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Activation of extracellular pattern recognition receptors by abundant and recurring bacteria in humans

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Vienna, 01.06.2023

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Abstract

Pattern recognition receptors are an important part of the innate immune system and responsible for identifying pathogenic molecular structures and initiating the immune response by proinflammatory cytokines, chemokines and interferons. One of the most essential pattern recognition receptors are Toll-like receptors that comprise a set of ten subgroups in humans, capable of recognizing a diverse set of conserved bacterial, viral and fungal structures. In this regard, bacterial activation of extracellular Toll-like receptors is of high interest. Recent studies showed that chronic nasal inflammation or viral infection may impact the composition of the host microbiome. Furthermore, an altered microbiome is linked to a variety of diseases and dysregulation of the immune system. For example, allergic disorders in children can be related to a deviated microbiome and changed signaling from Toll-like receptors, possibly resulting from differential exposures to microbes or viral infections. This is the reason why it is essential to understand the interactions of bacteria with the innate immune system and to further decipher altered signaling pathways.

To gain insights into the activation profile of Toll-like receptors by bacteria in the human respiratory tract, Toll-like receptor reporter cell lines were used. These cell lines are genetically modified to over-express a human Toll-like receptor and secrete an embryonic alkaline phosphatase upon activation of the respective receptor, which can be quantified photometrically. The selection of bacteria is based on a variety of publications concerning an altered microbiome in context of chronic nasal inflammatory disease or a viral infection. Bacterial species that occurred in an altered amount in ill patients compared to healthy individuals were analyzed.

Results showed very distinct activation patterns across all analyzed strains, even within strains of the same species. This demonstrates that the response of Toll-like receptors to each strain must be examined and evaluated independently, since a classification according to a certain species is not necessarily possible. Moreover, there was a significant difference in Toll-like receptor signaling between bacteria that were shown to have a higher or lower prevalence in patients with chronic nasal inflammation. These findings suggest a complex influence from the microbiome on the immune system and disease in humans.

Kurzfassung

Mustererkennungsrezeptoren sind ein wichtiger Bestandteil des angeborenen Immunsystems und verantwortlich für die Erkennung von konservierten molekularen Strukturen sowie in weiterer Folge die Initiation einer Immunantwort durch proinflammatorische Zytokine, Chemokine und Interferone. Eine Gruppe der wesentlichsten Mustererkennungsrezeptoren ist die Familie der Toll-like Rezeptoren. Der Mensch besitzt insgesamt zehn verschiedene Toll-like Rezeptoren, welche dafür verantwortlich sind molekulare Strukturen von Pathogenen zu erkennen. In dieser Arbeit liegt das Hauptaugenmerk auf der bakteriellen Aktivierung von extrazellulären Toll-like Rezeptoren. Neueste Studien zeigten, dass eine chronische Entzündung der oberen Atemwege oder eine virale Infektion die Zusammensetzung des humanen Mikrobioms beeinflussen kann. Außerdem wird eine Veränderung des Mikrobioms mit einer Reihe an Erkrankungen in Verbindung gebracht. Allergische Erkrankungen bei Kindern können zum Beispiel in Verbindung mit einem veränderten Mikrobiom oder gestörter Signalübertragung durch Toll-like Rezeptoren stehen.

Um einen Einblick in das Aktivierungsprofil von Toll-like Rezeptoren durch Bakterien der oberen Atemwege zu bekommen, wurden Toll-like Rezeptor Reporter Zelllinien verwendet. Diese Zelllinien sind genetisch modifiziert, sodass sie einen bestimmten humanen Toll-like Rezeptoren überexprimieren und gleichzeitig bei dessen Aktivierung eine Phosphatase sezernieren, welche anschließend photometrisch quantifiziert werden kann. Die Auswahl der Bakterien erfolgte aufgrund von Publikationen, die Änderungen des Mikrobioms als Folge einer Entzündung oder Infektion genauer untersuchten. Es wurden schließlich bakterielle Spezies ausgewählt, welche in einer veränderten Menge in kranken Patienten vorkamen.

Die Ergebnisse zeigen, dass es ein sehr diverses Aktivierungsmuster zwischen den unterschiedlichen Bakterien gibt. Dabei zeigen selbst unterschiedliche Stämme derselben Art signifikante Abweichungen, weshalb die Antwort des Immunsystems nicht zwingend nach Spezies eingeteilt werden kann. Außerdem gibt es einen signifikanten Unterschied im Signalverhalten von Toll-like Rezeptoren, wenn Bakterien verglichen werden, welche öfters beziehungsweise seltener in Patienten mit chronischer Entzündung der oberen Atemwege vorkommen. Das legt nahe, dass es einen komplexen Einfluss des Mikrobioms auf das Immunsystem und Krankheiten im Menschen gibt.

List of Abbreviations

AP-1	activating protein-1
CLR	C-type lectin receptor
CFU	colony forming units
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate-buffered saline
hTLR	human Toll-like receptor
IFN	interferon
IκB	inhibitor of NF-κB
IRAK4	interleukin-1 receptor-associated kinase 4
IRF3	interferon regulatory factor 3
LBS	LPS binding protein
LPS	lipopolysaccharides
LRR	leucine-rich repeats
MAL	MyD88-adapter like
MAMP	microbial associated molecular pattern
MyD88	myeloid differentiation primary response protein 88
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NLR	nucleotide-binding oligomerization domain-like receptor
NTC	no template control
PAMP	pathogen-associated molecular pattern
PRR	pattern recognition receptor
RLR	retinoic acid-inducible gene-I-like receptor
RSV	respiratory syncytial virus
SEAP	secreted embryonic alkaline phosphatase
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TRAM	TRIF-related adapter molecule
TRIF	TIR domain-containing adapter inducing IFNβ

Table of content

1 Introduction

The immunological response to pathogens is divided into two subgroups in vertebrates. A distinction is made between so called innate and adaptive immunity. While innate immunity is responsible for a more generalized and less-specific immune response, adaptive immunity is known for its specialized reaction (1). The primary function of the innate immune system is the detection of pathogens and mediation of their clearance through different innate and adaptive defense mechanisms. Innate immune defense mechanisms include immune cells like eosinophils, macrophages, antigen presenting cells, natural killer cells and non-specific immune mechanisms like complement system. Additionally, innate immune system leads to the activation of the adaptive immune system, resulting in the activation of T and B lymphocytes. There are several different subunits from T and B cells as well (2).

1.1 The role of pattern recognition receptors in innate immunity

Detection of pathogens by the innate immune system is from particular importance in order to start an adequate response. Therefore, nearly all cells possess so called pattern recognition receptors (PRRs). PRRs sense the presence of highly conserved patterns of pathogens and trigger the production of proinflammatory cytokines, interferons (IFNs) and chemokines (3). Those patterns are commonly referred to as pathogen-associated molecular patterns (PAMPs). Furthermore, PRRs can also recognize non-pathogenic microbes like commensal bacteria in the human gut, which is why it is sometimes more appropriate to refer to the term microbial associated molecular patterns (MAMPs) (4).

1.2 Types of pattern recognition receptors

Generally, PRRs can be differentiated into two main groups, namely membrane-bound and unbound intracellular receptors. To date, several studies have highlighted the importance of two intracellular receptors. They belong to the family of either retinoic acid-inducible gene-I-like receptors (RLRs) or nucleotide-binding oligomerization domain-like receptors (NLRs) and are present in the cytoplasm. Their main function is to survey for intracellular PAMPs and initialize an immune response. Whereas RLRs are known for identifying viruses, NLRs can recognize bacteria, viruses, fungi as well as molecules that are produced as a result of cell stress (5). RLRs can bind RNA via proteins of the DEAD-Box helicases and further differentiate between RNA of eukaryotic and non-eukaryotic origin. This distinction is made based on posttranscriptional modifications of RNA in eukaryotic cells. Since most RNA-viruses do not replicate in the nucleus, they miss a triphosphate on their 5’-end (6). NLRs are a diverse group

of receptors, which activation leads to either one of two possible pathways. The canonical pathway is known for the assembly of a protein complex called inflammasome, resulting in the release of proinflammatory cytokines and the induction of pyroptotic cell death, whereas the non-canonical pathway is controlling cell growth and the immune response via a range of transcription factors (7).

Furthermore, the group of membrane-bound PRRs consist of Toll-like receptors (TLRs) and C-type lectin receptors (CLRs). TLRs are a broad-ranging family, present on the outer cell membrane as well as on endosomes and lysosomes. As to date, TLRs are the best-characterized TLR-family, able to recognize a wide variety of pathogenic components (8). CLRs are only expressed on the plasma membrane and are known for identifying bacterial, viral and fungal infections. CLRs can recognize PAMPs with a so-called carbohydrate cognition domain, which leads to the production of proinflammatory transcription factors (9).

1.3 Toll-like receptors

The function of TLRs were first identified in *Drosophila melanogaster* by the team of J. Hoffmann in 1996 (10) until eventually homologous proteins were also found in mammals (11). To date there are ten subgroups of human TLR (hTLR)-genes, named continuously from hTLR1 to hTLR10, differing in their respective cellular localization, ligands and signal transduction (12).

1.3.1 Expression and localization within the cell

Since hTLRs are part of the innate immune system, they are expressed nearly on all cell types to a certain degree. The type of TLR and expression can vary depending on the cell type. Additionally, TLRs are present on innate immune cells like natural killer cells, dendritic cells, monocytes, neutrophils and adaptive immune cells like T and B cells (3).

From a cell biological point of view, hTLRs can either be located at the extracellular side of the plasma membrane or be part of the endolysosome. hTLR1, hTLR2, hTLR4, hTLR5 and hTLR6 are extracellular TLRs and able to identify pathogens outside of the cell. In contrast, hTLR3, hTLR7, hTLR8, hTLR9 and hTLR10 are membrane-bound PRRs in the endolysosome. They are only capable of recognizing PAMPs if the respective pathogen was phagocytosed and degraded in the acidic environment of the lysosome (4,5).

1.3.2 Structure and binding of ligands

TLRs are Type-I transmembrane proteins, hence they only pass the lipid bilayer once with the N-terminus on the extracellular side and the C-terminus on the cytoplasmatic side of the cell. The intracellular side is also known as Toll/interleukin-1 receptor (TIR) domain and is highly conserved. The TIR domain plays an important role in protein-protein interaction and downstream signaling. However, the N-terminus consists of a varying number of leucine-rich repeats (LRR), which play a main role in ligand binding and thus pathogen recognition (13). Generally, binding of ligands happens directly to the LRR domain of the receptor. The only exception involves TLR4 and the respective ligand lipopolysaccharides (LPS) from bacterial cell walls. Activation of TLR4 via LPS is only executed in collaboration with the adaptor protein MD-2. Beforehand, LPS needs to be detached from the cell wall and transported to CD14, this happens with the aid of the LPS binding protein (LBS). After transportation by LBS, LPS is split into monomeric molecules by CD14 and can be presented to the TLR4/MD-2 complex. Knockout mice deficient of TLR4 or MD-2 were found to be unable to recognize LPS (14,15).

Typically, TLRs exist as monomers on their respective membrane, but after activation by a ligand the receptor will form a dimer with another TLR. This dimerization can either take place with a receptor of the same subclass or from another. In general, most TLRs form homodimers after activation. The only exception pertains TLR1, TLR2 and TLR6 which mold into heterodimers. In this case, TLR2 dimerizes either with TLR1 or TLR6, homodimers do not exist within these three subclasses of TLRs (16).

1.3.3 Signaling of extracellular Toll-like receptors

All extracellular TLRs elicit highly conserved pathways that result in the activation of either nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB), activating protein-1 (AP-1) or interferon regulatory factor 3 (IRF3). NF-κB is a dimeric transcription factor which lies in its inactivated form in the cytoplasm. Upon activation, the inhibitor of NF-κB (IκB) will be phosphorylated and degraded whereby NF-κB can enter the nucleus and bind to the κB-site. AP-1 and IRF3 are transcription factors as well and get activated following phosphorylation by upstream signaling. Depending on the subtype of TLR and the activated transcription factor, the pathways result in the induction of inflammatory cytokines, type 1 IFNs and chemokines. Additionally, signaling promotes the maturation of dendritic cells which eventually represents an important link between innate and adaptive immunity (17).

Regarding the pathways leading to the activation of the various transcription factors, it can be differentiated between the myeloid differentiation primary response protein 88 (MyD88)-dependent pathway and the TIR domain-containing adapter inducing IFNβ (TRIF)-dependent pathway. The MyD88-dependent pathway is the most prominent one and consist of MyD88 and the adapter protein MyD88-adapter-like (MAL) with both binding to the TIR domain. This pathway is occurring in all extracellular TLRs, with the only exception of TLR5 where MyD88 binds solely to the TIR domain. After binding of a ligand and dimerization of the receptors, MyD88 and MAL initiate a cascade of kinases which eventually results in the activation of AP-1 as well as the degradation of IκB and hence the activation of NF-κB (18).

