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The Role of TRPM4 Channel in *Pseudomonas aeruginosa* Infection and the Regulation of the Inflammatory Response in Airway Epithelial Cells

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vorgelegt von: Ahmed Mahdy

geb. am: 11-07-1985 in Sharkia, Egypt

Tag der Promotion: 12.07.2021

Dekan: Univ.-Prof. Dr. med. Michael D. Menger

Berichterstatter: Prof. Robert Bals

Prof. Gabriela Krasteva-Christ

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Dedication

I dedicate my dissertation to my family: a special feeling of gratitude to **my parents**, whose words of encouragement and prayers have always been with me, I really appreciate all they have done for me. **My wife,** who has supported me and never left my side, proves her ability to take responsibility; I owe her love and special gratitude.

I dedicate this dissertation and give special thanks to **my kids** for bearing my absence throughout the entire doctorate program.

I also dedicate this dissertation to my mother-in-law, my siblings, my wife's siblings, my best friends; all of them have been my best cheerleaders.

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Zusammenfassung

Bakteriell verursachte Pneumonien zählen zu den häufigsten Todesursachen weltweit. Das vermehrte Auftreten von resistenten Stämmen verdeutlicht die Notwendigkeit der Entwicklung neuer Behandlungsstrategien. Ionenkanäle spielen eine wichtige Rolle bei vielen Vorgängen in der Zelle. TRPM4 gehört zu der Familie der TRP-Kanäle (transient receptor potential ion channels), die eine umfangreiche Familie von membranständigen Kationenkanälen bilden. TRPM4 wird durch Steigende Ca^{2+}-Konzentrationen in der Zelle aktiviert und ermöglicht durch den Transport der monovalenten Kationen Na^{+} und K^{+} die Depolarisation der Membran. TRPM4 ist für Ca^{2+} undurchlässig.

Im Rahmen dieser Arbeit soll untersucht werden, ob TRPM4 bei einer bakteriellen Entzündung der Lunge die Entzündungsreaktion und Immunabwehr beeinflusst. Untersuchungen mit einem spezifischen TRPM4-Inhibitor und bronchialen Epithelzelllinien, differenzierten primären Atemwegsepithelzellen und Makrophagen sollen zeigen, ob eine bakterielle Stimulation durch die Aktivität von TRPM4 beeinflusst wird.

Es konnte gezeigt werden, dass im Rahmen einer bakteriellen Stimulation eine Vorinkubation mit dem TRPM4-Inhibhitor 9-Phenanthrol (9-Ph.) zu einer signifikant niedrigeren Abgabe von IL-6, TNF-α, CXCL2 und S100A8 durch Atemwegsepithelzellen führt. Dieser Effekt war abhängig von der Konzentration des Inhibitors und konnte bei Konzentrationen von 1/4 bis 1/8 des IC_{50}-Wertes nicht mehr nachgewiesen werden. Im Gegensatz dazu wurde die Transkription von IL-6, TNF-a und CXCL2 durch die Stimulation mit *Pseudomonas aeruginosa* (Ps.a.) induziert, durch die zusätzliche Vorbehandlung mit 9-Ph. aber nicht inhibiert. Die Transkription von S100A8 wurde durch die Vorbehandlung mit 9-Ph. gehemmt, was auf eine direkte Abhängigkeit von Ca^{2+} hindeutet. Die Inhibition der Zytokinabgabe konnte durch die Verwendung von TRPM4-spezifischer siRNA bestätigt werden. Mit Hilfe einer FACS-Analyse und ELISA konnte gezeigt werden, dass es durch die Verwendung von 9-Ph. zu einer Hemmung des Exports von IL-6 kommt. Außerdem war die Inhibition durch 9-Ph. nach der Stimulation mit TLR4-, TRL1/2- und TLR5-Liganden in Atemwegepithelzelllinien und primären Bronchialepithelzellen nachweisbar.

Die antimikrobielle Aktivität von Atemwegsepithelzellen wird zu einem Teil durch die Expression von β-Defensin-2 (hBD-2) vermittelt. Die Stimulation von humanen differenzierten, primären Bronchialepithelzellen mit lebendigen Ps.a. führte zu einer erhöhten Transkription und Abgabe von hBD-2, die mit einer erhöhten antimikrobiellen Aktivität korrelierte. Die Vorinkubation mit 9-Ph. führte zu einer verringerten Transkription und Abgabe von hBD-2, die mit einer erhöhten bakteriellen Besiedelung korrelierte. Das zeigt, dass die Aktivität von TRPM4 unmittelbaren Einfluss auf die Reaktion des angeborenen Immunsystems hat.

Die Stimulation von TRPM4-defizienten Mäusen mit hitzeinaktivierten Ps.A führte zu einer geringeren Entzündungsreaktion, als bei den jeweiligen Wildtyp Kontrolltieren. Dies konnte durch einen geringeren Einstrom von neutrophilen Granulozyten und einer niedrigeren Abgabe von IL-6, TNF-α, CXCL1 und CXCL2 in die broncho-alveolare Lavage gezeigt werden. Zusätzlich war die Expression von mBD-4, dem murinen homolog zu hBD-2, in der Lunge von TRPM4-defizienten Tieren nach der Stimulation mit Ps.a. signifikant niedriger, als bei den entsprechenden wildtyp-Tieren. Die Stimulation von primären differenzierten murinen Trachealepithelzellen mit hitzeinaktivierten Ps.a. führte zu einer erhöhten Abgabe von KC und MIP-2, wenn die Zellen zuvor mit 9-Ph. inkubiert wurden. Die Entzündungsreaktion bei TRPM-4 defizienten Tieren nach der Stimulation mit LPS war höher als bei den vergleichbaren Wildtyp-Kontrollen.

Die Versuche zeigen, dass die Aktivität von TRPM4 für die Regulation und die Abgabe verschiedener Mediatoren des angeborenen Immunsystems der Lungen wichtig ist.

Summary

Bacterial pneumonia is among the leading causes of death worldwide. The emerging resistant strains require the development of new treatment strategies. Ion channels play a role in many signaling pathways of the cell. TRPM4 belongs to the family of TRP-channels (transient receptor potential ion channels), which are membrane bound ion channels expressed in a wide variety of cells. The activity of TRPM4 is induced by rising Ca^{2+} concentrations in the cytosol. The channel is permissive for Na^{+} and K^{+} ions, which lead to a depolarization of the cellular membrane. However, activated by Ca^{2+}, TRPM4 is not permissive for Ca^{2+}.

The goal of this work is to investigate the influence of the activity of TRPM4 on the pulmonary innate immune response in the course of bacterial infection. After detecting the expression of TRPM4 in the human bronchial epithelial cell line NCI-H292, the cytotoxicity of the pharmacologic TRPM4 inhibitor 9-Phenanthrol (9-Ph.) and bacterial stimulation with heat inactivated *Pseudomonas aeruginosa* (Ps.a.) was investigated. The stimulation with heat inactivated Ps.a. or 9-Ph. in the IC_{50}-range had no cytotoxic effects on the cells for at least 18 hr. Most interestingly, the release of IL-6, TNF-α, CXCL2, and S100A8 was inhibited after the pre-treatment with 9-Ph. and stimulation with Ps.a.. This effect was dependent on the concentration of 9-Ph. and was not observed at concentrations of 1/4 - 1/8 of the IC_{50} value of 9-Ph. Furthermore, the inhibition was not detected on transcriptional level, except for S100A8, whose transcription was also inhibited by the pre-incubation with 9-Ph. These findings were confirmed by TRPM4-specific siRNA, which also resulted in significantly less concentrations of IL-6 after the stimulation with Ps.a.. This indicates that the function of TRPM4 was necessary for the export of inflammatory mediators. This was proved by FACS-Analysis and ELISA. The bronchial-epithelial cell line NCI-H292 and human primary airway epithelial cells were also stimulated with TLR1/2, TLR4, and TLR5-ligands. These experiments showed that the treatment with distinct TLR-ligands can also be influenced by the inhibition of TRPM4 and that the effects observed so far are not due to the inhibition of a certain TLR-signaling pathway.

The antimicrobial activity of airway-epithelial cells is mostly mediated by the expression of antimicrobial peptides. The expression of the antimicrobial peptide hBD-2 (human beta-defensin-2) was highly induced in differentiated human primary airway epithelial cells after the stimulation with live Ps.a.. This correlated with increased concentrations of hBD-2 in the cell culture supernatant and increased antimicrobial activity of the cells. In contrast, the inhibition of TRPM4 significantly downregulated the expression of hBD2, the concentration in cell culture supernatant, and the antimicrobial activity of the cells. This indicates a direct connection of the activity of TRPM4 to the innate immune response of the lung.

To investigate the influence of TRPM4 on the release of inflammatory cytokines in different cell types and species, monocyte derived macrophages from the bone marrow of mice and the human monocyte cell line

U937 were used. Again, the inhibition of TRPM4 resulted in a significantly decreased release of IL-6, TNF-α, CXCL1, and CXCL2. These results were also confirmed with macrophages isolated from TRPM4-deficient mice.

To further explore these findings, the lungs of TRPM4-deficient mice were stimulated with heat inactivated Ps.a.. TRPM4-deficient mice showed less inflammation than the corresponding wildtype control animals. These findings are based upon less influx of neutrophilic granulocytes into the lung and lower concentrations of IL-6, TNF-a, CXCL1, and CXCL2 in the broncho-alveolar lavage (BALF) of the TRPM4-deficient animals. Furthermore, the expression of mBD-4, the murine homologue of the human beta-defensin-2, was significantly reduced in the TRPM4-deficient mice. The stimulation of differentiated primary murine tracheal epithelial cells with heat inactivated Ps.a. induced a higher release of KC and MIP-2 if TRPM4 was inhibited. Furthermore, the stimulation of TRPM4-deficient mice with LPS resulted in an increased inflammatory response from the TRPM4-knockout mice compared to the wildtype controls. These differences may result from the different stimulations or the reactivity of the different cell types and possible variations in the immune response of human and mouse cells.

These results show that the function of TRPM4 is important for the regulation of inflammation and the innate immune response of the lung.

List of Abbreviation

No.	Abb.	Full Name
1.	9-Ph.	9-phenanthrol
2.	AC	Adenylyl cyclase
3.	ALI	Airway-liquid interface
4.	AMPs	Antimicrobial peptides
5.	AP-1	Activator protein-1
6.	ATP	Adenosine triphosphate
7.	BALF	Bronchoalveolar fluid
8.	BCA	Bicinchoninic acid
9.	BMDMs	Bone-marrow-derived macrophages
10.	BSA	Bovine serum albumin
11.	CaM	Calmodulin
12.	CaMKII	Calcium/calmodulin-dependent protein kinase II
13.	cAMP	3',5'-cyclic AMP
14.	CCL	Chemokine ligand
15.	CCR	Chemokine receptor
16.	CD	Cluster of differentiation
17.	CF	Cystic fibrosis
18.	CFTR	Cystic fibrosis transmembrane conductance regulator
19.	CFU	Colony-forming unit
20.	CN	calcineurin
21.	COPD	Chronic obstructive pulmonary disease
22.	COX-2	Cyclooxygenase -2
23.	CTD	Carboxyterminal domain
24.	CXCL2	C-X-C motif ligand 2
25.	DAG	Diacylglycerol
26.	DCs	Dendritic Cells
27.	dsRNA	double-stranded RNA
28.	EAE	encephalomyelitis
29.	EC50	Half maximal effective concentration
30.	EDTA	Ethylenediaminetetraacetic acid
31.	ELISA	Enzyme-linked immunosorbent assay
32.	ENaC	epithelial sodium channel
33.	ER	Endoplasmic reticulum
34.	ERK1/2	Extracellular signal-regulated kinases 1/2
35.	FACS	Fluorescence-activated cell sorting
36.	FBS	Fetal Bovine Serum
37.	Fc	Fragment crystallizable
38.	FCS	Fetal calf serum
39.	GLP-1	glucagon-like-peptide-1
40.	hBD-2	human beta-defensin 2
41.	hBD-3	human beta-defensin 3
42.	HCE	human corneal epithelial
43.	hr./ hrs.	Hour/ Hours
44.	HRP	Horseradish peroxidase
45.	IFN-α	Interferon alpha
46.	IFN-γ	Interferon gamma

47.	IL	Interleukin
48.	IMIT	Intubation-mediated intratracheal technique
49.	IP	Immunoprecipitated
50.	IP3	Inositol 1,4,5-trisphophate
51.	IRAK	Interleukin-1 receptor-associated kinase
52.	IRF3	Interferon regulatory factor 3
53.	Jnk	c-Jun N-terminal kinase
54.	LB	luria-Bertani medium
55.	LDH	Lactate dehydrogenase
56.	LOX-1	lectin-like oxidized low density lipoprotein receptor
57.	LPS	Lipopolysaccharide
58.	MAPKs	Mitogen activated protein kinases
59.	mBD-1	murine β-defensin-1
60.	mBD-4	murine β-defensin-4
61.	MCP-1	Monocyte chemotactic protein-1
62.	MD-2	Myeloid differentiation factor 2
63.	MHR	Major homology region
64.	min.	Minutes
65.	MIP2-α	Macrophage inflammatory protein 2-alpha
66.	mRNA	messenger RNA
67.	MyD88	Myeloid differentiation primary response 88
68.	NC	Nitrocellulose membrane
69.	NCX2	Na^{+}/Ca^{2+}exchanger 2
70.	NE	Neutrophil elastase
71.	NFAT	activated T cell nuclear factor
72.	NF-kB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
73.	NHBE	normal human bronchial epithelial
74.	NK	Natural killer cells
75.	NLRP3	NLR family pyrin domain containing 3
76.	NLRs	NOD-like receptors
77.	Nox4	NADPH oxidase-4
78.	NTC	No template negative control
79.	O.D.	Optical density
80.	ox-LDL	oxidized low-density lipoprotein
81.	p38	p38 kinase
82.	PAGE	Polyacrylamide gel electrophoresis
83.	PAMPs	Pathogen-associated molecular pattern molecules
84.	PBS	Phosphate buffered saline
85.	PFA	Paraformaldehyde
86.	PFHBI	progressive familial heart block type I
87.	pH	potential of hydrogen
88.	PHA	phytohemagglutinin
89.	pHBEs	Primary human bronchial epithelial cells
90.	PIP2	Phosphatidylinositol 4,5-bisphosphate
91.	PKA	Protein kinase A
92.	PKC	Protein kinase C
93.	PLC	Phospholipase C
94.	PMA	phorbol 12-myristate 13-acetate
95.	PMNs	Polymorphonuclear leukocytes
96.	PRRs	Pattern recognition receptors

97.	Ps.a.	*Pseudomonas aeruginosa*
98.	PTECs	proximal tubular epithelial cells
99.	PVDF	Polyvinylidene fluoride membrane
100.	qPCR	Quantitative polymerase chain reaction
101.	RAGE	receptor for advanced glycation end products
102.	RBCs	Red blood cells
103.	RE	Recycling endosome
104.	RIPA	Radioimmunoprecipitation assay buffer
105.	RLRs	Retinoic acid-inducible gene-I-like receptors
106.	ROS	Reactive oxygen species
107.	RT	Room temperature
108.	RT-	Reverse transcriptase minus negative control
109.	RT-PCR	Real-time polymerase chain reaction
110.	s	Second
111.	S100A8	S100 calcium-binding protein A8
112.	shRNA	short hairpin ribonucleic acid
113.	SIM	Structured illumination microscopy
114.	siRNA	small interfering RNA
115.	SIT	Sample injection tube
116.	SNAP	Synaptosomal associated protein
117.	SNARE	Soluble N-ethylmelaimide-sensitive fusion protein attachment protein receptor
118.	ß-ME	ß-mercaptoethanol
119.	Strep-PE	Streptavidin-phycoerythrin conjugate
120.	SUMO	small ubiquitin-like modifier
121.	SUR-1	Sulfonylurea receptors
122.	T3SS	Type III secretion
123.	TBS	Tris-HCl buffered saline
124.	TBST	Tris-buffered saline with 0.1% tween 20 detergent
125.	TER	Trans-epithelial resistance
126.	TGF-β	Transforming growth factor beta
127.	TGN	Trans-Golgi network
128.	Th2	T helper 2
129.	TLRs	Toll like Receptors
130.	TMD	Transmembrane domain
131.	TMEM16A	transmembrane protein 16A
132.	TNF	Tumor necrosis factor
133.	TRAF	TNF receptor associated factor
134.	TRAM	TRIF-related adaptor molecule
135.	TRIF	TIR-domain-containing adapter-inducing interferon-β
136.	TRP	Transient receptor potential
137.	TRPC	Transient receptor potential Canonical
138.	TRPM	Transient receptor potential Melastatin
139.	TRPV	Transient receptor potential Vanilloid
140.	t-SNARE	target SNAREs
141.	UV	Ultraviolet
142.	VAMP3	Vesicle-associated membrane protein 3
143.	VDCC	voltage dependent Ca^{2+} channel
144.	v-SNARE	vesicle SNARE
145.	WR	Working Reagent

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1. Introduction

1.1 Innate immune response

The mammalian immune system consists of two divisions that function in co-ordination; to provide resistance to infection, the innate immune system and the adaptive immune system. The first line of host defense is the innate immune response which is responsible for immediate detection and control of microbial invasion **(Akira et al., 2006) (M. S. Lee & Kim, 2007) (Trinchieri & Sher, 2007)**.

The innate immune system provides the first line of protection against pathogens prevalent in the environment. It is a non-specific system, lacks memory, and is not impaired by prior exposure to pathogens or harmful substances, as opposed to adaptive immune system **(Guani-Guerra et al., 2010)**.

Innate immune cells include white blood cell community such as circulating dendritic cells (DCs), natural killer cells (NK), neutrophils, monocytes, eosinophils and basophils, in addition to macrophages and mast cells that are tissue-residents. These cells manipulate opportunistic invasions by a diverse variety of microbial and parasitic pathogens, throughout discharging a plenty of chemokines and cytokines, which enables them to interact with other cells and thus orchestrate immune responses. Tumor necrosis factor (TNF), Interferon gamma (IFN-γ), interleukin IL-1β, IL-18, IL-10, IL-4, IL-6, IL-12, chemokine ligand 4 (CCL4)/CCL5 (RANTES), and transforming growth factor beta (TGF-β) are also included in this series of soluble mediators secreted by various innate immune cells **(Iwasaki & Medzhitov, 2010)**.

Innate immune systems can identify pathogen-associated molecular pattern molecules (PAMPs) as the first defense against invading pathogens by means of germ line-encoded pattern recognition receptors (PRRs) in a non-specific but a rapid manner of detection acting as the first safeguard against invading pathogens **(Janeway & Medzhitov, 2002)**. PAMPs are detected by a wide range of PRRs. Consequently, they enable the downstream intracellular signalling machinery prerequisite to mediate host defence **(Gay et al., 2014)**, which results in the activation of antimicrobial responses required for pathogen eradication, such as antimicrobial peptides synthesis and secretion of different proinflammatory cytokines **(Finlay & McFadden, 2006)**.

Endocytic PRRs primarily facilitate phagocytic ingestion and microorganism destruction, whereas the intracellular signaling mediated by PRRs, such as Toll like Receptors (TLRs), NOD-like receptors (NLRs) and Retinoic acid-inducible gene-I-like receptors (RLRs) promotes the synthesis and release of cytokines and chemokines, acting as immune regulators and play a vital role in coordinating, maintenance and harmony interaction between innate and adaptive immunity **(Medzhitov, 2007) (Areschoug & Gordon, 2009)**. Inherent immune system cells cause precisely tuned balanced between host defence responses and host protection through mitigating collateral damage to it **(Iwasaki & Medzhitov, 2015)**.

1.2 Inflammation & host defense

Inflammation is a dynamic defense mechanism characterized by leukocyte migration from the vasculature into damaged tissues to destroy the injurious agents. Acute inflammation is a limited beneficial response particularly during infectious challenges, whereas chronic inflammation is a persistent phenomenon, which can develop to inflammatory diseases. One of the cornerstones of acute inflammation is that neutrophil is originally predominated in leukocyte infiltrate, but after 24 to 48 hour (hr.) the situation changed and the monocytic cells become prevalent **(G. B. Ryan & Majno, 1977) (Doherty et al., 1988)**. Mononuclear cells such as macrophages and lymphocytes are histologically correlated with chronic inflammation **(G. B. Ryan & Majno, 1977)**.

Neutrophils are granulocytic polymorphonuclear leukocytes (PMNs) that are considered as a key ingredient in innate immune defense **(Baggiolini & Dewald, 1985)**. Neutrophils are the first cells that aggregate in the tissues **(G. B. Ryan & Majno, 1977) (Doherty et al., 1988)**.

Because of their ability to synthesize oxygen metabolites and to release different enzymes, neutrophils are key cells in the protection of an organism against damage, especially infection. Nevertheless, these agents can also be toxic to normal surrounding tissues and possibly cause several inflammatory diseases. Subsequently neutrophil functions should be rapidly and adversely regulated. It is worth mentioning that this is accomplished by local death of aged PMN cells by apoptosis **(Savill et al., 1989)**.

The activation of PMN cells by bacterial phagocytosis or more broadly by the fragment crystallizable (Fc) mediated phagocytosis can induce up-regulation of apoptosis or so-called programmed cell death **(Kobayashi et al., 2002)**.

Death by apoptosis triggers the loss of biological functions of PMN cells, but prevents the release of intracellular toxic neutrophil material and further tissue injury **(Whyte et al., 1993)**.

Neutrophils from the inflammatory site are early depleted; while blood monocytes, on the other hand, accumulate and differentiate into inflammatory macrophages, performing its phagocytic activity and participating in killing of the harmful agents **(G. B. Ryan & Majno, 1977) (Doherty et al., 1988)**.

Contrary to neutrophils monocytes and macrophages do not die locally, but evacuate toward local lymph nodes after elapse of many days **(Bellingan et al., 1996)**. Also, some requisite changes take place throughout this migration process such as monocytes differentiation into dendritic cells, acquiring costimulatory molecules such as cluster of differentiation 80 (CD80), cluster of differentiation 86 (CD86) and upregulating the expression of human leukocyte antigen (HLA) class II antigen on its surface that is needed for further maturation and activation **(Randolph et al., 1998)**.

In addition to adhesion molecules, Leukocyte recruitment depends on the specificity of chemokines produced at the inflammatory site. The most important chemokines for the recruitment of PMN cells and monocytes are Interleukin 8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) **(Sallusto et al., 1999) (Yamashiro et al., 1999)**.

After inflammatory cytokines activate neutrophils or other cells, IL-8 is typically produced early and sustained for 24 hr., giving privilege for more attracting and activating neutrophils locally **(Sallusto et al., 1999) (Yamashiro et al., 1999)**. Furthermore, sustained development of IL-8 could generate a higher chemokine concentration in vessels, acting as an obstacle in neutrophil adhesion to endothelium and also affecting the extravasation process **(Gimbrone et al., 1989)**.

MCP-1 development by macrophages is caused by phagocytosis of apoptotic neutrophils **(McDonald et al., 1999)**. Thus PMN-cell apoptosis not only participates in the removal of neutrophils, but it also facilitates a shift in chemokines contributing to monocyte recruitment. Generally, development of MCP-1 is postponed but maintained for many days. Its accumulation contributes to late recruitment of monocytes; but at the same time, it does not desensitize the cells **(Sallusto et al., 1999) (Yamashiro et al., 1999)**.

Interleukin 6 (IL-6) plays a slightly surprising role in this scenario, which was involved in in-vivo recruitment of leukocytes; due to the fact that the soluble IL-6 receptor complex can stimulate the secretion of MCP-1 and IL-8 from the endothelial cells, in addition to enhancing the adhesion molecules expression **(Romano et al., 1997)**.

Endothelium or other stromal cells may be stimulated by thrombin (proinflammatory agent) to produce IL-8 and other chemo-attractants that favor early neutrophil recruitment at the inflammatory site. Activated PMN cells can release the soluble interleukin-6 receptor, which further interacts with locally generated IL-6. This will, in turn, lead to production of MCP-1, not IL-8 from endothelial cells, which give rise to reduction of neutrophil and to favor the recruitment of monocytes **(Xing et al., 1998)**.

IL-6 has a protective vital role in suppressing neutrophils and encouraging the recruitment of monocytes, contributing to inflammation recovery. Conversely, IL-6 can have a very harmful role in chronic inflammation through favoring mononuclear cell aggregation at the injury site. This occurred by the aid of continuous MCP-1 secretion, angio-proliferation and anti-apoptotic function on T cells **(Atreya et al., 2000)**.

The excessive aggregation and persistence of neutrophils cause an excessive inflammatory response which leads to serious damage of the lung as seen in cystic fibrosis (CF) patient **(Doring, 1994) (Dunlevy et al., 2012)**. The regulated development of *Pseudomonas aeruginosa* (Ps.a.) rhamnolipid controlled by quorum-sensing promotes severe necrotic destruction of penetrating neutrophils, which describes why neutrophils

aren't substantially engaged in Ps.a. removal from the CF lung **(Jensen et al., 2007) (Alhede et al., 2009) (Van Gennip et al., 2009)**.

Elastase is defined as a powerful serine protease which could be produced by various strains of Ps.a. and infiltrating neutrophils. It is demonstrated that it has numerous biological impacts that greatly contribute to progression of pulmonary disease in CF patient **(Wretlind & Pavlovskis, 1983) (Tirouvanziam, 2006)**. Elastase shows an effective antimicrobial activity against a wide variety of gram-negative bacteria, but surprisingly, it has no effect on Ps.a. **(Sonawane et al., 2006)**. Also subjected to elevated concentrations of neutrophil elastase (NE) up to 25 μM that is normal in CF lung, Ps.a. remains viable and morphologically unchanged **(Berger, 1991)**. Neutrophils surrender to apoptosis and eventual phagocytotic clearance by macrophages after a short life span **(Watt et al., 2005)**. Intriguingly, it was approved that IL-6 has been shown to induce apoptosis of PMN-cells **(Afford et al., 1992)**.

Cathepsins are cysteine proteases produced naturally by macrophages which are involved in extracellular matrix remodeling **(Wolters & Chapman, 2000)**. In reaction to the elevated levels of apoptotic neutrophils within the lungs of CF patients, pulmonary macrophage influx occurs to eradicate these apoptotic cells, and this contributes to extend cathepsin secretion into the bronchoalveolar fluid (BALF) of the CF lung **(Berger, 1991) (Ulrich et al., 2010)**. High concentration of cathepsins present in the BALF gives it flexibility to degrade beta-defensins, which have a preserved core structure of three disulfide bridges. This made it liable to cathepsins proteolytic cleavage **(Hoover et al., 2000)**. Particularly, cathepsins B, L and S were found to cleave human beta-defensin 2 (hBD-2) and human beta-defensin 3 (hBD-3) disulfide bonds, which caused their degradation and loss of antimicrobial activity **(Taggart et al., 2003)**. In addition, most cathepsins have an optimal acid potential of hydrogen (pH) proteolytic feature, low pH in CF bronchoalveolar fluid promotes optimal enzymatic activity for cathepsin proteolytic activity which lose their properties and it's proteolytic power at physiological or basic pH **(Wolters & Chapman, 2000)**. Due to impaired bicarbonate transport through the pulmonary epithelium caused by the cystic fibrosis transmembrane conductance regulator (CFTR) mutation, the bronchoalveolar fluid of CF patients become acidic **(Tate et al., 2002)**. In addition, the efficacy of hBD-2 is brought down due to the elevated Cl^- present in the bronchoalveolar fluid that resulted from a defect in functional CFTR. The Decent explanation for this phenomenon correlated with lowering in the electrostatic contact between the cationic hBD-2 peptide and the resting membrane of invasive pathogen which has anionic properties **(Bals et al., 1998)**. During the chronic pulmonary infection, overexpression of cathepsin can cause additional degradation of hBD-2, encouraging bacterial colonization and infection propagation **(Taggart et al., 2003)**.

1.3 Airway epithelial cells and cellular host defense

Airway epithelium is a physiological tissue barrier with two layers and two purposes. First, it owns a variety of mechanical and chemical defense factors such as mucus, antimicrobial agents, or beating cilia which shield the respiratory compartment of the lung from invading pathogens. Second, intact airway epithelium prevents inflammation initiation in response to microbes which are usually present in airway lumen by preventing interaction of bacteria with inflammatory subepithelial cells such as macrophages and DCs that are very sensitive to any kind of threat. Therefore, the main vital role of the airway epithelial barrier is to defend against pathogens without progression of inflammation. Moreover, epithelium cells are often equipped with autonomous program to protect their barrier integrity during any damage or attack caused by microbes **(Shaykhiev, 2007) (Proud & Leigh, 2011)**.

Epithelial cells not only prevent inflammation progression, but also play a vital role in inflammation management by producing anti-inflammatory molecules of many families, including cytokines such as (IL-10, TGF-ß), soluble cytokine receptors/receptor antagonists, inhibitory arachidonic acid metabolites, protease inhibitors, and other immunosuppressive surface cell molecules **(Janes et al., 2006) (K. S. Park et al., 2007)**. Many of such molecules are triggered by T helper (Th2) and proinflammatory cytokines, which indicate that negative feedback pathways can be regulated to mitigate inflammatory signals **(Janes et al., 2006) (Levine et al., 1997)**.

The unique component of respiratory epithelium, consisting of ciliary columnar cells, goblet cells, and clara cells, enables it to form a persistent and reasonably periodic lining of the airway and protects it against aeroallergens, toxins and pathogens that have been inhaled from the external environment **(Pohunek, 2004)**.

The respiratory epithelium provides a physical barrier between inner and outer milieu, and is essential for the protection of the interior part **(Khalmuratova et al., 2017) (Lambrecht & Hammad, 2014)**. The core components of epithelial cells physical barrier, which enable it to adhere to neighbouring cell through cell-cell junctions including tight junctions (TJs), adhering junctions (AJs), gap junctions, and desmosomes **(Soyka et al., 2012) (Nomura et al., 2014) (N. Zhang et al., 2016)**. In addition, mucus is considered one of the arsenal weapons of epithelial cell that shield the epithelium from microbes, allergens and toxic compounds **(Birchenough et al., 2015)**, and enable the inhaled foreign particles to be easily captured then decayed and destroyed by aiding of antimicrobial molecules such as defensins bactericidins, antiproteases and antioxidants. Finally, it is expelled via mucociliary movements **(Ganesan et al., 2013) (Loxham et al., 2014)**.

It has been shown that airway epithelial cells primarily express toll like receptors from TLR2-TLR6 **(Sha et al., 2004) (Homma et al., 2004)**. Bacterial components such as lipoprotein, peptidoglycan (PGN), and

lipoteichoic acid are detected by TLR2, that is mainly heterodimerized with TLR1 or TLR6. Viral double-stranded RNA (dsRNA) and also a synthetic analog of dsRNA could be recognized by TLR3, while TLR4 recognizes gram-negative bacterial lipopolysaccharide (LPS). Flagellin, a constituent of bacterial flagella, is detected by TLR5. Thus, these unique TLRs are strategically located on epithelial cells to enable microbes commonly encountered on the surface of the mucosa to be easily recognized **(Kato & Schleimer, 2007)**.

Airway epithelial cells usually are used to secrete a variety of molecules from different families which are involved in defense against bacterial, viral and fungal infections. Common antimicrobial products are naturally produced and presented by epithelial cells such as, LL-37, defensins, lactoferrin, collectins, lysozyme, pentraxins, serum amyloid A (SAA) and secretory leukocyte protease inhibitor (SLPI) that can bind and neutralize different types of infectious agent **(Schleimer et al., 2007)**.

In addition to mediating and triggering innate immune responses, airway epithelial cells also manage adaptive immune responses through interactions with DC, T cells and B cells, that are crucial in protection against airway inflammation and also viral and bacterial infection **(Lukacs et al., 2001) (Phadke et al., 2007)**. Adaptive immunity is influenced by local and infiltrating DC which play a critical role in responses to inhaled foreign antigens, Epithelial airway cells are also able to cause DC migration into the epithelium **(Upham & Stick, 2006)**.

1.4 Cytokine synthesis, release & exocytosis process

Cytokines from innate immune cells are developed and released as vital responses to infection and inflammation in the body **(Iwasaki & Medzhitov, 2010)**. Adequately, cytokine synthesis and release must be highly regulated, simultaneously and temporally synchronized. Thus, cascades of cytokines released by innate immune cells initially trigger inflammatory response or allergic reactions, and these reactions were guaranteed to be decayed in a time dependent manner **(X. Hu & Ivashkiv, 2009)**.

Cytokine secretion is a complex and tightly regulated process, and the intracellular pathways appropriate for release are also specific for each different type of cytokine and cell type dependent. In classical pathways, cytokines with signal peptides are incorporated into the endoplasmic reticulum (ER) for synthesis. These are transported in vesicles to the Golgi complex for further processing. At trans-Golgi network (TGN), they were loaded into vesicles or carriers for constitutive delivery to the cell surface or other organelle. Additional modes of secretion are provided in specialized cell types by loading cytokines and other cargo into granules for storage, and released later according to cellular demand and the surrounding circumstances **(Lacy & Stow, 2011)**.

Interleukin 6 (IL-6) is considered one of the most popular examples of soluble cytokine, which is released within hrs. after LPS stimulation **(Verboogen et al., 2017)**. LPS binds to TLR4 located in plasma

membranes of the immune cells and this binding prompts nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-kB) and other transcription factors activation, which in turn enhance IL-6 transcription and translation. Like many other cytokine, IL-6 traffics through the Golgi apparatus from the endoplasmic reticulum (ER) to the recycling endosome (RE) **(Murray & Stow, 2014) (Manderson et al., 2007)**. RE are tubulovesicular organelles that control the transport of secretory vesicles toward the plasma membrane **(Stanley & Lacy, 2010)**, which ultimately contributes to IL-6 release to the extracellular environment.

In addition, some trafficking proteins involved in the release of IL-6 and other cytokines are simultaneously upregulated **(Stanley & Lacy, 2010)**. Soluble N-ethylmelaimide-sensitive fusion protein attachment protein receptor (SNARE) proteins are detected to modulate IL-6 secretion, such as syntaxin-3 and vesicle-associated membrane protein 3 (VAMP3), knockdown and overexpression of these SNAREs complexes minimize and increase the release of IL-6, respectively **(Manderson et al., 2007) (Collins et al., 2015)**. Also, when dendritic cells are activated by LPS, it is noticed that there is an increased interaction of VAMP3 with syntaxin-4 at the plasma membrane and this correlates with an increase in IL-6 secretion **(Verboogen et al., 2017)**.

Three synaptic α-SNAREs **(Söllner et al., 1993)**, which include a VAMP called vesicle SNARE (v-SNARE), is located on the membrane of secretory vesicles, while synaptosomal associated protein (SNAP-23/SNAP-25), and syntaxin protein called target SNAREs (t-SNARE) are located on the plasma membrane. All of these three SNAREs are combined into a SNARE 4-helical protein complex in two adjacent membranes; in order to pull the membranes into close apposition. Thus, SNAREs assembly is believed to facilitate the membrane fusion **(Sutton et al., 1998) (Fasshauer, 2003)**. In this way, the essential mechanism for exocytosis is thus thought to involve these three basic SNARE proteins **(Weber et al., 1998)**. It is worth mentioning that calcium ion (Ca^{2+}) at even low micromolar concentrations can trigger SNARE complex formation **(K. Hu et al., 2002)**.

In human airway and lung epithelial cells, specifically in the airway goblet cell, VAMP8 an essential component of v-SNARE proteins were implicated in the regulation of mucin granule exocytosis **(Jones et al., 2012)**. Also, VAMP8 is associated with mucin granules purified from airway goblet cell-like Calu-3 cells **(Kreda et al., 2010)**.

SNARE and some other regulatory factors were also involved in the secretion of lung surfactant, which were synthesized in the endoplasmic reticulum in alveolar epithelial type II cells **(Abonyo et al., 2004; Abonyo et al., 2003) (Gou et al., 2004)**. It was proved that t-SNARE proteins, syntaxin 2 and SNAP-23, are required in lung surfactant secretion **(Abonyo et al., 2004)**. VAMP-2 as a part of v-SNARE may also be involved in the regulation of lung surfactant secretion **(Wang et al., 2012)**. In other types of immune cell, in both vitro and

ex-vivo, eosinophil degranulation was mediated by VAMP-7, and this correlated with increasing in airway hyperresponsiveness **(Willetts & Felix, 2018)**.

Regulated exocytose is temporarily monitored by a large increase in the amount of intracellular calcium. Fusion of the intracellular vesicle with the opposite plasma membrane and the subsequent shooting of the vesicle contents outside the cell is mainly a Ca^{2+} process dependent **(Zucker, 1999)**.

Calmodulin (CaM) and protein kinase C (PKC) are the main proteins that help the trigger process of the Ca^{2+} induced exocytosis. Adenosine triphosphate (ATP) could modulate the exocytosis process as PKC demands ATP to increase secretion, while calmodulin boosts secretion in the absence of ATP, implying that phosphorylation is crucial in PKC mediated stimulation. However, it doesn't have an effect on calmodulin stimulation. It is thus proposed that calmodulin and PKC increase exocytosis triggered by Ca^{2+} by directly modulating the exocytic machinery or cytoskeleton attached to the membrane **(Bernstein, 2015)**.

PKC is a serine/threonine kinase activated by calcium. It also could be activated through a diacylglycerol (DAG) signaling pathway. This make it a common research target for exocytosis control **(Malenka et al., 1999)**. PKC improves secretion in an ATP-dependent way in PC12 cells, supporting consecutive thoughts for phosphorylation requirement **(Y. A. Chen et al., 1999)**. PKC activation contributes in decreasing calcium threshold required for secretion **(Zhu et al., 2002)**. PKC minimize the quantal size of individual exocytotic events, increase kinetics **(M. E. Graham et al., 2002)** and accelerate the expansion of fusion pores **(Scepek et al., 1998)**.

In eukaryotic cells, CaM is the most prevalent calcium mediator, but its participation in membrane trafficking has not been proved. Some earlier studies reported that Ca^{2+} activated exocytosis was blocked by inhibitors of CaM **(R. D. Burgoyne et al., 1982) (Verhage et al., 1995)**, anti-CaM antibodies **(Kenigsberg & Trifaró, 1985)**, or CaM-binding inhibitory peptides **(Birch et al., 1992)**. Ca^{2+}/CaM has been documented to trigger the complete docking process and cause a late stage of vacuole fusion in yeast, indicating that Ca^{2+}/CaM plays an important role in fundamental intracellular membrane fusion **(Peters & Mayer, 1998).**

CaM is made up of two EF-hand domains proteins involved in controlling exocytosis. EF-hand domain proteins bind calcium via a short helix-loop-helix motif of 30 residues, where calcium binding to the loop results in a conformational change that shows its hydrophobic surface. So, it could then bind easily to a target protein **(Lewit-Bentley & Réty, 2000) (Robert D. Burgoyne & Clague, 2003)**. In both calcium-dependent and independent manner, CaM can exert its effects. CaM has a well-established role in exocytosis, promoting a phosphorylation of synapsins by calcium/calmodulin-dependent protein kinase II (CaMKII) activation. However; calmodulin also binds to synaptotagmin, SNARE complex, Rab3, **(Robert D. Burgoyne & Clague, 2003)** and Munc13 **(Junge et al., 2004)**.

CaMKII, a multifunctional Ca^{2+}/CaM dependent kinase identified in synaptic vesicles, can serve as a potential target of CaM **(Braun & Schulman, 1995)**. Following depolarization, calcium influx triggers CaM to activate CaMKII. Activation leads to an autophosphorylation mechanism that makes CaMKII able to work independently, rendering it constitutively active and capable of temporally and spatially exerting effects longer than the initial calcium signal itself **(Hudmon & Schulman, 2002)**.

Beside CaMKII observed for phosphorylation, it also might bind directly to many proteins apart from synapsin, which may correspond to its activity-dependent consequences. CaMKII can bind in vitro to Ca^{2+} sensory protein like synaptotagmin, VAMP and syntaxin, thus facilitate SNARE complex formation. And at calcium concentrations higher than 10^{-6}M, it could facilitate binding only autophosphorylated CaMKII to the syntaxin binding domain. In chromaffin cells and in neurons, the microinjected CaMKII-binding domain of syntaxin specifically affected exocytosis process **(Ohyama et al., 2002)**.

In addition to presynaptic calcium-binding proteins such as synaptotagmin, munc13, RIM, piccolo, rabphilin and doc2 that influence the effectiveness of synaptic process through direct interaction with the machinery of the exocytotic protein, raising residual calcium levels can concurrently have useful impacts by means of a calcium-dependent regulation of presynaptic protein phosphorylation. CaMKII and PKC were experimentally involved as they represent major calcium-activated kinases. It has been found that both of them have the ability to phosphorylate a series of relevant exocytotic proteins, and consequently transduce activity-dependent modification to the release process **(Barclay et al., 2005)**.

1.5 *Pseudomonas aeruginosa (Ps.a.)*

The genus *Pseudomonas* is a group of over 140 species of bacteria, all strictly aerobic Gram-negative rods, usually found in the environment, including sources of water. Ps.a. is the most common species which could cause problems in human health, where colonization estimates range from 3-5 percent among healthy people to 20 percent among hospitalized patients. Colonization, however, does not mean infection. In spite of these high rates of colonization and virulence potential; Ps.a. is believed to be responsible for less than 10 % of inpatient infections **(Moore et al., 2016)**.

Ps.a. is a widespread opportunistic gram-negative bacterium that can weaken the natural defenses of human beings and cause serious pulmonary disease especially in immunocompromised patients. It is one of the major pathogenic agents correlated with nosocomial infectious diseases. Ps.a. has a wide variety of virulence factors, such as a type III secretion (T3SS), various proteases, lipases, pyocyanin, phospholipases and rahmnolipids and other factors **(Sadikot et al., 2005)**, which enable it to promote adhesion, capable of modulates or interact with pathways of host cells thus disrupt it and also could target the extracellular matrix. Ps.a. tends to make biofilms acting as safeguards to protect it from antibiotics and host immune system **(Alhazmi, 2015)**.

Some specific strains of Ps.a. have the largest sequenced genome at 6.3 million base pairs **(Stover et al., 2000)**. This large genome of genetic machinery provides an extraordinary advantage that allows Ps.a. to undergo major genetic and phenotypic transformations easily, in response to several changes such as environmental changes giving it the flexible capacity for antibiotic resistance **(Oliver et al., 2000)**.

During the infectious process, Ps.a. arouses a potent inflammatory response. Exposure of the airway epithelium to Ps.a. triggers the robust neutrophil chemokines expressions such as IL-6 and IL-8, leading to neutrophil infiltration **(Greene et al., 2005).** Although the recruitment of leukocytes is useful for enhancing host defense, extreme neutrophil aggregation leads to life-threatening illnesses, like acute lung injury or evolvement of set of associated symptoms such as acute respiratory distress syndrome. Most mortality in immunocompromised patients, such as cystic fibrosis, can be due to the gradual deterioration in pulmonary function arising from Ps.a. chronic infections. Lung diseases caused by Ps.a. are considered as ones of the common causes of death in both immunocompromised individuals and infants **(Alhazmi, 2015)**. Ps.a., once obtained, almost always colonizes the lungs of CF patients for life **(Watt et al., 2005)**.

Many pathogens have developed adaptive strategies to subvert the host innate immune system through escaping from PRR detection or deteriorating the downstream cell signalling pathway **(Finlay & McFadden, 2006).** In the CF lung, in response to changes in the lung environment, Ps.a. undergoes major genetic and phenotypic transformations. Over the span of chronic pulmonary infection, Ps.a. mutates to a mucoid, flagella-deficient phenotype **(E. E. Smith et al., 2006) (Jelsbak et al., 2007)**. Ps.a. could take advantage of the PAMPs alteration as a technique to significantly impair and minimize the detection and signalling of the innate immune system, thereby preventing the eradication of infection **(Cigana et al., 2009).**

There is a correlation between Ps.a. virulence and hBD-2 production. The changes in the expression of the virulence factors of Ps.a. impair the expression of hBD-2 in the lung epithelium, which diminishes the lung's innate immune response **(Cobb et al., 2004)**.

Among most gram-negative bacteria, flagellum is a common structure that is derived from flagellin monomers that affords motility, facilitates adhesion, and is therefore a major bacterial virulence factor **(Soutourina & Bertin, 2003)**. Flagellum is a bacterial ligand identified by a TLR 5 **(Hayashi et al., 2001)**. Flagellum activation of TLR5 undergoes an inflammatory response that is correlated with hBD-2 up-regulation in airway epithelial cells through a nuclear factor NF-kB dependent pathway **(Z. Zhang et al., 2005a)**. During the transition to the mucoid phenotype, the loss of flagella expression helps Ps.a. to evade the antimicrobial action of hBD-2 by reducing TLR5 stimulation, leading to potent pathogenesis of Ps.a. in the CF lung **(Hayashi et al., 2001; Z. Zhang et al., 2005a)**.

1.6 Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) is the key component of the gram negative bacterial outer membrane, also known as endotoxin. LPS is a double-edged sword; because it could be beneficial by stimulating the innate immune system of the host, and in the same time detrimental or destructive in a way that causes inflammation, diffuse intravascular coagulation, diverse organ failure, shock and sometimes death **(Chaby, 2004)**. LPS could trigger acute inflammatory responses by inducing the secretion of a broad variety of inflammatory cytokines in different cell types **(Ngkelo et al., 2012)**, particularly in macrophages and B cells **(Stewart et al., 2006)**. Beside cytokines, LPS involves in adhesion molecules upregulation in endothelial cells, which further lead to apoptotic death activation **(Chaby, 2004)**.

LPS is considered as a strong proinflammatory PAMP found in the cell wall of gram-negative bacteria. LPS response initiated after its interaction with TLR4 in combination with the myeloid differentiation factor 2 (MD-2) accessory molecules and soluble or membrane-bound protein CD14 **(Beutler, 2002) (Borzęcka et al., 2013)**. The response is then transmitted through the IL-1 receptor signalling complex, which includes two important adaptor proteins, Myeloid differentiation primary response 88 (MyD88) and TNF receptor associated factor (TRAF6), as well as serine-threonine kinase defined as Interleukin-1 receptor-associated kinase (IRAK). Also, mitogen activated protein kinases (MAPKs) pathway could be involved in this signalling response, which includes extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun N-terminal kinase (Jnk), and p38 kinase (p38) **(Takeda et al., 2003) (O'Neill, 2002)**. This signal transduction pathway further manages the activation of various genes encode proinflammatory cytokines and co-stimulatory molecules **(Hoffmann et al., 2002)**.

LPS in large quantity may form a toxic sign to the cell; due to immune hyperactivation which leads to cellular and systemic deterioration. Sepsis progression requires initiation of a systemic inflammatory response whereas the host's response to infection influences the sepsis development **(Cohen, 2002; Nystrom, 1998)**. The rise in oxidative stress is a key feature found in the physiopathology of sepsis. Through reactive oxygen species (ROS) development, LPS and several cytokines increase the oxidative stress that promotes oxidative-mediated cellular injury **(F. Simon & Fernandez, 2009)**.

As pulmonary infection switches from the acute to chronic phase, the LPS cellular responsiveness declines while the LPS expression is not affected **(Dalcin & Ulanova, 2013)**.

TRPM4 plays a crucial role in controlling LPS-prompted endothelial cell death. It was proved that down-regulation of TRPM4 substantially reduces LPS-prompted endothelial cell death and also increases endothelial cell viability **(Becerra et al., 2011)**.

LPS is commonly known as potent monocytes/macrophages activator **(Takashiba et al., 1999)**. LPS may also bring about a temporary Ca^{2+} ion elevation **(Martin et al., 2006) (X. Liu et al., 2008)**, by releasing from intracellular Ca^{2+} stores. Intracellular calcium movement triggered synaptic vesicles, which are proved to be associated with exocytosis **(Kochubey et al., 2011)**.

1.7 Toll like Receptors (TLRs)

Toll-like receptors (TLRs) display a family of innate immune recognition receptors, that play a key role in detecting pathogens, activating innate immune responses and connecting innate and adaptive immunity **(Barton & Medzhitov, 2002) (Takeda & Akira, 2001)**. TLRs are a family of membrane-resident PRRs; due to its ectodomain senses. It could easily detect the presence of PAMPs, triggering cellular responses that match the infection pattern **(Gay et al., 2014)**. In humans, thirteen TLRs have been recognized as being able to react to lipids, proteins or nucleic acids. Except TLR3, all TLRs and IL-1R1 signal trigger activation of cytoplasmic TIR domain (Toll/Interleukin-1 Receptor) which in turn recruits the common adaptor molecule MyD88, leading to NF-kB activation and nuclear translocation. TLR3 and also TLR4 (beside MyD88 pathway) could activate the TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway that moderates antiviral responses through enhancing interferon regulatory factor 3 (IRF3) transcription factor and subsequent production of type I interferons (IFNs) **(Kawai & Akira, 2010)**.

TLRs activation by any types of agonists triggers the secretion of proinflammatory cytokines, chemokines, and IFNs, such as TNF- α and IFN-β, also IL-6, MCP-1, which play a vital role in recruitment of leucocyte to the infection site; in order to battle against invading pathogens **(Kawai & Akira, 2010)**.

TLR signalling caused by any part of bacterial component not only triggers classical immune responses through the traditional MAPK pathway, but also it could trigger a rapid elevation in cytosolic Ca^{2+} which in turn enhance ATP release, leading to stimulation of the immune cells in a different manner through autocrine and/or paracrine pathway. In fact, ATP production appears to be more important to the immune system, as TLR activation could enhance intracellular signalling only in immune cells. However; ATP can in short time deliver the warning signal easily to specific receptors of neighboring cells, in a way that is even faster, widespread and effective more than the cytokines production. Therefore, the release of ATP may be also considered as a form of regulatory feedback circle that may be essential to safeguard host against severe infection **(Ren et al., 2014)**.

Extracellular ATP encourages the macrophage-mediated phagocytosis and cytokine release; in order to clear invading bacteria by activating the P2 receptors (purinergic receptors P2X, P2Y, P2Z, P2U, and P2T). Thus, the concept for the upregulation of innate immune responses to bacterial infection by extracellular ATP releases is mediated by TLR activated calcium mobilization **(Ren et al., 2014)**.

When airway epithelial cells are exposed to two different bacterial infections, in the second bacterial challenge, it was noticed that TLR responsiveness is diminished. This may be attributed to down regulation of IRAK1 signaling protein, which is associated with activation of NF-kB. **(Wu et al., 2005)**. Phosphorylation of IRAK1 leads to activation of NF-kB and activator protein-1 (AP-1). These two transcription factors induce IL-8 and hBD-2 upregulation in epithelial airway cells **(Wehkamp et al., 2004)**. These findings could provide a realistic rationale for the decreased hBD-2 expression levels of in CF patients at the chronic stage of lung infection **(C. I. Chen et al., 2004)**. Moreover, in case of advanced chronic lung infection, the reduced expression of hBD-2 in the lung related to diminishment in TLR responsiveness gives advanced knowledge about why Ps.a. only colonizes in lung after *Haemophilus influenzae* and *Staphylococcus aureus* Infection. Also, in response to acute infection, a decreasing in TLR4 expression in the airway's epithelia was detected, this may contribute to a lower expression of hBD-2 and facilitate colonization of Ps.a. **(MacRedmond et al., 2005)**.

1.8 Antimicrobial peptide (AMPs)

Antimicrobial peptides (AMPs) are cationic endogenous antibiotic proteins distributed in the epithelium cells which are considered as effectors form of the innate immune system, In a concentration dependent manner, AMPs exert antimicrobial activity, making their expression a crucial factor in host defense mechanism **(Guani-Guerra et al., 2010)**.

AMPs's amphiphatic nature leads to their efficacy in coping with hydrophobic and anionic components of the bacterial membrane **(Moskowitz et al., 2004)**. Among the main groups of human AMPs are cathelicidins, alpha-defensins, and beta-defensins **(Pinheiro da Silva & Machado, 2012)**.

Beta-defensins are considered as the border line between innate and adaptive immunity. Beta-defensins display chemotactic behavior toward mast cells, immature dendritic cells, neutrophils primed with TNF-α, and memory T cells expressing chemokine receptor (CCR6) **(Boniotto et al., 2006)**. Beta-defensins have a unique antimicrobial activity. Among the different multiple kinds of defensive antimicrobial peptides, it has been detected that only hBD-2 and hBD-3 have been increasingly expressed after stimulation by the inflammatory cytokine. Regardless to other factors, all other defensive AMPs have been continuously expressed **(Tsutsumi-Ishii & Nagaoka, 2002)**.

However, while pro-inflammatory cytokines, like IL-6, TNF-α, IL-1β, IL-17, IFN-γ and IL-22, can stimulate the expression of hBD-2 and hBD-3, these antimicrobial peptides are still expressed in baseline quantities in unstimulated cells **(Bals & Hiemstra, 2004) (Z. Zhang et al., 2005a)**.

Another distinctive difference between these two humoral stimulation-induced AMPs seems to be that hBD-2 predominantly targets gram-negative bacteria, such as Ps.a. At the same time hBD-3 has substantial bacteriostatic activity against both gram-negative and gram-positive bacteria **(Zasloff, 2002)**.

HBD-2 is the first peptide antibiotic extracted from human epithelial cell, it is a monomeric protein with six residues of conserved cysteine that form three core disulfide bonds **(Schibli et al., 2002)**. Like other defensins, hBD-2 is abundant present in the mammalian epithelium cells. HBD-2 mRNA is found out expressed in the epithelia of tonsil, trachea, bronchia and lung and thus exhibits a crucial role in combating pulmonary infection **(Bals et al., 1998)**. The inducible characteristics of hBD-2 indicate that it plays a vital role in innate immune defense. The assumption that hBD-2 is committed to an energetic human host defense mechanism of respiratory epithelial is approved **(Harder et al., 2000)**.

The dynamic interaction happened between hBD-2 and invasive pathogen depends on an electrostatic amphipathic attraction between the bacterial phospholipid-bilayer groups that have been negatively charged and cationic anti-microbial peptide **(Powers & Hancock, 2003) (Zasloff, 2002)**. After initial electrostatic attraction, hBD-2 exerts its antimicrobial effects by disrupting the membrane integrity of the invading bacteria through penetration into the phospholipid bilayer, which results in the collapse of the membrane potential and thus death of the invading pathogen **(Corrales-Garcia et al., 2010)**.

