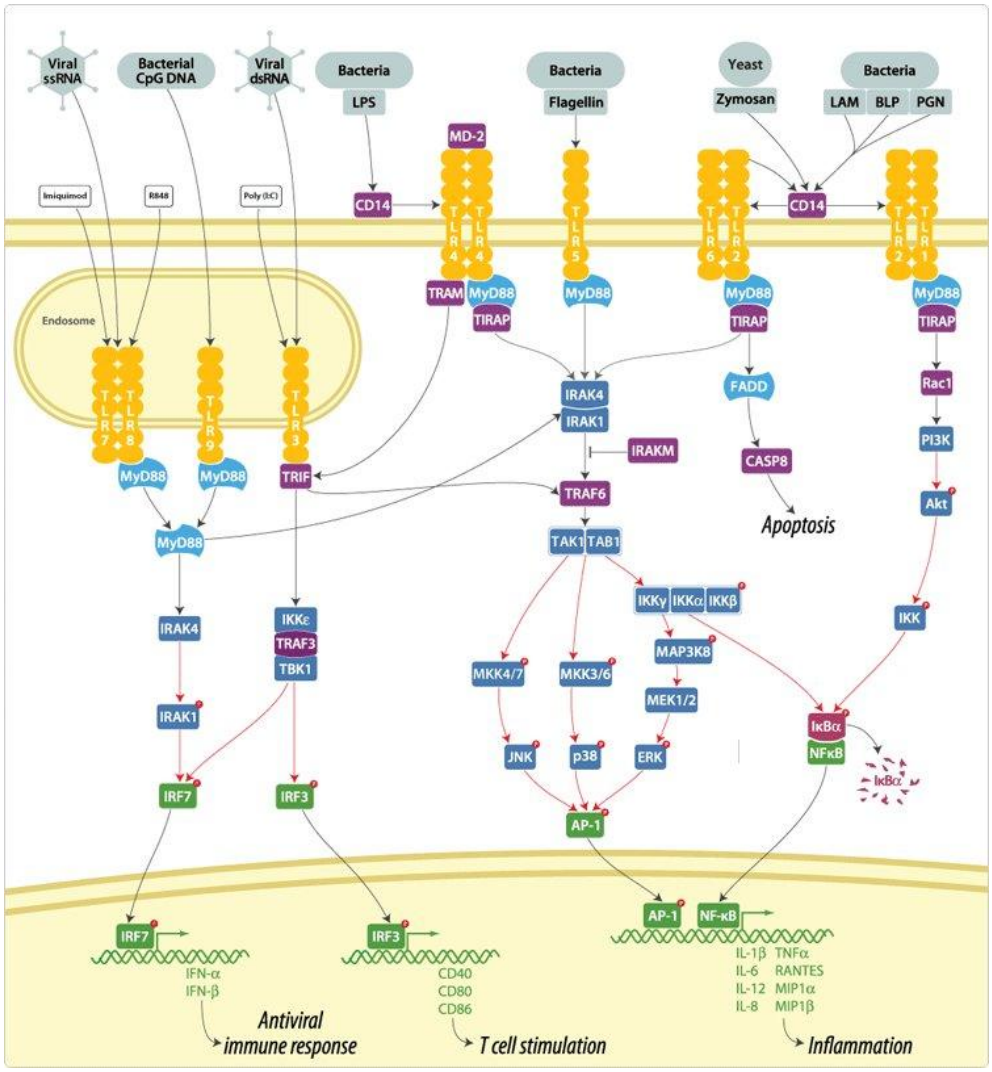

Figure 5: *Overview of Toll-like receptor signaling pathways.*

involved in the regulation of gene expression, including those encoding cytokines and chemokines, which trigger innate immune responses and prime the antigen-specific adaptive immunity [1, 7, 20, 23].

The TLRs are highly conserved class I transmembrane proteins, which consist of an extracellular ectodomain containing variable numbers of leucine-rich repeats mediating the PAMP recognition; a transmembrane domain; and a common cytoplasmic Toll-interleukin-1 receptor (TIR) domain required for the intracellular downstream signal transduction [7, 9, 24]. Upon ligand binding, TLRs dimerize and undergo conformational changes required for the recruitment of TIR-domain-containing adaptor molecules including the myeloid differentiation factor 88 (MyD88), the TIR-containing adaptor protein (TIRAP), the TIR-containing adaptor inducing IFN-β (TRIF), and the TRIF-related adaptor molecule (TRAM) (Figure 5) [9]. The signaling pathways eventually results in the selective activation of the transcription factors NF-κB and mitogen-activated protein kinases (MAPKs) leading to the transcription of genes encoding cytokines, chemokines and antimicrobial factors, such as β-defensin 2 [9-11, 23-25]. The specific response to microbial products appears to be due to the individual TLRs having their own signaling pathways [1].

There are ten TLRs that have been identified to be expressed in human cells [4, 11, 25]. TLRs recognize molecular structures derived from bacteria, viruses, fungi, or parasites (Table 1) [1, 4, 7, 23, 24]. Bronchial epithelial cells have been shown to express TLR1-6 [26], TLR7-8 [27], TLR9 [6], and TLR10 [27]. These receptors can be divided into subgroups according to their cellular localization: TLR1, TLR2, TLR4, TLR5, and TLR6 are surface receptors, while TLR3, TLR7, TLR8, and TLR9 are located in intracellular compartments. However, the observation of a low TLR2 expression in the airway epithelium coincides with a hyporesponsiveness to certain TLR2 ligands and Gram-positive bacteria [6, 28]. TLR2 expression has been also reported to be reduced in airway epithelial cells as well as the expression of co-receptors, such as the expression of CD36. It has been shown that TLR2 increased surface expression is upregulated by vitamin D_3 and during

Table 1: *Toll-like receptors and their ligands.*

Receptor	Ligand	Source of ligand
TLR1/TLR2	Triacyl lipopeptides	Gram-negative bacteria
TLR2	Lipoproteins and -peptides Peptidoglycan Lipoteichoic acid Zymosan	Bacteria, fungi, parasites and viruses
TLR3	dsRNA	Viruses
TLR4	LPS	Gram-negative bacteria
TLR5	Flagellin	Flagellated bacteria
TLR6/TLR2	Diacyl lipopeptides	Gram-positive bacteria
TLR7	ssRNA	Viruses
TLR8	ssRNA	Viruses
TLR9	CpG-containing DNA	Bacteria and viruses
TLR10	Unknown	

chronic inflammation, such as it has been shown to be the case with cystic fibrosis [6, 27]. The epithelial cell polarity and differential localization of TLRs on the apical and basolateral cell surfaces is also implicated to play a role in TLR sensitivity. TLR4 has been shown to be located at the basolateral cell surface [6]. These findings are well in line with the observed hyporesponsiveness of a healthy bronchial epithelium towards certain TLR ligands and especially Gram-positive bacteria. This may be a mechanism to regulate host responses. Since the airway epithelia are in constant contact with microorganisms, a constant activation of the surface receptors would result in constant inflammation. A basolateral localization ensures that the receptors are only activated by bacteria that have been able to breach of the epithelial barrier, resulting in appropriate innate and inflammatory responses.

Interestingly, TLRs may also mediate responses to endogenous ligands. These ligands include a number of host danger signals, such as heat shock proteins and extracellular matrix components that are generated during infection and inflammation [1, 10].

Other pattern recognition receptors

Additional PRRs have been shown to be expressed in airway epithelial cells, including NOD-like receptors (NOD1, NOD2 and NALP1), RIG-like receptors (RIG-I and MDA5) [6, 7, 24], and collectins. These are less well defined receptors and pathways that are nonetheless of importance to the innate immune responses of the airway epithelial cells [24].

The NOD-like receptor (NLR) family belongs to the class of cytosolic PRRs that are responsible for the intracellular detection of PAMPs, generated by intracellular pathogens, but also to host-derived and stress-associated DAMPs [7, 10, 18, 23]. Humans are known to express at least 22 NLR genes, which can be classified into three distinct subfamilies according to their phylogenetic relationships and domain structure similarities: the NODs (NOD1-5 and CIITA), the NLRPs (NLRP1-14), and the IPAFs (IPAF and NAIP). Most of the NLR are restricted to the leukocytes, but NOD1 and NOD2 are known to be expressed by lung epithelial cells [29]. The NLRs are part of multiprotein complexes, so-called inflammasomes that are able to mediate caspase-1-dependent processing of cytokines, such as IL1-β, a crucial pro-inflammatory mediator [10, 20]. NOD1 and NOD2 both recognize cytoplasmic breakdown products of bacterial cell walls and initiate pro-inflammatory responses. NOD2 can be seen as a more general sensor of bacteria, while NOD1 detects more specifically Gram-negative bacteria. The IPAF inflammasome is known to be activated by flagellin of Gram-negative bacteria possessing type III or IV secretion systems by which the bacteria inject their bacterial virulence factor into the cytoplasm of host cells [9, 10, 20].

The RIG-like receptors (RLRs) belong to a family of cytoplasmic PRRs that have been shown to be involved in TLR-independent recognition of RNA viruses and the associated production of type I interferons. There are three known members: the retinoic acid-induced gene-1 (RIG-I), the melanoma differentiation-associated gene-5 (MDA5) [7], and LGP2 [24]. RLRs are RNA helicases expressed by cells of the airway epithelium and their expression has been shown to be inducible by proinflammtory cytokines or by the viral infections themselves. Both receptors are crucial for the detection of viral infections, with RIG-I detecting non-capped 5'-triphosphate RNA, therefore detecting ssRNA viruses [30], and MDA5 recognizing dsRNA [9, 10, 20, 23, 31].

The C-type lectins, or also known as collectins, belong to a family of soluble PRRs that act as opsonins for bacterial products in plasma and tissues. The surfactant proteins (SP)-A and SP–D are two members of this PRR family which are associated with pulmonary surfactant and are expressed by the submucosal glands of the conducting airways. Both SP-A and SP-D recognize and bind microbial carbohydrates and thereby enhance phagocytosis by alveolar macrophages and neutrophils through immunoglobulin-mediated opsonization and agglutination of the bacteria and viruses. They are additionally thought to possess direct antimicrobial activities in addition to their immunomodulatory properties through their interactions with dendritic cells and T cells [4, 7, 10].

1.4 Antimicrobial peptides

In addition to serving as a physical barrier, the epithelia cells of the conducting airways and submucosal glands have the function of producing and modifying the ASL and thereby establishing a chemical barrier [7, 32]. The composition of the ASL provides an optimal environment for the function of the many secreted antimicrobial proteins that clear and inactivate invading microorganisms through several mechanisms, such as disrupting cell walls and sequestering nutrients [7]. These antimicrobial proteins produced by the airway epithelia act in a broad spectrum against Gram-positive and Gram-negative bacteria, enveloped viruses and certain fungi [7, 33, 34] and include lysozyme, lactoferrin, and SLPI, as well as the antimicrobial peptides [5, 7].

The antimicrobial peptides (AMPs) consists of two major subfamilies, the defensins and the cathelicidins, which both are expressed and secreted by airway epithelial cells and neutrophils [25, 39, 40]. Resident macrophages and recruited leukocytes further contribute to the constitutive presence of AMPs at the epithelial surface. Their expression is readily inducible in response to microbial products or other chemical signals [1, 7]. There are indications that AMPs play important immunomodulatory roles in the interactions between innate and adaptive immunity (Figure 6) [37, 38]. By secreting the AMPs into the ASL, the airway epithelial cells are able to chemoattract and activate cells of the innate as well as

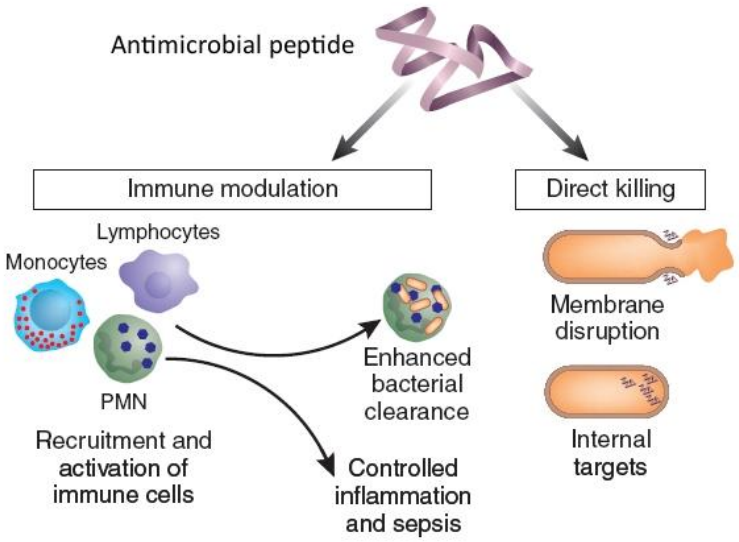

Figure 6: *Biological roles of antimicrobial peptides.*

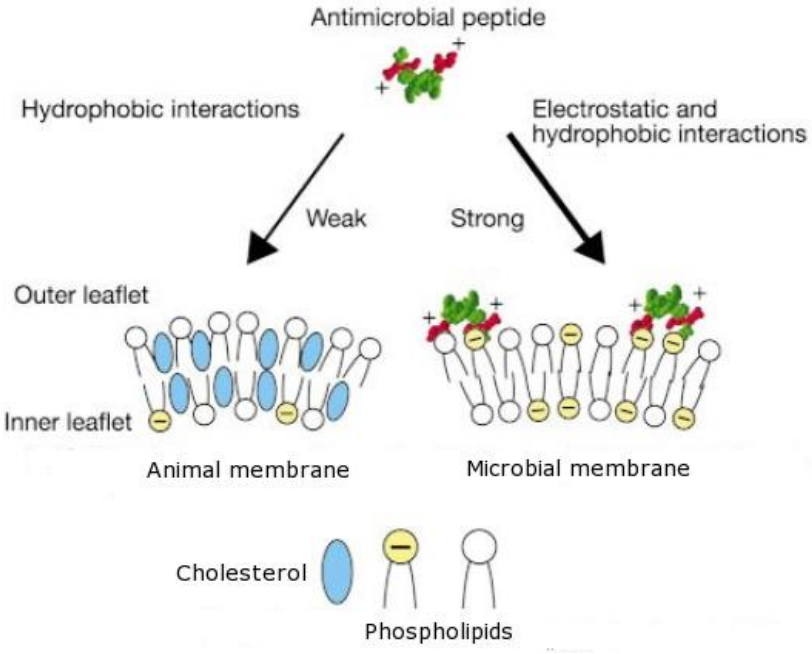

Figure 7: *Schematic representation of the membrane selectivity of antimicrobial peptides. The outer leaflet of microbial membranes is rich in negatively charged phospholipids facilitating the electrostatic interaction with antimicrobial peptides while the presence of cholesterol in the eukaryotic membranes reduces this interaction.*

adaptive immune systems. AMPs have the ability to act directly as chemoattractants for neutrophils, monocytes, mast cells and T-helper (T_H) cells in addition to stimulate the release of neutorphil and monocyte chemokines from the host cells, resulting in the recruitment of the immune cells to the site of infection. AMPs can also increase the production of integrins that are part of chemotactic responses and have been shown to promote non-opsonic phagocytosis. AMPs are able to immobilize and directly kill microorganisms. They have been additionally shown to promote tissue and wound repair, by promoting fibroblast chemotaxis and growth and stimulating angiogenesis in response to injury [25, 32, 37-40].