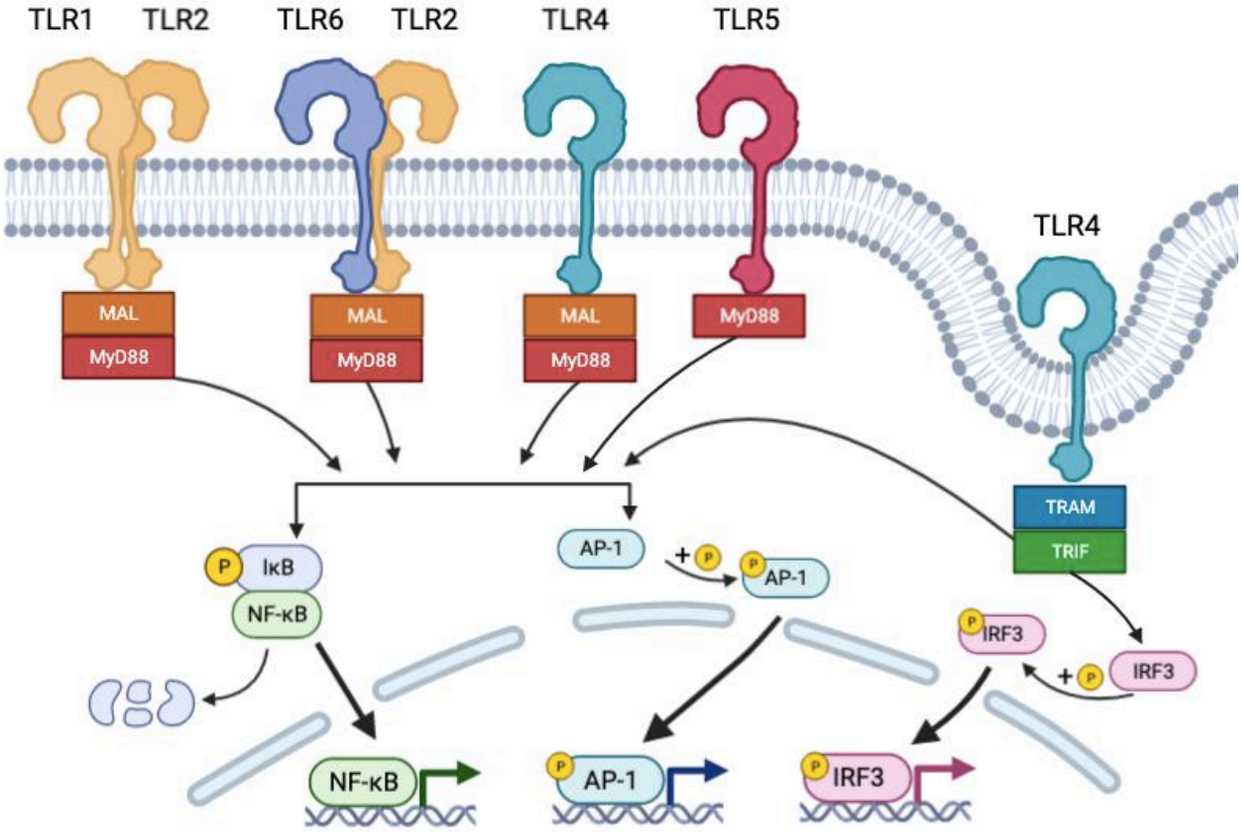

Figure 1: Signaling pathways of extracellular TLRs. TLR2-1, TLR2-6 and TLR4 signal through MAL and MyD88, resulting in the activation of transcription factors NF-κB and AP-1. TLR5 only signals through MyD88, nonetheless resulting in the activation of the same set of transcription factors. If TLR4 is internalized, signaling occurs through TRAM and TRIF, activating IRF3 as well. For the sake of convenience, homodimers are only shown as monomers, although it is important to note that signaling only occurs after dimerization. (Created with BioRender.com, adapted after Kawai & Akira (2006) (17) and Kagan et al. (2008) (19))

On the contrary, the TRIF-dependent pathway is composed of TRIF and the TRIF-related adapter molecule (TRAM) which connects TRIF to the TIR domain of TLR4. After dimerization, this pathway results in a similar multilayered kinase cascade, activating NF-κB, AP-1 and IRF3.

Equal to AP-1, IRF3 will get phosphorylated and move to the nucleus where it can operate as a transcription factor (18). However, it is important to note that signaling through the TRIF-dependent pathway is only the result of internalization of TLR4. TLR4 on the plasma membrane only makes use of the MyD88-dependent pathway (19). CD14 was found to be a key player in endocytosis of TLR4 and thus the signaling through TRIF and IRF3. Cells deficient of CD14 were unable to produce type 1 IFN which heavily relies on IRF3 (20). All relevant pathways are depicted in a simplified manner in Fig. 1.

1.4 Bacterial infections

Infections with bacteria pose a major threat to the healthcare system and public health in general. It was found that 56,2 % of all infection-related deaths in 2019 are caused by bacteria. Moreover, bacteria were accountable for 13,6 % of all deaths worldwide (21).

1.4.1 Bacterial ligands of Toll-like receptors

TLRs are capable of recognizing a variety of microbial PAMPs. Multiple structures of bacteria, viruses, protozoa and fungi are known to activate TLRs and subsequently trigger an immune response (22). For this thesis, bacterial compounds as are shown in Tab. 1 are of main interest and will be focused on. Generally, TLRs can either identify structural proteins from the cell wall, flagellin or DNA of bacterial origin. The most important bacterial components for extracellular TLRs are parts of the cell wall. The most potent immune stimulatory molecule is LPS from gram-negative bacteria which is recognized by TLR4. Additionally, it was shown that TLR4 reacts to heat-shock proteins which are expressed in a high number if a cell is under stress, for example by a bacterial infection (23). Other elements of bacterial cell walls can be identified in a large number by the TLR2 heterodimers with TLR1 or TLR6. Demonstrated molecules include lipoproteins, peptidoglycans, lipoteichoic acid and lipomannans. Whereas lipoproteins and peptidoglycans are abundant in gram-negative as well as gram-positive bacteria, lipoteichoic acid is only in the latter. However, lipomannans are only part of mycobacteria (24).

TLR5 distinct itself from other TLRs as its activation is only triggered by a conserved region within the flagellum and not from cell wall compartments like the other described extracellular TLRs (25,26). Regarding other ligands, the highly abundant unmethylated CpG motif in the microbial DNA is recognized by TLR9 and thus only after internalization of the microbe (27).

Table 1: Bacterial-related ligands and their respective TLRs. Ligands of TLR2 are equal to the heterodimers of TLR2, namely TLR2/1 and TLR2/6. It is important to note that heat-shock proteins are no bacterial ligand but much rather are expressed as a consequence of cell stress that can be caused by bacteria.

Ligand	Activated TLR	Note	Reference
Lipoprotein	TLR2	-	(28,29)
Peptidoglycan	TLR2	-	(30)
Lipoteichoic acid	TLR2/6	-	(31)
Lipomannan	TLR2	Only in mycobacteria	(32)
LPS	TLR4	Only in gram-negative bacteria	(33)
Flagellin	TLR5	Only in flagellated bacteria	(26)
Unmethylated CpG-DNA	TLR9	Only if bacteria are internalized	(27)
Heat-shock protein	TLR4	Indirect through cell stress	(23)

1.4.2 Commensal bacteria

Not only pathogenic bacteria may have an impact on TLRs, abundant commensal bacteria influence PRRs as well. A variety of bacterial species can be found in high abundancy on the skin, in the gut and on mucosal surfaces (34). It was shown that composition of these bacteria has an influence on immune homeostasis and how our body responds to pathogens (35). MAMPs of pathogenic as well as commensal bacteria can trigger TLRs, nonetheless the inflammatory response to the latter is limited. Studies showed that virulence factors, such as adhesion molecules, are needed to provoke an immune reaction in tissue highly exposed to innocuous bacteria (36). Treatment with antibiotics or a pathological altered microbiome can lead to up- or downregulation of certain TLRs, resulting in a defective immune response (37). Generally, dysbiosis of the host microbiome can lead to a wide set of respiratory diseases like viral infections and sinusitis. As a consequence of the altered microbiome, invasion of the nasal cavity is eased for viral pathogens (38). Moreover, expression of TLRs is mostly restricted to the basolateral side of epithelial cells, limiting a possible inflammatory response impending from commensal bacteria on the luminal side of cells. Dysregulation of this expression pattern could lead to auto-immune diseases (39).

Interaction between commensal bacteria and the immune system plays an important role in maintaining homeostasis and staying healthy. For example, development of the immune system during childhood is influenced by the composition of the host microbiome. Individuals exposed to poor microbial diversity during early infanthood show a heightened risk for allergies since their TLR-response is decreased and protective immune cells stay naïve (40).

Additionally, children with a respiratory syncytial virus (RSV) infection display an altered microbiome, which may lead to the development of childhood asthma in further consequence. Unfortunately, the pathomechanisms and pathways behind this effect are still understood poorly (41,42). Furthermore, recent studies showed that the host microbiome of patients with chronic nasal inflammation is highly altered in contrast to healthy individuals, suggesting an impact from commensal bacteria on dysregulation of the immune system (43).

1.5 Dysfunction and medical relevance of Toll-like receptors

Deficiencies or hyperactivity of TLRs and their signaling molecules can lead to a compromised immune response or adverse inflammatory events. It was found that patients with a deficient activity of MyD88 or the downstream enzyme interleukin-1 receptor-associated kinase 4 (IRAK4) have a heightened susceptibility to bacterial infections. Decreased signaling resulted in reduced B-cell proliferation and resulting in minimal antibody responses (44). However, increased signaling caused by mutations can lead to chronic inflammation and malignancies like B-cell lymphoma (45). Neonates show a naïve immune response and are highly susceptible to microbes. Particularly preterm newborns have a decreased expression of CD14 and MyD88 and in further consequence lower cytokine levels compared to adults. This suggest a correlation between dysfunctional signaling of TLRs and the naïve immune system of neonates (46).

If TLRs are targeted with pharmaceuticals, it is important to differentiate between a heightened or degraded pathological TLR response. In case of an excessive immune reaction, TLR blockers could be of help. This pertains diseases like sepsis, inflammatory bowel disease or rheumatoid arthritis. Currently there is no TLR-based drug against an inflammatory event on the market, but clinical trials are under way as pharmaceutical companies see huge potential in this approach (47). On the other side, sometimes inflammation is desired. It was shown that "*in situ* vaccination" of a tumor side with TLR ligands can have an immunostimulatory effect at cancer and lead to its clearance. Nevertheless, activation of TLRs and further of NF-κB has positive effects on cell proliferation as well and needs to be handled cautiously (48).

2 Aim

Bacterial infections pose a major threat in today's world (21) and even commensal bacteria may have an influence on health and disease in humans (40—43). It is well described that dysfunctional TLR-signaling plays a central role in the naïve immune system of neonates (46) and that the host microbiome has an important role in priming the juvenile immune system (40). Moreover, dysbiosis of nasopharyngeal microbiota was shown to be associated with a diverse set of respiratory diseases, especially viral infections (38). This is the reason why the TLR-response to bacteria in context of an infection or as part of an altered host microbiome is of particular interest.

The aim of this thesis is to determine interactions between extracellular TLRs and the nasal microbiome. To determine TLR activation, commercially available HEK-Blue™ TLR reporter cell lines will be stimulated with different quantities of bacteria, that are either commensal or pathogenic in the human nasal airway system, to provide a comprehensive insight into TLR activation by different bacteria capable of inhabiting the nasal airway system.

3 Material and Methods

3.1 Cell culture

3.1.1 Cell lines

To determine the response of TLRs, HEK-Blue™ TLR Cells (InvivoGen, Toulouse, France) were used. HEK-Blue™ TLR Cells co-express a specific human TLR and a NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP). Stimulated reporter cells secret SEAP upon NF-κB activation and will be quantified with HEK-Blue™ Detection media (InvivoGen). Reporter cells of hTLR2, hTLR2-1, hTLR2-6, hTLR4 and hTLR5 were used for the experiments. Whereas hTLR2-1 and hTLR2-6 only express the respective heterodimer, hTLR2 expresses both heterodimers and accounts for general activation of this receptor, independent from the second receptor needed for dimerization.

Furthermore, HEK-Blue™ Null1 and Null2 (InvivoGen) were the respective internal control cell lines, since HEK-Blue™ TLR Cells express endogenous levels of TLR3, TLR5 and NOD1, which stimulation also results in activation of NF-κB and AP-1. It is important to differentiate a false positive signal coming from these endogenous receptors or the examined receptor. Therefore, Null1 and Null2 serve as control cell line for unspecific activation.

3.1.2 Cell cultivation

Cells were cultured in tissue culture flasks (Sarstedt, Nümbrecht, Germany) in Dulbecco's modified Eagle's medium (DMEM, Gibco, Waltham, MA, USA) supplemented with additives according to the manufacturer. All cell lines were incubated at 37 °C and 5 % CO_2. Cells were split when reaching a confluency of 80–90 %. For hTLR5, Null1 and Null2 detaching was performed with Dulbecco's phosphate-buffered saline (DPBS, Gibco) and a TPP™ cell scraper (Thermo Scientific, Waltham, MA, USA). All other cells were detached with 5 mL of Accutase® solution (Merck, Darmstadt, Germany) while being incubated for three minutes at 37 °C. After detaching cells were centrifuged at room temperature for five minutes at 300 × g and finally resuspended in the desired splitting ratio.

3.1.3 Cell harvest

To harvest cells for stimulation, they were first washed twice with DPBS and finally detached with DPBS and a TPP™ cell scraper. An aliquot was used to determine cell count of harvested cells by Acridine Orange staining (Logos Biosystems, Anyang, South Korea). Stained cells were added on PhotonSlides™ (Logos Biosystems) and cell count was determined with a

LUNA-FL™ Dual Fluorescence Cell Counter (Logos Biosystems). Eventually, cells were diluted with HEK-Blue™ Detection media to a final concentration of 2,8 × 10^5 cells/mL for hTLR2, hTLR2-1, hTLR2-6, Null1 and Null2 and 1,4 × 10^5 cells/mL for hTLR4 and hTLR5.

3.2 Bacteria

In total 19 different commensal and pathogenic bacterial strains were chosen to be analyzed onto their ability to trigger a certain TLR. The selection of bacteria was based on recent papers that showed alterations in the host microsome upon different diseases and viral infections (41–43). Selected bacterial strains are listed in Tab. 2.

Table 2: List of bacteria that were analyzed upon their TLR-activation.

Corynebacterium minutissimum	*Corynebacterium simulans*
Corynebacterium striatum	*Corynebacterium tuberculostearicum*
Corynebacterium ulcerans	*Escherichia coli*
Haemophilus influenzae	*Klebsiella oxytoca*
Klebsiella pneumoniae	*Moraxella catarrhalis*
Staphylococcus aureus (two different strains)	*Staphylococcus capitis*
Staphylococcus epidermidis	*Staphylococcus haemolyticus*
Staphylococcus hominins	*Staphylococcus lugdunensis*
Streptococcus pneumoniae	*Streptococcus pyogenes*

Bacterial samples were provided by the Division of Clinical Microbiology at the Medical University of Vienna. Initial plating of freshly thawed bacterial samples was done on BD Columbia Agar with 5 % sheep blood (Becton Dickinson, Franklin Lakes, NJ, USA) and conducted two days before TLR stimulation experiments. All agar plates were incubated at 37 °C and 10 % CO_2. To ensure wildtype physiological properties of prokaryotic cells, samples were inoculated and plated approximately 24 h in advance of TLR stimulation experiments. To prepare samples for stimulation, multiple colonies were taken with a cotton swab and diluted in 0,9 % NaCl solution. The concentration of the solution was measured with a Densichek Plus McFarland calibration device (bioMérieux, Marcy-l'Étoile, France) and adjusted to be within 0,45—0,55 McF, which roughly corresponds to be 1—5 × 10^8 cells/mL, depending on the strain (50). Based on this assumption, serial dilution was performed to obtain the desired concentrations of bacteria. Additionally, the solution was plated on a Columbia blood agar plate, incubated for 24 h and colony forming units (CFU) were counted and calculated to CFU/ml to determine bacterial count.