HBD-2 as a major inducible antimicrobial factor is coordinated as a response to both microbial infectious agents and inflammatory mediators. Clear example of this, hBD-2 is released either by direct interaction with the mucoid Ps.a. in airway epithelial cells or through primary cytokines produced endogenously. It may be also critical in lung infections produced by mucoid Ps.a. like those observed in CF patients **(Harder et al., 2000)**.

In another aspect, mixed or co-pathogenic infection, such as rhinovirus and Ps.a. co-infection of airway epithelial cells results in synergistic hBD-2 expression opposed to either pathogen. The combination of virus and flagellin contribute in extending this synergistic effect. While this synergy was not shown by the flagella-deficient mutant Ps.a., the effects of Ps.a. have been mediated through flagellin interactions with TLR5. in epithelial cells extracted from smokers with retained pulmonary function, and in patients with smoking-related mild to moderate chronic obstructive pulmonary disease (COPD), the observed response was dramatically attenuated **(Arnason et al., 2017)**.

1.9 Transient receptor potential channel (TRP) & Transient receptor potential melastatin 4 (TRPM4) Channel

Transient receptor potential (TRP) proteins are a category of ion channels and were initially classified into three major subfamilies: TRPV "vanilloid", TRPM "melastatin" and TRPC "canonical" **(Clapham, 2003) (C. Harteneck et al., 2000) (Montell et al., 2002)**. Recently, TRP channels based on their structure, have been subdivided into six main subfamilies: the TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPP (polycystin), TRPML (mucolipin), and TRPC (canonical) **(Gees et al., 2010)**.

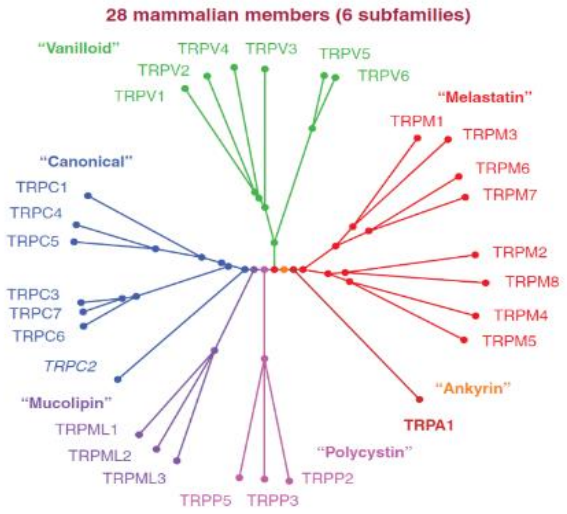

Fig. 1: Phylogenetic tree of the mammalian TRP channel superfamily.

The TRP channel family is divided into six subfamilies, which are closely related but can be discriminated upon their tertiary structure into a TRPV, TRPM, TRPA, TRPP, TRPML, and TRPC branch **(Gees et al., 2010).**

A topological structure of sensory TRP channels has six membrane-spanning transmembrane domains. The assembly of the channels is likely to engage these domains together. The pore loop that permits ionic stream is located between the transmembrane 5 & 6 domains. The adversely negative charged residues buildups on this pore loop might be necessary to conjugate and penetrate the cations **(Owsianik et al., 2006)**. Amino and carboxyl ends are situated in cytosol. In amino terminals, it contains several repeats of ankyrin. The transmembrane domains 4/5 linker is expected to be one of the main significant areas or locations for interacting with endogenous lipid regulators or lipophilic pharmacological agents. PIP2 could bind to some TRP channels' C-termini near the TRP domain **(S. Yoo et al., 2014).**

The largest and most diverse subfamily of the TRP superfamily is the TRPM (transient receptor potential melastatin) family **(Fleig & Penner, 2004)**. The TRPM subfamily comprises of eight individuals, from TRPM1 to TRPM8. On the basis of sequence homology, it can be classified into four groups as follows: TRPM1/3, TRPM2/8, TRPM4/5, and TRPM6/7. Ca^{2+} permeability in TRPM family varies from Ca^{2+}

impermeable as seen in TRPM4 and TRPM5 channels to extremely permeable to Ca^{2+} in other channels **(Gees et al., 2010)**.

Awareness of their physiological activity has quite recently started to emerge. These TRPM channels are widely expressed in various tissues, organs, and contribute to the signaling of cellular Ca^{2+} via encouraging Ca^{2+} passage into the cytosol responding to various physiological processes such as alteration in ion concentration, lipids and tiny molecules, which in turn act as intracellular second messengers. Thus, it facilitates Ca^{2+} influx through Ca^{2+} permeable channels such as (TRPM1, TRPM2, TRPM3, TRPM6/7, TRPM8) or by indirect influencing on other various channels such as modulating the membrane potential of voltage-gated Ca^{2+} channels. Several biological processes such as oxidative stress sensing, endothelial permeability regulation, cell death, magnesium homeostasis, myogenic reaction, and vascular tone control can be affected by variation in intracellular cellular $Ca^{2+/}Mg^{2+}$ ion concentration or a shift in the membrane potential and subsequent electrical activity of the cell **(Felipe Simon et al., 2013) (McNulty & Fonfria, 2005) (Earley et al., 2004)**.

This TRPM family members have thus drawn more attention during the last decade, to the promising drugs for treating of various disease such as cardiovascular diseases **(Abriel et al., 2012)**, type II diabetes **(Vennekens et al., 2018)**, neurodegenerative disorders **(Yuyang Sun et al., 2015)**, inflammation **(Zierler et al., 2017)**, and inflammatory pain **(Held et al., 2015)**.

TRPM channels architecture remained undetected until late 2017, the structure was resolved using single-particle cryo-electron microscopy, where several TRPM4 structures and one TRPM8 structure were revealed **(Winkler et al., 2017) (Guo et al., 2017) (Autzen et al., 2018) (Yin et al., 2018)**.

The TRPM channels classified among the largest individuals in the TRP superfamily as shown in **(Fig.1)**, it includes cytosolic domain of almost between 732 and 1611 amino acids per subunit. They have a standard characteristic structure consisting of a cytoplasmic transmembrane domain (TMD) comprising of six transmembrane helices that connect both N and C-terminal together as illustrated in **(Fig.2)**. At N-terminal there is a TRPM major homology region (MHR) domain. At C-terminal terminal, it has TRP helix, coiled-coil domain, and a carboxyterminal domain (CTD) which varies from one individual to another **(Huang et al., 2020)**.

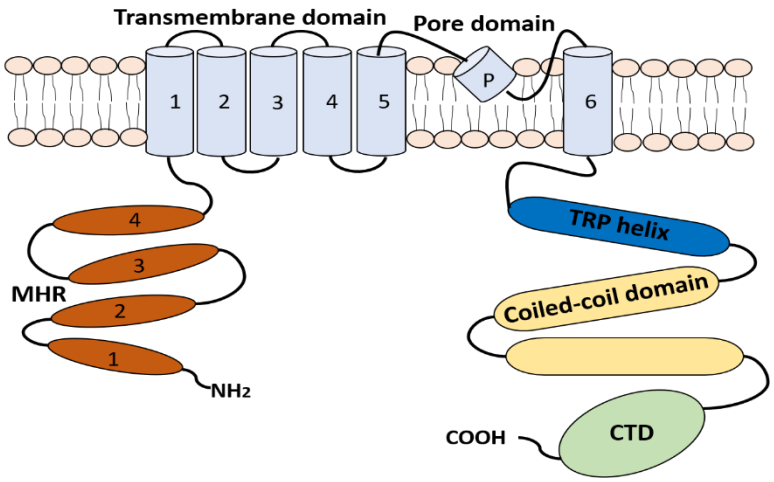

Fig. 2: Domain organization of TRPM family.

Domain organization of a monomer of the human TRPM family; N-terminal homology region domain (MHR), the C-terminal domain (CTD) differs from one family member to another, modified after **(Huang et al., 2020)**.

TRPM4 is one of the eight individuals from the TRPM subfamily branched from TRP channels **(Christian Harteneck, 2005) (Kraft & Harteneck, 2005)**. A crown-like tetrameric transmembrane core with a domain-swapped architecture reflects the TRPM4 structural topology. As shown in **(Fig.3)**, the tetrameric composition of TRPM4 is determined by N-terminal nucleotide-binding domain in NH_2 terminal and C-terminal coiled coil domain in other COOH terminal. Six transmembrane helices (S1-S6) contain a distinctive selectivity filter as a loop shape lying between S5 and S6. These six helices connect both sides of cytoplasmic N- and C-termini. **(Nilius et al., 2005a) (Clémençon et al., 2014) (Autzen et al., 2018)**. A lower gate is formed by a cluster of hydrophobic amino acids. Endogenous modulators that bind to the intracellular tails of TRPM4 will allosterically affect channel-gating. Additionally, several intracellular factors, which have binding sites on both terminal including Calmodulin (CaM), phosphatidylinositol 4,5-bisphosphate (PIP2), PKC, ATP, etc., could modulate and closely regulate the ultimate TRPM4 Ca^{2+} sensitivity **(Nilius et al., 2005b) (Nilius et al., 2006) (Vennekens & Nilius, 2007) (Guo et al., 2017)**.

In various cell types, the key difference between TRPM4 and TRPM5 is dependent on its sensitivity to smaller concentration of intracellular Ca^{2+} which have the ability to boost activation. The HEK-293 cell study showed that the Ca^{2+} half-maximum current (EC50) concentration for TRPM4 activation (20.2 ± 4.0 µM) was far higher than the EC50 of TRPM5 activation (0.70 ± 0.1 µM) **(Ullrich et al., 2005)**.

TRPM4 is expressed by about 90% of cells which also express TRPM5. In some cases, a desired dynamic coupling between both channel TRPM4 and TRPM5 is required to produce the appropriate cellular response. Even so, if this dynamic coupling occurs, the channels are able to operate independently via responding to higher stimulus concentrations. Coupling of TRPM4 and TRPM5 could encourage to evoke greater sensitivity to Ca^{2+} concentration. In other systems, TRP channels have been shown to complement functionally **(Chubanov et al., 2004) (Lintschinger et al., 2000) (X.-Z. S. Xu et al., 1997)**.

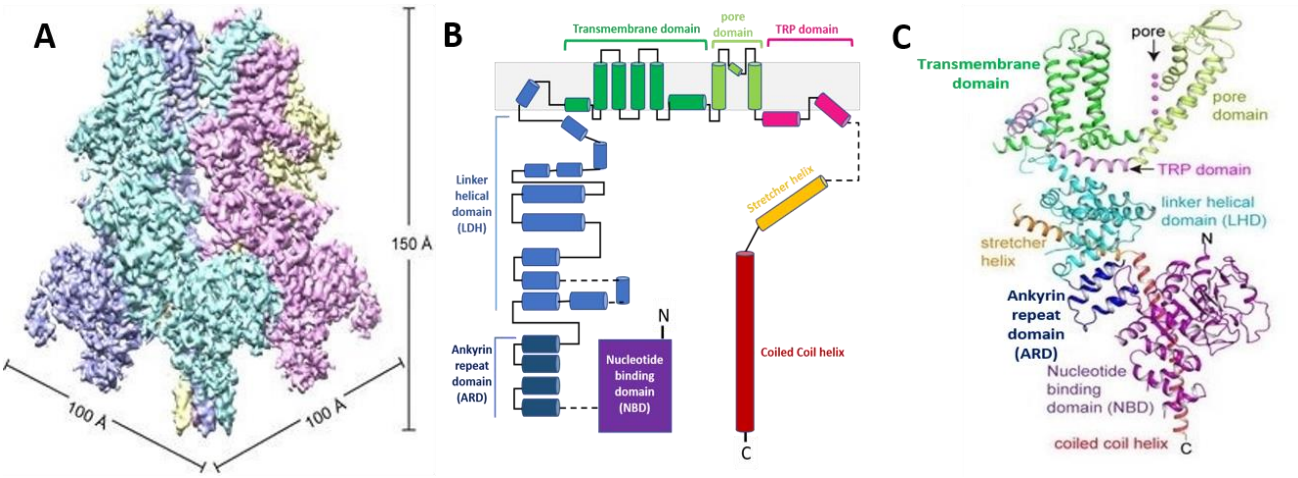

Fig. 3: Overall TRPM4 architecture

A, 3D reconstruction of TRPM4 with specific colour for each subunit. **B,** Schematic depiction of the domain arrangement in a single subunit of TRPM4. **C,** Structure of a single cyan colored subunit in the same orientation as present in the diagram A, modified after **(Guo et al., 2017)**.

A critical phase in determining of TRP channels performance validity and functionality tends to translocate to the plasma membrane. Numerous members of the TRP family, such as TRP-3 (TRPC homologs) **(X. Z. Xu & Sternberg, 2003)**, TRPC3 **(Singh et al., 2004)**, TRPC5 **(Bezzerides et al., 2004)**, TRPC6 **(Cayouette et al., 2004)** and TRPV1 **(Morenilla-Palao et al., 2004)** have been reported to translocate to the plasma membrane during exocytosis. Many cellular physiological processes such as fertilization, nociception and differentiation are implicated in recruitment of TRP channels to the plasma membrane **(Singh et al., 2004) (Bezzerides et al., 2004) (Morenilla-Palao et al., 2004)**. Translocation and activation of TRPM4 were conditioned to increase in intracellular Ca^{2+} ion concentration, in contrast with other channels such as TRPC3 and TRPC6 in which an independent Ca^{2+} mechanism was proposed for their activation **(Singh et al., 2004) (Cayouette et al., 2004)**.

TRPM4 channels have three isoforms resulting from alternative splicing: **(P. Launay et al., 2002)** the full-length TRPM4 (TRPM4b) **(Fleig & Penner, 2004)**, TRPM4a isoform with 174 amino acid deletion at N-terminal **(Vennekens & Nilius, 2007)**, and TRPM4c isoform without 537 amino acids **(Vennekens & Nilius, 2007) (J. C. Yoo et al., 2010) (C. H. Cho et al., 2014)**. However, *in vivo*, the specific functions of these splicing variants are still mysterious.

TRPM4b channels form the basis of a modulatory mechanism that manages the amount of the Ca^{2+} inflow by boosting the membrane potential and thereby subsequent driving the Ca^{2+} inflow via other pathways permeable to Ca^{2+}. The physiological effect of TRPM4b on Ca^{2+} signalling depends on the cellular sense in which it is expressed. Once intracellular Ca^{2+} increase, the voltage-dependent Ca^{2+} channels acting as a major activation for TRPM4b channel. Lacking of voltage-dependent Ca^{2+} channels in electrically non-excitable cells seems to minimize Ca^{2+} influx by the decrease in driving force for Ca^{2+} entry induced by depolarization.

TRPM4b in these types of the cells can function to regulate intracellular Ca^{2+} oscillations via oscillatory changes in membrane potential in coordination with K^+ and Cl^- channels **(P. Launay et al., 2002)**.

1.10 Ca^{2+} and TRPM4 Channel

TRPM4 is broadly described as a non-selective Ca^{2+} activated cation channel that is mainly permeable to monovalent ions such as K^+ and Na^+ without obvious noticeable permeation to divalent cations such as Ca^{2+} ions **(X. Z. Xu et al., 2001)**.

In comparison to other members of the TRP family, an elevated concentration or intracellular accumulation of Ca^{2+} can cause TRPM4 activation **(Nilius et al., 2003) (Nilius et al., 2005b) (Venkatachalam & Montell, 2007) (Vennekens et al., 2007)**. Furthermore, TRPM4 is feasible with the following ionic selectivity for monovalent cations: $Na^+ > K^+ > Cs^+ > Li^+$ **(Nilius et al., 2005a)**. TRPM4, however, demonstrates no Ca^{2+} permeability, it causes accumulation of intracellular Ca^{2+} leading to cell membrane depolarization, sustain Ca^{2+} overload could further contributes to cell damage or death **(Song & Yuan, 2010) (C.-H. Cho et al., 2015)**.

The changes in the concentration of intracellular Ca^{2+} are closely linked to a wide variety of physiological and pathological processes in significant tissue systems. For instance, proliferation and differentiation of epidermal cells in tissue culture are sharply affected by changes in Ca^{2+} concentration **(Hennings & Holbrook, 1983)**, TRPM4 has been associated with various physiological functions, including cell membrane depolarization as a defense against Ca^{2+} overload. Regulation of Ca^{2+} oscillations could also drive cytokine synthesis in leukocyte such as T lymphocytes and mast cells. Moreover, they have an impact on dendritic cell migration **(Barbet et al., 2008) (Pierre Launay et al., 2004) (P. Launay et al., 2002) (Vennekens et al., 2007)**. It has been reported that, proliferation, cytokine secretion, phagocytosis, and apoptosis, are regulated by intracellular Ca^{2+} concentration **(Lewis, 2001) (Dai et al., 2009)**. In macrophages and neutrophils Ca^{2+} mobilization through activated Ca^{2+} channels have been detected and appear to be a predominant factor for the activation of these phagocytic activity. Also tightly regulated Ca^{2+} mobilization and its control by TRPM4 are essential for the effective activation of monocytes and macrophages to combat acute infection **(C. Lee et al., 2003) (Schorr et al., 1999) (Serafini et al., 2012)**.

TRPM4 is implicated in the control of Ca^{2+} homeostasis **(Abriel et al., 2012)**, by decreasing the driving force of Ca^{2+} influx via the activated Ca^{2+} channel. In fact, under physiological conditions, higher intracellular Ca^{2+} concentration drives TRPM4 channel opening, which permits massive Na^+ ion influx and subsequent leading to membrane depolarization, thus reducing of an additional Ca^{2+} ions influx **(Pierre Launay et al., 2004)**. TRPM4 could acts in conjunction with the activated Ca^{2+} channels in various types of immune cells including T cells, mast cells and DC's to manipulate intracellular Ca^{2+} concentration, and thus subsequent physiological reactions **(Pierre Launay et al., 2004) (Barbet et al., 2008)**.

After TRPM4 is triggered by a rise in intracellular Ca^{2+} concentration, currents decay quickly occurred due to a rapid decrease in TRPM4 sensitivity to Ca^{2+} **(P. Launay et al., 2002) (Nilius et al., 2004) (Nilius et al., 2003)**.

TRPM4 is rapidly desensitized to Ca^{2+}, and TRPM4's Ca^{2+} sensitivity is controlled by several factors mainly binding at the C terminus, such as ATP, PIP2, PKC-dependent phosphorylation, and calmodulin. Cellular modulation and modification occur by these factors through interacting with distinctive residues located in the TRPM4 channel. Desensitization is noticed delayed when Mg-ATP is bound, the PKC phosphorylation of the channel, and the CaM is bound to the C-terminal region of TRPM4 channel **(Nilius et al., 2005b)**.

ATP plays a pivotal role in sustaining the Ca^{2+} sensitivity of the TRPM4 channels. ATP addition helps preserve TRPM4's Ca^{2+} sensitivity in the inside-out patches. TRPM4 could overcome the desensitization. This was noticed when the cytoplasmic membrane was treated with a Ca^{2+} free solution containing Mg-ATP. Intriguingly, all mutations that have altered putative ATP binding sites on TRPM4 have significantly accelerated Ca^{2+} channel desensitization. And at least a proportion of TRPM4's Ca^{2+} sensitivity is restored directly once ATP binding **(Nilius et al., 2005b)**.

ATP believed as the valuable key in differentiation between TRPM4 and TRPM5 channels, where TRPM4 showed sensitivity toward internal ATP which could lead to block it, while TRPM5 does not display the same feature and couldn't be blocked by internal ATP. This difference is possibly due to the presence of four ATP binding sites in the TRPM4 channel, while TRPM5 shows only one binding site which appears to be not easily accessible **(Ullrich et al., 2005)**.

PIP2 is a minor ingredient of the phospholipid cell membrane. It has been reported as the most significant regulator of TRPM4 channel. TRPM4 has two PIP2 binding sites at its C terminal, suggesting that the modification or depletion of these binding sites can affect the sensitivity of TRPM4 to both PIP2 and Ca^{2+}, thus acting as a possible factor of its desensitization **(Nilius et al., 2006) (Z. Zhang et al., 2005b)**.

PIP2 is a substrate for a variety of significant signaling proteins. It acts as a substrate of phospholipase C (PLC) **(Watschinger et al., 2008) (Hammond et al., 2006)**. PIP2 is a powerful TRPM4 enhancer which can lead to a desensitization impact on TRPM4 activity via Ca^{2+} activated PLC, which mediate PIP2 decomposition in the plasma membrane **(Nilius et al., 2006) (Z. Zhang et al., 2005b)**, thus turns TRPM4 channel progressively to Ca^{2+} insensitivity, leading to a transition towards the state of negative potential **(Nilius et al., 2006) (Nilius & Vennekens, 2006)**, Poly-L-lysine, which is a form of PIP2 scavenger, has been shown to cause sharp TRPM4 desensitization **(Z. Zhang et al., 2005b)**. PIP2 is unable to activate TRPM4 on its own, but able to rectify desensitization through increasing TRPM4's sensitivity to Ca^{2+}, and

limit TRPM4's voltage dependence **(Nilius et al., 2006) (Z. Zhang et al., 2005b) (Vennekens & Nilius, 2007)**.

PKC-dependent phosphorylation modulates the Ca^{2+} sensitivity of TRPM4. The EC50 value for TRPM4 activation by Ca^{2+} was reduced from 15 to 4 µM by the Phorbol 12-myristate 13-acetate (PMA), a PKC activator, which improves TRPM4 activity by increasing TRPM4's vulnerability to Ca^{2+}. This effect was eliminated when one of the two C-terminal Ser residues (Ser1152 and Ser1145) required for PKC phosphorylation was mutated **(Guinamard et al., 2004) (Nilius et al., 2005b)**.

CaM binding to the TRPM4 C terminus enhances its activation at high physiological voltages. In contrast to Ca^{2+} activated K^+ channels, it was demonstrated that Ca^{2+} binding is absolutely necessary for TRPM4 activation. However; in the absence of Ca^{2+}, TRPM4 fails to be activated even at extremely positive voltages **(Nilius et al., 2005b)**.

By using in-vitro binding assays, it was revealed that TRPM4 has five short CaM binding sites: two at the N terminus and three at C terminus. It was proved that TRPM4 CaM interaction is a Ca^{2+} dependent manner, In the presence of Ca^{2+}, all CaM-binding fragments showed a much stronger association with CaM than in the absence of Ca^{2+}, and only one region was detected which has the ability to bind to a measurable amount of CaM in the absence of Ca^{2+} ions. It is thought that CaM-binding sites tend to be preferentially participating in channel control. Since elimination of the C-terminal but not the N-terminal sites impacted TRPM4's Ca^{2+} sensitivity, in which the current amplitude was significantly decreased and rapid current decay was facilitated **(Nilius et al., 2005b)**.

The aim of this work is to investigate the contribution of the TRPM4 channel on the bacteria induced pulmonary host defence. To do this, bronchial epithelial cells, cell lines and monocyte derived macrophages were used to examine the inflammatory response and host defence after the inhibition of TRPM4 and additional bacterial stimulation. Additionally, the lungs of TRPM4-knockout mice were stimulated with heat inactivated bacteria to investigate the contribution of TRPM4 on the inflammatory response and the expression of host defence molecules.

During the scope of this work, the following questions will be answered:

1. Is TRPM4 expressed in airway epithelial cells and can its activity be inhibited without inducing cytotoxicity?
2. Is the activity or presence of TRPM4 necessary to induce the release of inflammatory cytokines after bacterial stimulation *in vitro* and *in vivo*?
3. Is the innate immune defence compromised after the inhibition of TRPM4 *in vitro* and *in vivo*?

2. Material and Methods

2.1 Material

2.1.1 Devices

Table (1): summarizes the devices uses in this study.

1.	Analytical balance, KERN ABS-N / ABJ-NM, Analytical balance, KERN EMB 200-2
2.	BD FACS Canto II
3.	Biological Safety Cabinets, Thermo Scientific™ Safe 2020 Class II, Biological safety cabinets, MSC-Advantage™ Class II
4.	Cellspin II, THARMAC
5.	Centrifuge Heraeus Fresco 21, Centrifuge Allegra X-30R, BECKMAN COULTER, Centrifgue, Heraeus Megafuge 1.0RS, Thermo Scientific, Centrifuge Heraeus Labofuge 400, Kobe.
6.	ChemiDocTM MP Imaging System
7.	ELISA Plate Washer, Capp W-12 Wash, 12 channel
8.	Epithelial Voltohmmeter, EVOM1, World-Precision-Instruments, Sarasota, FL, USA.
9.	FLUOstar Omega –Microplate Reader, BMG Labtech
10.	Freezer PHCbi TwinGuard MDF-DU502VX, -86°C,528L, Freezer, Thermo Scientific™ HERA ™ HFU T Series -86 ° C Ultra Low Freezers
11.	Handheld Magnetic Separator Block for 96 Well Flat Bottom Plates, Merck Millipore 40-285
12.	Heater & Stirrer, NeoLab, D-6010, Stirrer Neolab D-6011
13.	Heating block, Biozym Scientific GmbH
14.	HERAcell 240 CO_2-Incubator Thermo Scientific
15.	Homogenizer, ULTRA-TURRAX®, IKA
16.	Incubation shaker, Thriller, PeQLab
17.	Lonza-Amaxa Nucleofector I devices
18.	Luminex MAGPIX™
19.	Magnetic stirrer - with heating plate, Heidolph MR 3000
20.	Microscope Olympus BX51 + Camera Olympus DP72, Microscope Leica Microsystem CMS, Microscope Axiovert 25 Carl Zeiss Microscopy GmbH Structured Illumination Microscopy (SIM), Zeiss Elyra PS1 Zeiss, Oberkochen
21.	Microwave, Exquisit
22.	Multi-channel pipette, manifold dispenser, Eppendorf, Multichannel electronic pipette, EPPENDORF Research Pro/Plus, Multichannel electronic pipette, Thermo Scientific
23.	NanoDrop 8000 Spectrophotometer, Thermo Scientific
24.	Pipettes, Gilson™ PIPETMAN Neo
25.	Pipetting aid, NeoLab 8-5010
26.	Plate Shaker, Heidolph Titramax 101, Shaker, Heidolph Duomax 1030, Minishaker MS1, MS2 IKA®, Roller shaker, RST
27.	Spectrometer Ultraspec 2100 Pro, biochrom
28.	Structured Illumination Microscopy (SIM) Zeiss Elyra PS1 Zeiss, Oberkochen.
29.	Thermal cycler CFX96 Touch™ BioRAD, C1000 Touch, Thermal cycler T100 TM BioRAD

30.	Ultrapure water purification systems, Barnstead™ GenPure
31.	Vacuum filtration devices bottle-top filters, Type DS0320, Thermo Scientific
32.	Vertical Autoclave, ZIRBUS Technology
33.	Vortex, NeoLab, D-6012, Vortex, Genie 2, Scientific Industries, Vortex Janke & Kungel
34.	Water bath WB6, VWR, Water bath GFL Typ 1003 Kobe

Table 1: Used devices in this study.

2.1.2 Commercial Kits, Reagents and Chemicals

Table (2): summarizes the commercial kits that were used in this study

No.	Kits
1.	Pierce™ BCA Protein Assay Kit, Number 23225.
2.	RNA isolation (NucleoSpin® RNA, Machery-Nagel) REF. 740955.
3.	Thermo Scientific RevertAid RT Kit #K1691.
4.	SensiMix™ SYBR® & Fluorescein Kit, BioLine, Cat. QT615-05.
5.	LDH-Cytotoxicity Assay Kit II (ab65393 Abcam Company).
6.	Duo-Set ELISA kits (R&D Systems, Lille, France) Cat. DY406, DY410, DY452, DY453, DY206, DY210, DY208, DY4570, DY276.
7.	Ingenio® Electroporation Kits for Amaxa® Nucleofector®, MIR 50111.
8.	R&D System Human Premixed Multi-Analyte Kit; Cat. LXSAHM.
9.	R&D System Mouse Premixed Multi-Analyte Kit; Cat. LXSAMSM.

Table 2: Used commercial kits in this study.

Used reagents and chemicals were listed according to **Table (3).**

Protocols	Reagents and Chemicals
Cells and Cell lines	o **Medium DMEM1x, GlutaMAX**, Gibco 61965-026. o **RPMI medium 1640 (1x) + Gluta MAX^{TM-1}**, Gibco 61870-010. o **Medium DMEM/ F-12 (1:1), + L-Glutamin**, Gibco 11320-074. o **1:1 DMEM/F12**, Gibco 11320-074. o **Airway Epithelial Cell Growth Medium Kit**, PromoCell C-21160 o **Primocin**:Antimicrobial agent for primary cells, InvivoGen ant-pm1 o **Ultroser-G** serum substitute, Pall Life Science, Cytogen, Greven, Germany, No. 15950-017. o **RIPA Lysis buffer,** Thermo Scientific ™ 89900 o 25 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, 4% protease inhibitor. o **PBS 1x**, sterile, Dulbecco's, without Ca/Mg^{++}, PAA H15-002 o **Penicillin/Streptomycin** (100x), PAA P11-010 o **Trypsin-EDTA-Solution** (0,05% Trypsin/0,02% EDTA), 1x Invitrogen 25300-054 o **Fetal Bovine Serum (FBS)**, Gibco 10270-106 o **Trypan blue solution** (0.5g / 100mL) in sodium chloride solution (0.9g / 100mL), sterile filtered. PH 7.4 storage at **room temperature (RT)**
Human bronchial primary epithelial cell	o **DMEM/Hams F12 medium** (Gibco, ThermoFisher Scientific, Waltham, MA, USA, Cat.11320033) o **0.1% Pronase-E** (Sigma Aldrich, St. Louis, MI, USA, Cat. 9036-06-

	0) ○ **0.1% DNAse** (Sigma Aldrich, St. Louis, MI, USA, Cat. 10104159001) ○ **1% penicillin-streptomycin** (Gibco, ThermoFisher Scientific, Waltham, MA, USA, Cat. 15140-122) ○ **1% Ultroser G** (Pall Life Science, Cytogen, Greven, Germany, No. 15950-017) ○ **1 µM A83-01** (TGF-β inhibitor, Tocris Bioscience, GB, Cat. No. 2939) ○ **0.2 µM DMH-1** (BMP4 inhibitor, Tocris Bioscience, GB, Cat. No. 4126) ○ **5 µM Y27632** (ROCK inhibitor, Tocris Bioscience, GB, Cat. No. 1254)
Tracheal primary epithelial cells from mice	○ **Pronase E 20 mg/ml (Sigma-Aldrich)** Cat. No. 10165921001 Dissolved in Tris-HCl buffer (0.5 M; pH=7.5) at 37 °C. this solution made up prior to use, aliquoted and stored at- 20 °C. ○ **DMEM/Hams F12 medium** (Gibco, ThermoFisher Scientific, Waltham, MA, USA, Cat.11320033) ○ **Airway epithelial cell culture medium (PromoCell) (Ready-to-use)** Cat. No.C-21060
Bone-marrow-derived macrophages (BMDMs) from mice.	○ **BD Pharm Lyse** (BD Biosciences, Cat. No.555899) ○ **Ficoll-Paque plus** (GE Healthcare, Cat. 17144002) ○ **GM-CSF& M-CSF** (R&D Systems, Cat.415-ML-010& 416-ML-010) ○ **Medium DMEM1x**, **GlutaMAX** (Gibco 61965-026) ○ **PBS** 1x, sterile, Dulbecco's, without Ca/Mg^{++} (SIGMA D8537) ○ **Penicillin/Streptomycin** (100x)(Gibco 15140-122) ○ **Trypsin-EDTA**-Solution (0,05%) Trypsin/0,02% EDTA (Gibco 25300-054) ○ **Fatal Bovine Serum (FBS)** (Gibco 10270-106) ○ **CD68** antibody (Biolegend, Cat. 137007) ○ **F4/80+** antibody(Biolegend, Cat. 123125)
ELISA	○ **PBS:** 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH2PO4, pH 7.2-7.4, 0.2 µm filtered (R&D Systems, Cat. # DY006). ○ **Wash Buffer**: 0.05% Tween® 20 in PBS, pH 7.2-7.4 (R&D Systems, Cat. # WA126). ○ **Reagent Diluent:** 1% BSA in PBS, pH 7.2-7.4, 0.2 µm filtered (R&D Systems, Cat. # DY995). ○ **Substrate Solution:** ▪ **TMB Solution,** Calbiochem, CL07 ▪ Solution1:1 mixture of **Color Reagent A** (**H_2O_2**) and **Color Reagent B** (**Tetramethylbenzidine**) (R&D Systems, Cat. # DY999). ○ **Stop Solution:** 2 N H_2SO_4 (R&D Systems, Cat. # DY994).
FACS	○ **BD FACS Flow sheath fluid** (BD Biosciences, 342003) ○ **BD FACS Clean solution** (BD Biosciences, 340345) ○ **BD FACS shutdown solution** (BD Biosciences, 334224) ○ **BD FACS™ lysing solution** (BD Biosciences, 349202) ○ **25mM EDTA** ○ **BD Cytofix™ fixation buffer (**BD Biosciences, 554655) ○ **BD Perm/wash™ buffer (**BD Biosciences, 554723)

	○ **Human TruStain FcX ™ (Fc receptor blocking solution) (**Biolegend, 422301) ○ **Human IL-6 PE-conjugated antibody** (R&D Systems, Clone #1936, Cat. #IC206P) ○ **Stain buffer with FBS** (BD Biosciences, 554656) ○ **CS&T Beads (**BD Biosciences, 650622) ○ **FacsDiva Software**
Western Blotting	○ **10x blotting buffer** 0,048 M Tris Base, 0,039 M Glycine, dissolved in 600 ml distilled water and adjust pH to 9.2, 0,0013 M SDS, Distilled water filled up to 1 L. ○ **1x blotting buffer (1 litre)** 100 ml 10x blotting buffer, 700 ml Aqueous distilled water, 200 ml absolute Methanol. ○ **10X SDS-PAGE Running buffer** 0,25 M Tris Base, 1,92 M Glycin, 1% SDS, PH adjusted to 8.3 with HCl. ○ **1X SDS-PAGE Running buffer (1 litre)** 100 ml of 10X SDS-PAGE Running buffer, 900 ml distillated water. ○ **RIPA Lysis Buffer** NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH adjusted to 8.0 ○ **Dilution Buffer** Phosphate Buffered Saline (PBS) or Tris Buffered Saline (TBS) ○ **Washing Buffer** 5-10 mL of 10% Tween-20 were added to 1 Litre of Dilution Buffer to reach a final 0.05-0.1% Tween-20 concentration. ○ **Blocking Buffer** 5% Milk powder in TBST or 5% FBS in TBST ○ **Roti®-Load 1**, Carl Roth Gmbh, K929. ○ **cOmplete EDTA-free**, Protease Inhibitor Cocktail Tablets, Roche, Cat. No. 11 873 580 001. ○ **PhosSTOP,** Phosphatase Inhibitor Cocktail Tablets, Roche, Ref. 04 906 845 001 ○ **4-12% Tris-Glycine gel & 10- 20 % Tris-Tricine gel**, Anamed electophorese GmbH, TG 41212& TR 12012. ○ **Nitrocellulose membrane or polyvinylidene difluoride membrane,** Thermo Scientific™ 88018, 88518 ○ **SeeBlue® Plus2 Pre-Stained Protein Standard,** Invitrogen™ LC5925. ○ **Pierce™ ECL Western Blotting Substrate,** Thermo Scientific™ 32209. ○ **Film cassette** ○ **Developing and Fixing reagents** for processing autoradiographic film, ASN 110105. ○ **Restore™ Western Blot Stripping Buffer,** Thermo Scientific™ 21059.

Immunofluorescence for adherent cells line	○ **Coverslip/chamber slides** Coverslip 1.5, thickness (0.17 mm) or chamber slides coated with poly-L-lysine or collagen coating. ○ **Solutions & Antibody:** • **10× PBS:** NaCl 80g, KCl 2.0g, Na_2HPO4 14.4 g, KH_2PO_4 2.4 g, Distilled Water 800 mL, pH adjusted to 7.2-7.4 with HCl., adjust volume to 1 L with additional H_2O and sterilize by autoclaving. • **1xPBS** 100 ml of the 10xPBS were diluted in a total 1L volume of dd H2O. • **Washing Buffer:** 1 X PBS (or) PBST: add 0.5 ml of Tween-20 in 1L 1xPBS and mix well. • **Fixatives:** 4% Paraformaldehyde (PFA), pH 7.4 or 100% ice-cold methanol (or) Ice-cold1:1methanol/Acetone • **Blocking Buffer** 5-10% serum from host species of secondary antibody (blocking) (or) 1-3% BSA (stabilizer) in 1 x PBS (or) 1-3% BSA (stabilizer) in 1 x TBST • **Permeabilization Buffer** 0.05-0.3% Triton X-100 (or 100 µM digitonin or 0.5% saponin) in PBS • **Antibody Dilution Buffer** 1-2% BSA (for stabilizer and blocking) in 1 x TBST • **Phalloidin-iFluor 488 Reagent,** Abcam ab176753 • **ProLong® Gold Antifade Reagent with DAPI,** Invitrogen™ P36941 • **ProLong™ Diamond Antifade Mountant with DAPI,** Invitrogen™ P36966
Experimental Mice infection	○ Dissecting tools, Cannula (green, diff. color according to needle size), Thread, 50 ml falcon tubes, 8 ml screw capped bottle, Eppendorf tubes, Needles and syringes, PBS, Flushing solution (PBS+EDTA 0,5mM), 4% formaldehyde, 2-3% isoflurane, lidocaine solution, Bepanthen eye&nasal ointment, intubation platform with O-ring attached to Velcro strip, Teflon plunger syringe, cotton-wooden applicator, wire, catheter, heating pad, ketamine/xylazine anesthesia solution (5x for 10 mice): 437,5 µl Rompun® (xylazine) + 1312,5 µl Ketamin + 250µl Nacl or water.
Other chemicals and reagent used	○ TRPM4 inhibitor 9-Phenanthrol-CAS 484-17-3 – Calbiochem, Sigma Aldrich ○ TRPM4 antibody 9-Phenanthrol, TOCRIS 484-17-3 ○ TRPM4 - Control Antigen, Alomone Labs ACC-044 ○ DAPI, Roth 6335.1 ○ LPS-B5 Ultrapure, Cat. # tlrl-pb5lps, Lot# B5P-41-01, InvivoGen ○ FLA-PA Ultrapure, Cat. # tlrl-pafla, Lot# FPA-40-02, InvivoGen ○ Pam3CSK4, Cat. # tlrl-pms, Lot# 5930-41-05, InvivoGen ○ Dimethylsulfoxid, ≥99,5 % (DMSO), Carl Roth ○ β-Mercaptoethanol, Sigma-Aldrich ○ cOmplete ULTRA Tablets, Mini, EDTA-free, EASYpack, Roche ○ Pierce™ Phosphatase Inhibitor Mini Tablets; Thermo Scientific™

	o Ethanol absolute, Sigma-Aldrich o Methanol, >99,9%, Fisher Scientific GmbH o Agarose, Sigma-Aldrich o Agar Powder, VWR o LB Agar, Carl Roth o LB Broth, Carl Roth o Pseudomonas Isolation Agar, Difco, 500g, BD Biosciences o Triton X-100, Roche o Diff Quick or Quick Giemsa stain, Medion Diagnostics. o Giemsa May-Grünwald, RAL Diagnostics. o TRIzol® Reagent, Life Technologies o Trypan Blue, Carl Roth o Tryptic Soy Broth, Fluka o Tryptone, Applichem o Tween 20, AppliChem

Table 3: Reagents and chemicals used in this study.

2.1.3 Consumables

Lab consumables used in lab work were gathered in **Table (4).**

1.	Beakers with spout, low form, measuring beakers, with handle, polypropylene, Measuring cylinder, borosilicate glass, class A, blue graduated
2.	Pasteur pipettes, glass VWR 612-1701, Graduated pipettes & Serological pipettes 5 ml, 10 ml, 25 ml sterile, CELLSTAR, greiner bio-one
3.	Erlenmeyer flasks, narrow neck, wide neck, Laboratory flasks with blue screw caps, bottles, with screw cap and retrace code, Conical flasks, narrow neck, wide neck, Culture flasks, borosilicate glass, Wide-necked flasks, low-density polyethylene natural, high-density polyethylene brown, Tissue culture flasks, TC flask, sterile, 75 cm2 (250 ml), vented cap (red) Sarstedt 703230
4.	Thermo Scientific™ Pierce™ 96-Well Plates, Product No. 15041 96 well microplates (R&D Systems, Catalog # DY990). 12-well transwell cell culture plates with a polyester membrane with 0,4 µm pore size (Corning Inc., Lowell, MA, USA) Axon Labortechnik, 25 x 96-well PCR plate with half frame, segmented 4 times, suitable for cutting Nr. 28242 Microplate BRAND plates®, immunoGrade, size 96 wells, flat bottom, transparent
5.	Microlitre syringes, for removable needles Hemocytometer counting chamber
6.	Falcon Tubes 50 ml, sterile GBO 227261, Culture glass tubes, screw cap, Soda-lime glass, Storage cases for culture tubes
7.	Standard reaction tubes, 3810 X,0.2 cm cuvettes (Ingenio® Cuvettes), Cryotubes, CryoPure cryovial 1.6 ml Sarstedt REF 72.380, Centrifuge tubes, PP, Polypropylene test tubes, Polystyrene test tubes BD Biosciences, High speed centrifuge tube, borosilicate glass
8.	Reaction tube stand, PP, Safe-lock reaction tubes, PP, Tube racks 4-Way, PP, Wire Racks, epoxy-coated steel, Microtube Racks, 20-Well, 80-Well, 96-Well PP, Test tube racks, wire/nylon, Test tube racks, stainless steel, Floating cryovial and tubes racks

9.	Collapsible boxes, PP, Jars with screw cap, PE-HD, Transport and storage containers, PE-HD, Nalgene Cryo 1°C Freezing Container
10.	Spray bottles, wide neck, LaboPlast®, PE/PP, Aspirator Bottles, wide mouth, PE-HD, narrow neck, PE-HD, with stopcock
11.	Laboratory trays LaboPlast®, PP, Laboratory spoon, stainless steel, Reagent spoons, double-ended, 18/10 steel.
12.	Spatulas, round grooved, 18/10 steel, Spoon spatulas, 18/10 steel, deep form, Measuring scoops, PP, Scoops, stainless steel, Square weighing boats, antistatic, PS
13.	Forceps, 18/10 steel, Forceps, curved end, stainless steel, Cover glass forceps, stainless 18/10 steel
14.	Pipette tips, racked in TipBox, Pipette tips refill TipStack™, sterile, BIO-CERT®, Pipetman DIAMOND® tips – Towerpack™
15.	Pipettes, Pasteur, plastic, Manual repetitive pipette, Petri dishes, Bottle-top dispenser, Circular vinyl-coated Lab ring, Funnels, PP
16.	Magnetic stirring bar, octagonal, PTFE, Magnetic Stirring Bar Retrievers, PTFE-coated
17.	Needles and waste containers, Biohazard Disposal Bags, PP, red, Holder for waste sacks, Lab. trolleys, stainless steel, Pressure atomizer LaboPlast®, PE-HD, Dewar carrying flasks, cylindrical, for CO2 and LN2, Stopcocks LaboPlast®, Angled stopcocks, PE-HD
18.	Filter discs, Filter paper, Absorbent disposable paper tissue, Quantitative filter paper, circles, Syringe filters, disposable, Chromatography paper/Ion exchange papers
19.	Parafilm® M sealing film, Adhesive label tape Write-on™, writable, Autoclavable adhesive Tape, Cryogenic boxes, slip lid with adaptable height, PP, Plate sealers (R&D Systems, Catalog # DY992).
20.	laboratory coats, Safety spectacles, Surgical face masks, Infrared Thermometers
21.	Latex gloves "Select Blue", powder free, Latex medical examination gloves "Comfort", powder free, Latex medical examination gloves "Comfort", powder free, Disposable nitrile gloves, powder free, Cryogenic Gloves water proof
22.	Lab Notebook, Lab markers, 1.0 mm point, Permanent markers, edding 404, 0.75mm, CD/DVD/BD markers, edding 8400, 0.5mm to 1mm, grease pen (Dako-pen) pencil, cover slip, Batteries Energizer, Label printer BMP™71, Labels for label printers BMP™71, Dual short period timer WB 388 TR 118 OS, Paper-cutting scissors, stainless steel.

Table 4: Lab consumables used in this study.

2.1.4 Nucleic Acids

2.1.4.1 Primers

Primers specific for target genes were list in **Table (5).**

Gene		Sequence (5'->3')	Amplicon Size (bp)
S100 A8	F	TTC TGT TTT TCA GGT GGG GC	190
	R	TCT GCA CCC TTT TTC CTG ATA TAC T	
TLR4	F	CAT CCC TGG GTG TGT TTC CA	670
	R	ACC ACA CTT ACA TGT AGC ACG	
TLR4-EXON	F	GAT AGC GAG CCA CGC ATT CA	167
	R	TTA GGA ACC ACC TCC ACG CAG	
TNF-α	F	TGA AAG CAT GAT CCG GGA CG	287
	R	CAG CTT GAG GGT TTG CTA CAA C	
CXCL2	F	TAA AAG GGG TTC GCC GTT CTC	346
	R	CCA TTC TTG AGT GTG GCT ATG AC	
IL-6	F	GAT GGC TGA AAA AGA TGG ATG C	230
	R	TGG TTG GGT CAG GGG TGG TT	
IL-1β	F	CAA CAG GCT GCT CTG GGA TT	175
	R	GTC CTG GAA GAA GCA CTT CAT	
IL-8	F	TCT GTG TGA AGG TGC AGT TTT G	309
	R	ATT TGC TTG AAG TTT CAC TGG CA	
TRPM4	F	CTG AAT GAC CGG CCT GAG TT	267
	R	AGA GCA GAT ACA TCT CGG CG	
TRPM5	F	GAC CAG AAG GTC GTC ACC TG	321
	R	TTC CCA GCC ATC TAA ACC ACC	
TRPV1	F	CAG TCC GGG AAA CAC TTC AGT TCT A	490
	R	CTG GGA CAG CAG CCT GG	
TRPV4	F	GAT TCA GGA AGC GCG GAT CTC	478
	R	GCG GCT GCT TCT CTA TGA TCT	
hBD2	F	ACC AGG GAC CAG GAC CTT TAT	190
	R	GCT CCA CTC TTA AGG CAG GT	
hBD1	F	GCC TCA GGT GGT AAC TTT CTC A	180
	R	GCG TCA TTT CTT CTG GTC ACT	
mBD1	F	ACA TCT GCC TGG TCC TGA GT	138
	R	CAG GAA GCC TGT GTA CCG TG	
mBD4	F	GAG CCA TAT GCT GGG GTC C	144
	R	ATG GAG GAG CAA ATT CTG GCA A	
House Keeping gene			
HPRT1	F	CCT GGC GTC GTG ATT AGT GA	505
	R	ATC CAA CAC TTC GTG GGG TC	
hGAPDH	F	GAT CAT CAG CAA TGC CTC CT	97
	R	TGT GGT CAT GAG TCC TTC CA	
hbAct	F	AAG ATC AAG ATC ATT GCT CCT CCT G	428
	R	TGT AAC AAC GCA TCT CAT ATT TGG AA	
mBact	F	AGA TCA AGA TCA TTG CTC CTC CTG AGC G	176
	R	AAA CGC AGC TCA GTA ACA GTC CGC	

Table 5: The used primers in this study.

2.1.4.2 Short hairpin RNA (shRNA) plasmid

The gene expression of TRPM4 was inhibited by the use of pre-designed and validated shRNA Plasmids (Mission RNAi, Merck KGaA, Darmstadt, Germany). The constructs were designed and validated by the RNAi Consortium (TRC, Broad Institute).

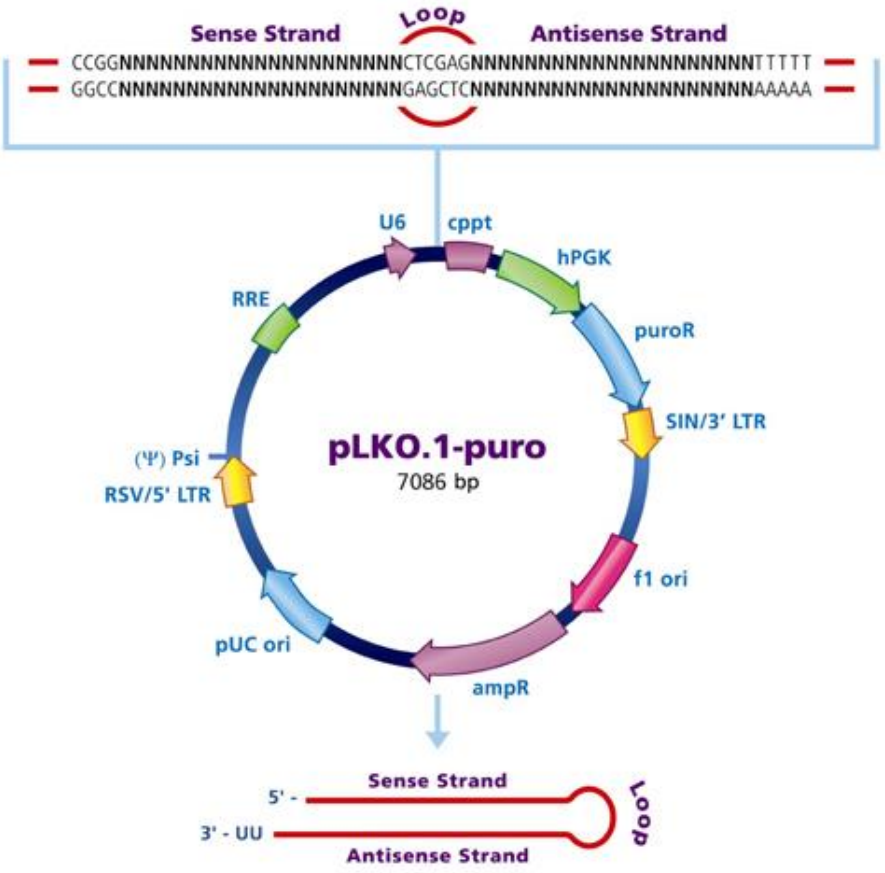

Fig. 4: Plasmid map of pLKO.1-puro plasmid.

The shape on top view shows the general structure of the shRNA-construct, that hybridizes to the shRNA-structure after transcription (bottom design). Image by **Merck KGaA**.

The following shRNA constructs were used listed in **Table (6).**

Plasmid	Abbreviation	Function	Sequence	Target
TRCN0000044923	siRNA1	TRPM4-shRNA	GCTGCTCTATTTCTGGGCTTT	Exon 17
TRCN0000437533	siRNA2	TRPM4-shRNA	GTTCGTGCGCTTGCTCATTTC	Exon 11
SHC016	ctr-siRNA	non-target shRNA		

Table 6: The used shRNA in this study.

The specificity of the small interfering RNA (siRNA) sequences was selected to cover different exons of human TRPM4. To visualize their specificity, the RNA-sequences were pasted to the webpage "splice center, siRNA Check" at the National Cancer Institute, Genomics and Bioinformatics Group **(**http://projects.insilico.us/SpliceCenter/siRNACheck**, (M. C. Ryan et al., 2008))**. The sequence of siRNA 1 hybridizes on exon 17, near the ion transporter domain (red rectangle in **Fig.5**), while siRNA2 is binding to the n-terminal part of exon 11.

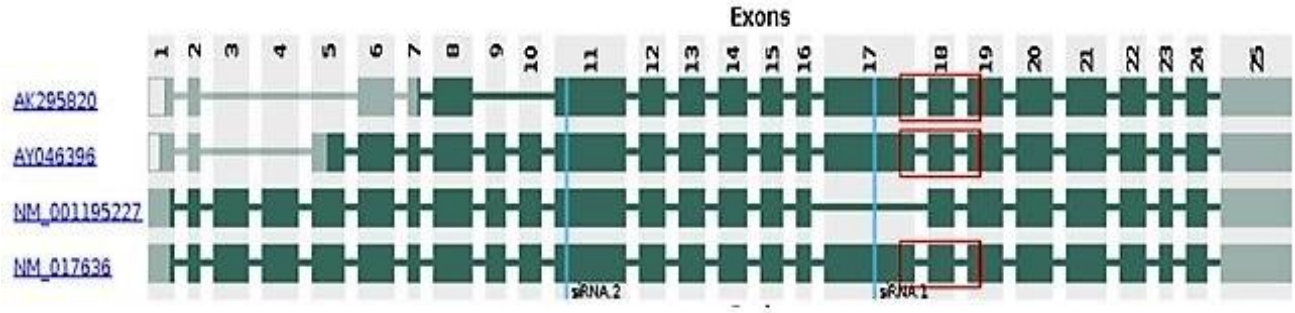

Fig. 5: Schemata of the proposed binding sites of the selected siRNAs on the genomic DNA of human TRPM4.

Image by Splice Center **(M. C. Ryan et al., 2008)**.

2.1.5. Antibodies and peptides

2.1.5.1 Primary antibody

Primary antibodies are summarized in **Table (7).**

No.	Antibody	Company	Article-No.	Mouse/Human	Sec. Antibody
1.	PLC ß2	Santa Cruz Biotechnology	sc-515912	**H M R**	Mouse
2.	TRPM4	Alomone Labs	ACC-044	H M R	Rabbit
3.	Actin / β-actin	Cell signaling	4967L	H M R	Rabbit
4.	Actin / β-actin	Abcam	ab8226	H M R	Mouse
5.	IL-6	R&D	MAB206	Human/Primate	Mouse
6.	NF-κB p65	Cell signaling	8242S	H M R	Rabbit
7.	p44/42 (ERK1/2)	Cell signaling	4695S	H M R	Rabbit
8.	Phospho-p44/42 (Erk1/2)	Cell signaling	4370S	H M R	Rabbit
9.	Human IL-6 PE-conjugated Antibody	R&D	IC206P	Human	----------

Table 7: Primary antibody used in this study.