All AMPs have in common that they are small in size and cationicaly charged [5, 39]. Their antimicrobial activity results from difference in lipid composition of membranes of microbial and multicellular organisms resulting in the membrane selectivity. Their amphipathic structure allows them to specifically target by electrostatically interacting with the anionic outer leaflet of the microbial membranes that lack cholesterol but are rich in negatively charged headgroups of phospholipids (Figure 7) [9, 32, 37, 39]. This peptide-membrane interaction leads to the displacements of lipids, the membrane destabilization, and the formation of physical pores. All of which eventually result in the disruption of the microbial membrane and the leakage of cellular content and the microbial cell death [10, 36, 39, 41]. Certain AMPs have been shown to translocate across the microbial membrane to interact and influence intracellular functions of the host, such as the inhibition of cell division or the induction of autolysis [42, 43].

1.4.1 Defensins

The defensins are a class of broad-spectrum amphipathic antimicrobial peptides, with known activity against an array of bacterial, viral, and fungal pathogens [7, 44], and are secreted from leukocytes and the respiratory epithelial cells [39, 45]. These peptides are characterized by a β-sheet-rich fold which contains six highly conserved cysteine residues giving rise to three intramolecular disulfide bonds [5, 8, 39, 46]. Similar to cathelicidin, defensins have been shown to require proteolytic processing from an inactive precursor [5, 39]. The mammalian defensins can be classified into three major subfamilies according to the structural differences in the spacing of these three cysteine residues and the resulting tertiary structures: the α-defensins, the β-defensins and the θ-defensins [5, 8, 10, 21, 36]. The α-defenisns are mainly expressed by neutrophils, while the β-defensins are known to be secreted by the lung epithelia [47]. However, the human θ-defensins only exists as expressed pseudogene due to a premature stop codon within the signaling sequence which prevents subsequence translation [48]. In the airways, human β-defensin-1 (hBD-1) is constitutively expressed by the respiratory epithelium and secreted into the ASL, whereas hBD-2, -3, and -4 are induced in response to infection and inflammation [1, 5, 25, 36, 49]. In addition to their antimicrobial activity, both hBD-1 and hBD-2 serve as chemoattractants for immature dendritic cells and memory T cells through the chemokine receptor CCR6 [5, 49].

1.4.2 Cathelicidin

Cathelicidins are members of another class of cationic antimicrobial peptides. The human genome is known to have only one cathelicidin gene, the cathelicidin antimicrobial peptide *(CAMP)* [37, 39, 51]. The *CAMP* gene contains four exons, with the first three encoding the N-terminal signal sequence and the cathelin domain and the forth exon encoding the peptide sequence (Figure 8). The resulting pre-pro-form of the peptide is characterized by a N-teminal signal sequence, which is cleaved off during the process of translocation through the endoplasmic reticulum. This pro-form of the peptide consists of a conserved cathelin domain and highly variable C-terminal domain, often referred to as the 18-kDa human cationic antimicrobial protein 18 (hCAP18) [5, 32, 37, 39]. Depending on the cell type and state, this pro-form is further transported and stored in secretory granules, or directly secreted onto the cellular surface. Extracellular proteolytic processing by the neutrophil-derived proteinase 3 has been shown to release and activate the C-terminal antimicrobial peptide [39, 40, 52-54]. However, the exact activation mechanism and its regulation still remain unclear. The resulting AMP, LL-37, adopts a amphipathic α-helical structure, allowing the AMP-bacterial membrane interaction [55]. LL-37 has been shown

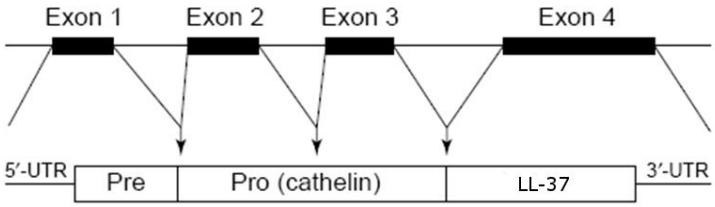

Figure 8: *Schematic depiction of the* CAMP *gene and mRNA encoding regions. The first three exons encode the signal peptid, the cathelin domain and a processing site within the cathelin domain, while the forth exon encodes the actual peptide.*

to be synthesized and secreted by epithelia that are constantly exposed to environmental microorganism [34], such as the airway epithelia [25], as well as being expressed by immune cells [56]. This AMP is present on unstimulated epithelial surfaces at a concentration of approximately 2-5 µg/ml which can increase to a concentration of about 30 µg/ml during epithelial inflammation [57]. The expression of this antimicrobial peptide is inducible by TLR agonists in synergy with vitamin D_3. LL-37 is able to kill relevant airway pathogens such as the Gram-negative *Pseudomonas aeruginosa* and other bacteria that are resistant to antibiotics [25]. In addition, LL-37 has been shown to inhibit the formation of *P. aeruginosa* biofilm at very low concentrations [57] and has been shown to have additional important functions in airway antimicrobial defenses, such as having immunomodulatory properties by stimulating cytokine and chemokine release [58], chemotaxsis of neutrophils, monocytes and T cells [56, 59], LPS neutralization [60], angiogenesis [61], and wound repair [5, 10, 37, 40, 62].

1.4.3 Regulation of AMP expression in airway epithelia

The expression and secretion of the antimicrobial peptides is a tightly regulated process. The regulation of expression of AMPs occurs both at the transcriptional level, such as the gene expression, and post-transcriptional level, with the required proteolytic processing of the stored pro-form to release the activated peptides [39]. Some peptides are produced constitutively, such as the hBD-1. The epithelial expression of hBD-2, hBD-3, hBD-4, and several other AMPs has been shown to be increased upon injury or infection [39, 63-65], as well as in response to contact with microbial products or pro-inflammatory mediators after TLR activation [66, 67]. However, cathelicidin expression is to a lesser extent regulated by TLR activation [68], but is directly induced by vitamin D and the histone deacetylase inhibitor butyrate [39, 69]. Growth factors involved in wound healing have been seen to increase expression of various AMPs, including *CAMP* [70]. The regulation of *CAMP* expression in epithelial cells has been additionally shown to depend on the differentiation status of the cells [1].

1.4.4 Inducers of *CAMP* expression

Short-chain fatty acids

The short-chain fatty acids (SCFAs), such as acetate, propionate and butyrate, are organic acids produced by anaerobic microbial fermentation of complex carbohydrates and undigested dietary fiber in the human intestine [71, 72]. The majority of the produced SCFAs is rapidly absorbed and metabolized as energy source by the colonic epithelium. However, it is known that low concentrations of SCFAs are present in the blood circulation. The cellular absorption of the SCFAs across the apical membrane is thought to occur through diffusion and active transport by SCFA-transporters, such as the monocarboxylate transporter isoform 1 (MCT1) and the SLC5A3, a Na^+-coupled co-transporter. The basolateral transport of SCFAs still remains unclear. Butyrate is known to influence cell function by affecting the regulation of gene expression. Although this exact mechanism still remains unknown, it is thought that butyrates ability to modulate gene expression is attributed to its inhibition of histone deacetylase (HDAC) and thereby affecting the accessibility of transcription factors to the nucleosomal DNA by inducing chromatin remodeling [32, 73]. Butyrate is known to be involved in immunity and has been shown to modulate the host immune response. Butyrate has been shown to reduce mucosal inflammation and to improve epithelial integrity and barrier function [32, 74].

Butyrate has direct immunomodulatory effects by stimulating the release of antimicrobial peptides and by affecting the cytokine-mediated gene expression [75, 76]. This anti-inflammatory effect of butyrate has been linked to the suppression of NF-κB activation, believed to result from the inhibition of HDAC [77, 78]. However, butyrate´s anti-inflammatory activity may also be linked to the inhibition of the intereferon-γ synthesis and signaling [79] as well as the upregulation of the peroxisome proliferator-activated receptor-γ (PPARγ) [80] and vitamin D receptor (VDR) [81], which is as well involved in anti-inflammatory regulation. In addition, butyrates as well as other SCFAs are ligands for specific G-protein-coupled receptor, GPR41 and GPR43 [82] that are thought to play a role in immune regulation. Butyrate has been shown to increase the expression of *CAMP* [83, 84]. However the exact mechanism of the SCFA-induced epithelial expression of *CAMP* still remains unclear.

Vitamin D_3

Vitamin D_3 is a steroid hormone that is involved in the regulation of innate immunity. Many epithelial cells express the VDR [85] and thus are able to respond to the hormone. Humans obtain vitamin D_3 from the exposure of the skin to UVB radiation and to a much smaller extent from dietary intake [86]. For vitamin D_3 to become active there are two sequential hydroxylase steps required. The first step takes place in the liver, where the 25-hydroxylase converts vitamin D_3 into 25-hydroxyvitamin D_3, the primary circulating form of vitamin D_3 [87]. The second necessary hydroxylation step primarily occurs in the kidneys [88], but also in immune cells and epithelial cells [41] including respiratory epithelial cells [89], which have been found to express 1α-hydroxylase that converts the vitamin D_3 the circulating inactive form of vitamin D_3 to the active from, 1,25-dihydroxyvitamin D_3 ($1,25D_3$) [86]. $1,25D_3$ effects the regulation of gene expression by interacting with the VDR, a transcription factor belonging to the steroid/hormone receptor family [86]. Active $1,25D_3$ binds to VDR and together with the retinoid X receptors (RXRs) they bind to the vitamine D-responsive element (VDRE), which can be found in the promoter regions of vitamin D-responsive genes [41, 90, 91], such as within the promoter region of CAMP [92, 93]. $1,25D_3$ thereby regulates the expression of many genes in a cell- and tissue-specific manner, many of which being involved in immunity, including cathelicidin [89, 94] and β-defensin 2 [92]. $1,25D_3$ has long been known to promote recovery from tuberculosis. A vitamin D_3-deficiency results in a higher susceptibility to *Mycobacterium tuberculosis* infections [89]. $1,25D_3$ has been demonstrated to trigger the induction of *CAMP* expression in monocytes in response to TLR-mediated recognition of intracellular *M. tuberculosis* [68].

1.4.5 Therapeutic potential of AMPs

The emergence of bacterial multiple resistance to conventional antibiotics due to their widespread usage, has created problems in nosocomial infections and are of increasing importance in community acquired diseases [95]. In the light of these developments, antimicrobial peptides are becoming increasingly interesting candidates for investigation and development of therapeutic agents against systemic and topical infections. Both the fact that there is only a low incidence of bacterial resistance subsequent to repeated exposure to AMPs [5] as well as their broad spectrum of antimicrobial activity and their immunomodulatory function are crucial aspects to their promising role as future antimicrobials [42, 95]. The therapeutic usage of the peptides could have diverse applications: they could be used as single antimicrobials or in combination with other

antibiotics or additional AMPs for a synergistic effect as well as immunomodulatory or priming factors [42]. The most important aspect might be their ability to kill multi-drug resistant bacteria, when the treatment with conventional antibiotics fails.

Both the pharmaceutical direct local application of synthetic AMPs [96] as well as the application of inducers, such as butyrate and $1,25D_3$, to locally augment AMP expression, could be possible strategies to increase epithelial defenses and to treat infections by multi-drug resistant microorganisms. However, such a development is still in its early stages [96].

Therapeutic administered peptides could also bear the risk of promoting the resistance to the peptides of the innate immunity system, which would certainly lead to peptide-resistant infections. However, synthetic peptides which currently are in clinical trails for drug approval seem to have no effect on the microbial peptide susceptibility nor on the innate immune system. But there are known cases of microbial resistance to AMPs. Some microbes have evolved strategies to downregulate the expression of AMPs [97] or to directly degrade AMPs by releasing proteases [95]. However, effective microbial strategies to evade AMPs are claimed to be less likely. This is partly due to the strict regulation of AMP expression, which leads to very high AMP concentration at the site of infection. Then there is the simultaneous expression and secretions of a variety of different AMPs with diverse microbial targets providing a broad antimicrobial defense strategy.

1.5 Respiratory infections and disease

All major diseases affecting the respiratory system often result from conditions with an impaired innate and adaptive immunity, such as it the case with cystic fibrosis (CF) and primary ciliary dyskinesa. These conditions are accompanied by chronic infections and colonization of the airways by a multiple number of opportunistic pathogens, such as *P. aeruginosa, Streptococcus pneumonia* and *M. tuberculosis* [7].