3.3 Experimental setup

Stimulations of HEK-Blue™ TLR Cells were performed in NuncTM MicroWellTM 96-Well Microplates (Thermo Scientific). Each bacterial strain was tested in three concentrations on each of the cell lines. Concentrations were 1×10^8 cells/mL, 1×10^6 cells/mL and 1×10^4 cells/mL. Concentrations on the day of stimulation were based on the McFarland standards and are therefore not exact (50). Exact concentrations of microbes/mL were determined the next day by counting CFU on agar plates. A constant multiplicity of infection could not be applied through all bacterial strains due to the mentioned technical limitations.

180µL cell-detection-medium-suspension was added in each well and stimulated with 20µL of each bacterial concentration. Each condition was tested in quadruplicates. Additionally, each cell line was stimulated with three negative as well as positive controls respectively. Negative controls were composed of cell culture media and positive controls were ligands specific for each cell line (listed in Tab. 3). Plates were incubated at 37 °C and 10 % CO_2. After approximately 22 h plates were photometrically analyzed at 620 nm with a Multiskan FC photometer (Thermo Scientific) and SkanIt Software (Version 7.0.2, Thermo Scientific). Results were exported to Excel (Version 16.72, Microsoft, Redmond, Washington, USA) for statistical analysis.

Table 3: Positive controls of each cell lines and their respective final concentration.

HEK-Blue™ TLR Cell Line	**Positive Control**	**Source**	**Final Concentration**
hTLR2	*Heat-killed E. coli*	Invivogen	10^8 cells/mL
hTLR2-1	Heat-killed *E. coli*	Invivogen	10^8 cells/mL
hTLR2-6	FSL-1	Invivogen	200 ng/mL
hTLR4	LPS	Invivogen	10 ng/mL
hTLR5	Ultrapure flagellin from *Salmonella typhimurium*	Invivogen	10 ng/mL
Null1	TNF-α	PeproTech, London, United Kingdom	10 ng/mL
Null2	TNF-α	Peprotech	10 ng/mL

3.4 Statistical analysis

Mean value and standard deviation of every condition was calculated with Excel. If the standard deviation of replicates was larger than 20 % of the mean value, the replicate furthest from the mean value was defined as outlier, removed from the data set and mean value as well as standard deviation were calculated again. All corrected data sets are listed in Add. Tab. 1—10. Fold changes of activation intensity were calculated as the mean values of each condition were divided by the respective no template control (NTC). Additionally, fold change of the internal control cell line was subtracted in order to correct for activation that is not attributable to the examined receptor. Null1 is the control cell line for hTLR2, hTLR2-1, hTLR2-6 and hTLR5, whereas Null2 is related to hTLR4. Eventually, this data was analyzed with R (Version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria) and RStudio (RStudio PBC, Boston, MA, USA). Heatmaps were generated with the "ComplexHeatmap"-package (51) and graphs with "ggplot2" (52). To test for statistically significant differences between two groups of bacteria a student's t-test was used. To test for significant differences between multiple groups of bacteria, a one-way ANOVA was conducted. To determine that ANOVA- and t-test-assumptions are ensured, Levene's test to check for homogeneity of variances and Shapiro-Wilk test to check for normality of data were carried out. If the described conditions could not be ensured, a Kruskal-Wallis-Test was conducted instead of a one-way ANOVA and a Whitney-Mann-U-test instead of student's t-test. A p-value of < 0.05 was chosen to define statistical significance.

4 Results

To determine TLR stimulation, SEAP was photometrically quantified at 620nm, data was transferred to Excel and the mean (Add. Tab. 1—5) and standard deviation (Add. Tab. 6—10) of each condition was calculated. Furthermore, negative and positive controls were controlled for their respective stimulation. Negative controls of hTLR2-6 showed high alterations and were not constant. hTLR2-6 negative controls displayed the same absorption levels as the positive controls in some cases, thus no true activation of hTLR2-6 by bacteria could be determined and this cell line was excluded from analysis. Data from the other cell lines showed clear differences in the respective controls and were used for further analysis. Absorption values were used to calculate the fold change as the mean value of each condition was divided by the respective negative control. These values were further subtracted by the fold change of the related internal control cell line in order to correct for unspecific activation by endogenous expressed receptors.

After incubation for 22 h, *K. pneumoniae* was the only bacteria that showed excessive biofilm formation. Moreover, the color of the cell culture media changed to a very unusual green, rather than the common red or blue which corresponds to a negative or positive reaction respectively (Fig. 2). Photometrical quantification at 620 nm showed exceptionally strong signals compared to all other strains, which suggests that measurement is influenced by this color shift. Since the origin of this color tone is not known and the effects on the photometrical measurements are unclear, it was decided to remove *K. pneumoniae* from analysis.

Figure 2: Comparison of cells stimulated with K. pneumoniae (left) and C. tuberculostearicum (right). The greenish color tone only appeared upon stimulation with K. pneumoniae, whereas C. tuberculostearicum shows an expected and common result.

4.1 Absorption values after stimulation

Absorption values of all conditions and controls were compared in a heatmap to depict a possible activation of the cells. This dataset is not corrected for any unspecific activation.

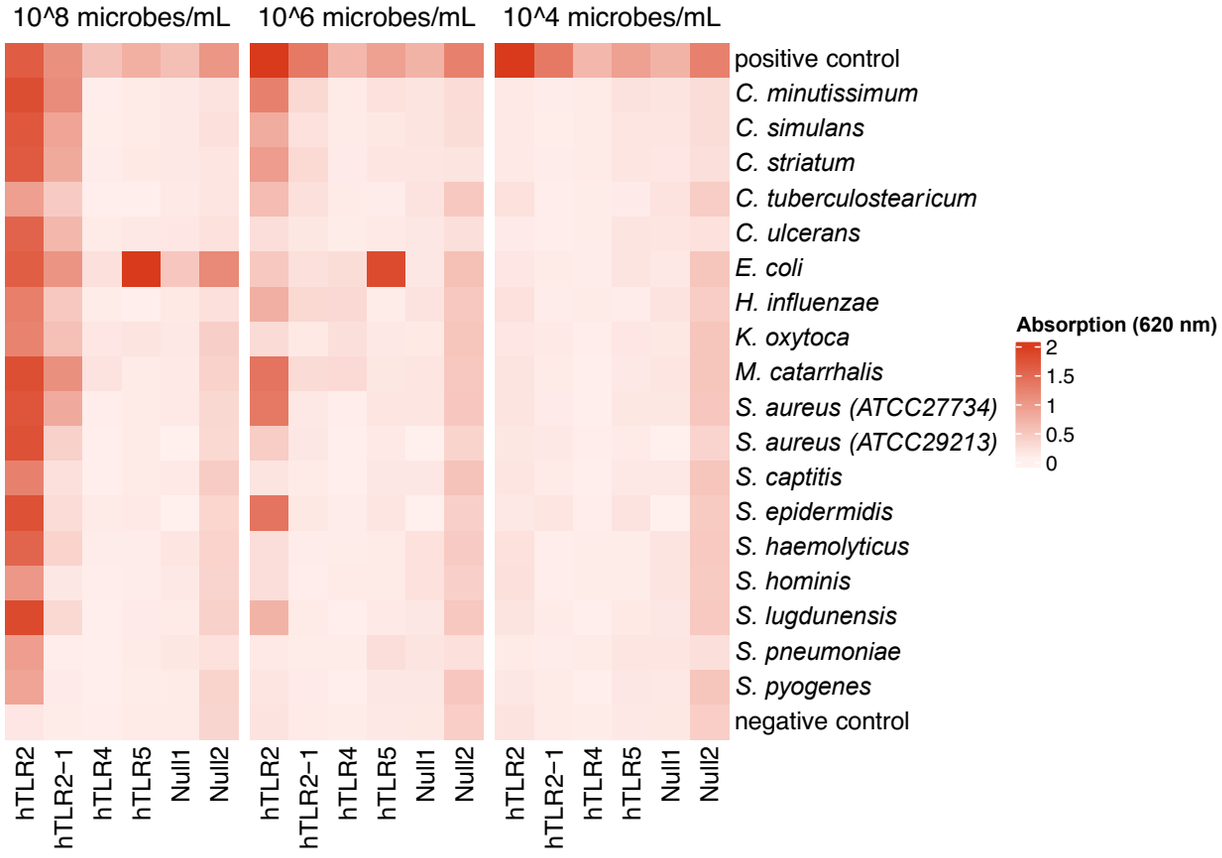

Figure 3: Absorption values of each receptor if stimulated by the respective bacterial strain. Mean positive and negative controls are added for orientation. This dataset is not corrected for the values of the internal control cell lines.

Results show a constant activation of hTLR2 reporter cells through all tested strains. hTLR2-1 reporter cells are activated in a high amount as well, although some strains do not trigger a strong response. Stimulation of hTLR4 reporter cells is hardly visible in Fig. 3, due to general low values compared to other cell lines. This includes the positive controls for hTLR4, displaying a rather weak signal. hTLR5 reporter cells only produce a strong signal upon a small number of strains. Whereas control cells Null1 show a minor amount of activation, Null2 displays a relatively strong signal. Generally, all reporter cells generated a stronger signal upon stimulation with a high quantity of microbes than compared with a low amount (Fig. 3).

4.2 Activation of Toll-like receptors

To further correct for a general low response as it was seen with hTLR4 reporter cells, the fold change relative to the NTC was calculated. Fold changes across all concentrations were

plotted together for each receptor of interest. Furthermore, the fold changes of Null1 for hTLR2, hTLR2-1 and hTLR5 and Null2 for hTLR4 were marked in the respective bars, correcting for unspecific activation and indicating the threshold above which a receptor is considered activated.

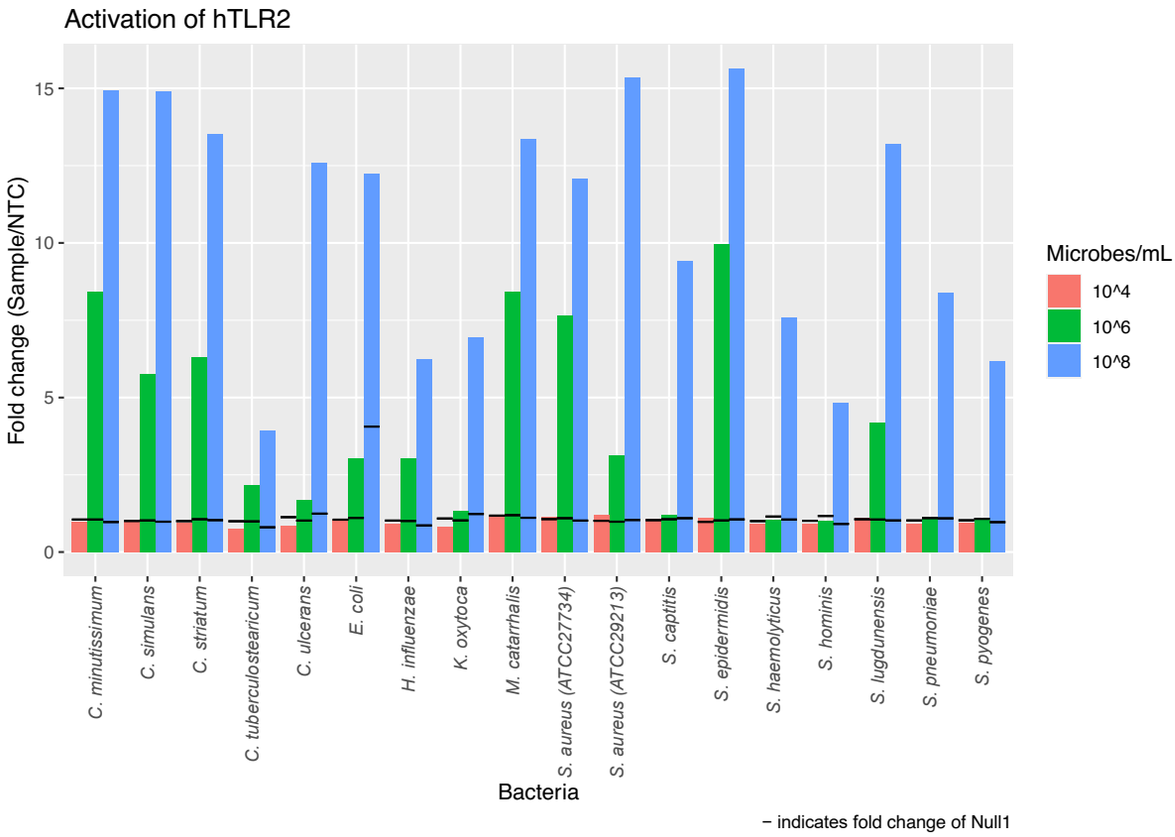

Figure 4: Fold changes of hTLR2 reporter cells when stimulated with the respective bacteria. Black lines indicate the fold change of the internal control cell line Null1.

A strong response of hTLR2 across all strains was observed, especially at a concentration of 10^8 microbes/mL. Activation of this TLR is attributable to the heterodimers hTLR2-1 and hTLR2-6. Whereas most genera also show a positive activation pattern at 10^6 microbes/mL, some strains of *Staphylococcus* like *S. haemolyticus* and *S. hominis* evoke no signal. This appears for both analyzed strains of *Streptococcus* as well, with *S pneumoniae* and *S. pyogenes* only eliciting a signal at the highest tested concentration. Fold changes of Null1 are nearly constant across all conditions, only *E. coli* shows a strong response in this cell line, possibly as a result of unspecific activation of the endogenous expressed TLR5. Therefore, no definite statement can be made about activation in the reporter cell line coming from the examined receptor or rather from unspecific endogenous PRRs (Fig. 4).