2.1.5.3 Secondary antibody

Secondary antibodies used were collected in **Table (8).**

No.	Secondary Antibody	Company	Article-No.	Host	Conjugated with
1.	Anti-Mouse IgG	R&D	NL007	Donkey	NorthernLights 557 Fluorochrome
2.	Anti-Mouse IgG	N-Histofine	414131F	Goat	HRP
3.	Anti-Rabbit IgG	N-Histofine	414341F	Goat	HRP
4.	Anti-Rabbit IgG	Santa Cruz Biotechnology	sc-362270	Bovine	Alexa 555 Orange
5.	Anti-Rabbit IgG	Invitrogen	A10523	Goat	Cy5 Red
6.	Anti-Mouse IgG	Dako	P0161	Rabbit	HRP
7.	Anti-Rabbit IgG	Dako	P0448	Goat	HRP

Table 8: Peptide and reagent used in this study.

2.1.6 9-phenanthrol TRPM4 inhibitor

The 9-phenanthrol (9-Ph.) phenanthrene derivative is a recently reported TRPM4 channel inhibitor, subsequent experiments on other ion channels support its specificity toward TRPM4 channel **(Guinamard et al., 2014)**.

9-phenanthrol 's ultimate strength lies in its efficiency to discriminate between TRPM4 and TRPM5, which are otherwise closely similar **(Ullrich et al., 2005) (Guinamard et al., 2011)**. The discovery of 9-Ph. provided a valuable opportunity to differentiate between them in native preparations; because these two ion channels hold ionic currents with quite similar characters.

Primarily, 9-Ph. inhibitor was tested on the TRPM5 channel, the nearest TRPM4 family relevant, it was found that about 10^{-4} M concentration of 9-Ph. in HEK-293 cells did not influence a recombinant TRPM5 current, this is a promising indication of its high selectivity for the TRPM4 channel **(T. Grand et al., 2008)**.

Furthermore, TRPM4 current inhibition by 9-Ph. modifies a wide range of physiological processes, and has a significant impact in many pathological conditions. 9-Ph. regulates smooth muscle contraction in the bladder and cerebral arteries, affects neuronal and cardiac spontaneous function, and decreases cell death caused by lipopolysaccharide. Imminent applications 9-Ph. could be a successful prospective application in term of cardioprotective effects against ischaemia-reperfusion injuries and in lowering ischaemic stroke injuries **(Guinamard et al., 2014)**.

Results obtained from patch recordings and inside-out configuration suggest that, due to 9-Ph. hydrophobicity, the molecule is capable of crossing the membrane of the molecule, it is more probably to interact with the channel on both sides, although the specific site of interaction within the channel is not recognized **(T. Grand et al., 2008) (Seung Kyoon Woo et al., 2013)**.

2.1.7 Software, statistics and data work

The Microsoft office software package was used for texts and calculations.

Results are expressed as mean ±SEM. Statistical significance was analyzed using the T test, then "Unpaired t test" or One-way ANOVA, then One-Way Analysis of Variance and posttest "Tukey's Multiple Comparison Test" according to the number of groups to be analyzed. Statistical analysis and diagrams were created with GraphPad Prism 8 software (La Jolla, CA, USA) and statistically evaluated. A p-value $p < 0.0332$, $p < 0{,}0021$, $p < 0{,}0002$, $p < 0{,}0001$ was considered significant.

2.2 Methods

2.2.1 Molecular biological and microbiological methods

2.2.1.1 Preparation of lysate from cell culture for protein extraction

The treated cell culture plates were put on ice, supernatant media was aspirated, then the cells were twice washed with ice-cold PBS, PBS aspirated, and then 300 µl of ice-cold radioimmunoprecipitation assay buffer (RIPA) lysis buffer was applied to each well within 6 well plates, protease and phosphatase inhibitor tablets were added to prepared lysis buffer, also 1mM (1.7 milligrams) of serine protease inhibitor Phenylmethylsulfonyl fluoride (PMSF) (serine hydrolase inactivator) could be added, which was widely used for lysate preparation. Constant shaking was maintained for 30 minutes (min.) up to 1 hour (hr.) at 4°C to detach the adherent cells. By using a plastic cell scraper, the cells were scraped off and gently moved to a pre-cooled microcentrifuge tube, then centrifuged for 30 min. at 14,000 xG or maximum speed for 10 min. at 4°C. The tubes were carefully removed from the centrifuge and put on ice. The supernatant was transferred to a fresh tube kept on ice, while the pellet was discarded. For longer preservation, supernatant tubes were stored at -80°C for the future usage.

2.2.1.2 Protein concentration measurement

The BCA protein is a bicinchoninic acid (BCA) dependent technique for sensitive and optimized colorimetric detection and total protein quantification. This approach uses a special reagent containing bicinchoninic acid to detect cuprous cation (Cu^{+1}) after reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (biuret reaction). The purple-colored reaction product is obtained by the chelation of two BCA molecules with one cuprous ion. this water-soluble complex at 562 nm give a linear relationship with increasing protein concentrations over a large working range between (20-2000µg/mL).

It is reported that the macromolecular protein structure, peptide bonds number, and the existence of four unique amino acids (tyrosine, tryptophan, cystine, and cysteine) are necessary for BCA color evolution. Studies of the two, triple and quaternary peptides indicate that the intensity of color formation results from more than just the sum of the individual functional groups producing color. Protein concentrations are therefore usually calculated and monitored with regards to common protein standers, such as bovine serum albumin (BSA). Serial dilution of known protein concentration was prepared, that is processed together with unknown one. Then the concentration is easily calculated based on the standard curve.

2.2.1.2.1 Preparation of diluted albumin (BSA) standards

The components of one albumin standard (BSA) ampule was diluted in multiple dry and clean vials to prepare a set of protein standards (standard curve) as shown in **table (9),** where RIPA buffer is used as a diluent and blank for measurement correction.

Preparation of diluted protein standards were made according to the **Table (9).**

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

Table 9: diluted albumin (BSA) standards used in this study.

2.2.1.2.2 Preparation of the BCA working reagent (WR)

Working reagent was obtained by dissolving 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B), thereafter 25 µl of each standard or unknown sample was pipetted in replicate or triplicate into a microplate well, then 200µl of the WR (200:25 = 1:8) was applied to each well and the plate mixed gently for 30 second (s) on a plate shaker. The plate was subsequently sealed and incubated at 37°C for 30 min., then cooled to room temperature (RT). The absorbance was assessed at or around 562 nm on the plate reader, corrected by a blank. The mean blank standard 562 nm absorbance measurement is subtracted from all other standard or unknown sample. The standard curve was used to determine the concentration of protein in each unknown sample which was designed by plotting the average BSA standard blank-corrected measurement compared to its concentration in µg/mL. Eventually samples were diluted with Lysis buffer or PBS without inhibitor to obtain protein concentration adjusted to 10-20 µg per 20 µl.

2.2.1.3 RNA isolation

For cultured cell line cells, lysis were performed according to the used protocol by adding 350 µl lysis buffer RA1 and 3.5 µl ß-mercaptoethanol (ß-ME) to the well plate and robustly vortexed for about 30 min. in cold room. Filtration was done to lessen viscosity and clear the lysate through nucleospin violet ring filter which was placed in a collection tube (2 mL), then the mixture was applied to it, and centrifuged at 11,000 xG for 1 min. The nucleospin filter was discarded and 350 µl ethanol (70%) was applied to the homogenized lysate and mixed by pipetting up and down 5 times, or flow-through alternatively transferred into a new 1.5 mL centrifuge tube, 350 µl ethanol (70%) was added and mixed by vortexing (2x5) s. Lysate was pipetted 2-3 times up and down and loaded to the light blue ring nucleospin RNA column in collection tube. Centrifugation carried out at 11,000 xG for 30 s, then the column transferred to fresh collection tube (2 mL).

Then 350 µl MDB (Membrane Desalting Buffer) were added which work on salt removal for further efficient rDNase digestion then centrifugation occurred at 11,000 xG for 1 min. to dry the membrane, 95 µl DNase (used for DNA digestion) reaction mixture was applied directly to the center of the column's silica membrane and incubated for 15 min. at RT. Washing carried out on 3 steps as follows: in 1st step 200 µl of washing buffer RAW2 (which will inactivate the rDNase) were loaded to the nucleospin RNA column and centrifuged at 11,000 xG for 30 s. Then the column transferred to 2 ml a new collection tube. On 2nd wash, 600 µl washing buffer RA3 were loaded to the nucleospin RNA column and centrifuged at 11,000 xG for 30 s. Flow-through was discarded and the column returned back to the collection tube. On 3rd wash, 250 µl washing buffer RA3 were loaded to the nucleospin RNA column and centrifuged at 11,000 xG for 2 min. For completely drying the membrane, the column then transferred to a nuclease-free collection tube. RNA is eluted into 60 µl RNase-free H_2O and centrifuged for 1 min. at 11,000 xG. The elution can be made in 40 µl if higher RNA concentration is needed.

2.2.1.3.1 RNA concentration measurement

Concentration of RNA were assessed using Nano-drop device, 1µl of the samples dispensed onto the lower measurement pedestal ensuring the samples are laid in the lower pedestal. Then the obtained result exported to excel file and the final concentration adjusted to 1µg/10µl.

2.2.1.3.2 cDNA synthesis

The following reagents were applied to a sterile, nuclease-free tube on ice in this specified order: total RNA 5µg, random hexamer primer 1µg, water, nuclease-free 6µl, thus the total volume was 12µl.

The vial was centrifuged and incubated at 65 °C for 5 min. cooled on ice, spun down and returned back on ice. Then the following ingredient were added consecutively: 5X Reaction Buffer 4µg, RiboLock RNase Inhibitor (20 U/µl) 1µg, 10 mM dNTP Mix 2µl, RevertAid Reverse Transcriptase (RT) (200 U/µl) 1µl, thus the final volume was 20µl. Contents mixed carefully, centrifuged and incubated in thermal cycler for 60 min. at 42 °C. The reaction was then stopped through heating at 70 °C for 5 min. Finally, 180µl of nuclease free water added to each sample and stored at -80 °C for further usage.

Some control reactions are often required to validate the effects of cDNA synthesis like positive and negative control, also to test RNA sample for genomic DNA contamination, reverse transcriptase minus (RT-) negative control is essential in real-time polymerase chain reaction (RT-PCR) or quantitative polymerase chain reaction (qPCR), which includes every reagent for the reverse transcription reaction, except for the RT-enzyme. To evaluate the reagent contamination, no template negative control (NTC) is required, which includes every reagent for the reverse transcription reaction except RNA template.

2.2.1.4 Real-time polymerase chain reaction (RT-PCR) or quantitative polymerase chain reaction (qPCR)

The most sensitive and accurate tool for detecting and quantifying nucleic acids (DNA, cDNA, & RNA) levels is real-time or quantitative PCR. It is based on fluorescence detection and quantification in real time emitted from a reporter molecule. This detection was reported at each amplification cycle through the accumulation of the PCR product, thus allowing the PCR reaction to be tracked during the early & exponential phase, where the first significant growing in the PCR product is associated with the original target template amount.

The SensiMix™ SYBR®& Fluorescein Kit is a high-performance product laid out for outstanding specificity and sensitivity on real-time PCR with optional use of a passive fluorescein reference signal. It is inactivated and has no polymerase activity during the initialization of the reaction, which avoids non-specific amplifications, including the formation of primary dimers.

SensiMix™ SYBR®& Fluorescein Kit is supplied as a 2x master mix including; SYBR Green I dye, dNTPs, stabilizers and enhancers. SYBR I could not bind to single-strand DNA. But it has the ability to bind with double-strand DNA. Upon binding to double-strand DNA it emits very brightly fluorescence. The SYBR I signal strength correlates with the amplified DNA and thus the initial sample input quantities.

The following components are added in semi-skirted 96-well PCR plate in the following order, 25 µl of 2x SensiMix™ SYBR®& Fluorescein, 0.5 µl 25 µM forward primer, 0.5 µl 25 µM reverse primer, H_2O up to 45 µl, and 5 µl cDNA template, thus the final volume was 50 µl. Then PCR programme is adjusted and run according to the protocol as illustrated in **table (10)**, then the result was exported and analyzed for gene expression using ΔΔCt quantification.

Table (10): PCR designed thermal cycler programme.

Cycles	Temperature	Time	Notes
1	95 °C	10 min	Polymerase activation
40	95 °C	15 s	Temp. depends on the Tm of
	55-60 °C	25 s	primers
	72 °C	25 s	Acquire at end of step

Table 10: The used thermal cycler conditions in PCR.

2.2.1.5 LDH cytotoxicity assay

Lactate dehydrogenase (LDH) is a stable enzyme that is present in all forms of cells and upon damage to the plasma membrane, it is easily released into the cell culture medium. Hence, LDH is the most commonly used marker in the assessment of cytotoxicity.

To make sure of reagent effectivity and also ensure and validate the result, some controls should be included such as positive control which could be utilized to verify whether all reagents are properly working in response to active LDH enzyme by adding 5 µl of LDH solution in triplicate cultured well. Background control, in which 100 µl of culture medium without cells were added in triplicates per each well, for normalization, the obtained value from background control has to be subtracted from all other outcome. Low control was achieved by adding 100 µl of cells in triplicate wells, while for high control, 10 µl cell lysis solution were added to 100 µl of cells in triplicates well and mixed well.

The desired tissue cultured cells were collected from 75 cm^2 (250 ml), vented cap culture flask, washed twice with sterile PBS, and then seeded into a 96-well plate of 100 µl cells in triplicate approximately corresponding to 2-10x10^4 cells. Well plates were left in an incubator (5 % CO_2, 90 % humidity, 37°C) overnight for full attachment. Then tested substances were added in a different concentration to each well and mixed well. Well plates were incubated in an incubator (5 % CO_2, 90 % humidity, 37°C) for different time point 3, 6, 18 hr. At the end of the incubation, the plate was gently shaken to ensure that LDH in the culture medium is distributed uniformly and centrifuged at 600 xG for 10 min. to precipitate the cells. 10 µl from each transparent medium solution was transferred to new 96-well plate. Then LDH reaction mix was prepared by mixing WST substrate mix with LDH assay buffer. 100 µl of fresh prepared LDH reaction mix was applied to each well, thoroughly mixed and incubated for about 30 min. at RT, the reaction stopped by adding 10 µl stop solution to each well, mixed and readed using a plat reader fitted with a 450 nm (440-490 nm) filter assessed absorption for all controls and samples. The reference wavelength was adjusted at 650 nm. Finally, the equation shown below were used for data analysis:

Cytotoxicity (%) = (Test Sample – Low Control) / (High Control – Low Control) X 100

2.2.1.6 Microbiological methods- preparing of heat inactivated *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (Ps.a.) stored in glycerol in -80°C was thawed and streaked onto previous prepared agar plates in a biosafety cabinet and incubated at 37°C for 24 hr. For additional confirmation step, another streak is done on cetrimide agar that is used for the selective isolation of *Pseudomonas aeruginosa,* cetrimide is considered as selective agent that inhibits a wide variety of microbial flora. It also enhances the production of pyocyanin and fluorescein pigment, which display a distinctive blue-green and yellow-green color. Then the two isolated colonies were picked up from agar plate and placed into 100 mL of sterilized Luria-Bertani (LB) medium and incubated in rotatory shaking position for 18 hr. at 37°C. Next day, after centrifugation at maximum speed, supernatant was discarded to obtain the pellets which were re-suspended in PBS, and bacterial suspension concentration were measured using spectrophotometer at optical density (O.D.) 600 nm

wavelength. Then the suspension was reconstituted and measured again till reach 1.0 O.D. value which approximately corresponded to 2.04×10^8 colony-forming unit (CFU)/ml **(D. J. Kim et al., 2012)**. then suspension was aliquoted in 500 µl Eppendorf tubes. Heat inactivation was carried out using heating block at 75°C for 30 min. and finally stored in -80°C for longer preservation.

2.2.2 Cell culture methods

2.2.2.1 Cell lines and cell culture

In general, deep freeze cell lines were thawed and grown in 75 cm^2 (250 ml) tissue culture flasks with 25 ml appropriate medium containing 10% FBS+1% streptomycin& penicillin antibiotic till reached 80% confluent growth. Then media removed, cell lines washed twice with PBS, detached using Trypsin/EDTA, and cell number were counted using hemocytometer counting chamber. After that the cell lines were seeded in 12 well plates at a density of 4 $x10^5$ per well or 6 well plates at a density of 10 $x10^5$ per well, then incubated overnight to settle down and fully attachment in a cell culture incubator at 37°C, 5 % CO_2, and 95% humidity, after that cell lines were starved for at least 6 hr. in a suitable media containing 1% FBS, the cell lines were now ready for treatment.

The experiment was divided into 4 sets, 4 wells per each group, one group untreated cells as a control group, 2^{nd} group treated with 20 µM TRPM4 inhibitor, 3^{rd} group treated with 100 µl heat inactivated Ps.a., 4^{th} group pre-treated with 20 µM TRPM4 inhibitor for 3 or 6 hr. prior to heat inactivated Ps.a. treatment. Cells were incubated in a cell culture incubator at 37°C, 5 % CO_2, and 95% humidity for 18 hr. Then the supernatant was collected for measuring different inflammatory cytokines and chemokines using ELISA or LUMINEX, the cells were trypsinized, collected, transferred to Eppendorf tubes and stored at -80°C for further protein expression measurement and messenger RNA (mRNA) activity detection using suitable picked primers and real-time PCR procedure.

In a separate set of experiments to be used for western blot, cells were treated for maximum 4 hr. to detect the phosphorylation or nuclear translocation of different protein involved in inflammatory signaling pathway. After treatment, media removed, cells washed with PBS in 4°C, RIPA Lysis buffer containing two tablets (anti-proteases and anti-phosphorous) were added to the cells. Plates were put on plate shaker at 4°C for 30 min. up to 1 hr. Then the cells were collected with cell scraper and transferred to Eppendorf tubes. Centrifugation carried out for 30 min. at 14,000 xG or maximum speed for 10 min. then supernatant discarded and cells stored at -80°C for further usage.

Protein concentration were measured using Pierce™ BCA Protein Assay Kit. Concentration of each unknown samples is determined based on the standard curve of a series of dilutions of known prepared protein concentration as described before, and adjusted to 10-20 µg per 20 µl. SDS-PAGE and western blotting were carried out according to later described protocols.

2.2.2.1.1 NCI-H292 & U937 cell line

NCI-H292 is human cell line derived from a 32-year-old female lymph node metastasis of pulmonary mucoepidermoid carcinoma. It derived during studies of enzyme deficiencies in squamous cell cancers. In tissue culture, the cells maintain their mucoepidermoid features as recognized by their ultrastructure and expression of multiple squamous differentiation markers **(Banks-Schlegel et al., 1985) (Carney et al., 1985)**.

U937 cell lines were derived from of a 37-year-old caucasian male patient with diffuse histiocytic lymphoma. It was used to study the behavioral characteristics and differential propagation of monocytes because it is one of few cell lines which has the ability for continuous display of monocytic features. In response to a variety of soluble stimuli, U937 cell lines mature and differentiate acquiring the morphological and characteristic features of mature macrophages **(Sundström & Nilsson, 1976)**.

NCI-H292 epithelial cell lines and U937 macrophage cell lines were obtained and grown in 75 cm^2 tissue culture flasks with RPMI medium 1640(1x) + GlutaMAX™ containing 10% FBS + 1% streptomycin/penicillin antibiotic till reached 80% confluent of growth. Cell lines were seeded in 12 well plates at a density of 4 $x10^5$ per well, then incubated overnight in a cell culture incubator at 37°C, 5 % CO_2, and 95% humidity, after that cell lines were starved for at least 6 hr. in RPMI medium 1640(1x) + GlutaMAX™ containing 1% FBS, the cell lines were now ready for treatment.

2.2.2.2 Isolation and culture of human bronchial primary epithelial cell

Human primary bronchial epithelial cells were isolated from small airways obtained during surgical lung resections. The samples were collected freshly after obtaining the patients informed consent. After surgery and evaluation by a pathologist only healthy tissue samples were selected for the isolation of cells. The cells were isolated by enzymatic digestion of small airways as described by **(Bals et al., 2004)**. Briefly, small airways were separated from connective tissue by mechanical dissection. The small airways were incubated over night at 4°C in DMEM/Hams F12 medium (Gibco, ThermoFisher Scientific, Waltham, MA, USA), including 0.1% Pronase-E (Sigma Aldrich, St. Louis, MI, USA) and 0.1% DNAse (Sigma Aldrich, St. Louis, MI, USA). On the next day the cells were separated from the surrounding tissue by mechanical detachment, after centrifugation the cells were re-suspended in complete growth medium for adherent growth. Complete growth medium consists of airway epithelial cell growth medium, containing growth factor supplements (both Promocell, Heidelberg, Germany), 1% penicillin-streptomycin (Gibco, ThermoFisher Scientific, Waltham, MA, USA), 1 µM A83-01 (TGF-b inhibitor, Tocris Bioscience, GB), 0.2 µM DMH-1 (BMP4 inhibitor, Tocris Bioscience, GB), and 5 µM Y27632 (ROCK inhibitor, Tocris Bioscience, GB). Cells were cultured in a cell culture incubator at 37°C, 5 % CO_2, and 95% humidity. After 3 to 4 days, floating cells were discarded and the medium changed thereafter every two days until reached confluent growth. After that media removed and cells washed with PBS, trypsinized, counted and transferred to 12-well plates with a

DMEM/Hams F12 medium contain 1% Ultroser G, serum substitute for animal cell culture, (Pall Life Science, Cytogen, Greven, Germany). The cells were now ready for treatment with different reagent and bacteria.

2.2.2.3 Isolation and culture of tracheal primary epithelial cells from mice

On first day, Germ-free mice (specific pathogen-free (SPF)) were killed by cervical dislocation or gassing with isoflurane. Tracheae were cleaned from surrounded muscles and organs (thyroid, esophagus, etc.), dissected, cuted between the larynx and bifurcation, and mounted on scissor tip. The rests of surrounding tissues were removed with tweezers and the tracheae were cuted lengthwise. Tracheae were washed in sterile phosphate buffered saline (PBS) and put into ice-cold Ham's F12 (penicillin+ streptomycin+ nystatin) medium. From this point, all further steps were carried out under sterile bank. Every 2 tracheae were put in 5 ml Ham's F12 medium (penicillin+ streptomycin+ nystatin) containing 1.5 mg/ml of pronase E and incubated for 18 hr. at 4 °C. This enzyme tends to hydrolyze peptide bonds on the carboxyl side of aspartic or glutamic acid.

On second day, the digestion stopped by adding 10 % fetal calf serum (FCS). The tubes containing cell mixtures were gently inverted 12 times to disassociate the epithelial cells from tracheae. The medium was transferred into a new sterile 50 ml tube. The tracheae were washed with the same volume of Ham's F12, (penicillin+ streptomycin+ nystatin) supplied with 10 % FCS, inverted again 12 times and combined with the first part of cell suspension. Tracheae were washed one more time with 1.7 ml Ham's F12 (penicillin+ streptomycin+ nystatin) supplied with 10 % FCS, tubes also were inverted 12 times and finally tracheae were discarded. The cell suspension of washed trachea was combined together and centrifuged 5 min. at 400 xG. Supernatant were removed and the cell pellet were re-suspended in 400 µl of Ham's F12 (penicillin+ streptomycin+ nystatin) containing 0.5 mg/ml DNase (The DNase degrade the DNA released during the cell lysis) and 10 mg/ml BSA**.** the mixture was incubated on ice for 5 min. and centrifuged at 400 xG for 5 min., supernatant removed, the cell pellets were resuspended in 5 ml of airway epithelial cell culture medium (PromoCell) containing 10 % of FCS, the cell suspension transferred into a petri dish and incubated for 3 hr. at 37°C & 5 % CO_2. The non-epithelial cells will adhere to the dish surface more rapidly. The supernatant contained epithelial cells was removed carefully and centrifuged for 5 min. at 400 xG. Then fresh medium was added to the pellet, cell counted. The cells were now ready to be applied for airway-liquid interface (ALI) culture system and subsequent reagent and bacterial treatment.

2.2.2.4 Air Liquid interface culture of human bronchial & mice tracheal epithelial cells

Differentiated epithelial-like structures were obtained by cultivation of primary airway epithelial cells at an air-liquid-interface. Cells from a cell culture flask at a passage number (n<10) were trypsinized and transferred to 12-well plates equipped with transwell cell culture inserts (Corning Inc., Lowell, MA, USA) with a polyester membrane with 0,4 µm pore size. The cells were seeded at a density of $2x10^5$ cells/well in complete airway epithelial cell growth medium. After reaching confluency, the medium in the upper compartment was removed. The medium in the lower compartment was exchanged to differentiation medium, which consists of 1:1 DMEM/F12 (Gibco, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 2 % Ultroser-G serum substitute (Pall Life Science, Cytogen, Greven, Germany). The medium was exchanged every two day. Cell differentiation in this cell culture model correlates with the development of an increased electrical resistance across the cells. To evaluate cellular differentiation, the trans-epithelial resistance (TER) was measured after every medium exchange with an epithelial voltohmmeter (EVOM1, World-Precision-Instruments, Sarasota, FL, USA). Primary bronchial epithelial cells are considered to be differentiated after reaching a TER of more than 1000 Ohm/cm^2.

2.2.2.5 Air Liquid interface tissue culture infection with live *Pseudomonas aeruginosa*

Two or three colonies of sub-cultured Ps.a. are incubated in the 20-25 ml of liquid broth overnight at 37°C, 2 or 3 ml were taken from overnight culture & transferred to 20 ml fresh liquid broth. Mixed well, the optical density was measured using spectrophotometer and the dilution was adjusted to 0.3 O.D., when needed few drops of overnight culture added to reach the desired concentration. The suspension was incubated for 1-2 hr. at 37 °C, for growth stimulation & enhancement, and the O.D. was re-measured at regular time interval till reach the 1 O.D. concentration; then was diluted using sterile PBS to 1:10 000 dilution or 1:100 (2X), thus the bacterial suspension are ready for treatment. The confluent air liquid interphase tissue culture cell line was treated from apical side with 15 µl per each well (equal to 10^4 CFU/ml), and incubated for 6 hr. at 37°C. The cells were flushed apically using 85 µl PBS, the bacterial flushing suspension solution was collected and loaded to small Eppendorf tubes, then was cultured on agar plate for counting (serial dilution 1:10 or 1:100 fold was made till 1:100 000 dilution & cultured on two agar plates). Down compartment filtrate can be used also for measuring different inflammatory cytokines using ELISA technique.

2.2.2.6 Isolation and differentiation of bone-marrow-derived macrophages (BMDMs).

Wild type (WT) mice, 6-8-week-old were used to collect bone marrow cells by separating and flushing tibias and femurs with DMEM, GlutaMAX™ containing 10 % heat-inactivated FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin. Flushing solution was transferred to 15 ml falcon tube, and then centrifuged at 1500 rpm for 5 min. The supernatant was discarded and the pellets were dissolved in 3 ml DMEM media. BD Pharm Lyse was used as an optional choice to dissolve red blood cells. 2 ml of fresh media was added to 3 ml pellets and mixed by pipetting up and down 2-3 times and loaded drop by drop to the wall of falcon tube

containing 3 ml Ficoll-Paque, then centrifuged at room temperature for 30 min. It is necessary to avoid vigorous movement and refrained from centrifugation brake usage. The upper layer was removed till reaching mononuclear cells buffy ring layer. mononuclear cells were loaded to new falcon tube, re-suspended in 5 ml DMEM containing 10 % heat-inactivated FBS, 50 U/ml penicillin, 50 mg/ml streptomycin, beside 20 ng/ml GM-CSF and 20 ng/ml M-CSF (special growth factor for propagation and differentiation of monocytes). Then the cell was counted, seeded into 5 ml small plastic flask at a density relative to $27x10^6$ cells and incubated in a humidified environment containing 5 % CO_2 at 37 ºC overnight. On next day, firmly adherent cells were discarded and non-or weakly-adherent cells were recovered, transferred to 15 ml falcon tube, centrifuged, the supernatant was discarded and the pellets were re-suspended in 75ml culture flask containing fresh media and then allowed to culture under the same conditions to give it a chance for propagation and differentiation. After 2 days, the culture medium was changed and adherent BMDMs were gently washed with cold PBS. After 5 days of seeding, cells were harvested by limited time exposure to trypsin-EDTA. The 75ml culture flask was then scratched using cell scraper in one direction and the cells were collected, washed; Consequently, the cells were ready to be used for further experiment. The percentage of the monocyte cell surface marker $CD68^+$ and $F4/80^+$ was examined using FACS (fluorescence-activated cell sorting) to investigate the purity of the cells.

2.2.2.7 Transfection using shRNA

NCI-H292 cell line were passaged 24 hr. before electroporation process in 50 ml tissue culture flasks with RPMI medium 1640 (1x) + GlutaMAX™ containing 10% FBS + 1% streptomycin & penicillin antibiotic till reaching 80% confluent growth at the time of electroporation. Starvation carried out overnight with RPMI medium 1640 (1x) + GlutaMAX™ containing 1% FBS. Then the cell is now ready for treatment. Ingenio® electroporation solution, Trypsin-EDTA and growth medium were warmed to room temperature. Cell lines were harvested and counted, in addition the cell density was adjusted to $5x10^6$ cells/ml. Cell volume equivalent to desired density were pipetted in clean tubes, and centrifuged at 300 xG for 5 min. Supernatant was aspirated, then the obtained cells were resuspended in 100 µl of Ingenio® electroporation solution to form Ingenio® cell mixture. 2 µl of TRPM4-siRNA plasmid & control siRNA plasmid as listed in **(table 6)** was added to Ingenio® cell mixture, and mixed gently but thoroughly to avoid air bubbles formation. Ingenio® cell mixture containing nucleic acid were applied to 0.2 cm cuvettes, thus the cells were ready for electroporation. The electroporation program setting was adjusted to T-020 programme with 80% efficiency and 70% resultant viable cells. 100 µl from electroporated cells were seeded into 2ml culture medium per each well of 12 well plate, and were incubated in a cell culture incubator at 37°C, 5 % CO_2, and 95% humidity for about 48-72 hr. A culture medium was changed after 24 hr. The cells were divided into 4 groups for treatment as follows: the 1st group is untreated TRPM4-siRNA plasmid electroporated cells, 2nd group is heat inactivated Ps.a. treated TRPM4-siRNA plasmid electroporated cells, 3rd group is untreated

control siRNA plasmid electroporated cells and the 4th group is the heat inactivated Ps.a. treated control siRNA plasmid electroporated cells. The cells then were incubated for 18 hr. The supernatant was collected for measuring different inflammatory cytokines and chemokines using ELISA or LUMINEX. The cells were trypsinized, collected, transferred to Eppendorf tubes and stored at -80°C for further handle.

2.2.3 Immunobiological methods

2.2.3.1 The enzyme-linked immunosorbent assay (ELISA)

Sandwich ELISAs commonly used to measure natural and recombinant different types of interleukin. It has high specificity, involves two antibodies that detects different epitopes on the same antigen. Two antibodies include; one capture and one detection, identify the same antigen, thereby forming a complex like "sandwich" structure.

The capture antibody is adsorbed onto the ELISA plate against its specific antigen. after washing and blocking the immobilized capture antibody, sample containing antigen is applied to the microplate. A detection antibody with a linked conjugated enzyme was added, which will bind to another region of the antigen present in the sample. If the detection antibody is not conjugated, another enzyme conjugated antibody is utilized to reveal the detection antibody. After washing, substratum for the enzyme is applied and followed by a short time incubation to give chance for color development, then the reaction was stopped by adding stop solution. The signal will usually be measured using a plate reader.

Methods

Capture Antibody was diluted in PBS to reach the desired working concentration. 100 µl of diluted capture antibody were instantly applied to each well of 96-well microplate. At RT, the sealed plate was incubated overnight. The plate was aspirated and washed three times with the washing buffer via filling each well with 400 µl washing buffer using a multiple dispenser. For good performance and accurate results, complete removal of liquid at every step is necessary. After the last washing step, any remaining washing buffer has been totally removed by aspirating and subsequent blotting the inverted plate against clean paper towels, then blocking carried out by applying 300 µl of reagent diluent to each well and was incubated for a minimum of 1 hr. at RT. Washing and aspiration were repeated as happened before. The plates are now ready for the sample addition. 100 µl of samples or standards were applied per each well. It could be used directly or diluted in reagent diluent to a desired concentration. Plate was covered with the adhesive strip and incubated at RT for 2 hrs. or preferably an overnight incubation at 4°C. Washing and aspiration were repeated as done in the previous steps, then 100 µl of the detection antibody diluted in reagent diluent was loaded to each well, then plate was covered with adhesive strip and incubated at RT for 2 hrs. Washing and aspiration were repeated as done in the previous steps. 100 µl of streptavidin-HRP working dilution was applied per each well. The plate was then sealed and incubated away from direct light exposure at RT for 20

min. washing & aspiration was repeated as done in the previous steps, 100 µl of substrate solution was applied and incubated away from direct light at room temperature for 20 min. or until the color converted to dim to avoid precipitation or crystal formation, 50 µl of stop solution was applied, the plate sealed and gently shaked for 30 s to ensure full mixing. Using a microplate reader was adjusted to 450 nm and a secondary 540 nm or 570 nm, the optical density of each well was immediately measured. The readings at 540 nm or 570 nm were subtracted from the 450 nm result. this subtraction will correct any optical imperfections or deviations occurred during plate reading.

2.2.3.2 Luminex assay

Luminex Assays can be utilized to evaluate and quantify any chosen biomarkers levels in a single sample. Analyte-specific antibodies are pre-coated onto magnetic color-coded fluorophores microparticles at a specific ratio of each unique microparticle. Standards, samples and microparticles are loaded into wells and thus immobilized antibodies could easily bind to the analytes of interest. A biotinylated antibody cocktail unique to the relevant analytes was applied to each well, then streptavidin-phycoerythrin conjugate (Strep-PE) is applied to each well and thus binds to the biotinylated antibody after that microparticles were resuspended in a suitable buffer and reading was carried out using the Luminex® MAGPIX analyzer. The superparamagnetic microparticles were kept in a monolayer due to the action of the magnet. These microparticles were illuminated by two spectrally distinctive light emitting diodes (LEDs). Micro particle zone or region was defined via one laser or LED that excites the dyes within each microparticle, and the second laser or LED excites the PE to quantify the number of analytes bound to the microparticle. A charge coupled device (CCD) camera equipped a set of filters to detect the sample in each well and was able to distinguish different excitation levels. Another way of detection is through using a photomultiplier tube (PMT) and an avalanche photodiode. All fluorescence emissions from each microparticle are then analyzed to distinguish emission levels.

Methods

As stated in the protocol, all reagents, standards, and samples were prepared according to used protocol. 50 µl of standard or sample were applied to each well, then 50 µl of the microparticle cocktail were loaded to microplate. The plate is then tightly sealed with a foil sealer, incubated in a horizontal orbital microplate shaker and placed at 800 ± 50 revolutions per minute (rpm) for 2 hr. at RT. Washing is achieved by applying the magnet to the microplate bottom, allowing to stand for 1 min. before liquid removal, filling each well with a 100 µl wash buffer and again allowing to stand for 1 min. before liquid removal. Washing is preferably done on three times. For favorable performance and good results, complete removal of liquid is mandatory but without plate blotting which may cause loss of microparticles. Thereafter, 50 µl of diluted biotin-antibody cocktail was applied to each well, then the plate was tightly sealed with a foil plate sealer and

incubated on a shaker adjusted at 800 ± 50 rpm for 1 hr. at RT. After washing away any unbound substances, another wash was carried out to make sure of any unbound biotinylated antibody elimination, then 50 µl of diluted Strept-PE was applied to each well. The plate was also tightly sealed with a foil plate sealer and incubated on a shaker adjusted at 800 ± 50 rpm for 30 min. at RT. Final washes were performed to remove any unbound Strep-PE, then 100 µl of washing buffer was loaded to each well for microparticles re-suspension and incubated for 2 min. on the shaker set at 800 ± 50 rpm. It is preferable to do microparticles re-suspension directly before reading step. Reading is conducted by using a Luminex® analyzer within 90 min.

2.2.3.3 Fluorescence-activated cell sorting (FACS)

Cytometry is a technology through which cells pass through a light beam in a fluid stream that simultaneously calculates and analyzes several physical properties of single particles using an optical-to-electronic coupling system that tracks how the cell or particle emits fluorescence and scatters incident laser light. These properties include the relative size of a particle, granularity or internal complexity, and relative intensity of fluorescence.

Three major structures are composed of a flow cytometer: fluidics, optics, and electronics. The fluidic system conveys particles into a laser beam stream for interrogation. The optics device consists of lasers for particles illumination and optical filters to guide the emitted light signals to a convenient detector. The light signals were transformed into electronic signals via the electronic system, that subsequently the computer can easily interpret it. Some devices are equipped with a sorting feature, in which the electronic system has the ability to initiate sorting decisions via charge and deflect the desired or needed particles.

In the flow cytometer, the sample core is the section of the fluid stream where particles are positioned, gathered and passed through the laser beam. When particles pass through the laser intercept, they scatter laser light and trigger fluorescence of any fluorescent molecules present on the particle. Through properly positioned lenses, the scattered and fluorescent light is captured by a mixture of splitters and filters arranged in a specific configuration and directed to the proper detectors, which create electronic signals that are proportional to the optical signals hitting them. Adequate analysis occurred to any particle has size ranged from 0.2–150 micrometers. However, segregation is necessary for solid tissue cell before initiating analysis.

Complete data are collected about each event or particle. Each event’s features or characteristics are dependent on its light scattering and fluorescent properties which could provide information about subpopulations within the sample. All these gathered data were saved and processed on the server.

Methods

Cell line collection

In vitro, after cell line were cultured and treated as previously explained in 6 well plated, the culture medium was aspirated and the cells were rinsed twice with stain buffer with PBS. Cell was detached using 25mM ethylenediaminetetraacetic acid (EDTA) (1:20 fold from stock solution 0.5 M EDTA) and gently pipetted up and down to disperse the cells. The cells were collected and filtrated through membrane filter 40 µm mash. The cells were counted and about one million cells transferred to each flow test tube. These cells were then centrifuged at 250 xG for 10 min. at RT. All next procedures throughout staining and storage should be performed at 4°C on ice and the cells were protected from direct light exposure.

Fixation

The supernatant was aspirated and the cells were thoroughly re-suspended in 1ml of cold fixation BD cytofix buffer, incubated for 20 min. at RT, then centrifuged at 250 xG for 10 min. at RT, and washed twice with stain buffer with PBS, centrifuged again at 250 xG for 10 min at RT. Cells could be stored in stain buffer at 4°C up to 72 hr. Repetitive centrifugation and aspiration processes may loosen the cells, so special care should be taken when aspirating wash buffer from the tube by not aspirating all buffer, about 100-150 µl of buffer left in the tube to avoid cell loose for all subsequent washing steps.

Permeabilization

Cells in stain buffer were centrifuged at 250 xG for 10 min. at RT, and stain buffer was aspirated. The cells were re-suspended in 1x BD Per/wash buffer and incubated for 15 min. at RT, then centrifuged at 250 xG for 10 min. at RT and supernatant was aspirated.

Blocking

5 µl of human TruStain FcX was added per million cells in 100 µl staining buffer, mixed and incubated for 10 min. at RT. It is not necessary to wash the cells after blocking step. This FcX blocking step is beneficial to avoid false positive or negative result; due to Fc receptor mediated Ig Fc binding.

Preparing of untreated and unstained control

50 µl of stain buffer was added to unstained control cell (cells without staining), and also to untreated cell (cell without treatment) which will be used as a reference control in flow cytometric analysis.

Antibody addition

10 µl of human IL-6 PE-conjugated antibody were added to all tubes except for unstained control, incubated for 30 min. at RT, then centrifuged at 250 xG for 10 min. at RT and supernatant was aspirated. Cells were re-suspended in 200 µl stain buffer, kept on ice away from light prior to FACS analysis.

Flow cytometric analysis

The CS&T beads were prepared for device performance check by pipetting 350 µl of FACS flow solution in a tube, and one drop of the CS&T beads was added and gently vortexed at about 1000 RPM before being loaded on sample injection tube (SIT). After finishing CS&T beads calibration, the system was connected to facsDiva software, where new folder could be created for a new experiment. The desired parameter

(fluorochromes) was chosen to be measured in the “Cytometer” window and the non-required items were deleted to save disk space. Dot plots, histograms and contour plots were created by using the buttons at the top of the “Worksheet” window. “Statistics view” and “Population hierarchy” can be created by right-clicking on a dot plot. Sample and control tube were vortexed then applied to the sample injection tube (SIT) one by one. Then “acquisition dashboard” was used for acquiring and recording data. Before recording samples “acquire data” button was clicked, and either after 2 or 3 s or as soon as the first dots appear in the dot plot, “record data” was clicked. The voltage of the forward scatter (FSC) and side scatter (SSC) was adjusted while acquiring samples and before recording started and the selected value were kept the same during all further measurement. The “threshold” was defined in the “cytometer” window. Different gate-buttons in the “worksheet” toolbar were used to define populations and subpopulations in the dot plots. Interval gates are the only ones that can be used in histograms. Afterwards the cytometer performance report could be viewed, stored and exported.

2.2.3.4 Western Blotting

Western blotting is a technology that enables researchers to identify modifications of specific concerned protein, such as phosphorylation, concentration changes, conformational changes, insertion or the elimination of minor chemical changes and alteration in interaction with other proteins.

In this technique, proteins are initially separated from the rest of the cell's components via chemical and/or physical methods; in order to disrupt the cell's plasma membrane and release its proteins component.

Chemical-based lysis employs a variety of detergents to allow proteins easily accessible. Weak detergents, for example, are used within the cell to extract water-soluble proteins without disrupting intracellular membrane compartments. While strong detergents could solubilize the proteins present in all membranes.

The properly regulated cellular environment is disrupted by cell lysis. This happens by providing certain cellular enzymes unrestricted access to their targets, this may result in undesired protein modifications, unfolding, and/or degradation. So, lysis is carried out at a low temperature in the presence of enzymatic inhibitors to avoid any presumably proteins alteration.

After lysis step, all cellular contents are spun in a microcentrifuge. This yields an aqueous supernatant containing solubilized proteins, as well as a pellet containing membranes, organelles, nucleic acids, and any remaining insoluble proteins. Supernatant is separated from the pellet, which is now referred to as protein lysate that used for western blotting.

A "whole cell lysate" is the most common form of protein lysate, since it includes all of the proteins found inside the cell. Nuclear and cytoplasmic lysates considered also as a two other usually prepared forms of lysates, that are often used to monitor and quantify protein localization to these two compartments. A third

mostly used lysate type is called immunoprecipitated or (IP), in which proteins that may interfere with a protein of interest are identified and recognized. In this manner, the researcher isolates not only the protein of interest but also other proteins which it interacts physically with it by using specific primary antibody, followed by secondary beads conjugated antibody; in order to precipitate the protein-antibody complex out of the lysate solution.

Western blotting consists of two main phases: In the first phase, proteins are loaded onto a gel and separated from one another using an electrical current during "polyacrylamide gel electrophoresis" or PAGE. In the second phase, the separated proteins are transferred onto a membrane and the protein of interest is detected using a labelled specific antibody and visualized using photographic or other imaging techniques.

Methods

Sample preparation for electrophoresis

During sample preparation, a Roti®-Load1 buffer were used, which is a special gel loading buffer for protein gel electrophoresis. This buffer system already contains denaturing and reducing reagents which are engineered to protect protein from degradation and to stabilize peptide bonds responsive. Furthermore, it contains a dye and glycerol as density increasing reagent. Roti®-Load1 buffer was added to the sample (one-third of sample volume) for example: 60 µl sample + 20 µl Roti®-Load1 (4x-concentrated) and mixed well by pipetting. Then the samples were heated for 3-5 min. at 80-95 °C. to unfold protein. Roti®-Load1 prevents degradation of proteins while heating, then samples were centrifuged in high speed pulse, after that it become ready to be loaded onto the gel.

Polyacrylamide gel electrophoresis or PAGE

The gel-cassette was taken out from gel-pouch and rinsed with distilled water, the well was marked with a marker. The tape strip was removed from the lower portion of the cassette. Gel-cassette then fixed in electrophoresis cell with side clips, wells side should be positioned towards up and stripped tap side towards outside. Then the running buffer were added to upper compartment to check for fluid leakage. After that the comb removed with in a quick and vertical manner. Using a pasteur-pipette, the wells were flushed by gently filled up wells completely with washed running-buffer, it is necessary to avoid air-bubbles during flushing process. Samples were loaded (20-25 µl) per each well & 5-7 µl of SeeBlue®Plus2 (pre-stained protein standard protein marker) was applied. samples were added to the bottom of each well, it is recommended to avoid swirls during sample adding. Then running-buffer filled electrophoresis cell, on both sides of cathode and node, then cell closed and cables connected to the power supply. The running program adjusted on two steps: the first step for 15 min. at 80 Volt (for stacking gel) and the second one for 120 min. at 100 Volt (for running gel). The time and voltage may require optimization: depending on types of used gel as illustrated in **table (11)** and also relying on our observation, time could be increased to 130 min. for more protein separation, after runtime was elapsed, instrument was switched off, power cables were disconnected and the

cassette was carefully taken out. A gel-knife used to open the cassette, and strong spatula utilized to crack in-between the front and posterior cassette plate. then the cassette positioned horizontally on the table and front plate removed. The lower portion of the gel and also comb-wells were cut using gel-knife, then carefully take one edge of the gel with spatula and shake the plate slightly to free the gel from the cassette.

Table (11): shows the voltage and time required for different gels.

Gel Type	Voltage (V)	Current (mA)	Duration (min.)
Tris-Glycine, SDS	125	60	90
Tris-Tricine, SDS	125	100	70-90

Table 11: Gels and conditions used in this study.

Immunoblotting (Transfer the protein from the gel to the membrane)

Protein band on the gel should be transferred to nitrocellulose membrane (NC) or polyvinylidene fluoride membrane (PVDF). Prior to blotting the membrane, it is preferable to be placed in methanol for 5 min. for its activation and rinsed with transfer buffer before usage. A sandwich blotting was carried out in a stacking manner from negative to positive current. The set were arranged in the following order; black sponge-2x filter pad-Gel-NC membrane-2x filter pad-black sponge, then all of them were pressed together by a support grid, the black side of the support grid's was positioned downward. The sandwiched gel is positioned vertically in a transfer tank between stainless steel/platinum wire electrodes. Transfer apparatus was filled with 1x blotting buffer and ice freezer block was added to counteract the heating released from generated current or the whole apparatus were placed in a cold room during blotting process. Protein transferred is carried out at 20 Watt (60-80 V under 300 mA) for 1 hr. The membrane was then removed carefully and blocked using 5% milk powder in tris-buffered saline with 0.1% tween® 20 detergent (TBST) or 5% FBS in TBST for 1 hr. at RT or 4°C overnight, then the membrane was washed 3 times with TBST for 5 min. for each wash.

Antibody staining

Primary antibody was added in 50 ml falcon tube containing (5 ml blocking buffer + 2.5 or 5 µl primary antibody according to its working concentration) and incubated for 2-3 hr. in RT or 4°C overnight in shaking or rotating position, then the membrane was washed 3 times with TBST for 5 min. for each wash. Horseradish peroxidase (HRP)-conjugated secondary antibody was added in 50 ml falcon tube containing (5 ml blocking buffer + 2.5 or 5 µl primary antibody according to its working concentration) and incubated for 1 hr. at RT in rotating position, then the membrane was washed 3 times with TBST for 5 min. for each wash.

Visualization

The substrate working solution (Pierce™ ECL Western Blotting Substrate) is an extraordinarily sensitive nonradioactive, it enables the detection of picogram amounts of antigen through boosting luminol-dependent chemiluminescent substrate for disclosure of HRP on immunoblots using photographic or other imaging

methods. For best results, working solution was prepared immediately before use; which remains stable for 1 hr. at RT.

The substrate was prepared by mixing equal parts of detection reagents 1 and 2 (1ml+1ml). Blotted membrane was incubated with working solution for 5 min. at RT. After incubation, the membrane taken out, the excess liquid was dried up using an absorbent tissue and then placed in a plastic sheet protector or clear plastic wrap. After that any bubbles formed between the blot and the membrane protector was carefully pressed out. Sheet protected membrane with the protein side facing up were placed in a film cassette and fixed with adhesive tape, then all lights were switched off, excluding those necessary for X-ray film viewing (e.g., a red safelight). X-ray film was carefully placed on top of the membrane. To achieve the optimal desired outcomes, the exposure time was verified to different time point. The emission of light is quite intense within the first 5-30 min. after incubation of the substrate. The emission of light persists for several hours, but gradually declines with time. As time passes, longer exposure duration could be crucial to obtain a valuable result. Film was created with adequate developing and fixative solution, and was finally rinsed with water. The film was then scanned and saved as an image. If the signal is too intense, the exposure time was reduced or the blot was stripped and re-probed with reduced concentrations of antibodies.

Stripping

Blotted membrane can be preserved at 4°C in PBS or tris-HCl buffered saline (TBS) before the stripping process be continued. The membrane was washed with stripping buffer (Restore Western Blot Stripping Buffer, Thermo Scientific) 2 times for 15 min. then washed with PBS 2 times for 10 min. and with TBST 2 times for 5 min. sequentially. Then to make sure of complete removal of the immunodetection reagents some test is required, such as HRP label removal test (i.e., secondary antibody removal), in which the membrane was incubated with new working solution and exposed to film. The successful HRP conjugate removal from the antigen or primary antibody correlated with no signal detection after a 5 min. exposure. Another test for complete removal of the primary antibody is carried out, in which the membrane was incubated with the HRP-labelled secondary antibody, then washed by washing buffer and incubated in new substrate working solution and exposed to film. If there is no signal detected, thus the primary antibody has been successfully removed from the antigen, if the signal is still detected special manipulation is required for fully stripping such as longer stripping incubation time and an elevated temperature which should be optimized to ensure complete removal of antibodies, but with simultaneous protection and reduction of antigen damage that should be taken into consideration. Then the membrane was blocked using 5% milk powder in TBST or 5% FBS in TBST for 1 hr. at RT. or 4°C overnight. The membrane was now ready for another immunoblotting.

2.2.3.5 Immunofluorescence for adherent cells line

Coverslip preparation and coating:

Coverslip was sterilized by washing in ethanol or exposing to ultraviolet (UV) in a biological safety cabinet for at least 60 min., then coated by 50-100 µg/ml poly-lysine for about 1hr. at RT, washed 3 times by H_2O, dried completely overnight in the cell culture hood, and for full sterilization it is recommended to be exposed to UV light for at least 4 hrs.

Fixation:

The culture medium of pre-cultured cell line was aspirated and the cells were rinsed twice with PBS. The cells were then fixed on coverslip with 2-4% paraformaldehyde (PFA) for 10-20 min. at RT or with -20 °C ice-cold methanol or ice cold 1:1 methanol/ acetone for 1 to 10 min., then washed by PBS 3 times for 5 min. to each wash.

Permeabilization:

It is important to permeabilize the cells to help the antibodies to get access to intracellular located target epitopes inside the cells. After PBS aspiration, 0.05-0.25% triton X-100 (or 100 µM digitonin or 0.5% saponin) dissolved in PBS were applied to the samples and incubated for 10-15 min. at RT. then washed 3 times by PBS for 5 min. to each wash.

In other instances, permeabilization step is not necessary, for example, if the sample has been previously fixed with methanol or acetone, also, if the epitopes of primary antibodies located in the extracellular region of proteins. In permeabilization step triton X-100 seems to be the famous detergent enhancing the penetration of the antibody. However; the cell membrane can be broken or dissociated; so, it is not ideal to use it in case of membrane-associated protein staining. So digitonin or saponin could be used an alternative choice in such case.

Blocking:

PBS was aspirated and the cells were incubated with blocking buffer (1-3% BSA in PBS) for 30-60 min. at RT.

Primary antibody staining:

Blocking buffer was aspirated; the cells were incubated with the primary antibody in a recommended working dilution with dilution buffer for 1-4 hr. at RT, or overnight at 4°C. Coverslips were kept in a humid chamber to protect samples from drying out. After that the cells were washed 3 times by PBS for 5 min. to each wash.

Secondary antibody staining:

Washing buffer was aspirated; and the cells then were incubated with the secondary antibody in a recommended working dilution with dilution buffer for 30-60 min. at RT. Coverslips were kept in a humid chamber to protect samples from drying out. After that the cells were washed 3 times by PBS for 5 min. to each wash.

Counterstain staining:

The cells were incubated with Phalloidin for staining actin filaments (also known as F-actin) for about 30-60 min. then the cells were washed by PBS 3 times for 5 min. to each wash.

Mounting and DAPI Counterstaining:

Washing buffer was aspirated and excess liquid was dried up using fiber free paper or paper towel. The coverslips were mounted upside down on slides with a small drop of proLong®gold antifade reagent with DAPI. It is important to avoid bubbles formation beneath coverslips, then nail polish were used to seal it with the slide. Then the slides were left to dry for about 3 hr., or overnight incubation stored in a covered box to keep it away from light at 4°C. At that moment, slides were ready to be examined under microscope.

Checked under structured illumination microscopy (SIM)

The SIM used was a Zeiss Elyra PS1 Zeiss, Oberkochen. Cells were imaged using a 63x Plan-Apochromat with laser excitation at 488, 561, and 635 nm for each corresponding fluorophore, then higher resolution images were obtained by processing the images without background using Zen software **(Zen 2012; Carl Zeiss).**

2.2.4 Animal experiments

2.2.4.1 Mice housing & maintenance

Mouse experiments were approved by the Landesamt für Soziales, Gesundheit und Verbraucherschutz of the State of Saarland in accordance with the national guidelines for animal treatment (17/2016). The TRPM4-knockout mice, the corresponding wildtype and heterogenous littermates were kindly provided by the group of Prof. Trese Leinders-Zufalls (School of Medicine, Department of Physiology). The animals were kept under standard conditions with a 12/12 hours dark/light cycle and an access to food, water ad libitum. The mice carry a global TRPM4-knockout, that was generated by cre-lox recombination technology and that resulted in the deletion of the exon 15 and 16 of the TRPM4 gene **(Vennekens et al., 2007)**. The mice were generated on a 129/SvJ background and crossed back on a C57Bl/6N strain for more than 10 generations.

2.2.4.2 Bacterial suspension preparation

For Ps.a. suspension solution preparation, single bacterial colony aseptically picked up from previously prepared cultured plates and transferred to 5 ml LB culture medium, incubated on shaker at 37°C for 18 hr., then centrifuged at 12,000 xG for 30 s, supernatant discarded and pellet re-suspended in 1ml PBS, bacterial stock suspension diluted 1:10 in PBS and measured using spectrophotometry device at O.D. 600 to determine concentration, finally diluted in PBS to desired concentration.

