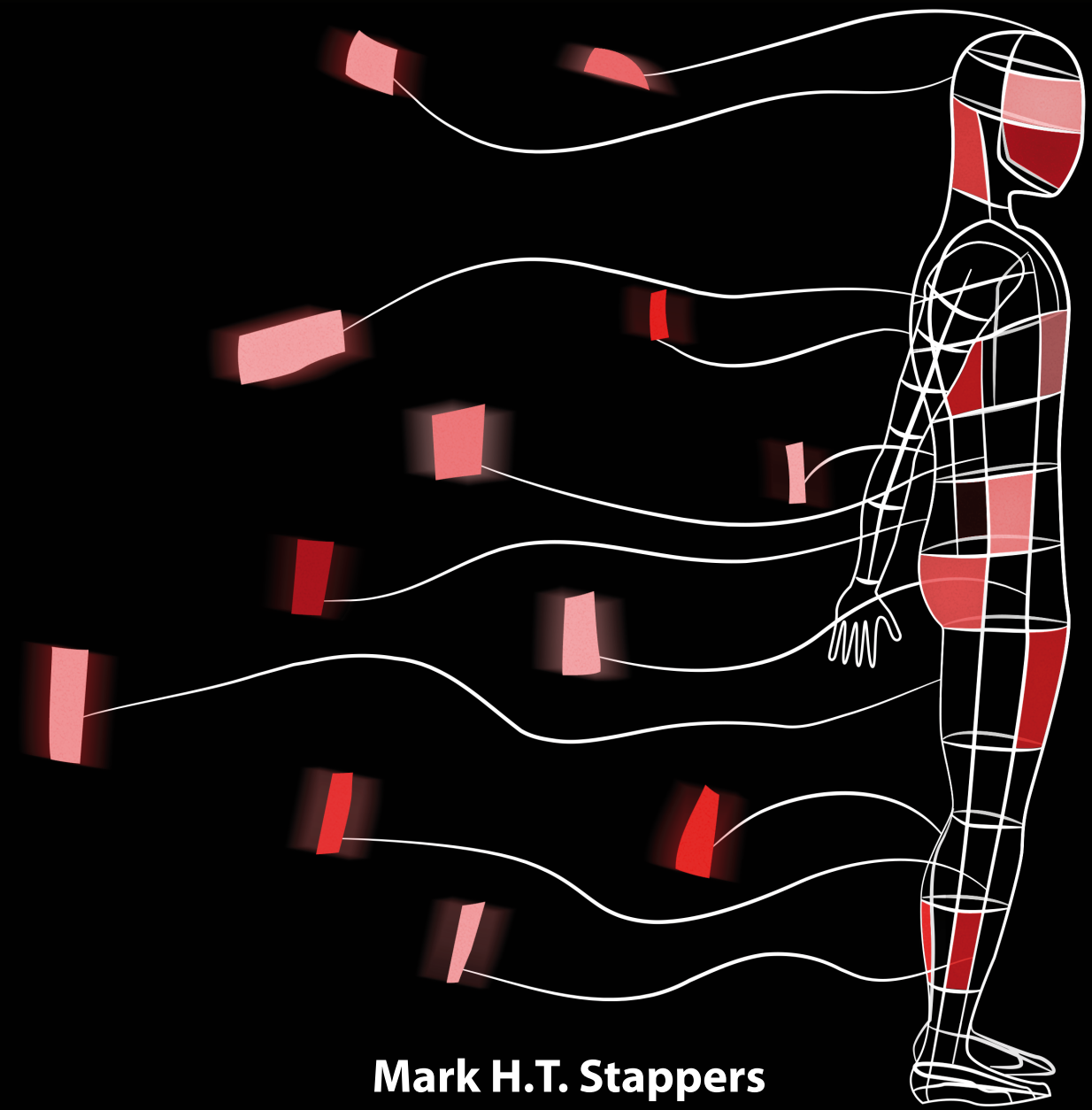
Complicated skin and skin structure infections

from host to pathogen



Mark H.T. Stappers

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Colofon
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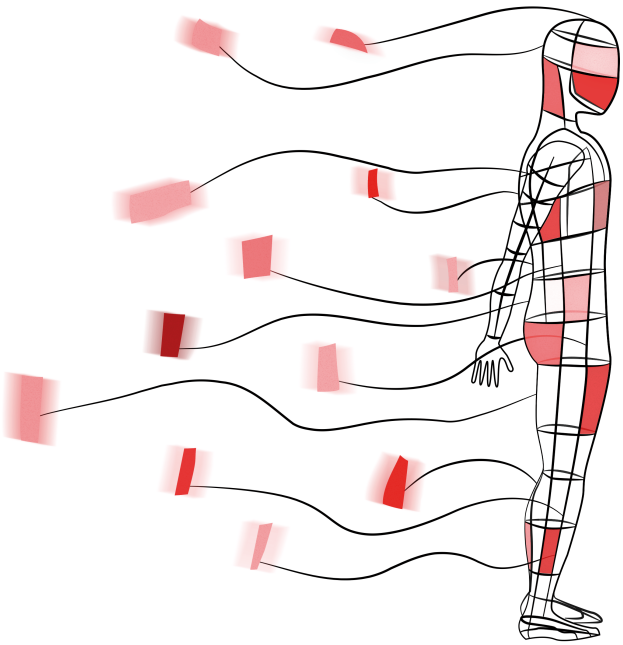
Chapter 1

General introduction and outline of the thesis



Introduction

Colonization of the human body by microorganisms occurs at the very beginning of life. Most of these microorganisms are commensals, and as such play a major role in bodily functions such as digestion and protection against disease [1, 2]. The skin is the largest organ in the human body and acts as a physical barrier with the environment, protecting the body against bacterial invasion. Unfortunately, this barrier does not always suffice and when breached may result in the development of skin and skin structure infections (SSSIs). SSSIs encompass a spectrum of clinical conditions with varying presentation, etiology and severity, caused by microbial invasion of the epidermis, dermis and subcutaneous tissues [3]. In clinical practice, SSSIs are among the most commonly observed human infections. Most often, presentation is mild and resolves either without antibiotic treatment or with oral antimicrobial therapy. However, severe cases result in either long-lasting or acute, life-threatening infections, which require immediate medical and surgical intervention. Perhaps due to this diversity in clinical conditions and duration of symptoms, the prevalence of SSSIs has been difficult to determine. Studies from the USA estimated increasing health care visits for SSSIs, with 48.1 per 1000 population per year in 2005, reaching a total of 14.2 million health care visits [4]. Incidence of hospital admissions due to SSSIs is also increasing and are estimated to account for ~10% of all hospitalizations [5-7].

Classification of skin and skin structure infections

There is, to date, no universally accepted terminology and classification for SSSIs. Synonyms for SSSIs are skin and soft-tissue infections and acute bacterial skin and skin structure infections. Various attempts have been made to classify SSSIs based on: primary (infection of the otherwise normal skin) and secondary infection (complication of a chronic skin condition), acute versus chronic infection, local versus systemic infection, focal versus diffuse location, depth of the invasion, community-acquired versus nosocomial infection, or causative organism [3, 8-12].

In this thesis we will adhere to the classification developed by the US Food and Drug Administration (FDA) [3]. In their 1998 guidance document for developing antimicrobial drugs for treatment of SSSIs, the FDA classified the different conditions in 2 broad categories: uncomplicated (uSSSIs) and complicated skin and skin structure infections (cSSSIs). The FDA based this classification on the observation that SSSIs tend to share common pathogens and show comparable responses to antimicrobial therapy. uSSSIs included superficial conditions such as minor abscesses, impetigo, ecthyma, folliculitis, furunculosis, erysipelas and cellulitis. cSSSIs included infections of the deeper soft tissue, involving surgical intervention or a significant underlying disease state that complicates

the response to treatment, such as major abscesses, infected ischemic or diabetic foot ulcers, wound and burn infections. Superficial infections located in an anatomical site in which the chance of involvement of anaerobic or gram-negative pathogens is high should also be considered as cSSSIs [3, 7].

Clinical presentation of skin and skin structure infections

The hallmark of SSSIs is redness, warmth, edema and tenderness of the skin, often accompanied by (purulent) discharge. At the more severe end of the spectrum of SSSIs, infections involve the deeper soft tissue, including the subcutaneous fat and/or fascia and muscle, requiring surgical intervention or involving a significant underlying disease state that complicates the response to treatment [3]. These deeper SSSIs can be subdivided based on clinical presentation in major abscesses, wound and burn infections or infected ischemic and diabetic foot ulcers. Major abscesses are characterized by collections of pus within the deeper skin and soft tissues, often surrounded by extensive cellulitis. Wound infections can originate from a variety of wounds such as post-surgical, post-traumatic, human or animal bite wounds, whereas burn infections occur after thermal injury of the skin. Infected ischemic ulcers occur in peripheral vascular disease, and diabetic foot ulcers are defined as infections occurring below the ankle in patients with confirmed diabetes mellitus [3].

Causative organisms of skin and skin structure infections

The microbiology of SSSIs is diverse, with presence of gram-positive, gram-negative aerobic or anaerobic bacteria, either alone or as part of a polymicrobial infection. Reviewing the major clinical trials for antibiotic registration over the last 15 years, performed according to the FDA guidance documents for developing antimicrobial drugs for treatment of SSSIs indicated that the large majority of SSSIs are caused by gram-positive bacteria, mainly *Staphylococcus aureus* followed by *Streptococcus* species (*S. pyogenes*, *S. agalactiae*, *S. dysgalactiae* and *S. anginosus* group) and *Enterococcus faecalis*. When reported, gram-negatives *Escherichia coli* and *Pseudomonas aeruginosa* are also frequently found in the top 5 of causative organisms. Other bacteria, including *Bacteroides fragilis*, *Proteus mirabilis*, *Enterobacter cloacae* and *Staphylococcus epidermidis* only incidentally reached the top 5 of causative organisms [13-24].

Pathogenesis

The skin is of crucial importance for fluid and temperature regulation, sensation, metabolism of vitamin D and defense against bacterial invasion. It consists of 2 distinct layers, the epidermis and dermis. The epidermis is the outermost layer of the skin which comprises several strata of epithelium and forms the main layer of protection against bacterial invasion. The dermis is the layer underneath the epidermis and contains

the connective tissue responsible for strength and elasticity of the skin. This layer also contains the hair follicles, glands, blood and lymphatic vessels. Beneath these layers of the skin, the subcutaneous tissues (fat and fascia), muscle, and bone are positioned (Figure 1). The skin controls bacterial invasion by maintaining a low pH, secreting sebum (containing antioxidants and antimicrobial peptides) and salty sweat, limiting the presence of moisture and maintaining a low surface temperature to prevent growth of pathogens [25, 26]. In addition, the skin is covered with its own microflora, thereby preventing unwanted pathogenic colonization [27]. Upon breaching of this physical barrier, microorganisms may invade below the skin and result in the development of SSSIs. Often, these infections are caused by commensal bacteria that have escaped their normal habitat and invade after physical or chemical trauma to the skin surface. Spread from other invasive bacterial infections to the skin rarely occurs, except for septic emboli from certain bloodstream infections and endocarditis. Depending on the depth of the microbial invasion and the degree of tissue damage, different clinical entities can be described ranging from mild superficial infection to deep life-threatening disease (Figure 1) [3]. The pathogenesis of SSSIs is an interplay between virulence of the pathogen and the immune response of the host.