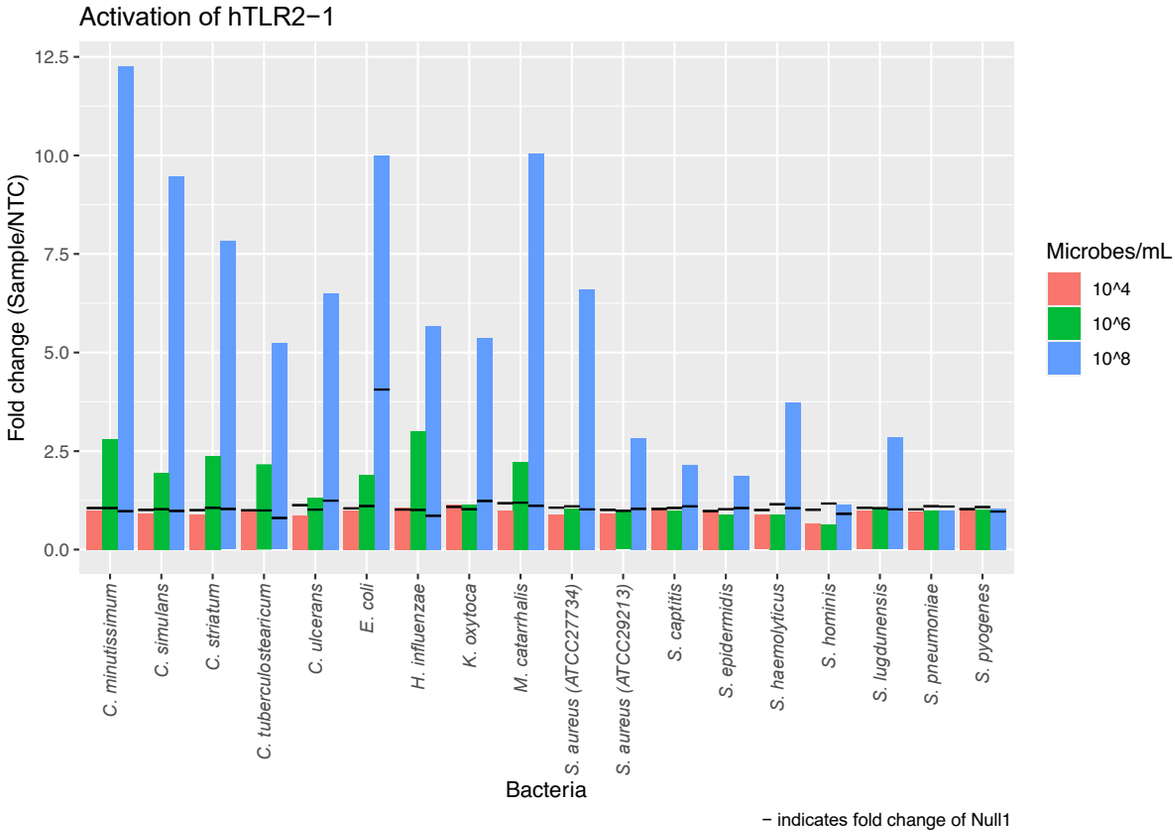

Figure 5: Fold changes of hTLR2-1 reporter cells when stimulated with the respective bacteria. Black lines indicate the fold change of the internal control cell line Null1.

As observed in hTLR2 reporter cell lines, hTLR2-1 elicits a strong response in most strains as well. *Staphylococci* display a rather downscaled response in comparison to hTLR2 cells, suggesting a stronger impact from the hTLR2-6 heterodimers than from hTLR2-1. Both analyzed *Streptococcus* strains do not activate hTLR2-1 at all, proposing that recognition by hTLR2 only happens in combination with hTLR6 rather than hTLR1 (Fig. 5).

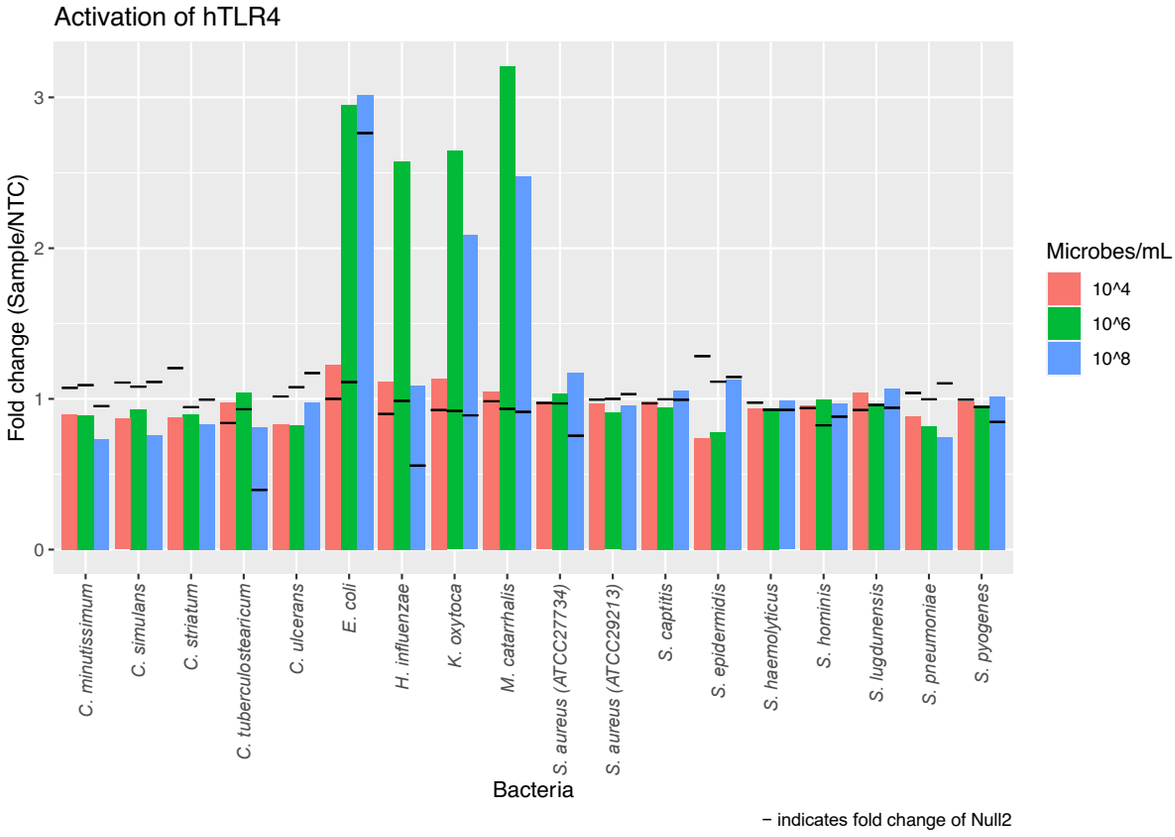

Figure 6: Fold changes of hTLR4 reporter cells when stimulated with the respective bacteria. Black lines indicate the fold change of the internal control cell line Null2.

hTLR4 is primarily known for recognizing LPS from gram-negative bacteria. As expected, only gram-negative bacteria evoked a positive response in hTLR4 reporter cell lines. Each of the four analyzed gram-negative bacteria *E. coli*, *H. influenzae*, *K. oxytoca* and *M. catarrhalis* showed a positive response. However, fold changes are rather low in this cell line compared to other reporter cell lines. Moreover, gram-positive bacteria *C. tuberculostearicum* and *S. aureus* (ATCC27734) induced a minimal response at 10^8 microbes/mL, which cannot be triggered by LPS but can much rather be the result of cell stress or natural fluctuation (Fig. 6).

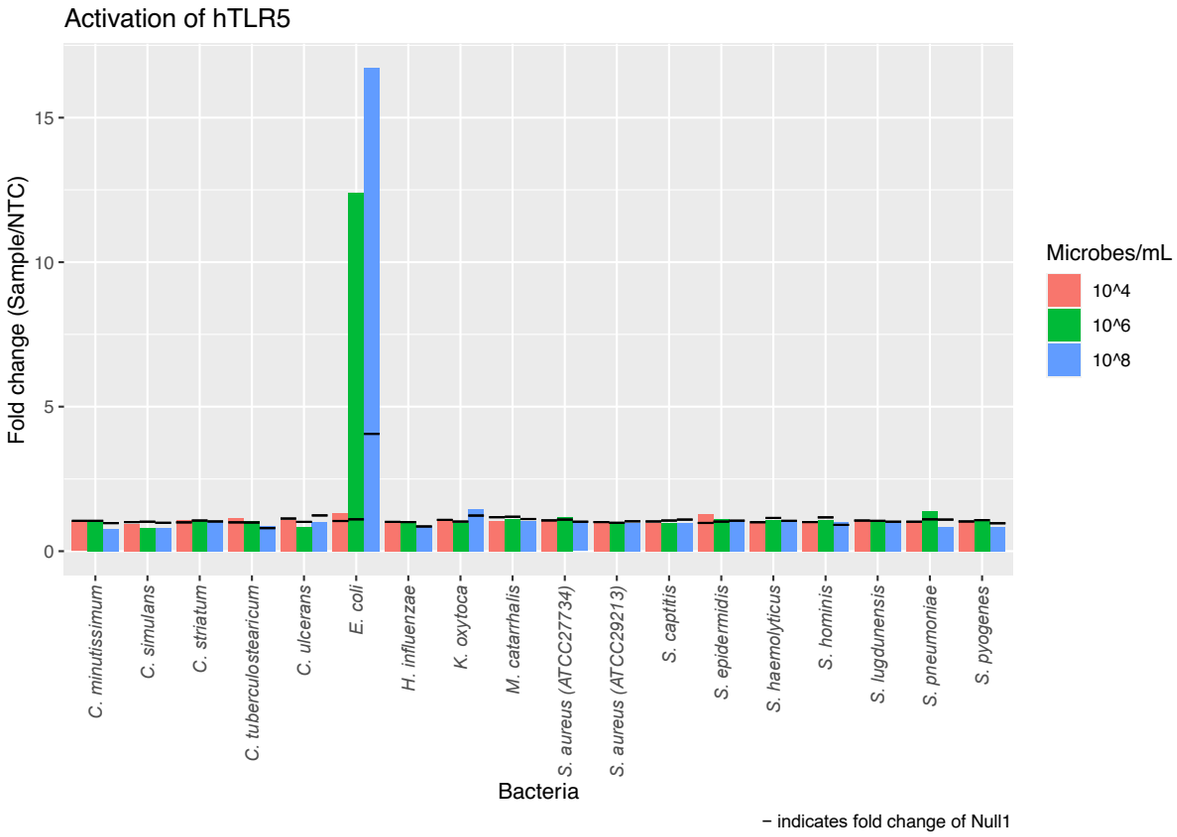

Figure 7: Fold changes of hTLR5 reporter cells when stimulated with the respective bacteria. Black lines indicate the fold change of the internal control cell line Null1.

Reporter cells of hTLR5 only displayed a response upon stimulation with *E. coli*. All other strains show a minimal signal above the internal control cell line Null1 at most, which is probably due to unspecific activation and a certain fluctuation range rather than the consequence of a suitable ligand for hTLR5 (Fig. 7).

4.3 Activation overview of all Toll-like receptors

To create an overview of all activated TLRs, a heatmap with all fold changes was generated. Fold changes are already corrected for the respective fold changes of the internal control cell lines Null1 and Null2. A response with a fold change lower than two was considered not biologically relevant and thus the receptor as not activated. Since hTLR4 reporter cell lines showed very weak signaling, the cut-off value for this cell line was lowered in order to see an activation pattern. However, all activated TLRs were divided into three groups of weak, medium and strong activation. Different levels were generated by plotting all fold changes above the cut-off value in a histogram and splitting them into three ranges of the same size.

This classification is very arbitrary and should only give a brief summary of which bacteria are eliciting a strong response.

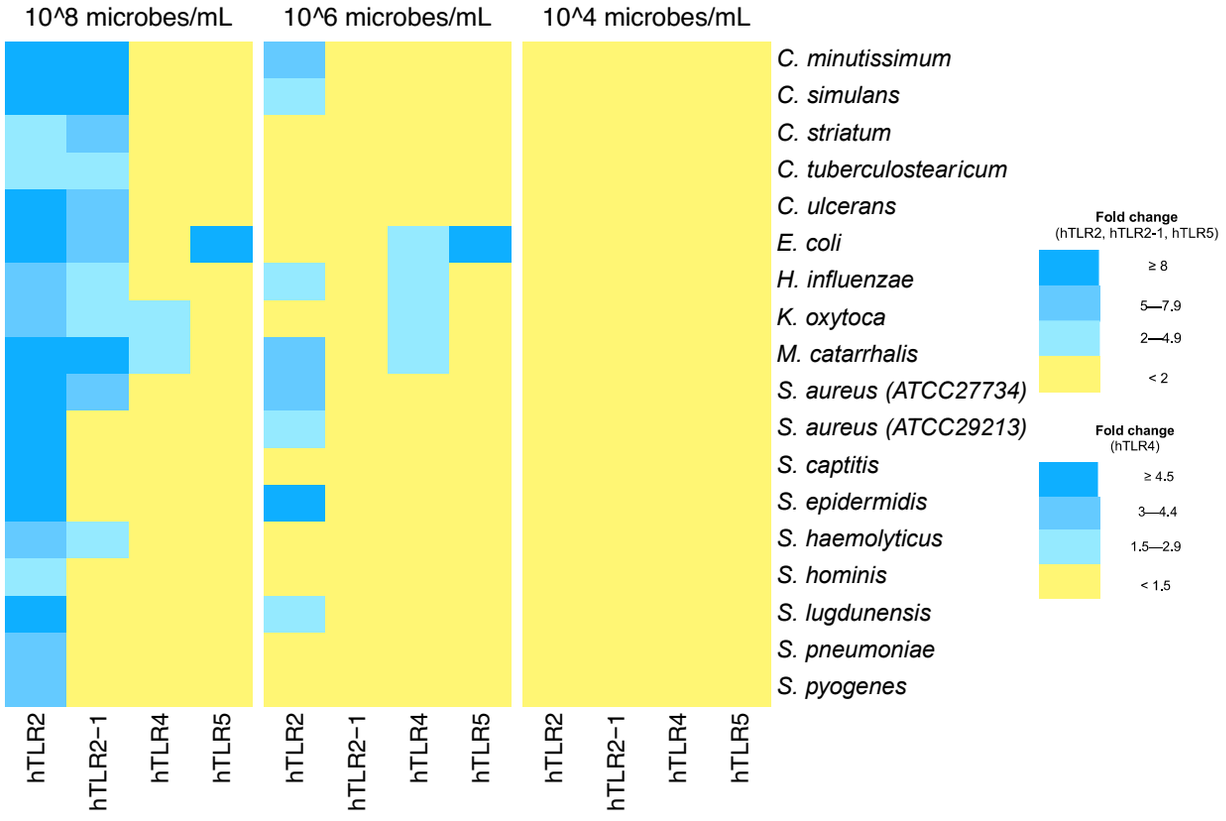

Figure 8: Fold changes of all receptors divided into four groups according to their strength of activation. The fold change is already corrected for the fold change of the internal control cell lines Null1 and Null2. Since hTLR4 showed weak signaling, the cut-off values were lowered in order to see a possible activation.