2.2.4.3 Mice anesthesia, treatment and euthanasia

Techniques mainly used to induce respiratory infection include; intranasal distillation, bronchoscopy intubation and evaporation methods. we aimed to use intubation-mediated intratracheal (IMIT) technique as a bacterial suspension delivery technique, due to it ascertain reaching to lower respiratory tract and minimize the incidence of digestive system delivery. This technique ascertains over 98% of reagent delivery effectiveness into the lungs with an outstanding distribution throughout the lung. IMIT therefore provides a novel approach to the study of lower respiratory tract disease and therapeutic delivery into the lung directly **(Lawrenz et al., 2014)**.

Mice were anesthetized using 40 µl of previously prepared ketamine/xylazine anesthesia, or by placing them into closed chamber saturated with 2-3% isoflurane. After onset of sedation symptoms, 10 µl of 2% of local anesthetic lidocaine solution were applied to the throat, and allowed 5 min. before intubation to reach full effectivity.

One mouse picked up and fixed on intubation platform from his incisors using O-ring attached to Velcro strip. Using Teflon plunger syringe, 150 µl of air were draw up prior to 50 µl of prepared bacterial suspension, air helps to distribute suspension throughout the lung.

The tongue was retracted with a rolling motion by aiding of cotton-wooden applicator, with one hand, otoscope fit with an intubation specula used to visualize the glottis and keep tongue retracted, with another hand, mice was intubated using wire passed through the catheter, which goes inside mouse trachea till 10 mm in depth, then otoscope and wire were removed.

Catheter was fixed with nondominant hand, and syringe containing suspension was inserted carefully inside catheter using other hand. Then solution- air dispensed in one motion, and removed directly after injection. Mice eyes were moisture with Bepanthen eye & nasal ointment to protect conjunctiva and cornea from injuries and dryness, the mice were kept on heating pad till wake up, then returned back to cage for full recovery. For euthanize, mice were subjected to CO_2 asphyxia or injected intra-peritoneally with a suitable dose (200 µl) of anesthesia solution after 18hr. post-inoculation; the mice were then left a little enough time until anesthesia effect work.

2.2.4.4 Mice dissection

After euthanized mouse lay down, it fixed on dissection tray and chest and abdomen were sprayed with 70% alcohol. The incision in the skin was made with fine scissors about a couple of millimeters above the orifice. The incision must have proceeded from this opening up to the chin on the middle ventral side. Two lateral incisions extended toward the forelimbs and hindlimbs extremities, then the skin was detached from the underlying muscular layer and fixed to the sides, abdominal muscles must be lifted and incised up to the base of the thorax, then to transversal incisions were made. At this level to complete the abdominal cavity opening, muscular folding was pinned on the sides. The blood sample was carefully drawn from the heart, it is important to avoid making lung puncture. The needle was removed before spilling the blood in Eppendorf tubes; to avoid red blood cells (RBCs) destruction. Then the cannula was inserted down in trachea and 1 ml of flushing PBS solution was injected and withdrawn for 3 times to obtain bronchoalveolar lavage (BALF), transferred to Eppendorf tubes and placed on ice. In another instance, whole lung needed to be excised by tying thread around the trachea and then pulling it carefully out, which subsequently hanged to be inflated and preserved with 4% paraformaldehyde. It is important to be taken into consideration that 3 times lung flushing, may cause vary or destroy lung internal structure. In this case; it no longer makes sense to take the lung out for further histopathological examination.

2.2.4.5 Sample processing

2.2.4.5.1 Bronchoalveolar lavage (BALF)

After returning to the lab., BALF Eppendorf tubes were centrifuged at 300 xG for 10 min. at 4°C, supernatant transferred to new Eppendorf tubes and placed in -80°C freezer for longer preservation. It could be used for ELISA, Luminex, culture on agar plates to detect bacterial count. Re-suspension of cell pellets in 1 ml PBS make it ready for cytospin; in which 50 µl of cell suspension was added to 150 µl PBS (1:4 dilution), transferred to the funnel part of cyto-funnel apparatus which connected with slide in closure caps and was centrifuged at 600 xG for 6 min. After that slides were directly fixed using -20° C cold methanol for 10 min. for further staining. Diff Quick or Quick Giemsa stain is usually used for BALF staining. In Diff Quick staining, fixation carried out using fast green or blue in methanol for 10 min., then the slides were immersed 5 min. in solution 1 (Eosin G in phosphate buffer) to stain cytoplasm with red color, then 1 min. in

solution 2 (Thiazine dye in phosphate buffer) to stain nucleus with blue color, which was followed by water rinse and drying. In Quick Stain Giemsa, air dried film was placed in undiluted giemsa stain for 1-2 min., and placed in deionized water for 2-4 min. depending upon desired color preference, then slides were rinsed in deionized water, and left in the air to dry.

2.2.4.5.2 Blood

Blood samples in Eppendorf tubes were centrifuged at 2500 xG for 10 min. to obtain plasma for further investigation and quantification of different inflammatory cytokine. leukocytes cell counting in counting chamber is possible by adding 10 µl in 20-fold 5% diluted acetic acid for RBCs precipitation. Blood smear was made using one drop (about 15-20 µl) on the slide edge and spread it along the slide using a wider second slide for further staining. Fixation carried out using cold methanol for 10 min. Slides could be placed in rack, covered with methanol and be left to dry for later usage. Then the slides were stained with Giemsa May-Grünwald. Initially, slides were placed in May-Grünwald-Eosin stain for 5 min., then placed in working phosphate buffer pH 7.2, for 1.5 min., after that transferred into 1:20 fold diluted Giemsa solution for 15-20 min. with deionized water or buffered water at pH 7.2, then the slides were immersed in working phosphate buffer two times and rinsed carefully using deionized water. Finally slides let to air dry, after totally drying xylol aqueous mounting medium was applied for long-term storage and preservation. Before mounting medium added, a quick check could be done to look at color degree, if the stain is too dark, the slides in rack could be immersed several times in glass container with 500 µl to 1 ml of 35% HCL/EtOH (1:1) in 200 ml water, till reach to the desired degree or left in the solution for a long time to remove the stain completely and then staining process repeated again.

2.2.4.5.3 Lung tissue

The right lung is divided into four lobes and the left lung is made of only one large lobe. Left lung can be used in 1-2 ml TRIzol Reagent which is a perfect, fully prepared reagent for high-quality isolation of total RNA from a wide variety of biological samples for further RT-PCR reaction. Right lung could be homogenized in 1 ml PBS, centrifuged, and the obtained supernatant could be used for ELISA, Luminex, and culture on agar plates.

3. Results

3.1 Bronchial epithelial cells express TRPM4

The main goal of this work is to explore the contribution of TRPM4 to the innate immune response of the lung. To confirm that lung epithelial cells express TRPM4, specific primers were designed and tested alongside TRPM5, TRPV1, and TRPV4. TRPM4 belongs to the TRPM-family of ion channels; and TRPM5 is functionally and structurally a close relative to TRPM4. TRPV1 and TRPV4 had been detected in lung epithelial cells by other research labs beforehand. NCI-H292 cells were cultured and RNA was isolated as described in the Material and Methods section. After cDNA-synthesis intron-crossing specific primers for TRPM4, TRPM5, TRPV1, and TRPV4 were used in separate reactions and normalized to HPRT1 expression.

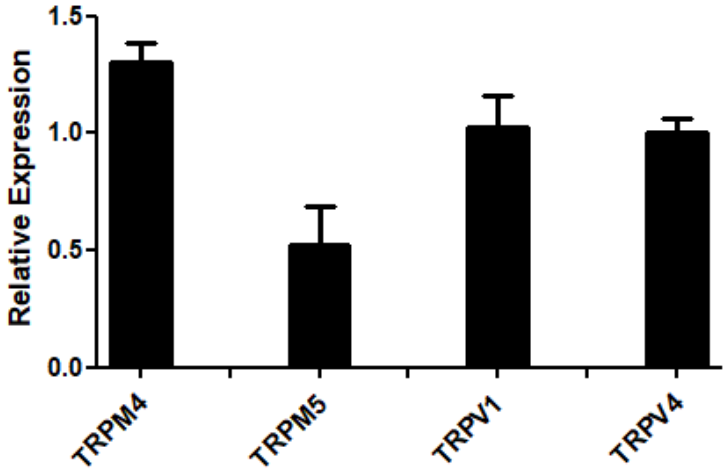

Fig. 6: Expression of different ion channels in NCI-H292 cell line.

Specific primers for TRPM4, TRPM5, TRPV1, and TRPV4 were used to detect the expression of the corresponding genes. The expression was normalized to HPRT1 as the housekeeping gene. N=3, values are depicted as mean +/- SEM.

The expression of TRPM4 was approximately 1,25-fold of the housekeeping gene, while TRPV1 and TRPV4 were expressed at similar strength as HPRT1 **(Fig.6)**. While the expression of TRPM5 was very weak **(Fig.6)**. TRPM5 has been shown to be expressed in brush cells only **(Kaske et al., 2007)**. Since NCI-H292 don't share properties with brush cells, a low expression level of TRPM5 was expected.

To confirm the expression of TRPM4 in NCI-H292 cells, the cells were grown on chamber slides and used for immunofluorescent microscopy. A blocking peptide specific for TRPM4 was used to test for specificity. The cells were fixed and permeabilized as described in the Material & Methods section and incubated with an TRPM4 specific antibody. The blocking peptide was pre-incubated together with the antibody before

applied to the cells. After incubation with DAPI-containing mounting medium and curing, the specimen was visualized by SIM (structure illumination microscopy).

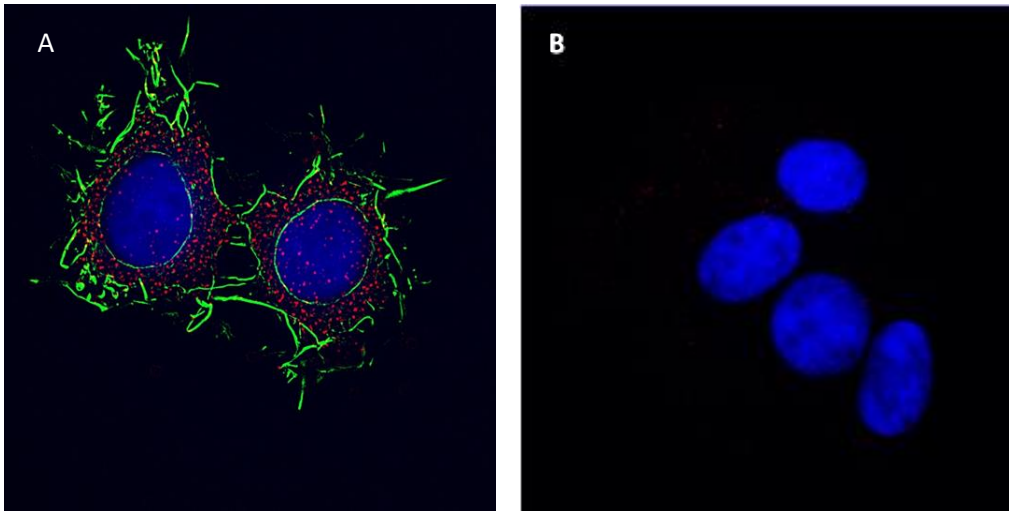

Fig. 7: Immunofluorescence localization of TRPM4 in NCI-H292 cell line.

(A) The overlay of the TRPM4 signal (red channel, Cy5 Red), filamentous actin (green channel, Phalloidin-iFluor 488), and nuclear DNA (blue channel, DAPI) revealed a cytoplasmic localization of TRPM4. (B) The TRPM4-blocking peptide blunted the detection of TRPM4 almost completely.

Out of the 13 sections that were obtained by SIM, similar sections were chosen for the analysis of TRPM4-localization. **Fig.7A** shows that TRPM4 is distributed throughout the cytoplasm and nucleoplasm. The pre-incubation with the blocking peptide resulted in an almost complete quenching of the TRPM4 signal (**Fig.7 B**). The detection of TRPM4 by qRT-PCR and immunofluorescence in NCI-H292 bronchial epithelial cells was successfully determined.

3.2 TRPM4 inhibition and stimulation with heat inactivated bacteria does not induce cytotoxicity

Bacteria and their components are prominent danger signals that lead to the activation of the innate immune response. During this work the contribution of TRPM4 signaling after bacterial stimulation on the innate immune response was investigated. Therefore, a specific inhibitor for TRPM4 was used before the stimulation of airway epithelial cells with heat inactivated Ps.a., which represents a typical pathogen in cystic fibrosis. To exclude possible cytotoxic effects of the treatment, NCI-H292 cells were incubated with increasing concentrations of the TRPM4-specific inhibitor 9-Phenantrol (9-Ph.) and heat inactivated Ps.a. Previous studies have shown a high specificity of 9-Ph. for TRPM4 based on ion current measurements. The half maximal inhibitory concentration for 9-Ph. has been shown to be IC50=20µM **(T. Grand et al., 2008)**.

The authors detected no inhibition of the structurally related TRPM5 or the CFTR channel at 1 mM or 0,25 mM respectively. Based on this study, concentrations ranging from 2,5 µM – 40 µM were used in the following experiments. To test for cytotoxic effects the release of the enzyme LDH was quantified, which is rapidly released from apoptotic cells.

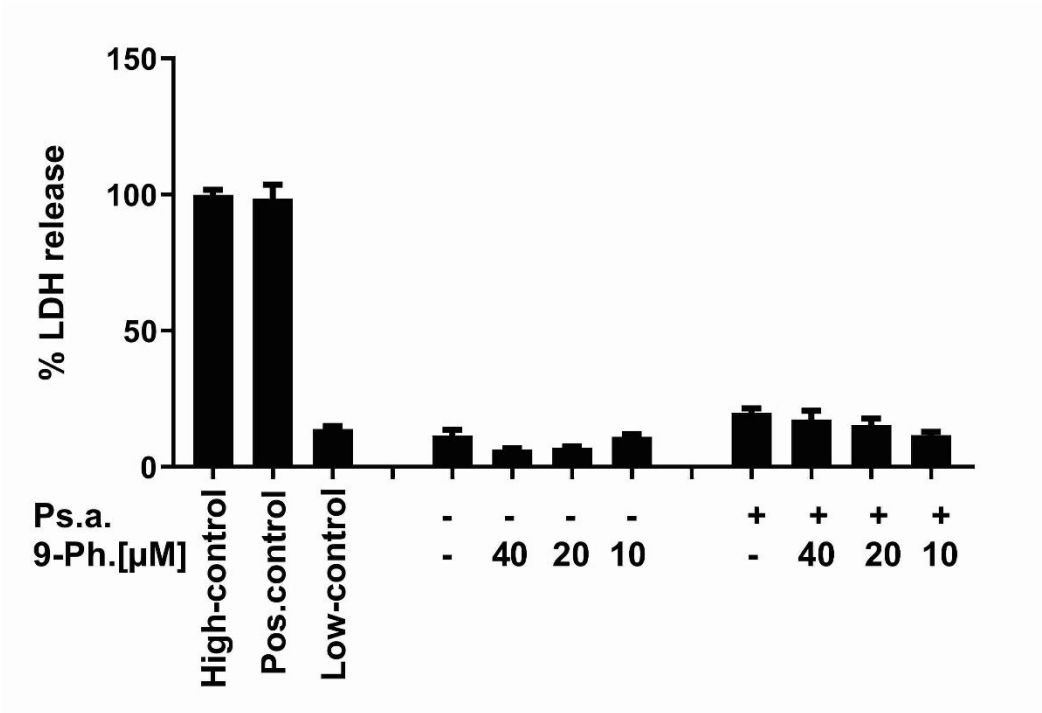

Fig. 8: LDH release from NCI-H292 cell line

LDH release from NCI-H292 cells after 9-Ph. treatment and stimulation with heat inactivated Ps.a. 18 hr., after stimulation the release of LDH in cell-free cell culture supernatant was determined. High-control – cell lysate, Pos.control (positive-control) – LDH enzyme contained in the test kit, Low-control – fresh cell culture medium. Ps.a. - heat inactivated *P.aeruginosa* PAOI, 9-Ph. – 9-Phenantrol. Experiment was repeated twice (N=4). Data was shown as mean +/- SEM.

Compared to the positive controls, the treatment with 9-Ph. and the combination with Ps.a. induced a release of LDH that was merely above the values of fresh cell culture medium. Therefore, the treatment with 9-Ph. and Ps.a. was considered to be not toxic for 18 hr. **(Fig.8)**. These findings proved that there is no increase in LDH-release after 18 hr. of treatment with the combination of 9-Ph. and Ps.a., which is a prerequisite for further investigations of the inhibition of TRPM4 in combination with Ps.a. stimulation on the activation of the innate immune response of airway epithelial cells.

3.3 The inhibition of TRPM4 decreases the release of pro-inflammatory mediators from NCI-H292 cells after bacterial stimulation

The release of pro-inflammatory mediators is an important hallmark in the course of the initiation of the innate immune response. On the one hand; they attract other immune cells to the site of infection but on the other hand it also acts directly on the pathogens. An exaggerated inflammation is known to have severe side effects on tissue integrity. To investigate the influence of TRPM4 signaling during the initiation of the innate immune response, NCI-H292 cells were incubated for 6 hr. with 9-Ph. and stimulated with heat inactivated Ps.a. for an additional 6 hr. The concentration of 9-Ph. was adjusted to the published IC_{50} value at 20 µM. The concentrations of IL-6, TNF-α, CXCL-2 (MIP-2α), and S100A8 were determined by ELISA in cell free supernatant.

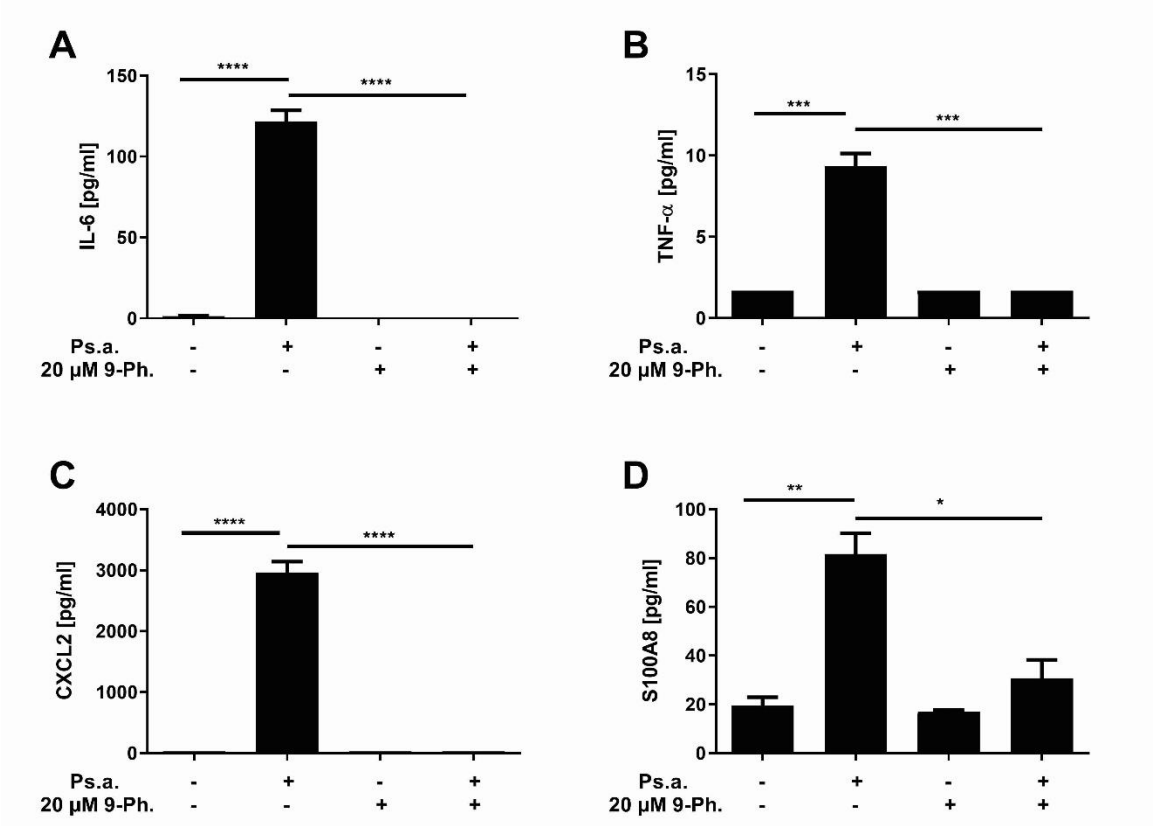

Fig. 9: The concentration of different pro-inflammatory mediators in cell culture supernatant of NCI-H292 cells after 6 hr. treatment.

Pro-inflammatory mediators were quantified with ELISA assay after 6 hr. pre-incubation with 9-Ph. and additional 6 hr. incubation with Ps.a.. IL-6, Interleukin 6; TNF-α, tumor necrosis factor alpha; CXCL2, C-X-C motif ligand 2, macrophage inflammatory protein 2-alpha (MIP2-α); S100A8, S100 calcium-binding protein A8. Experiments were carried out in duplicates, N =3; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0,0021$, *** $p < 0,0002$, **** $p < 0,0001$. Data are shown as mean ± SEM.

There was a significantly increased concentration of IL-6, TNF-α, CXCL2, and S100A8 after stimulation with heat inactivated Ps.a. compared to the untreated controls **(Fig.9 A-D)**. In contrast, the sequential treatment with 9-Ph. and Ps.a. reduced the amount of pro-inflammatory mediators in the cell culture supernatant almost to that of the untreated controls. The treatment with 9-Ph. alone did not induce an increased cytokine release. To confirm that these findings were present after longer incubation times, the cells were incubated for 18 hr. with heat inactivated Ps.a. after 6 hr. pre-incubation with 9-Ph. Additionally, lower gradual concentrations of 9-Ph. were used to test, if the inhibitory effect of 9-Ph. is dose dependent.

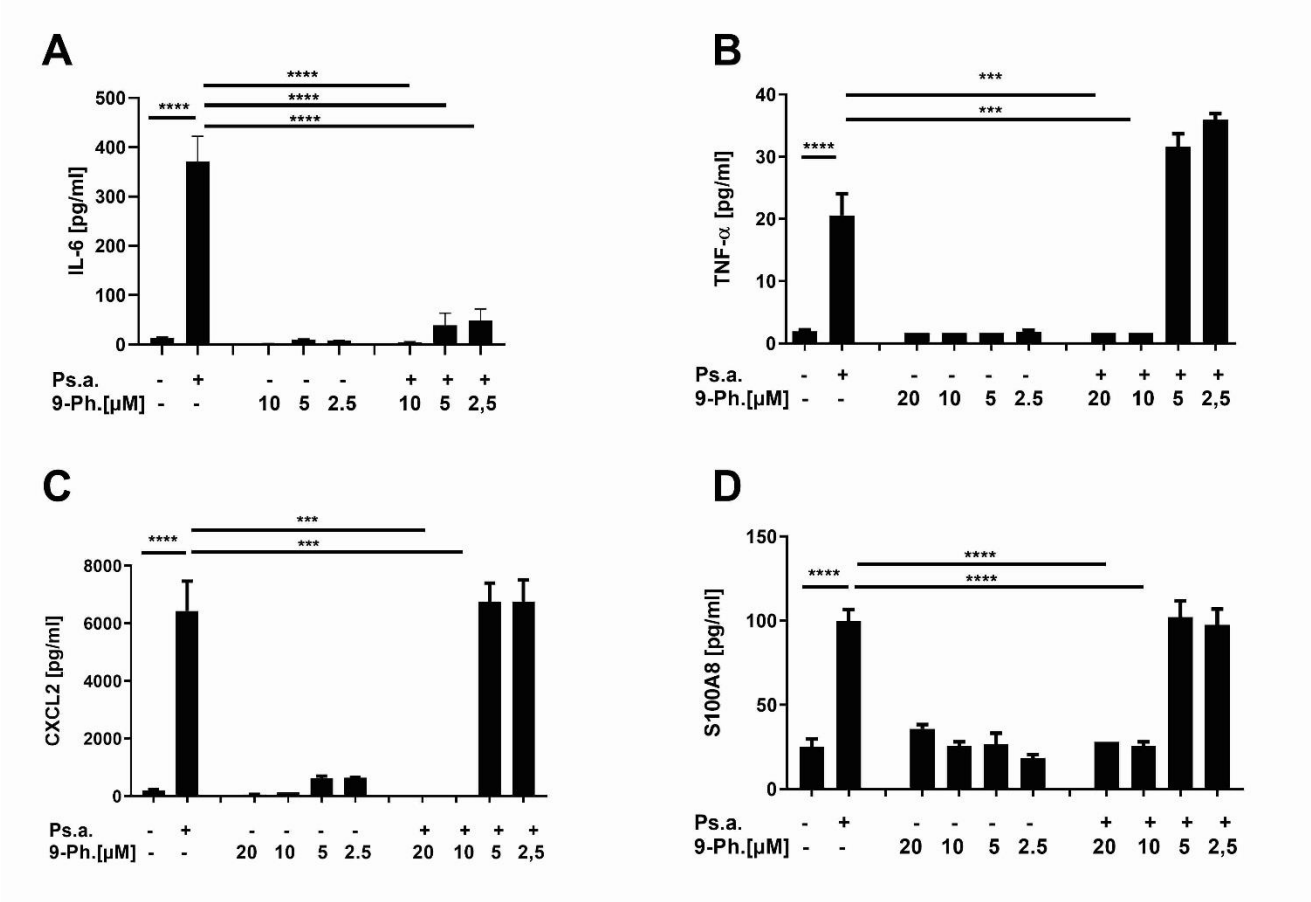

Fig. 10: The concentration of different pro-inflammatory mediators in cell culture supernatant of NCI-H292 cells after 18 hr. treatment.

NCI-H292 cells were incubated for 6 hr. with the designated concentrations of 9-Ph. and stimulated with heat inactivated Ps.a. for 18 hr. The concentration of the different analytes was determined by a multiplex Luminex assay. TNF-α, CXCL2, and S100A8 showed a strong inhibition of cytokine release with 10 µM and 20 µM 9-Ph. (B-D) while the release of IL-6 was completely inhibited in a range of 2,5 – 10 µM 9-Ph. (A). Experiments were carried out in duplicates, N =3; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0,0021$, *** $p < 0,0002$, **** $p < 0,0001$. Data was shown as mean ± SEM.

As already observed after 6 hr. of incubation with Ps.a. **(Fig.9)**, the release of all investigated analytes increased strongly 18 hr. after stimulation with Ps.a. **(Fig.10)**. Since a strong inhibition of IL-6 secretion was observed after 6 hr. of incubation with 9-Ph. and additional Ps.a. treatment **(Fig.9 A)**, lower gradual concentrations of the inhibitor were used to test whether a dose dependent inhibitory effect could be observed. While the inhibitor alone induced no increased concentration of inflammatory mediators in the cell culture supernatant, the pre-incubation with 10 µM and 20 µM 9-Ph. resulted in a strong inhibition of cytokine release in the case of TNF-α, CXCL2, and S100A8 **(Fig.10 B-D)**. The release of IL-6 was strongly inhibited in a range of 2,5-10 µM 9-Ph. **(Fig.10 A)**, while the Ps.a.-induced release of TNF-α, CXCL2, and S100A8 was not influenced by 2,5 – 5 µM 9-Ph.

Taken together; these results showed that the inhibition of TRPM4 by 9-Ph. inhibited the release of several pro-inflammatory cytokines after stimulation with heat inactivated Ps.a. Furthermore, the effect is dose dependent for the concentration of the inhibitor.

3.4 TRPM4 inhibition does not influence the transcription of selected inflammatory mediators after stimulation with Ps.a.

The pre-incubation of NCI-H292 cells with 9-Ph. resulted in a highly reduced release of IL-6, TNF-α, CXCL2, and S100A8 after stimulation with heat inactivated Ps.a. TRPM4 represents a Ca^{2+}-activated cation channel which plays a role in smooth muscle contraction, neuronal activity, and insulin secretion. The inhibition of such a channel may therefore, depending on the cell type, with several cellular processes. To find out if the transcription of the previously investigated analytes is influenced by 9-Ph., the expression of the corresponding genes was investigated after 6 hr. pre-incubation with 9-Ph. and 6 hr. stimulation with heat inactivated Ps.a. Intron crossing primers were used whenever possible.

The expression of IL-6, TNF-α, CXCL2 was significantly increased after 6 hr. stimulation with Ps.a. **(Fig.11 A, B, D)**. The pre-incubation with 9-Ph. didn't reduce the Ps.a. induced expression. Surprisingly, in the case of TNF-α the expression was even increased after pre-incubation with 9-Ph. and subsequent treatment with Ps.a. **(Fig.11 B)**. S100A8 is the only cytokine investigated here, that is directly dependent on the complex formation with Ca^{2+}. After stimulation with heat inactivated Ps.a. its expression is mildly but significantly induced and inhibited by a pre-incubation with 9-Ph. **(Fig.11 C)**. The pre-incubation with 9-Ph. alone had no influence on the expression of IL-6, TNF-α, S100A8, and CXCL2 **(Fig.11 A-D)**.

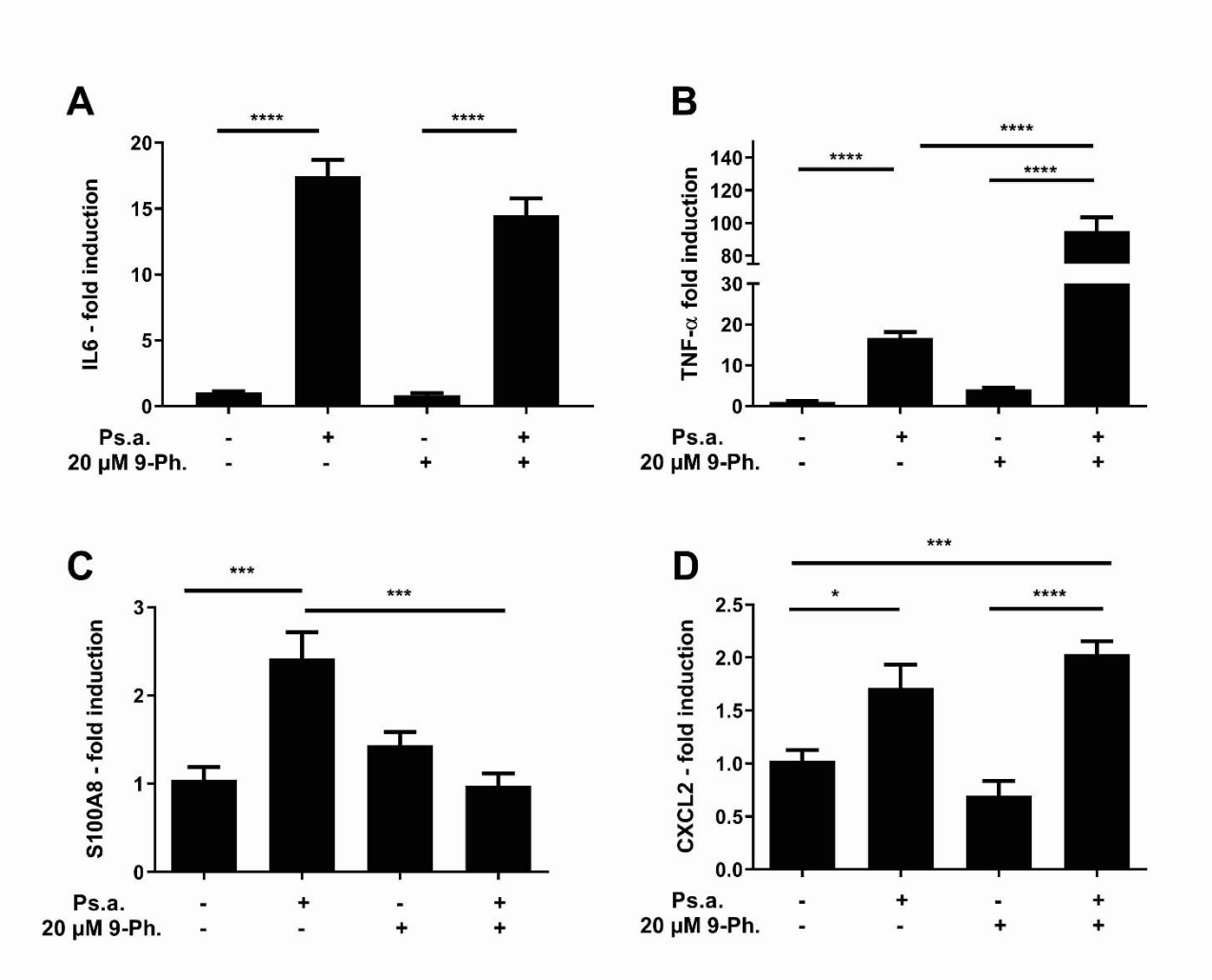

Fig. 11: Gene expression of different pro-inflammatory mediators in NCI-H292 cells

Gene expression in NCI-H292 after pre-incubation with 9-Ph. and stimulation with heat inactivated Ps.a. The expression of IL-6 (A) and TNF-α (B) is strongly induced after stimulation with Ps.a.. The pre-incubation with 9-Ph. did not decrease the expression, in case TNF-α increased more than 5 folds (B). The expression of CXCL2 in only mildly induced by Ps.a. and was not inhibited by additional pre-incubation with 9-Ph. (D). In contrast to other investigated genes, the expression of S100A8 was significantly inhibited after pre-incubation with 9-Ph. (C). Experiments were carried out in duplicates, N =3; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0{,}0021$, *** $p < 0{,}0002$, **** $p < 0{,}0001$. Data are shown as mean ± SEM.

This data shows that the pre-incubation with 9-Ph. and subsequent stimulation with heat inactivated Ps.a. leads to up-regulation of transcription of IL-6, TNF-α, and CXCL2. The expression of S100A8, as the only directly Ca^{2+} dependent mediator in our analysis, is inhibited after the pre-incubation with 9-Ph. and subsequent Ps.a. thus correlates with the reduced release of this mediator in the cell-culture supernatant.

3.5 TRPM4-specific shRNA inhibits the release of IL-6 from NCI-H292 cells

After showing that the TRPM4-inhibitor 9-Ph. inhibits the release of several pro-inflammatory mediators from NCI-H292 cells, it is important to prove that other modes of inhibition lead to the similar results. While 9-Ph. inhibits the ion conducting properties of TRPM4, shRNA will lead to a degradation of mRNA and a reduced protein concentration of TRPM4.

Validated TRPM4-specific shRNA was used and electroporated together with a non-target control shRNA. 24 hr. after electroporation the cells were stimulated with heat-inactivated Ps.a. and analyzed 18 hr. later.

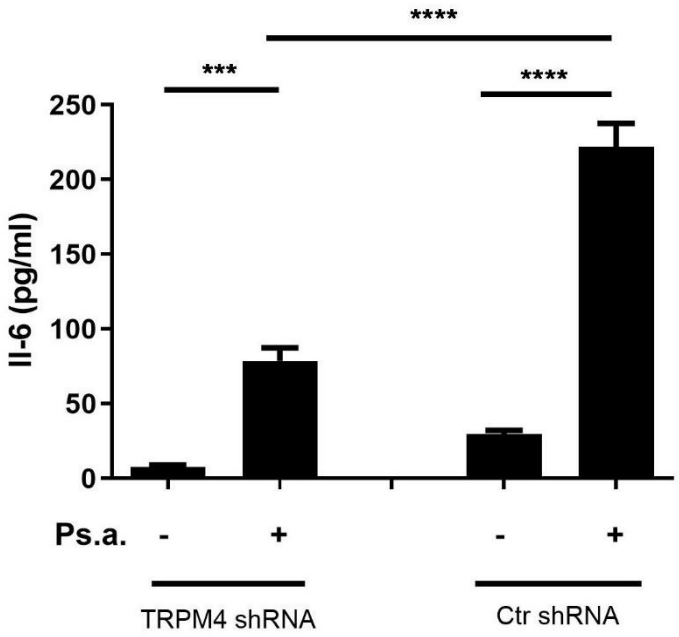

Fig. 12: IL-6 level in the cell culture supernatant of NCI-H292 cells treated with TRPM4-specific shRNA and non-target shRNA (ctr.shRNA)

The treatment with Ps.a. induced an increased release of IL-6, which was significantly lower in cells with TRPM4-specific shRNA than those treated with non-target shRNA. N =3; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0,0021$, *** $p < 0,0002$, **** $p < 0,0001$. Data are shown as mean ± SEM.

The transfection of shRNAs induced no significant increased IL-6 concentrations in the cell culture supernatant of unstimulated cells. The release of IL-6 increased significantly after the treatment with Ps.a. and was significantly lower from samples treated with TRPM4-specific shRNA compared to non-target shRNA **(Fig.12)**.

These results show, that by inhibiting the ion-conducting properties with 9-Ph. and the protein synthesis by shRNA both lead to a reduced release of pro-inflammatory mediators from bronchial epithelial cells.

3.6 The inhibition of TRPM4 does not influence its expression or the expression of TLR4

As the inhibition of TRPM4 by 9-Ph. or specific shRNA induced a lower release of different pro-inflammatory mediators from Ps.a. stimulated cells, it may be, that this effect due to changing or altering of the TRPM4 expression itself or inhibition of expression of other receptors, that recognize bacterial components.

To verify, if the expression of TRPM4 or TLR4 is influenced by the treatment with 9-Ph., NCI-H292 cells were pre-incubated with 9-Ph. for 6 hr. and stimulated with heat inactivated Ps.a. The expression of TRPM4 and TLR4 was analyzed by qRT-PCR after normalization to the housekeeping gene HPRT1.

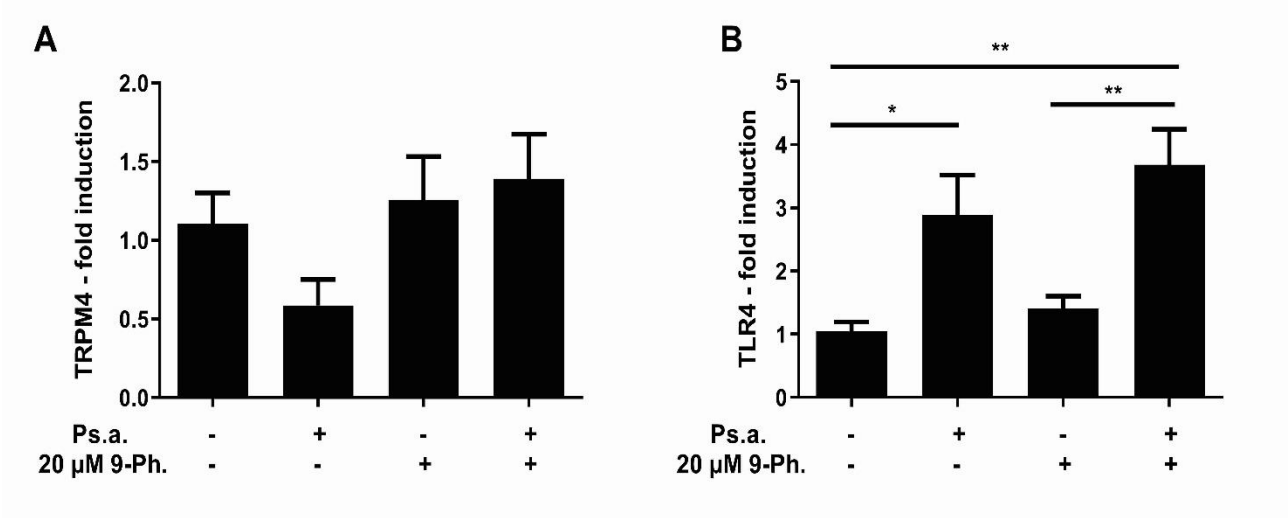

Fig. 13: The expression of TRPM4 and TLR4 in NCI-H292 cells

TRPM4 (A) and TLR4 (B) after the pre-treatment with 9-Ph. and stimulation with Ps.a. N =3; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0,0021$, *** $p < 0,0002$, **** $p < 0,0001$. Data are shown as mean ± SEM.

Although the expression of TRPM4 slightly decreased after the stimulation with Ps.a., the expression was not influenced by the pre-treatment with 9-Ph. or the additional incubation with Ps.a. **(Fig.13 A)**. The expression of TLR4, the prototypical receptor for gram-negative bacterial LPS, is significantly induced after the stimulation with heat inactivated Ps.a. The inhibition of TRPM4 had no additional effect compared to untreated or Ps.a. stimulated cells **(Fig.13 B)**.

These results indicate, that the inhibition of TRPM4 has no influence on the expression of the channels itself or the expression of the prototypical receptor for gram-negative bacteria, TLR4.

3.7 The inhibition of TRPM4 attenuates the export of IL-6 from the cytoplasm after bacterial stimulation

The translocation of proteins from the cytoplasm is a highly regulated process that involves different routes of transport and signaling pathways through various cellular compartments. Since the TRPM4-inhibitor 9-Ph. inhibited the release of various inflammatory mediators into the cell culture supernatant but not their transcription, it would now be interesting to show, if those peptides would accumulate in the cytoplasm of the cells.

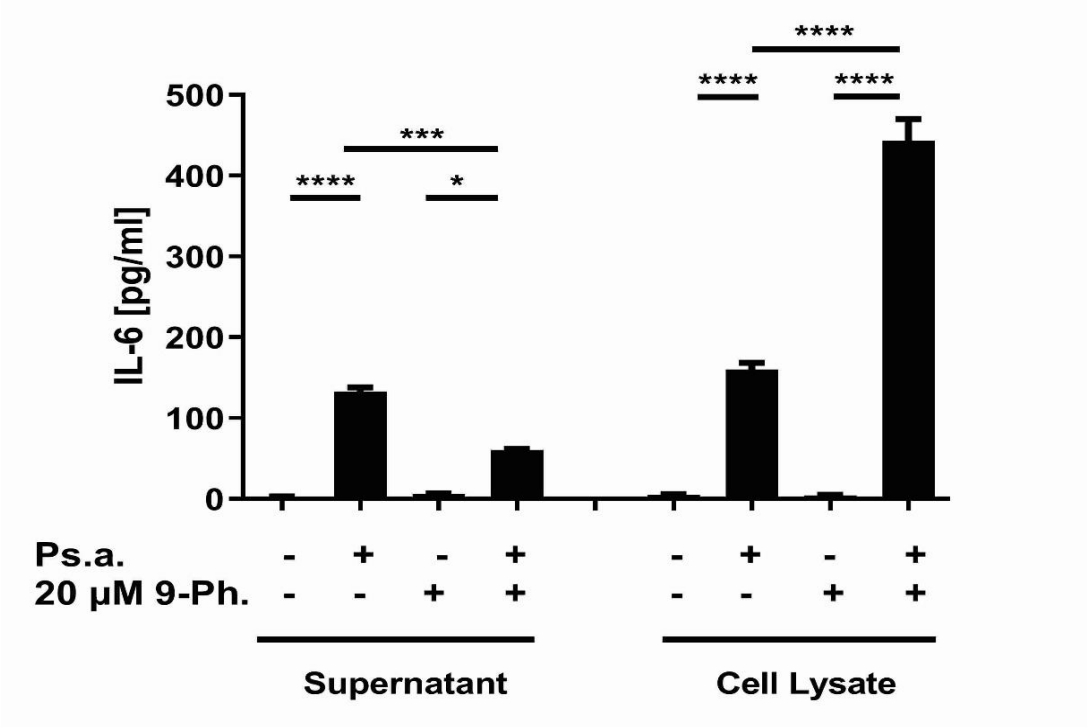

Fig. 14: IL-6 concentration in cell culture supernatant and cell lysate of NCI-H292 cells

IL-6 concentration analyzed in cell culture supernatant and cell lysate of NCI-H292 cells after stimulation with heat inactivated Ps.a. and pre-incubation with 9-Ph. N =3; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0{,}0021$, *** $p < 0{,}0002$, **** $p < 0{,}0001$. Data are shown as mean ± SEM.

To investigate, if the analyte accumulated more inside the cells after 9-Ph. pre-treatment and stimulation with Ps.a., the cell lysate and cell culture supernatant was analyzed separately in an ELISA. Therefore, the cells were pre-treated with 20 µM of 9-Ph. for 6 hr. and stimulated with heat inactivated Ps.a. for additional 18 hr. The supernatant was removed carefully and centrifuged to remove cell-remains. The cells in the tissue culture plate were washed twice with PBS and resuspended in lysis buffer RIPA after the last washing step. Cell lysis was fortified by vigorous pipetting and vortexing. Cell fragments were removed by centrifugation. The cell lysate and supernatant were analyzed by ELISA.

The concentration of IL-6 in the cell culture supernatant increased as expected significantly after the stimulation with Ps.a. **(Fig.14)**. As already observed before, the pre-incubation with 9-Ph. resulted in a significantly lower concentration in the supernatant compared to the stimulation with Ps.a. alone. In contrast, the concentration in the cell lysate highly increased after the pre-incubation with 9-Ph. and subsequent stimulation with Ps.a. **(Fig.14)** compared to the stimulation with heat inactivated bacteria only. This indicates, that IL-6 accumulates in the cell, when the TRPM4 channel is inhibited by 9-Ph.

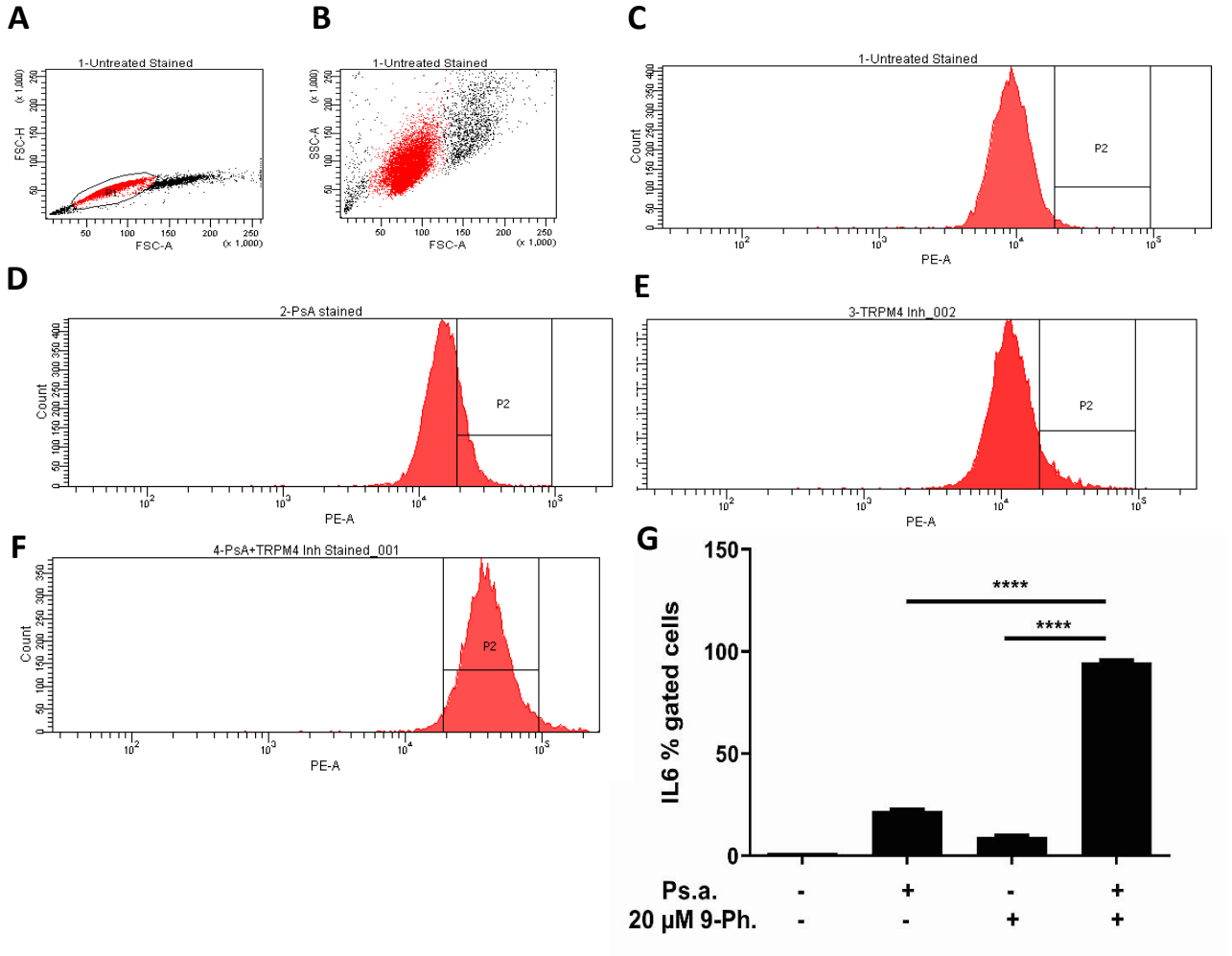

Fig. 15: FACS analysis of IL-6 release in NCI-H292 cells

FACS analysis of IL-6 release in NCI-H292 cells after stimulation with Ps.a. and pre-incubation with 20 µM 9-Ph. Gating strategy for single cells with no treatment (A-C). (D) After the stimulation with Ps.a. the number of IL-6 positive cells increased slightly, while the pre-treatment with 9-Ph. induced a low number of IL-6 positive cells (E). The highest number of IL-6 positive cells was detected after pre-treatment with 9-Ph. and stimulation with heat inactivated Ps.a. (F). Graphical evaluation of the FACS-results (G). N = 3; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0{,}0021$, *** $p < 0{,}0002$, **** $p < 0{,}0001$. Data are shown as mean ± SEM.

To confirm these results, the cells were analyzed by FACS in a separate experiment using an IL-6-specific antibody. The cells were grown in 12-well plates like in the previous experiments. To increase the cell number, 12 wells were used for each experimental condition and each 4 wells combined in one FACS-analysis (n=3). After the experiment, the cells were detached by ice-cold 0,1 M EDTA in PBS and a cell scraper. The cells were gated based on their size and granularity. The number if IL-6 positive cells in the untreated group was very low and increased after the treatment with heat inactivated Ps.a. **(Fig.15 C-D)**. The treatment with 9-Ph. didn't increase the number of IL-6 positive cells **(Fig.15 E)**, while the combination of pre-treatment with 9-Ph. and Ps.a. stimulation resulted in a significantly increased number of IL-6 positive cells compared to the other groups **(Fig.15 F-G)**.

These results confirm the hypothesis that the inhibition of TRPM4 by 9-Ph. leads to an accumulation of IL-6 inside the cells and probably also TNF-α, and CXCL2.

3.8 The inhibition of cytokine release by 9-Ph. is not dependent on a distinct TLR pathway

The recognition of microbial components by airway epithelial cells is accomplished by a set of TLR, that in turn activate downstream signaling events like cytokine synthesis or the release of antimicrobial peptides. Heat inactivated Ps.a. contains a mixture of different TLR-ligands like LPS (TLR4), bacterial DNA (TLR9) or flagellin (TLR5), which all activate distinct TLR-receptors.

To investigate whether primary human bronchial epithelial cells (pHBEs) respond to the inhibition of TRPM4 as seen in the cell line NCI-H292, they were pre-treated with 9-Ph. and stimulated 6 hr. later with heat inactivated Ps.a. PHBE shows high responsive of IL-6 release compared to NCI-H292, while the pre-treatment with 9-Ph. led to a decreased release of IL-6 compared to bacterial stimulation alone in both types of cells **(Fig.16 A-C)**. The stimulation with LPS and flagellin induced a release of IL-6, while the pre-treatment with 9-Ph. was able to reduce the concentration in cell culture supernatant **(Fig.16 B)**. Although pHBEs were not responsive to Pam3CSK4 (Pam3CysSerLys4, tri-acetylated lipopeptide), a synthetic TLR2/TLR1 ligand, NCI-H292 responded with IL-6 release, that was significantly reduced after pre-incubation with 9-Ph. **(Fig.16 C)**.

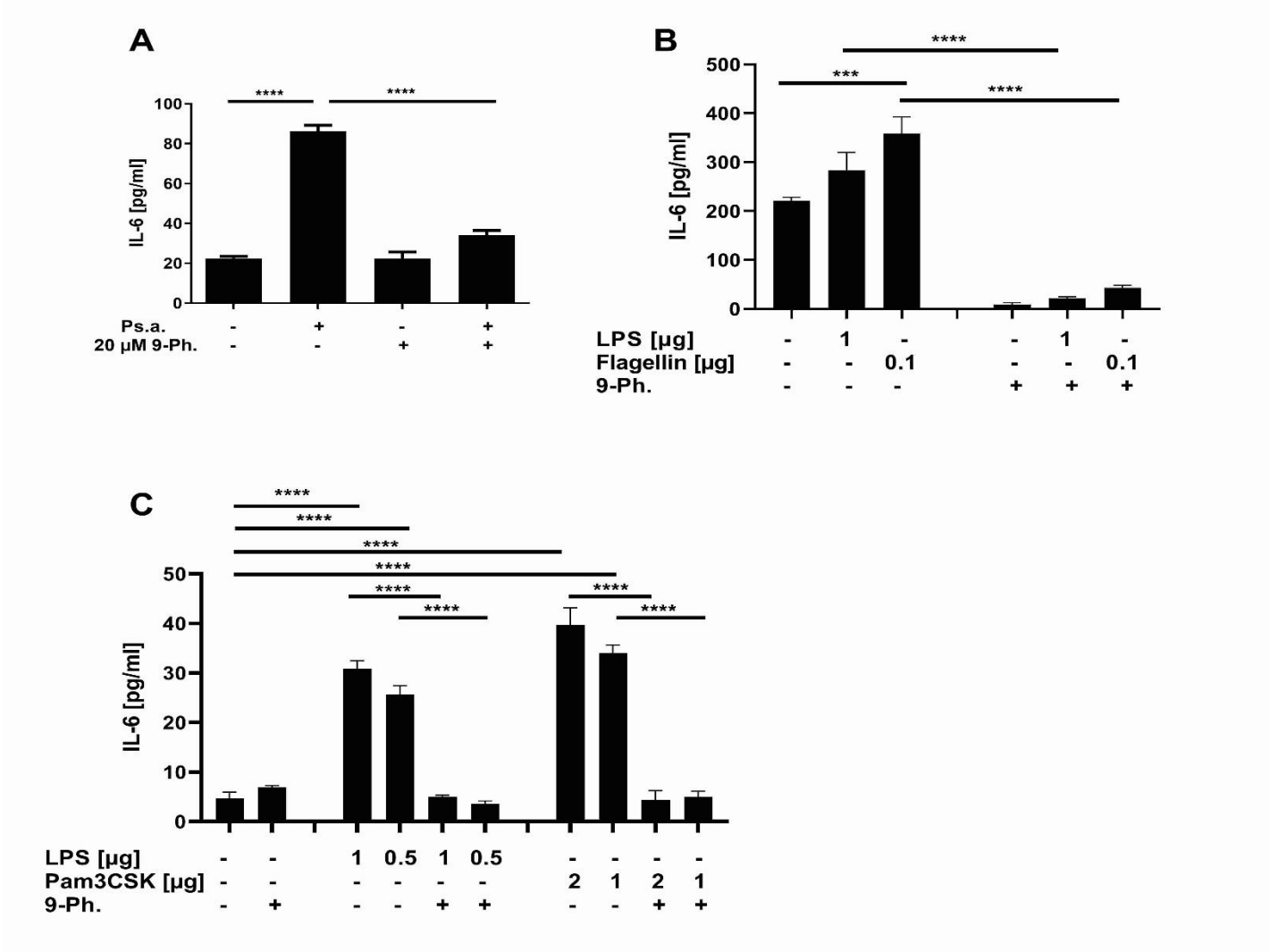

Fig. 16: The release of IL-6 from pHBE & NCI-H292 cells

(A) The release of IL-6 is inhibited by 9-Ph. in primary bronchial epithelial cells (pHBE) after the pre-incubation with 9-Ph. and stimulation with heat inactivated Ps.a. 9-Ph. equally inhibited the Il-6 release after stimulation with LPS and flagellin from pHBEs (B). In NCI-H292 cells 9-Ph. inhibited IL-6 release after stimulation with LPS and Pam3CSK (C). Experiments were carried out in duplicates, N = 3; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0{,}0021$, *** $p < 0{,}0002$, **** $p < 0{,}0001$. Data are shown as mean ± SEM.