Bacterial virulence

In order for bacteria to successfully induce SSSIs, they have to overcome several obstacles: first, they must be able to adhere to and colonize the skin; secondly, they have to invade and cause disease to the skin and underlying tissue; and finally, they need to escape

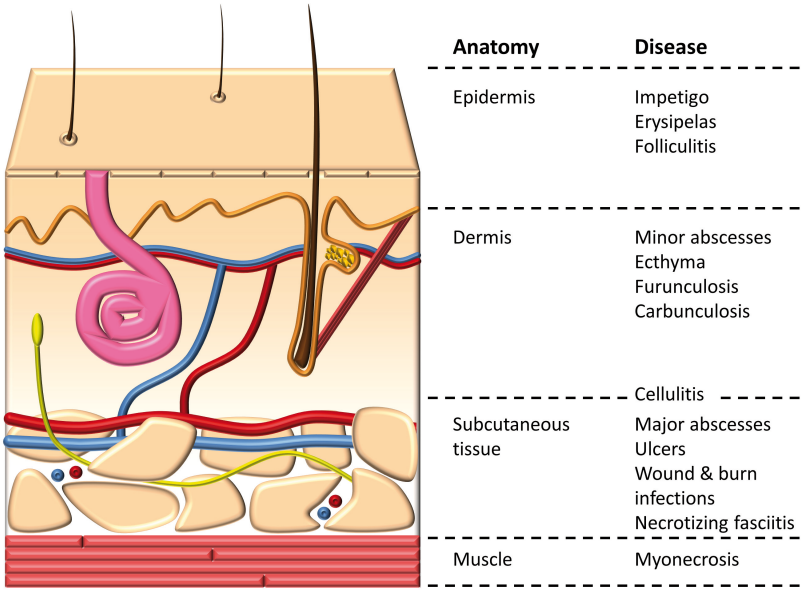


Figure 1. Schematic representation of the skin and presence of skin and skin structure infections.

destruction by the host immune response. Virulence factors are small molecules that are expressed or secreted by bacteria that help the bacteria to induce SSSIs. According to function, virulence factors can be divided in several classes, such as: factors that assist in adherence and invasion, the capsule which protects against opsonization and phagocytosis, cell-surface endotoxins and secreted exotoxins. Each species possesses their own set of virulence factors as extensively reviewed for major cSSSI pathogens *S. aureus* (O'Riordan *et al.* [28], Dinges *et al.* [29] and Foster *et al.* [30]) and *Streptococcus* species (Cuningham *et al.* [31], Walker *et al.* [32] and Lindahl *et al.* [33]).

Host immune response

When a pathogen breaches the physical barrier of the skin, cells of the host immune system come into play. The immune system can be divided into innate and adaptive immunity. Cells of the innate immune system are first to respond after invasion of microbes. They recognize these invaders by conserved microbial structures, called pathogen-associated molecular patterns (PAMPs), using specific pattern-recognition receptors (PRRs). Five different classes have been described to date: Toll-like receptors (TLRs), Nucleotide-binding domain (NOD)-like receptors (NLRs), C-type lectin receptors, Retinoic acid-inducible gene I (RIG-I) like receptors and absent in melanoma 2 (AIM2)-like cytoplasmic DNA receptors. Activation of PRRs, induces downstream intracellular signal transduction cascades which result in gene transcription, synthesis and secretion of effector molecules such as cytokines, chemokines and antimicrobial peptides, recruitment of other innate immune cells (e.g. neutrophils), and activation of an adaptive immune response [34]. Each microbe, depending on its composition, activates a distinct set of PRRs, resulting in tailored immune responses for each pathogen, for example as reviewed for cSSSI pathogen *S. aureus* (Fournier *et al.* [35], Krishna *et al.* [36], Miller *et al.* [37]).

Outline of this thesis

The overall aim of this thesis is to study the contribution of the innate immunity diversity of the host and presence of relevant pathogens in the development of complicated skin and skin structure infections.

The first part of this thesis focuses on the role of the innate immune system of the host during complicated skin and skin structure infections (**Chapters 2-6**). Following breaches of the skin, microorganisms can invade and cause infection. Cells of the innate immune system recognize through PRRs and phagocytose these microorganisms. In **Chapter 2**, we provide an overview of the different PRR families (Toll-like receptors, C-type lectin receptors, NOD-like receptors, RIG-I-like receptors and AIM2-like receptors), describe their signaling pathways and roles during infection. We also reviewed the influence of

variation in genes encoding these PRRs on their function and susceptibility to infectious disease. *Bacteroides fragilis*, a commensal of the human gut microbiome, is the most frequently isolated anaerobic microorganism from SSSIs [38], but evidence regarding the pattern recognition of *B. fragilis* is lacking. In **Chapter 3**, we investigated the PRRs involved in the recognition of *B. fragilis* and characterised the cytokine profile induced by this microorganism. Although several genes have been associated with susceptibility to infectious disease (Chapter 2), the role of genetic variation in susceptibility to complicated skin and skin structure infections remained to be clarified. In **Chapters 4 and 5**, we investigated the influence of genetic variation in genes encoding PRRs on the susceptibility to cSSSIs in a cohort of cSSSI patients versus healthy, and the functional consequences of sequence variants associated with disease were studied. In addition, the role of TLR10, recently proposed as an anti-inflammatory receptor [39], was studied in the innate immune response to frequent cSSSI pathogens in **Chapter 5**. Recognition of the microorganism by cells of the innate immune system results in activation of an immune response and production of various pro- and anti-inflammatory cytokines [34]. In **Chapter 6**, the influence of sequence variants in genes encoding cytokines on the susceptibility to cSSSIs, and the functional consequences of sequence variants associated with disease, were studied.

The second part of this thesis focuses on the identification of pathogens present in cSSSIs (**Chapters 7 and 8**). Routine assessment of bacterial presence currently relies upon culture of biopsies. However, in recent years various molecular methods have been developed to improve the detection of pathogens, as reviewed by [40, 41]. In **Chapters 7 and 8** we evaluate the potential benefit of direct, pathogen-specific real-time PCR assays on clinical samples in the detection of the causative organisms of cSSSIs for the gram-positive *S. aureus* and *Streptocccus* species and for the gram-negative *B. fragilis*, respectively.

A summary of the findings and conclusions described in this thesis is presented in **Chapter 9**.

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Part 1

The role of the innate immune system of the host during complicated skin and skin structure infections

Chapter 2

Genetic variation in pattern-recognition receptors: functional consequences and susceptibility to infectious disease

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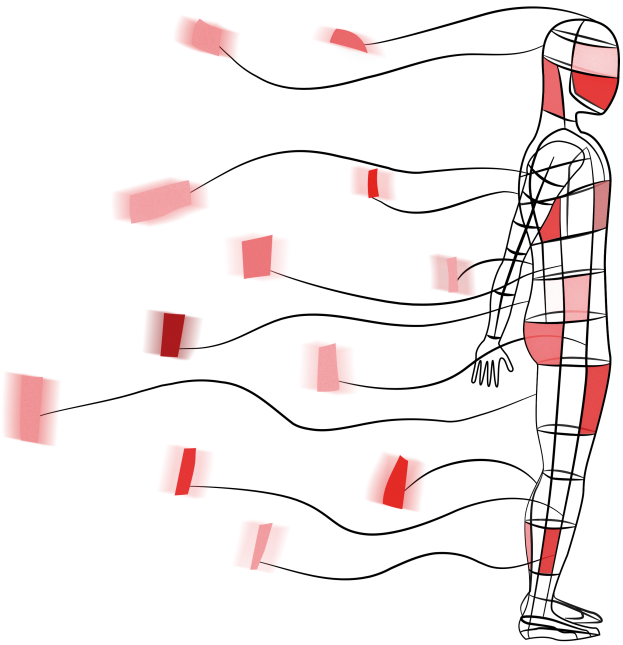

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Abstract

Cells of the innate immune system are equipped with surface and cytoplasmic receptors for microorganisms called pattern-recognition receptors (PRRs). PRRs recognize specific pathogen-associated molecular patterns and as such are crucial for the activation of the immune system. Currently, 5 different classes of PRRs have been described: Toll-like receptors, C-type lectin receptors, nucleotide-binding oligomerization domain-like receptors, retinoic acid-inducible gene I-like receptors and absent in melanoma 2-like receptors. Following their discovery, many sequence variants in PRR genes have been uncovered and shown to be implicated in human infectious diseases. In this review, we will discuss the effect of genetic variation in PRRs and their signalling pathways on susceptibility to infectious diseases in humans.

Introduction

Infectious diseases are a major cause of morbidity and mortality worldwide [1]. The genetic makeup of an individual has a strong influence on the risk of death from infection, as demonstrated by studies correlating the cause of death in adopted children with biological or adoptive parents [2]. To defend the host against infections, components of the innate and adaptive immune system collaborate to eliminate intruding pathogens. Genetic variation in both components of the immune system influences the susceptibility to infectious disease. For example, individuals with severe combined immunodeficiency suffer from recalcitrant opportunistic infections due to deficiencies in T and B cells of the adaptive immune system [3], and defects in the innate interferon-γ pathway are associated with recurrent infections with mycobacteria and *Salmonella* spp. [4].

In this review, we will discuss the major findings concerning genetic variation in pattern-recognition receptors of the innate immune system and molecules involved in their signalling pathways. We will focus on the relation between genetic variation and susceptibility to infectious disease and review the functional consequences induced by these genetic variants.

Pattern recognition of the innate immune system

The human body is constantly exposed to potential harmful threats such as bacteria, fungi, viruses, parasites and protozoa. Upon intrusion of a pathogen in a normally sterile environment, the innate immune system is the first line of defense against these invaders. Cells of the innate immune system are equipped with membrane and cytoplasmic receptors called pattern-recognition receptors (PRRs), a concept first proposed by Charles Janeway Jr. [5]. These highly conserved, germ-line encoded receptors are able to recognize specific pathogen-associated molecular patterns (PAMPs) that are essential structures of microorganisms [6, 7]. Although highly conserved, small interspecies variation in PAMPs have been shown to result in altered immune responses mediated by the same PRR. One relevant example in that respect is that of bacterial muramyl dipeptide (MDP): it has been shown that the glycolyl-MDP from mycobacteria and other *Actinomycetes* is much more active for stimulation of immune responses compared to acetyl-MDP from other bacterial species [8-10]. Recognition of PAMPs by PRRs activates intracellular signal transduction cascades followed by gene transcription, secretion of distinctive effector molecules and generation of an adaptive immune response. Depending on the structure and composition of the microbe, different combinations of PRRs are activated, inducing specific signaling pathways, which results in tailored responses to infectious agents [6, 7]. At present, 5 main classes of PRRs have been described: Toll-like receptors (TLRs),

C-type lectin receptors (CLRs), nucleotide-binding domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and absent in melanoma 2 (AIM2)-like receptors (ALRs) (Figure 1) [7, 11]. Most PRRs recognize cell wall components of microorganisms, but DNA, RNA or metabolites of microbial origin can also act as PAMPs [7]. Table 1 summarizes important PRRs of the innate immune system with corresponding PAMPs and their microbial sources.

Genetic variation in pattern-recognition receptors

Changes in the nucleotide sequence of genes encoding PRRs can affect the susceptibility to infectious disease. Most often, the susceptibility to infections is multifactorial, with the odds of developing the disease increasing with every risk sequence variant. In a minority of cases, single sequence variants are able to induce a strong increase in susceptibility to certain infections. The presence of such a mutation thus results in a monogenetic or primary immunodeficiency.

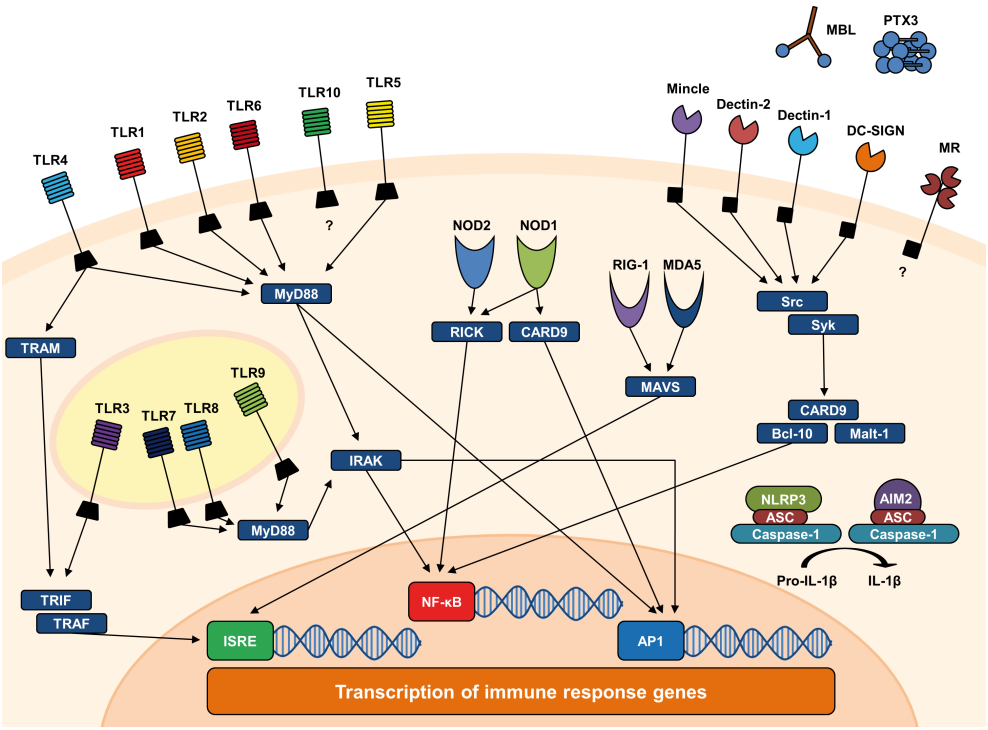

Figure 1. Overview of pattern-recognition receptors and downstream signalling pathways.