1.5.1 *Pseudomonas aeruginosa*

P. aeruginosa is a motile Gram-negative aerobic bacterium with a single flagellum and polar pili, which aid the adherence to respiratory epithelial cells [22]. *P. aeruginosa* also possesses a type III secretion system that allows the bacterium to inject virulence factors and toxins into its host cell to interfere with its signal transduction, resulting in alterations in host immune response or even apoptosis [22]. These type III toxins have been additionally shown to interact with cytoskeletal components of the host cell, thereby further aiding the disruption of the epithelial barrier function [98]. It has a wide environmental distribution and can be found in soil, water, on plants and animals, as well as in various foods. It is a major opportunistic human pathogen that was able to develop resistance to many commonly used antibiotics and disinfectants. *P. aeruginosa* is the leading cause of nosocomial infections and can cause severe burn and urinary tract infections in immunocompromised patients. It is additonaly known to establish chronic lifelong colonization of the airway epithelium of cystic fibrosis patients, with an eventual fatal outcome [57]. Such *P. aeruginosa* infections are difficult to treat due to the limited susceptibility to antibiotics and the high frequency of emergence of antibiotic resistance during the course of treatment eventually leading to a multidrug resistance [99]. *P. aeruginosa* has been shown to have a larger genome size and genetic complexity compared to other bacteria [100]. This genome size difference is thought to contribute to the

advantage *P. aeruginosa* has over other bacteria and allows it to adapt to ever changing environmental condition by being metabolic versatile and flexible [22, 100]. Many of the identified genes are involved in the regulation, catabolism and transport of organic compounds [101]. Although it has been shown that repeated exposure of *P. aeruginosa* to AMPs results in a slight induction of resistance [59], it is thought to be unlikely that *P. aeruginosa* would be able to naturally develop a resistance to AMPs due to the nature of AMP-microbial membrane interaction that would require a rearrangement of the membrane [9, 42]. Additionally, the peptides might have multiple targets making a bacterial AMP-resistance rather unlikely [42]. *P. aeruginosa* has recently been shown to be able to modulate flagellin-induced pro-inflammatory responses of human airway epithelial cells [102]. In addition, *P. aeruginosa* is known to be able to open up tight junctions and thereby impair epithelial integrity to gain access into the host [3, 103, 104]. The ability to form a biofilm allows *P. aeruginosa* to grow in microcolonies and thereby survive in the hostile environment of the lung [3, 12, 105, 106].

1.5.2 Cystic fibrosis

CF is a genetic disorder that impairs innate immune mechanisms of the airway epithelia. It is a autosomal recessive disease that is characterized by the impaired function of the CF transmembrane conductance regulator (CFTR) protein due to mutations [3]. CFTR is a member of the ATP-binding cassette transporters and forms an apical membrane anion channel which is regulated by cAMP and nucleotides [5].

Early CF is characterized by intermitted bacterial infections and the onset of inflammation of the airway caused by various bacteria, including *P. aeruginosa* [5]. Characteristics of the chronic CF lung disease include persistent bacterial infection with microorganisms living in biofilms, chronic neutrophilic inflammation, and progressive bronchiectasis [7]. This chronic infection and inflammation associated with CF causes epithelial injury and repair responses [5] leading to impaired epithelial functions.

There are two CF-specific defects in the airway epithelial ion transport. In normal airway epithelia the ASL volume maintenance is mediated by an active Na^+ absorption (Figure

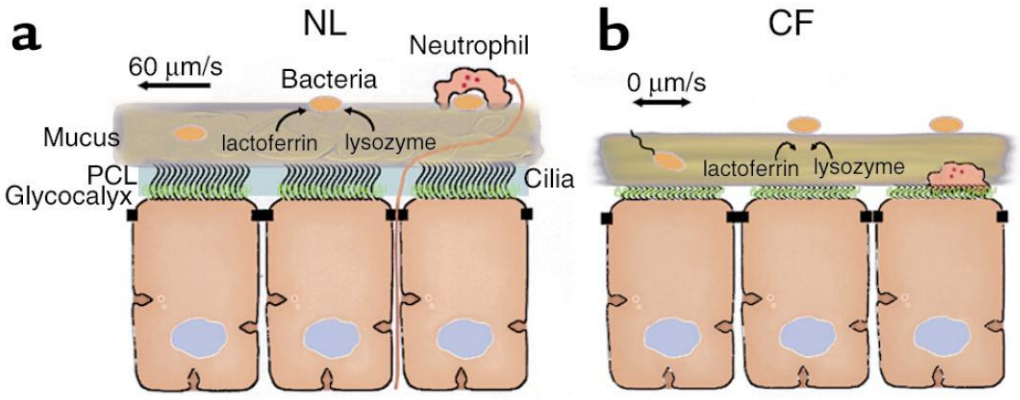

Figure 9: *ASL characteristics in normal lungs (a) and CF lungs (b). The ASL volume depletion on the airway surface leads to an impaired mucuciliary transport of the mucus layer in CF lungs.*

9a). In case of ASL volume depletion, normal airway epithelial cells can slow the Na^+ absorption by inducing Cl^- secretion. However, in CF, both the Na^+-absorption and Cl^--secretion mechanisms appear to be affected. It has been proposed that CFTR mutations cause increased NaCl and liquid absorption across the airway epithelia, thereby dramatically decreasing the ASL volume and impairing ciliary function, by removing the liquid in which cilia can extend and beat freely (Figure 9b) [3, 5, 12]. In addition, goblet cells in CF epithelia hypersecrete mucus to the apical surface, which results in a additional mucosal load [3]. Together the ASL volume depletions and the additional mucosal load result in impaired mucociliary clearance, which facilitates bacterial adherence and colonization. These volume depletions additionally compromise the PCL's lubricant function, thus allowing the mucus layer to adhere to the cell surface and inhibiting cough clearance. In addition, AMPs, such as β-defensins and LL-37, have been shown to be inactivated by the epithelial surface fluid in CF lungs, which might be due to high salt concentration or through other present components [25, 35, 107-109].

2 Aims of this thesis

The aim of this thesis was to characterize the VA10 cell line in respect to its suitability as a model system for innate immunity of bronchial epithelia. Our aim was to determine the expression of relevant receptors in VA10 cells as well as possible signaling pathways of antimicrobial defenses. It was furthermore of interest to determine whether and how the stimulation with Toll-like receptor ligands in addition to the stimulation with 4-PBA and $1,25D_3$ affects the innate gene expression in VA10 cells. Finally our goal was to determine if bacterial clearance can be enhanced through these stimulations.

3 Materials and Methods

3.1 Cell culture

The bronchial epithelial cell line (VA10), immortalized by retroviral transduction with E6 and E7 viral oncogenes, was cultured as previously described [110]. Briefly, the cell line was maintained in Gibco® LHC-9 medium (Invitrogen, Carlsbad, California, USA) in 75 cm^2 nuclon™ plastic flasks (Nunc™, Roskilde, Denmark) at 37°C in 5% CO_2. For experiments, 2.0 x 10^5 cells were seeded in six well Nunclon™ surface plates (Nunc™). On the first day after seeding medium was changed to bronchial epithelium basal medium (BEBM®) supplemented with SingleQuots® bronchial epithelial growth medium supplements (BEGM®) (Lonza, Walkersville, MD, USA) consisting of 2 ml bovine pituitary extract, 0.5 ml insulin, 0.5 ml hydrocortisone, 0.5 ml retinoic acid, 0.5 ml transferrin, 0.5 ml triiodothyronin, 0.5 ml epinephrine, and 0.5 ml human epidermal growth factor in 500 ml basal medium. The supplied gentamicin (GA-1000) was omitted, and penicillin (25 U/ml) and streptomycin (25 µg/ml) were utilized (Gibco®, Invitrogen, Carlsbad, California, USA). Cultures were rinsed with phosphate-buffered saline (PBS) every 2-3 days. Cells at 90% confluence were stimulated with 4 mM Sodium 4-Phenylbutyrate (4-PBA) dissolved in MilliQ H_2O (Tocris Bioscience, Ellisville, MO, USA) and 1α,25-Dihydroxyvitamin D_3 (VitD) dissolved in ethanol (Fluka®, Sigma-Aldrich, St. Louis, MO, USA), *Pseudomonas aeruginosa* lipopolysaccharide (LPS) dissolved in MilliQ H_2O (Sigma®, Sigma-Aldrich), *P. aeruginosa* flagellin, and whole *P. aeruginosa* in BEGM without antibiotics inactivated by repeated freeze-thaw cycles. For air-liquid interface (ALI) cultures 3.0 x 10^5 VA10 cells were seeded onto 12-well Transwell permeable filter supports with a pore size of 0.4 µm (Corning Costar Corporation, Acton, MA, USA). On the second day after seeding, the medium was changed from BEGM to 50:50 DMEM-Ham's F-12 medium (Gibco®) supplemented with 2% Ultroser G (Pall, Port Washington, NY, USA) as well as penicillin (25 U/ml) and streptomycin (25 µg/ml) (Gibco®) on both apical and basolateral sides. After reaching full confluence the medium was removed from the apical side of the epithelium to establish the air-liquid interface. Cultures were rinsed with PBS every 2-3 days. The cells were cultured at the air-liquid interface for 1 week before RNA isolation.

3.2 Flagellin isolation

P. aeruginosa flagellin has been isolated as previously described [111]. Briefly, two starter cultures of 5.0 ml LB broth were inoculated with PKS-1 (a wild-type *P. aeruginosa* strain (PAO1) that was kindly provided by Prof. Pradeep K. Singh, Seattle, Washington, USA) and incubated for 4 h at 37°C and 200 rpm. These starter cultures were then transferred to 500 ml LB broth each, and incubated at overnight in the shaker incubator. The bacterial cultures were then centrifuged at 9.000 x g for 20 min at 4°C to pellet the bacteria. LB broth was removed and bacterial pellets were resuspend in 30 ml PBS. The resuspended bacteria were carefully pulled through a 18 G syringe to shear off the flagella. After syringing the suspension was centrifuged at 6.800 x g for 30 min at 4°C to pellet the bacteria. The resulting supernatant was centrifuged at 100.000 x g in a Optima™ L-100 xP

Ultracentrifuge (Beckman Coulter, Brea, CA, USA) with the SW-41 rotor for 3 h at 4°C. The PBS was removed and flagella pellets were resuspended in 500 µl H_2O. These samples were freeze-dried overnight and pooled together, prior to being finally resuspended in 200 µl PBS. The protein concentration was determined by using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). The absorbance was measured at 595 nm in a spectrophotometer (Ultrospec 1100 pro, GE Healthcare Life Siences, Little Chalfont, Buckinghamshire, UK). The purity of the flagellin isolation was confirmed by running the samples on a SDS gel. The samples were prepared by adding 1.5 µl β-mercaptoethanol and 3.5 µl NuPAGE® LDS sample buffer (Invitrogen) with 10.0 µl sample. These preparations as well as the Page Ruler unstained protein ladder (Fermentas, St. Leon-Rot, Germany) were heated to 70°C for 15 min on a heating block. 5.0 µl of the ladder and 15.0 µl of the samples were loaded on a SDS NuPAGE® 4-12% Bis-Tris Gel (Invitrogen) in NuPAGE® MES running buffer (Invitrogen), the first 15 min at 90 Volt and 150 Volt for approximately 1 h. The gel was washed three times with approximately 200 ml MilliQ H_2O. Then 20.0 ml PageBlue protein staining solution (Fermentas) was applied to cover the gel. This was heated briefly in a microwave and incubated on a rocker for 20 min at room temperature. The gel was again rinsed three times with MilliQ H_2O and incubated over night on a rocker at room temperature before pictures were taken.

3.3 Total RNA isolation

Total RNA was isolated by using the Nucleospin® RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The total RNA was quantified on a spectrometer (Nanodrop 1000, Thermo Scientific). One µg total RNA was reverse transcribed to cDNA by using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to manufacturer's protocol, modified by using 100 units of reverse transcriptase per reaction.

3.4 Quantitative real-time PCR

The cDNA of stimulated cells of three expreimnets was quantified relative to controls, using either Power SYBR® green Master Mix (Applied Biosystems, Foster City, CA, USA) or TaqMan® Universal PCR Master mix (Applied Biosystems) with respective primers and probes (Table 2). The reaction setup per reaction was: 5.0 µl SYBR® green master mix, 0.5 µl 5.0 µM forward primer, 0.5 µl 5.0 µM reverse primer, 2.0 µl MilliQ H_2O, and 2.0 µl cDNA for a SYBR® green reaction; and 5.0 µl TaqMan® master mix, 0.5 µl primer and probe, 3.5 µl MilliQ H_2O and 1.0 µl cDNA for a TaqMan® reaction. The quantity of gene transcripts in each sample was measured in duplicates on a 7500 Real Time PCR System (Applied Biosystems) as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Data was collected and recorded by the 7500 Software v2.0 (Applied Biosystems) at 60°C during every cycle. The data was expressed as a function of the threshold cycle (Ct). Further data analyses were done using Microsoft Excel. Figure 2 and 3 were generated by calculating the average Ct. The ΔΔCt value was obtained by calibrating the sample to the control (ΔΔCt = average Ct Sample – average Ct control). The fold difference in RNA quantity and therefore in gene expression was determined by $2^{(-\Delta\Delta Ct)}$. The standard error of the mean (SEM) was obtained by first calculating the standard deviation (stdev) of the samples followed by the formula