These results indicated that the decreased release of cytokine after the inhibition of TRPM4 in bronchial epithelial cells is not restricted to a distinct TLR-signaling pathway, but a general mechanism of airway cell biology. These results also indicated that the response of pHBE in terms of IL-6 release toward flagellin differs from NCI-H292 cell line, which doesn't give any flagellin responsiveness.

3.9 The increased co-localization of IL-6 with filamentous β-Actin after TRPM4 inhibition correlates with the increased cellular retardation

After showing that the decreased IL-6 release after bacterial stimulation and inhibition of TRPM4 is not a feature of a distinct TLR-pathway and present in both cell lines and primary cells, a more experimental approach to visualize the IL-6 release was tried. The goal was to quantify the IL-6 specific immunofluorescent staining in high resolution structure illumination microscopy with the staining for a "house-keeping" protein like β-actin. NCI-H292 cells were grown in chamber-slides at low density and stimulated as described for the previous experiments.

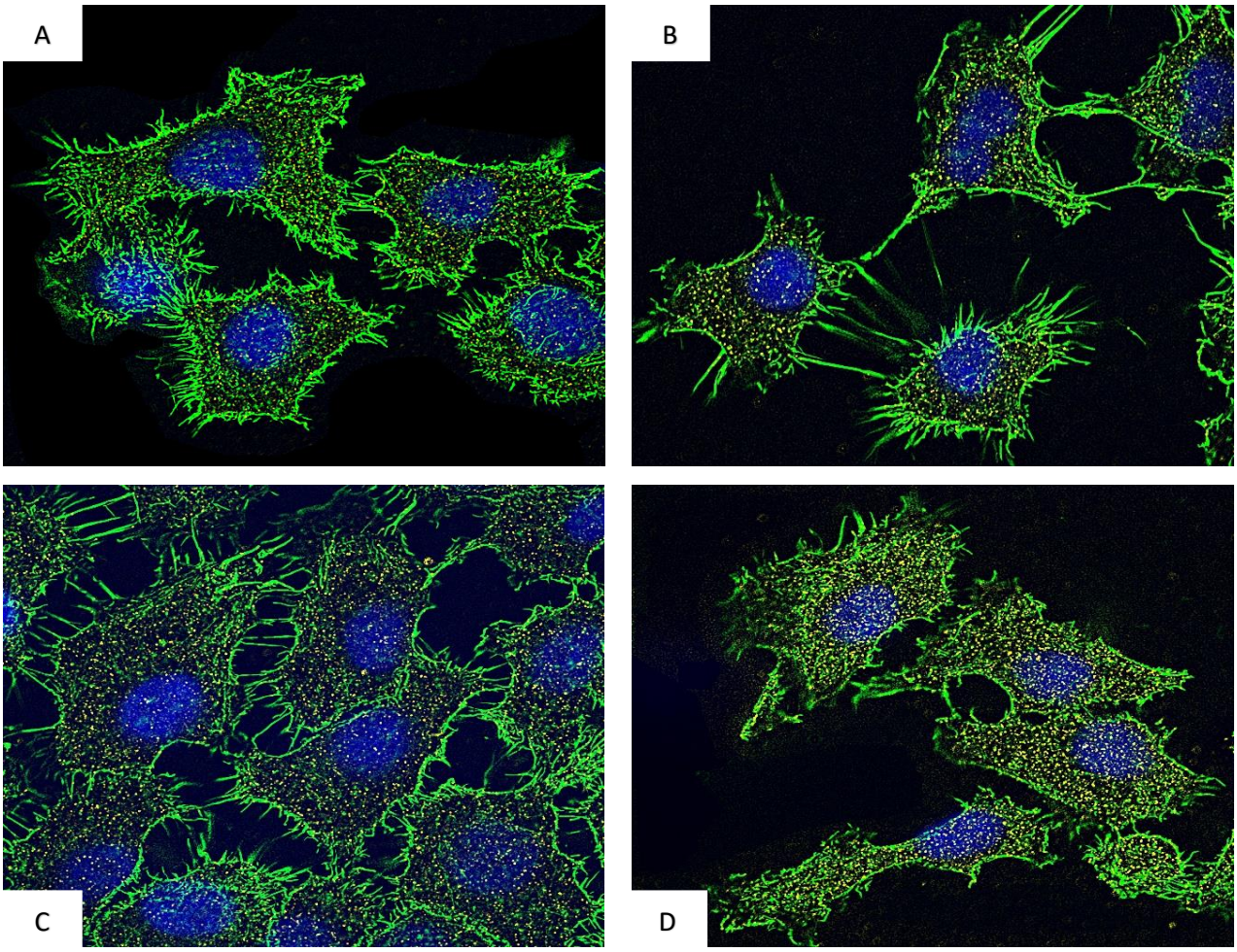

Fig. 17: Structure illumination microscopy of NCI-H292 cells

without treatment (A), after the stimulation with Ps.a. (B), the treatment with 9-Ph. (C), and the pre-treatment with 9-Ph. and following stimulation with Ps.a. (D). The cells were stained with a primary antibody against IL-6 (yellow), filamentous β-actin (Phalloidin-F488, green), and nuclear staining (DAPI, blue).

The co-localization of a protein, expressed independently of the treatment, should correlate with the intracellular concentration of the protein which is changed by the treatment. There are mainly two methods available for confocal-laser scanning microscopy to calculate co-localization. The Pearson's correlation coefficient (PCC) and the Manders' colocalization coefficient (MCC). In this work; MCC was used due to its straight forward workflow and intuitive measure of co-localization.

All images were taken with the same exposure settings and the number of optical planes. The analysis was performed on the same optical slice for all images.

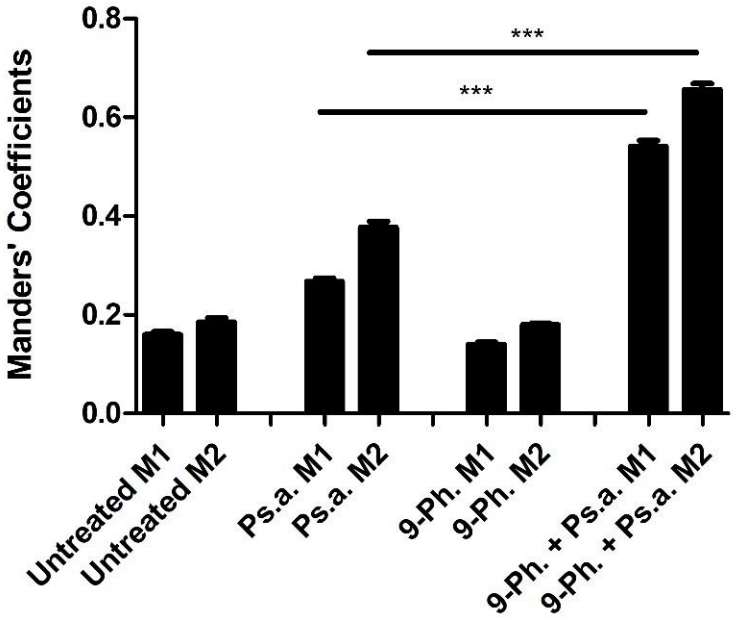

Fig. 18: Co-localization analysis of IL-6 and β-actin in NCI-H292 cells

Co-localization analysis of IL-6 and filamentous β-actin in images of NCI-H292 cells after different treatments. While untreated and TRPM4-inhibitor (9-Ph.) treated cells showed a co-ocalization of about 20%, the treatment with Ps.a. induced an increased co-localization to 30-40%. The highest co-localization was calculated for the cells treated with 9-Ph. and stimulated with Ps.a., N = 10; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0{,}0021$, *** $p < 0{,}0002$, **** $p < 0{,}0001$. Data was shown as mean ± SEM.

MCC has to be displayed for both directions, i.e. the co-localization of IL-6 with β-actin (M1), and the co-localization of β-actin with IL-6 (M2). The MCC calculated for untreated and 9-Ph. treated cells was calculated to be around 20%. This increased to about 30-40% in Ps.a. treated samples and raised to about 60% in cells that were pre-treated with 9-Ph. and stimulated with Ps.a. **(Fig.18)**.

The results of the MCC correlate with the previous findings obtained by ELISA and FACS analysis.

3.10 The inhibition of TRPM4 interferes with the activation of major transcription factors

The activation of transcription factors is an indispensable step in the regulation of gene transcription and expression. The activation occurs mostly by the specific phosphorylation of one or more serine or threonine residues, that may lead to dimerization or structural changes that modify the DNA-binding of the transcription factor-complex. In the case of Nf-kB, the majority of un-phosphorylated complex will stay in the cytoplasm, while the phosphorylation of serine residues on the p65-sbunit will lead to a pre-dominant localization in the nucleus and promote binding to the respective p65-DNA binding site; to enable transcription of the target genes. A lot of the steps that lead to the activation of transcription factor, require divalent cations like $Ca^{2+;}$ and therefore will depend, at least in part, on the distributions of ions in the cytoplasm. This process has already been shown to be influenced by TRPM4.

To investigate the activation of the Nf-kB transcription factor, NCI-H292 cells were pre-incubated for 6 hr. with 9-Ph. and stimulated with Ps.a. for 20 min. The amount of the p65-subunit was investigated by western blot in the cytoplasm and nuclear extracts **(Fig.19 A)**. While p65 was nearly not detectable in the cytoplasm of untreated cells (control cytoplasmic extract), there was already certain amount of p65 detectable in the nuclear extract (control nuclear extract). The treatment with 9-Ph. induced a slight increase in the amount of cytoplasmic p65, that was slightly decreased when the cells were additionally treated with Ps.a. compared to cell treated with Ps.a. alone **(Fig.19 A)**. As expected, the stimulation with Ps.a. induced high translocation of p65 to the nuclear space, as seen in the samples from the nuclear extract. This amount was reduced by the treatment with 9-Ph. and also in Ps.a. stimulation after pre-treated with 9-Ph. **(Fig.19 A)**. The signal intensity of the nuclear extract from samples treated with 9-Ph. was comparable with nuclear extracts from untreated cells.

These results showed that the treatment with 9-Ph. inhibits the nuclear translocation of the p65-subunit of NF-kB. This implictes that the activation of the canonical NF-kB pathway is inhibited by the treatment with 9-Ph. This could have an impact on the transcription of NF-kB-dependent genes.

The MAPK1 is mostly activated by growth factors or mitogenic stimuli and leads to the phosphorylation dependent activation of ERK1/2 complex.

The pre-treatment of NCI-H292 cells with 9-Ph. leads to an increased phosphorylation of ERK1/2, which was not influenced by the subsequent stimulation with Ps.a. **(Fig.19 B)**. The treatment with Ps.a. alone induced a phosphorylation of ERK1/2 that was comparable to untreated cells. This result showed that the treatment with 9-Ph. induces the transcriptional activation of MAPK1 dependent genes.

Many signaling pathways in the cell require Ca^{2+} as a co-factor. Through the inhibition of TRPM4 the influx of Na^{+} from the extracellular space is impaired, which may also indirectly change the concentration of Ca^{2+} inside the cell. The formation of inositol 1,4,5-trisphophate (IP3) and DAG from PIP2 is catalyzed by the enzyme PLC-β2.

The stimulation of NCI-H292 cells with Ps.a. leads to an increased protein detection of PLC-β2 compared to untreated cells **(Fig.19 B)**. The pre-treatment with 9-Ph. didn't increase the amount of PLC-β2, which also stayed at a comparable level after the subsequent stimulation with Ps.a.

This result showed that the inhibition of TRPM4 by 9-Ph. leads to a decreased expression of PLC-β2 even after subsequent stimulation with Ps.a., which may have an impact on the synthesis of IP3 and subsequent internal Ca^{2+} ions release.

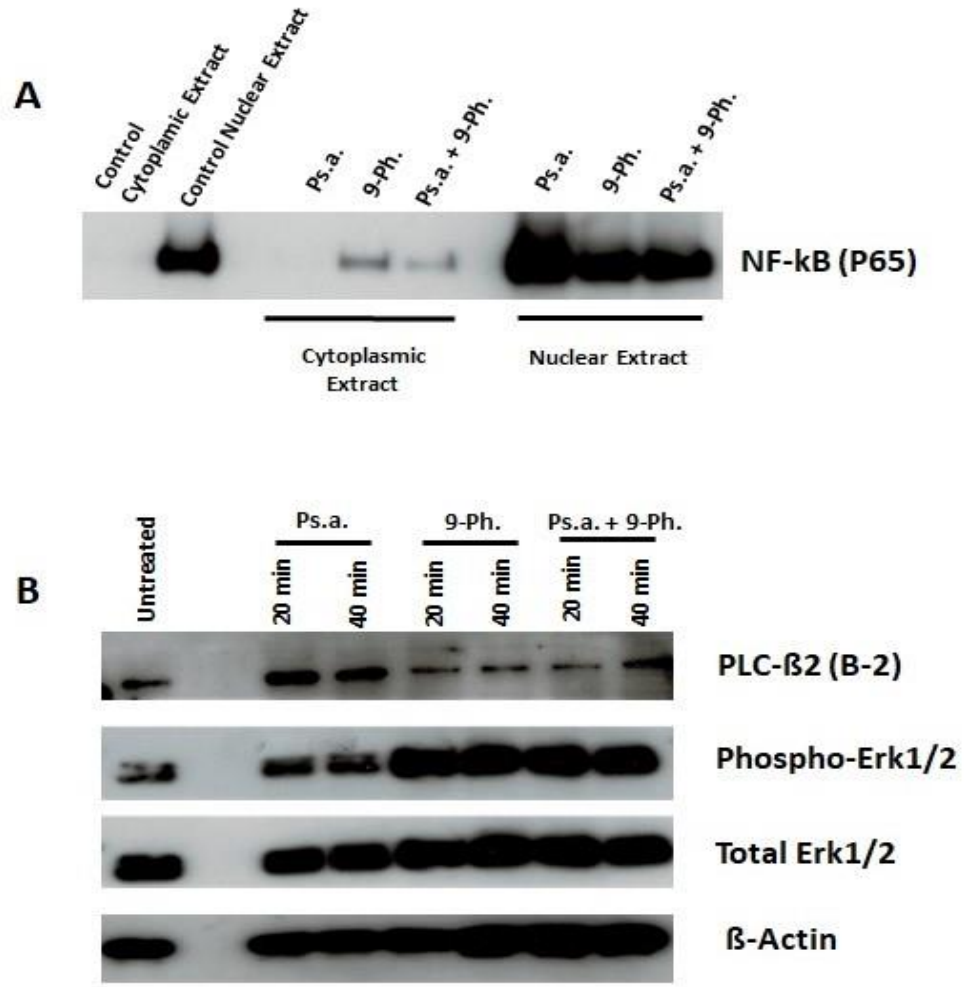

Fig. 19: Activation and localization analysis of the transcription factors in NCI-H292 cells

Analysis of the activation and localization of transcription factors in NCI-H292 cells after the pre-incubation with 9-Ph. and the stimulation with Ps.a.. Cytosolic and nuclear extracts were prepared after the separation and lysis of the nucleus from the cytoplasm. (A) The localization of the p65 subunit of Nf-kB in NCI-H292 cells after the pre-incubation with 9-Ph. for 6 hr. and the treatment with Ps.a. for 20 min. in the cytoplasm and nuclear extract is shown. Equal loading was accomplished by normalizing to the total protein content. (B) The detection of Phospholipase-C-β2, phosphor-Erk1/2, total Erk1/2 and β-actin in cell lysate of NCI-H292 cells after the pre-incubation with 9-Ph. for 6 hr. and the subsequent treatment with Ps.a. for 20 and 40 min. Experiments were carried out in duplicates.

3.11 TRPM4 inhibition leads to decreased eradication of Ps.a. and β-defensin-2 production from human bronchial epithelial cells

Chronic infections of the lung with Ps.a. are an important driver for cystic fibrosis pathology and exacerbations of COPD. Bronchial epithelial cells actively contribute to innate immune response by the expression of anti-microbial peptides like β-defensin-1, β-defensin-2, and cathelicidin.

The experiment was carried out with pHBEs, cultured at air-liquid interface, and differentiated until a trans-epithelial resistance of more than 1000 Ω/cm^2. The cells were pre-treated with 9-Ph. in the lower compartment (basolateral) and infected with $1x10^3$ CFU live Ps.a. from upper compartment for 6 hr. The treatment with 9-Ph. didn't influence the expression of hBD-1 compared to untreated control, while Ps.a. treatment induce a lower hBD-1 expression but did not reach significance level **(Fig.20 A)**. Since hBD-1 is known to be a constantly expressed b-defensin, it was expected not to be induced by Ps.a. infection. More interestingly, the expression of hBD-2 was highly significantly induced by the infection with Ps.a. and repressed to baseline by pre-incubation with 9-Ph. **(Fig.20 B)**. The decreased expression of hBD-2 correlated with lower concentration of the peptide in cell culture supernatant **(Fig.20 C)** and an increased survival of Ps.a. after pre-treatment with 9-Ph. **(Fig.20 D)**. The influence of TRPM4-inhibition on the release hBD-2 and the survival of Ps.a. correlated with the duration of the pre-treatment with 9-Ph. A co-treatment of the cells at the same time point with 9-Ph. and Ps.a. reduced the release of hBD-2 into the cell culture supernatant, but had nearly no effect on the antimicrobial activity of the cells. On the other hand, 3 hr. and 18 hr. 9-Ph. pre-treatment showed the highest efficiency in reducing the release of hDB-2 which correlates with suppression of antimicrobial activity of the cells after 6 hr. incubation with live Ps.a.

These results show a functional correlation of TRPM4 inhibition and the antimicrobial activity of differentiated pHBEs.

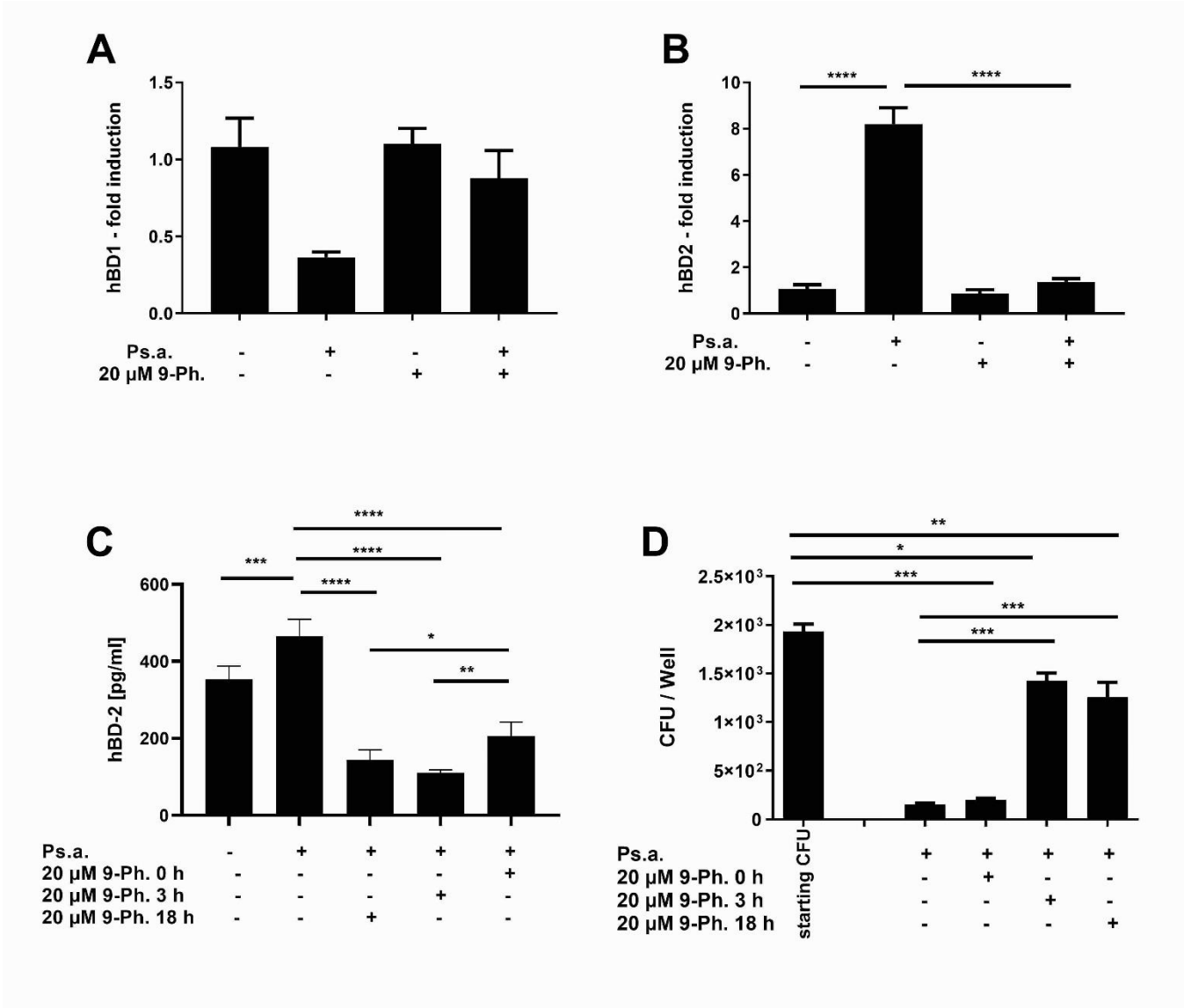

Fig. 20: hBD-1 & hBD-2 secretion and expression in pHBEs

The treatment of pHBEs with 9-Ph. modulates the expression and release of hBD-2 after the stimulation with live Ps.a. While (A) the expression of hBD-1 was not affected by the treatment with 9-Ph. or live Ps.a., the expression of hBD-2 (B) was highly significantly induced after bacterial stimulation. The additional pre-treatment with 9-Ph. resulted in an inhibition of the hBD-2 expression. The reduced expression of hBD-2 in response to bacterial infection and TRPM4-inhibition correlates with (C) decreased release of hBD-2 into the cell culture supernatant, and (D) an increased survival of Ps.a. Additionally, the pre-treatment with 9-Ph. shows a time dependent influence on the concentration of hBD-2 (C) and the survival of Ps.a.(D). Experiments were carried out in duplicates, N = 3; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0{,}0021$, *** $p < 0{,}0002$, **** $p < 0{,}0001$. Data are shown as mean ± SEM.

3.12 The inhibition of TRPM4 leads to reduced release of IL-6 from human and murine macrophages

During this work it has been shown that the inhibition of TRPM4 led to a reduced release of pro-inflammatory mediators from human bronchial epithelial cell lines **(Fig.9, 10)** and human primary cells **(Fig.16)**. Since the mouse is the most important organisms for pre-clinical models of different diseases, it was important to know, whether the same effects would also be true for this species. This would also prove whether it is a general property or a specific one for human TRPM4. Furthermore, the innate immune response is driven by the recognition of pathogens by epithelial cells and immune cells like macrophages.

To investigate whether the inhibition of TRPM4 leads to a reduced release of IL-6 from macrophages, human U937 cells and murine BMDM were pre-incubated with 9-Ph. and stimulated with heat inactivated Ps.a.

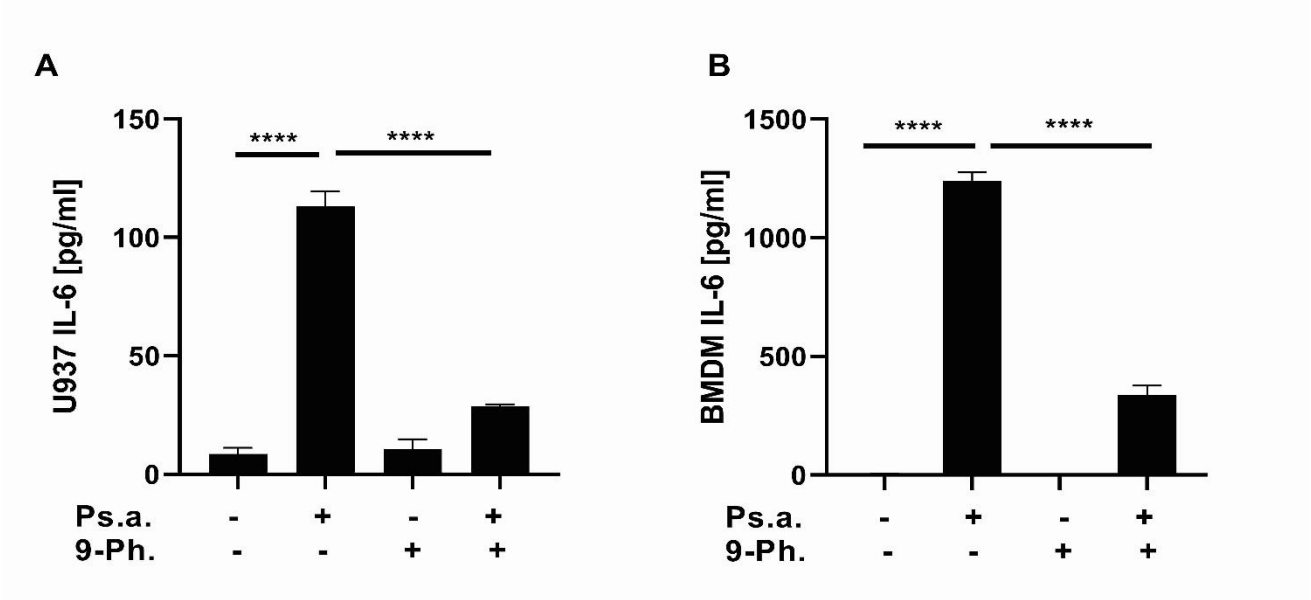

Fig. 21: IL-6 secretion from U937 cells and BMDM

Human U937 cells and murine BMDM were pre-incubate with 9-Ph. and stimulated with Ps.a. IL-6 was measured in cell-free supernatant. Experiments were carried out in duplicates, N = 4; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0{,}0021$, *** $p < 0{,}0002$, **** $p < 0{,}0001$. Data are shown as mean ± SEM.

Human and murine macrophages responded after the stimulation with heat inactivated Ps.a. with a highly significant release of IL-6 **(Fig.21)**. The pre-treatment with 9-Ph. significantly reduced the release of IL-6 into the cell culture supernatant. The inhibitor without the bacterial stimulation had no effect on the concentration of IL-6 in the cell culture medium.

These results show that U937 is as human macrophage like cells, and mouse BMDM responds in a similar way as the human epithelial cells investigated so far.

Next, the release of inflammatory mediators was compared between BMDMs from TRPM-knockout animals and cells, that were isolated from wildtype littermates and subsequent treated with 9-Ph. This was important to verify that the BMDMs isolated from the genetically modified animals show the same phenotype as cells, isolated from wildtype animals and treated with a pharmacological inhibitor.

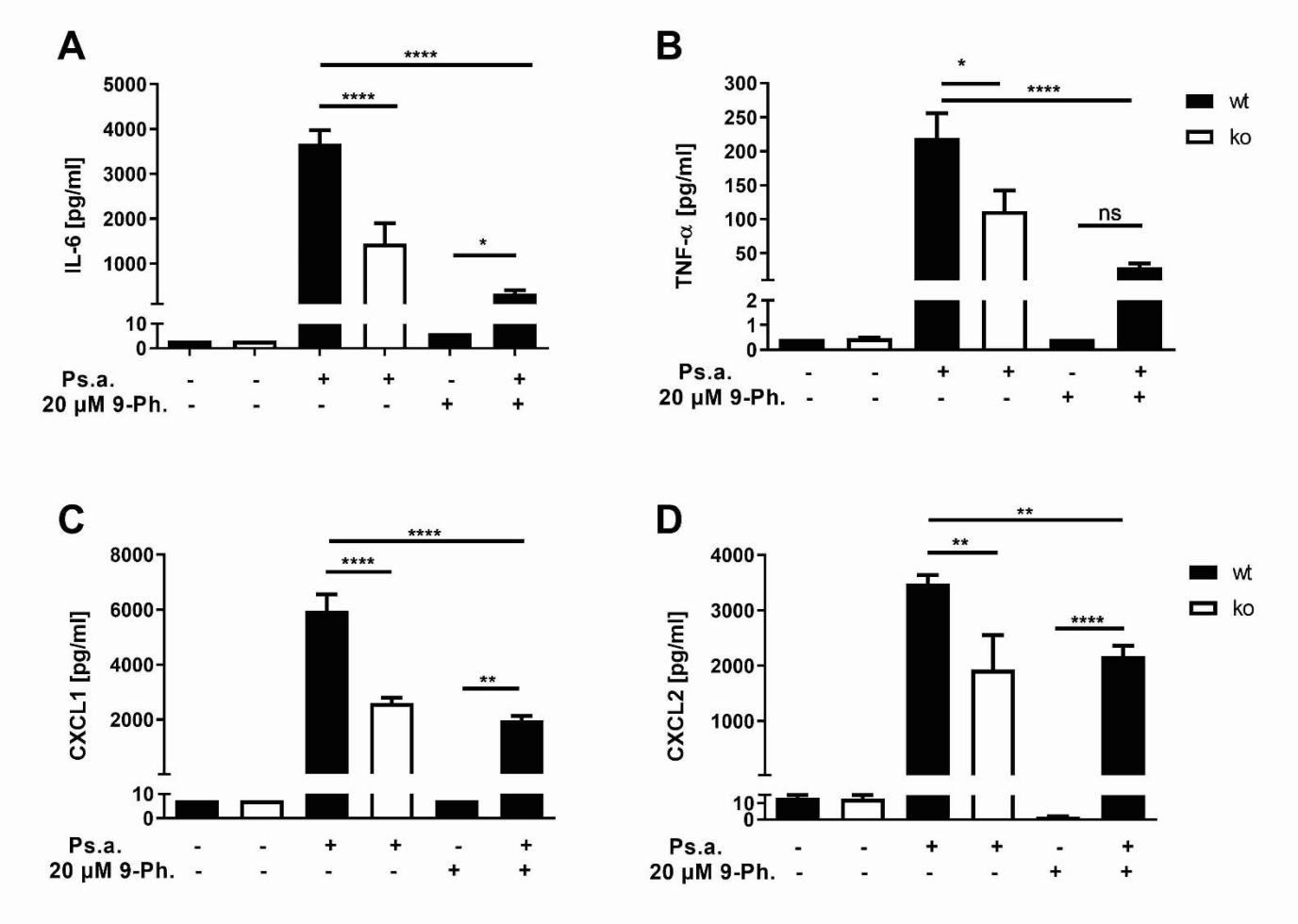

Fig. 22: Pro-inflammatory mediators secretion from BMDMs of wildtype and TRPM4-konckout mice

The release of pro-inflammatory mediators is inhibited from BMDMs of TRPM4-konckout mice (ko) and wildtype BMDM treated with 9-Ph. The release of IL-6 (A), TNF-a (B), CXCL1 (C), and CXCL2 (D) was significantly reduced from BMDMs derived from TRPM4-Knockout and wildtype mice (wt) treated with 9-Ph. prior to Ps.a. stimulation compared to wt cells stimulated with Ps.a., N = 4; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0{,}0021$, *** $p < 0{,}0002$, **** $p < 0{,}0001$. Data are shown as mean ± SEM.

The obtained results confirmed the previous findings **(Fig.21 B)**, that the pre-treatment of BMDM from wild type mice with 9-Ph. for 6 hr. and the subsequent stimulation with Ps.a. for 18 hr., significantly reduced the concentration for IL-6, TNF-a, CXCL1, and CXCL2 in cell culture supernatant, compared to specimen that received no pre-treatment **(Fig.22)**. The response from BMDMs isolated from genetically modified animals was comparable to the pre-treatment with 9-Ph. which means that the pharmacological TRPM4-inhibitor had the same effect as the deletion of the ion-conduction domains in the genetically modified mice (TRPM4-knockout).

These results show that 9-Ph. and the deletion of the ion-conducting domains in TRPM4 led to a reduced cytokine response after bacterial stimulation in macrophage like cells in mice.

3.13 TRPM4 channel participate in inflammatory cytokine induction in isolated mice tracheal epithelial cell after heat inactivated Ps.a. Infection.

Bronchial epithelial cells are an important component of the innate immune response; because they actively contribute to antimicrobial defense and chemotaxis of leukocytes. It was shown that human bronchial epithelial cells release less pro-inflammatory cytokines and anti-microbial peptide hBD2 after the inhibition of TRPM4 in response to the bacterial stimulation.

The murine tracheal epithelial cells were isolated by enzymatic digestion and cultured in 12-well plates. The inflammatory response was compared between wildtype and knockout mice.

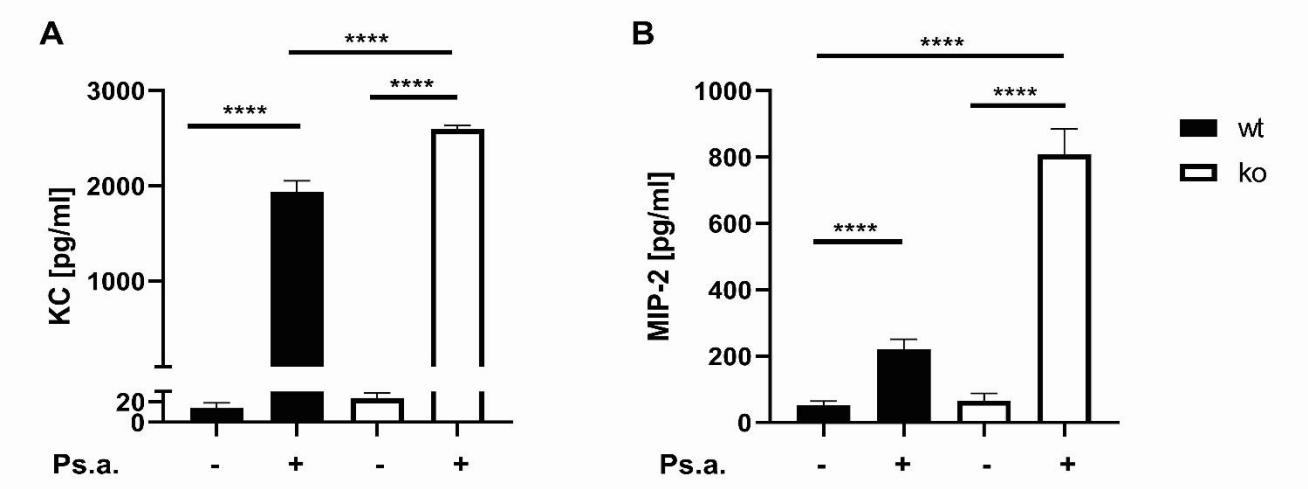

Fig. 23: The cytokine release from murine tracheal epithelial cells

The cytokine response from murine tracheal epithelial cells after stimulation with Ps.a. (A) The release of KC and (B) MIP-2 (CXCL2) was quantified by ELISA in cell free cell culture supernatant 18 hr. after stimulation. The experiment was performed in duplicate. wt – wildtype mice, ko – TRPM4-knockout. N = 4; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0{,}0021$, *** $p < 0{,}0002$, **** $p < 0{,}0001$. Data are shown as mean ± SEM.

After the stimulation; the released cells significantly increased amounts of KC and CXCL2 (MIP-2) **(Fig.23)**. The detection of IL-6 after stimulation with heat inactivated Ps.a. was not possible. In contrast to the human bronchial epithelial cells and cell lines, the release of pro-inflammatory cytokines was significantly higher from cells of TRPM4-knockout mice than from cell of their wildtype littermates. This was somehow surprising; because the inhibition of cytokine release was confirmed by different methods in human cells. This result also differs from the data obtained from murine BMDM.

3.14 Reduced inflammation and antimicrobial response in TRPM4-knockout mice after the stimulation with Ps.a.

The innate immune response in the lung is triggered by different cell types. The epithelial cells come into contact primarily with inhaled bacteria, thereafter the macrophages (interstitial and alveolar) are stimulated by bacteria and chemokines released from epithelial cells. It is a complex and regulated procedure. After obtaining contradictory results from TRPM4-knockout tracheal epithelial cells from mice and human, it was interesting to know how the inflammatory response in TRPM4-knockout mice would develop after the stimulation with heat inactivated Ps.a.

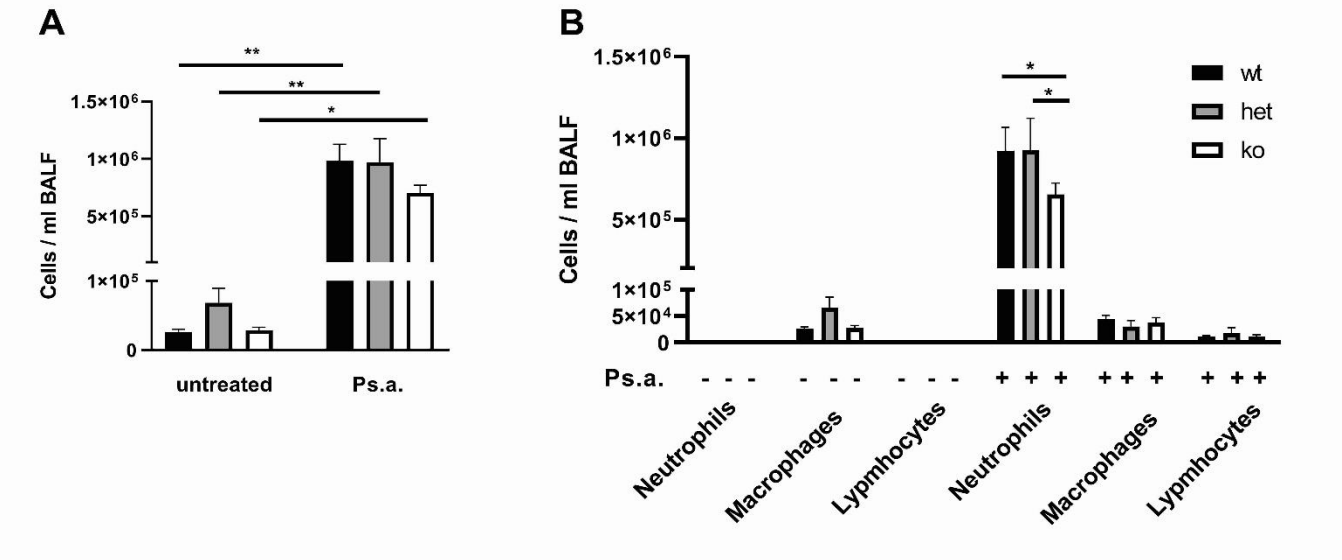

Fig. 24: Leukocyte counts from the BALF of TRPM4-knockout (ko) and corresponding littermate controls (wt, het) treated with heat inactivated Ps.a.

The counts of all leukocytes were compared between the groups (A). After May-Grünwald staining the leukocytes were quantified based upon their morphology (B). wt – wildtype mice, ko – TRPM4-knockout mice, het – TRPM4-heterozuygous mice. N = 5; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0{,}0021$, *** $p < 0{,}0002$, **** $p < 0{,}0001$. Data are shown as mean ± SEM.

For the experiment, the mice were selected to be 8-10 weeks of age and from the same breeding. Only male mice were used for the experiments; because other groups have been found that the expression of TRPM4 is regulated by the female estrus cycle **(Eckstein et al., 2020)**. The mice were stimulated by tracheal intubation with 40 µl of heat inactivated Ps.a. solution.

18 hr. after the stimulation, the BALF was collected, blood was taken by cardiac puncture and the lung was resected. The treatment with Ps.a. induced a significant increase in the number of leukocytes in the BALF, which was lightly reduced and was less significant in the TRPM4-knockout mice **(Fig.24 A)**. Based on their morphology, the cells in the BALF were mainly neutrophilic granulocytes. The number of neutrophils was significantly lower in the TRPM4-ko mice, compared to Ps.a. treated wildtype and heterozygous animals **(Fig.24 B)**.

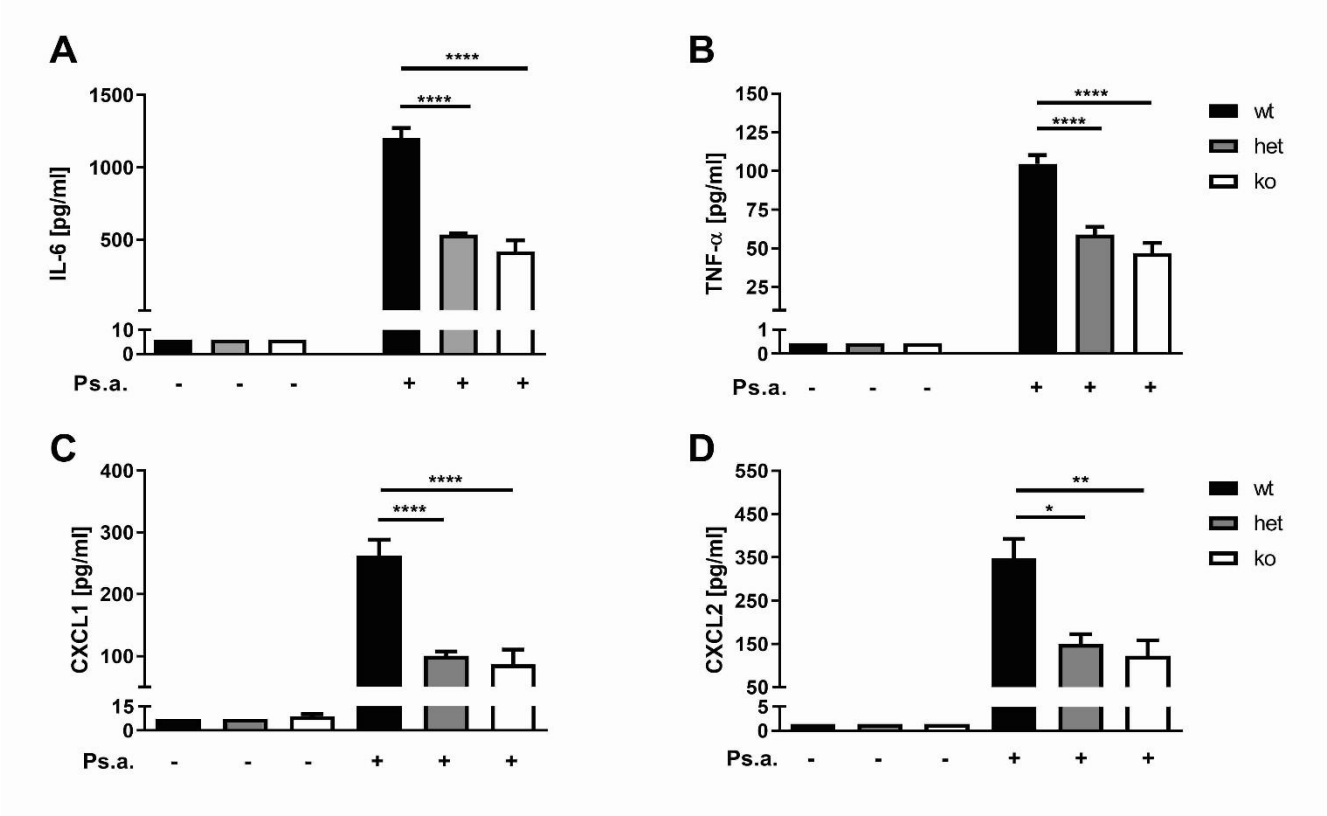

Fig. 25: Inflammatory cytokines in the BALF of mice treated with heat inactivated Ps.a.

The concentration of IL-6 (A), TNF-α (B), CXCL1 (C), and CXCL2 (D) was quantified in cell BALF with a multiplex Luminex immunoassay. wt – wildtype mice, ko – TRPM4-knockout mice, het – TRPM4-heterozuygous mice. N = 5; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0{,}0021$, *** $p < 0{,}0002$, **** $p < 0{,}0001$. Data are shown as mean ± SEM.

The reduced number of neutrophils in the BALF of TRPM4-knockout mice after stimulation with heat inactivated Ps.a. correlated with significantly lower concentrations of IL-6, TNF-α, CXCL1 (KC), and CXCL2 (MIP-2) in the knockout and heterozygous mice **(Fig.25 A-D)**.

Although the stimulation of isolated tracheal epithelial cells from TRPM4-knockout animals with Ps.a. induced a higher cytokine response than that from their wild type littermate controls **(Fig.23)**, this effect seemed to be superseded by the response of macrophages and other immune cells like neutrophils. despite not shown in alveolar macrophages, the release of inflammatory cytokines from BMDMs of TRPM4-knockout mice was lower compared to stimulated wild type controls **(Fig.22)**.

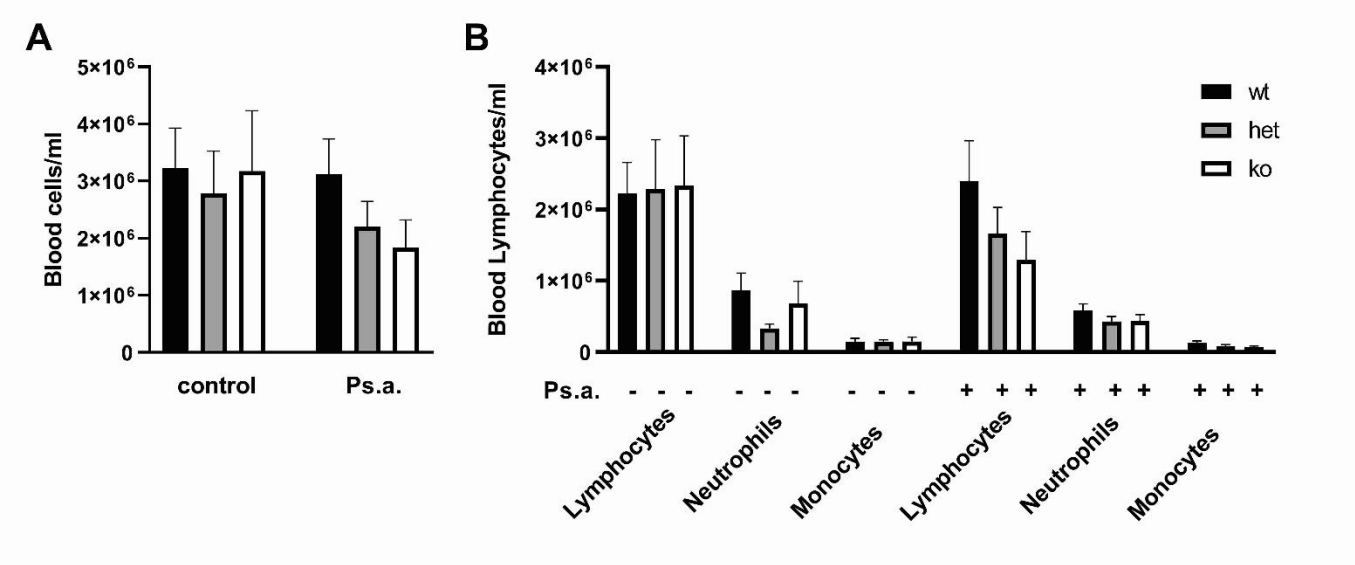

Fig. 26: leukocytes number in the blood from mice treated with heat inactivated Ps.a.

The number of leukocytes in EDTA-stabilized blood from mice after the stimulation with heat inactivated Ps.a., The total number of leukocytes (A) and the number of different leukocyte populations, quantified from blood smears (B). wt – wildtype mice, ko – TRPM4-knockout mice, het – TRPM4-heterozuygous mice. N = 5; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0{,}0021$, *** $p < 0{,}0002$, **** $p < 0{,}0001$. Data are shown as mean ± SEM.

The number of leukocytes in blood was only slightly reduced after the stimulation with Ps.a. **(Fig.26 A)**. This was mostly due to a reduced number of lymphocytes in the blood of TRPM4-knockout mice after Ps.a. stimulation **(Fig.26 B)**. However, the differences observed reached no significance. In contrast the number of neutrophils in the BALF was significantly reduced in TRPM4-knockout mice after Ps.a. stimulation **(Fig.24 B)**.

3.15 Reduced expression of mouse beta defensin (mBD) in the lungs of TRPM4-knockout mice after the stimulation with Ps.a.

Antimicrobial peptides are an important effector of the innate immune response. They are released from epithelial cells and leukocytes after the stimulation with TLR-ligands, like bacterial components. As already shown, the inhibition of TRPM4 in primary human bronchial epithelial cells, resulted in a decreased expression and release of hBD-2, that correlated with an increased survival of Ps.a. **(Fig.20)**. The functional murine homologue to hBD-1 is murine β-defensin-1 (mBD-1) and for hBD-2 is murine β-defensin-4 (mBD-4). To quantify the expression of both peptides after the stimulation with Ps.a., the lung tissue was homogenized in TRIZOL and used for the isolation of RNA. After cDNA-synthesis, the samples were used for the analysis of gene expression for the antimicrobial peptides mBD-1, and mBD-4 by semiquantitative real-time-PCR. The expression of mBD1 and mBD4 in lung homogenate significantly increased after the stimulation with Ps.a. **(Fig.27)**. The expression of mBD1 didn't change significantly in the TRPM4-knockout animals, while the expression of mBD4 was highly significantly reduced in the knockout mice **(Fig.27)**.

These data showed that the knockout of TRPM4 results in a lower inflammatory response and less expression of antimicrobial peptides after the stimulation with heat inactivated Ps.a. in mice.

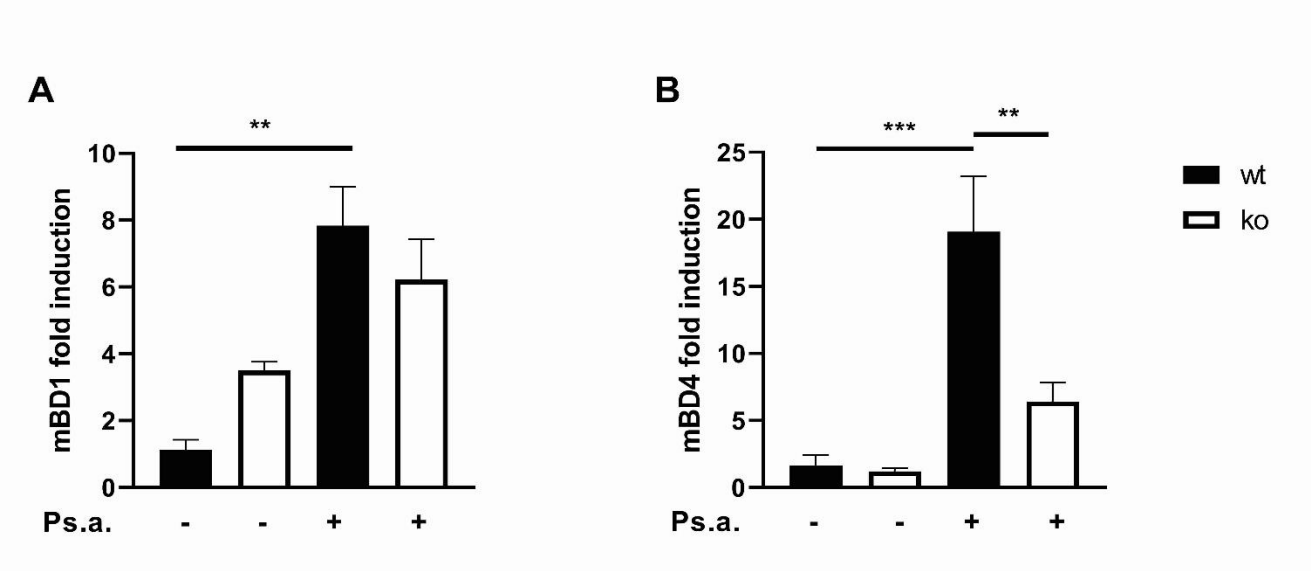

Fig. 27: mBD-1 & mBD-4 expression in mice after treated with heat inactivated Ps.a.

The expression of murine beta-defensin 1 (mBD-1) (A) and murine beta-defensin 4 (mBD4-) (B) in homogenized lung tissue of mice after the stimulation with heat inactivated Ps.a.. The gene-expression was normalized to the expression of β-actin. N = 5; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0,0021$, *** $p < 0,0002$, **** $p < 0,0001$. Data are shown as mean ± SEM.