The heatmap shows that there is no relevant activation of any receptor at a concentration of 10^4 microbes/mL. However, hTLR2 produces a very strong response and is able to recognize all tested bacteria at the highest concentration. Moreover, hTLR2-1 reacts to most bacteria as well, although bacteria of the family *Staphylococcus* and *Streptococcus* are recognized rarely. Interestingly, both strains of *S. aureus* show a distinct activation pattern in hTLR2 and hTLR2-1. hTLR4 only generates a weak fold change as *M. catarrhalis* and *K. oxytoca* are the only gram-negative bacteria that are activated at the highest concentration. However, all four gram-negative bacteria elicit a weak response at 10^6 microbes/mL. This can be the result of general weak signaling of this reporter cell line or some kind of unique negative feedback mechanism in response to a higher number of microbes. As expected, hTLR5 was only activated once by *E. coli* (Fig. 8).

4.4 Correlation between strength of response and amount of bacteria

In order to validate if there is a correlation between strength of the response and the number of bacteria that was used for stimulation, the fold change of each receptor was plotted against the number of microbes that were used. The fold change is already subtracted by the fold change of Null cell lines, thus it is corrected for any possible unspecific activation. For a better overview, only the responses to six distinct bacterial strains were analyzed. Strains were primarily selected so that a high number of different families are depicted. Additionally, strains that showed very distinct responses regarding the strength in Fig. 8 were favored.

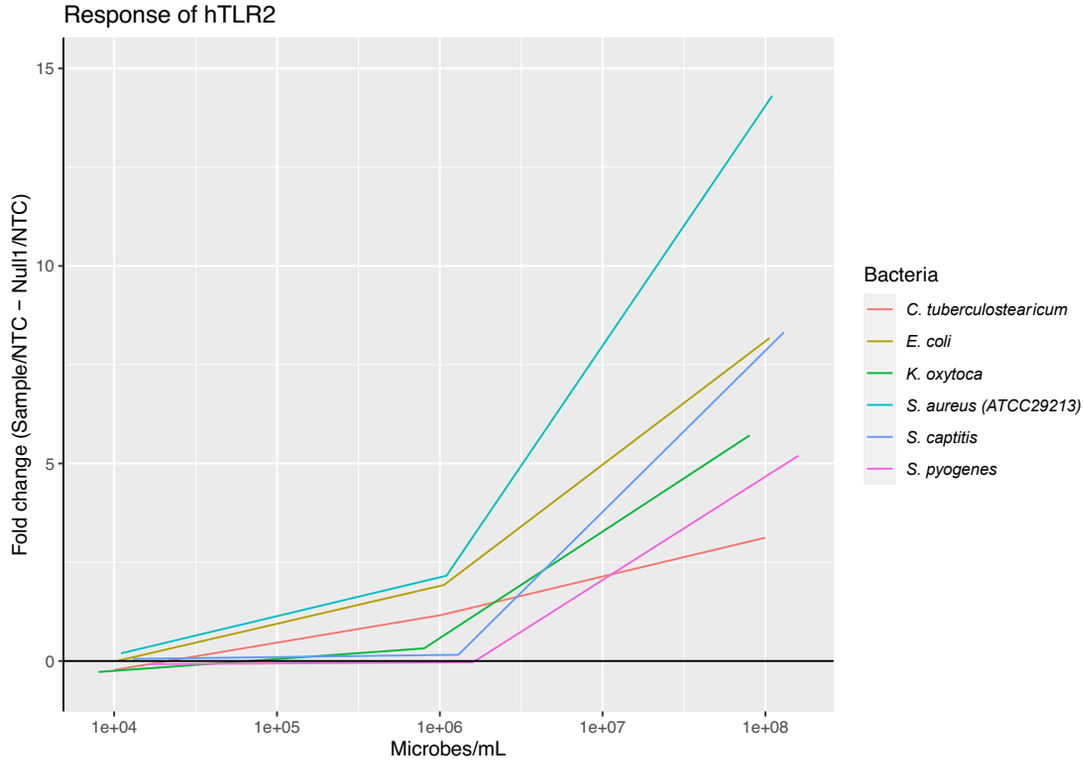

Figure 9: Fold change already corrected for the value of the internal cell line Null1 plotted against the number of microbes used for stimulation of hTLR2 reporter cell line.

The activation of hTLR2 is very heterogenous since it is able to recognize a variety of different bacterial strains. Some bacteria like *K. oxytoca*, *S. capitis* and *S. pyogenes* only show a response upon stimulation with a high concentration of bacteria. Although each strain activates hTLR2 in a diverse quantity, it can be seen that the fold change increases each time with a rising number of bacteria (Fig. 9).

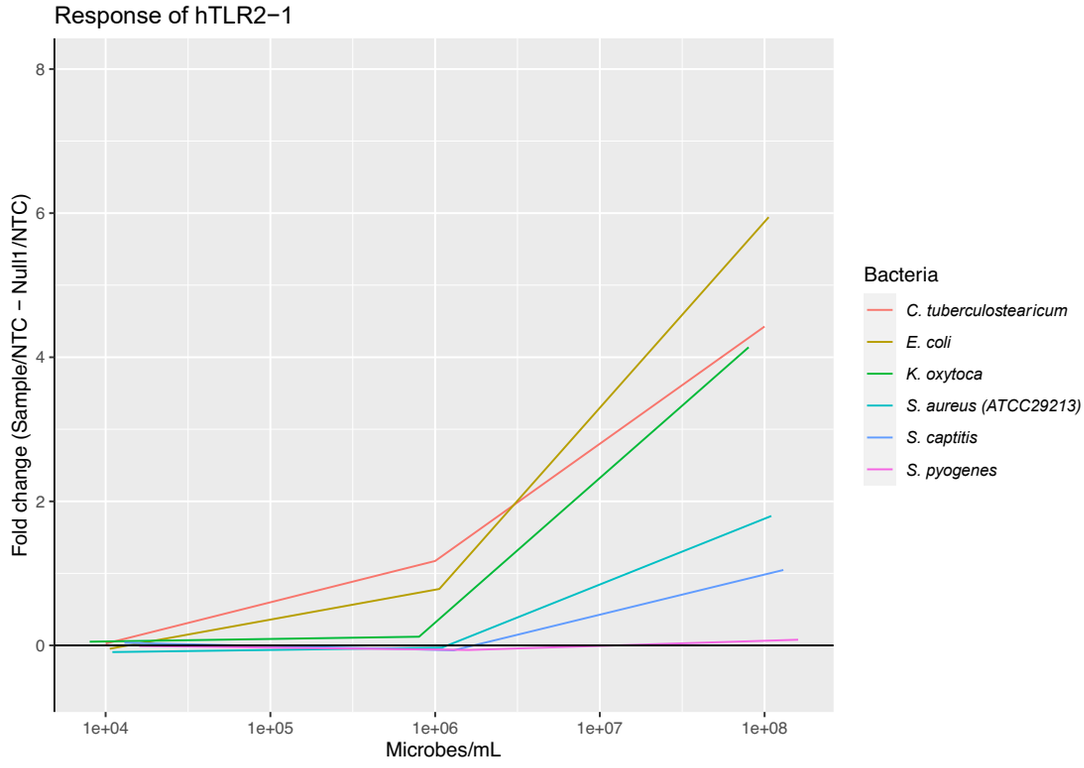

Figure 10: Fold change already corrected for the value of the internal cell line Null1 plotted against the number of microbes used for stimulation of hTLR2-1 reporter cell line.

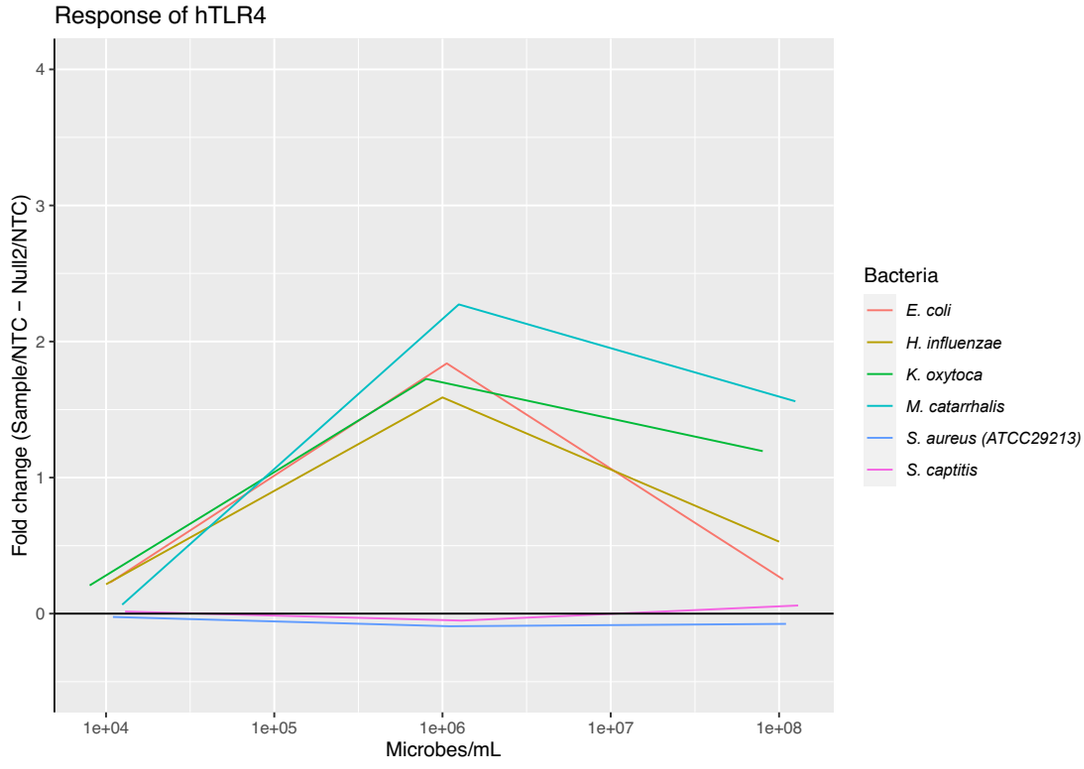

Figure 11: Fold change already corrected for the value of the internal cell line Null2 plotted against the number of microbes used for stimulation of hTLR4 reporter cell line.

Not every bacterial strain activates heterodimer hTLR2-1. If recognized, all bacteria elicit a stronger response upon an increased concentration of bacteria. In contrast to hTLR2, hTLR2-1 is not able to recognize each strain as *S. pyogenes* triggers no hTLR2-1 response at all (Fig. 10).

Since hTLR4 is well described for primarily recognizing LPS, as expected, only activation by gram-negative bacteria was detected. Gram-positive bacteria trigger no response at all, as they seem to do not have any fitting ligand for hTLR4. This is the reason why all four analyzed gram-negative strains were used for this graph. If activated, the strength of the response is not very strong compared to other reporter cell lines and does not increase with the number of microbes used for stimulation as it was observed in hTLR2 and hTLR2-1 reporter cell lines. Moreover, the strength of the response even decreases from 10^6 to 10^8 microbes/mL in all strains. This can be the result of a unique negative feedback mechanism or saturation of the receptor at a higher concentration (Fig. 11).

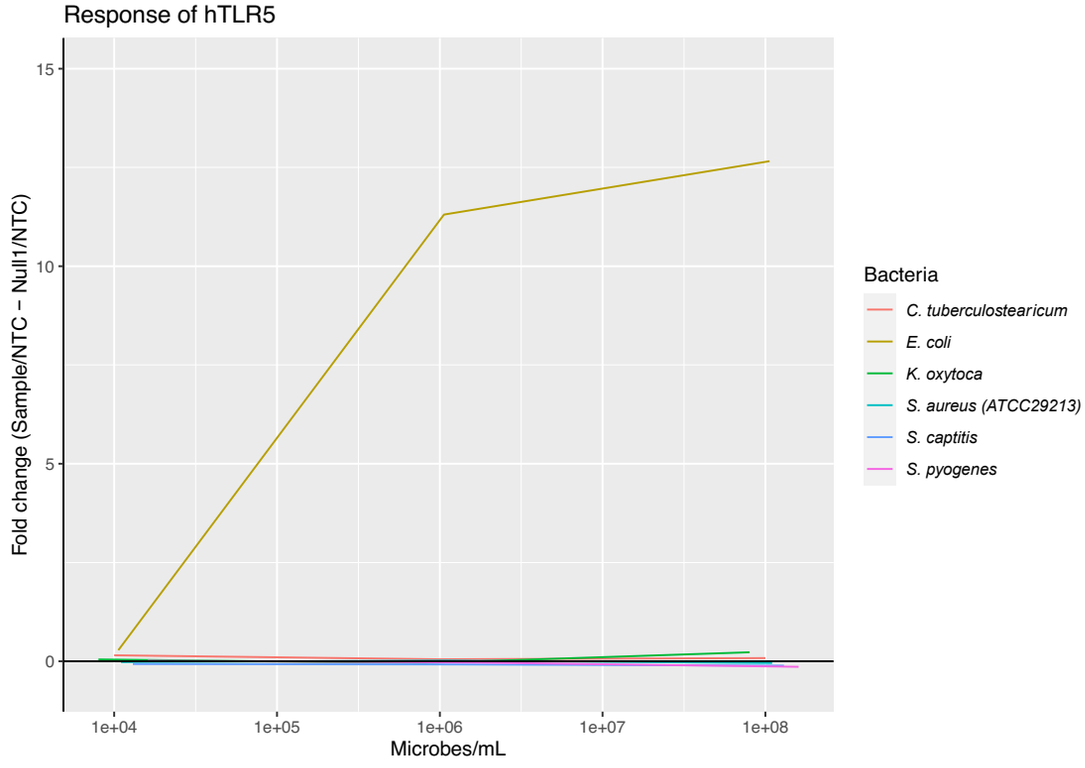

Figure 12: Fold change already corrected for the value of the internal cell line Null1 plotted against the number of microbes used for stimulation of hTLR5 reporter cell line.

Activation of hTLR5 was only detected in *E. coli*. Even at the intermediate level of tested concentration, the response occurs strongly and increases even more with a rising number of

microbes. All other strains do not activate hTLR5, independent on the quantity of microbes, suggesting no suitable ligand for hTLR5 in these strains (Fig. 12).