Table 1. Overview of pattern-recognition receptors and corresponding pathogen-associated molecular patterns.

Receptor	PAMP	Source
Toll-like receptors		
TLR2/TLR1	Triacyl lipopeptides	Bacteria
TLR2	Peptidoglycans Glycolipids Envelope proteins	Bacteria Bacteria, fungi Viruses
TLR2/TLR6	Diacyl lipopeptides Lipoteichoic acid Zymosan	Bacteria Bacteria Fungi
TLR2/TLR10	Unknown	Unknown
TLR3	Single-stranded RNA Double-stranded RNA	Viruses Viruses
TLR4	Lipopolysaccharide Mannans Envelope proteins	Bacteria Fungi Viruses
TLR5	Flagellin	Bacteria
TLR7	Single-stranded RNA	Viruses
TLR8	Single-stranded RNA	Viruses
TLR9	Unmethylated CpG motifs Double-stranded DNA	Bacteria, fungi, viruses Viruses
C-type lectin receptors		
Dectin-1	Beta (linked)-glucans	Fungi, bacteria
Dectin-2	High mannose structures	Fungi, bacteria
Mannose receptor	Mannose, fucose	Fungi, bacteria, viruses
DC-SIGN	Mannan, high mannose structures	Fungi, bacteria, viruses
Mannose-binding lectin	High mannose, N-acetylglucosamine structures	Fungi, bacteria, viruses
NOD-like receptors		
NOD1	Muramyl tripeptide	Bacteria
NOD2	Muramyl dipeptide	Bacteria
NLRP3	Uric acid crystals, silica, different PAMPs	Bacteria, fungi, viruses
RIG-I-like receptors		
RIG-I	Short double-stranded RNA	Viruses
MDA5	Long double-stranded RNA	Viruses
AIM2-like receptors		
AIM2	Double-stranded DNA	Bacteria, viruses, host

PAMP, pathogen-associated molecular pattern.

The majority of genetic variation is represented by variants such as substitutions (single-nucleotide polymorphisms), insertions, deletions, inversions and duplications and micro- and minisatellites, in which only 1 or a few nucleotides are affected. Less often, large-scale variants in which whole chromosomes or regions are altered can also be encountered [12]. Depending on the type of sequence variant and its location in the gene, it can have different functional consequences. Variants in the coding region can affect the amino acid sequence, and as such, the potential to influence the function of the protein. By contrast, variation in the noncoding region of the gene can still affect the functionality of the gene by alterations in promoter binding sites, splicing of the gene, stability and degradation of the messenger RNA.

As a result, one may expect that genetic variation in genes encoding for PRRs, key mediators in innate immune recognition, affects susceptibility to infectious disease, a hypothesis confirmed in recent years. Here we will discuss the major genetic polymorphisms in TLR, CLR, NLR, RLR and ALR genes, their association in susceptibility to infectious disease and functional consequences for activation of the innate immune response.

Toll-like receptors

Following the discovery of the Toll-signaling pathway in *Drosophila*, implicated in the host defense against fungal infections [13], 10 Toll homologues termed TLRs (TLR1-10) have been characterized in humans. TLRs are primarily expressed in cells of the immune system such as monocytes, neutrophils, basophils, eosinophils and NK cells, but also cells of epithelial, endothelial and stromal origin [14]. TLRs recognize a variety of different microbes based on the presence of specific PAMPs (Table 1). A broad division can be made between TLRs primarily expressed on the surface of cells (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10), mainly targeting microbial lipids and proteins; and those located in intracellular compartments of the endocytic pathway (TLR3, TLR7, TLR8 and TLR9), mainly involved in the recognition of microbial nucleic acids (Table 1).

All TLRs consist of an extracellular domain with varying amounts of leucine-rich repeats for recognition of PAMPs, a transmembrane region, and a cytoplasmic Toll/Interleukin-1R (TIR) homology domain involved in signal transduction. Recognition of its ligand induces the formation of homo- or heterodimers between TLRs and subsequent recruitment of downstream adaptor proteins, activation of transcription factors, resulting in the production of cytokines and interferons and induction of an adaptive immune response. Most TLRs depend on the activation of adaptor protein myeloid differentiation primary response gene 88 (MyD88), which via Interleukin-1 receptor-associated kinase (IRAK) induces activation of transcription factors such as nuclear factor kappa-light-chain-

enhancer of activated B-cells (NF-κB) and activating protein-1 (AP1). In contrast, TLR3 signals via the TIR domain containing adaptor protein inducing IFN-β (TRIF). TLR4 has, besides MyD88, a second signalling pathway via TRIF-related adaptor molecule (TRAM). Both TRIF and TRAM pathways activate TNF receptor associated factor (TRAF)3, resulting in the activation of IFN-stimulated response elements (ISRE) [15]. For the last discovered member, TLR10, the exact signaling pathway remains to be elucidated. Table 2 summarizes the sequence variants in TLRs and their association in susceptibility to infectious diseases.

Toll-like receptor 2 subfamily

TLR1, *TLR2*, *TLR6* and *TLR10* are located in a cluster on chromosome 4, and for convenience are termed the TLR2 subfamily. Besides significant sequence similarity [16], members of the TLR2 subfamily form heterodimers that cooperate for the recognition of microbes. TLR2 is the key receptor in this process, due to its ability to form homodimers as well as heterodimers with TLR1 and TLR6 for the recognition of various ligands from bacterial, fungal and viral origin (Table 1). Until recently, TLR10 was the only TLR without known ligand and function. However, molecular modelling indicated Pam_3Cys as a possible ligand for the TLR10/2 heterodimer and PamCysPam for the TLR10/1 heterodimer and TLR10 homodimer [17]. Recent studies have suggested that the function of TLR10 differs from other TLRs, as it was shown to inhibit TLR2-driven innate immune activation [18-20]. By contrast, TLR10 has also been described as a stimulatory receptor in the innate immune recognition [21, 22].

Toll-like receptor 1

TLR1 sequence variant rs5743618 (Ile602Ser) leads to the substitution of isoleucine for serine at amino-acid position 602 of the TLR1 protein. Presence of the 602Ser variant results in aberrant trafficking of TLR1 to the cell surface and results in decreased NF-κB activation and proinflammatory cytokine responses to TLR1 agonists [23, 24]. *TLR1* sequence variant rs5743618 is associated with susceptibility to tuberculosis [25], leprosy [23, 26], candidemia [27] and malaria [28].

TLR1 sequence variant rs4833095 (Ser248Asn) leads to the substitution of serine for asparagine at amino-acid position 248 of the TLR1 protein. *TLR1* rs4833095 is in strong linkage with the above described *TLR1* rs5743618 in populations of European descent [24, 27], which may partially explain why the 248Asn variant is associated with reduced cytokine responses after exposure to TLR2 ligand Pam_3Cys and *Staphylococcus aureus* [27, 29]. However, slight reduction of NF-κB activation was detected in the presence of 248Asn, irrespective of the presence of *TLR1* rs5743618 [30]. *TLR1* sequence variant rs4833095 is associated with susceptibility to tuberculosis [25], leprosy [31], complicated skin and skin structure infections [29], aspergillosis [32] and candidemia [27].

Table 2. Overview of genetic variation in Toll-like receptors and susceptibility to infectious diseases.

Gene	Sequence variant			Disease	Risk allele	Reference
	Rs-number	Nucleotide change#	Amino acid change#			
TLR1	rs5743618	T > G	Ile602Ser	Tuberculosis	T	[25]
				Leprosy	T	[23, 26]
				Candidemia	G	[27]
				Malaria	G	[28]
TLR1	rs4833095	C > T	Ser248Asn	Tuberculosis	C	[25]
				Leprosy	C	[31]
				Complicated skin and skin structure infection	T	[29]
				Aspergillosis	C	[32]
				Candidemia	T	[27]
TLR2	rs5743708	G > A	Arg753Gln	Tuberculosis	A	[35, 36]
				Lyme disease	G	[37]
				CMV infection	A	[38, 39]
TLR2	rs5743704	C > A	Pro631His	Tuberculosis	A	[40]
				Complicated skin and skin structure infection	A	[29]
				Recurrent vulvo vaginal candidiasis	A	[41]
TLR6	rs5743810	C > T	Pro249Ser	Tuberculosis	T	[25]
				Complicated skin and skin structure infection	T	[29]
				Aspergillosis	T	[32]
				Malaria	T	[28]
TLR10	rs4129009 & rs11096955	A > C & A > C	Ile775Leu & Ile369Leu	Complicated skin and skin structure infection	A & A	[44]
TLR4	rs4986790 & rs4986791	A > G & C > T	Asp299Gly & Thr399Ile	Sepsis	G & T	[57-59]
				Urinary tract infection	A & C	[60]
				Brucellosis	G & T	[61]
				Legionnaires´ disease	A & C	[62]
				Meningococcal disease	G & T	[63, 64]
				Tuberculosis	G & T	[65, 66]
				Leprosy	A & C	[67]
				Aspergillosis	G & T	[68-71]
				Candidiasis	G & T	[72]
				RSV infection	G & T	[75]
				Malaria	G & T	[73, 74]
TLR5	rs5744168	C > T	Arg392Ter	Legionnaires' disease	T	[76]
				Urinary tract infection	T	[60]
TLR3	rs3775291	G > A	Leu412Phe	HCV infection	A	[82]
				HSV infection	G	[83]
				HIV infection	G	[81]
				Tick-borne encephalitis virus infection	G	[84]
TLR7	rs179008	A > T	Gln11Leu	HCV infection	T	[86]
				HIV infection	T	[85]

TLR8	rs3764880	G > A	Val1Met	Tuberculosis	A	[90, 91]
				Crimean-Congo hemorrhagic fever	G	[93]
				HCV infection	A	[89, 92]
TLR9	rs5743836	C > T	N/A	Tuberculosis	T	[96]
				Aspergillosis	C	[69]
				Malaria	C	[97, 98]

[#] The first nucleotide (and corresponding amino acid) is the ancestral nucleotide and therefore is considered the wild-type allele. N/A, not applicable; CMV, cytomegalovirus; RSV, respiratory syncytial virus; HCV, hepatitis C virus; HSV, herpes simplex virus; HIV, human immunodeficiency virus.

Toll-like receptor 2

TLR2 sequence variant rs5743708 (Arg753Gln) leads to the substitution of arginine for glutamine at amino-acid position 753 of the TLR2 protein. Presence of the 753Gln variant does not influence the expression of TLR2, but instead causes deficient tyrosine phosphorylation, compromises the assembly of the TLR2-TLR6 complex and impairs recruitment of transcription factors MyD88 and Mal [33]. Engagement of a receptor encoded by *TLR2* 753Gln results in decreased NF-κB activation and proinflammatory cytokine secretion after exposure to specific TLR2 ligands (Pam_3Cys, lipoteichoic acid and macrophage-activating lipopetide-2) and whole cell stimuli such as *Mycobacterium tuberculosis* or *S. aureus* [33, 34]. *TLR2* sequence variant rs5743708 is associated with susceptibility to tuberculosis [35, 36], Lyme disease [37] and cytomegalovirus infection [38, 39].