Table 2: *Primer and probe sequences.*

Target		Sequences	Amplicon [bp]
TLR1	F	5′-CAA GAC TGT AGC AAA TCT GG-3′	89
	R	5′-GTT TCG CCA GAA TAC TTA GG-3′	
TLR2	F	5′-TCA CCT ACA TTA GCA ACA GTG-3′	365
	R	5′-GTA GAT CTG AAG CAT CAA TCT C-3′	
TLR4	F	5′-CCT AAG GAA ACC TGA TTA ACA C-3′	148
	R	5′-GAT ATT AGC TTA TAG GCA AGA C-3′	
TLR5	F	5′-CCT CTA GAC CAT CCT CAC AGT CAC-3′	355
	R	5′-GGC TTC AAG GCA CCA GCC ATC TC-3′	
TLR6	F	5′-TAT CCT GCC ATC CTA TTG TG-3′	90
	R	5′-AGT TGC CAA ATT CCT TAC AG-3′	
TLR9	F	5′-CAT ACC AAC ATC CTG ATG CT-3′	90
	R	5′-CTT GTA ATA ACA GTT GCC GT-3′	
IPAF	F	5′-TCA TGA ACT GAT CGA CAG GA-3′	236
	R	5′-TTT CCC GCC AAA TTC AAC TG-3′	
SLC5A8	F	5′-CCC TGG CTT TGA ATC AAG TC-3′	98
	R	5′-CTT TAA GAC CAC CCA GTG TG-3′	
GPR41	F	5′-ATC TAC GTG ACG CTT CTC AG-3′	100
	R	5′-CTC AGC AGC TCA TGA AAG TC-3′	
GPR43	F	5′-AGA ACT TCA CCG ATA ACC AG-3′	100
	R	5′-GTA GCA GAA GAT GGT GAC TG-3′	
CAMP	F	5′-TCA CCA GAG GAT TGT ACT TCA A-3′	67
	R	5′-TGA GGG TCA CTG TCC CCA TAC-3′	
	Probe	5′-FAM-AAG GAC GGG CTG GTG -BHQ1-3′	
DEFB1	F	5′-TGC TGT TTA CTC TCT GCT TAC TTT TGT-3′	67
	R	5′-CCA AGG CCT GTG AGA AAG TTA CC-3′	
	Probe	5′-FAM-CTG AGA TGG CCT CAG GT-BHQ1-3′	
IL8	F	5′-ACA TAC TCC AAA CCT TTC CAC-3′	88
	R	5′-CAA TAA TTT CTG TGT TGG CGC-3′	
TNF	F	5′-CCA GGC AGT CAG ATC ATC TT-3′	72
	R	5′-GCT TGA GGG TTT GCT ACA AC-3′	
TUBB	F	5′-CAG CTG GAC CGC ATC TCT GT-3′	64
	R	5′-TGG CAC GAG GAA CAT ATT TGC-3′	

SEM $= \frac{\text{stdev}}{\sqrt{n}}$. Figure 6 and 7 were generated by the calculations according to the ΔΔCt method described by [112]. From the obtained gene-specific Ct values of each sample the average Ct and the standard deviation of the average of each sample were determined. To determine the ΔCt values the average Ct of the samples (Sample) was normalized to the average Ct of the housekeeping gene β-tubulin (*TUBB)* (ΔCt = average Ct Sample – average Ct *TUBB*). The standard deviation for the ΔCt was calculated by the formula stdev $\Delta Ct = \sqrt{(\text{stdev TUBB})^2 + (\text{stdev Sample})^2}$. The ΔCt value of the unstimulated control samples were used as reference samples (Control) in order to calculate the ΔΔCt for each stimulated sample (ΔΔCt = ΔCt Sample - ΔCt Control). The standard deviation for the

ΔΔCt values are the same as the standard deviation of the ΔCt. The fold difference in gene expression relative to the control samples was obtained by $2^{(-\Delta\Delta Ct)}$. The resulting fold difference values were plotted in a bar graph. The standard deviation of the fold difference was calculated (stdev Fold difference = (ln2)(stadev ΔΔCt)($2^{(-\Delta\Delta Ct)}$)) in order to determine the standard error of the mean (SEM = $\frac{\text{stdev Fold difference}}{\sqrt{n}}$).The most stable housekeeping gene in VA10 cells was determined with the Human Reference Gene Panel (Tataa Biocenter, Gothenburg, Sweden). Specific primer and probes were designed using PrimerExpress™ v2.0.0 (Applied Biosystems) and PerlPrimer v1.1.19 (Marshall O, 2007). Nucleic acid synthesis was performed by Microsynth (*CAMP* and *DEFB1*) (Balgach, Switzerland) and TAG Copenhagen (Copenhagen, Denmark).

3.5 Receptor expression

To determine receptor expression in VA10 cells in both simple cell cultures and at an air-liquid interface, total RNA was isolated and reverse transcribed as described above. The PCR products were produced as following: 5.5 µl ThermoPol Buffer (New England BioLabs, Ipswich, MA, USA), 1.1 µl dNTP (Invitrogen), 0.44 µl *Tag* polymerase (New England BioLabs), 2.2 µl forward primer, 2.2 µl reverse primer (Table 1), 2.5 µl cDNA, and 11.06 µl MilliQ H_2O for 10 min at 95°C, followed by 30 cycles of 15 sec at 95°C and 1 min at 60°C, and a final 10 min extension step at 60°C. The 10.0 µl of the resulting DNA fragments were loaded together with 2.0 µl Xylene loading dye and 3.0 µl 100 bp ladder (Fermentas) on a 2% agarose gel and electrophorized for 40 min at 80 Volt.

3.6 Cloning

In order to confirm the resulting receptor bands by sequencing these receptor DNA fragments were cloned into a gateway recombinational cloning entry vector using the pCR®8/GW/TOPO® TA cloning® kit (Invitrogen). Briefly, for the TOPO® Cloning reaction 1.5 µl cDNA, 0.3 µl salt solution and 0.25 µl TOPO® vector were gently mixed and incubated for 15 min at room temperature. The resulting constructs were then transformed into 50.0 µl of "homemade" competent *Escherichia coli* and gently mixed. The cells were incubated on ice for 15 min, followed by being heat-shocked for 40 sec in a 42°C water bath. The cells were then immediately transferred to ice were 250.0 µl of room temperature S.O.C. medium were added. The cells were incubated horizontally at 37°C for 1 h at 200 rpm. 50.0 µl and 250.0 µl of the transformations were then spread on pre-warmed selective LB agar plates containing 100.0 µg/ml Spectinomycin (Sigma®, Sigma-Aldrich), and incubated overnight at 37°C. The clone insert fragments were amplified from individual colonies using the described PCR reaction, appropriate primers and confirmed by electrophoresis on a 2% agarose gel.

3.7 Plasmid isolation

Plasmids were isolated by using the Nucleospin® Plasmid DNA Purification kit (Mackerey-Nagel) according to manufacturer´s protocol with the exception of eluting the DNA in two 25.0 µl MilliQ H_2O elution steps. The DNA concentration was quantified on a spectrometer (Nanodrop) at 230 nm.

3.8 Sequencing

The 0.8 µg of plasmid DNA were then precipitated as follows: to the volume of 0.8 µg of DNA MilliQ H_2O was added to give a total volume of 50.0 µl. 5.0 µl of 3 M sodium acetate with a pH 5.2 were added along with 150.0 µl of -20°C cold 100% ethanol. The samples were incubated at -20°C for approximately 2 hours followed by centrifugation at 14.000 rpm at 4°C for 15 min. The supernatant was removed by pipetting and 200.0 µl of -20°C 70% ethanol was added to rinse the DNA pellets. All the ethanol was removed by pipetting and the samples were shortly centrifuged at 14.000 rpm at 4°C to collect and remove most of the ethanol. The remaining ethanol was evaporated by placing the samples in a heating block at 35°C for approximately 5 min. The samples were sent to Microsynth for sequencing (Cycle sequencing/Capillary electrophoresis). The forward M13 (5´- TGT AAA ACG ACG GCC AG-3´) primer was provided by Microsynth.

3.9 Immunohistochemistry

Approximately 2.5 x 10^4 VA10 cells were seeded into the wells of a 8-well chamber slide and cultured in BEGM as described above until they were well confluent. The growth medium was removed and the cells were rinsed with PBS before being fixated with 100% methanol at -20°C for 5 min. The methanol was removed and chambers taken off the slides. The fixated cells were rinsed two times with PBS before proceeding with the protocol of the EnVision+ System-HRP (DAB) kit (DakoCytomation, Glostrup, Denmark) for use with rabbit primary antibodies. Briefly, the cells were covered with the supplied peroxidase block and incubated for 5 min at room temperature. After rinsing with PBS, the rabbit polyclonal NFκB p50 (H-119) antibody (sc-7178, Santa Cruz Biothechnology, Santa Cruz, California, USA) dilution in a ratio of 1/100 was applied to cover the cells and incubated for 30 min at room temperature, followed by a rinsing step with PBS. The cells were incubated with the supplied peroxidase labelled polymer solution for 30 min at room temperature. After washing the cells with PBS, the cells were incubated with the DAB+ substrate-chromogen solution for 10 min at room temperature. At last the cells were once more rinsed with PBS and then with MilliQ H_20 and covered with cover glasses to prevent the cells to dry out on the microscope during the picture taken at a 40 x magnification.

3.10 Statistical analysis

The results are presented as mean of gene expression together with the standard error of the mean, from three independent experiments. Outliners were not excluded from the statistical analysis. To compare differences between groups, the two-sided Student´s t-test was used. Differences with $p < 0.05$ were considered statistically significant. Tests were performed in Microsoft Excel.

4 Results

4.1 Receptor expression in VA10 cells

To determine the expression of bacteria-relevant PRRs by VA10 cells, total RNA was isolated from VA10 cells as well as from ALI VA10 cells. Total RNA isolated from THP1 and HT29 cell cultures was used as control. The RNA was reverse transcribed prior to the PCR reactions that were performed with the appropriate primer pair (Table 2). The resulting fragments were analyzed according to size by electrophoresis on a 2% agarose gel.

We detected DNA fragments for *TLR1*, *TLR2*, *TLR5* and *TLR6* of expected size in the VA10 cells. The amplified fragments for *TLR4* and *TLR9* were weaker but of expected size (Figure 10). The fragments generated from ALI VA10 total RNA were cloned into *E. coli*. The amplified plasmids were isolated and sequenced. The obtained sequences have been analyzed by NCBI BLAST searches within the human genome confirming the identity of *TLR1*, *TLR2*, *TLR4*, *TLR5*, *TLR6*, and *TLR9* in ALI VA10 cells (Appendix A).

We were additionally interested in the expression of the intracellular *IPAF*. This NLR family member is known to recognize cytoplasmic flagellin. However, there was no detectable expression of *IPAF* in VA10 cells.

The mechanism through which 4-PBA affects the gene expression in VA10 cells still remains to be determined. We were therefore interested in the expression of the G-protein-coupled receptors GPR41 and GPR43, which have been shown to recognize SCFAs such as butyrate. The analysis of the PCR-generated fragments indicates that *GPR43* might be

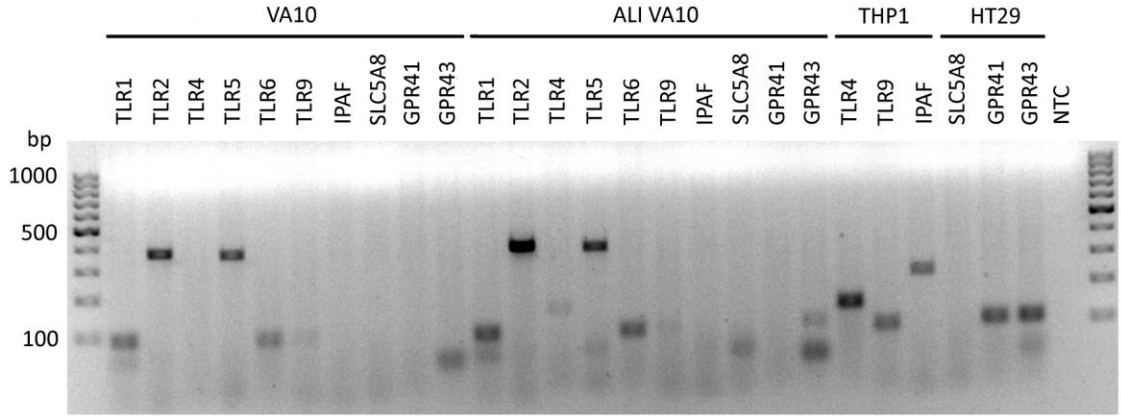

Figure 10: *Receptor expression in VA10 cells. Cells were grown to confluency before total RNA was isolated and reverse transcribed. PCR reactions with the appropiate primers were performed and the resulting fragments were analyzed according to their expecetd size by 2 % agarose gel electrophoresis. RNA from THPI and HT29 cell cultures served as control.*

expressed by ALI VA10 cells (Figure 10). However, the sequencing of the fragment generated a nonsense sequence, despite the right-sized PCR-generated fragment and clone insert.

Furthermore, the Na^+-co-transporter SLC5A8 was considered of interest as it is known to co-import butyrate together with sodium across the cellular membrane. However, we were unable to detect any fragments corresponding to *SLC5A8* both in VA10 cells and our control cell line (Figure 10).