4.5 Comparison of two different strains of *Staphylococcus aureus*

To determine differences in the response between different strains of the same species, two strains of *S. aureus* were used for stimulation. For this purpose, *S. aureus* (ATCC27734) and (ATCC29213) were compared in a bar graph regarding their activation of each receptor at different concentrations. The fold change of displayed numbers is already corrected for the fold change of respective internal control cell lines Null1 and Null2.

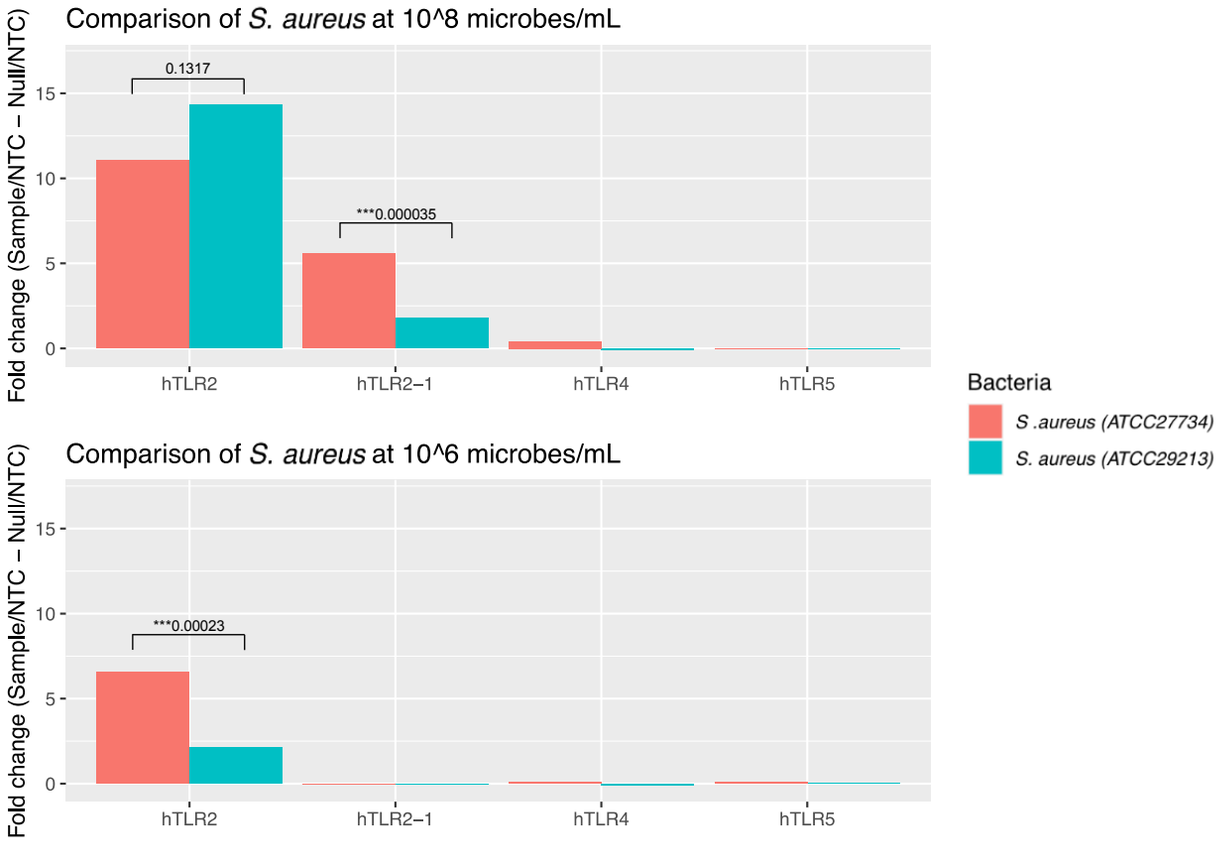

Figure 13: Comparison of two different strains of S. aureus regarding their activation of hTLRs at different concentrations. p-values were calculated with a Welch two-sample t-test.

As shown in Fig. 13, each strain activates the same set of receptors, nevertheless the strength of the response is slightly diverging. Whereas strain ATCC27734 evokes a significant stronger response in hTLR2-1, ATCC29213 is eliciting a stronger activation in hTLR2, although not significant. hTLR4 and hTLR5 only have a minimal to nonexistent response to *S. aureus* in general. At a lower concentration, hTLR2 still gets activated strongly, however strain ATCC27734 evokes a significantly stronger response compared to ATCC29213. In contrast to

the concentration of 10^8 microbes/mL, hTLR2-1 is not activated in neither strain in this case. At the lowest tested concentration neither strain seems to activate any receptor.

4.6 Comparison of different groups of bacteria

Whereas hTLR4 and hTLR5 only showed a positive response upon stimulation with certain groups of bacteria, namely LPS- and flagellin-positive microbes respectively, hTLR2 and hTLR2-1 recognized a wide variety of distinct species. The response of these cell lines to different strains was very diverse regarding the fold change. This is the reason why bacteria were categorized into different groups and compared regarding their activation of hTLR2 and hTLR2-1.

4.6.1 Variations between commensal, opportunistic and pathogenic bacteria

To determine if there is a difference between abundant commensal and recurring pathogenic bacteria regarding their potential to evoke an immune response, microbes were categorized into commensal, opportunistic and pathogenic bacteria of the human airway and plotted together. This classification is very generalizing and only shows the most common role of a microbe in the population. As for this analysis, commensal bacteria are considered to occur regularly in the human airway and do not cause any diseases in humans with the exception of immunocompromised patients. In contrast, pathogenic bacteria that are not being present regularly in the human airway and cause diseases in most people according to this classification. However, opportunistic bacteria present the intermediate position, existing in a non-pathogenic or pathogenic state, differing from individual to individual (53).

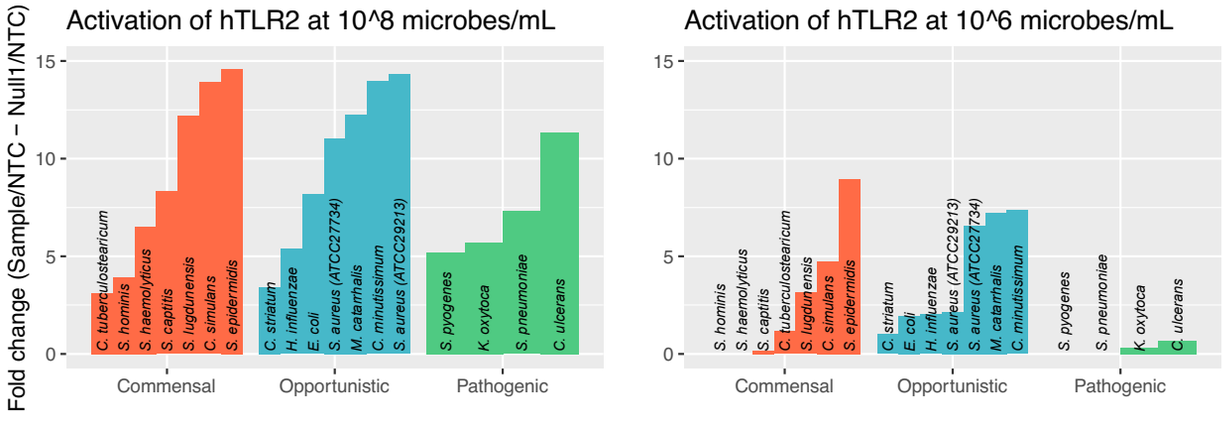

Figure 14: Commensal, opportunistic and pathogenic bacteria plotted together regarding their response to hTLR2.

At a concentration of 10^8 microbes/mL no obvious distinction is perceivable across the different classes. A one-way ANOVA showed no significant differences between the three different classes. The response to pathogenic bacteria at 10^6 microbes/mL is very low to nonexistent,

compared to commensal or opportunistic bacteria. Some strains of these classes still show a heightened immune answer, whereas some are not recognized at all. However, a one-way ANOVA did not show any significant differences between all three classes, suggesting that bacteria cannot be differentiated in their pathogeny according to the hTLR2 response (Fig. 14). There is no positive response in any class at 10^4 microbes/mL, which is why this graph is not shown.

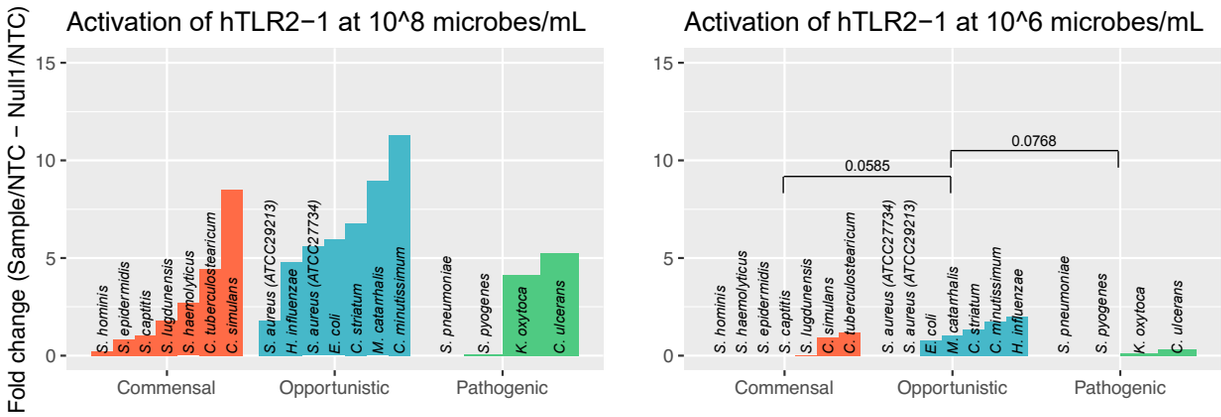

Figure 15: Commensal, opportunistic and pathogenic bacteria plotted together regarding their response to hTLR2-1. Only p-values close to the threshold of 0.05 are shown.

Likewise to hTLR2, hTLR2-1 shows a potent response at 10^8 microbes/mL in most strains, although some bacteria are not recognized at all by this heterodimer. A one-way ANOVA did not reveal any significant differences between both groups. However, fold changes at 10^6 microbes/mL are weaker compared to the higher concentration and only some bacterial strains are recognized, whereby the class of opportunistic bacteria is recognized the most. Nevertheless, the p-values of one-way ANOVA were not below 0.05 when the differences between all classes were evaluated (Fig 15).

4.6.2 Comparison of altered microbiota upon chronic nasal inflammation

A publication by Kaspar et al. (2016) revealed differences in the bacterial microbiome in patients with chronic nasal inflammation compared to healthy individuals (43). Tested bacterial strains that appeared in that publication were grouped and compared according to their decreased or increased prevalence in patients.

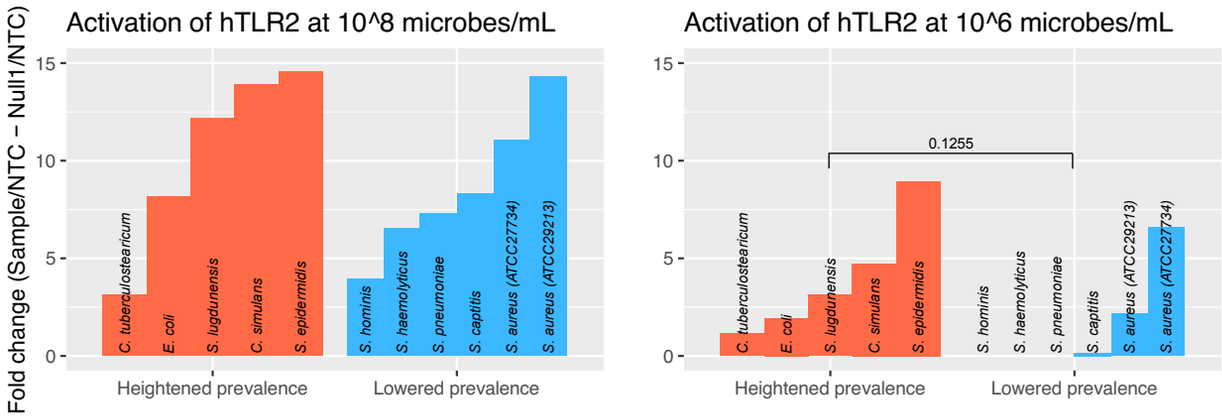

Figure 16: Response of hTLR2 to bacteria that were shown to either have decreased or increased prevalence in patients with chronic nasal inflammation. Only p-values close to the threshold of 0.05 are shown.

Once again, the signal was very strong across all bacterial strains at 10^8 microbes/mL and no significant difference between these two classes is detectable. However, all strains with a higher abundancy in nasal inflammation elicit a positive response in hTLR2 at 10^6 microbes/mL, whereas some bacteria that appear less in patients with inflammation evoke no response at all. Nonetheless, differences were measured with a Mann-Whitney-U-test and showed no significant difference between both groups (p-value: 0.1255) (Fig. 16).

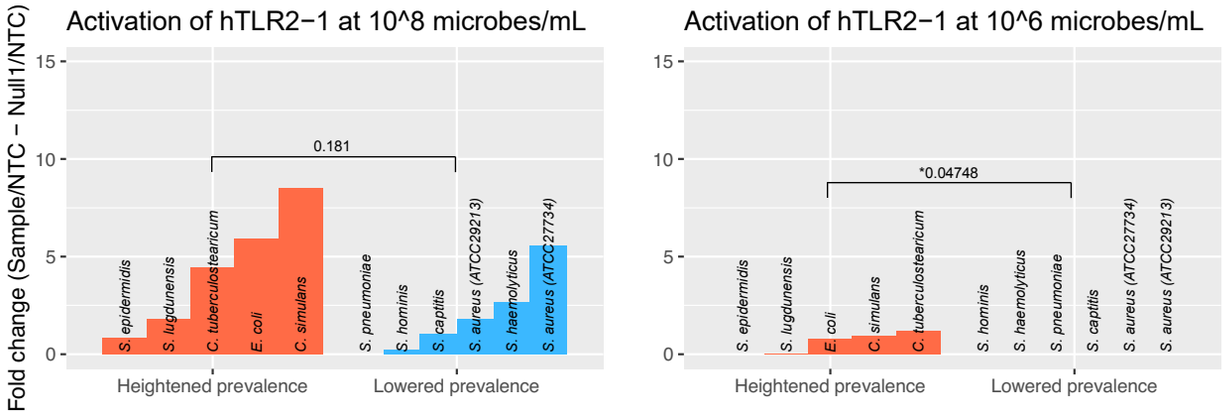

Figure 17: Response of hTLR2-1 to bacteria that were shown to either have a decreased or increased prevalence in patients with chronic nasal inflammation.