TLR2 sequence variant rs5743704 (Pro631His) leads to the substitution of proline for histidine at amino-acid position 631 of the TLR2 protein. Presence of the 631His variant is associated with decreased internalization of the TLR2 complex after recognition of its ligand [40], attenuated NF-κB activation and proinflammatory cytokine secretion after exposure to specific TLR2 ligands (Pam_3Cys, lipoteichoic acid, lipomannan and follistatin-like 1) and *Mycobacterium avium* [30, 40]. *TLR2* sequence variant rs5743704 is associated with susceptibility to tuberculosis [40], complicated skin and skin structure infections [29], and recurrent vulvovaginal infections with *C. albicans* (RVVC) [41].

Toll-like receptor 6

TLR6 sequence variant rs5743810 (Pro249Ser) leads to the substitution of proline for serine at amino-acid position 249 of the TLR6 protein. Presence of the 249Ser variant resulted in decreased NF-κB activation and cytokine secretion after exposure to TLR6/2 ligands and *Mycobacterium tuberculosis* [42]. The Pro249Ser variant is located in the extracellular domain of TLR6. Therefore, this variant may influence ligand recognition or affect assembly of a TLR6/2 heterodimer, but the exact mechanism remains unknown. *TLR6* sequence variant rs5743810 is associated with susceptibility to tuberculosis [25], complicated skin

and skin structure infections [29], aspergillosis [32] and malaria [28].

Toll-like receptor 10

TLR10 sequence variant rs4129009 (Ile775Leu) leads to the substitution of isoleucine to leucine at amino-acid position 775 of the TLR10 protein. *TLR10* sequence variant rs11096955 (Ile369Leu) also leads to the substitution of isoleucine to leucine at amino-acid position 369 of the TLR10 protein. Presence of the 775Leu or 369Leu variant is associated with increased cytokine secretion in the presence of TLR1/2 ligand Pam_3Cys, TLR6/2 ligand follistatin-like 1 and various pathogens [19, 20, 43]. How these *TLR10* sequence variants precisely exert their effect remains unknown, but the location of rs4129009 in the LRR region and rs11096955 in the TIR domain could indicate involvement in the recognition of PAMPs or signal transduction, respectively. Both these *TLR10* sequence variants have been associated with susceptibility to complicated skin and skin structure infections [44].

Toll-like receptor 4

TLR4 forms homodimers for the recognition of various ligands of bacterial, fungal and viral origin (Table 1). The *TLR4* gene is located on chromosome 9. *TLR4* sequence variant rs4986790 (Asp299Gly) leads to the substitution of aspartic acid for glycine at amino-acid position 299 of the TLR4 protein. *TLR4* sequence variant rs4986791 (Thr399Ile) leads to the substitution of threonine for isoleucine at amino-acid position 399 of the TLR4 protein. Both *TLR4* sequence variants lead to alterations in the leucine-rich repeat region of the protein, and as such, possible influence ligand recognition [45]. The presence of both *TLR4* variants differs between populations [46]. In populations from European descent, *TLR4* rs4986790 and rs4986791 are found to be present in cosegregation, whereas in African populations mainly presence of 299Gly and almost no 399Ile is observed. In Asian populations both 299Gly and 399Ile are practically absent [46]. Several studies have investigated the functional consequences of *TLR4* sequence variants rs4986790 and/or rs4986791. Presence of the TLR4 299Gly allele, but not the 399Ile allele, is associated with diminished recruitment of MyD88 and TRIF, decreased NF-κB activation and cytokine responses after exposure to LPS in transfection experiments with different cell lines [47-49]. On the other hand, most studies investigating the effect of TLR4 299Gly and/or 399Ile on LPS-stimulated whole-blood or isolated primary cells, showed no differences [50-55]. These contrasting results are possibly due to type of stimulation used or the fact that the cosegregated nature of these variants in different populations was not taken into account [56]. *TLR4* sequence variants rs4986790 and rs4986791 are associated with susceptibility to sepsis [57-59], urinary tract infections [60], brucellosis [61], Legionnaires' disease [62], meningococcal disease [63, 64], tuberculosis [65, 66], leprosy [67], aspergillosis [68-71], candidiasis [72], malaria [73, 74], respiratory syncytial virus infection [75].

Toll-like receptor 5

TLR5 forms homodimers for the recognition of bacterial flagellin (Table 1). The *TLR5* gene is located on chromosome 1. *TLR5* sequence variant rs5744168 (Arg392Ter) leads to the substitution of arginine to a stop codon at amino-acid position 392 of the TLR5 protein. Presence of the 392Ter variant results in premature truncation in the extracellular domain of the TLR5 protein with complete loss of the transmembrane and cytoplasmic region. Cells expressing the 392Ter variant were not able to induce NF-κB activation and reduced cytokine secretion was observed in response to TLR5 ligand flagellin [76]. *TLR5* sequence variant rs5744168 is associated with susceptibility to Legionnaires' disease [76] and urinary tract infections [60].

TLRs recognizing nucleic acids

TLR3, TLR7, TLR8 and TLR9 are all located in intracellular compartments of the cell where they recognize different microbial nucleic acids structures (Table 1). TLR3, TLR7 and TLR8, as homodimers, recognize single-stranded RNA of viral origin. In addition, TLR3 is also involved in the recognition of viral double-stranded RNA. TLR9 forms homodimers for the recognition of DNA motifs of bacterial, fungal or viral origin. The *TLR3* gene is located on chromosome 4, and the *TLR9* gene is located on chromosome 3. The *TLR7* and *TLR8* genes are located in close proximity of one other on chromosome X.

Toll-like receptor 3

TLR3 sequence variant rs3775291 (Leu412Phe) leads to the substitution of leucine for phenylalanine at amino-acid position 412 of the TLR3 protein. Presence of the 412Phe variant is associated with decreased NF-κB activation and proinflammatory cytokine secretion after exposure to TLR3 ligand Poly I:C [77-79]. Decreased intracellular expression of TLR3 [79], deficient cell surface expression and receptor shedding [77, 80] have all been suggested as possible mechanisms for the described functional consequences of the 412Phe variant. By contrast, 1 study reported increased cytokine responses after exposure to Poly I:C in the presence of the 412Phe variant [81]. *TLR3* sequence variant rs3775291 is associated with susceptibility to hepatitis C virus (HCV) infection [82], human immunodeficiency virus (HIV) infection [81], herpes simplex virus (HSV) infection [83] and tick-borne encephalitis virus infection [84].

Toll-like receptor 7

TLR7 sequence variant rs179008 (Gln11Leu) leads to the substitution of glutamine for leucine at amino-acid position 11 of the TLR7 protein. Presence of the 11Leu variant is associated with decreased IFNα secretion after exposure to TLR7 ligand Imiquimod, whereas IL-6 remained unaltered [85]. Although the exact mechanism remains unknown, it is speculated that due to its location in the signal sequence region, sequence variant

rs179008 could influence post-translational modification, localization, quantity and therefore functionality of TLR7 [85]. *TLR7* sequence variant rs179008 is associated with susceptibility to HCV infection [86] and HIV infection [85].

Toll-like receptor 8

The *TLR8* gene encodes 2 different splice variants, TLR8v1 and TLR8v2, which share a similar sequence, with the exception of the longer N-terminal section of TLR8v1 [87]. *TLR8* sequence variant rs3764880 (Val1Met) influences TLR8v2, but not TLR8v1, and leads to the substitution of valine for methionine at amino-acid position 1, resulting in alteration of the start codon and truncation of the TLR8 protein. Different functional consequences have been described for *TLR8* sequence variant rs3764880. Presence of the variant 1Val is associated with decreased NF-κB activation, but increased cytokine secretion, after exposure to TLR8 ligands CL075 and R848 [88, 89]. By contrast, another study reported no differences in TLR8 function and cellular distribution for *TLR8* sequence variant rs3764880 [87]. This study suggested that the *TLR8* sequence variant rs3764880 influences fine-tuning of translation of both TLR8 isoforms, indicated by decreased TLR8v2 and increased TLR8v1 [87]. *TLR8* sequence variant rs3764880 is associated with susceptibility to tuberculosis [90, 91], HCV infection [89, 92], and Crimean-Congo hemorrhagic fever [93].

Toll-like receptor 9

TLR9 sequence variant rs5743836 (-1237C/T) is located in the promoter region of the *TLR9* gene. Presence of the -1237C variant is associated with increased transcription of the *TLR9* gene after exposure to proinflammatory cytokines or TLR ligands [94, 95]. The incorporation of cytosine is suggested to create a novel NF-κB transcriptional binding site [94] or IL-6 binding site [95] in the promoter region of the *TLR9* gene. *TLR9* sequence variant rs5743836 is associated with susceptibility to tuberculosis [96], aspergillosis [69] and malaria [97, 98].

Primary immunodeficiencies in the TLR pathway

Primary immunodeficiencies in the TLR signaling pathway are rare, but novel genetic variants are continuously discovered. Some of the most important examples are the deficiencies in MyD88 and IRAK4 of the TLR signaling pathway [99, 100]. Different sequence variants have been described in both genes including deletions, insertions and substitutions resulting in loss-of-expression or loss-of-function phenotypes [99]. Deficiencies in the MyD88/IRAK4 pathway result in defective downstream signaling of most TLRs and failure to induce proinflammatory cytokines in response to known TLR ligands and whole organisms [101, 102]. Patients with MyD88 or IRAK4 deficiency are predisposed to life-threatening infections with invasive pyogenic bacteria, such as *Streptococcus pneumoniae*, *S. aureus* and *Pseudomonas aeruginosa* [99]. Despite antibiotic

treatment, these infections lead to a mortality up to 30 - 40% during infancy and childhood, but decrease in frequency and severity of infections after adolescence [103].

Another cluster of primary immunodeficiencies discovered over the last decade are the defects in the TLR3/TRIF/TRAF3/UNC93B pathway [104-107]. The TLR3 pathway is important for the recognition of single- and double stranded RNA of viral origin (Table 1), and patients with primary immunodeficiencies in this pathway have loss-of-expression or loss-of-function phenotypes, due to sequence variations such as deletions and substitutions. TLR3 pathway deficiencies are characterized by a failure to induce interferons after stimulation with HSV-1. Patients with TLR3 pathway deficiencies are predisposed to development of recurrent herpes simplex encephalitis, a rare complication of the common viral infection HSV-1.

C-type lectin receptors

A prominent role in host immunity in general and antifungal immunity in particular has been attributed to CLRs, as they are able to recognize fungal cell wall components as β-glucans or mannans [108]. Most, but not all, CLRs are Ca^{2+} dependent and recognize carbohydrate-containing PAMPs through a C-type-lectin-like-domain. Recognition leads to subsequent signaling via Src and spleen tyrosine kinase (Syk), which in turn signal downstream adaptor molecules to induce a NF-κB dependent immune response through secretion of cytokines such as (pro-)IL-1β, IL-6, IL-10 and TNFα [109-111]. Moreover, signaling leads to reactive oxygen species (ROS) production and subsequent activation of inflammasomes, which in turn process the inactive pro-IL-1β and pro-IL-18 into their biologically active forms [112]. Although most CLRs are membrane-bound, there are also soluble CLRs such as the complement activating mannose-binding-lectin (MBL) and pentraxin-3. Table 3 summarizes the sequence variants in CLRs and their association in susceptibility to infectious diseases.

Dectin-1

The β-glucan receptor Dectin-1 (C-type lectin domain family 7 member A, CLEC7A) is a prominent member of the CLR family. By recognizing its ligand, different adaptor proteins such as Caspase recruitment domain-containing protein 9 (CARD9), Syk, as well as Bcl-10 and Malt-1 are recruited which leads to the activation of NF-κB and subsequent transcription of proinflammatory cytokines. In addition, Dectin-1 is known to synergize with TLRs to amplify cytokine production after pathogen recognition in both humans and mice [108, 113-115].

Table 3. Overview of genetic variation in C-type lectin receptors and susceptibility to infectious diseases.