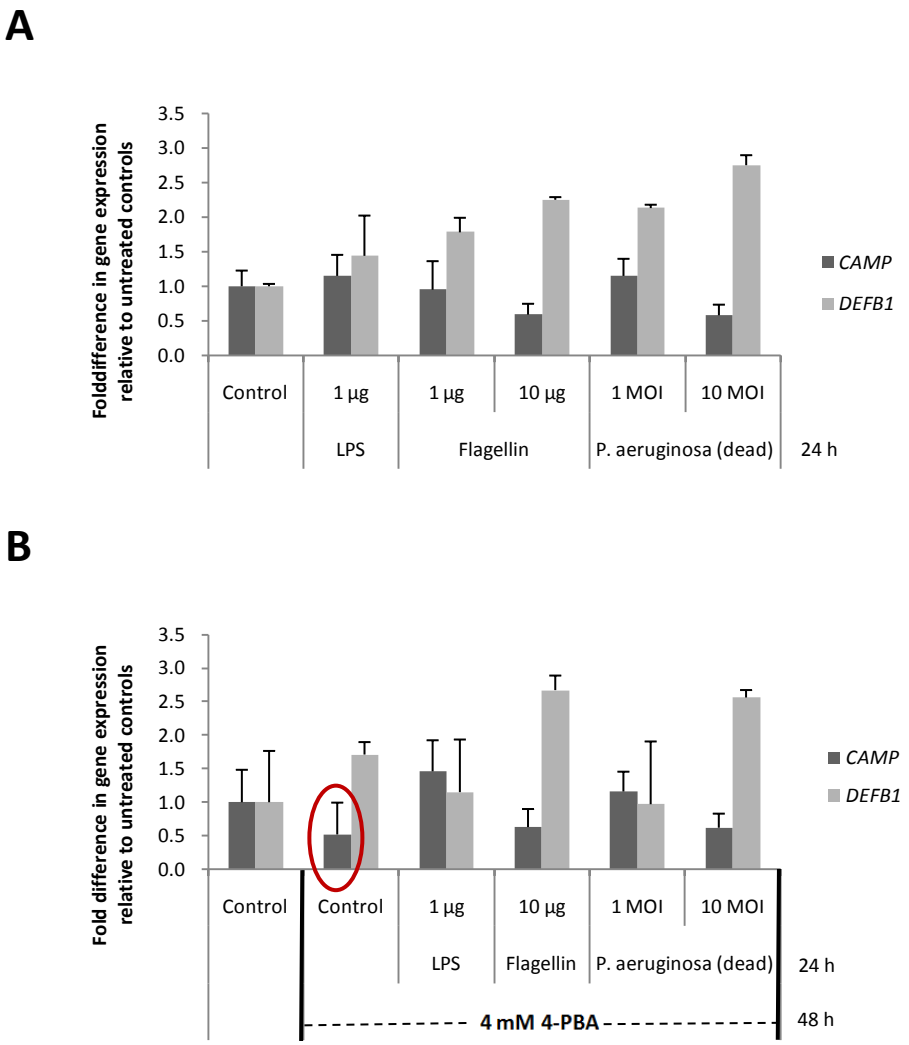

Figure 11: *Fold changes in* CAMP *and* DEFB1 *mRNA expression in VA10 cells cultured in LHC-9. A) VA10 cells were treated with the indicated concentrations of stimulants for 24 hours. B) VA10 cells with the indicated concentrations of stimulants for 24 hours after the prestimulation with 4 mM 4-PBA for 24 hours.* CAMP *and* DEFB1 *mRNA levels were determined by quantitative RT-PCR. The quantity of the RNA input (1 µg) was the same for all individual reactions. Results were normalized to expression in control samples, where controls were given the arbitary value of one. Data of three independent experiments is presented as mean change in gene expression together with the standard error of the mean (n = 3).*

4.2 Gentamicin affects innate immunity gene expression in VA10 cells

To determine whether TLR stimulation has an effect on the AMP gene expression in bronchial epithelia cells, VA10 cell cultures were stimulated with indicated concentrations of LPS, flagellin or dead *P. aeruginosa* for 24 hours. In another experiment the VA10 cells

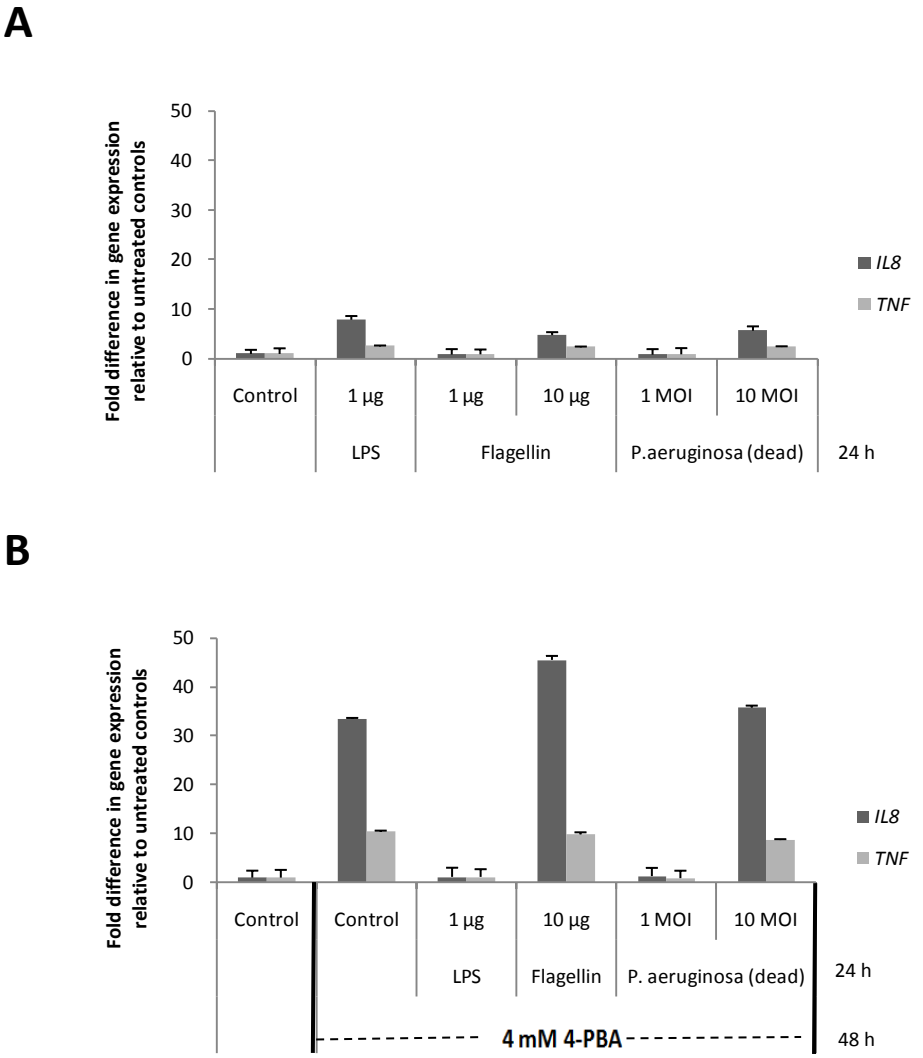

Figure 12: *Fold changes in* IL8 *and* TNF *mRNA expression in VA10 cells cultured in LHC-9. A) VA10 cells were treated with the indicated concentrations of stimulants for 24 hours. B) VA10 cells with the indicated concentrations of stimulants for 24 hours after the prestimulation with 4 mM 4-PBA for 24 hours.* IL8 *and* TNF *mRNA levels were determined by quantitative RT-PCR. The quantity of the RNA input (1 µg) was the same for all individual reactions. Results were normalized to expression in control samples, where controls were given the arbitary value of one. Data of three independent experiments is presented as mean change in gene expression together with the standard error of the mean (n = 3).*

Table 3: *Overview of the supplemental components of the bronchial epithelial cell culture media used for culturing VA10 cells.*

LHC-9	**BEGM**
LHC basal medium	BEBM basal medium
Supplemented with:	
Retionic acid	Retionic acid
Epinepherine	Epinepherine
Gentamicin	-
Insulin	Insulin
Hydrocortisone	Hydrocortisone
EGF	hEGF
Transferrin	Transferrin
Bovine pituitary extract	Bovine pituitary extract
Triiodothyronine	Triiodothyronine
plus penicillin and streptomycin	

were first prestimulated with 4 mM 4-Phenylbutyrate (4-PBA) for 24 hour prior to the stimulation with the TLR ligands for additional 24 hours. Total RNA was isolated from these cells and reverse transcribed before quantitative RT-PCR analysis of *CAMP* and *DEFB1* as well as *IL8* and *TNF* gene expression (Figure 11 and 12).

However, the reduction of *CAMP* gene expression in VA10 cells subsequent to stimulation with 4 mM 4-PBA for a total of 48 hours was not according to the previous results obtained in a similar experimental setup, when a 40 fold induction of *CAMP* expression was detected [84] (Figure 11 B).

The only difference from the previous experimental setup was the growth medium used for culturing VA10 cells. In these experiments we had used LHC-9 medium but BEGM medium before. The VA10 cells certainly appeared to grow in both bronchial epithelial media similarly well.

After a comparison of the supplemental components of both the bronchial epithelial cell culture media, the pre-added gentamicin in the LHC-9 medium caught our attention (Table 3). We were used to omit the addition of gentamicin to the BEGM and replace it with penicillin and streptomycin.

To determine whether gentamicin affected the gene expression in VA10 cells, we cultured the cells in LHC-9 medium and in BEGM medium with and without gentamicin. The cells were stimulated with 4 mM 4-PBA or with 100 nM 1,25-Dihydroxyvitamin D_3 ($1,25D_3$)

for 24 hours, followed by total RNA isolation and quantitative RT-PCR analysis of the *CAMP* expression.

Gentamicin affected the baseline *CAMP* expression in unstimulated control VA10 cell cultures. There was a low expression of *CAMP* in cells cultured in BEGM without gentamicin (high Ct value), while there was a similarly induced expression in both the cells cultures grown in media with gentamicin (low Ct value) (Figure 13 A). The stimulation with 4-PBA and $1,25D_3$ induced *CAMP* expression. These results were normalized to the gene expression in the unstimulated control VA10 cell cultures, and show different fold induction values. However, the gene expression was induced to very similar Ct values both in media with and without gentamicin. The gentamicin affected the baseline *CAMP* expression in the control cells resulting in different fold induction values (Figure 13 B).

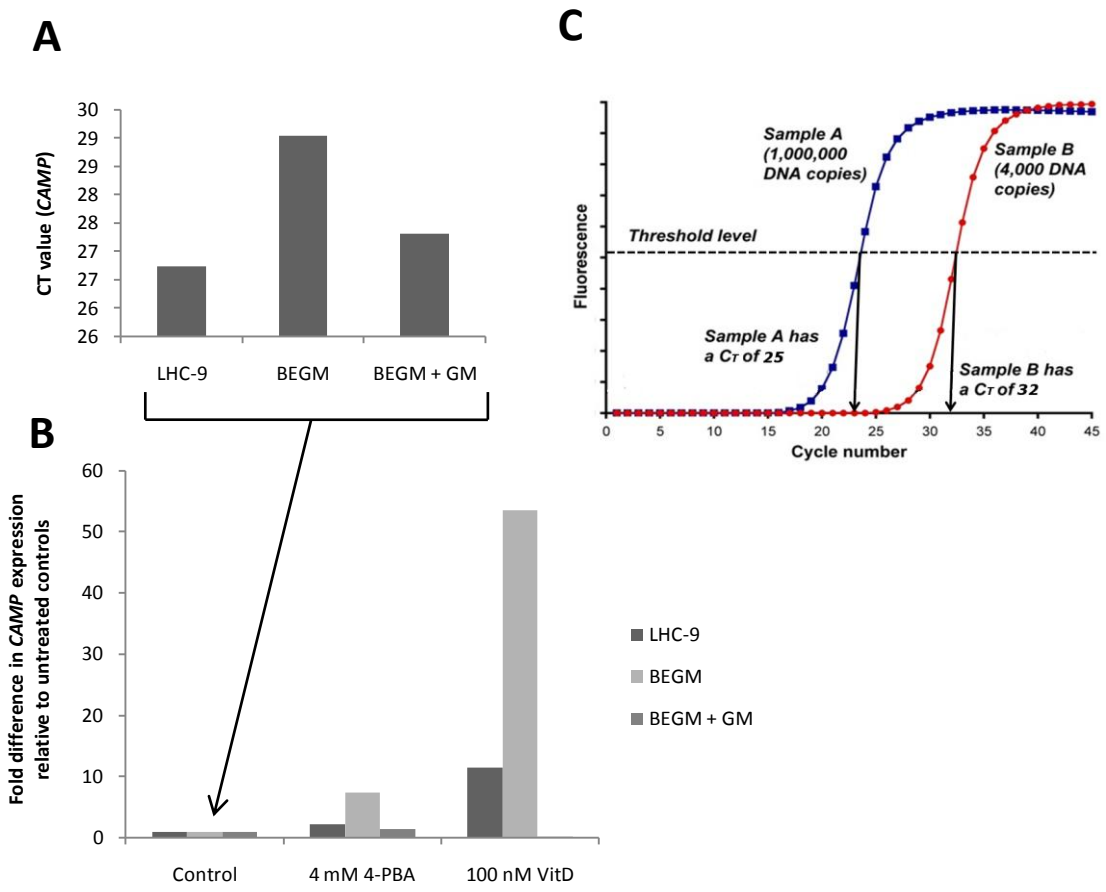

Figure 13: *The effects of gentamicin on the expression of* CAMP *mRNA in VA10 cells. Gentamicin induces* CAMP *expression in unstimultaed cells (A), resulting in different results of* CAMP *expression in 4-PBA and* $1,25D_3$ *stimulated cells despite similar CT values (B). Schematic representation of quantitative RT-PCR data. The CT (cycle threshold) value is defined as the number of cylces required for the fluorescent signal to crosss the threshold, depending on the amount of target nuclic acid in the sample. Thus, a low CT value indicates an abundant target nucleic acid, while a high CT value indicates a lower amount of nucleic acid in the sample (C). GM = Gentamicin.*

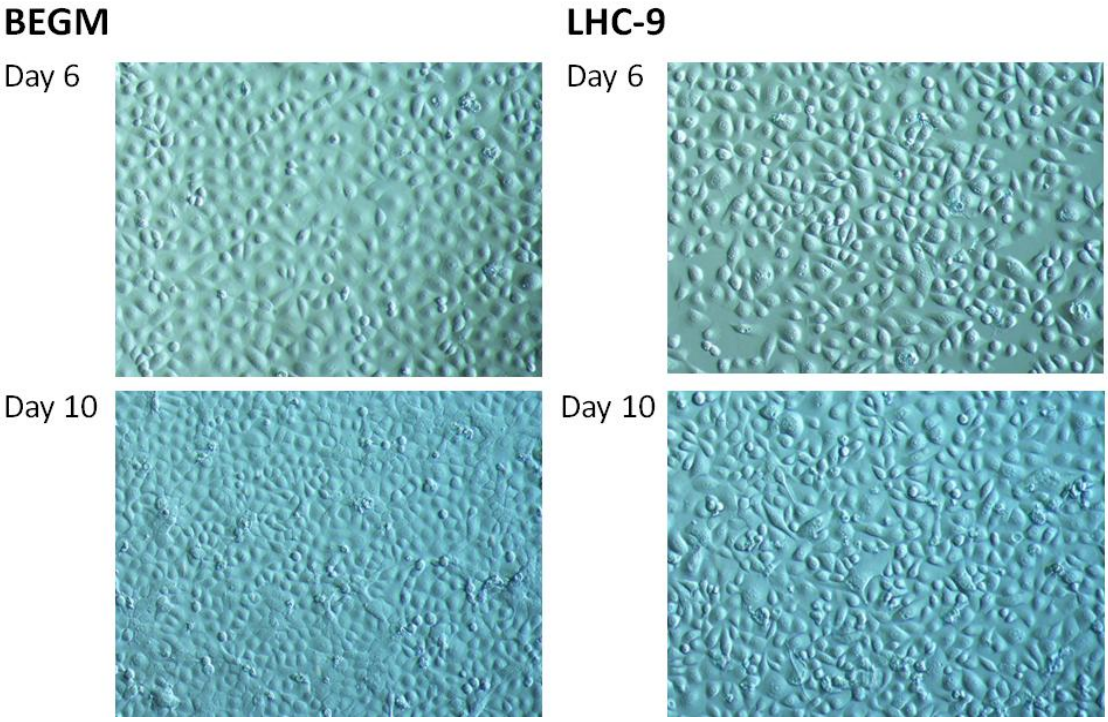

Figure 14: *Difference in visual appearance of VA10 cell cultures cultured in different growth media. VA10 cells were grown in BEGM or LHC-9 for 6 and 10 days.*

Further visual comparison of VA10 cell cultures grown in BEGM and in LHC-9 medium did show differences. The VA10 cells cultured in BEGM did form a evenly confluent monolayer of cells with smooth cellular boundaries, while the cells cultured in LHC-9 medium appeared more separated with sharper cellular edges (Figure 14).