The differences between bacteria that have a higher prevalence in chronic nasal inflammation and those that are decreased are once again not significant at 10^8 microbes/mL. Regarding the response of hTLR2-1 at 10^6 microbes/mL, it can be seen that the fold change of bacteria that appear more often in inflammation is significantly higher compared to the ones that were shown to be decreased, since none of them activate hTLR2-1 at the tested concentration (Fig. 17).

4.6.3 Differences of altered microbes in viral infections

A publication by Rosas-Salazar et al. (2016) showed that the human microbiome is heavily altered upon infection with RSV compared to healthy individuals (42). Tested bacterial strains that appeared in that publication were grouped according to their decreased or increased abundancy in patients with RSV infection.

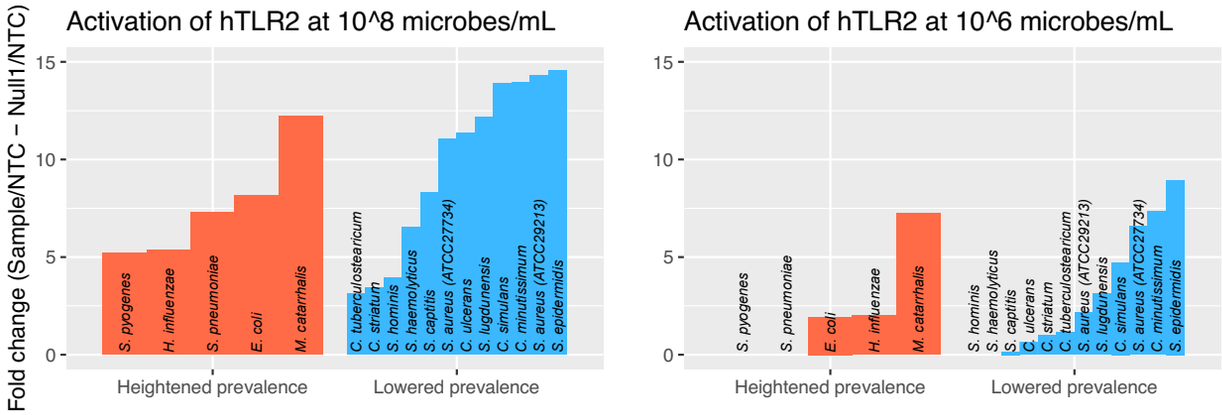

Figure 18: Response of hTLR2 to bacteria that were shown to either have a decreased or increased prevalence in patients upon infection with RSV.

When stimulated at 10^8 microbes/mL, hTLR2 reporter cells showed a strong response in both groups, as no significant difference occurs. However, bacteria induced a varying response from strain to strain at 10^6 microbes/mL across both classes, as the students t-test showed no significant differences as well (Fig. 18).

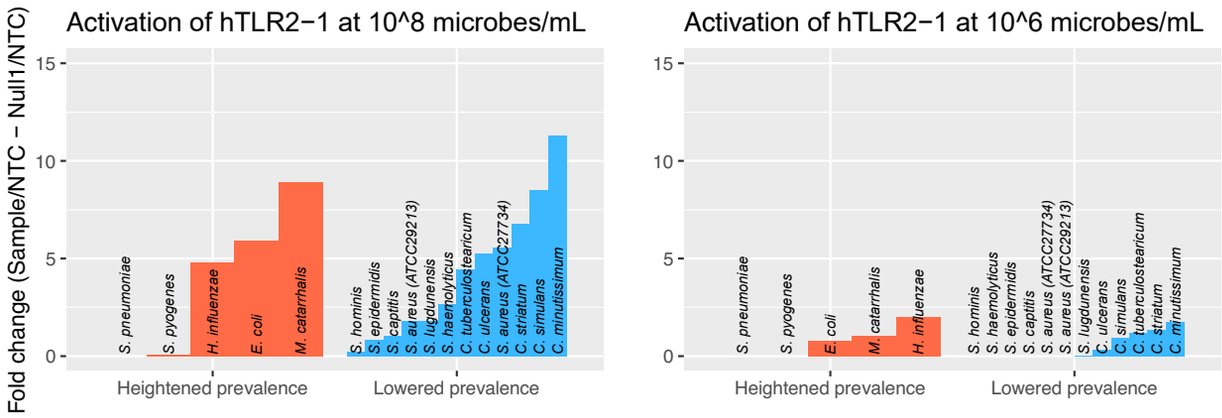

Figure 19: Response of hTLR2-1 to bacteria that were shown to either have a decreased or increased prevalence in patients upon infection with RSV.

hTLR2-1 reporter cell lines were activated very differently to the various bacterial strains as no pattern between both observed groups is perceptible. The response at 10^6 microbes/mL is generally lower as some strains activate hTLR2-1 and some do not. The students t-test showed no significant difference between both classes across both tested concentrations (Fig. 19).

5 Discussion

As expected, hTLR2 generated the most potent response of all exogenous TLRs and was able to recognize all analyzed bacteria. This is probably the result of the formation of a heterodimer with either hTLR1 or hTLR6, leading to a high variance of possible ligands (24). Reporter cells with hTLR2-1 showed a less potent response, still recognizing a wide variety of strains, although some tested bacteria from the family of *Staphylococcus* or *Streptococcus* do not provoke a strong response of hTLR2-1, suggesting that recognition of these strains primarily depends on the heterodimer hTLR2-6. On the contrary, hTLR4 and hTLR5 reporter cell lines only produced a signal upon stimulation to a small range of bacteria. As expected, hTLR4 only reacted to gram-negative bacteria which cell wall is primarily constituted of LPS, the main ligand of hTLR4 (18). Gram-positive bacteria did not cause any signal. Generally, the response of hTLR4 was rather weak, which is why fold changes can hardly be compared to those of other reporter cell lines. On the other side, hTR5 only reacted to bacteria that are known to possess a flagellum (26). There were no unexpected responses in those two cell lines, as they seem to only have a very small group of suitable ligands compared to hTLR2 and hTLR2-1, which have very common ligands that appeared in all analyzed bacteria (17).

Upon activation, hTLR2 showed an increased response to a heightened concentration of bacteria. If activated, hTLR2-1 and hTLR5 increased their response with a higher concentration of microbes as well. However, hTLR4 did not necessarily produce a stronger response at a higher concentration, the response was much more downgraded at 10^8 microbes/mL than when stimulated with 10^6 microbes/mL. This suggests a unique control mechanism for hTLR4 compared to other extracellular TLRs. The most possible reason includes a negative feedback mechanism. There are multiple ways TLR-signaling can be downscaled, for example via degradation of MyD88 (54). Moreover, it was found that monocytic cells express microRNAs that suppress proinflammatory genes in response to LPS, which could lead to a decreased expression of SEAP in hTLR4 reporter cells (55). The specificity of this mechanism in hTLR4 reporter cells could be at the basis of the TRIF-dependent pathway. TLR4 is the only extracellular TLR that makes use of the TRIF-dependent pathway (18), which is why negative regulation of proinflammatory genes may only happen downstream of signaling via TRIF.

The comparison of two different strains of *S. aureus* showed that different strains of the same bacteria can elicit a diverse response in the same receptor. This backs other publications that showed that different strains of the same bacterial species can induce a variety of distinct immune responses (56,57). Although the same set of receptors were activated, the intensity of the response was significantly diverse, which is evidence that no generalizing statement can be made about a bacterial species. The presence of certain extrachromosomal plasmids in microbes was shown to have a positive influence on the virulence of a strain (58). This may result in a heightened amount of PAMPs, which is why one strain is more strongly recognized than the other. Interestingly, this difference was mainly seen at 10^6 and not at 10^8 microbes/mL. A possible reason is quorum sensing, the mechanism whereby bacteria communicate among themselves and adjust their gene expression according to cell density (59). Bacteria may only express virulence factors and PAMPs if a certain concentration of bacteria is reached (60). It is already proven in *Pseudomonas* that the threshold of the required concentration to express certain PAMPs differs from strain to strain (61). This could be the case in *S. aureus* as well and explain why the responses of TLRs do not differ at the highest concentration, since the threshold is already reached in both strains, whereas at 10^6 microbes/mL only one strain actively expresses certain PAMPs.

The response of hTLR2 and hTLR2-1 were of particular interest, since they showed a very diverse response across all strains in contrast to hTLR4 and hTLR5, which only reacted to gram-negative and flagellin-positive bacteria respectively. When bacteria were grouped by their pathogeny, the response of hTLR2 and hTLR2-1 at 10^8 microbes/mL was very strong across all classes. It seems that all strains express a great amount of PAMPs at the highest concentration (60) and thus are recognized exponentially stronger compared to lower concentrations. A comparison at 10^6 microbes/mL showed that especially pathogenic bacteria provoked a narrowed response in hTLR2 and hTLR2-1 compared to commensal and opportunistic bacteria, which seem to be more tolerated by TLRs. It is possible that pathogenic bacteria developed certain immune escape mechanisms, which is why recognition by the immune system is decreased compared to commensal or opportunistic bacteria (62). Another reason why this effect is only perceptible at 10^6 microbes/mL may once again be quorum sensing. Pathogenic bacteria may only express virulence factors and PAMPs above a much higher threshold than non-pathogenic microbes. This is why they become pathogenic in the first place and it is so difficult for the immune system to eliminate them (63). Although this alteration was visually noticeable, it could not be confirmed statistically.

When bacteria were grouped together according to their increased or decreased occurrence in chronic nasal inflammation as was demonstrated by Kaspar et al. (2016) (43), it can be seen that the response from hTLR2 at a concentration of 10^6 microbes/mL is very diverse. Only *S. aureus* triggered a positive reaction from the group of bacteria that have a decreased abundancy in nasal inflammation, whereas bacteria that were shown to be increased were constantly activated. Nevertheless, these findings were not statistically significant. However, the response of hTLR2-1 at 10^6 microbes/mL was significantly higher in bacteria that have a higher prevalence in patients with chronic nasal inflammation when compared to those that were decreased. This finding suggests that the composition of the microbiome in chronic nasal inflammation can amplify the already present immune answer in patients, since signaling of TLRs is increased and as a consequence more proinflammatory genes are expressed (43). Nevertheless, each clinical case must be handled individually since different strains can elicit variable responses and thus may have a different impact.

Additionally, bacteria were compared according to their altered occurrence in RSV infection as was demonstrated by Rosas-Salazar et al. (2016) (42). Regarding the response of hTLRs, it can be seen that there are no significant differences across all conditions between bacteria that have an increased or decreased abundancy in patients with RSV infection. However, the effect of a mixture of multiple bacterial strains remains unknown. Studies showed that a mixture of multiple probiotic bacteria can protect against RSV (64), so the impact of a single strain may be negligible. Since it was already demonstrated that each strain must be examined and evaluated independently, the same should apply to a composition of multiple bacteria. Nevertheless, it is possible that the altered microbiome in those cases is rather based on the presence of RSV than on the response of the immune system (65). However, the exact mechanism behind an altered microbiome upon RSV infection cannot be explained based on the response of TLRs to single bacterial strains.

5.1 Limitations

HEK-Blue™ TLR Cells do not depict a complex organism with lots of different immune reactions. Therefore, no general statement about the systemic impact of a tested reaction can be made. Additionally, photometrical quantification of SEAP is a very sensitive approach as well, leading to complications rather quick. As was seen with hTLR2-6 reporter cell lines, unspecific activation can happen quickly and very randomly, making it difficult to see a pattern.

Moreover, growth and biofilm-formation from microbes, as it was seen with *K. pneumoniae*, may alter the results as well.

5.2 Outlook

Based on these findings, further experiments will be performed. Since *S. aureus* showed a diverse response across different strains, it will be interesting, if this circumstance is the case in other bacterial species as well. Moreover, since stimulation with *K. pneumoniae* caused an unexpected color change and excessive biofilm formation, experiments will be conducted where bacteria will be heat-killed before stimulation to prevent further growth. Nevertheless, some PAMPs may be destroyed in this approach, which needs to be considered. Additionally, as a significant difference in response was seen between bacteria that have a heightened or lowered prevalence in patients with chronic nasal inflammation, further bacteria occurring in the regarding paper (43) will be analyzed.

Ultimately, the goal is to move to a biologically more realistic system like human infant primary nose epithelial cells. The differentiation assay is already well established in this research group (66) and would provide insight into multiple pathomechanisms in infants. Furthermore, these cells can be used to study downstream signaling and immunometabolism, which is a further step to understanding the effect of the human microbiome on health and disease in humans.

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7 List of Figures

8 List of Tables

9 Addendum

Add. Tab. 1: Mean-values of all technical replicates at a concentration of 10^8 microbes/mL.

Bacteria	*hTLR2*	*hTLR4*	*hTLR2-1*	*hTLR5*	*Null1*	*Null2*
C.minutissimum	1,813	0,094	1,138	0,131	0,145	0,199
C.simulans	1,694	0,095	0,886	0,129	0,147	0,224
C.striatum	1,680	0,100	0,820	0,142	0,148	0,167
C.tuberculostearicum	0,930	0,080	0,459	0,078	0,137	0,172
C.ulcerans	1,581	0,114	0,676	0,144	0,162	0,217
E.coli	1,639	0,228	1,051	2,024	0,497	1,172
H.influenzae	1,274	0,108	0,492	0,084	0,141	0,226
K.oxytoca	1,234	0,163	0,580	0,186	0,149	0,416
M.catarrhalis	1,801	0,191	1,097	0,127	0,137	0,392
S.aureus (ATCC27734)	1,725	0,091	0,815	0,127	0,134	0,319
S.aureus (ATCC29213)	1,773	0,083	0,395	0,117	0,060	0,313
S.captitis	1,253	0,083	0,226	0,132	0,138	0,447
S.epidermidis	1,776	0,350	0,275	0,138	0,066	0,347
S.haemolyticus	1,556	0,100	0,381	0,102	0,166	0,379
S.hominis	1,015	0,099	0,154	0,103	0,143	0,361
S.lugdunensis	1,856	0,079	0,312	0,112	0,129	0,401
S.pneumoniae	0,950	0,088	0,094	0,1386	0,159	0,216
S.pyogenes	0,882	0,078	0,111	0,106	0,120	0,369

Add. Tab. 2: Mean-values of all technical replicates at a concentration of 10^6 microbes/mL.