Gene	Sequence variant			Disease	Risk allele	Reference
	Rs-number	Nucleotide change[#]	Amino acid change[#]			
CLEC7A	rs16910526	T > G	Tyr238Ter	*Candida* colonization Recurrent vulvovaginal candidiasis Onychomycosis	G G G	[119] [118] [118]
CLEC7A	rs16910527	A > C	Ile223Ser	Oropharyngeal candidiasis Aspergillosis	A C	[124] [121, 122]
CLEC7A	rs7309123	G > C	N/A	Aspergillosis	G	[123]
CLEC7A	rs3901533	G > T	N/A	Aspergillosis	T	[123]
CD209	rs4804803	G > A	N/A	Tuberculosis HIV infection Dengue fever	G G G	[127] [128] [129]
CD209	rs735239	A > G	N/A	Tuberculosis	A	[127]
CD209	rs4804800	A > G	Unknown	Aspergillosis	G	[123]
CD209	rs11465384	C > T	Unknown	Aspergillosis	T	[123]
CD209	rs7248637	G > A	Unknown	Aspergillosis	A	[123]
CD209	rs7252229	G > C	Unknown	Aspergillosis	C	[123]
CD206	rs1926736	C > T	Gly396Ser	Tuberculosis Leprosy	C T	[133] [132, 134]
CD206	rs692527	T > C	Unknown	Leprosy	C	[134]
CD206	rs34856358	C > T	Unknown	Leprosy	T	[134]
PTX3	h2/h2 (G-A/G-A)			Aspergillosis	h2/h2 haplotype	[140]
PTX3	rs2305619	A > G		Tuberculosis	Haplotype	[142]
PTX3	rs1840680	C > T		Tuberculosis *Pseudomonas aeruginosa* colonization	Haplotype Haplotype	[142] [143]
MBL	rs1800450, rs5030737, rs1800451		Arg52Cys, Gly54Asp, Gly57Glu	Recurrent vulvovaginal candidiasis	Haplotype	[149-152]
CARD9	rs121918338	C > T	Gln295Ter	Chronic mucocutaneous candidiasis	T	[116]
CARD9	rs398122363	C > T	Gln289Ter	Dermatophytic disease	T	[157]
CARD9	rs398122364	C > T	Arg101Cys	Dermatophytic disease	T	[157]
CARD9	Unknown	T > C	Tyr91His	*C. albicans* meningoencephalitis	C	[158]

[#] The first nucleotide (and corresponding amino acid) is the ancestral nucleotide and therefore is considered the wild-type allele. N/A, not applicable; HIV, human immunodeficiency virus.

Dectin-1's importance in β-glucan recognition was shown in different mice knock-out studies as well as in patients with fungal infections who carried a specific mutation in the *CLEC7A* gene [116, 117]. The sequence variant responsible for the altered phenotypes is rs16910526 (Tyr238Ter) in the *CLEC7A* gene, located in exon 6 of chromosome 12. The sequence variant leads to a change from tyrosine into a stop codon resulting in the loss of amino acids within the carbohydrate-recognition domain [118]. Subsequently, this sequence variant results in decreased surface expression of the receptor accompanied by low levels of IL-17 and IL-23 after stimulation with either β-glucan or *C. albicans* [118]. Contradictory results regarding susceptibility to different infectious diseases have been observed for *CLEC7A* sequence variant Y238X. However, independent studies show an increase in mucosal and gastrointestinal fungal colonization as a consequence of the sequence variant [118-120]. In addition, several studies show a correlation between this sequence variant and invasive as well as pulmonary aspergillosis [121-123]. Finally, Sainz *et al.* observe a correlation with susceptibility to aspergillosis in 2 other intronic *CLEC7A* sequence variants (rs7309123 and rs3901533). Presence of the GG genotype for sequence variant rs7309123 leads to decreased Dectin-1 mRNA expression, whereas the functional consequences of rs3901533 remains unknown [123]. Plantinga *et al.* observed an association of rs16910527 (Ile223Ser) and oropharyngeal candidiasis in HIV patients which is accompanied with lower IFNγ levels as well as a reduced capacity to bind zymosan [124].

DC-SIGN

DC-SIGN (CD209) is mainly present in dendritic cells and macrophages and recognizes mannose glycoproteins. In addition, it has an important function in the process of adhesion and was shown to modulate TLR function [125]. Several studies link genetic variation in DC-SIGN to various infectious diseases. *DC-SIGN* sequence variant rs4804803 (336A/G), located in the promoter region is linked with susceptibility to tuberculosis, HIV and dengue fever [126-129]. By contrast, both -336A as well as -871G (rs735239) have been shown to have a protective effect in the development of tuberculosis [130]. These sequence variants most likely affect transcription levels leading to lower expression of CD209 [127, 129]. However, 1 meta-analysis of the -336A/G polymorphism of *DC-SIGN* did not show any association with susceptibility to tuberculosis [131]. Sainz *et al.* observed 4 sequence variants in *DC-SIGN* (rs4804800, rs11465384, rs7248637, rs7252229) that are associated with increased risk to develop invasive pulmonary aspergillosis [123].

Mannose receptor

The mannose receptor (CD206) recognizes different high mannose structures present on the surface of various pathogens. After recognition and subsequent internalization, the pathogen is degraded in the lysosome and an immune response is initiated. Very little

is known about sequence variants in *CD206* and susceptibility to infectious disease in humans. Nevertheless, sequence variant rs1926736 (Gly396Ser) in exon 7 of the *MRC1* gene on chromosome 10 is suggested to be associated in susceptibility to leprosy and tuberculosis [132-134].

Soluble C-type lectin receptors

Pentraxin-3

The soluble and circular pentraxin-3 (PTX3) belongs to the pentraxin superfamily that also includes C-reactive protein as well as serum amyloid P component. It is released by several immune and nonimmune cells, such as dendritic cells and endothelial cells. By forming complexes with different pathogenic antigens and components of the complement system it enhances cellular processes as antigen recognition and phagocytosis. Interaction of PTX3 with *Aspergillus fumigatus* and *Candida albicans* has been reported [135-139]. In a cohort of patients with hematopoietic stem-cell transplantation Cunha *et al.* showed that the h2/h2 haplotype (G-A/G-A) in *PTX3* is associated with invasive aspergillosis [140], whereas another study did not find an association for *PTX3* with susceptibility to aspergillosis [141]. The increased risk for aspergillosis is most likely due to a reduced expression of PTX3 in neutrophils, which might therefore lead to reduced phagocytosis of the fungus [140]. In a study of African tuberculosis cases and controls, Olesen *et al.* found an association between sequence variants rs2305619 and rs1840680 in *PTX3* and pulmonary tuberculosis [142]. In addition, a haplotype containing these 2 sequence variants has been shown to be associated with *P. aeruginosa* colonization in patients suffering from cystic fibrosis [143].

Mannose-binding-lectin

Mannose-binding-lectin (MBL) is able to recognize carbohydrate patterns present on the surface of different microorganisms. Upon binding to its ligand, MBL activates the lectin pathway of the complement system, initiating a cascade of complement activation [144, 145]. Three polymorphisms located in exon 1 of the *MBL2* gene rs5030737 (Arg52Cys), rs1800450 (Gly54Asp) and rs1800451 (Gly57Glu) have been shown to decrease circulating levels of MBL2 [146, 147]. All 3 polymorphisms are linked to recurrent vulvovaginal infections with *C. albicans* (RVVC), although there is still some debate for the role of MBL2 as a marker for this disease since genotype distribution strongly depends on ethnic background [148-153]. Decreased levels of MBL and complete MBL deficiency are associated with invasive and pulmonary aspergillosis [154, 155]. Moreover, Hibberd *et al.* showed that homo and/or heterozygous combinations of *MBL2* genotypes are more frequently present in patients suffering from meningococcal disease [156].

CARD9

CARD9 functions as adapter protein downstream of most CLRs, for example, Dectin-1, Dectin-2 and Mincle. Upon engagement of these CLRs with their ligands typically present on the cell walls of various fungi, signaling via CARD9 leads to the production of proinflammatory cytokines. Several sequence variants in the *CARD9* gene are associated with fungal infections in humans [116, 157]. Sequence variants rs121918338 (Gln295Ter) and rs398122363 (Gln289Ter) lead to the incorporation of a stop codon, whereas rs398122364 (Arg101Cys) leads to the substitution of arginine for cysteine. Homozygosity for these variants results in absence (289Ter, 295Ter) or decreased levels of CARD9 protein (101Cys), and it is associated with reduced numbers of Th17 cells and altered cytokine profiles, essential for the antifungal defense [116, 157]. Gavino *et al.* recently reported a novel mutation in the CARD domain of *CARD9* (Tyr91His) in a patient with relapsing *C. albicans* meningoencephalitis. Interestingly, presence of 91His is associated with decreased levels of CARD9 protein and impaired GM-CSF expression but not reduced numbers of Th17 cells [158].

NOD-like receptors

NOD-like receptors are intracellular receptors that recognize mainly bacterial PAMPs, and they are able to induce proinflammatory cytokines and activation of host defense. Interestingly, it has also been shown that NLRs also participate in the antiviral immune response [159]. Moreover, a central role in the induction of autophagy was ascribed to members of this receptor family. Although NLRs form a heterozygous group of receptors, in general they contain a C-terminal leucine-rich repeat (LRR) domain, a NACHT (and NACHT associated domain, NAD) and an effector domain. Based on the N-terminal effector domain, which can be either PYD, CARD or BIR2, several subfamilies can be distinguished [160]. However, regarding phylogenetic aspects of NLRs, 3 subfamilies (NALPs, IPAF/NAIP and NODs) have been proposed [161, 162].

Besides their role in the direct signaling and stimulation of cytokine production, some family members form molecular complexes known as inflammasomes that are able to recruit inflammatory caspases. The classical example is the recruitment of caspase-1 into the NLRP3 inflammasome that processes the inactive pro-IL-1β and pro-IL-18 into their bioactive forms. Two well-known cytosolic NLRs are NOD1 (CARD4) and NOD2 (CARD15). Both recognize different parts of the bacterial cell wall component peptidoglycan [163, 164]. Table 4 summarizes the sequence variants in NLRs and their association in susceptibility to infectious diseases.

Table 4. Overview of genetic variation in NOD-like receptors and susceptibility to infectious diseases.

Gene	Sequence variant			Disease	Risk allele	Reference
	Rs-number	Nucleotide change[#]	Amino acid change[#]			
CARD15	rs2066845	G > C	Gly908Arg	Crohn's disease	C	[165, 166]
CARD15	rs2066844	C > T	Arg702Trp	Sepsis Tuberculosis	T C	[167] [168]
CARD15	rs2066847	insC	Leu1007insC	Crohn's disease	1007C	[165, 166]
CARD15	rs9302752	A > G	Unknown	Leprosy	G	[171, 172]
CARD15	rs7194886	G > A	Unknown	Leprosy	A	[171]
CARD15	rs8057341	A > G	Unknown	Leprosy	G	[171]
CARD15	rs3135499	A > C	Unknown	Leprosy	C	[171]
CARD15	rs5743289	C > T	Unknown	Leprosy	T	[173]
CARD15	rs2066842	C > T	Pro268Ser	Tuberculosis	C	[168]
CARD15	rs5743278	C > G	Ala725Gly	Tuberculosis	G	[168]
CARD4	rs2075820 (E266K)	G > A	Glu796Lys	*H. pylori* gastric inflammation	A	[180]
CIAS1	rs74163773	12,9,7,6	Tandem repeat	*Mycoplasma* infection-associated infertility Recurrent vulvovaginal candidiasis	Allele 7 Allele 7	[184] [183]
CIAS1	rs4925663	C > T	Gly223Asp	*Chlamydia trachomatis* infection	T	[185]

[#] The first nucleotide (and corresponding amino acid) is the ancestral nucleotide and therefore is considered the wild-type allele. N/A, not applicable.

NOD2

Frameshift variant rs2066847 (Leu1007fsinsC) in *CARD15* encoding the leucine-rich repeat of NOD2, as well as 2 sequence variants in *CARD15* rs2066844 (Arg702Trp) and rs2066845 (Gly908Arg) are associated with susceptibility to Crohn's disease and sepsis [163, 165-168]. These sequence variants most likely affect antigen binding at the site of the LRR and, therefore, downstream signaling via NF-κB. This, in turn, may lead to a diminished production of proinflammatory cytokines. Genome-wide association studies suggest that genetic variants in *CARD15* (also *CARD4*) may affect autophagy function and thereby contribute to the development and severity of various diseases, such as Crohn's disease [169, 170].