3.16 The stimulation of TRPM4-knockout mice with LPS leads to an increased inflammatory response

The recognition of bacteria by cells of the innate immune system is mostly accomplished by toll-like receptors, specific for a distinct set of ligands. It has already been shown that bronchial epithelial cells express and respond to TLR-stimulation by different microbial ligands **(Mayer et al., 2007)**. The previous results in this work also showed that human bronchial epithelial cells respond to different TLR-ligands and that the inhibition of TRPM4 led to a reduced release of IL-6 **(Fig.16)**. To test, if the LPS-stimulation of TRPM4-knockout mice would also lead to a decreased inflammatory response, littermates were stimulated with 1 µg LPS for 18 hr.

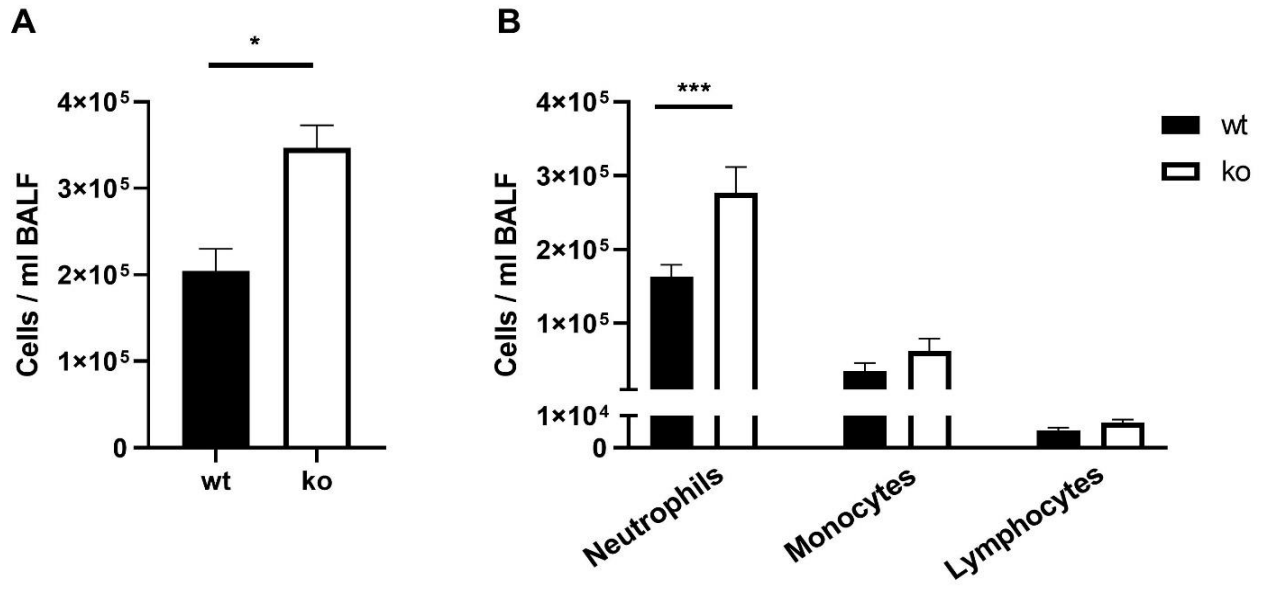

Fig. 28: leukocytes number in BALF of TRPM4-knockout mice (ko) and the corresponding wild type littermates (wt) treated with with LPS.

The stimulation of TRPM4-knockout mice (ko) and the corresponding wild type littermates (wt) with 1 µg LPS. The number of leukocytes in BALF was counted (A) and differentiated based on their morphology after staining (B). N = 4; one-way ANOVA with Turkey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0,0021$, *** $p < 0,0002$, **** $p < 0,0001$. Data was shown as mean ± SEM.

In contrast to the animals that were stimulated with heat inactivated bacteria, after the stimulation with LPS a higher number of leukocytes was present in the BALF of TRPM4-knockout mice compared to their littermate wildtype mice **(Fig.28 A)**. This was mostly induced by a significantly higher influx of neutrophilic granulocytes in the TRPM4-knockout mice **(Fig.28 B)**. Accordingly, the concentration of inflammatory cytokines in the BALF was significantly higher in the TRPM4-knockout mice. The stimulation with LPS induced a significant increase in the treated mice compared to the untreated controls. The cytokines were

quantified in cell free BALF supernatant with a multiplex Luminex-based immunoassay. The amount of IL-6, TNF-α, CXCL1, and CXCL2 **(Fig.29)** was significantly higher in the LPS-treated TRPM4-knockout mice compared to the treated wildtype mice.

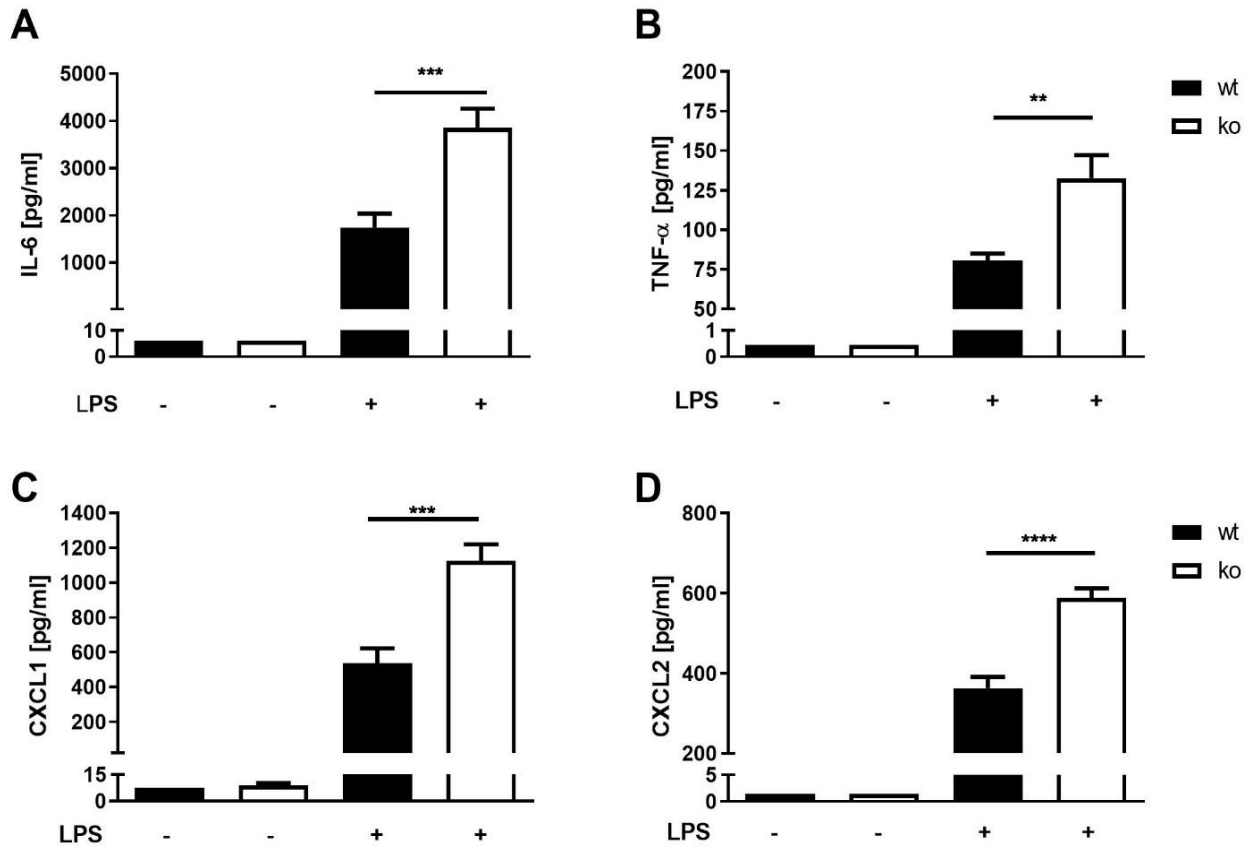

Fig. 29: Cytokines concentration in BALF of TRPM4-knockout (ko) and control mice (wt) treated with LPS.

The concentration of IL-6 (A), TNF-α (B), CXCL1 (C), and CXCL2 (D) in cell-free BALF of TRPM4-knockout (ko) and control mice (wt), 18 hr. after LPS stimulation. The cytokines were quantified with the help of a multiplex Luminex-based immunoassay. N = 4; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0{,}0021$, *** $p < 0{,}0002$, **** $p < 0{,}0001$. Data are shown as mean ± SEM.

The analysis of the number of leukocytes in the blood of LPS-treated mice showed that numbers of leukocytes and the number of the different leukocyte-populations did not change between stimulated TRPM4-knockout and wild type mice **(Fig.30)**. which support the hypothesis, that the stimulation with LPS led to a strictly pulmonary inflammation.

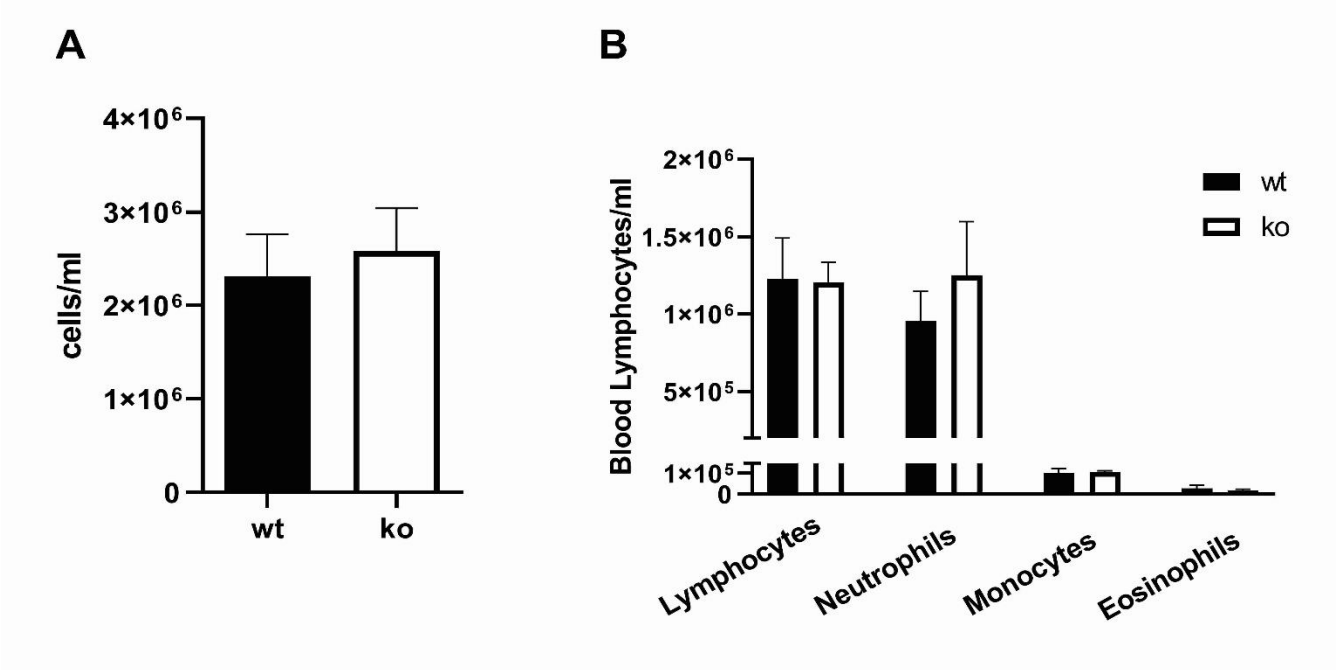

Fig. 30: leukocytes number in blood of TRPM4-knockout (ko) and wild type littermates (wt) treated with LPS.

The number of leukocytes in the blood of LPS-stimulated TRPM4-knockout (ko) and wild type littermates (wt) (A) was counted. The number of different leukocyte populations in the blood was differentiated based on their size and morphology (B). N = 4; one-way ANOVA with Tukey's multiple comparison post-hoc test, * $p < 0.0332$, ** $p < 0,0021$, *** $p < 0,0002$, **** $p < 0,0001$. Data are shown as mean ± SEM.

The number of neutrophilic granulocytes slightly increased in the LPS-treated TRPM4-ko animals but did not reach significance level.

This data shows that during a stimulus with TLR4-ligands, the inflammatory response in the TRPM4-knockout mice is higher than in the corresponding wild type control animals. This is in contrast to the results obtained after the stimulation with heat inactivated Ps.a. **(Fig.24, 25)**, but in line with the response of isolated tracheal epithelial cells **(Fig.23)**. Up to now it is not completely clear why the response is completely different after the stimulation with a mixture of TLR-ligands that are already present in the preparation of heat inactivated Ps.a., and the stimulation with a purified TLR4-ligand. This will be the subject of future investigations and will also be elaborated in the discussion of this work.

Discussion

The main findings of this study were as follows:

1. TRPM4 is expressed in airway epithelial cells.
2. The inhibition of TRPM4 leads to a decreased release of inflammatory mediators from bronchial epithelial cells and macrophage like cells, but it does not influence the expression of TRPM4 or TLR4.
3. TRPM4 is necessary for the secretion of a number of cytokines, while the related transcription is not inhibited.
4. *In vivo*, the inhibition of TRPM4 leads to a reduced expression of antimicrobial peptides, and reduces release and subsequent host defense *in vitro*.
5. TRPM4 knockout mice develop a reduced inflammatory reaction in response to the stimulation with Ps.a.. In contrast, the stimulation with LPS leads to an increased inflammatory response of TRPM4 knockout mice.

During this project we could show that the non-selective monovalent cation channel TRPM4 is an important regulator of the inflammatory response after the stimulation with Ps.a. or bacterial components like LPS, flagellin or nucleotide-mimics (Pam3CSK, Poly-IC) in airway epithelial cells and monocyte derived macrophages.

The inhibition of the TRPM4 channel by the 9-Phenantrol specific inhibitor or siRNA highly significantly reduced the export of typical immune mediators like IL-6, TNF-a, CXCL2, and S100A8. These findings were confirmed in primary human bronchial epithelial cells, epithelial cell line, macrophage cell line and murine monocyte derived macrophages. The reduced cytokine concentrations in the supernatant after the treatment of the cells with the TRPM4-inhibitor and additional bacterial stimulation did not correlate with the decreasing in corresponding transcription. The anti-microbial peptide hBD2 and the alarmin S100A8 were the only mediators during this project that were inhibited on transcriptional level after the inhibition of TRPM4 and bacterial stimulation. This behavior could be related to their dependency on divalent cations.

In vivo, the inflammatory response of TRPM4 knockout mice was reduced compared to the corresponding wild type littermates after the pulmonary stimulation with heat inactivated Ps.a. In contrast, the stimulation with LPS induced a higher inflammatory reaction in the TRPM4 knockout animals than in their wildtype littermates. This finding may be related to the differential recognition of heat killed bacteria and LPS, however it needs to be further investigated.

Most of the previous studies have been performed on TRPM4 channel in different types of tissues, while fewer studies have focused on TRPM4 channel in the epithelial cell, and rarely on airway epithelial cell, that is why TRPM4 channel was chosen for our current study.

TRPM4 and TRPM5 are two closely related cation channels that are expressed in a wide variety of the tissue. TRPM4 is usually highly expressed in the heart, pancreas, and placenta, while TRPM5 expression is located predominantly in the intestine, pancreas and taste buds, and can also be found in the stomach, lung, testis, and brain **(Fonfria et al., 2006)**.

TRPM4 channels are engaged in a wide variety of physiological processes such as T-cell activation **(Pierre Launay et al., 2004)**, allergic reactions, myogenic vasoconstriction **(Crnich et al., 2010)** and neurotoxicity **(Schattling et al., 2012)**. Furthermore TRPM4 plays a role in smooth muscle contraction **(A. C. Smith et al., 2013)**, insulin secretion by pancreatic beta cells **(Cheng et al., 2007)**, cardiac action potentials **(Hof et al., 2013; Simard et al., 2013)**, immune system responses **(Barbet et al., 2008; Vennekens et al., 2007)** and cerebral artery constriction **(Earley, 2013)**. In addition, the channel is involved in pathologies, such as cardiac hypertrophy **(Guinamard et al., 2006)**, human cardiac-genetic diseases **(Kruse et al., 2009; H. Liu et al., 2013)**, autoimmune encephalomyelitis, human multiple tissue sclerosis **(Schattling et al., 2012)**, and ischaemia-reperfusion injuries in heart or brain **(Loh et al., 2014; Simard et al., 2012)**. So, understanding the regulatory function of the TRPM4 channels on the plasma membrane may open a therapeutic window for interference with diseases associated with TRPM4 channel.

Throughout this work, it has been shown that the TRPM4 channel is expressed in the bronchial epithelial cell line NCI-H292. This finding was proved by structured illumination microscopy (SIM), which shows that TRPM4 channels are localized to the nucleoplasm and plasma membrane of the bronchial epithelial cell line NCI-H292 **(Fig.7 A)**.

The release of cytokines is an important step during the activation of cells in the course of a bacterial infection **(Kube et al., 2001; Malhotra et al., 2019)**. Chemokines and cytokines released from epithelial cells attract immune cells or directly interact with the bacteria. Since calcium plays a central role in many processes of the cell, we speculated that an interference of this pathway would lead to a different response of the cells.

We could show that the inhibition of TRPM4 by 9-Phenantrol (9-Ph.) reduced the release of different inflammatory mediators and cytokines in the NCI-H292 cell line. The treatment of the cells with the inhibitor or with heat inactivated Ps.a. had no impact on the release of LDH **(Fig.8)**. During this initial toxicity testing, concentrations of 10 – 40 µM were used, which correspond to 0.5 times to 2 times the reported IC_{50} value of 20 µM **(T Grand et al., 2008)**. The stimulation of the cells with heat inactivated Ps.a. after the pre-

incubation with 9-Ph. blunted the release of IL-6, TNF-α, MIP2-α, and S100A8 nearly completely. This was clearly observed when 9-Ph. was used at a concentration that corresponded to its reported IC_{50} value of 20 µM **(Fig.9)**. This effect was concentration dependent; because at concentrations equal to $1/4^{th}$ the IC_{50} value (= 5µM) the release of the above-mentioned mediators went back to concentrations similar to those, observed in samples without 9-Ph. **(Fig.10)**. The inhibition of TRPM4 by 9-Ph. was mostly affected the release, while the transcription of IL-6, TNF-α, and MIP2-α was not influenced. Surprisingly, the transcription of S100A8 and hBD-2 were inhibited by 9-Ph. in cells, that were subsequently stimulated with heat inactivated or live Ps.a. **(Fig.11 C, Fig.20 B)**. Since TRPM4 is activated by changes in the membrane potential of the cell, PIP2, and an increase of the concentration of Ca^{2+}, these findings implicate that the expression of S100A8 and hBD-2 depends on one of these factors, while the expression of IL-6, TNF-α, and MIP2-α is not affected by Ca^{2+}, PIP2 or changes in membrane potential.

We choose 9-phenanthrol as a specific TRPM4 inhibitor. Although there are many other compounds, including MPB-104, quinidine, quinine, flufenamic acid, and intracellular spermine that have the ability to inhibit TRPM4 channel, nevertheless their use has been limited; due to their weak selectivity toward TRPM4 channel among other ion channels **(Simard et al., 2013)**. 9-Ph. has been described as a selective inhibitor for TRPM4 channel, with an IC_{50} of 1 µM in endothelial cells and an IC_{50} of 20 µM in HEK293 transfected cells **(Guinamard et al., 2014)**.

Additionally it was shown that 9-Ph. inhibits the chloride channel TMEM16A (transmembrane protein 16A), which is mainly found in smooth muscle cells **(Burris et al., 2015)**. Since the IC_{50} of 9-Ph. for TMEM16A is 12 µM in arterial myocytes, it is most likely that after the use of 9-Ph. in an animal model, artery smooth muscle contraction may be impaired before the TRPM4 channel is inhibited. *In vivo* usage of 9-phenanthrol remains controversial and a source of anxiety, due to its toxicity effect as a member of polycyclic aromatic hydrocarbons.

The specificity of 9-Ph. has initially been described in TRPM4-transfected HEK-293 cells **(T Grand et al., 2008)**. The author could show no inhibitory activity of 9-Ph. at 10^{-4} M on the closely related TRPM5 channel. Others also showed no inhibition of TRPC3 or TRPC6 at 3×10^{-5} M in HEK-293 cells **(Gonzales et al., 2010)**. They also confirmed the IC_{50} value of the endogenous TRPM4 in isolated cerebral artery myocytes, which was comparable to the value obtained for the HEK-293 transfected TRPM4 channel in previous publication. Therefore, it is very likely that the influence on the release of cytokines and host defense molecules in airway epithelial cells is due to the lack of activity of TRPM4.

To confirm the results obtained after the inhibition of TRPM4 with 9-Ph., NCI-H292 cells were transfected with a plasmid that expresses TRPM4-specific shRNA, driven by a U6 promoter. The shRNA was obtained from a commercial supplier and had been validated by the RNAi-consortium at the Broad-Insitute (MIT,

Havard, Cambridge, MA, USA). The shRNA selectively binds to exon 11 (shRNA2) and exon 17 (shRNA1), which are in close proximity of the ion-transducing domain, spanning exon 17-19 (**Fig.5**). The control in this experiment was a non-target shRNA-plasmid from the same supplier in the same vector-backbone. The use of the TRPM4-specific shRNA inhibited the release of IL-6 in a similar way as already observed in experiment, where the pharmacologic inhibition of TRPM4 was used. This shows that the presence and function of TRPM4 are necessary for an appropriate release of IL-6 in the course of a bacterial stimulation.

Bronchial epithelial cells respond to bacteria through different pathways. They express a distinct set of TLRs, that form hetero- or homodimers and bind a specific class of bacterial ligands **(Thorley et al., 2011)**.

Heat inactivated bacteria consist of a mixture of different TLR-ligands, like lipopeptides, flagella, bacterial DNA, and cytoplasmic components. To evaluate, if the reduced release of cytokines is associated with the activation of a distinct TLR-stimulation, the cells were stimulated with either LPS, flagellin, or Pam3CSK. It is also important to evaluate if the activity of TRPM4 is necessary for the release of cytokines from primary cells. Heat inactivated Ps.a., LPS, and flagellin induced the release of IL-6 from primary human bronchial epithelial cells. LPS and Pam3CSK also induced the release of IL-6 from NCI-H292 cells. In all of those conditions, the activity of TRPM4 was necessary for the release of IL-6 into the cell culture supernatant, because the pre-incubation with 9-Ph. significantly inhibited the Ps.a. induced release of IL-6 **(Fig.16)**.

One possible explanation of the reduced cytokine release after TRPM4 inhibition and bacterial stimulation could be a reduced expression of a TLR or a reduced expression of TRPM4. TLR4 is one of the most prominent TLRs, specific for bacterial LPS and required for an antimicrobial response of airway epithelial cells **(MacRedmond et al., 2005)**. Ps.a. is able to activate airway epithelial cells either by flagellin via TLR5 **(Z. Zhang et al., 2005a)** or through its lipid core of LPS **(Hajjar et al., 2002)**.

The expression of TLR4 or TRPM4 was not inhibited by 9-Ph., conversely heat inactivated Ps.a. significantly induced the expression of TLR4 **(Fig.13)** in NCI-H292 cells. Although it may be possible that the expression of other TLRs like TLR5 could be influenced by the inhibition of TRPM4, the main focus of this work is the interplay between bacterial stimulation, the activity of TRPM4, and the resulting host response.

These findings, together with the finding that the gene expression of IL-6, MIP2-α, and TNF-α was not inhibited by reduced TRPM4 activity, led to the hypothesis that the function of TRPM4 is necessary for export of these mediators out of the cell. To validate this finding, the concentration of IL-6 was examined in cell lysate and supernatant. After the inhibition of TRPM4 and the stimulation with heat inactivated Ps.a., the supernatant of the cells was collected separately from the cell lysate and analyzed by ELISA. Our result revealed that after bacterial stimulation, the concentration of IL-6 in cells with functional TRPM4 showed

equilibrium between the concentration found in supernatant and cell lysate. In contrast, the inhibition of TRPM4 prior to bacterial stimulation induced a significantly higher concentration of IL-6 inside the cell, compared to the supernatant **(Fig.14)**, although the expression of IL-6 was not influenced by the inhibition of TRPM4 **(Fig.11 A)**. To confirm this finding, cells were again treated with 9-Ph. and stimulated with heat inactivated Ps.a.. The detection of intracellular IL-6 was performed by FACS analysis using a phycoerythrin coupled IL-6-specific antibody.

The results show that while in the untreated group only 1.4 % of the cells were stained positive for IL-6, this value raised to more than 23% in the Ps.a. stimulated cells. In contrast, the inhibition of TRPM4 and the additional stimulation with Ps.a. resulted in nearly 95% positive staining for IL-6 **(Fig.15)**.

These results confirmed that the function of TRPM4 is necessary for a successful export of IL-6, TNF-α, and MIP2-α after a bacterial stimulation. Since it has been shown by other publications that the inhibition of TRPM4 results in an accumulation of Ca^{2+} in the cytosol, and a change in the membrane potential **(T Grand et al., 2008; P. Launay et al., 2002)**, it is highly possible that the Ca^{2+} concentration and thereby the charge of the cell plays an important role in the export of cytokines from non-excitable cells.

A more experimental approach consisted in the quantification of immunofluorescence images obtained by SIM. This is usually not a straight-forward approach, since the normalization is most likely difficult. Mander's coefficient is used to determine the colocalization of two different proteins of interest. Here, the colocalization of IL-6 with β-Actin was used, with the assumption that the staining for β-Actin was not influenced by the experimental conditions. β-Actin is also frequently used for normalization in the analysis of western blots or qRT-PCRs. The results show that the colocalization increased after the stimulation with Ps.a. compared to the untreated cells. These values increased even more in the samples, that had been treated with the TRPM4 inhibitor prior to Ps.a. stimulation **(Fig.17)**. These results correlate with the previous analysis and confirm that the export of IL-6 requires the function of TRPM4.

The export of cytokines is a highly regulated process and has been investigated in more detail in macrophage cell lines (RAW264.7) **(Manderson et al., 2007)** and dendritic cells **(Verboogen et al., 2018)**. They showed that IL-6 and TNF-α accumulate within 1 hr. after LPS-stimulation in the perinuclear Golgi complex and are transported further through the trans-Golgi network to recycling endosomes; suggesting that the Golgi could serve as a bottleneck restricting the secretion of the newly synthesized IL-6. In the recycling endosome, IL-6 and TNF-α localize to separate regions. IL-6 is transported directly to the cell surface while TNF-α is exported through phagocytic cups **(Manderson et al., 2007)**.

Since airway epithelial cells are less actively secreting cells compared to macrophages, these mechanisms may not take place to the same extent. Nevertheless, the finding of this thesis indicates that the traffic of

vesicles from the endoplasmic reticulum to the trans-Golgi network towards the recycling endosome may be dependent on the activity of TRPM4, since the release of IL-6 and TNF-α is reduced after the inhibition of TRPM4.

The influence of TRPM4 on the regulation of secretion has been investigated in more detail for insulin. It has been shown that the expression of a dominant negative form of TRPM4 significantly reduced the secretion of insulin from rat pancreatic β-cells **(Cheng et al., 2007)**. It was also shown by another group that 9-Ph. inhibits the glucose or the glucagon-like-peptide-1 (GLP-1) induced insulin secretion from isolated rat pancreatic islets in a dose dependent manner **(Ma et al., 2017)**. Another publication showed that the mechanism of glucose-stimulated insulin secretion from pancreatic islets is driven by the activation of PLC by GLP-1. The activity of PLC, in turn, leads to the synthesis of DAG and IP_3. This pathway is dependent on the Ca^{2+} mobilization from intracellular Ca^{2+} stores. The Ca^{2+} mobilization is mainly mediated by TRPM4 and TRPM5. Accordingly they could show that the GLP-1 mediated insulin secretion was severely impaired in TRPM4 and TRPM5 knockout mice **(Shigeto et al., 2015)**.

Protein synthesis involves a fine regulated network of transcription factors and upstream activating kinase-pathways. The transcription factor complex Nf-kB is one of the most prominent ones, which is also involved in downstream signaling events of TLR-signaling and host-response. The signalling generated by LPS activates many post-translational modifications in the Nf-kB p65 subunit (Nf-kB p65) prior to nuclear translocation **(Vallabhapurapu & Karin, 2009)**. Nf-kB p65 phosphorylation is correlated with nuclear translocation in mice **(Zhong et al., 1997)** and consequently with transcriptional transactivation **(Yang et al., 2003)**.

The stimulation of NCI-H292 cells with heat inactivated Ps.a. induced the translocation of Nf-kB to the nucleus. The treatment with 9-Ph. alone induced the activation of Nf-kB, that was comparable to untreated controls. Surprisingly the translocation of Nf-kB was not increased in cells that were treated with 9-Ph. 6 hr. prior to Ps.a. stimulation **(Fig.19 A)**. This indicates that the function of TRPM4 is necessary for the activation of Nf-kB in airway epithelial cells. For hBD-2 it is known that for an efficient transcription all Nf-kB binding sites on the promoter need to be occupied **(Kao et al., 2008; Tsutsumi-Ishii & Nagaoka, 2002)**. Additionally, we observed that the expression of PLC-β2 was downregulated after the inhibition of TRPM4. The amount of PLC-β2 detected by western blotting was higher in cells that were stimulated with heat inactivated Ps.a.. In contrast, the concentration was lower in cells that had been treated with 9-Ph. prior to bacterial stimulation **(Fig.19 B)**. This is mostly due to an increased concentration of Ca^{2+} inside the cells, which has also been shown for dendritic cells of TRPM4-knockout mice after the stimulation with *Escherichia coli* **(Barbet et al., 2008)**. The decreased expression of PLC-β2 after the inhibition of TRPM4

also indicates that the inhibition of TRPM4 leads to a change in the intracellular Ca^{2+} concentration in NCI-H292 airway epithelial cells.

Members of the PLC-family are important for the signaling of G-protein coupled chemokine receptors (reviewed by **(Hubbard & Hepler, 2006)**). The stimulation with Ps.a. led to an upregulation of PLC-β2, which is known to induce the synthesis of IP3, which in turn binds to the endoplasmic reticulum receptors and induces intracellular Ca^{2+} release. It was also shown that the reduced PLC-β2 expression in the TRPM4-knockout cells correlated with a decreased CCL21-mediated chemotaxis of mature dendritic cells **(Barbet et al., 2008)**.

The activation of the MAPK p38, Erk1/2, and Jnk by LPS in the airway epithelial cell line Beas2B has been already shown **(Guillot et al., 2004)**. However, after the stimulation with heat inactivated Ps.a. in NCI-H292, only a slightly increased phosphorylation of Erk1/2 was detected **(Fig.19 B)**. The inhibition of TRPM4 induced an increased phosphorylation, that was not further increased by the additional stimulation with Ps.a.. This indicates that the activity of TRPM4 independently of Ps.a. stimulation leads to an increased activation of Erk1/2.

A direct involvement of TRPM4 in Nf-kB mediated transcriptional initiation has not been published so far; however, it could provide an explanation for the finding of this work that the expression of hBD-2 was highly suppressed after the inhibition of TRPM4. The expression of antimicrobial peptides after bacterial stimulation is a first and immediate response of bronchial epithelial cells. Our result showed that the expression of hBD-2 was highly induced after the stimulation with heat inactivated Ps.a. and it correlated with increased antibiotic activity and extracellular concentration of hBD-2. In contrast, the pre-incubation with 9-Ph. decreased the antimicrobial activity in a time dependent manner which correlates with a decreased expression and extracellular concentration of hBD-2. In contrast to IL-6. TNF-α, and MIP2-α, the inhibition of TRPM4 had a direct influence on the transcription of hBD-2, which resulted in an impaired anti-microbial defense of the cells. The time dependent reduction of gene expression indicates that the transcriptional activation of hBD-2 involves factors influenced by Ca^{2+}. It has been shown that the inhibition of TRPM4 leads to an increased concentration of Ca^{2+} in the cell **(Rixecker et al., 2016)**. This mechanism seems to be specific for hBD-2, since the transcription of hBD-1 was not influenced by the activity of TRPM4. Another important aspect of this result is that cellular stimulation whether by live bacteria, heat inactivated bacteria or bacterial components is dependent on TRPM4-activity.

Macrophages play an important role in the innate immune defense of the lung. They are involved in the phagocytosis and the direct antimicrobial response. Alveolar macrophages represent population of specialized tissue resident macrophages that persist in the lung and act as a sentinel cell population to act immediately upon the contact with microbes or small particles. Alveolar macrophages belong to a group of

tissue resident macrophages that develop before the hematopoietic system develops and colonize fetal tissue; to differentiate into specialized, tissue resident and self-renewing macrophage populations **(Takahashi et al., 1989)**. Upon a bacterial stimulation, additional blood derived monocytes will be attracted and support the host defense reaction. Additionally, blood derived monocytes may give rise to different lung macrophage populations, like interstitial, alveolar, pulmonary intravascular, and intermediate state macrophages **(Evren et al., 2020)**. Many publications have used cell lines, bone marrow derived or alveolar macrophages interchangeably; to investigate the immune response after stimulation with LPS or bacteria with comparable results **(Rayees et al., 2019; Woods et al., 2020)**. Also different results between primary cells and cell lines have been reported after the stimulation with bacteria **(Andreu et al., 2017)**.

In the course of this project, the inhibition of TRPM4 had similar effects on macrophages of human and mouse origin. The inhibition of TRPM4 with 9-Ph. in the human macrophage-like cell line U937 and in murine BMDM led to a highly significantly decreased extracellular concentration of IL-6 after the stimulation with heat inactivated Ps.a. **(Fig.21)**. This indicates that the involvement of TRPM4 in the export of cytokines is not restricted to human cells. To further investigate the role of TRPM4 in the innate immune response, TRPM4-knockout mice were used to isolate cells and to investigate the function to TRPM4 in a pneumonia model.

Knockout animals have been very useful in other studies to investigate the function of different signaling pathways *in vivo*. TRPM4-knockout mice have been generated by different groups, either by excising exon 3-6 **(Barbet et al., 2008)** or exon 15-16 that includes the first transmembrane spanning domain **(Vennekens et al., 2007)**. During this work mice with deleted exons 15-16 were used.

It has been shown that TRPM4 decreases the anaphylactic response and IgE-dependent mast cell activation **(Vennekens et al., 2007)**, and that TRPM4 is necessary for the migration of dendritic cells **(Barbet et al., 2008)**. Additionally, it has been shown *in vivo* that the TRPM4 channel is necessary for the function of macrophages and monocytes, and is beneficial for the survival in a murine sepsis model **(Serafini et al., 2012)**.

To confirm the previous results obtained by the 9-Ph. TRPM4 inhibitor, macrophages from TRPM4-knockout mice were isolated from the bone marrow and differentiated in the presence of M-CSF to generate macrophage like cells. Depending on our finding, we could assume that the function and presence of TRPM4 were necessary; to induce the release of IL-6. In this experiment, the cells that were treated with the pharmacologic TRPM4 inhibitor 9-Ph. or isolated from TRPM4-knockout mice released lower concentrations of IL-6, TNF-α, CXCL-1, and CXCL-2 in the cell culture supernatant in response to bacterial infection compared to cells that were isolated from wildtype mice **(Fig.22)**. These results are consistent with

the findings that TRPM4 is needed to initiate an inflammatory response in human airway epithelial cells and monocyte derived macrophages cell lines **(Fig.16, Fig.21)**.

Next, a pneumonia model with heat inactivated Ps.a. was used to investigate the contribution of TRPM4 on the inflammatory response in TRPM4-knockout mice, where the samples were obtained and analyzed 18 hr. after stimulation. In concordance with the previous in vitro experiments, the stimulated knockout animals displayed a reduced inflammatory response compared to the wildtype littermates. The stimulation with heat inactivated Ps.a. induced an influx of leukocytes into the lung, which predominantly contained neutrophilic granulocytes, where the number of cells was significantly lower in TRPM4-knockout mice compared to wildtype and heterozygous animals **(Fig.24)**. The decreased cellular influx correlated with significantly lower concentration of IL-6, TNF-α, CXCL1, and CXCL2 in the BALF of TRPM4-knockout mice. Although the number of cells was not significantly decreased in the heterozygous animals, the concentration of inflammatory markers was significantly lower than in the wildtype mice and comparable to the knockout animals. This indicates that the influx of leukocytes was not affected but their activation may have been impaired, this assumption needs to be proven by further investigation. Additionally, the expression of the antimicrobial peptides mBD1 and mBD4 was significantly induced after the stimulation with heat inactivated Ps.a. in wildtype mice **(Fig.27)**. In line with the results from the experiment with differentiated primary human airway epithelial cells, the expression of mBD4 is significantly reduced in the TRPM4-knokcout animals, where the defensin mBD4 is the murine homologue to the human defensin hBD2.

In contrast to our previous findings, the pulmonary stimulation of mice with LPS induced the influx of a higher number of leukocytes in the lung of knockout littermates compared to wild type mice. The leukocytes isolated from the BALF were predominantly neutrophilic granulocytes (**Fig.28**). Their number was also significantly higher after LPS stimulation in the TRPM4 knockout mice than in the wildtype-littermates and correlates with increased concentrations of IL-6, TNF-α, MIP1, and MIP2 in the BALF (**Fig.29**).

In line with these results, tracheal epithelial cells that were isolated from TRPM4-knockout mice and differentiated at the air-liquid interface, produced significantly higher concentrations of KC and CXCL-2 than their corresponding wildtype controls after the stimulation with heat inactivated Ps.a. **(Fig.23)**. The release of IL-6 was not detectable in cells from wildtype or TRPM4-knockout mice. This is in contrast to the previous results obtained from human primary cells and cell lines that showed reduced secretion of IL-6, TNF-a, MIP1, and other host defense molecules after inhibition of TRPM4 and additional stimulation with TLR-ligands or heat inactivated Ps.a. **(Fig.9, Fig.16)**.

The findings; that the inflammatory response after the stimulation with heat inactivated Ps.a. is lower in the absence of TRPM4, is in contrast to the results from the work that investigated the function of macrophages and monocytes in a murine sepsis model **(Serafini et al., 2012)**. In their sepsis model the inflammatory

response is significantly increased, and the survival rate decreased in the absence of TRPM4. This may be a result of the disease model that they used. It's also possible that the reactivity of intestinal macrophages differs from pulmonary macrophages. While the intestine gets in contact with microbes more frequently, the lung is far more less colonized by microbes and may therefore display a different sensitivity towards microbial stimulation.

In contrast to the stimulation with Ps.a., these results indicate that LPS might trigger a different response in mouse cells. Additionally, LPS used in this experiment was isolated from *Escherichia coli*, which is different in structure than LPS isolated from Ps.a.. A recent publication showed similar results obtained from TRPM5-knokcout mice **(Sakaguchi et al., 2020)**. They could show that in the absence of TRPM5 the severity of an LPS induced inflammation in a sepsis model is significantly higher than inflammation in the corresponding wildtype animals. Since TRPM4 and TRPM5 are two closely related channels with similar properties, their results are also important for the current experiment. While pure LPS mainly activates TLR4-signaling pathways, heat inactivated Ps.a. stimulates a much wider spectrum of TLR-pathways. This may lead to different effects in respect to the calcium concentration and overall charge of the cells.

Actually, different cellular response toward LPS could be attributed to many factors such as types of airway epithelial cells, cell culture conditions and heterogeneous cell calcium responses **(Paradiso et al., 1999)**. However the LPS used in other research did not generate a lethal effect on 16HBE14o cells and the structure of LPS could influence various cellular responses, LPS lipid A of Ps.a. shows less toxicity than *Escherichia coli* lipid A attributable to its penta-acylated form **(Pier, 2007)**. It was demonstrated that A hexa-acylated lipid A is related to a stronger inflammatory response **(Ernst et al., 2003)**. In addition, the O-antigen length of the LPS also acts as a virulence determining factor **(Cryz et al., 1984)**.

Another main reason for LPS unresponsiveness is attributable to lack of TLR4 **(Kumar et al., 2006) (Ueta et al., 2004)**. On the one hand some researchers postulate that respiratory epithelial cells express a complex of LPS receptors comprising TLR4 and MD-2. Furthermore, production of proinflammatory mediator in respond to LPS is mediated by intracellular TLR4 **(Guillot et al., 2004)**. On the other hand others claim that human corneal epithelial cells react to TLR2, TLR3, and TLR5 ligands *in vitro*, but do not respond to LPS **(Y. Sun & Pearlman, 2009) (Ueta et al., 2004) (Visintin et al., 2006)**. While other research shows that TLR4 is not present on the surface of intestinal epithelial cells, they demonstrate that it is distributed intracellularly on Golgi apparatus, where in respond to internalized LPS, TLR4 co-localization could be affected. This is, in turn, contrary to monocytes which could express TLR4 on its surface membrane **(Hornef et al., 2002)**.

In accordance with the absence of TLR4 expression on the cell surface of the pulmonary epithelial cells, it has been observed that there is no effect on LPS activation after addition of the TLR4 blocking antibody in

the extracellular medium, where the assessment based on the measurement of IL-8 secretion. By comparison, the TLR4 blocking antibody decreased the activation of LPS-induced U-937 macrophage cell lines by 68 %. Intracellular compartmentalization of TLR4 may play a vital role in preventing inopportune activation of pulmonary epithelial cells, which is suggested to be due to frequent exposure to air containing trace amounts of LPS and as a consequence a chronic inflammatory state **(Guillot et al., 2004)**. Therefore, TLR4 signalling can only be activated when exposed to a massive quantity of considerable or bacteria-related LPS as happens in infectious diseases **(Schulz et al., 2002) (Simpson et al., 1999)**.

MD-2 & CD14, as a co-stimulatory accessory molecule, plays a vital role in LPS responsiveness. TLR4 in conjunction with its accessory molecules facilitates the identification of even very low picomolar concentration of LPS **(Gioannini et al., 2004)**. In mice, neither TLR4 deficient nor MD-2 deficient mice, couldn't show LPS responsiveness. So, it is concluded that TLR4/MD-2 complexes are crucial for LPS response **(Hoshino et al., 1999) (Shimazu et al., 1999) (Nagai et al., 2002)**. It has been proved that MD-2 is not expressed in human corneal epithelial cells and that exogenous MD-2 or transfection with plasmid-expressing MD-2 confers LPS responsiveness **(Y. Sun & Pearlman, 2009) (Visintin et al., 2006)**. In agreement with the latter observations, it has been showed that TLR4 is substantially expressed on the surface of human corneal epithelial cells, while MD-2 is not detected at either RNA or protein level **(Roy et al., 2011)**. Nevertheless, LPS responsiveness was detected in the presence of MD-2 surface receptor in primary human corneal epithelial (HCE) cells and HCE cell lines. In line with our assumption that the cellular response towards Ps.a. is different from LPS, it was found that IFN-γ which is usually produced during Ps.a. corneal infection, could induces MD-2 expression and LPS responsiveness in HCE cell lines by JAK-2-dependent STAT1 activation and direct binding to the MD-2 promoter results in activation of transcriptional factor, MD-2 gene expression and subsequent surface expression. These data were confirmed by external trails, which prove that IFN-γ was able to induce MD-2 mRNA, MD-2 cell surface expression, and LPS responsiveness in primary HCE cells and HCE cell lines **(Roy et al., 2011)**.

Although many researchers postulate that TLR4 activation depends on combination between LPS and CD14 to form monomeric endotoxin, the CD14/LPS complexes are then transferred to MD-2. This binding contributes to homodimerization of the TLR4/MD-2 complex, resulting in TLR4 downstream signalling activation **(Shimazu et al., 1999) (Viriyakosol et al., 2001) (Visintin et al., 2001) (Ohto et al., 2007) (H. M. Kim et al., 2007)**. Others claim that CD14 protein staining couldn't be identified in lung epithelial specimens and also in the pulmonary epithelial cell line A549 **(Guillot et al., 2004)** Therefore, the debate that exists regarding the expression and function of CD14 in LPS-induced lung epithelial activation is currently not dispelled. Several authors indicated that these cells are CD14 negative **(Hedlund et al., 2001) (Pugin et al., 1993)**, While others have shown both CD14 mRNA and cell surface protein could be

expressed in human epithelial airway cells **(G. Diamond et al., 2000) (Schulz et al., 2002) (Becker et al., 2000)**. These differences could be explained by the different stimulation or differentiation conditions used for epithelial cells throughout these different studies. In reality, these contradictory findings need further investigations to be approved.

LPS concentration is one of the major factors affecting cellular response. Relatively elevated levels of LPS (0.1-1 µg/ml) were required for inflammatory cytokines production by epithelial cells, such as IL-8 and IL-6. Compared to phagocytic cells where it was found to be induced by a lower concentration ranging from 1 to 10 ng/ml LPS **(Guha & Mackman, 2001) (Zarember & Godowski, 2002) (F. X. Zhang et al., 1999)**. Therefore, in the pulmonary epithelium, initiation and coupling to downstream signalling events tend to be less successful than those in myeloid cells **(Guillot et al., 2004)**.

On the other hand, heat inactivated Ps.a. contains a mixture of different TLR-activating components. LPS is the specific ligand for TLR4. Ps.a., and more general, pathogenic bacteria produce virulence factors that are beneficial for the survival of the bacteria, however they are mostly detrimental to the infected host. In the case of Ps.a., these factors are composed of T3SS, various proteases, lipases, pyocyanin, phospholipases and rahmnolipids and other factors **(Sadikot et al., 2005)**. They induce cell damage directly or by binding to different TLR-receptors or other danger-associated molecule receptors like RAGE (receptor for advanced glycation end products) **(Lutterloh et al., 2007; Ramsgaard et al., 2011)**.

It is also likely that part of the different inflammatory responses in TRPM4-knockout mouse cells and human cells originate from the different development of the immune system between mouse and humans during evolution. One good example for this diverging evolution is the different stimulation of LPS from *Neisseria meningitis* LpxL1 by the TLR4/MD2 complex in human and mouse dendritic cells **(Steeghs et al., 2008)**. In contrast to the wild type strain, the LpxL1 strain contains a mutated Lipid-A, that is penta-acetylated in contrast to the wild type hexa-acetylated form. While mouse dendritic cells are activated by the mutated and the wild type LPS, human dendritic cells are only activated by the wild type form of LPS from *Neisseria meningitis*. Furthermore, the authors of this study could show that this was mostly due to a different recognition of LPS by the human TLR4 and that MD2 had less impact on the TLR-4 dependent signaling. Lipid-A of bacterial LPS is the major endotoxicity inducing part **(Galanos et al., 1985)**. The biological activity of LPS can be further modified either by phosphorylation, length alteration or composition modification of the fatty acyl part of Lipid-A **(Takada & Kotani, 1989)**.

It has also been shown that after the stimulation with LPS, mouse and human macrophages activate different downstream signaling cascades **(J. Sun et al., 2016)**. They showed that the stimulation of mouse macrophages with LPS mostly activates IRAK4 and IRAK2 while the pro-inflammatory signaling of human macrophages was dependent on IRAK1.

Another explanation could be that heat inactivated Ps.a. contains molecules that modulate the activity of other signaling pathways, which may lead to the opposing outcomes when compared with LPS stimulation. It has been shown that rhamnolipids from Ps.a. are able to suppress the absorption of Na^+ in epithelial cells **(A. Graham et al., 1993)**. Flagellin has been shown to inhibit Na^+ absorption in murine tracheal epithelium **(Kunzelmann et al., 2006)** and promote the chloride secretion in Calu-3 (human airway epithelial cell-line) **(Illek et al., 2008)**.

In vivo, the airway epithelium is capable of chloride secretion and sodium absorption. Surprisingly, LPS extracted from different bacteria has different action on the cell in regulating epithelial ion transport. For example, LPS from Ps.a. has been documented to alter the ion transport function in mammalian alveolar epithelial cells via inhibition of the sodium absorption through the epithelial Na^+ channel (ENaC) **(Boncoeur et al., 2010)**. In comparison, in guinea pig tracheal epithelium, *Salmonella enterica* LPS induced sodium absorption by enhancing the expression of Na^+/K^+ ATPase **(Dodrill et al., 2011)**. While in human airway epithelial cells *Escherichia coli* LPS reported to increase the expression of chloride channel **(Hauber et al., 2007)**.

Many studies have already shown the involvement of TRPM4 in different physiological processes, which may be important in diseases. Since TRPM4 is necessary for the glucose induced secretion of insulin from pancreatic beta-cells **(Cheng et al., 2007)**, the development of drugs that would specifically promote the activity of TRPM4 in the absence of glucose would mitigate the insulin deficiency in diabetic patients.

Many studies show an involvement of TRPM4 in cardiovascular and endothelial functions. It has been shown that TRPM4 is needed for pharmacologically induced endothelial cell contraction. Furthermore, the authors of that study already suggested TRPM4 a valuable target in the treatment of overactive bladder **(A. C. Smith et al., 2013)**. TRPM4 plays a role in the vasoconstriction and influencing the contractibility of rat artery cerebral smooth muscle cells, which are important for the regulation of the cerebral blood flow **(Earley et al., 2004)**. In addition, the phorbol 12-myristate 13-acetate (PMA) induced constriction of rat isolated cerebral arteries was significantly reduced after the inhibition of TRPM4 with siRNA or 9-Ph. **(Crnich et al., 2010)**.

TRPM4 considered as a key regulator in coordination with Na^+/Ca^{2+}exchanger 2 (NCX2) for the release of both types of mucin MUC2 and MUC5AC. In differentiated normal human bronchial epithelial (NHBE) cells and tracheal cells obtained from cystic fibrosis patients, it was demonstrated that blocking of TRPM4 or NCX channel in these cells leads to abolishment of MUC5AC mucin secretion. Also knocking down the particular TRPM and NCX isoform has a greater impact on the mucin MUC2 and MUC5AC secretion from colonic epithelial cells and bronchial epithelial cells respectively **(Cantero-Recasens et al., 2019)**.

T-cells are part of the adaptive immune system. It has been shown the knockout of TRPM4 in the human T-cell-line Jurkat leads to an increased Ca^{2+} influx that resulted in significantly higher IL-2 secretion after the T-cell receptor stimulation with phytohemagglutinin (PHA) (reagent for in vitro stimulation of human and mouse leukocytes) **(Pierre Launay et al., 2004)**. These results highlight that the involvement of TRPM4 in the immune response depends on the cell type and the stimulus.

It has also been shown that TRPM4 may mitigate the axonal and neuronal degeneration in autoimmune encephalomyelitis (EAE) **(Schattling et al., 2012)**. They showed that the neurons from TRPM4-knockout mice were protected against excitotoxic stress and energy deficiency, which play a role in neuronal degeneration in EAE.

Human TRPM4 gene mutations and their concomitant dysfunction have been associated with several diseases. It was documented that TRPM4 expression was highly increased following hypoxia/ischemic stroke in vascular endothelium, and also following spinal cord injury in endothelial cells of capillary vessels **(Gerzanich et al., 2009) (Loh et al., 2014)**.

The participation of TRPM4 mutations in diseases is still not fully understood. One study identified a missense mutation of TRPM4 in a South African cohort that is associated with progressive familial heart block type I (PFHBI) syndrome **(Kruse et al., 2009)**. This mutation leads to a constitutive SUMOylation of the TRPM4 channel, which causes elevated TRPM4 channel densities due to impaired endocytosis. The increased presence of TRPM4 on the cell surface blunted the conduction of cardiac action potentials that is responsible for the development of PFHBI. Three different heterozygous missense mutations in TRPM4 were found in one Lebanese and 2 French families also caused a cardiac conduction block **(Hui Liu et al., 2010)**. These mutations were identified as gain of function mutations that lead to an increased TRPM4-mediated current density.

The participation of TRPM4 in the innate immune response opens up new possibilities in the treatment of chronic inflammatory diseases like cystic fibrosis or COPD. One the one hand, reducing the inflammatory reaction would decrease the inflammation associated tissue damage. On the other hand, the insufficient chemotaxis of professional immune cells and the decreased antimicrobial defense would possibly lead to increased damage through colonizing bacteria.

Finally, it is worth mentioning that TRPM4 could interact with another protein that affects its behavioural and physiological function; in order to achieve protein-protein interactions post-translational modification are required such as phosphorylation, SUMOylation, and glycosylation **(C.-H. Cho et al., 2015)**. All of these changes need to be further investigated in response to different types of infection and also their influence on subsequent cellular response. So far, there is no enough research in this area.

One of the main proteins interacting with TRPM4 is 14-3-3γ trafficking chaperone, which acts as a label for TRPM4 channel, that upon interaction, it could drive it to the membrane **(C. H. Cho et al., 2014)**. It was also found out that Ser88 is essential for 14-3-3γ binding at the N-terminus of TRPM4b that regulates forward trafficking to plasma membrane. It is probable, in a phosphorylation-dependent manner, these findings were approved by TRPM4b-S88A mutant form, which were unable to reach the plasma membrane **(C.-H. Cho et al., 2015)**.

Phosphorylation plays a pivotal role in TRPM4 regulation, where Ca^{2+}/CAM binding sites have been reported at the TRPM4 C-terminus. It was proved that Ca^{2+} sensitivity of TRPM4 channel was influenced by PKC-mediated phosphorylation at Ser1145 and Ser1152 binding sites **(Nilius et al., 2005b)**.

SUMOylation is a post-translational modification which modulates protein function by binding the target protein to a member of SUMO (small ubiquitin-like modifier) protein family **(Luo et al., 2013)**. SUMOylation has been seen in patients with progressive cardiac bundle branch disease, in which missense mutation brought forth to cytoplasmic N-terminal (Glu7Lys) of TRPM4 channel, that contributes to defect in endocytosis process resulting in increased plasma membrane channel levels **(Kruse et al., 2009)**.

Moreover, TRPM4 has been shown to be affected by glycosylation **(Seung Kyoon Woo et al., 2013) (Syam et al., 2014)**. N-glycosylation is necessary for proper growth, maturation and appropriate delivery of ion channels to the plasma membrane **(Baycin-Hizal et al., 2014)**.

Interestingly, it has been shown that TRPM4 associates with various types of other ion channel subunits **(J. Y. Park et al., 2008) (S. K. Woo et al., 2013)**, such as SUR-1 (Sulfonylurea receptors) or TRPC3. SUR-1-TRPM4 heteromerization leads to a change in biophysical properties of TRPM4 which showed double affinity to calmodulin and double sensitivity to intracellular calcium **(S. K. Woo et al., 2013)**. Also, TRPM4 heteromerization with TRPC3 (Ca^{2+} activated, Ca^{2+} permeable TRP family member) leads to suppression of TRPC3 channel **(Lichtenegger & Groschner, 2014)**.