Together these findings led to the decision to change the medium used during experimental setups from LHC-9 back to BEGM.

4.3 Toll-like receptor stimulation has little effect AMP expression in VA10 cells

In order to assess whether TLR stimulation affects AMP gene expression, VA10 cell cultures were cultured in BEGM and stimulated with indicated concentrations of LPS, flagellin and dead *P. aeruginosa* for 24 hours. In addition we used prestimulation with 4 mM 4-PBA, 20 nM $1,25D_3$ or 4 mM 4-PBA together with 20 nM $1,25D_3$ for 24 hour prior to the stimulation with the TLR ligands for additional 24 hours. Total RNA was isolated from these cells and reverse transcribed before quantitative RT-PCR analysis of *CAMP* and *DEFB1* gene expression. Statistical differences between the 4-PBA or $1,25D_3$ stimulated control samples and the TLR stimulated samples were analyzed by the Student´s t-test (Figure 15).

The stimulation with LPS, flagellin or dead *P. aeruginosa* alone had only minor effect on *CAMP* and *DEFB1* expression in VA10 cells (Figure 15 A).

The prestimulation with 4-PBA significantly induced the *CAMP* gene expression in all stimulated samples compared to the gene expression in unstimulated control VA10 cell

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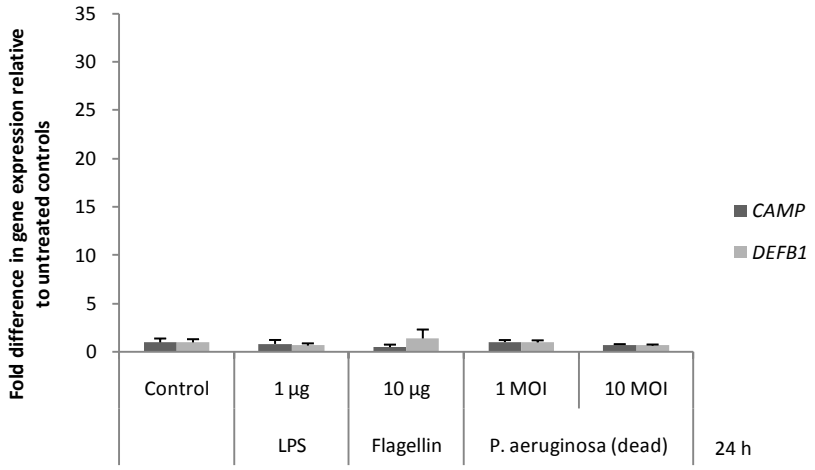

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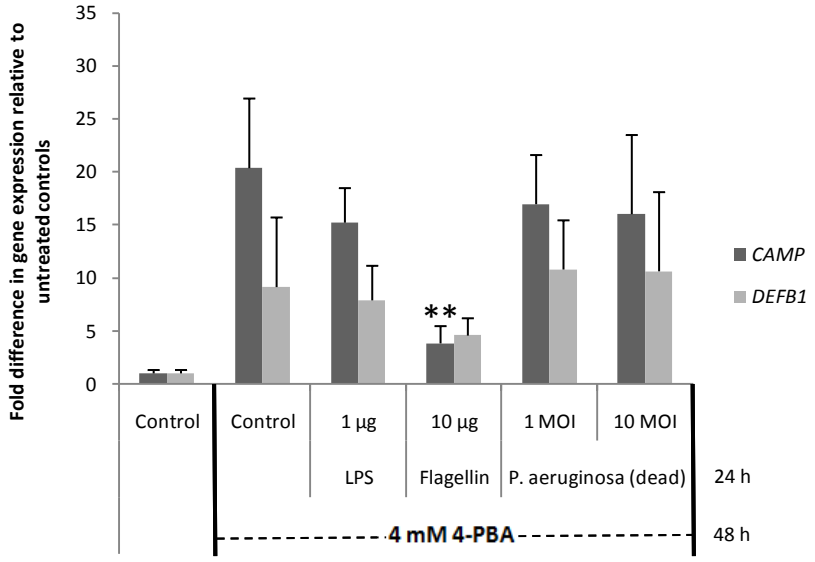

Figure 15: *Fold induction of* CAMP *and* DEFB1 *mRNA expression in VA10 cells cultured in BEGM. VA10 cells were stimulated with the indicated concentrations of TLR ligands for 24 hours (A). VA10 cells were prestimulated with 4 mM 4-PBA (B) prior the stimulation with the indicated concentrations of TLR ligands for additional 24 hours.* CAMP *and* DEFB1 *mRNA levels were determined by quantitative RT-PCR. The quantity of the RNA input (1 µg) was the same for all individual reactions. The expression of the housekeeping gene* TUBB *was used as internal reference. Results were normalized to expression in control samples, where controls were given the arbitary value of one. Data of three independent experiments is presented as mean together with the standard error of the mean (n = 3). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.*

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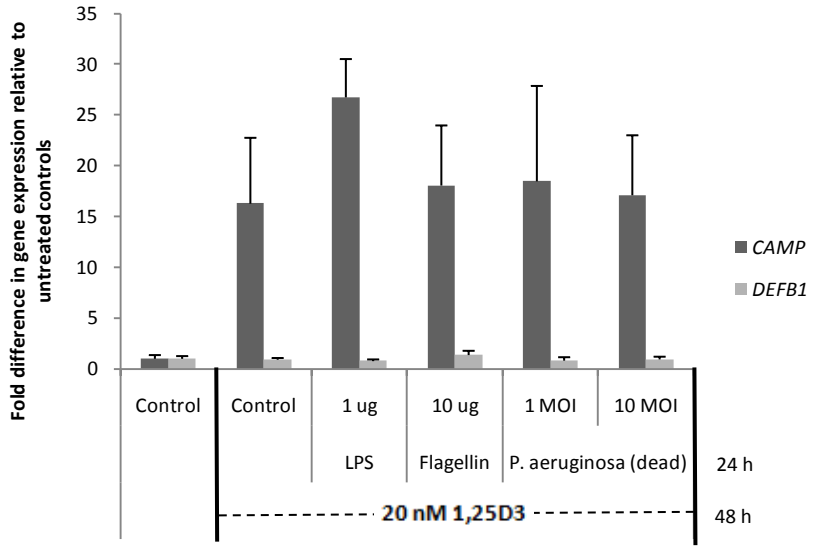

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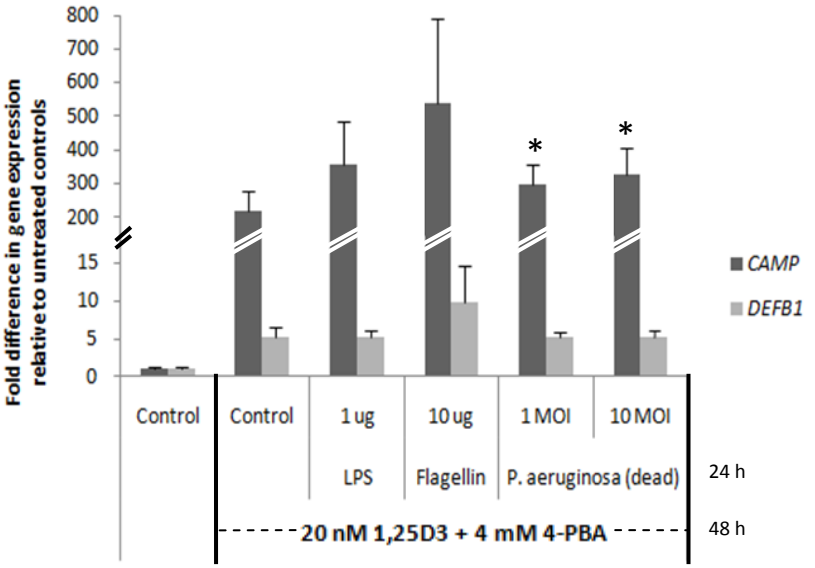

Figure 15: *Fold induction of* CAMP *and* DEFB1 *mRNA expression in VA10 cells cultured in BEGM. VA10 cells were prestimulated with 20 nM VitD (C) or with 20 nM 1,25D$_3$ and 4 mM 4-PBA (D) for 24 hours prior the stimulation with the indicated concentrations of TLR ligands for additional 24 hours.* CAMP *and* DEFB1 *mRNA levels were determined by quantitative RT-PCR. The quantity of the RNA input (1 µg) was the same for all individual reactions. The expression of the housekeeping gene* TUBB *was used as internal reference. Results were normalized to expression in control samples, where controls were given the arbitary value of one. Data of three independent experiments is presented as mean with the standard error of the mean (n = 3). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.*

cultures. Compared to unstimulated VA10 cells, the prestimulaion with 4-PBA alone led to significant induction in *DEFB1* expression. The stimulation with LPS and dead *P. aeruginosa* had no significant effect on the 4-PBA-mediated induction of AMP expression in the VA10 cells. Only the stimulation with flagellin significantly reduced the 4-PBA-induced *CAMP* expression (Figure 15 B).

The prestimulation with $1,25D_3$ significantly induced the *CAMP* gene expression in all stimulated samples, while the *DEFB1* expression was unaffected by $1,25D_3$. Stimulation with TLR ligands did not significantly affect the $1,25D_3$-mediated induction of AMP expression in the VA10 cells (Figure 15 C).

Both the *CAMP* gene and the *DEFB1* gene expression was significantly induced by the prestimulation with 4-PBA and $1,25D_3$. Additional stimulation with TLR ligands did not significantly affect the expression of *DEFB1*. For the *CAMP* gene, the additional induction by TLR ligands LPS and flagellin was not significant, but the treatment with dead *P. aeruginosa* mediated a significant induction of *CAMP* expression (Figure 15 D).

Taken together, these findings show, that there is no direct interaction between TLR activation and AMP expression in VA10 cells when stimulated with TLR ligands alone. However, the stimulation with flagellin interfered with the 4-PBA-mediated induction of AMP expression. The stimulation with the TLR ligands enhanced the 4-PBA and $1,25D_3$-mediated induction of *CAMP* expression.

4.4 4-PBA and $1,25D_3$ reduce TLR-mediated expression of *IL8* and *TNF* in VA10 cells

In order to be certain about the actual TLR activation by the TLR ligands, we decided to analyze the expression of *IL8* and *TNF* genes. Both the chemokine IL-8 and the cytokine TNF-α are early effector molecules of the innate immune system that are known to be induced by the TLR-mediated NF-κB activation.

In order to assess whether the TLRs were actually activated in VA10 cells by the stimulation with the ligands, VA10 cell cultures were cultured in BEGM and stimulated with indicated concentrations of LPS, flagellin and dead *P. aeruginosa* for 24 hours. In additional experiments the VA10 cells were first prestimulated with 4 mM 4-PBA, 20 nM $1,25D_3$ or 4 mM 4-PBA together with 20 nM $1,25D_3$ for 24 hour prior to the stimulation with the TLR ligands for additional 24 hours. The total RNA was isolated from these cells and reverse transcribed before quantitative RT-PCR analysis of *IL8* and *TNF* gene expression. Differences between the 4-PBA or vitamin D stimulated control samples and the TLR stimulated samples were analyzed by the Student´s t-test (Figure 16).

The TLR stimulation significantly induced the *IL8* and *TNF* gene expression in VA10 cells. Flagellin especially caused a pronounced induction in both *IL8* and *TNF* gene expression indicating the activation of TLR pathways (Figure 16 A).