In addition, sequence variants in *CARD15* (NOD2) are linked to susceptibility to leprosy and tuberculosis. Using a genome-wide association study approach, a couple of sequence variants in *CARD15* (rs9302752, rs7194886, rs8057341, rs3135499) and downstream genes were identified to be associated with the susceptibility to leprosy [171, 172]. In addition, Berrington *et al.* identified an intronic sequence variant rs5743289 that is also associated

with susceptibility to leprosy [173]. Finally, rs5743278 (Ala725Gly) is shown to increase the risk for tuberculosis, whereas rs2066842 (Pro268Ser) as well as rs2066844 (Arg702Trp) are shown to have a protective effect [168]. The protective effect is most likely due to a more stable NOD2 protein with increased functionality [168].

NOD1

Two different sequence variants rs2075820 (G796A) and +32656 (rs number unknown) have linked CARD4 (NOD1) to various inflammatory diseases such as inflammatory bowel disease, Crohn's disease and asthma [174-179]. In addition, Kim *et al.* found an association with sequence variant rs2075820 (Gly796Ala) and *H. pylori* associated gastric mucosal inflammation [180].

NACHT, LRR and PYD domains-containing protein 3

NLRP3 is encoded by the *CIAS1* gene and was extensively studied due to its capacity to form active inflammasomes. Several danger signals can activate the NLRP3 inflammasome, leading to activation of caspase-1, which in turn processes the inactive forms of pro-IL-1β and pro-IL-18 into their biologically active forms [160, 181]. Numerous genetic variations have been described within the *CIAS1* locus that lead to various different diseases, commonly summarized as cryopyrin-associated periodic syndrome. Depending on the genetic variant, most (but not all) share an increased inflammasome activity that leads to excessive IL-1β production and tissue damage [181, 182].

A few genetic variants in *NLRP3* have been described that are associated with bacterial or fungal infections. Lev-Sagie *et al.* showed that presence of allele 7 of tandem repeat rs74163773 in intron 4 of the *CIAS1* gene is increased in patients suffering from recurrent vulvovaginal infections with *C. albicans*. Furthermore, the risk genotype is associated with reduced levels of IL-1β, pointing toward a reduced inflammasome activity [183]. In addition, the same variant was described by Witkin *et al.* who showed a significant correlation with mycoplasma infection-associated infertility [184]. Wang *et al.* proposed a negative correlation between rs4925663 in *NLRP3* and the course of *Chlamydia trachomatis* infections [185].

RIG-I-like receptors

RLRs are intracellular DEAD-box containing RNA helicases that are important for antiviral defense [186, 187]. RIG-I and melanoma differentiation-associated protein 5 (MDA5) recognize double stranded RNA (dsRNA) of viral origin. RIG-I is known to recognize relatively short dsRNA strands, whereas MDA5 recognizes longer dsRNA strands [188]. Recognition and downstream signaling through mitochondrial antiviral-signaling protein

followed by interferon regulatory factors leads to the production of type 1 interferons. Laboratory of genetics and physiology 2 (LGP2), the third member of the RLR family, is thought to have a regulatory function on RIG-I and MDA5, as it misses the N-terminal CARD domain essential for downstream signaling [189, 190]. Table 5 summarizes the sequence variants in RLRs and their association in susceptibility to infectious diseases.

RIG-I

RIG-I is encoded by *DDX58* (DEAD (Asp-Glu-Ala-Asp) box polypeptide 58) which is located on chromosome 9. A sequence variant in the CARD domain of RIG-I rs10813831 (Arg7Cys) has been shown to increase transcription of IFNB1 and DDX58 [191]. In addition, Ovsyannikova *et al.* showed that the same variant and RIG-1 sequence variant rs669260 is associated with differences in rubella antibody levels [192]. A frameshift mutation in *DDX58* rs36055726 (p229fs) was reported to lead to a truncated and constitutively active protein, resulting in excessive cytokine production. In addition, sequence variant rs11795404 (Ser183Ile) leads to impaired antiviral signaling due to disrupted interactions of RIG-I with downstream molecules. Both sequence variants most likely lead to an altered immune response against dsRNA viruses [193].

MDA5

MDA is encoded by the *IFIH1* gene located on chromosome 2. MDA5 polymorphisms have been mainly associated with autoimmune diseases such as Type-1 diabetes mellitus and SLE, or with IgA deficiency [194-197]. In this context, the sequence variant rs1990760 (Ala946Thr) is proposed to have a major contribution to disease development and progression, due to increased (baseline) expression levels of MDA5 [198]. Recently, 2 MDA5 variants rs1990760 (Ala946Thr) and rs3747517 (His843Arg) have been associated with susceptibility to candidemia [199]. However, it remains to be investigated which

Table 5. Overview of genetic variation in RIG-1-like receptors and susceptibility to infectious diseases.

Gene	Sequence variant			Disease	Risk allele	Reference
	Rs-number	Nucleotide change[#]	Amino acid change[#]			
DDX58	rs10813831	G > A	Arg7Cys	IgG antibody levels	A	[191]
DDX58	rs669260	A > G	Unknown	IgG antibody levels	G	[192]
DDX58	rs36055726	Frameshift	p229fs	Immune response against viruses		[193]
DDX58	rs11795404	G > T	Ser183Ile	Immune response against viruses	T	[193]
IFIH1	rs1990760	C > T	Ala946Thr	Candidemia	T	[199]
IFIH1	rs3747517	T > C	His843Arg	Candidemia	C	[199]

[#]The first nucleotide (and corresponding amino acid) is the ancestral nucleotide and therefore is considered the wild-type allele. N/A, not applicable.

PAMP of *C. albicans* is recognized by MDA5.

AIM2-like receptors

The AIM2 protein is encoded by the *AIM2* gene located on chromosome 1. It recognizes cytosolic DNA derived from the host, bacterial and viral origin [200, 201]. Antigen recognition by a specific C-terminal domain of AIM2 is followed by formation of an inflammasome platform. The pyrin domain of AIM2 interacts with ASC, which contains a CARD domain that subsequently recruits caspase-1 to the complex responsible for processing of pro-IL-1β and pro-IL-18 [11, 202]. Although no specific polymorphisms have been found that link AIM2 to infectious diseases so far, it is likely that more research on this recently discovered receptor will identify genetic variants that contribute to infectious disease susceptibility.

Conclusion

Since the discovery of PRRs, sequence variants in the genes encoding them have been recorded, and evidence regarding their associations with susceptibility to infectious disease has mounted. In this review, we have discussed major examples of genetic variation in PRRs, their association with susceptibility to infectious diseases, with a specific focus on their functional consequences. As such, this review provides strong arguments that genetic variation in PRRs has a profound impact on innate immune responses to infectious stimuli and affects infectious disease susceptibility.

However, this review is subject to several limitations. Although very important for the understanding of the consequences of sequence variants, negative associations and numerous attempts to validate sequence variants received less prominence. In addition, generalization of the results of individual studies is difficult and reproducibility often fails in independent validation studies. At least part of the differences between studies may be due to variations in ethnic background of the populations, often the small sample sizes of patient cohorts, the type of analyses performed, as well as possible confounding factors such as age, gender and co-morbidities which were not always taken into account.

Nonetheless, the study of genetic variation in PRRs has been of crucial importance for several reasons. First, genetic variation in PRRs has increased our understanding of the role that these receptors play in infectious diseases. Second, this information may represent a first step in an approach toward personalized diagnosis and treatment. Finally, this type of research provides a human model to study PRR pathways to complement experimental animal models of infection.

Future perspective

Within the last decades extensive research in the field of immunogenetics has led to a better understanding of the functional immune pathways and interactions underlying infectious diseases. Studying the genetic background of patients with infections has allowed us to pinpoint components of the immune system that contribute to disease susceptibility, as well as the influence on disease progression and outcome. Advances in technology, such as next generation sequencing, will enable the extensive study of large-scale genomic information rather than single sequence variants. Moreover, structural variation as well as gene-gene interactions and gene expression changes will be more easily detected and may be linked to different diseases. Finally, technical improvement will allow the study of the epigenetic changes, and add an additional layer of complexity to the field of immunogenetics. Understanding the multiple pathways and factors that underlie susceptibility to infectious disease may lead to personalized diagnosis and treatment in the future. Such personalized treatment might involve patient-adjusted prophylaxis in those deemed at high risk of developing a severe infection due to a combination of genetic risk factors, or inhibition of an overactive immune system due to genetic variants in order to restore a normal cytokine balance.

Executive summary

- Pattern-recognition receptors (PRRs) recognize specific pathogen-associated molecular patterns (PAMPs) and are involved in the initiation of the immune response against microorganisms.
- Five different classes of PRRs have been described: Toll-like receptors, C-type lectin receptors, NOD-like receptors, RIG-1-like receptors and AIM2-like receptors.
- Sequence variants are changes in the nucleotide sequence of DNA.
- Depending on the type of sequence variant and its location in the gene, it can have different functional consequences.
- Genetic variation in PRRs influences susceptibility to infectious disease.
- Genetic variation in PRRs has increased our understanding in the pathological mechanism of infectious diseases and may lead toward personalized diagnosis and treatment in the future.

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Chapter 3

A role for TLR1, TLR2 and NOD2 in cytokine induction by *Bacteroides fragilis*

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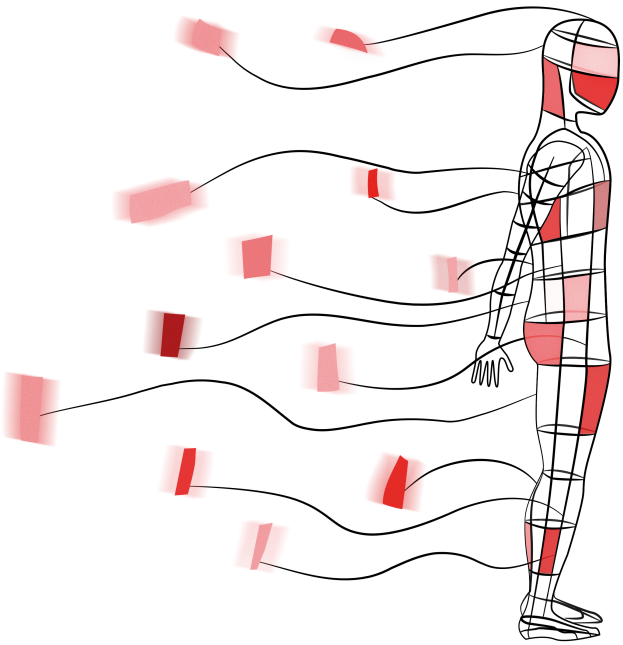

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Abstract

Bacteroides fragilis, an intestinal flora commensal microorganism, is frequently isolated from abscesses and soft tissue infections. This study aimed to identify pattern-recognition receptors (PRRs) involved in *B. fragilis* recognition and to characterize the induced cytokine profile. Human PBMCs were stimulated with heat-killed *B. fragilis* and cytokine levels were determined by ELISA. Roles of individual PRRs were assessed using specific blockers of receptor signaling pathways and PBMCs carrying single-nucleotide polymorphisms of PRR genes. Cell lines expressing human TLR2 or TLR4 were employed to assess TLR-specificity of *B. fragilis*. TLR1, TLR2 and NOD2 were the main PRRs responsible for recognition of *B. fragilis*, while TLR4, TLR6, NOD1 and Dectin-1 were not involved. *B. fragilis* induced strong IL-6 and IL-8, moderate IL-1β and TNFα, and poor IL-10, IL-17, IL-23 and IFNγ production. This study identifies the receptor pathways of the innate immune response to *B. fragilis*, and thus provides new insights in the host defense against *B. fragilis*.

Introduction

The gram-negative, obligate anaerobic, rod-shaped bacterium *Bacteroides fragilis* normally resides within the colon as part of the commensal flora and is present in the majority of humans [1]. Species of the genus *Bacteroides* account for 20 - 30% of the species in human fecal flora and are an important component of the human microbiome [2]. As such, *B. fragilis* plays important biological roles in the induction but also modulation of the gut immune response. An immunomodulatory feature of *B. fragilis* has been shown to be mediated by one of its capsular components, polysaccharide A (PSA) [3, 4]. However, when *B. fragilis* spreads to normally sterile body sites, it can lead to serious infections with high associated mortality unless treated [5, 6]. This potential is underlined by the fact that *B. fragilis* is the most common anaerobic organism isolated from clinical infections [6, 7], e.g. intra-abdominal abscesses, bacteraemia and complicated skin and skin structure infections [8]. Furthermore, *B. fragilis* shows increasing resistance against several antibiotics [9, 10]. Abscesses caused by *B. fragilis* are often polymicrobial and it has been shown that live *B. fragilis* and *Escherichia coli* act synergistically in promoting larger abscesses and bacterial persistence in a rat model [11].