In conclusion, this work shows that the activity of TRPM4 is necessary to initiate an inflammatory host response reaction after the stimulation with heat inactivated Ps.a. in vitro and in vivo. In the absence of TRPM4, cytokines and other host defence molecules are not efficiently exported from the cells. It was shown with differentiated pHBEs that this leads to a decreased host defence; due to an insufficient synthesis and export of the antimicrobial peptide hBD2. The results obtained from mouse cells and in vivo are not conclusive so far and need further investigation.

Outlook

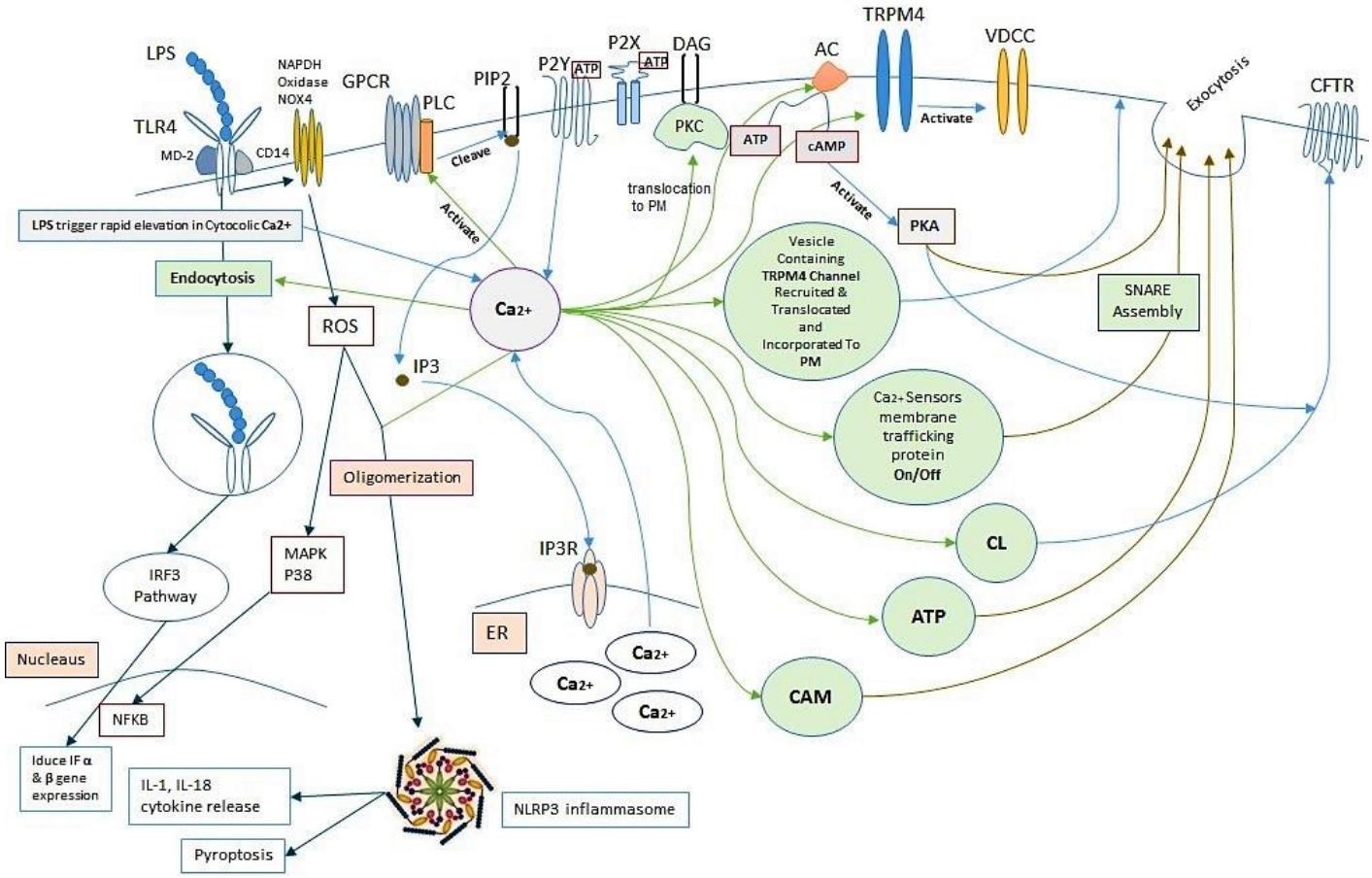

Fig. 31: Overview of intracellular Ca^{2+} dependent signalling pathway.

TRPM4 is a Ca^{2+} dependent channel and the higher internal Ca^{2+} ion concentration leads to its activation. There are many factors that affect Ca^{2+} ion concentration and thus lead to the subsequent TRPM4 activation. In excitable cells, TRPM4 regulates Ca^{2+} influx by causing membrane depolarization that leads to activation of different form of voltage dependent Ca^{2+} channel (VDCC) and causing more Ca^{2+} ion influx. In non-excitable cells, Ca^{2+} could access the cells through a variant TRP or L type Ca^{2+} channel. Once intracellular Ca^{2+} increased, many signalling pathways could be subsequently activated inside the cell as shown in (**Fig.31)**.

Ca^{2+} could activate PLC, which in turn cleaves PIP2, to IP3 and DAG. IP3 once released, it attaches with IP3R receptor on ER membrane leading to increase of intercellular Ca^{2+} release from ER. DAG which remains in the plasma membrane due to its hydrophobic properties, can activate and facilitate translocation of PKC from cytosol to plasma membrane, which plays a pivotal role in activation of TRPM4 channel through its phosphorylation at different N-terminal sites **(Nilius et al., 2005b) (Cerda et al., 2015) (Crnich**

et al., 2010) (C. H. Cho et al., 2014). In the airway epithelial cell, the involvement of PLC in producing calcium release has been established **(Boncoeur et al., 2010)**. Furthermore In epithelial cell, integrins that are reported to be activated during cell adhesion could mediate PLC activation **(Gilcrease, 2007)**.

Ca^{2+} could activate AC (adenylyl cyclase) membrane enzyme, which catalyses the conversion of ATP to 3',5'-cyclic AMP (cAMP) and pyrophosphate group. Protein kinase A (PKA) is also known as a cAMP-dependent protein kinase, whose activity is dependent on cellular levels of cAMP. It also plays an important role in the exocytosis process and in regulating and trafficking of CFTR. In the airway epithelium, beside intracellular Ca^{2+} concentration, CFTR channel is activated by cAMP. Chloride current evoked by calcium agonists in human bronchial epithelial cells is mediated by CFTR through a mechanism involving calcium activation of AC1 and cAMP/PKA signalling pathway **(Namkung et al., 2010)**. Intracellular Ca^{2+} modulation can boost cAMP production by means of various calcium-sensitive AC isoforms **(Willoughby & Cooper, 2007)**. LPS could induce intracellular Ca^{2+} ion increase, which enhances the chloride secretion through CFTR in human bronchial epithelial cells **(Buyck et al., 2013)**. Also, in the human airway epithelial cells, Ps.a. was identified to trigger a store-operated cAMP signalling in which the content of calcium stores is correlated with cAMP signalling and CFTR activation **(Schwarzer et al., 2010)**. Lack of CFTR decreases the Cl^- secretion leading to an air way dehydration, a decreased muco-ciliary clearance, and an excessive mucus production.

In pancreatic β-cells, Ca^{2+} could enhance TRPM4 translocation from vesicular pool to plasma membrane through Ca^{2+} dependent exocytosis process. Overexpression of TRPM4 in plasma membrane enhances the cellular depolarization that drives the activation of voltage-dependent Ca^{2+} channel and thus the insulin secretion **(Cheng et al., 2007)**.

LPS has long been established to induce a dramatic rise in the cytosolic Ca^{2+} **(Letari et al., 1991)** by releasing it from Ca^{2+} stores rather than extracellular Ca^{2+} influx **(X. Liu et al., 2008)**. However, it still remains a controversial identity of the ion channel that involved in Ca^{2+} signalling route. In contrast, Ca^{2+} ion could promote the endocytosis of LPS bounded to TLR4 in macrophage **(Schappe et al., 2018)**, which triggers the association of TLR4 to TRIF and its co-adaptor TRIF-related adaptor molecule (TRAM), leading to activation of nuclear translocation of transcription factor IRF3 pathway, which induces IF type1 α & β gene expression **(Kagan et al., 2008)**. Thus, TLR4 endocytosis could modulate cellular response through additional activation of IRF3 beside NF-kB, which could be also activated through both TRIF and MyD88 pathway. Crucial evidence has been given for the intracellular localization of TLR4 in pulmonary epithelial cells **(Guillot et al., 2004).**

A sustained increase in Ca^{2+} ions influx caused by sulfonylureas, led to enhancing synthesis of reactive oxygen species (ROS) which drives the cell to accelerate apoptosis **(Efanova et al., 1998) (Iwakura et al., 2000) (Tsubouchi et al., 2005)**. Increasing intracellular ROS could be capable of activating TRPM4, which led to acceleration of the cell death process **(Felipe Simon et al., 2010)**. LPS binding to TLR4 Launch signalling cascade leads to the stimulation of both dependent and independent MyD88 pathways **(Dauphinee & Karsan, 2006)**. In a MyD88-dependent pathway, LPS-TLR4 could trigger the intracellular ROS formation mediated by NADPH oxidase (Nox4) and Cyclooxygenase (COX-2) membrane enzyme. ROS acts as an intermediate factor in p38 MAPK and NF-κB pathway activation which leads to the lectin-like oxidized low density lipoprotein receptor (LOX-1) expression in endothelial cell. The enhanced LOX-1 expression promotes oxidized low-density lipoprotein (ox-LDL) endocytosis, which plays a vital role in atherosclerosis initiation and propagation. And it also acts as an adhesion molecular to engage in endothelial monocytes interaction, which is regarded as one of the initial event in early stage of atherosclerosis **(Zhao et al., 2014)**. ROS formed via Nox4 or as a response to different PAMPs or DAMPs could also induce assembly of NLR family pyrin domain containing 3 (NLRP3) inflammasome through oligomerization with Ca^{2+} ion **(Crane et al., 2014) (Rajanbabu et al., 2015)**.

In general, TLR could recognize many types of PAMPs or DAMPs, leading to activation NF-κB mediated signalling. Then NF-Kβ translocates to the nucleus to promote the transcription of pro-inflammatory gene such as IL-1β, IL-6, IL-18, TNF-α **(Bauernfeind et al., 2009)**. TLR signaling pathway, it activates not only classic immune response such as MAPK pathway involved in inflammation response, but also regulates the innate immunity through increasing extracellular ATP release via Ca^{2+} mobilization dependent exocytosis process.

In macrophage, extracellular ATP plays a protective role during the bacterial infection, where it could bind to all purinergic P2X and most of P2Y receptors such as (P2Y1, P2Y2, P2Y4, and P2Y11 receptors). Upon binding, receptors transduce intracellular signaling through the activation of ERK pathway and drive AP-1 nuclear translocation and subsequent CCL-2 and IL-1β cytokine production, which enhance bacterial phagocytosis **(Ren et al., 2014)**. Upon stimulation P2Y also could activate PLC enzyme, which in turn hydrolyses PIP2 to IP3 and DAG **(von Kügelgen & Wetter, 2000)**.

TLR signaling pathway could also regulate the transcription of inflammasome component, which includes inactive NLRP3, proIL-1β, and proIL-18 **(Bauernfeind et al., 2009)**. NLRP3 inflammasome could be activated by k^{+} efflux and intracellular Ca^{2+} ion influx from ER **(Ketelut-Carneiro et al., 2015) (Schmid-Burgk et al., 2015)**. This complex, in turn; catalyses the conversion of procaspase-1 into caspase-1 which takes part in the mature IL-1β and IL-18 production and secretion. In a second pathway, called non-canonical

inflammasome pathway, caspase 11 could detect cytosolic LPS, lead to inflammasome formation, and trigger pyroptosis (inflammation related cell death) **(C. E. Diamond et al., 2015)**.

TRPM4 acts as a key regulator in coordination with Na^{+}/Ca^{2+}exchanger 2 (NCX2) for the release of both types of mucin MUC2 and MUC5AC from many human epithelial cell lines**.** Upon activation, TRPM4/TRPM5 permeates the intracellular sodium ion increase into the cytoplasm, thus enhances NCX to act a reverse mode leading to sustain increase in Ca^{2+} ion concentration, which in cooperation with synaptotagmin 2 (calcium sensor protein) could facilitate the fusion of mucin granules into the plasma membrane **(Cantero-Recasens et al., 2019)**.

TRPM4 was found to associate with different types of membrane receptors or other ion channel such as TRPC3 (Ca activated, Ca permeable TRP family member) or SUR-1 (Sulfonylurea receptors). TRPM4 heteromerization with TRPC3 lead to suppress TRPC3 channel **(Lichtenegger & Groschner, 2014)**.

Increasing intracellular Ca^{2+} activates TRPM4 co-association with Sur-1, which is tied directly to the membrane depolarization, generating negative feedback that forbids additional entry of Ca^{2+}. Co-assembly with Sur1 leads to alteration of biophysical properties of TRPM4 which showed double affinity to calmodulin and double sensitivity to intracellular Ca^{2+} concentration, thus enhancing the function of TRPM4 as a negative Ca^{2+} entry regulator **(S. K. Woo et al., 2013)**. Sur1-TRPM4 heteromerization resulted in activation of calcineurin (CN) and the activated T cell nuclear factor (NFAT), lead to dependent Nos2/NOS2 expression **(not shown in Fig.31)**. Glibenclamide blockage to Sur1-TRPM4 led to massive intracellular Ca^{2+} entry, which in turn preferentially activates CaMKII, then inhibits CN/NFAT and decreases Nos2/NOS2 expression **(Kurland et al., 2016)**.

Increasing intracellular Ca^{2+} concentration governed by KATP channels could macerate the autophagy process, which is related to neurodegenerative diseases **(Hambrock et al., 2006)**, where autophagy protects proximal tubular epithelial cells (PTECs) from injury and apoptosis **(Dong et al., 2015) (Y. Xu et al., 2016)**. Autophagy dysfunction or impairment induced by high glucose **(X. Q. Zhang et al., 2017)** or sulfonylureas **(R. Zhang et al., 2018)** plays a vital role in PTEC apoptosis.

Intracellular Ca^{2+} also involved in CAM activation, which plays an important role in exocytosis process **(Bernstein, 2015)**. Also, Intracellular calcium mobilization and intracellular calcium that activated synaptic vesicles have been documented to be involved in exocytosis **(Kochubey et al., 2011)**.

Publication

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Internal Medicine V – Pulmonology, Allergology, Intensive Care Medicine, Saarland University and Medical Faculty, Homburg, Germany;
*Department of Physiology, Saarland University, Homburg, Germany.

Conference contributions

Lecture

Inhibition of TRPM4 in airway epithelial cells leads to reduced inflammation and host defense

Mahdy A, Beisswenger C, Bals R, Herr C.
Internal Medicine V – Pulmonology, Allergology, Intensive Care Medicine, Saarland University and Medical Faculty, Homburg, Germany

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References

Abonyo, B. O., Gou, D., Wang, P., Narasaraju, T., Wang, Z., & Liu, L. (2004). Syntaxin 2 and SNAP-23 are required for regulated surfactant secretion. *Biochemistry, 43*(12), 3499-3506. doi: 10.1021/bi036338y

Abonyo, B. O., Wang, P., Narasaraju, T. A., Rowan, W. H., 3rd, McMillan, D. H., Zimmerman, U. J., & Liu, L. (2003). Characterization of alpha-soluble N-ethylmaleimide-sensitive fusion attachment protein in alveolar type II cells: implications in lung surfactant secretion. *Am J Respir Cell Mol Biol, 29*(3 Pt 1), 273-282. doi: 10.1165/rcmb.2002-0189OC

Abriel, H., Syam, N., Sottas, V., Amarouch, M. Y., & Rougier, J.-S. (2012). TRPM4 channels in the cardiovascular system: Physiology, pathophysiology, and pharmacology. *Biochemical Pharmacology, 84*(7), 873-881. doi: https://doi.org/10.1016/j.bcp.2012.06.021

Afford, S. C., Pongracz, J., Stockley, R. A., Crocker, J., & Burnett, D. (1992). The induction by human interleukin-6 of apoptosis in the promonocytic cell line U937 and human neutrophils. *J Biol Chem, 267*(30), 21612-21616.

Akira, S., Uematsu, S., & Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell, 124*(4), 783-801. doi: 10.1016/j.cell.2006.02.015

Alhazmi, A. (2015). Pseudomonas aeruginosa – Pathogenesis and Pathogenic Mechanisms. *International Journal of Biology, 7*(2). doi: 10.5539/ijb.v7n2p44

Alhede, M., Bjarnsholt, T., Jensen, P. O., Phipps, R. K., Moser, C., Christophersen, L., . . . Givskov, M. (2009). Pseudomonas aeruginosa recognizes and responds aggressively to the presence of polymorphonuclear leukocytes. *Microbiology, 155*(Pt 11), 3500-3508. doi: 10.1099/mic.0.031443-0

Andreu, N., Phelan, J., de Sessions, P. F., Cliff, J. M., Clark, T. G., & Hibberd, M. L. (2017). Primary macrophages and J774 cells respond differently to infection with Mycobacterium tuberculosis. *Sci Rep, 7*, 42225.

Areschoug, T., & Gordon, S. (2009). Scavenger receptors: role in innate immunity and microbial pathogenesis. *Cellular Microbiology, 11*(8), 1160-1169. doi: 10.1111/j.1462-5822.2009.01326.x

Arnason, J. W., Murphy, J. C., Kooi, C., Wiehler, S., Traves, S. L., Shelfoon, C., . . . Leigh, R. (2017). Human β-defensin-2 production upon viral and bacterial co-infection is attenuated in COPD. *PLoS One, 12*(5), e0175963. doi: 10.1371/journal.pone.0175963

Atreya, R., Mudter, J., Finotto, S., Müllberg, J., Jostock, T., Wirtz, S., . . . Neurath, M. F. (2000). Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: Evidence in Crohn disease and experimental colitis in vivo. *Nature Medicine, 6*(5), 583-588. doi: 10.1038/75068

Autzen, H. E., Myasnikov, A. G., Campbell, M. G., Asarnow, D., Julius, D., & Cheng, Y. (2018). Structure of the human TRPM4 ion channel in a lipid nanodisc. *Science, 359*(6372), 228. doi: 10.1126/science.aar4510

Baggiolini, M., & Dewald, B. (1985). The Neutrophil. *International Archives of Allergy and Immunology, 76(suppl 1)*(Suppl. 1), 13-20. doi: 10.1159/000233730

Bals, R., Beisswenger, C., Blouquit, S., & Chinet, T. (2004). Isolation and air-liquid interface culture of human large airway and bronchiolar epithelial cells. *J Cyst Fibros, 3 Suppl 2*, 49-51. doi: 10.1016/j.jcf.2004.05.010

Bals, R., & Hiemstra, P. S. (2004). Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. *Eur Respir J, 23*(2), 327-333. doi: 10.1183/09031936.03.00098803

Bals, R., Wang, X., Wu, Z., Freeman, T., Bafna, V., Zasloff, M., & Wilson, J. M. (1998). Human beta-defensin 2 is a salt-sensitive peptide antibiotic expressed in human lung. *J Clin Invest, 102*(5), 874-880. doi: 10.1172/jci2410

Banks-Schlegel, S. P., Gazdar, A. F., & Harris, C. C. (1985). Intermediate filament and cross-linked envelope expression in human lung tumor cell lines. *Cancer Res, 45*(3), 1187-1197.

Barbet, G., Demion, M., Moura, I. C., Serafini, N., Léger, T., Vrtovsnik, F., . . . Launay, P. (2008). The calcium-activated nonselective cation channel TRPM4 is essential for the migration but not the maturation of dendritic cells. *Nat Immunol, 9*(10), 1148-1156. doi: 10.1038/ni.1648

Barclay, J. W., Morgan, A., & Burgoyne, R. D. (2005). Calcium-dependent regulation of exocytosis. *Cell Calcium, 38*(3), 343-353. doi: https://doi.org/10.1016/j.ceca.2005.06.012

Barton, G. M., & Medzhitov, R. (2002). Toll-like receptors and their ligands. *Curr Top Microbiol Immunol, 270*, 81-92. doi: 10.1007/978-3-642-59430-4_5

Bauernfeind, F. G., Horvath, G., Stutz, A., Alnemri, E. S., MacDonald, K., Speert, D., . . . Latz, E. (2009). Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol, 183*(2), 787-791. doi: 10.4049/jimmunol.0901363

Baycin-Hizal, D., Gottschalk, A., Jacobson, E., Mai, S., Wolozny, D., Zhang, H., . . . Betenbaugh, M. J. (2014). Physiologic and pathophysiologic consequences of altered sialylation and glycosylation on ion channel function. *Biochem Biophys Res Commun, 453*(2), 243-253. doi: 10.1016/j.bbrc.2014.06.067

Becerra, A., Echeverria, C., Varela, D., Sarmiento, D., Armisen, R., Nunez-Villena, F., . . . Simon, F. (2011). Transient receptor potential melastatin 4 inhibition prevents lipopolysaccharide-induced endothelial cell death. *Cardiovasc Res, 91*(4), 677-684. doi: 10.1093/cvr/cvr135

Becker, M. N., Diamond, G., Verghese, M. W., & Randell, S. H. (2000). CD14-dependent lipopolysaccharide-induced beta-defensin-2 expression in human tracheobronchial epithelium. *J Biol Chem, 275*(38), 29731-29736. doi: 10.1074/jbc.M000184200

Bellingan, G. J., Caldwell, H., Howie, S. E., Dransfield, I., & Haslett, C. (1996). In vivo fate of the inflammatory macrophage during the resolution of inflammation: inflammatory macrophages do not die locally, but emigrate to the draining lymph nodes. *J Immunol, 157*(6), 2577-2585.

Berger, M. (1991). Inflammation in the lung in cystic fibrosis. A vicious cycle that does more harm than good? *Clin Rev Allergy, 9*(1-2), 119-142. doi: 10.1007/978-1-4612-0475-6_8

Bernstein, L. H. (2015). Ca2+-Stimulated Exocytosis: The Role of Calmodulin and Protein Kinase C in Ca2+ Regulation of Hormone and Neurotransmitter. *Archives of Medicine*.

Beutler, B. (2002). TLR4 as the mammalian endotoxin sensor. *Curr Top Microbiol Immunol, 270*, 109-120. doi: 10.1007/978-3-642-59430-4_7

Bezzerides, V. J., Ramsey, I. S., Kotecha, S., Greka, A., & Clapham, D. E. (2004). Rapid vesicular translocation and insertion of TRP channels. *Nat Cell Biol, 6*(8), 709-720. doi: 10.1038/ncb1150

Birch, K. A., Pober, J. S., Zavoico, G. B., Means, A. R., & Ewenstein, B. M. (1992). Calcium/calmodulin transduces thrombin-stimulated secretion: studies in intact and minimally permeabilized human umbilical vein endothelial cells. *The Journal of cell biology, 118*(6), 1501-1510. doi: 10.1083/jcb.118.6.1501

Birchenough, G. M., Johansson, M. E., Gustafsson, J. K., Bergström, J. H., & Hansson, G. C. (2015). New developments in goblet cell mucus secretion and function. *Mucosal Immunol, 8*(4), 712-719. doi: 10.1038/mi.2015.32

Boncoeur, E., Tardif, V., Tessier, M. C., Morneau, F., Lavoie, J., Gendreau-Berthiaume, E., . . . Berthiaume, Y. (2010). Modulation of epithelial sodium channel activity by lipopolysaccharide in alveolar type II cells: involvement of purinergic signaling. *Am J Physiol Lung Cell Mol Physiol, 298*(3), L417-426. doi: 10.1152/ajplung.00170.2009

Boniotto, M., Jordan, W. J., Eskdale, J., Tossi, A., Antcheva, N., Crovella, S., . . . Gallagher, G. (2006). Human beta-defensin 2 induces a vigorous cytokine response in peripheral blood mononuclear cells. *Antimicrob Agents Chemother, 50*(4), 1433-1441. doi: 10.1128/aac.50.4.1433-1441.2006

Borzęcka, K., Płóciennikowska, A., Björkelund, H., Sobota, A., & Kwiatkowska, K. (2013). CD14 mediates binding of high doses of LPS but is dispensable for TNF-α production. *Mediators Inflamm, 2013*, 824919. doi: 10.1155/2013/824919

Braun, A. P., & Schulman, H. (1995). The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. *Annu Rev Physiol, 57*, 417-445. doi: 10.1146/annurev.ph.57.030195.002221

Burgoyne, R. D., & Clague, M. J. (2003). Calcium and calmodulin in membrane fusion. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1641*(2), 137-143. doi: https://doi.org/10.1016/S0167-4889(03)00089-2

Burgoyne, R. D., Geisow, M. J., Barron, J., & Gray, E. G. (1982). Dissection of stages in exocytosis in the adrenal chromaffin cell with use of trifluoperazine. *Proceedings of the Royal Society of London. Series B. Biological Sciences, 216*(1202), 111-115. doi: 10.1098/rspb.1982.0064

Burris, S. K., Wang, Q., Bulley, S., Neeb, Z. P., & Jaggar, J. H. (2015). 9-Phenanthrol inhibits recombinant and arterial myocyte TMEM16A channels. *Br J Pharmacol, 172*(10), 2459-2468.

Buyck, J. M., Verriere, V., Benmahdi, R., Higgins, G., Guery, B., Matran, R., . . . Urbach, V. (2013). P. aeruginosa LPS stimulates calcium signaling and chloride secretion via CFTR in human bronchial epithelial cells. *J Cyst Fibros, 12*(1), 60-67. doi: 10.1016/j.jcf.2012.06.007

Cantero-Recasens, G., Butnaru, C. M., Brouwers, N., Mitrovic, S., Valverde, M. A., & Malhotra, V. (2019). Sodium channel TRPM4 and sodium/calcium exchangers (NCX) cooperate in the control of Ca(2+)-induced mucin secretion from goblet cells. *J Biol Chem, 294*(3), 816-826. doi: 10.1074/jbc.RA117.000848

Carney, D. N., Gazdar, A. F., Bepler, G., Guccion, J. G., Marangos, P. J., Moody, T. W., . . . Minna, J. D. (1985). Establishment and identification of small cell lung cancer cell lines having classic and variant features. *Cancer Res, 45*(6), 2913-2923.

Cayouette, S., Lussier, M. P., Mathieu, E. L., Bousquet, S. M., & Boulay, G. (2004). Exocytotic insertion of TRPC6 channel into the plasma membrane upon Gq protein-coupled receptor activation. *J Biol Chem, 279*(8), 7241-7246. doi: 10.1074/jbc.M312042200

Cerda, O., Cáceres, M., Park, K. S., Leiva-Salcedo, E., Romero, A., Varela, D., . . . Stutzin, A. (2015). Casein kinase-mediated phosphorylation of serine 839 is necessary for basolateral localization of the Ca^{2+}-activated non-selective cation channel TRPM4. *Pflugers Arch, 467*(8), 1723-1732. doi: 10.1007/s00424-014-1610-3

Chaby, R. (2004). Lipopolysaccharide-binding molecules: transporters, blockers and sensors. *Cell Mol Life Sci, 61*(14), 1697-1713. doi: 10.1007/s00018-004-4020-4

Chen, C. I., Schaller-Bals, S., Paul, K. P., Wahn, U., & Bals, R. (2004). Beta-defensins and LL-37 in bronchoalveolar lavage fluid of patients with cystic fibrosis. *J Cyst Fibros, 3*(1), 45-50. doi: 10.1016/j.jcf.2003.12.008

Chen, Y. A., Duvvuri, V., Schulman, H., & Scheller, R. H. (1999). Calmodulin and Protein Kinase C Increase Ca2+-stimulated Secretion by Modulating Membrane-attached Exocytic Machinery. *Journal of Biological Chemistry, 274*(37), 26469-26476. doi: 10.1074/jbc.274.37.26469

Cheng, H., Beck, A., Launay, P., Gross, S. A., Stokes, A. J., Kinet, J.-P., . . . Penner, R. (2007). TRPM4 controls insulin secretion in pancreatic beta-cells. *Cell Calcium, 41*(1), 51-61. doi: 10.1016/j.ceca.2006.04.032

Cho, C.-H., Lee, Y.-S., Kim, E., Hwang, E. M., & Park, J.-Y. (2015). Physiological functions of the TRPM4 channels via protein interactions. *BMB Rep, 48*(1), 1-5. doi: 10.5483/bmbrep.2015.48.1.252

Cho, C. H., Kim, E., Lee, Y. S., Yarishkin, O., Yoo, J. C., Park, J. Y., . . . Hwang, E. M. (2014). Depletion of 14-3-3γ reduces the surface expression of Transient Receptor Potential Melastatin 4b (TRPM4b) channels and attenuates TRPM4b-mediated glutamate-induced neuronal cell death. *Mol Brain, 7*, 52. doi: 10.1186/s13041-014-0052-3

Chubanov, V., Waldegger, S., Schnitzler, M. M. y., Vitzthum, H., Sassen, M. C., Seyberth, H. W., . . . Gudermann, T. (2004). Disruption of TRPM6/TRPM7 complex formation by a mutation in the TRPM6 gene causes hypomagnesemia with secondary hypocalcemia. *Proc Natl Acad Sci U S A, 101*(9), 2894. doi: 10.1073/pnas.0305252101

Cigana, C., Curcurù, L., Leone, M. R., Ieranò, T., Lorè, N. I., Bianconi, I., . . . Bragonzi, A. (2009). Pseudomonas aeruginosa exploits lipid A and muropeptides modification as a strategy to lower innate immunity during cystic fibrosis lung infection. *PLoS One, 4*(12), e8439-e8439. doi: 10.1371/journal.pone.0008439

Clapham, D. E. (2003). TRP channels as cellular sensors. *Nature, 426*(6966), 517-524. doi: 10.1038/nature02196

Clémençon, B., Fine, M., Lüscher, B., Baumann, M. U., Surbek, D. V., Abriel, H., & Hediger, M. A. (2014). Expression, purification, and projection structure by single particle electron microscopy of functional human TRPM4 heterologously expressed in Xenopus laevis oocytes. *Protein Expr Purif, 95*, 169-176. doi: 10.1016/j.pep.2013.11.017

Cobb, L. M., Mychaleckyj, J. C., Wozniak, D. J., & Lopez-Boado, Y. S. (2004). Pseudomonas aeruginosa flagellin and alginate elicit very distinct gene expression patterns in airway epithelial cells: implications for cystic fibrosis disease. *J Immunol, 173*(9), 5659-5670. doi: 10.4049/jimmunol.173.9.5659

Cohen, J. (2002). The immunopathogenesis of sepsis. *Nature, 420*(6917), 885-891. doi: 10.1038/nature01326

Collins, L. E., DeCourcey, J., Rochfort, K. D., Kristek, M., & Loscher, C. E. (2015). A role for syntaxin 3 in the secretion of IL-6 from dendritic cells following activation of toll-like receptors. *Frontiers in immunology, 5*, 691-691. doi: 10.3389/fimmu.2014.00691

Corrales-Garcia, L., Possani, L., & Corzo, G. (2010). Expression systems of human β-defensins: Vectors, purification and biological activities. *Amino acids, 40*, 5-13. doi: 10.1007/s00726-010-0493-7

Crane, D. D., Bauler, T. J., Wehrly, T. D., & Bosio, C. M. (2014). Mitochondrial ROS potentiates indirect activation of the AIM2 inflammasome. *Front Microbiol, 5*, 438. doi: 10.3389/fmicb.2014.00438

Crnich, R., Amberg, G. C., Leo, M. D., Gonzales, A. L., Tamkun, M. M., Jaggar, J. H., & Earley, S. (2010). Vasoconstriction resulting from dynamic membrane trafficking of TRPM4 in vascular smooth muscle cells. *Am J Physiol Cell Physiol, 299*(3), C682-694. doi: 10.1152/ajpcell.00101.2010

Cryz, S. J., Jr., Pitt, T. L., Fürer, E., & Germanier, R. (1984). Role of lipopolysaccharide in virulence of Pseudomonas aeruginosa. *Infect Immun, 44*(2), 508-513. doi: 10.1128/IAI.44.2.508-513.1984

Dai, X., Jayapal, M., Tay, H. K., Reghunathan, R., Lin, G., Too, C. T., . . . MacAry, P. A. (2009). Differential signal transduction, membrane trafficking, and immune effector functions mediated by FcgammaRI versus FcgammaRIIa. *Blood, 114*(2), 318-327. doi: 10.1182/blood-2008-10-184457

Dalcin, D., & Ulanova, M. (2013). The Role of Human Beta-Defensin-2 in Pseudomonas aeruginosa Pulmonary Infection in Cystic Fibrosis Patients. *Infectious Diseases and Therapy, 2*(2), 159-166. doi: 10.1007/s40121-013-0015-5

Dauphinee, S. M., & Karsan, A. (2006). Lipopolysaccharide signaling in endothelial cells. *Laboratory Investigation, 86*(1), 9-22. doi: 10.1038/labinvest.3700366

Diamond, C. E., Khameneh, H. J., Brough, D., & Mortellaro, A. (2015). Novel perspectives on non-canonical inflammasome activation. *ImmunoTargets and therapy, 4*, 131-141. doi: 10.2147/ITT.S57976

Diamond, G., Legarda, D., & Ryan, L. K. (2000). The innate immune response of the respiratory epithelium. *Immunol Rev, 173*, 27-38. doi: 10.1034/j.1600-065x.2000.917304.x

Dodrill, M. W., Beezhold, D. H., Meighan, T., Kashon, M. L., & Fedan, J. S. (2011). Lipopolysaccharide increases Na(+),K(+)-pump, but not ENaC, expression in guinea-pig airway epithelium. *Eur J Pharmacol, 651*(1-3), 176-186. doi: 10.1016/j.ejphar.2010.10.088

Doherty, D. E., Downey, G. P., Worthen, G. S., Haslett, C., & Henson, P. M. (1988). Monocyte retention and migration in pulmonary inflammation. Requirement for neutrophils. *Laboratory investigation; a journal of technical methods and pathology, 59*(2), 200-213.

Dong, G., Liu, Y., Zhang, L., Huang, S., Ding, H.-F., & Dong, Z. (2015). mTOR contributes to ER stress and associated apoptosis in renal tubular cells. *American journal of physiology. Renal physiology, 308*(3), F267-274. doi: 10.1152/ajprenal.00629.2014

Doring, G. (1994). The role of neutrophil elastase in chronic inflammation. *Am J Respir Crit Care Med, 150*(6 Pt 2), S114-117. doi: 10.1164/ajrccm/150.6_Pt_2.S114

Dunlevy, F. K., Martin, S. L., de Courcey, F., Elborn, J. S., & Ennis, M. (2012). Anti-inflammatory effects of DX-890, a human neutrophil elastase inhibitor. *J Cyst Fibros, 11*(4), 300-304. doi: 10.1016/j.jcf.2012.02.003

Earley, S. (2013). TRPM4 channels in smooth muscle function. *Pflugers Arch, 465*(9), 1223-1231. doi: 10.1007/s00424-013-1250-z

Earley, S., Waldron Brian, J., & Brayden Joseph, E. (2004). Critical Role for Transient Receptor Potential Channel TRPM4 in Myogenic Constriction of Cerebral Arteries. *Circulation Research, 95*(9), 922-929. doi: 10.1161/01.RES.0000147311.54833.03

Eckstein, E., Pyrski, M., Pinto, S., Freichel, M., Vennekens, R., & Zufall, F. (2020). Cyclic regulation of Trpm4 expression in female vomeronasal neurons driven by ovarian sex hormones. *Mol Cell Neurosci, 105*, 103495. doi: 10.1016/j.mcn.2020.103495

Efanova, I. B., Zaitsev, S. V., Zhivotovsky, B., Köhler, M., Efendić, S., Orrenius, S., & Berggren, P. O. (1998). Glucose and tolbutamide induce apoptosis in pancreatic beta-cells. A process dependent on intracellular Ca2+ concentration. *J Biol Chem, 273*(50), 33501-33507. doi: 10.1074/jbc.273.50.33501

Ernst, R. K., Hajjar, A. M., Tsai, J. H., Moskowitz, S. M., Wilson, C. B., & Miller, S. I. (2003). Pseudomonas aeruginosa lipid A diversity and its recognition by Toll-like receptor 4. *J Endotoxin Res, 9*(6), 395-400. doi: 10.1179/096805103225002764

Evren, E., Ringqvist, E., Tripathi, K. P., Sleiers, N., Rives, I. C., Alisjahbana, A., . . . Willinger, T. (2020). Distinct developmental pathways from blood monocytes generate human lung macrophage diversity. *Immunity*.

Fasshauer, D. (2003). Structural insights into the SNARE mechanism. *Biochim Biophys Acta, 1641*(2-3), 87-97. doi: 10.1016/s0167-4889(03)00090-9

Finlay, B. B., & McFadden, G. (2006). Anti-Immunology: Evasion of the Host Immune System by Bacterial and Viral Pathogens. *Cell, 124*(4), 767-782. doi: https://doi.org/10.1016/j.cell.2006.01.034

Fleig, A., & Penner, R. (2004). The TRPM ion channel subfamily: molecular, biophysical and functional features. *Trends in Pharmacological Sciences, 25*(12), 633-639. doi: https://doi.org/10.1016/j.tips.2004.10.004

Fonfria, E., Murdock, P. R., Cusdin, F. S., Benham, C. D., Kelsell, R. E., & McNulty, S. (2006). Tissue distribution profiles of the human TRPM cation channel family. *J Recept Signal Transduct Res, 26*(3), 159-178. doi: 10.1080/10799890600637506

Galanos, C., Lüderitz, O., Rietschel, E. T., Westphal, O., Brade, H., Brade, L., . . . Yoshimura, H. (1985). Synthetic and natural Escherichia coli free lipid A express identical endotoxic activities. *Eur J Biochem, 148*(1), 1-5.

Ganesan, S., Comstock, A. T., & Sajjan, U. S. (2013). Barrier function of airway tract epithelium. *Tissue Barriers, 1*(4), e24997. doi: 10.4161/tisb.24997

Gay, N. J., Symmons, M. F., Gangloff, M., & Bryant, C. E. (2014). Assembly and localization of Toll-like receptor signalling complexes. *Nature Reviews Immunology, 14*(8), 546-558. doi: 10.1038/nri3713

Gees, M., Colsoul, B., & Nilius, B. (2010). The role of transient receptor potential cation channels in Ca2+ signaling. *Cold Spring Harbor perspectives in biology, 2*(10), a003962-a003962. doi: 10.1101/cshperspect.a003962

Gerzanich, V., Woo, S. K., Vennekens, R., Tsymbalyuk, O., Ivanova, S., Ivanov, A., . . . Simard, J. M. (2009). De novo expression of Trpm4 initiates secondary hemorrhage in spinal cord injury. *Nature Medicine, 15*(2), 185-191. doi: 10.1038/nm.1899

Gilcrease, M. Z. (2007). Integrin signaling in epithelial cells. *Cancer Lett, 247*(1), 1-25. doi: 10.1016/j.canlet.2006.03.031

Gimbrone, M. A., Jr., Obin, M. S., Brock, A. F., Luis, E. A., Hass, P. E., Hébert, C. A., . . . et al. (1989). Endothelial interleukin-8: a novel inhibitor of leukocyte-endothelial interactions. *Science, 246*(4937), 1601-1603. doi: 10.1126/science.2688092

Gioannini, T. L., Teghanemt, A., Zhang, D., Coussens, N. P., Dockstader, W., Ramaswamy, S., & Weiss, J. P. (2004). Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations. *Proc Natl Acad Sci U S A, 101*(12), 4186-4191. doi: 10.1073/pnas.0306906101

Gonzales, A. L., Garcia, Z. I., Amberg, G. C., & Earley, S. (2010). Pharmacological inhibition of TRPM4 hyperpolarizes vascular smooth muscle. *Am J Physiol Cell Physiol, 299*(5), C1195-1202. doi: 10.1152/ajpcell.00269.2010

Gou, D., Narasaraju, T., Chintagari, N. R., Jin, N., Wang, P., & Liu, L. (2004). Gene silencing in alveolar type II cells using cell-specific promoter in vitro and in vivo. *Nucleic acids research, 32*(17), e134-e134. doi: 10.1093/nar/gnh129

Graham, A., Steel, D. M., Wilson, R., Cole, P. J., Alton, E. W., & Geddes, D. M. (1993). Effects of purified Pseudomonas rhamnolipids on bioelectric properties of sheep tracheal epithelium. *Exp Lung Res, 19*(1), 77-89. doi: 10.3109/01902149309071082

Graham, M. E., O'Callaghan, D. W., McMahon, H. T., & Burgoyne, R. D. (2002). Dynamin-dependent and dynamin-independent processes contribute to the regulation of single vesicle release kinetics and quantal size. *Proc Natl Acad Sci U S A, 99*(10), 7124-7129. doi: 10.1073/pnas.102645099

Grand, T., Demion, M., Norez, C., Mettey, Y., Launay, P., Becq, F., . . . Guinamard, R. (2008). 9-phenanthrol inhibits human TRPM4 but not TRPM5 cationic channels. *Br J Pharmacol, 153*(8), 1697-1705. doi: 10.1038/bjp.2008.38

Grand, T., Demion, M., Norez, C., Mettey, Y., Launay, P., Becq, F., . . . Guinamard, R. (2008). 9-phenanthrol inhibits human TRPM4 but not TRPM5 cationic channels. *Br J Pharmacol, 153*(8), 1697-1705.

Greene, C. M., Carroll, T. P., Smith, S. G., Taggart, C. C., Devaney, J., Griffin, S., . . . McElvaney, N. G. (2005). TLR-induced inflammation in cystic fibrosis and non-cystic fibrosis airway epithelial cells. *J Immunol, 174*(3), 1638-1646. doi: 10.4049/jimmunol.174.3.1638

Guani-Guerra, E., Santos-Mendoza, T., Lugo-Reyes, S. O., & Teran, L. M. (2010). Antimicrobial peptides: general overview and clinical implications in human health and disease. *Clin Immunol, 135*(1), 1-11. doi: 10.1016/j.clim.2009.12.004

Guha, M., & Mackman, N. (2001). LPS induction of gene expression in human monocytes. *Cell Signal, 13*(2), 85-94. doi: 10.1016/s0898-6568(00)00149-2

Guillot, L., Medjane, S., Le-Barillec, K., Balloy, V., Danel, C., Chignard, M., & Si-Tahar, M. (2004). Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. *J Biol Chem, 279*(4), 2712-2718. doi: 10.1074/jbc.M305790200

Guinamard, R., Chatelier, A., Lenfant, J., & Bois, P. (2004). Activation of the Ca2+-Activated Nonselective Cation Channel by Diacylglycerol Analogues in Rat Cardiomyocytes. *Journal of Cardiovascular Electrophysiology, 15*(3), 342-348. doi: 10.1046/j.1540-8167.2004.03477.x

Guinamard, R., Demion, M., Magaud, C., Potreau, D., & Bois, P. (2006). Functional Expression of the TRPM4 Cationic Current in Ventricular Cardiomyocytes From Spontaneously Hypertensive Rats. *Hypertension, 48*(4), 587-594. doi: 10.1161/01.HYP.0000237864.65019.a5

Guinamard, R., Hof, T., & Del Negro, C. A. (2014). The TRPM4 channel inhibitor 9-phenanthrol. *Br J Pharmacol, 171*(7), 1600-1613. doi: 10.1111/bph.12582

Guinamard, R., Sallé, L., & Simard, C. (2011). The non-selective monovalent cationic channels TRPM4 and TRPM5. *Adv Exp Med Biol, 704*, 147-171. doi: 10.1007/978-94-007-0265-3_8

Guo, J., She, J., Zeng, W., Chen, Q., Bai, X. C., & Jiang, Y. (2017). Structures of the calcium-activated, non-selective cation channel TRPM4. *Nature, 552*(7684), 205-209. doi: 10.1038/nature24997

Hajjar, A. M., Ernst, R. K., Tsai, J. H., Wilson, C. B., & Miller, S. I. (2002). Human Toll-like receptor 4 recognizes host-specific LPS modifications. *Nat Immunol, 3*(4), 354-359. doi: 10.1038/ni777

Hambrock, A., de Oliveira Franz, C. B., Hiller, S., & Osswald, H. (2006). Glibenclamide-induced apoptosis is specifically enhanced by expression of the sulfonylurea receptor isoform SUR1 but not by expression of SUR2B or the mutant SUR1(M1289T). *J Pharmacol Exp Ther, 316*(3), 1031-1037. doi: 10.1124/jpet.105.097501

Hammond, G. R., Dove, S. K., Nicol, A., Pinxteren, J. A., Zicha, D., & Schiavo, G. (2006). Elimination of plasma membrane phosphatidylinositol (4,5)-bisphosphate is required for exocytosis from mast cells. *J Cell Sci, 119*(Pt 10), 2084-2094. doi: 10.1242/jcs.02912

Harder, J., Meyer-Hoffert, U., Teran, L. M., Schwichtenberg, L., Bartels, J., Maune, S., & Schröder, J. M. (2000). Mucoid Pseudomonas aeruginosa, TNF-alpha, and IL-1beta, but not IL-6, induce human beta-defensin-2 in respiratory epithelia. *Am J Respir Cell Mol Biol, 22*(6), 714-721. doi: 10.1165/ajrcmb.22.6.4023

Harteneck, C. (2005). Function and pharmacology of TRPM cation channels. *Naunyn-Schmiedeberg's Archives of Pharmacology, 371*(4), 307-314. doi: 10.1007/s00210-005-1034-x

Harteneck, C., Plant, T. D., & Schultz, G. (2000). From worm to man: three subfamilies of TRP channels. *Trends Neurosci, 23*(4), 159-166.

Hauber, H. P., Goldmann, T., Vollmer, E., Wollenberg, B., Hung, H. L., Levitt, R. C., & Zabel, P. (2007). LPS-induced mucin expression in human sinus mucosa can be attenuated by hCLCA inhibitors. *J Endotoxin Res, 13*(2), 109-116. doi: 10.1177/0968051907079168

Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., . . . Aderem, A. (2001). The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature, 410*(6832), 1099-1103. doi: 10.1038/35074106

Hedlund, M., Frendéus, B., Wachtler, C., Hang, L., Fischer, H., & Svanborg, C. (2001). Type 1 fimbriae deliver an LPS- and TLR4-dependent activation signal to CD14-negative cells. *Molecular Microbiology, 39*(3), 542-552. doi: 10.1046/j.1365-2958.2001.02205.x

Held, K., Voets, T., & Vriens, J. (2015). TRPM3 in temperature sensing and beyond. *Temperature, 2*(2), 201-213. doi: 10.4161/23328940.2014.988524

Hennings, H., & Holbrook, K. A. (1983). Calcium regulation of cell-cell contact and differentiation of epidermal cells in culture: An ultrastructural study. *Experimental Cell Research, 143*(1), 127-142. doi: https://doi.org/10.1016/0014-4827(83)90115-5

Hof, T., Simard, C., Rouet, R., Sallé, L., & Guinamard, R. (2013). Implication of the TRPM4 nonselective cation channel in mammalian sinus rhythm. *Heart Rhythm, 10*(11), 1683-1689. doi: https://doi.org/10.1016/j.hrthm.2013.08.014

Hoffmann, E., Dittrich-Breiholz, O., Holtmann, H., & Kracht, M. (2002). Multiple control of interleukin-8 gene expression. *J Leukoc Biol, 72*(5), 847-855.

Homma, T., Kato, A., Hashimoto, N., Batchelor, J., Yoshikawa, M., Imai, S., . . . Matsumoto, K. (2004). Corticosteroid and cytokines synergistically enhance toll-like receptor 2 expression in respiratory epithelial cells. *Am J Respir Cell Mol Biol, 31*(4), 463-469. doi: 10.1165/rcmb.2004-0161OC

Hoover, D. M., Rajashankar, K. R., Blumenthal, R., Puri, A., Oppenheim, J. J., Chertov, O., & Lubkowski, J. (2000). The structure of human beta-defensin-2 shows evidence of higher order oligomerization. *J Biol Chem, 275*(42), 32911-32918. doi: 10.1074/jbc.M006098200

Hornef, M. W., Frisan, T., Vandewalle, A., Normark, S., & Richter-Dahlfors, A. (2002). Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J Exp Med, 195*(5), 559-570. doi: 10.1084/jem.20011788

Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., . . . Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol, 162*(7), 3749-3752.

Hu, K., Carroll, J., Fedorovich, S., Rickman, C., Sukhodub, A., & Davletov, B. (2002). Vesicular restriction of synaptobrevin suggests a role for calcium in membrane fusion. *Nature, 415*(6872), 646-650. doi: 10.1038/415646a

Hu, X., & Ivashkiv, L. B. (2009). Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases. *Immunity, 31*(4), 539-550. doi: 10.1016/j.immuni.2009.09.002

Huang, Y., Fliegert, R., Guse, A. H., Lü, W., & Du, J. (2020). A structural overview of the ion channels of the TRPM family. *Cell Calcium, 85*, 102111. doi: https://doi.org/10.1016/j.ceca.2019.102111

Hubbard, K. B., & Hepler, J. R. (2006). Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins. *Cell Signal, 18*(2), 135-150.

Hudmon, A., & Schulman, H. (2002). Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. *Biochem J, 364*(Pt 3), 593-611. doi: 10.1042/BJ20020228

Illek, B., Fu, Z., Schwarzer, C., Banzon, T., Jalickee, S., Miller, S. S., & Machen, T. E. (2008). Flagellin-stimulated Cl- secretion and innate immune responses in airway epithelia: role for p38. *Am J Physiol Lung Cell Mol Physiol, 295*(4), L531-542. doi: 10.1152/ajplung.90292.2008

Iwakura, T., Fujimoto, S., Kagimoto, S., Inada, A., Kubota, A., Someya, Y., . . . Seino, Y. (2000). Sustained enhancement of Ca(2+) influx by glibenclamide induces apoptosis in RINm5F cells. *Biochem Biophys Res Commun, 271*(2), 422-428. doi: 10.1006/bbrc.2000.2616

Iwasaki, A., & Medzhitov, R. (2010). Regulation of Adaptive Immunity by the Innate Immune System. *Science, 327*(5963), 291. doi: 10.1126/science.1183021

Iwasaki, A., & Medzhitov, R. (2015). Control of adaptive immunity by the innate immune system. *Nat Immunol, 16*(4), 343-353. doi: 10.1038/ni.3123

Janes, K. A., Gaudet, S., Albeck, J. G., Nielsen, U. B., Lauffenburger, D. A., & Sorger, P. K. (2006). The response of human epithelial cells to TNF involves an inducible autocrine cascade. *Cell, 124*(6), 1225-1239. doi: 10.1016/j.cell.2006.01.041

Janeway, C. A., & Medzhitov, R. (2002). Innate Immune Recognition. *Annual Review of Immunology, 20*(1), 197-216. doi: 10.1146/annurev.immunol.20.083001.084359

Jelsbak, L., Johansen, H. K., Frost, A. L., Thogersen, R., Thomsen, L. E., Ciofu, O., . . . Molin, S. (2007). Molecular epidemiology and dynamics of Pseudomonas aeruginosa populations in lungs of cystic fibrosis patients. *Infect Immun, 75*(5), 2214-2224. doi: 10.1128/iai.01282-06

Jensen, P. O., Bjarnsholt, T., Phipps, R., Rasmussen, T. B., Calum, H., Christoffersen, L., . . . Hoiby, N. (2007). Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by Pseudomonas aeruginosa. *Microbiology, 153*(Pt 5), 1329-1338. doi: 10.1099/mic.0.2006/003863-0

Jones, L. C., Moussa, L., Fulcher, M. L., Zhu, Y., Hudson, E. J., O'Neal, W. K., . . . Kreda, S. M. (2012). VAMP8 is a vesicle SNARE that regulates mucin secretion in airway goblet cells. *J Physiol, 590*(3), 545-562. doi: 10.1113/jphysiol.2011.222091

Junge, H. J., Rhee, J. S., Jahn, O., Varoqueaux, F., Spiess, J., Waxham, M. N., . . . Brose, N. (2004). Calmodulin and Munc13 form a Ca2+ sensor/effector complex that controls short-term synaptic plasticity. *Cell, 118*(3), 389-401. doi: 10.1016/j.cell.2004.06.029

Kagan, J. C., Su, T., Horng, T., Chow, A., Akira, S., & Medzhitov, R. (2008). TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-β. *Nat Immunol, 9*(4), 361-368. doi: 10.1038/ni1569

Kao, C.-Y., Kim, C., Huang, F., & Wu, R. (2008). Requirements for Two Proximal NF- B Binding Sites and I B- in IL-17A-induced Human -Defensin 2 Expression by Conducting Airway Epithelium. *J Biol Chem, 283*(22), 15309-15318.