While there seems to be little to no induction of *IL8* and *TNF* gene expression by 4-PBA alone, the prestimulation with 4-PBA reduced the flagellin-mediated induction in gene

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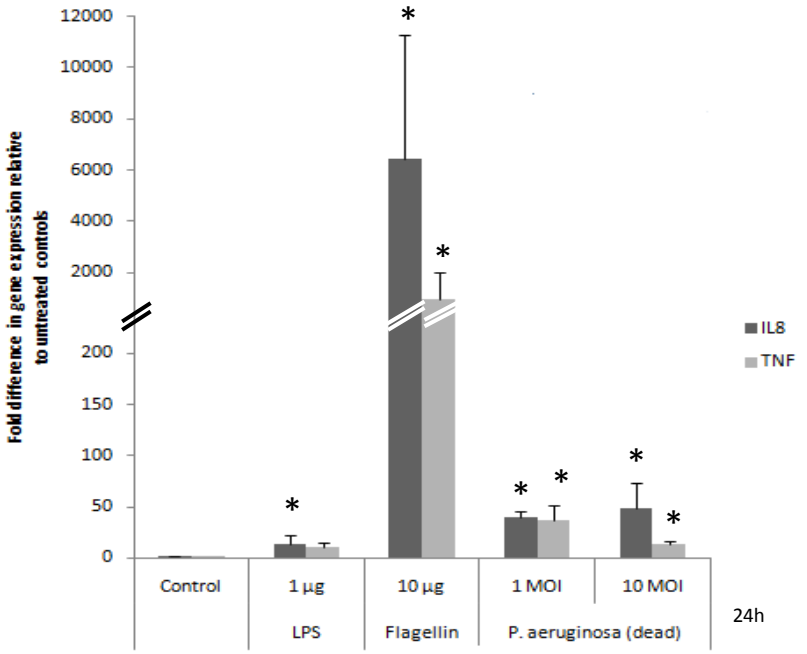

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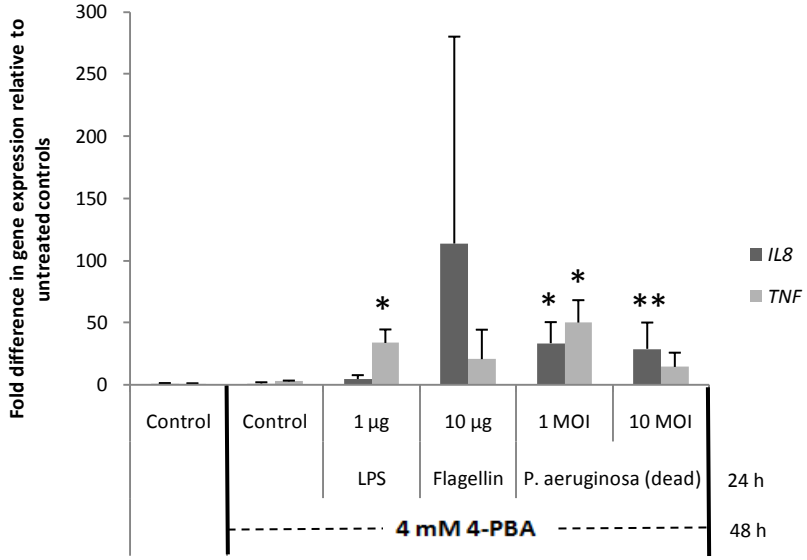

Figure 16: *Fold induction in* IL8 *and* TNF *mRNA expression in VA10 cells cultured in BEGM. The cells were stimulated with the indicated concentrations of TLR ligands for 24 hours (A). VA10 cells were prestimulated with 4 mM 4-PBA prior the stimulation with the indicated concentrations of TLR ligands for additional 24 hours (B).* IL8 *and* TNF *mRNA levels were determined by quantitative RT-PCR. The quantity of the RNA input (1 µg) was the same for all individual reactions. The expression of the housekeeping gene* TUBB *was used as internal reference. Results were normalized to expression in control samples, where controls were given the arbitary value of one. Data of three independent experiments is presented as mean together with the standard error of the mean (n = 3). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.*

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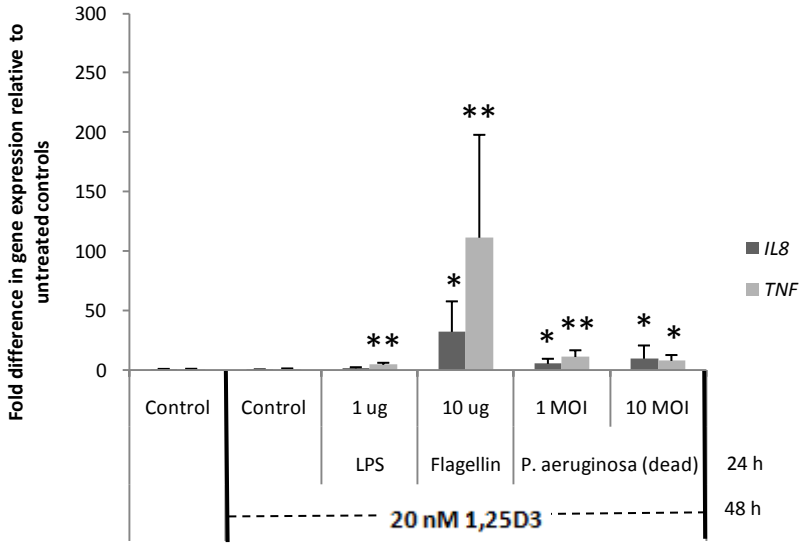

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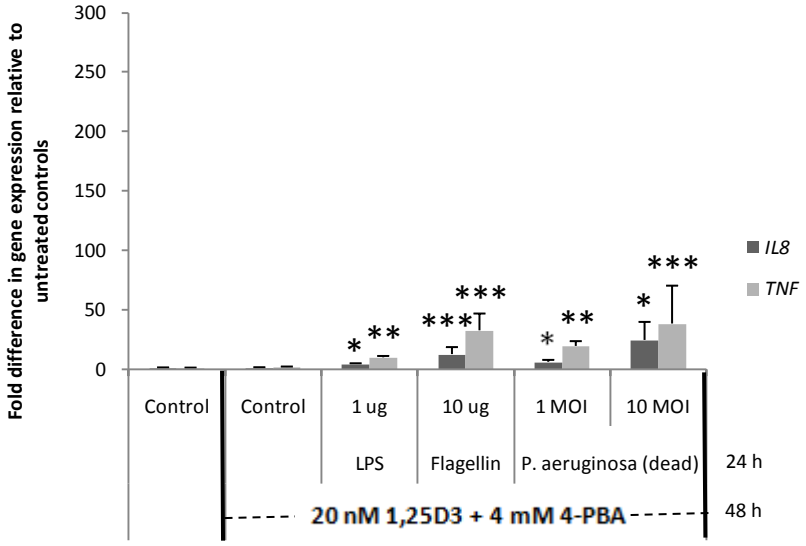

Figure 16: *Fold induction in* IL8 *and* TNF *mRNA expression in VA10 cells cultured in BEGM. VA10 cells were prestimulated with 20 nM* $1,25D_3$ *(C) or with 20 nM* $1,25D_3$ *and 4 mM 4-PBA (D) for 24 hours prior the stimulation with the indicated concentrations of TLR ligands for additional 24 hours.* IL8 *and* TNF *mRNA levels were determined by quantitative RT-PCR. The quantity of the RNA input (1 µg) was the same for all individual reactions. The expression of the housekeeping gene* TUBB *was used as internal reference. Results were normalized to expression in control samples, where controls were given the arbitary value of one. Data of three independent experiments is presented as mean with the standard error of the mean (n = 3). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.*

expression. The LPS and *P. aeruginosa*-induced gene expression was affected to a lesser extent by 4-PBA than the flagellin-mediated induction in gene expression (Figure 16 B).

The same observation was on gene expression in VA10 cells stimulated with $1,25D_3$. No detectable induction of *IL8* and *TNF* expression after stimulation with vitamin D alone, but an overall reduced TLR-mediated gene expression (Figure 16 C).

The prestimulation with 4-PBA together with $1,25D_3$ had additive inhibitory effects on the TLR-mediated induction of *IL8* and *TNF* gene expression in VA10 cells, especially on the flagellin-mediated induction. While the flagellin-mediated expression of *IL8* and *TNF* was affected by the 4-PBA and $1,25D_3$ stimulation, the flagellin and *P. aeruginosa*-stimulated cells appeared to be affected to a lesser extent (Figure 16 D).

In conclusion, flagellin stimulation induced *IL8* and *TNF* gene expression in VA10 cells, while the stimulation with LPS and dead *P. aeruginosa* resulted in a less pronounced induction in gene expression. Both 4-PBA and $1,25D_3$ reduced the flagellin-mediated induction. Both these agents had no effect on the expression of the chemokine and the cytokine when applied alone.

4.5 4-PBA and $1,25D_3$ affect NF-κB translocation

These findings might indicate an effect of 4-PBA and $1,25D_3$ on NF-κB-activated gene expression. We therefore decided to assess the activation status of NF-κB in the stimulation experiments according to its cellular localization. The cellular location of NF-κB was determined by immunohistochemical analysis. VA10 cells were cultured in BEGM on chamber slides. Unstimulated cell cultures were stimulated with 4 mM 4-PBA, 20 nM $1,25D_3$ or 4 mM 4-PBA and 20 nM $1,25D_3$ for 48 hours as controls. A identical series of VA10 cell cultures was stimulated with 10 µg flagellin for 24 hours after the prestimulation with 4-PBA and $1,25D_3$ for 24 hours. These cultures were then immunohistochemically analyzed with antibodies against NF-κB.

The results of a first pilot indeed indicated effects of 4-PBA and $1,25D_3$ on NF-κB activation (Data not shown). This could be an explanation for the reduced induction of *IL8* and *TNF* expression but certainly require further investigating.

4.6 Stimulation of AMP can prevent bacterial growth in VA10 cell cultures

During one stimulation experiment we made the observation that VA10 cells are able to inhibit bacterial growth when stimulated with 4-PBA and $1,25D_3$ in addition to TLR stimulation. In fact, this was our first observation of bacterial clearance upon AMP induction in VA10 cells.

VA10 cells were cultured in BEGM and stimulated with 4 mM 4-PBA, 20 nM $1,25D_3$, or 4 mM 4-PBA together with 20 nM $1,25D_3$ for 24 hours before 10 MOI dead *P. aeruginosa* were added.

However, we found that approximately 2 % of the bacteria had survived the inactivation process. These few surviving *P. aeruginosa* were able to grow in the untreated control cell

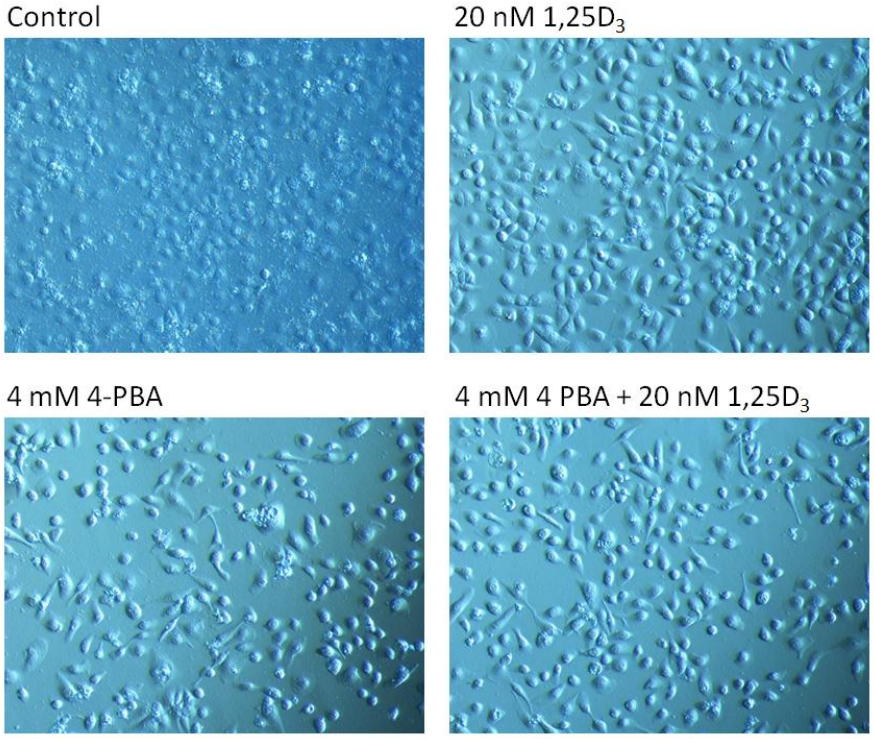

Figure17: *TLR stimulation in addition to 4-PBA and 1,25D$_3$ prestimulation enhances bacterial clearance. VA10 cells were cultured in BEGM and prestimulated with 4 mM 4-PBA, 20 nM 1,25D$_3$ or 4 mM 4-PBA together with 20 nM 1,25D$_3$ for 24 h prior to the stimulation with 10 MOI „dead" P. aeruginosa of which approximately 2% survived the inactivation process.*

culture, only containing the "dead" *P. aeruginosa*. The bacterial growth was clear and confirmed by microscopy. However, in VA10 cell cultures that have been prestimulated with 4-PBA or 1,25D$_3$ the bacterial growth was inhibited. In this case the growth medium was clear and there were no visible bacteria in the culture (Figure 17).

5 Discussion

The bronchial epithelium provides the first line of defense against inhaled pathogens. For the epithelial cells it is crucial to be able to recognize microbial presence and to initiate appropriate innate immune responses.

The characterization and optimization of the VA10 as model system was one of our aims. The culture conditions were established and the determination of a suitable housekeeping gene for quantitative RT-PCR analysis. A literature search for a commonly used housekeeping gene in bronchial epithelial cells came up with conflicting results. We decided therefore to screen for several common housekeeping genes, and found that β-tubulin (TUBB) was one of the genes with the smallest variance between samples and that was expressed in similar quantity as our genes of interest (Appendix B).

In this study we show that several bacterial-relevant TLRs are expressed by VA10 cells. These results are well in line with the results of other groups regarding the TLR expression in bronchial epithelial cells [26, 27].

In the light of the confirmed TLR expression in VA10 cells we were interested how the stimulation with TLR agonists might affect the innate gene expression, particularly with regard to the expression of AMPs. In fact, the literature was not clear on the connection between TLR-mediated signaling pathways and *CAMP* expression. Our results show that TLR stimulation with LPS, flagellin and whole dead *P. aeruginosa* had minor direct effects on the AMP expression in bronchial epithelial cells. Additionally, the 4-PBA and $1,25D_3$-mediated inductions of *CAMP* and *DEFB1* expression was not affected by TLR stimulation, except the stimulation with flagellin, which significantly reduced the AMP expression in the VA10 cells, indicating an interference of signaling pathways.