On the side of the host encountering *B. fragilis*, the innate immune system is an early line of defense against invading microorganisms. Cells of the innate immune system recognize pathogen-associated molecular patterns (PAMPs) on the surface of these microorganisms through pattern-recognition receptors (PRRs). PRR families include the Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and C-type lectin receptors (CLRs), which recognize specific microbial ligands and lead to specific innate immune responses such as cytokine production [12]. A fourth family of PRRs, the RIG-I-helicases, are believed to be mainly involved in antiviral host defense [13].

Apart from the intriguing immunological characteristics of *B. fragilis* mentioned above, not much is known about its interactions with the innate immune system at a molecular level. Despite studies showing that heat-killed *B. fragilis* is able to induce tumour necrosis factor (TNF)α, interleukin-6 (IL-6), IL-10 and transforming growth factor (TGF)β production from mononuclear cells [14, 15], only fragmentary information is available regarding the pattern recognition of *B. fragilis*. Lipopolysaccharide (LPS) and polysaccharide A (PSA) isolated from *B. fragilis* are recognized by TLR2 [15-18], and a biased proinflammatory cytokine response induced by *B. fragilis* is observed in cells from Crohn's disease patients with NOD2 mutations [15].

The aim of this study was to systematically explore the PRRs recognizing *B. fragilis*, characterize the cytokine profile induced by *B. fragilis*, and assess the impact of known

loss of function PRR single-nucleotide polymorphisms (SNPs) on cytokine production induced by *B. fragilis*.

Materials and methods

Bacterial strains and culture methods

Six different strains of *B. fragilis* were used: ATCC 25285, ATCC 23745, NCTC 10584 and 3 clinically isolated strains JY 1032005, JY 3042143 and JY 2033990, selected randomly from 95 *B. fragilis* isolates obtained in a recent clinical trial on complicated skin and skin structure infections [8]. All strains were confirmed to be *B. fragilis* by 16S rRNA polymerase chain reaction. Briefly, DNA was isolated using an automated DNA extraction platform (MagNA Pure, Roche Diagnostics, Almere, The Netherlands). Isolated DNA was amplified using primers targeting part(s) of the ribosomal gene cassette (forward: 5'-CCTAACACATGCAAGTCGARCG-3', reverse: 5'-CGTATTACCGCGGCTGCT-3', Eurogentec, Seraing, Belgium). Amplified products were purified using SPRI chemistry (Beckman Coulter, Mijdrecht, The Netherlands) and subjected to DNA sequence analysis using the DYEnamic ET dye terminator kit (GE Healthcare, Diegem, Belgium) and a MegaBACE 500 automated DNA analysis platform (GE Healthcare). Identification of the isolates was performed by BLAST analysis of the obtained DNA sequences.

B. fragilis, grown anaerobically overnight at 37°C on *Brucella* blood agar plates (BD Biosciences, Franklin Lakes, NJ, USA), was inoculated in 20 mL pre-warmed and pre-reduced Brain Heart Infusion broth (BD Diagnostics, Basel, Switzerland) and again grown anaerobically overnight at 37°C until reaching stationary growth phase mimicking growth conditions in abscesses. Bacterial suspensions were washed 3 times in phosphate-buffered saline (PBS; B. Braun Medical B.V., Melsungen, Germany) and heat-killed at 95°C for 30 minutes. Before heat-killing, aliquots of bacterial suspensions were taken to determine colony-forming unit (cfu) counts. Heat-killed bacteria were washed again and after adjusting the concentration in PBS to 1 x 10^8 cfu/mL, stored at -80°C. When comparing *B. fragilis* with other microorganisms, an inoculum of 1 x 10^6 cfu/mL was used. All other experiments were performed with an inoculum of 1 x 10^7 cfu/mL.

Isolation of peripheral blood mononuclear cells and cytokine induction

Peripheral blood mononuclear cells (PBMCs) were isolated from either freshly obtained venous blood in EDTA tubes (Monoject, s-Hertogenbosch, The Netherlands) or from buffy coats of healthy individuals (after informed consent), as described earlier [19]. Briefly, PBMCs were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare) and collecting the white interphase. Next, PBMCs were washed twice in cold PBS and concentrations were adjusted to 5 x 10^6 cells/mL for experiments in RPMI-1640

Dutch Modified culture medium (RPMI supplemented with 2 mM L-glutamine, 1 mM pyruvate and 50 µg/mL gentamicin; GIBCO Invitrogen, Carlsbad, CA, USA). PBMCs (5 x 10^5 cells) and stimuli were added to wells of a 96-well round-bottom plate (Greiner, Nurnberg, Germany) in a final volume of 200 µL. For induction of IL-1β, TNFα, IL-6, IL-8, IL-10 and IL-23, PBMCs were incubated for 24 hours at 37°C and 5% CO_2, after which the plates were centrifuged and supernatants were collected. For induction of IL-17 and interferon (IFN) γ, PBMCs and stimuli were supplemented with 10% pooled human serum and incubated for 7 days at 37°C and 5% CO_2 before supernatant collection. All supernatants were stored at -20°C. In addition to stimulations with *B. fragilis* strains, the following specific PRR ligands were used, in various concentrations depending on the experiment: Pam_3Cys (EMC Microcollections Tübingen, Germany) for TLR2/1, *E. coli* LPS (Sigma-Aldrich, St Louis, MO, USA) for TLR4, FSL-1 (EMC Microcollections) for TLR2/6 and MDP (Sigma-Aldrich) for NOD2.

Blocking pattern-recognition receptors and receptor signaling

PRR-mediated recognition was blocked using *Bartonella quintana* LPS (obtained as described previously [20]) for TLR4, anti-TLR2 blocking antibody (eBioscience, Halle-Zoersel, Belgium) for TLR2, anti-TLR1 blocking antibody (eBioscience) for TLR1, anti-TLR6 blocking antibody (Biolegend, San Diego, CA, USA) for TLR6, glucan-phosphate (kindly provided by Dr. David Williams, University of Tennessee, Knoxville, TN, USA) for blocking Dectin-1 and the RICK-inhibitor SB202190 (Sigma-Aldrich, St Louis, MO, USA) as an inhibitor of NOD1/2-induced stimulation. In these experiments, PBMCs (5 x 10^5 cells) were pre-incubated for 1 hour (30 min for RICK inhibitor) at 37°C and 5% CO_2 with the various blocking reagents in 96-well round-bottom plates. Thereafter, stimuli were added (among which *B. fragilis*) and plates were incubated for 24 hours before collection of supernatants.

Measurement of cytokine concentrations

Cytokine concentrations were determined by commercially available enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer's instructions. Concentrations of IL-1β, TNFα, IL-17 (R&D Systems, Inc., Minneapolis, MN, USA), IL-6, IL-8, IL-10, IFNγ (Sanquin Reagents, Amsterdam, The Netherlands) and IL-23 (eBioscience) were measured.

Transfected Chinese hamster ovary cell line stimulation experiments

Chinese hamster ovary (CHO) cell lines stably transfected with human CD14 alone (3E10), human CD14 and TLR2 (3E10/TLR2) and human CD14 and TLR4 (3E10/TLR4) were kindly provided by Robin R. Ingalls (Boston University, Boston, MA, USA). These cell lines have been transfected with a reporter gene (Tac/CD25) under transcriptional control of a segment of the human ELAM promoter, which contains a binding site for nuclear factor

κB (NF-κB). Upon TLR recognition of the microorganism, the subsequent TLR signaling cascade results in NF-κB translocation to the nucleus of the CHO cell. In the nucleus, NF-κB activates transcription of the reporter gene Tac/CD25 and drives the expression of membrane-bound CD25. Upregulation of membrane bound CD25 is detected by flow cytometry using anti-CD25 antibodies and represents recognition of the microorganism by the transfected TLR [21, 22]. Cells were grown at 37°C and 5% CO_2 in Ham's F-12 medium (GIBCO Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (GIBCO Invitrogen), 50 µg/mL gentamicin, 400 U/mL hygromycin B, and either 500 µg/mL G418 (geneticin) (for 3E10/TLR2) or 50 µg/mL puromycin (for 3E10/TLR4) as an additional selection antibiotic, as described previously [15]. For stimulation experiments, 5 x 10^4 cells in 500 µL culture medium were plated in 24-well tissue culture plates (Greiner) and incubated overnight. Next, cells were incubated with control culture medium, 10 µg/mL Pam_3Cys (EMC Microcollections Tübingen, Germany), 1 µg/mL purified *E. coli* LPS (Sigma-Aldrich) or 1 x 10^7 cfu/mL of heat-killed *B. fragilis* for 20 hours at 37°C and 5% CO_2. Subsequently, cells were harvested using trypsin-EDTA (GIBCO Invitrogen), prepared for flow cytometry and stained for CD25 expression with FITC-labeled mouse anti-human CD25 monoclonal antibody (Dako, Glostrup, Denmark). CD25 expression was assessed using a Coulter Epics XL-MCL flow cytometer (Beckman Coulter).

Genomic DNA isolation and single-nucleotide polymorphism analysis

Venous blood was obtained after informed consent from a cohort of 74 healthy foresters from the 'Geldersch Landschap' department in The Netherlands (cohort details: 23-73 years of age, 77% male, 23% female). DNA was isolated using the Gentra Pure Gene Blood kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. SNPs of the analyzed PRR genes were selected upon previously published functional effects on protein function and/or gene expression and characteristics are described in detail in Table 1 [23-26]. PCR amplification of *TLR1*, *TLR6*, *Dectin-1*, *NOD1* and *NOD2* gene fragments bearing the polymorphisms S248N (*TLR1*), P249S (*TLR6*), Y238X (*Dectin1*), ND1 +32656 (*NOD1*) and 1007finsC (*NOD2*) was performed using a pre-designed TaqMan® SNP genotyping assay (Applied Biosystems Inc., Carlsbad, CA, USA). Fluorescence intensities were corrected using a pre-read/post-read method for 1 min at 60°C before and after the amplification. The software used automatically plotted genotypes based on a 2-parameter plot with an overall success rate of >95%. Intermediate samples were excluded from analysis. Conventional PCR amplification of the *TLR1* gene fragment containing SNP I602S and *TLR2* gene fragment containing SNP R753Q was performed on a iCycler detection system (Bio-Rad Laboratories, Hercules, CA, USA). After sequencing using the Sanger method, samples were analyzed using the 3730 Sequence analyzer and Chromas 2.33 software (Technelysium Pty Ltd., Tewantin, QLD, Australia). PCR amplification of *TLR4* gene fragments containing SNPs D299G and T399I was performed as described previously [27].

Statistical analysis

Paired cytokine data from PRR blocking experiments were analyzed using the Wilcoxon signed rank test. Comparison of cytokine levels between different genotype/SNP groups was performed using either the Mann-Whitney U test or the Kruskal-Wallis test with Dunns post-test comparison. A P-value <0.05 was considered to be statistically significant. All statistical analyses were performed using GraphPad Prism 4.00 software (GraphPad Software Inc., La Jolla, CA, USA). Data are presented as means + SEM, unless stated otherwise.

Table 1. Genotyped single-nucleotide polymorphisms (SNPs) in genes coding for pattern-recognition receptors.

Gene	SNP ID	Mutation	Amino acid change[#]	Function
TLR1	rs4833095	Missense	S248N	Loss of function [23]
TLR1	rs5743618	Missense	I602S	Loss of function [23]
TLR2	rs5743708	Missense	R753Q	Loss of function [23]
TLR4	rs4986790	Missense	D299G	Loss of function [26]
TLR4	rs4986791	Missense	T399I	Loss of function [26]
TLR6	rs5743810	Missense	P249S	Loss of function [23]
NOD1 *	-	Ins/Del ND1+32656	-	Loss of function [25]
NOD2	rs5743293	Frameshift 1007finsC	-	Loss of function [25]
Dectin1	rs16910526	Nonsense	Y238X	Loss of function [24]

[#] First described amino acid (AA) is considered wild-type.

* NOD1 insertion/deletion (Ins/Del) polymorphism ND1+32656, partially identified as rs6958571.