Kaske, S., Krasteva, G., König, P., Kummer, W., Hofmann, T., Gudermann, T., & Chubanov, V. (2007). TRPM5, a taste-signaling transient receptor potential ion-channel, is a ubiquitous signaling component in chemosensory cells. *BMC Neuroscience, 8*(1), 49. doi: 10.1186/1471-2202-8-49

Kato, A., & Schleimer, R. P. (2007). Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity. *Current opinion in immunology, 19*(6), 711-720. doi: 10.1016/j.coi.2007.08.004

Kawai, T., & Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol, 11*(5), 373-384. doi: 10.1038/ni.1863

Kenigsberg, R. L., & Trifaró, J. M. (1985). Microinjection of calmodulin antibodies into cultured chromaffin cells blocks catecholamine release in response to stimulation. *Neuroscience, 14*(1), 335-347. doi: https://doi.org/10.1016/0306-4522(85)90183-6

Ketelut-Carneiro, N., Silva, G. K., Rocha, F. A., Milanezi, C. M., Cavalcanti-Neto, F. F., Zamboni, D. S., & Silva, J. S. (2015). IL-18 triggered by the Nlrp3 inflammasome induces host innate resistance in a pulmonary model of fungal infection. *J Immunol, 194*(9), 4507-4517. doi: 10.4049/jimmunol.1402321

Khalmuratova, R., Park, J.-W., & Shin, H.-W. (2017). Immune Cell Responses and Mucosal Barrier Disruptions in Chronic Rhinosinusitis. *Immune network, 17*(1), 60-67. doi: 10.4110/in.2017.17.1.60

Kim, D. J., Chung, S. G., Lee, S.-H., & Choi, J.-W. (2012). Relation of microbial biomass to counting units for Pseudomonas aeruginosa. *African journal of microbiology research, 6*(21), 4620-4622. doi: 10.5897/AJMR10.902

Kim, H. M., Park, B. S., Kim, J.-I., Kim, S. E., Lee, J., Oh, S. C., . . . Lee, J.-O. (2007). Crystal Structure of the TLR4-MD-2 Complex with Bound Endotoxin Antagonist Eritoran. *Cell, 130*(5), 906-917. doi: https://doi.org/10.1016/j.cell.2007.08.002

Kobayashi, S. D., Voyich, J. M., Buhl, C. L., Stahl, R. M., & DeLeo, F. R. (2002). Global changes in gene expression by human polymorphonuclear leukocytes during receptor-mediated phagocytosis: Cell fate is regulated at the level of gene expression. *Proceedings of the National Academy of Sciences, 99*(10), 6901. doi: 10.1073/pnas.092148299

Kochubey, O., Lou, X., & Schneggenburger, R. (2011). Regulation of transmitter release by Ca2+ and synaptotagmin: insights from a large CNS synapse. *Trends Neurosci, 34*(5), 237-246. doi: https://doi.org/10.1016/j.tins.2011.02.006

Kraft, R., & Harteneck, C. (2005). The mammalian melastatin-related transient receptor potential cation channels: an overview. *Pflugers Arch, 451*(1), 204-211. doi: 10.1007/s00424-005-1428-0

Kreda, S. M., Seminario-Vidal, L., van Heusden, C. A., O'Neal, W., Jones, L., Boucher, R. C., & Lazarowski, E. R. (2010). Receptor-promoted exocytosis of airway epithelial mucin granules containing a spectrum of adenine nucleotides. *J Physiol, 588*(Pt 12), 2255-2267. doi: 10.1113/jphysiol.2009.186643

Kruse, M., Schulze-Bahr, E., Corfield, V., Beckmann, A., Stallmeyer, B., Kurtbay, G., . . . Pongs, O. (2009). Impaired endocytosis of the ion channel TRPM4 is associated with human progressive familial heart block type I. *J Clin Invest, 119*(9), 2737-2744. doi: 10.1172/JCI38292

Kube, D., Sontich, U., Fletcher, D., & Davis, P. B. (2001). Proinflammatory cytokine responses to P. aeruginosa infection in human airway epithelial cell lines. *American Journal of Physiology-Lung Cellular and Molecular Physiology, 280*(3), L493-L502.

Kumar, A., Zhang, J., & Yu, F. S. (2006). Toll-like receptor 2-mediated expression of beta-defensin-2 in human corneal epithelial cells. *Microbes Infect, 8*(2), 380-389. doi: 10.1016/j.micinf.2005.07.006

Kunzelmann, K., Scheidt, K., Scharf, B., Ousingsawat, J., Schreiber, R., Wainwright, B., & McMorran, B. (2006). Flagellin of Pseudomonas aeruginosa inhibits Na+ transport in airway epithelia. *The FASEB Journal, 20*(3), 545-546. doi: 10.1096/fj.05-4454fje

Kurland, D. B., Gerzanich, V., Karimy, J. K., Woo, S. K., Vennekens, R., Freichel, M., . . . Simard, J. M. (2016). The Sur1-Trpm4 channel regulates NOS2 transcription in TLR4-activated microglia. *Journal of Neuroinflammation, 13*(1), 130. doi: 10.1186/s12974-016-0599-2

Lacy, P., & Stow, J. L. (2011). Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. *Blood, 118*(1), 9-18. doi: 10.1182/blood-2010-08-265892

Lambrecht, B. N., & Hammad, H. (2014). Allergens and the airway epithelium response: gateway to allergic sensitization. *The Journal of allergy and clinical immunology, 134*(3), 499-507. doi: 10.1016/j.jaci.2014.06.036

Launay, P., Cheng, H., Srivatsan, S., Penner, R., Fleig, A., & Kinet, J.-P. (2004). TRPM4 Regulates Calcium Oscillations After T Cell Activation. *Science, 306*(5700), 1374. doi: 10.1126/science.1098845

Launay, P., Fleig, A., Perraud, A. L., Scharenberg, A. M., Penner, R., & Kinet, J. P. (2002). TRPM4 is a Ca2+-activated nonselective cation channel mediating cell membrane depolarization. *Cell, 109*(3), 397-407. doi: 10.1016/s0092-8674(02)00719-5

Lawrenz, M. B., Fodah, R. A., Gutierrez, M. G., & Warawa, J. (2014). Intubation-mediated Intratracheal (IMIT) Instillation: A Noninvasive, Lung-specific Delivery System. *JoVE*(93), e52261. doi: doi:10.3791/52261

Lee, C., Liu, Q. H., Tomkowicz, B., Yi, Y., Freedman, B. D., & Collman, R. G. (2003). Macrophage activation through CCR5- and CXCR4-mediated gp120-elicited signaling pathways. *J Leukoc Biol, 74*(5), 676-682. doi: 10.1189/jlb.0503206

Lee, M. S., & Kim, Y. J. (2007). Signaling pathways downstream of pattern-recognition receptors and their cross talk. *Annu Rev Biochem, 76*, 447-480. doi: 10.1146/annurev.biochem.76.060605.122847

Letari, O., Nicosia, S., Chiavaroli, C., Vacher, P., & Schlegel, W. (1991). Activation by bacterial lipopolysaccharide causes changes in the cytosolic free calcium concentration in single peritoneal macrophages. *The Journal of Immunology, 147*(3), 980-983.

Levine, S. J., Wu, T., & Shelhamer, J. H. (1997). Extracellular release of the type I intracellular IL-1 receptor antagonist from human airway epithelial cells: differential effects of IL-4, IL-13, IFN-gamma, and corticosteroids. *J Immunol, 158*(12), 5949-5957.

Lewis, R. S. (2001). Calcium signaling mechanisms in T lymphocytes. *Annu Rev Immunol, 19*, 497-521. doi: 10.1146/annurev.immunol.19.1.497

Lewit-Bentley, A., & Réty, S. (2000). EF-hand calcium-binding proteins. *Curr Opin Struct Biol, 10*(6), 637-643. doi: 10.1016/s0959-440x(00)00142-1

Lichtenegger, M., & Groschner, K. (2014). TRPC3: a multifunctional signaling molecule. *Handb Exp Pharmacol, 222*, 67-84. doi: 10.1007/978-3-642-54215-2_4

Lintschinger, B., Balzer-Geldsetzer, M., Baskaran, T., Graier, W. F., Romanin, C., Zhu, M. X., & Groschner, K. (2000). Coassembly of Trp1 and Trp3 proteins generates diacylglycerol- and Ca2+-sensitive cation channels. *J Biol Chem, 275*(36), 27799-27805. doi: 10.1074/jbc.M002705200

Liu, H., Chatel, S., Simard, C., Syam, N., Salle, L., Probst, V., . . . Bouvagnet, P. (2013). Molecular genetics and functional anomalies in a series of 248 Brugada cases with 11 mutations in the TRPM4 channel. *PLoS One, 8*(1), e54131. doi: 10.1371/journal.pone.0054131

Liu, H., El Zein, L., Kruse, M., Guinamard, R., Beckmann, A., Bozio, A., . . . Bouvagnet, P. (2010). Gain-of-function mutations in TRPM4 cause autosomal dominant isolated cardiac conduction disease. *Circ Cardiovasc Genet, 3*(4), 374-385.

Liu, X., Yao, M., Li, N., Wang, C., Zheng, Y., & Cao, X. (2008). CaMKII promotes TLR-triggered proinflammatory cytokine and type I interferon production by directly binding and activating TAK1 and IRF3 in macrophages. *Blood, 112*(13), 4961-4970. doi: 10.1182/blood-2008-03-144022

Loh, K. P., Ng, G., Yu, C. Y., Fhu, C. K., Yu, D., Vennekens, R., . . . Liao, P. (2014). TRPM4 inhibition promotes angiogenesis after ischemic stroke. *Pflugers Arch, 466*(3), 563-576. doi: 10.1007/s00424-013-1347-4

Loxham, M., Davies, D. E., & Blume, C. (2014). Epithelial function and dysfunction in asthma. *Clin Exp Allergy, 44*(11), 1299-1313. doi: 10.1111/cea.12309

Lukacs, N. W., Prosser, D. M., Wiekowski, M., Lira, S. A., & Cook, D. N. (2001). Requirement for the chemokine receptor CCR6 in allergic pulmonary inflammation. *J Exp Med, 194*(4), 551-555. doi: 10.1084/jem.194.4.551

Luo, J., Ashikaga, E., Rubin, P. P., Heimann, M. J., Hildick, K. L., Bishop, P., . . . Wilkinson, K. A. (2013). Receptor trafficking and the regulation of synaptic plasticity by SUMO. *Neuromolecular Med, 15*(4), 692-706. doi: 10.1007/s12017-013-8253-y

Lutterloh, E., Opal, S., Pittman, D., Keith, J., Tan, X., Clancy, B., . . . Kessimian, N. (2007). Inhibition of the RAGE products increases survival in experimental models of severe sepsis and systemic infection. *Crit Care, 11*(6), R122.

Ma, Z., Björklund, A., & Islam, M. S. (2017). A TRPM4 Inhibitor 9-Phenanthrol Inhibits Glucose- and Glucagon-Like Peptide 1-Induced Insulin Secretion from Rat Islets of Langerhans. *J Diabetes Res, 2017*, 5131785.

MacRedmond, R., Greene, C., Taggart, C. C., McElvaney, N., & O'Neill, S. (2005). Respiratory epithelial cells require Toll-like receptor 4 for induction of human beta-defensin 2 by lipopolysaccharide. *Respiratory research, 6*(1), 116-116. doi: 10.1186/1465-9921-6-116

Malenka, R. C., Nicoll, & Roger, A. (1999). Long-Term Potentiation--A Decade of Progress? *Science, 285*(5435), 1870. doi: 10.1126/science.285.5435.1870

Malhotra, S., Hayes, D., & Wozniak, D. J. (2019). Mucoid Pseudomonas aeruginosa and regional inflammation in the cystic fibrosis lung. *J Cyst Fibros, 18*(6), 796-803.

Manderson, A. P., Kay, J. G., Hammond, L. A., Brown, D. L., & Stow, J. L. (2007). Subcompartments of the macrophage recycling endosome direct the differential secretion of IL-6 and TNFalpha. *The Journal of cell biology, 178*(1), 57-69. doi: 10.1083/jcb.200612131

Martin, L., Pingle, S. C., Hallam, D. M., Rybak, L. P., & Ramkumar, V. (2006). Activation of the Adenosine A₃ Receptor in RAW 264.7 Cells Inhibits Lipopolysaccharide-Stimulated Tumor Necrosis Factor-α Release by Reducing Calcium-Dependent Activation of Nuclear Factor-κB and Extracellular Signal-Regulated Kinase 1/2. *Journal of Pharmacology and Experimental Therapeutics, 316*(1), 71. doi: 10.1124/jpet.105.091868

Mayer, A. K., Muehmer, M., Mages, J., Gueinzius, K., Hess, C., Heeg, K., . . . Dalpke, A. H. (2007). Differential recognition of TLR-dependent microbial ligands in human bronchial epithelial cells. *J Immunol, 178*(5), 3134-3142. doi: 10.4049/jimmunol.178.5.3134

McDonald, P. P., Fadok, V. A., Bratton, D., & Henson, P. M. (1999). Transcriptional and Translational Regulation of Inflammatory Mediator Production by Endogenous TGF-β in Macrophages That Have Ingested Apoptotic Cells. *The Journal of Immunology, 163*(11), 6164-6172.

McNulty, S., & Fonfria, E. (2005). The role of TRPM channels in cell death. *Pflügers Archiv, 451*(1), 235-242. doi: 10.1007/s00424-005-1440-4

Medzhitov, R. (2007). Recognition of microorganisms and activation of the immune response. *Nature, 449*(7164), 819-826. doi: 10.1038/nature06246

Montell, C., Birnbaumer, L., Flockerzi, V., Bindels, R. J., Bruford, E. A., Caterina, M. J., . . . Zhu, M. X. (2002). A unified nomenclature for the superfamily of TRP cation channels. *Mol Cell, 9*(2), 229-231.

Moore, L. S., Cunningham, J., & Donaldson, H. (2016). A clinical approach to managing Pseudomonas aeruginosa infections. *Br J Hosp Med (Lond), 77*(4), C50-54. doi: 10.12968/hmed.2016.77.4.C50

Morenilla-Palao, C., Planells-Cases, R., García-Sanz, N., & Ferrer-Montiel, A. (2004). Regulated exocytosis contributes to protein kinase C potentiation of vanilloid receptor activity. *J Biol Chem, 279*(24), 25665-25672. doi: 10.1074/jbc.M311515200

Moskowitz, S. M., Ernst, R. K., & Miller, S. I. (2004). PmrAB, a two-component regulatory system of Pseudomonas aeruginosa that modulates resistance to cationic antimicrobial peptides and addition of aminoarabinose to lipid A. *J Bacteriol, 186*(2), 575-579. doi: 10.1128/jb.186.2.575-579.2004

Murray, R. Z., & Stow, J. L. (2014). Cytokine Secretion in Macrophages: SNAREs, Rabs, and Membrane Trafficking. *Frontiers in immunology, 5*, 538-538. doi: 10.3389/fimmu.2014.00538

Nagai, Y., Akashi, S., Nagafuku, M., Ogata, M., Iwakura, Y., Akira, S., . . . Miyake, K. (2002). Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat Immunol, 3*(7), 667-672. doi: 10.1038/ni809

Namkung, W., Finkbeiner, W. E., & Verkman, A. S. (2010). CFTR-adenylyl cyclase I association responsible for UTP activation of CFTR in well-differentiated primary human bronchial cell cultures. *Molecular biology of the cell, 21*(15), 2639-2648. doi: 10.1091/mbc.E09-12-1004

Ngkelo, A., Meja, K., Yeadon, M., Adcock, I., & Kirkham, P. A. (2012). LPS induced inflammatory responses in human peripheral blood mononuclear cells is mediated through NOX4 and Giα dependent PI-3kinase signalling. *J Inflamm (Lond), 9*(1), 1. doi: 10.1186/1476-9255-9-1

Nilius, B., Mahieu, F., Prenen, J., Janssens, A., Owsianik, G., Vennekens, R., & Voets, T. (2006). The Ca2+-activated cation channel TRPM4 is regulated by phosphatidylinositol 4,5-biphosphate. *Embo j, 25*(3), 467-478. doi: 10.1038/sj.emboj.7600963

Nilius, B., Prenen, J., Droogmans, G., Voets, T., Vennekens, R., Freichel, M., . . . Flockerzi, V. (2003). Voltage dependence of the Ca2+-activated cation channel TRPM4. *J Biol Chem, 278*(33), 30813-30820. doi: 10.1074/jbc.M305127200

Nilius, B., Prenen, J., Janssens, A., Owsianik, G., Wang, C., Zhu, M. X., & Voets, T. (2005a). The selectivity filter of the cation channel TRPM4. *J Biol Chem, 280*(24), 22899-22906. doi: 10.1074/jbc.M501686200

Nilius, B., Prenen, J., Tang, J., Wang, C., Owsianik, G., Janssens, A., . . . Zhu, M. X. (2005b). Regulation of the Ca2+ sensitivity of the nonselective cation channel TRPM4. *J Biol Chem, 280*(8), 6423-6433. doi: 10.1074/jbc.M411089200

Nilius, B., Prenen, J., Voets, T., & Droogmans, G. (2004). Intracellular nucleotides and polyamines inhibit the Ca2+-activated cation channel TRPM4b. *Pflugers Arch, 448*(1), 70-75. doi: 10.1007/s00424-003-1221-x

Nilius, B., & Vennekens, R. (2006). From cardiac cation channels to the molecular dissection of the transient receptor potential channel TRPM4. *Pflugers Arch, 453*(3), 313-321. doi: 10.1007/s00424-006-0088-z

Nomura, K., Obata, K., Keira, T., Miyata, R., Hirakawa, S., Takano, K.-i., . . . Kojima, T. (2014). Pseudomonas aeruginosa elastase causes transient disruption of tight junctions and downregulation of PAR-2 in human nasal epithelial cells. *Respiratory research, 15*(1), 21. doi: 10.1186/1465-9921-15-21

Nystrom, P. O. (1998). The systemic inflammatory response syndrome: definitions and aetiology. *J Antimicrob Chemother, 41 Suppl A*, 1-7. doi: 10.1093/jac/41.suppl_1.1

O'Neill, L. A. (2002). Signal transduction pathways activated by the IL-1 receptor/toll-like receptor superfamily. *Curr Top Microbiol Immunol, 270*, 47-61.

Ohto, U., Fukase, K., Miyake, K., & Satow, Y. (2007). Crystal Structures of Human MD-2 and Its Complex with Antiendotoxic Lipid IVa. *Science, 316*(5831), 1632. doi: 10.1126/science.1139111

Ohyama, A., Hosaka, K., Komiya, Y., Akagawa, K., Yamauchi, E., Taniguchi, H., . . . Igarashi, M. (2002). Regulation of exocytosis through Ca2+/ATP-dependent binding of autophosphorylated Ca2+/calmodulin-activated protein kinase II to syntaxin 1A. *J Neurosci, 22*(9), 3342-3351. doi: 10.1523/jneurosci.22-09-03342.2002

Oliver, A., Canton, R., Campo, P., Baquero, F., & Blazquez, J. (2000). High frequency of hypermutable Pseudomonas aeruginosa in cystic fibrosis lung infection. *Science, 288*(5469), 1251-1254. doi: 10.1126/science.288.5469.1251

Owsianik, G., Talavera, K., Voets, T., & Nilius, B. (2006). Permeation and selectivity of TRP channels. *Annu Rev Physiol, 68*, 685-717. doi: 10.1146/annurev.physiol.68.040204.101406

Paradiso, A. M., Brown, H. A., Ye, H., Harden, T. K., & Boucher, R. C. (1999). Heterogeneous responses of cell Ca2+ in human airway epithelium. *Exp Lung Res, 25*(4), 277-290. doi: 10.1080/019021499270196

Park, J. Y., Hwang, E. M., Yarishkin, O., Seo, J. H., Kim, E., Yoo, J., . . . Hong, S. G. (2008). TRPM4b channel suppresses store-operated Ca2+ entry by a novel protein-protein interaction with the TRPC3 channel. *Biochem Biophys Res Commun, 368*(3), 677-683. doi: 10.1016/j.bbrc.2008.01.153

Park, K. S., Korfhagen, T. R., Bruno, M. D., Kitzmiller, J. A., Wan, H., Wert, S. E., . . . Whitsett, J. A. (2007). SPDEF regulates goblet cell hyperplasia in the airway epithelium. *J Clin Invest, 117*(4), 978-988. doi: 10.1172/jci29176

Peters, C., & Mayer, A. (1998). Ca2+/calmodulin signals the completion of docking and triggers a late step of vacuole fusion. *Nature, 396*(6711), 575-580. doi: 10.1038/25133

Phadke, A. P., Akangire, G., Park, S. J., Lira, S. A., & Mehrad, B. (2007). The role of CC chemokine receptor 6 in host defense in a model of invasive pulmonary aspergillosis. *Am J Respir Crit Care Med, 175*(11), 1165-1172. doi: 10.1164/rccm.200602-256OC

Pier, G. B. (2007). Pseudomonas aeruginosa lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity. *Int J Med Microbiol, 297*(5), 277-295. doi: 10.1016/j.ijmm.2007.03.012

Pinheiro da Silva, F., & Machado, M. C. (2012). Antimicrobial peptides: clinical relevance and therapeutic implications. *Peptides, 36*(2), 308-314. doi: 10.1016/j.peptides.2012.05.014

Pohunek, P. (2004). Development, structure and function of the upper airways. *Paediatr Respir Rev, 5*(1), 2-8. doi: 10.1016/j.prrv.2003.09.002

Powers, J. P., & Hancock, R. E. (2003). The relationship between peptide structure and antibacterial activity. *Peptides, 24*(11), 1681-1691. doi: 10.1016/j.peptides.2003.08.023

Proud, D., & Leigh, R. (2011). Epithelial cells and airway diseases. *Immunol Rev, 242*(1), 186-204. doi: 10.1111/j.1600-065X.2011.01033.x

Pugin, J., Schürer-Maly, C. C., Leturcq, D., Moriarty, A., Ulevitch, R. J., & Tobias, P. S. (1993). Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. *Proceedings of the National Academy of Sciences, 90*(7), 2744. doi: 10.1073/pnas.90.7.2744

Rajanbabu, V., Galam, L., Fukumoto, J., Enciso, J., Tadikonda, P., Lane, T. N., . . . Kolliputi, N. (2015). Genipin suppresses NLRP3 inflammasome activation through uncoupling protein-2. *Cellular immunology, 297*(1), 40-45. doi: 10.1016/j.cellimm.2015.06.002

Ramsgaard, L., Englert, J., Manni, M., Milutinovic, P., Gefter, J., Tobolewski, J., . . . Oury, T. (2011). Lack of the receptor for advanced glycation end-products attenuates E. coli pneumonia in mice. *PLoS One, 6*(5), e20132.

Randolph, G. J., Beaulieu, S., Lebecque, S., Steinman, R. M., & Muller, W. A. (1998). Differentiation of monocytes into dendritic cells in a model of transendothelial trafficking. *Science, 282*(5388), 480-483. doi: 10.1126/science.282.5388.480

Rayees, S., Joshi, J. C., Tauseef, M., Anwar, M., Baweja, S., Rochford, I., . . . Mehta, D. (2019). PAR2-Mediated cAMP Generation Suppresses TRPV4-Dependent Ca Signaling in Alveolar Macrophages to Resolve TLR4-Induced Inflammation. *Cell Rep, 27*(3), 793-805.e794.

Ren, H., Teng, Y., Binghe, T., Zhang, X., Jiang, W., Liu, M., . . . Qian, M. (2014). Toll-Like Receptor-Triggered Calcium Mobilization Protects Mice against Bacterial Infection through Extracellular ATP Release. *Infect Immun, 82*(12), 5076-5085. doi: 10.1128/IAI.02546-14

Rixecker, T., Mathar, I., Medert, R., Mannebach, S., Pfeifer, A., Lipp, P., . . . Freichel, M. (2016). TRPM4-mediated control of FcεRI-evoked Ca(2+) elevation comprises enhanced plasmalemmal trafficking of TRPM4 channels in connective tissue type mast cells. *Sci Rep, 6*, 32981.

Romano, M., Sironi, M., Toniatti, C., Polentarutti, N., Fruscella, P., Ghezzi, P., . . . Mantovani, A. (1997). Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity, 6*(3), 315-325. doi: 10.1016/s1074-7613(00)80334-9

Roy, S., Sun, Y., & Pearlman, E. (2011). Interferon-gamma-induced MD-2 protein expression and lipopolysaccharide (LPS) responsiveness in corneal epithelial cells is mediated by Janus tyrosine kinase-2 activation and direct binding of STAT1 protein to the MD-2 promoter. *J Biol Chem, 286*(27), 23753-23762. doi: 10.1074/jbc.M111.219345

Ryan, G. B., & Majno, G. (1977). Acute inflammation. A review. *The American journal of pathology, 86*(1), 183-276.

Ryan, M. C., Zeeberg, B. R., Caplen, N. J., Cleland, J. A., Kahn, A. B., Liu, H., & Weinstein, J. N. (2008). SpliceCenter: a suite of web-based bioinformatic applications for evaluating the impact of alternative splicing on RT-PCR, RNAi, microarray, and peptide-based studies. *BMC Bioinformatics, 9*, 313. doi: 10.1186/1471-2105-9-313

Sadikot, R. T., Blackwell, T. S., Christman, J. W., & Prince, A. S. (2005). Pathogen–Host Interactions in Pseudomonas aeruginosa Pneumonia. *Am J Respir Crit Care Med, 171*(11), 1209-1223. doi: 10.1164/rccm.200408-1044SO

Sakaguchi, T., Okumura, R., Ono, C., Okuzaki, D., Kawai, T., Okochi, Y., . . . Takeda, K. (2020). TRPM5 Negatively Regulates Calcium-Dependent Responses in Lipopolysaccharide-Stimulated B Lymphocytes. *Cell Rep, 31*(10), 107755.

Sallusto, F., Palermo, B., Lenig, D., Miettinen, M., Matikainen, S., Julkunen, I., . . . Lanzavecchia, A. (1999). Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur J Immunol, 29*(5), 1617-1625. doi: 10.1002/(sici)1521-4141(199905)29:05<1617::aid-immu1617>3.0.co;2-3

Savill, J. S., Wyllie, A. H., Henson, J. E., Walport, M. J., Henson, P. M., & Haslett, C. (1989). Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest, 83*(3), 865-875. doi: 10.1172/JCI113970

Scepek, S., Coorssen, J. R., & Lindau, M. (1998). Fusion pore expansion in horse eosinophils is modulated by Ca2+ and protein kinase C via distinct mechanisms. *Embo j, 17*(15), 4340-4345. doi: 10.1093/emboj/17.15.4340

Schappe, M. S., Szteyn, K., Stremska, M. E., Mendu, S. K., Downs, T. K., Seegren, P. V., . . . Desai, B. N. (2018). Chanzyme TRPM7 Mediates the Ca(2+) Influx Essential for Lipopolysaccharide-Induced Toll-Like Receptor 4 Endocytosis and Macrophage Activation. *Immunity, 48*(1), 59-74.e55. doi: 10.1016/j.immuni.2017.11.026

Schattling, B., Steinbach, K., Thies, E., Kruse, M., Menigoz, A., Ufer, F., . . . Friese, M. A. (2012). TRPM4 cation channel mediates axonal and neuronal degeneration in experimental autoimmune encephalomyelitis and multiple sclerosis. *Nat Med, 18*(12), 1805-1811. doi: 10.1038/nm.3015

Schibli, D. J., Hunter, H. N., Aseyev, V., Starner, T. D., Wiencek, J. M., McCray, P. B., Jr., . . . Vogel, H. J. (2002). The solution structures of the human beta-defensins lead to a better understanding of the potent bactericidal activity of HBD3 against Staphylococcus aureus. *J Biol Chem, 277*(10), 8279-8289. doi: 10.1074/jbc.M108830200

Schleimer, R. P., Lane, A. P., & Kim, J. (2007). Innate and acquired immunity and epithelial cell function in chronic rhinosinusitis. *Clin Allergy Immunol, 20*, 51-78.

Schmid-Burgk, J. L., Gaidt, M. M., Schmidt, T., Ebert, T. S., Bartok, E., & Hornung, V. (2015). Caspase-4 mediates non-canonical activation of the NLRP3 inflammasome in human myeloid cells. *Eur J Immunol, 45*(10), 2911-2917. doi: 10.1002/eji.201545523

Schorr, W., Swandulla, D., & Zeilhofer, H. U. (1999). Mechanisms of IL-8-induced Ca2+ signaling in human neutrophil granulocytes. *Eur J Immunol, 29*(3), 897-904. doi: 10.1002/(sici)1521-4141(199903)29:03<897::aid-immu897>3.0.co;2-5

Schulz, C., Farkas, L., Wolf, K., Kratzel, K., Eissner, G., & Pfeifer, M. (2002). Differences in LPS-induced activation of bronchial epithelial cells (BEAS-2B) and type II-like pneumocytes (A-549). *Scand J Immunol, 56*(3), 294-302. doi: 10.1046/j.1365-3083.2002.01137.x

Schwarzer, C., Wong, S., Shi, J., Matthes, E., Illek, B., Ianowski, J. P., . . . Machen, T. E. (2010). Pseudomonas aeruginosa Homoserine lactone activates store-operated cAMP and cystic fibrosis transmembrane regulator-dependent Cl- secretion by human airway epithelia. *J Biol Chem, 285*(45), 34850-34863. doi: 10.1074/jbc.M110.167668

Serafini, N., Dahdah, A., Barbet, G., Demion, M., Attout, T., Gautier, G., . . . Launay, P. (2012). The TRPM4 Channel Controls Monocyte and Macrophage, but Not Neutrophil, Function for Survival in Sepsis. *The Journal of Immunology, 189*(7), 3689. doi: 10.4049/jimmunol.1102969

Sha, Q., Truong-Tran, A. Q., Plitt, J. R., Beck, L. A., & Schleimer, R. P. (2004). Activation of airway epithelial cells by toll-like receptor agonists. *Am J Respir Cell Mol Biol, 31*(3), 358-364. doi: 10.1165/rcmb.2003-0388OC

Shaykhiev, R. (2007). *CO-OPERATIVE REGULATION OF EPITHELIAL HOMEOSTASIS AND IMMUNITY*.

Shigeto, M., Ramracheya, R., Tarasov, A. I., Cha, C. Y., Chibalina, M. V., Hastoy, B., . . . Rorsman, P. (2015). GLP-1 stimulates insulin secretion by PKC-dependent TRPM4 and TRPM5 activation. *J Clin Invest, 125*(12), 4714-4728.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., & Kimoto, M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med, 189*(11), 1777-1782. doi: 10.1084/jem.189.11.1777

Simard, C., Hof, T., Keddache, Z., Launay, P., & Guinamard, R. (2013). The TRPM4 non-selective cation channel contributes to the mammalian atrial action potential. *Journal of Molecular and Cellular Cardiology, 59*, 11-19. doi: https://doi.org/10.1016/j.yjmcc.2013.01.019

Simard, C., Sallé, L., Rouet, R., & Guinamard, R. (2012). Transient receptor potential melastatin 4 inhibitor 9-phenanthrol abolishes arrhythmias induced by hypoxia and re-oxygenation in mouse ventricle. *Br J Pharmacol, 165*(7), 2354-2364. doi: 10.1111/j.1476-5381.2011.01715.x

Simon, F., & Fernandez, R. (2009). Early lipopolysaccharide-induced reactive oxygen species production evokes necrotic cell death in human umbilical vein endothelial cells. *J Hypertens, 27*(6), 1202-1216. doi: 10.1097/HJH.0b013e328329e31c

Simon, F., Leiva-Salcedo, E., Armisén, R., Riveros, A., Cerda, O., Varela, D., . . . Stutzin, A. (2010). Hydrogen peroxide removes TRPM4 current desensitization conferring increased vulnerability to necrotic cell death. *J Biol Chem, 285*(48), 37150-37158. doi: 10.1074/jbc.M110.155390

Simon, F., Varela, D., & Cabello-Verrugio, C. (2013). Oxidative stress-modulated TRPM ion channels in cell dysfunction and pathological conditions in humans. *Cellular Signalling, 25*(7), 1614-1624. doi: https://doi.org/10.1016/j.cellsig.2013.03.023

Simpson, J. C., Niven, R. M., Pickering, C. A., Oldham, L. A., Fletcher, A. M., & Francis, H. C. (1999). Comparative personal exposures to organic dusts and endotoxin. *Ann Occup Hyg, 43*(2), 107-115.

Singh, B. B., Lockwich, T. P., Bandyopadhyay, B. C., Liu, X., Bollimuntha, S., Brazer, S. C., . . . Ambudkar, I. S. (2004). VAMP2-dependent exocytosis regulates plasma membrane insertion of TRPC3 channels and contributes to agonist-stimulated Ca2+ influx. *Mol Cell, 15*(4), 635-646. doi: 10.1016/j.molcel.2004.07.010

Smith, A. C., Parajuli, S. P., Hristov, K. L., Cheng, Q., Soder, R. P., Afeli, S. A. Y., . . . Petkov, G. V. (2013). TRPM4 channel: a new player in urinary bladder smooth muscle function in rats. *American Journal of Physiology-Renal Physiology, 304*(7), F918-F929. doi: 10.1152/ajprenal.00417.2012

Smith, E. E., Buckley, D. G., Wu, Z., Saenphimmachak, C., Hoffman, L. R., D'Argenio, D. A., . . . Olson, M. V. (2006). Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. *Proc Natl Acad Sci U S A, 103*(22), 8487-8492. doi: 10.1073/pnas.0602138103

Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., & Rothman, J. E. (1993). SNAP receptors implicated in vesicle targeting and fusion. *Nature, 362*(6418), 318-324. doi: 10.1038/362318a0

Sonawane, A., Jyot, J., During, R., & Ramphal, R. (2006). Neutrophil elastase, an innate immunity effector molecule, represses flagellin transcription in Pseudomonas aeruginosa. *Infect Immun, 74*(12), 6682-6689. doi: 10.1128/iai.00922-06

Song, M. Y., & Yuan, J. X. (2010). Introduction to TRP channels: structure, function, and regulation. *Adv Exp Med Biol, 661*, 99-108. doi: 10.1007/978-1-60761-500-2_6

Soutourina, O. A., & Bertin, P. N. (2003). Regulation cascade of flagellar expression in Gram-negative bacteria. *FEMS Microbiol Rev, 27*(4), 505-523. doi: 10.1016/s0168-6445(03)00064-0

Soyka, M. B., Wawrzyniak, P., Eiwegger, T., Holzmann, D., Treis, A., Wanke, K., . . . Akdis, C. A. (2012). Defective epithelial barrier in chronic rhinosinusitis: The regulation of tight junctions by IFN-γ and IL-4. *Journal of Allergy and Clinical Immunology, 130*(5), 1087-1096.e1010. doi: https://doi.org/10.1016/j.jaci.2012.05.052

Stanley, A. C., & Lacy, P. (2010). Pathways for cytokine secretion. *Physiology (Bethesda), 25*(4), 218-229. doi: 10.1152/physiol.00017.2010

Steeghs, L., Keestra, A. M., van Mourik, A., Uronen-Hansson, H., van der Ley, P., Callard, R., . . . van Putten, J. P. M. (2008). Differential activation of human and mouse Toll-like receptor 4 by the adjuvant candidate LpxL1 of Neisseria meningitidis. *Infect Immun, 76*(8), 3801-3807.

Stewart, I., Schluter, P. J., & Shaw, G. R. (2006). Cyanobacterial lipopolysaccharides and human health - a review. *Environ Health, 5*, 7. doi: 10.1186/1476-069x-5-7

Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., . . . Olson, M. V. (2000). Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunistic pathogen. *Nature, 406*(6799), 959-964. doi: 10.1038/35023079

Sun, J., Li, N., Oh, K.-S., Dutta, B., Vayttaden, S. J., Lin, B., . . . Fraser, I. D. C. (2016). Comprehensive RNAi-based screening of human and mouse TLR pathways identifies species-specific preferences in signaling protein use. *Sci Signal, 9*(409), ra3.

Sun, Y., & Pearlman, E. (2009). Inhibition of corneal inflammation by the TLR4 antagonist Eritoran tetrasodium (E5564). *Invest Ophthalmol Vis Sci, 50*(3), 1247-1254. doi: 10.1167/iovs.08-2628

Sun, Y., Sukumaran, P., Schaar, A., & Singh, B. B. (2015). TRPM7 and its role in neurodegenerative diseases. *Channels, 9*(5), 253-261. doi: 10.1080/19336950.2015.1075675

Sundström, C., & Nilsson, K. (1976). Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *International Journal of Cancer, 17*(5), 565-577. doi: https://doi.org/10.1002/ijc.2910170504

Sutton, R. B., Fasshauer, D., Jahn, R., & Brunger, A. T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature, 395*(6700), 347-353. doi: 10.1038/26412

Syam, N., Rougier, J.-S., & Abriel, H. (2014). Glycosylation of TRPM4 and TRPM5 channels: molecular determinants and functional aspects. *Frontiers in cellular neuroscience, 8*, 52-52. doi: 10.3389/fncel.2014.00052

Taggart, C. C., Greene, C. M., Smith, S. G., Levine, R. L., McCray, P. B., Jr., O'Neill, S., & McElvaney, N. G. (2003). Inactivation of human beta-defensins 2 and 3 by elastolytic cathepsins. *J Immunol, 171*(2), 931-937. doi: 10.4049/jimmunol.171.2.931

Takada, H., & Kotani, S. (1989). Structural requirements of lipid A for endotoxicity and other biological activities. *Crit Rev Microbiol, 16*(6), 477-523.

Takahashi, K., Yamamura, F., & Naito, M. (1989). Differentiation, maturation, and proliferation of macrophages in the mouse yolk sac: a light-microscopic, enzyme-cytochemical, immunohistochemical, and ultrastructural study. *J Leukoc Biol, 45*(2), 87-96.

Takashiba, S., Van Dyke, T. E., Amar, S., Murayama, Y., Soskolne, A. W., & Shapira, L. (1999). Differentiation of monocytes to macrophages primes cells for lipopolysaccharide stimulation via accumulation of cytoplasmic nuclear factor kappaB. *Infect Immun, 67*(11), 5573-5578. doi: 10.1128/iai.67.11.5573-5578.1999

Takeda, K., & Akira, S. (2001). Roles of Toll-like receptors in innate immune responses. *Genes to Cells, 6*(9), 733-742. doi: 10.1046/j.1365-2443.2001.00458.x

Takeda, K., Kaisho, T., & Akira, S. (2003). Toll-like receptors. *Annual Review of Immunology, 21*, 335-376. doi: 10.1146/annurev.immunol.21.120601.141126

Tate, S., MacGregor, G., Davis, M., Innes, J. A., & Greening, A. P. (2002). Airways in cystic fibrosis are acidified: detection by exhaled breath condensate. *Thorax, 57*(11), 926-929. doi: 10.1136/thorax.57.11.926

Thorley, A. J., Grandolfo, D., Lim, E., Goldstraw, P., Young, A., & Tetley, T. D. (2011). Innate immune responses to bacterial ligands in the peripheral human lung--role of alveolar epithelial TLR expression and signalling. *PLoS One, 6*(7), e21827.

Tirouvanziam, R. (2006). Neutrophilic inflammation as a major determinant in the progression of cystic fibrosis. *Drug News Perspect, 19*(10), 609-614. doi: 10.1358/dnp.2006.19.10.1068008

Trinchieri, G., & Sher, A. (2007). Cooperation of Toll-like receptor signals in innate immune defence. *Nat Rev Immunol, 7*(3), 179-190. doi: 10.1038/nri2038

Tsubouchi, H., Inoguchi, T., Inuo, M., Kakimoto, M., Sonta, T., Sonoda, N., . . . Nawata, H. (2005). Sulfonylurea as well as elevated glucose levels stimulate reactive oxygen species production in the pancreatic beta-cell line, MIN6-a role of NAD(P)H oxidase in beta-cells. *Biochem Biophys Res Commun, 326*(1), 60-65. doi: 10.1016/j.bbrc.2004.10.201

Tsutsumi-Ishii, Y., & Nagaoka, I. (2002). NF-kappa B-mediated transcriptional regulation of human beta-defensin-2 gene following lipopolysaccharide stimulation. *J Leukoc Biol, 71*(1), 154-162.

Ueta, M., Nochi, T., Jang, M. H., Park, E. J., Igarashi, O., Hino, A., . . . Kiyono, H. (2004). Intracellularly expressed TLR2s and TLR4s contribution to an immunosilent environment at the ocular mucosal epithelium. *J Immunol, 173*(5), 3337-3347. doi: 10.4049/jimmunol.173.5.3337

Ullrich, N. D., Voets, T., Prenen, J., Vennekens, R., Talavera, K., Droogmans, G., & Nilius, B. (2005). Comparison of functional properties of the Ca2+-activated cation channels TRPM4 and TRPM5 from mice. *Cell Calcium, 37*(3), 267-278. doi: 10.1016/j.ceca.2004.11.001

Ulrich, M., Worlitzsch, D., Viglio, S., Siegmann, N., Iadarola, P., Shute, J. K., . . . Doring, G. (2010). Alveolar inflammation in cystic fibrosis. *J Cyst Fibros, 9*(3), 217-227. doi: 10.1016/j.jcf.2010.03.001

Upham, J. W., & Stick, S. M. (2006). Interactions between airway epithelial cells and dendritic cells: implications for the regulation of airway inflammation. *Curr Drug Targets, 7*(5), 541-545. doi: 10.2174/138945006776818647

Vallabhapurapu, S., & Karin, M. (2009). Regulation and Function of NF-κB Transcription Factors in the Immune System. *Annual Review of Immunology, 27*(1), 693-733. doi: 10.1146/annurev.immunol.021908.132641

Van Gennip, M., Christensen, L. D., Alhede, M., Phipps, R., Jensen, P. O., Christophersen, L., . . . Bjarnsholt, T. (2009). Inactivation of the rhlA gene in Pseudomonas aeruginosa prevents rhamnolipid production, disabling the protection against polymorphonuclear leukocytes. *Apmis, 117*(7), 537-546. doi: 10.1111/j.1600-0463.2009.02466.x

Venkatachalam, K., & Montell, C. (2007). TRP channels. *Annu Rev Biochem, 76*, 387-417. doi: 10.1146/annurev.biochem.75.103004.142819

Vennekens, R., Mesuere, M., & Philippaert, K. (2018). TRPM5 in the battle against diabetes and obesity. *Acta Physiologica, 222*(2), e12949. doi: 10.1111/apha.12949

Vennekens, R., & Nilius, B. (2007). Insights into TRPM4 function, regulation and physiological role. *Handb Exp Pharmacol*(179), 269-285. doi: 10.1007/978-3-540-34891-7_16

Vennekens, R., Olausson, J., Meissner, M., Bloch, W., Mathar, I., Philipp, S. E., . . . Freichel, M. (2007). Increased IgE-dependent mast cell activation and anaphylactic responses in mice lacking the calcium-activated nonselective cation channel TRPM4. *Nat Immunol, 8*(3), 312-320. doi: 10.1038/ni1441

Verboogen, D. R. J., Gonzalez Mancha, N., Ter Beest, M., & van den Bogaart, G. (2017). Fluorescence Lifetime Imaging Microscopy reveals rerouting of SNARE trafficking driving dendritic cell activation. *eLife, 6*, e23525. doi: 10.7554/eLife.23525

Verboogen, D. R. J., Ter Beest, M., Honigmann, A., & van den Bogaart, G. (2018). Secretory vesicles of immune cells contain only a limited number of interleukin 6 molecules. *FEBS Lett, 592*(9), 1535-1544. doi: 10.1002/1873-3468.13036

Verhage, M., Hens, J. J., De Grann, P. N., Boomsma, F., Wiegant, V. M., da Silva, F. H., . . . Ghijsen, W. E. (1995). Ba2+ replaces Ca2+/calmodulin in the activation of protein phosphatases and in exocytosis of all major transmitters. *Eur J Pharmacol, 291*(3), 387-398. doi: 10.1016/0922-4106(95)90081-0

Viriyakosol, S., Tobias, P. S., Kitchens, R. L., & Kirkland, T. N. (2001). MD-2 binds to bacterial lipopolysaccharide. *J Biol Chem, 276*(41), 38044-38051. doi: 10.1074/jbc.M105228200

Visintin, A., Halmen, K. A., Khan, N., Monks, B. G., Golenbock, D. T., & Lien, E. (2006). MD-2 expression is not required for cell surface targeting of Toll-like receptor 4 (TLR4). *J Leukoc Biol, 80*(6), 1584-1592. doi: 10.1189/jlb.0606388

Visintin, A., Mazzoni, A., Spitzer, J. A., & Segal, D. M. (2001). Secreted MD-2 is a large polymeric protein that efficiently confers lipopolysaccharide sensitivity to Toll-like receptor 4. *Proc Natl Acad Sci U S A, 98*(21), 12156-12161. doi: 10.1073/pnas.211445098

von Kügelgen, I., & Wetter, A. (2000). Molecular pharmacology of P2Y-receptors. *Naunyn-Schmiedeberg's Archives of Pharmacology, 362*(4), 310-323. doi: 10.1007/s002100000310

Wang, P., Howard, M. D., Zhang, H., Chintagari, N. R., Bell, A., Jin, N., . . . Liu, L. (2012). Characterization of VAMP-2 in the lung: implication in lung surfactant secretion. *Cell Biol Int, 36*(9), 785-791. doi: 10.1042/cbi20110146

Watschinger, K., Horak, S. B., Schulze, K., Obermair, G. J., Wild, C., Koschak, A., . . . Striessnig, J. (2008). Functional properties and modulation of extracellular epitope-tagged Ca(V)2.1 voltage-gated calcium channels. *Channels (Austin), 2*(6), 461-473. doi: 10.4161/chan.2.6.6793

Watt, A. P., Courtney, J., Moore, J., Ennis, M., & Elborn, J. S. (2005). Neutrophil cell death, activation and bacterial infection in cystic fibrosis. *Thorax, 60*(8), 659-664. doi: 10.1136/thx.2004.038240

Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., . . . Rothman, J. E. (1998). SNAREpins: Minimal Machinery for Membrane Fusion. *Cell, 92*(6), 759-772. doi: https://doi.org/10.1016/S0092-8674(00)81404-X

Wehkamp, J., Harder, J., Wehkamp, K., Wehkamp-von Meissner, B., Schlee, M., Enders, C., . . . Stange, E. F. (2004). NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium. *Infect Immun, 72*(10), 5750-5758. doi: 10.1128/IAI.72.10.5750-5758.2004

Whyte, M. K., Meagher, L. C., MacDermot, J., & Haslett, C. (1993). Impairment of function in aging neutrophils is associated with apoptosis. *J Immunol, 150*(11), 5124-5134.

Willetts, L., & Felix, L. C. (2018). Vesicle-associated membrane protein 7-mediated eosinophil degranulation promotes allergic airway inflammation in mice. *1*, 83. doi: 10.1038/s42003-018-0081-z

Willoughby, D., & Cooper, D. M. (2007). Organization and Ca2+ regulation of adenylyl cyclases in cAMP microdomains. *Physiol Rev, 87*(3), 965-1010. doi: 10.1152/physrev.00049.2006
Winkler, P. A., Huang, Y., Sun, W., Du, J., & Lü, W. (2017). Electron cryo-microscopy structure of a human TRPM4 channel. *Nature, 552*(7684), 200-204. doi: 10.1038/nature24674
Wolters, P. J., & Chapman, H. A. (2000). Importance of lysosomal cysteine proteases in lung disease. *Respiratory research, 1*(3), 170-177. doi: 10.1186/rr29
Woo, S. K., Kwon, M. S., Ivanov, A., Geng, Z., Gerzanich, V., & Simard, J. M. (2013). Complex N-glycosylation stabilizes surface expression of transient receptor potential melastatin 4b protein. *J Biol Chem, 288*(51), 36409-36417. doi: 10.1074/jbc.M113.530584
Woo, S. K., Kwon, M. S., Ivanov, A., Gerzanich, V., & Simard, J. M. (2013). The sulfonylurea receptor 1 (Sur1)-transient receptor potential melastatin 4 (Trpm4) channel. *J Biol Chem, 288*(5), 3655-3667. doi: 10.1074/jbc.M112.428219
Woods, P. S., Kimmig, L. M., Meliton, A. Y., Sun, K. A., Tian, Y., O'Leary, E. M., . . . Mutlu, G. M. (2020). Tissue-Resident Alveolar Macrophages Do Not Rely on Glycolysis for LPS-induced Inflammation. *Am J Respir Cell Mol Biol, 62*(2), 243-255.
Wretlind, B., & Pavlovskis, O. R. (1983). Pseudomonas aeruginosa elastase and its role in pseudomonas infections. *Rev Infect Dis, 5 Suppl 5*, S998-1004. doi: 10.1093/clinids/5.supplement_5.s998
Wu, Q., Lu, Z., Verghese, M. W., & Randell, S. H. (2005). Airway epithelial cell tolerance to Pseudomonas aeruginosa. *Respiratory research, 6*(1), 26. doi: 10.1186/1465-9921-6-26
Xing, Z., Gauldie, J., Cox, G., Baumann, H., Jordana, M., Lei, X. F., & Achong, M. K. (1998). IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest, 101*(2), 311-320. doi: 10.1172/JCI1368
Xu, X.-Z. S., Li, H.-S., Guggino, W. B., & Montell, C. (1997). Coassembly of TRP and TRPL Produces a Distinct Store-Operated Conductance. *Cell, 89*(7), 1155-1164. doi: https://doi.org/10.1016/S0092-8674(00)80302-5
Xu, X. Z., Moebius, F., Gill, D. L., & Montell, C. (2001). Regulation of melastatin, a TRP-related protein, through interaction with a cytoplasmic isoform. *Proc Natl Acad Sci U S A, 98*(19), 10692-10697. doi: 10.1073/pnas.191360198
Xu, X. Z., & Sternberg, P. W. (2003). A C. elegans sperm TRP protein required for sperm-egg interactions during fertilization. *Cell, 114*(3), 285-297. doi: 10.1016/s0092-8674(03)00565-8
Xu, Y., Guo, M., Jiang, W., Dong, H., Han, Y., An, X. F., & Zhang, J. (2016). Endoplasmic reticulum stress and its effects on renal tubular cells apoptosis in ischemic acute kidney injury. *Ren Fail, 38*(5), 831-837. doi: 10.3109/0886022x.2016.1160724
Yamashiro, S., Kamohara, H., & Yoshimura, T. (1999). MCP-1 is selectively expressed in the late phase by cytokine-stimulated human neutrophils: TNF-alpha plays a role in maximal MCP-1 mRNA expression. *J Leukoc Biol, 65*(5), 671-679. doi: 10.1002/jlb.65.5.671
Yang, F., Tang, E., Guan, K., & Wang, C.-Y. (2003). IKKβ Plays an Essential Role in the Phosphorylation of RelA/p65 on Serine 536 Induced by Lipopolysaccharide. *The Journal of Immunology, 170*(11), 5630-5635. doi: 10.4049/jimmunol.170.11.5630
Yin, Y., Wu, M., Zubcevic, L., Borschel, W. F., Lander, G. C., & Lee, S.-Y. (2018). Structure of the cold- and menthol-sensing ion channel TRPM8. *Science, 359*(6372), 237. doi: 10.1126/science.aan4325
Yoo, J. C., Yarishkin, O. V., Hwang, E. M., Kim, E., Kim, D. G., Park, N., . . . Park, J. Y. (2010). Cloning and characterization of rat transient receptor potential-melastatin 4 (TRPM4). *Biochem Biophys Res Commun, 391*(1), 806-811. doi: 10.1016/j.bbrc.2009.11.142
Yoo, S., Lim, J. Y., & Hwang, S. W. (2014). Sensory TRP channel interactions with endogenous lipids and their biological outcomes. *Molecules, 19*(4), 4708-4744.

Zarember, K. A., & Godowski, P. J. (2002). Tissue Expression of Human Toll-Like Receptors and Differential Regulation of Toll-Like Receptor mRNAs in Leukocytes in Response to Microbes, Their Products, and Cytokines. *The Journal of Immunology, 168*(2), 554. doi: 10.4049/jimmunol.168.2.554

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature, 415*(6870), 389-395. doi: 10.1038/415389a

Zhang, F. X., Kirschning, C. J., Mancinelli, R., Xu, X.-P., Jin, Y., Faure, E., . . . Arditi, M. (1999). Bacterial Lipopolysaccharide Activates Nuclear Factor-κB through Interleukin-1 Signaling Mediators in Cultured Human Dermal Endothelial Cells and Mononuclear Phagocytes. *Journal of Biological Chemistry, 274*(12), 7611-7614. doi: 10.1074/jbc.274.12.7611

Zhang, N., Van Crombruggen, K., Gevaert, E., & Bachert, C. (2016). Barrier function of the nasal mucosa in health and type-2 biased airway diseases. *Allergy, 71*(3), 295-307. doi: 10.1111/all.12809

Zhang, R., Zhou, X., Shen, X., Xie, T., Xu, C., Zou, Z., . . . Liao, L. (2018). Different sulfonylureas induce the apoptosis of proximal tubular epithelial cell differently via closing K(ATP) channel. *Mol Med, 24*(1), 47. doi: 10.1186/s10020-018-0042-5

Zhang, X. Q., Dong, J. J., Cai, T., Shen, X., Zhou, X. J., & Liao, L. (2017). High glucose induces apoptosis via upregulation of Bim expression in proximal tubule epithelial cells. *Oncotarget, 8*(15), 24119-24129. doi: 10.18632/oncotarget.15491

Zhang, Z., Louboutin, J. P., Weiner, D. J., Goldberg, J. B., & Wilson, J. M. (2005a). Human airway epithelial cells sense Pseudomonas aeruginosa infection via recognition of flagellin by Toll-like receptor 5. *Infect Immun, 73*(11), 7151-7160. doi: 10.1128/iai.73.11.7151-7160.2005

Zhang, Z., Okawa, H., Wang, Y., & Liman, E. R. (2005b). Phosphatidylinositol 4,5-bisphosphate rescues TRPM4 channels from desensitization. *J Biol Chem, 280*(47), 39185-39192. doi: 10.1074/jbc.M506965200

Zhao, W., Ma, G., & Chen, X. (2014). Lipopolysaccharide induced LOX-1 expression via TLR4/MyD88/ROS activated p38MAPK-NF-κB pathway. *Vascular Pharmacology, 63*(3), 162-172. doi: https://doi.org/10.1016/j.vph.2014.06.008

Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P., & Ghosh, S. (1997). The Transcriptional Activity of NF-κB Is Regulated by the IκB-Associated PKAc Subunit through a Cyclic AMP–Independent Mechanism. *Cell, 89*(3), 413-424. doi: https://doi.org/10.1016/S0092-8674(00)80222-6

Zhu, H., Hille, B., & Xu, T. (2002). Sensitization of regulated exocytosis by protein kinase C. *Proceedings of the National Academy of Sciences, 99*(26), 17055. doi: 10.1073/pnas.232588899

Zierler, S., Hampe, S., & Nadolni, W. (2017). TRPM channels as potential therapeutic targets against pro-inflammatory diseases. *Cell Calcium, 67*, 105-115. doi: https://doi.org/10.1016/j.ceca.2017.05.002

Zucker, R. S. (1999). Calcium- and activity-dependent synaptic plasticity. *Curr Opin Neurobiol, 9*(3), 305-313. doi: 10.1016/s0959-4388(99)80045-2

C.V.

Aus datenschutzrechtlichen Gründen wird der Lebenslauf in der elektronischen Fassung der Dissertation nicht veröffentlicht.