For a more complete picture and as a control for the actual TLR activation by the applied TLR ligands we looked at the chemokine and cytokine expression. Both IL-8 and TNF-α are known pro-inflammatory mediators that are readily inducible by TLR-mediated activation and translocation of NF-κB into the nucleus with the subsequent gene expression [1]. We observed pronounced flagellin-induced changes in *IL8* and *TNF* gene expression in VA10 cells. This stimulation with flagellin alone clearly led to a pro-inflammatory response. Interestingly, both the co-stimulation with 4-PBA and $1,25D_3$ led to a reduction of this inflammatory response. The inhibitory effect of butyrate and $1,25D_3$ on NF-κB activation has been previously shown, although in other model systems [80, 113]. The 4-PBA-mediated inhibition in bronchial epithelial cells has been shown here for the first time as well as the addative inhibitory effect of the stimulation with 4-PBA and $1,25D_3$ together. These results indicate that both 4-PBA and $1,25D_3$ could strengthen the innate defenses of the bronchial epithelial cells by inducing AMP expression to quickly inactivate and clear potential pathogens. At the same time, they both reduced the expression of the two pro-inflammatory genes investigated, indicating that both 4-PBA and $1,25D_3$ dampen the development of inflammation. Interestingly, 4-PBA has been shown to induce *in vivo* bacterial clearance in a rabbit infection model [Sarker 2011, in press *PLoS One*]. However, the molecular details are not clear and can be approached in a cell culture model system,

such as VA10. Further experiments could include immunohistochemical analysis of the cellular localization of NF-κB both in unstimulated and stimulated cell cultures with the nuclear localization being an indicator for NF-κB activation. In addition, western blot analysis could be utilized to determine the NF-κB activation by monitoring the quantity of IκB, as IκB is degraded during the process of NF-κB activation. And finally, the electrophoretic mobility shift assay (EMSA) could be utilized to determine the activation status of NF-κB through its interaction with DNA. In order to determine possible signaling pathways a kinase inhibition experiment could provide valuable insight into the signaling pathways between the 4-PBA and $1,25D_3$ stimulation and the induced NF-κB inhibition.

Up until now the sole stimulation with 4-PBA, $1,25D_3$ or both agents together have been shown to result in the cellular release of only the inactive pro-form of LL-37 [84]. We made the accidental observation that the additional co-stimulation with TLR ligands results in a successful prevention of bacterial infection. Whether the bacterial growth was really inhibited by the activated LL-37 or by other antimicrobial factors still has to be determined. We are currently working on defining the optimal experimental setup, which allows the stimulation of VA10 cell cultured grown in medium without antibiotics with live *P. aeruginosa*. Once these optimal conditions have been established we plan to assess the secretion and activation of LL-37 by western blot analysis and antimicrobial inhibition zone assays. These results might also give some indications about the exact processing mechanism of the peptide that still remains unclear. One might speculate if the TLR stimulation is needed for the expression of a required protease to activate the peptide or maybe one of the proteases provided by the bacteria themselves activates the secreted inactive pro-form of LL-37.

Earlier results indicated that the 4-PBA-mediated induction in *CAMP* expression might be regulated through secondary responses involving the expression of other genes activated by histone acetylation [84]. Despite their known inhibitory effects on HDAC activity, the exact molecular mechanism through which SCFAs such as butyrate or its derivate 4-PBA affect gene expression in bronchial epithelial cells still remains to be determined. We were therefore interested in how bronchial epithelial cells are able to recognize the presence of SCFAs. Several human epithelia have been shown to express G-protein coupled receptors, GPR41 and GPR43, both cell surface receptors for SCFAs [114]. To our best knowledge these receptors have not been associated with the airway epithelia. We detected both the receptors in the HT29 cells and we did find a possible but unconfirmed GPR43 expression in ALI VA10 cells. This possible *GPR43* expression in VA10 cells requires further investigating and could be confirmed by the analysis of the fragments by quantitative RT-PCR with a specific probe first, before re-cloning and re-sequencing. GPR inhibitors could be utilized to assess the involvement of GPRs in 4-PBA-mediated *CAMP* expression in bronchial epithelial cells. However, it should be noted that until now we primarily worked with the undifferentiated VA10 because this experimental setup does not require as much preparation time before the actual experiment. But clearly, VA10 cells are able to recognize SCFAs which might be an indication for another yet still unknown receptor. Another possibility could be that the SCFAs are imported into the cell for intracellular recognition. This import could be mediated by SLC5A8, a ABC transporter that co-imports butyrate as well as other SCFAs in a Na^+-dependent manner [115]. The focus of research about this transporter has been on colon epithelial and cancer cells. But SLC5A8 has been shown to be expressed by lung epithelial cells [116]. However, we were unable to detect any expression of *SLC5A8* in VA10 cells as well as in our control cells, leaving us

wondering, whether the primers were actually usable. Needless to say, the molecular mechanism through which 4-PBA acts on bronchial epithelial cells still remains unclear.

One interesting observation made during this study was the unexpected effect gentamicin had on gene expression in bronchial epithelial cells. Our results did show that the usage of gentamicin in the growth medium caused an elevated baseline *CAMP* expression level in VA10 cells. The cytokine and chemokine gene expression appeared to be affected as well, observing the different results obtained from VA10 cells cultured with or without gentamicin. The inability of cells grown in growth medium with genatmicin to form an even epithelium , certainly raises the question whether gentamicin not only effects the gene expression but also intercellular communication and thereby differentiation process. As gentamicin has a known toxicity, these observations are certainly relevant for the future experimental setup and approaches. But they leave one wondering whether and how other antibiotics might affect the gene expression of the host cells. This question will certainly be subject of future experiments to determine whether other commonly used antibiotics affect the host cell by interfering with certain signaling pathways.

In conclusion, the VA10 cell line can be considered as suitable model system for studies on innate immunity. Both 4-PBA and $1,25D_3$ exert interesting and certainly promising properties in connection with the treatment of bacterial infections. In fact, the first observation of inducible bacterial clearance through stimulation might provide with further investigation an important insight into the mechanism of 4-PBA-mediated AMP expression.

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Figure references

Figure 1: Modified from www.newworldencyclopedia.org/entry/Lung, 17.03.11

Figure 2: Modified from http://www.gla.ac.uk/ibls/US/cal/anatomy/paranasal/respiratory epithelium.html, 25.04.11

Figure 3: Adapted from **M.R. Knowles and R.C. Boucher (2002).** Mucus clearance ad a primary innate defense mechanism for mammalian airways. *The Journal of Clinical Investigation*, **109**, 571-577.

Figure 4: Adapted from **R. Bals and P.S. Hiemstra (2004).** Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. *European Respiratory Journal*, **23**, 327-333.

Figure 5: Adapted from http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-and-Tissue-Analysis/Signaling-Pathways/Toll-like-Receptor-TLR.html, 20.04.11

Figure 6: Modified from **R.E.W. Hancock and H.-G. Sahl (2006).** Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nature Biotechnology*, **24**, 1551-1558.

Figure 7: Modified from **M. Zasloff (2002).** Antimicrobial peptides of multicellular organisms. *Nature*, **425**, 389-395.

Figure 8: Modified from **R.E. Hancock and G. Diamond (2000).** The role of cationic antimicrobial peptides in innate host defenses. *Trends in Microbiolgy*, **8**, 402-410.

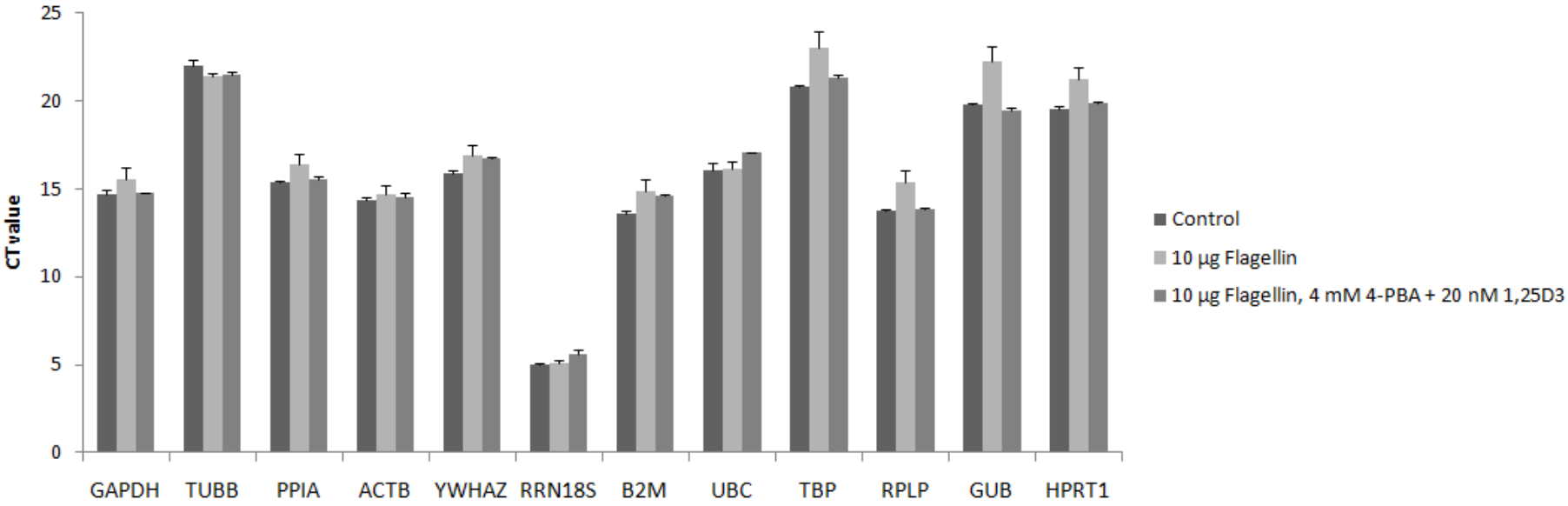
Figure 9: Adapted from **M.R. Knowles and R.C. Boucher (2002).** Mucus clearance ad a primary innate defense mechanism for mammalian airways. *The Journal of Clinical Investigation*, **109**, 571-577.

Figure 13 C: Modified from http://www.langfordvets.co.uk/images/ct_values.jpg, 19.04.11

Appendix

Receptor	Sequence	Blast
TLR1	CCTTCAAGACTGTAGCAAATCTGGAACTATCTAATATCAAATGTGTGCTAGAAGATAACA AATGTTCTTACTTCCTAAGTATTCTGGCGAAACAAGGG	Confirmed
TLR2	CCCTTCACCTACATTAGCAACAGTGACCTACAGAGGTGTGTGAACCTCCAGGCTCTGGT GCTGACATCCAATGGAATTAACACAATAGAGGAAGATTCTTTTTCTTCCCTGGGCAGTCT TGAACATTTAGACTTATCCTATAATTACTTATCTAATTTATCGTCTTCCTGGTTCAAGCCCC TTTCTTCTTTAACATTCTTAAACTTACTGGGAAATCCTTACAAAACCCTAGGGGAAACATC TCTTTTTTCTCATCTCACAAAATTGCAAATCCTGAGAGTGGGAAATATGGACGCCTTCAC TAAGATTCAAAGAAAAGATTTTGCTGGACTTACCTTCCTTGAGGAACTTGAGATTGATGC TTCAGATCTACAAGG	
TLR4	CCTTGATATTAGCTTATAGGCAAGACGTAAAAAAATGAAAACTGGATATATAAAAATAA AAATATATCAGGGGTGATTAGTTAAAAAATAGAACATGCTCGAGAATGACCAGGATGG TTGTGAGCATGTGTTAATCAGGTTTCCTTAGGAAGG	Confirmed
TLR5	CCTTCCTCATGACCATCCTCACAGTCACAAAGTTCCGGGGCTTCTGTTTTATCTGTTATAA GACAGCCCAGAGACTGGTGTTCAAGGACCATCCCCAGGGCACAGAACCTGATATGTAC AAATATGATGCCTATTTGTGCTTCAGCAGCAAAGACTTCACATGGGTGCAGAATGCTTT GCTCAAACACCTGGACACTCAATACAGTGACCAAAACAGATTCAACCTGTGCTTTGAAG AAAGAGACTTTGTCCCAGGAGAAAACCGCATTGCCAATATCCAGGATGCCATCTGGAAC AGTAGAAAGATCGTTTGTCTTGTGAGCAGACACTTCCTTAGAGATGGCTGGTGCCTTGA AGCCAAGG	
TLR6	CCTTTATCCTGCCATCCTATTGTGAGTTTCAGGCATTTAGATCTCTCATTCAATGATTTCA AGGCCCTGCCCATCTGTAAGGAATTTGGCAACTAAGG	Confirmed
TLR9	CCTTCATACCAACATCCTGATGCTAGACTCTGCCAGCCTCGCCGGCCTGCATGCCCTGCG CTTCCTATTCATGGACGGCAACTGTTATTACAAGAAGG	Confirmed

Appendix A: *Confirmation of TLR sequences expressed by ALI VA10 cells. Total RNA of unstimulated ALI VA10 cells was amplified by PCR with the appropiate primers. The PCR products were cloned into TOPO plasmid vector. Plasmids were isolated from transformed E. coli and sent for sequencing. Obtained sequences were confirmed through NCBI BLAST searches within the human genome*.



Appendix B: *Expression of common housekeeping genes in VA10 cells. Cells were either unstimulated (Control), stimulated with 10 µg* P. aeruginosa *flagellin (10 µg Flagellin) for 24 h or 10 µg* P. aeruginosa *flagellin for24 h after being prestimulated with 4 mM 4-PBA and 20 nM VitD for 24 h (10 µg Flagellin, 4mM 4-PBA + 20 nM VitD). CT value was obtained by quantitative RT-PCR. Data of three independent experiments are presents as mean with the standard error of mean (n = 3).*