Bacteria	*hTLR2*	*hTLR4*	*hTLR2-1*	*hTLR5*	*Null1*	*Null2*
C.minutissimum	1,021	0,114	0,259	0,183	0,157	0,228
C.simulans	0,654	0,115	0,183	0,130	0,154	0,218
C.striatum	0,784	0,108	0,249	0,145	0,152	0,159
C.tuberculostearicum	0,509	0,103	0,190	0,092	0,170	0,406
C.ulcerans	0,211	0,096	0,138	0,119	0,133	0,199
E.coli	0,494	0,223	0,198	1,502	0,135	0,471
H.influenzae	0,619	0,255	0,261	0,098	0,166	0,400
K.oxytoca	0,239	0,207	0,124	0,125	0,124	0,430
M.catarrhalis	1,135	0,248	0,244	0,136	0,147	0,401
S.aureus (ATCC27734)	1,095	0,080	0,129	0,149	0,144	0,410
S.aureus (ATCC29213)	0,363	0,079	0,133	0,118	0,057	0,304
S.captitis	0,162	0,074	0,105	0,132	0,133	0,449
S.epidermidis	1,133	0,090	0,130	0,144	0,064	0,337
S.haemolyticus	0,213	0,095	0,091	0,110	0,182	0,380

S.hominis	0,212	0,102	0,087	0,112	0,184	0,337
S.lugdunensis	0,590	0,072	0,116	0,117	0,133	0,408
S.pneumoniae	0,122	0,097	0,095	0,212	0,160	0,196
S.pyogenes	0,150	0,073	0,108	0,133	0,134	0,412

Add. Tab. 3: Mean-values of all technical replicates at a concentration of 10^4 microbes/mL.

Bacteria	*hTLR2*	*hTLR4*	*hTLR2-1*	*hTLR5*	*Null1*	*Null2*
C.minutissimum	0,118	0,115	0,092	0,173	0,157	0,224
C.simulans	0,120	0,108	0,086	0,152	0,152	0,224
C.striatum	0,119	0,106	0,093	0,1190	0,143	0,202
C.tuberculostearicum	0,182	0,096	0,090	0,101	0,170	0,366
C.ulcerans	0,109	0,098	0,090	0,157	0,147	0,188
E.coli	0,142	0,093	0,105	0,161	0,128	0,424
H.influenzae	0,185	0,111	0,092	0,093	0,167	0,365
K.oxytoca	0,144	0,089	0,123	0,144	0,131	0,433
M.catarrhalis	0,159	0,081	0,108	0,127	0,145	0,422
S.aureus (ATCC27734)	0,162	0,075	0,109	0,138	0,140	0,410
S.aureus (ATCC29213)	0,139	0,085	0,128	0,117	0,059	0,303
S.captitis	0,145	0,077	0,113	0,130	0,130	0,436
S.epidermidis	0,126	0,086	0,146	0,141	0,061	0,389
S.haemolyticus	0,188	0,095	0,090	0,097	0,159	0,399
S.hominis	0,191	0,097	0,090	0,099	0,159	0,384
S.lugdunensis	0,157	0,077	0,108	0,124	0,135	0,394
S.pneumoniae	0,104	0,104	0,092	0,148	0,148	0,204
S.pyogenes	0,136	0,075	0,109	0,133	0,128	0,434

Add. Tab. 4: Mean-values of the technical replicates of all negative controls.

Bacteria	*hTLR2*	*hTLR4*	*hTLR2-1*	*hTLR5*	*Null1*	*Null2*
C.minutissimum	0,121	0,128	0,093	0,167	0,149	0,209
C.simulans	0,114	0,124	0,093	0,159	0,150	0,202
C.striatum	0,124	0,120	0,105	0,138	0,143	0,168
C.tuberculostearicum	0,214	0,100	0,103	0,097	0,163	0,415
C.ulcerans	0,126	0,117	0,104	0,144	0,131	0,185
E.coli	0,148	0,077	0,114	0,125	0,125	0,438
H.influenzae	0,214	0,100	0,103	0,097	0,163	0,415
K.oxytoca	0,148	0,077	0,114	0,125	0,125	0,438
M.catarrhalis	0,148	0,077	0,114	0,125	0,125	0,438
S.aureus (ATCC27734)	0,148	0,077	0,114	0,125	0,125	0,438
S.aureus (ATCC29213)	0,115	0,101	0,143	0,124	0,060	0,303

S.captitis	0,148	0,077	0,114	0,125	0,125	0,438
S.epidermidis	0,115	0,087	0,143	0,124	0,060	0,303
S.haemolyticus	0,214	0,100	0,103	0,097	0,163	0,415
S.hominis	0,214	0,100	0,103	0,097	0,163	0,415
S.lugdunensis	0,148	0,077	0,114	0,125	0,125	0,438
S.pneumoniae	0,113	0,118	0,095	0,152	0,145	0,196
S.pyogenes	0,148	0,077	0,114	0,125	0,125	0,438

Add. Tab. 5: Mean-values of the technical replicates of all positive controls.

Bacteria	*hTLR2*	*hTLR4*	*hTLR2-1*	*hTLR5*	*Null1*	*Null2*
C.minutissimum	1,839	1,357	1,440	0,766	0,967	1,013
C.simulans	1,832	1,326	1,370	0,767	0,957	1,015
C.striatum	1,705	1,247	1,157	0,553	0,894	0,848
C.tuberculostearicum	1,602	0,268	1,175	0,323	0,653	0,968
C.ulcerans	1,711	1,080	1,294	0,632	0,657	0,874
E.coli	1,564	0,239	0,997	0,847	0,378	1,103
H.influenzae	1,602	0,268	1,175	0,323	0,653	0,968
K.oxytoca	1,564	0,239	0,997	0,847	0,378	1,103
M.catarrhalis	1,564	0,239	0,997	0,847	0,378	1,103
S.aureus (ATCC27734)	1,564	0,239	0,997	0,847	0,378	1,103
S.aureus (ATCC29213)	1,305	0,552	0,465	1,283	0,044	0,297
S.captitis	1,564	0,239	0,997	0,847	0,378	1,103
S.epidermidis	1,303	0,622	0,642	1,503	0,049	0,331
S.haemolyticus	1,602	0,268	1,175	0,323	0,653	0,968
S.hominis	1,602	0,268	1,175	0,323	0,653	0,968
S.lugdunensis	1,564	0,239	0,997	0,847	0,378	1,103
S.pneumoniae	1,765	1,202	1,421	0,744	0,926	1,000
S.pyogenes	1,564	0,239	0,997	0,847	0,378	1,103

Add. Tab. 6: SD-values of all technical replicates at a concentration of 10^8 microbes/mL.

Bacteria	*hTLR2*	*hTLR4*	*hTLR2-1*	*hTLR5*	*Null1*	*Null2*
C.minutissimum	0,020	0,001	0,034	0,019	0,002	0,008
C.simulans	0,029	0,002	0,083	0,008	0,002	0,004
C.striatum	0,043	0,001	0,068	0,024	0,006	0,022
C.tuberculostearicum	0,054	0,002	0,091	0,002	0,002	0,007
C.ulcerans	0,027	0,003	0,012	0,002	0,002	0,007
E.coli	0,020	0,005	0,005	0,109	0,016	0,031
H.influenzae	0,055	0,004	0,066	0,002	0,003	0,020

K.oxytoca	0,029	0,004	0,054	0,003	0,009	0,009
M.catarrhalis	0,047	0,021	0,096	0,009	0,009	0,010
S.aureus (ATCC27734)	0,041	0,008	0,048	0,014	0,008	0,010
S.aureus (ATCC29213)	0,011	0,001	0,046	0,008	0,002	0,006
S.captitis	0,034	0,002	0,006	0,003	0,002	0,002
S.epidermidis	0,024	0,068	0,021	0,025	0,002	0,028
S.haemolyticus	0,028	0,002	0,033	0,010	0,011	0,011
S.hominis	0,092	0,005	0,009	0,013	0,006	0,048
S.lugdunensis	0,049	0,003	0,014	0,002	0,001	0,006
S.pneumoniae	0,050	0,002	0,010	0,027	0,021	0,013
S.pyogenes	0,061	0,001	0,002	0,005	0,010	0,010

Add. Tab. 7: SD-values of all technical replicates at a concentration of 10^6 microbes/mL.

Bacteria	*hTLR2*	*hTLR4*	*hTLR2-1*	*hTLR5*	*Null1*	*Null2*
C.minutissimum	0,018	0,002	0,008	0,009	0,004	0,004
C.simulans	0,019	0,004	0,015	0,015	0,003	0,005
C.striatum	0,060	0,003	0,023	0,011	0,003	0,011
C.tuberculostearicum	0,016	0,002	0,003	0,005	0,001	0,063
C.ulcerans	0,016	0,002	0,003	0,005	0,001	0,010
E.coli	0,085	0,004	0,010	0,093	0,000	0,017
H.influenzae	0,022	0,009	0,005	0,002	0,002	0,005
K.oxytoca	0,008	0,010	0,013	0,006	0,002	0,007
M.catarrhalis	0,043	0,003	0,021	0,007	0,010	0,006
S.aureus (ATCC27734)	0,063	0,003	0,010	0,006	0,006	0,014
S.aureus (ATCC29213)	0,008	0,000	0,006	0,007	0,001	0,005
S.captitis	0,005	0,004	0,012	0,001	0,004	0,009
S.epidermidis	0,022	0,006	0,004	0,017	0,002	0,023
S.haemolyticus	0,002	0,002	0,001	0,002	0,003	0,010
S.hominis	0,005	0,003	0,003	0,010	0,007	0,027
S.lugdunensis	0,026	0,002	0,003	0,010	0,002	0,009
S.pneumoniae	0,006	0,006	0,006	0,011	0,009	0,008
S.pyogenes	0,011	0,002	0,003	0,013	0,008	0,002

Add. Tab. 8: SD-values of all technical replicates at a concentration of 10^4 microbes/mL.

Bacteria	*hTLR2*	*hTLR4*	*hTLR2-1*	*hTLR5*	*Null1*	*Null2*
C.minutissimum	0,005	0,001	0,004	0,014	0,002	0,009
C.simulans	0,003	0,003	0,004	0,021	0,001	0,004
C.striatum	0,004	0,006	0,003	0,003	0,005	0,011
C.tuberculostearicum	0,003	0,004	0,001	0,007	0,001	0,016
C.ulcerans	0,003	0,004	0,001	0,007	0,001	0,016
E.coli	0,008	0,010	0,007	0,007	0,003	0,013
H.influenzae	0,006	0,004	0,001	0,003	0,004	0,010
K.oxytoca	0,006	0,006	0,003	0,012	0,003	0,004
M.catarrhalis	0,003	0,004	0,007	0,010	0,004	0,004
S.aureus (ATCC27734)	0,011	0,001	0,004	0,011	0,003	0,009
S.aureus (ATCC29213)	0,014	0,002	0,017	0,006	0,000	0,011
S.captitis	0,004	0,001	0,005	0,002	0,004	0,011
S.epidermidis	0,005	0,007	0,024	0,016	0,001	0,018
S.haemolyticus	0,002	0,003	0,002	0,002	0,003	0,005
S.hominis	0,003	0,002	0,004	0,012	0,010	0,007
S.lugdunensis	0,023	0,001	0,003	0,005	0,002	0,019
S.pneumoniae	0,002	0,004	0,006	0,004	0,004	0,004
S.pyogenes	0,003	0,002	0,003	0,005	0,002	0,008

Add. Tab. 9: SD-values of the technical replicates of all negative controls.

Bacteria	*hTLR2*	*hTLR4*	*hTLR2-1*	*hTLR5*	*Null1*	*Null2*
C.minutissimum	0,012	0,003	0,002	0,003	0,004	0,005
C.simulans	0,002	0,003	0,002	0,011	0,005	0,006
C.striatum	0,019	0,003	0,010	0,016	0,003	0,011
C.tuberculostearicum	0,010	0,005	0,013	0,005	0,006	0,010
C.ulcerans	0,017	0,005	0,001	0,006	0,004	0,010
E.coli	0,003	0,001	0,001	0,001	0,000	0,005
H.influenzae	0,007	0,003	0,001	0,001	0,004	0,008
K.oxytoca	0,021	0,001	0,008	0,007	0,005	0,006
M.catarrhalis	0,003	0,001	0,005	0,003	0,004	0,006
S.aureus (ATCC27734)	0,002	0,002	0,018	0,005	0,006	0,009
S.aureus (ATCC29213)	0,006	0,003	0,020	0,001	0,000	0,005
S.captitis	0,003	0,002	0,001	0,004	0,002	0,022
S.epidermidis	0,003	0,001	0,010	0,012	0,001	0,003
S.haemolyticus	0,003	0,001	0,006	0,004	0,003	0,012
S.hominis	0,004	0,001	0,014	0,002	0,010	0,007

S.lugdunensis	0,004	0,002	0,002	0,005	0,001	0,010
S.pneumoniae	0,002	0,003	0,008	0,009	0,000	0,012
S.pyogenes	0,005	0,002	0,003	0,002	0,001	0,002

Add. Tab. 10: SD-values of the technical replicates of all positive controls.

Bacteria	*hTLR2*	*hTLR4*	*hTLR2-1*	*hTLR5*	*Null1*	*Null2*
C.minutissimum	0,023	0,012	0,008	0,028	0,013	0,005
C.simulans	0,040	0,011	0,032	0,049	0,018	0,025
C.striatum	0,045	0,044	0,143	0,100	0,049	0,024
C.tuberculostearicum	0,046	0,001	0,033	0,022	0,092	0,046
C.ulcerans	0,105	0,024	0,017	0,031	0,036	0,154
E.coli	0,012	0,008	0,014	0,058	0,007	0,006
H.influenzae	0,043	0,010	0,041	0,015	0,050	0,046
K.oxytoca	0,094	0,007	0,058	0,032	0,039	0,012
M.catarrhalis	0,029	0,016	0,018	0,021	0,007	0,039
S.aureus (ATCC27734)	0,014	0,016	0,028	0,035	0,005	0,022
S.aureus (ATCC29213)	0,007	0,023	0,068	0,247	0,001	0,014
S.captitis	0,019	0,005	0,013	0,100	0,013	0,006
S.epidermidis	0,025	0,020	0,076	0,287	0,001	0,014
S.haemolyticus	0,048	0,008	0,038	0,016	0,038	0,023
S.hominis	0,037	0,009	0,093	0,045	0,052	0,016
S.lugdunensis	0,023	0,026	0,009	0,069	0,016	0,156
S.pneumoniae	0,028	0,032	0,042	0,041	0,009	0,072
S.pyogenes	0,026	0,006	0,017	0,048	0,006	0,010