Results

Cytokine induction by *B. fragilis*

In order to characterize the cytokine profile induced by *B. fragilis*, PBMCs of 74 healthy individuals were stimulated with an inoculum of heat-killed 1 x 10^6 cfu/mL *B. fragilis*, *E. coli* (gram-negative bacterium) or *S. aureus* (gram-positive bacterium, also a potent abscess inducer) and medium as a control. *B. fragilis* stimulated PBMCs induced a significantly higher response for each cytokine, except for IFNγ compared to the negative control. *B. fragilis* was a strong inducer of IL-6 and IL-8, a moderate inducer of IL-1β and TNFα and a poor inducer of IL-10 and IFNγ. For each of these cytokines, the *B. fragilis* response was in between both positive controls, significantly higher than *S. aureus*, but lower than *E. coli*. In addition, recognition of *B. fragilis* resulted in an unexpected low IL-17 induction compared to other cytokines, with a higher response than *E. coli*, but significantly lower than the known IL-17 inducer *S. aureus* (Figure 1A). To further investigate the cause of the low IL-17 induction by *B. fragilis*, dose-response curves were obtained by stimulating PBMCs with increasing concentrations of 6 different *B. fragilis* strains and IL-17 (after 7

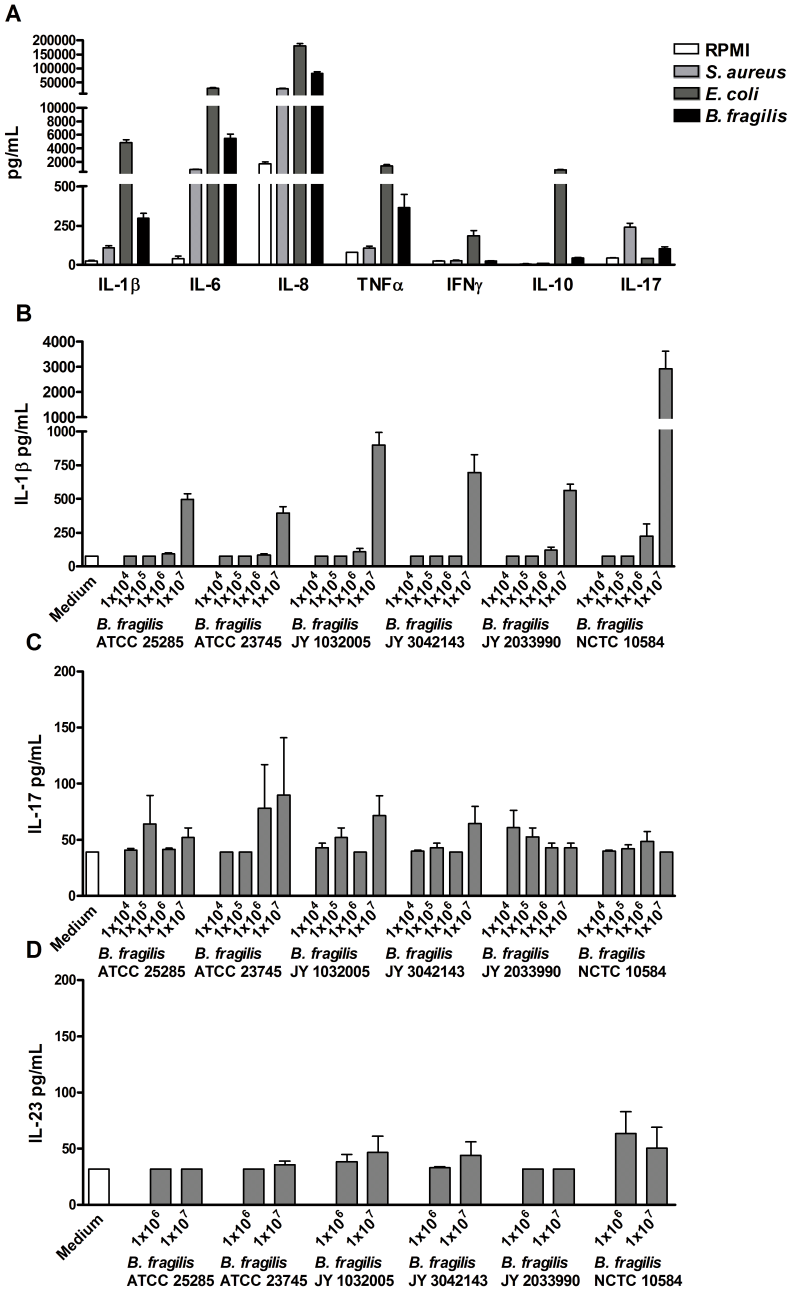

Figure 1. Cytokine induction by *B. fragilis*. (A) PBMCs (5 x 10^6/mL) were incubated with heat-killed *B. fragilis* NCTC 10584 (1 x 10^6 cfu/mL), heat-killed *S. aureus* ATCC 29213 (1 x 10^6 cfu/mL), *E. coli* ATCC 25922 (1 x 10^6 cfu/mL) or culture medium (negative control). After incubation for 24 hours (IL-1β, IL-6, IL-8, TNFα and IL-10) or 7 days (IL-17 and IFNγ), cytokine levels were measured in supernatants by ELISA (n=74). (B - D) PBMCs (5 x 10^6/mL) were incubated with increasing concentrations of 6 different strains of heat-killed *B. fragilis* (ATCC 25285, ATCC 23745, JY 1032005, JY 3042143, JY 2033990 and NCTC 10584) and cytokine levels of IL-1β (B), IL-17 (C) and IL-23 (D) were measured in supernatants by ELISA (n=4).

days) and IL-17 inducing cytokines IL-1β and IL-23 (after 24 hours) were measured. Again, a moderate induction of IL-1β (Figure 1B) and poor induction of IL-17 (Figure 1C) was confirmed for all strains, although the IL-1β induction was variable between strains. In addition, *B. fragilis* proved to be a very poor inducer of IL-23 (Figure 1D), providing the most likely explanation for its low IL-17 inducing potential.

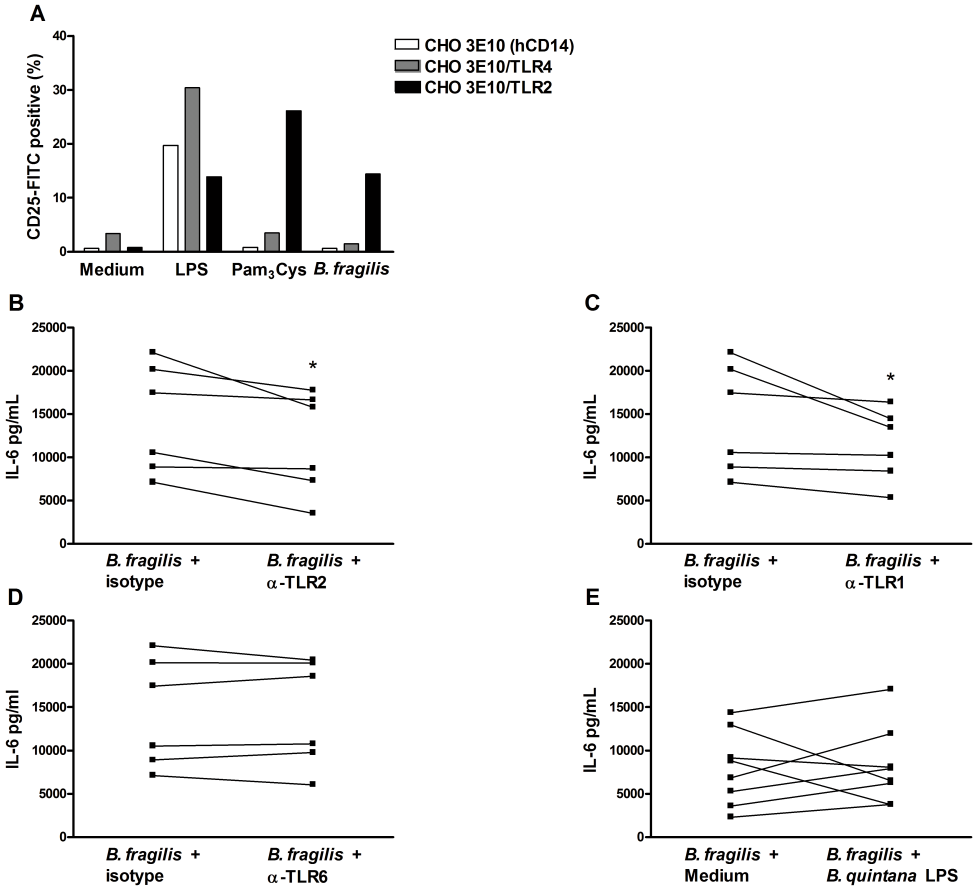

Figure 2. Role of Toll-like receptors in immune recognition of *B. fragilis*. (A) CHO 3E10 cells (1 x 10^5/mL) stably transfected with CD14 alone (3E10), human CD14 and TLR2 (3E10/TLR2) or human CD14 and TLR4 (3E10/TLR4) were incubated with medium, Pam_3Cys (10 μg/mL), *E. coli* LPS (1 μg/mL) or heat-killed *B. fragilis* JY 1032005 (1 x 10^7 cfu/mL) for 20 hours. Subsequently, cells were stained with FITC-labeled mouse anti-human CD25 monoclonal antibody, a measure for TLR signaling, and the percentage of CD25 positive cells was assessed by flow cytometry. Data are representative of an experiment performed in duplicate. (B - E) PBMCs (5 x 10^6/mL) were pre-incubated for 1 hour with TLR inhibitors, followed by 24 hours incubation with heat-killed *B. fragilis* JY 1032005 (1 x 10^7 cfu/mL). Subsequently, levels of IL-6 were measured in supernatants by ELISA. Wilcoxon signed-rank test; * $P<0.05$. Individual data are presented. TLR inhibitors: (B) α-TLR2 (10 μg/mL) or isotype control antibody (10 μg/mL), n=6. (C) α-TLR1 (10 μg/mL) or isotype control antibody (10 μg/mL), n=6. (D) α-TLR6 (10 μg/mL) or isotype control antibody (10 μg/mL), n=6. (E) *B. quintana* LPS (TLR4 inhibitor; 1 μg/mL) or medium control, n=8.

Role of Toll-like receptors in immune recognition of *B. fragilis*

To determine which TLRs are involved in immune recognition of *B. fragilis*, CHO cells transfected with human (h)CD14 alone, with hCD14 and hTLR2, or with hCD14 and hTLR4 were stimulated with medium (negative control), Pam_3Cys (TLR2 agonist), *E. coli* LPS (TLR4 agonist) or heat-killed *B. fragilis*. Induction of TLR signaling in these cells leads to translocation of NF-κB to the nucleus which drives the expression of membrane-bound CD25. *B. fragilis* strongly induced CD25 expression in hTLR2 transfected cells. In contrast, CHO cells transfected with hTLR4 and hCD14 or hCD14 alone did not respond to *B. fragilis* (Figure 2A). CD25 expression represents immune recognition of *B. fragilis* by the transfected TLR suggesting that TLR2, but not TLR4, has a major role in the binding and recognition of *B. fragilis*. However, they are not informative concerning the effects of this recognition on the induced cytokine responses.

In a subsequent set of experiments, we performed PRR and receptor signaling blocking experiments using IL-6 as a read-out cytokine. Using anti-TLR2 antibodies in order to inhibit TLR2 resulted in a significant decrease of IL-6 production by PBMCs stimulated with *B. fragilis* (Figure 2B). Recognition of TLR2 ligands can occur through heterodimeric association of TLR2 with either TLR1 or TLR6, therefore we also studied blocking these receptors with anti-TLR1 and anti-TLR6 antibodies. IL-6 production was significantly inhibited using anti-TLR1 antibodies, but not by anti-TLR6 antibodies (Figure 2C, D). Inhibiting TLR4 using *B. quintana* LPS had no significant effect on induction of IL-6 (Figure 2E) in PBMCs stimulated with *B. fragilis*, whereas production of IL-6 (data not shown) was significantly reduced in *E. coli* LPS stimulated PBMCs in the presence of *B. quintana* LPS. These findings suggest that TLR1 and TLR2, but not TLR4 and TLR6, are involved in the recognition of *B. fragilis*.

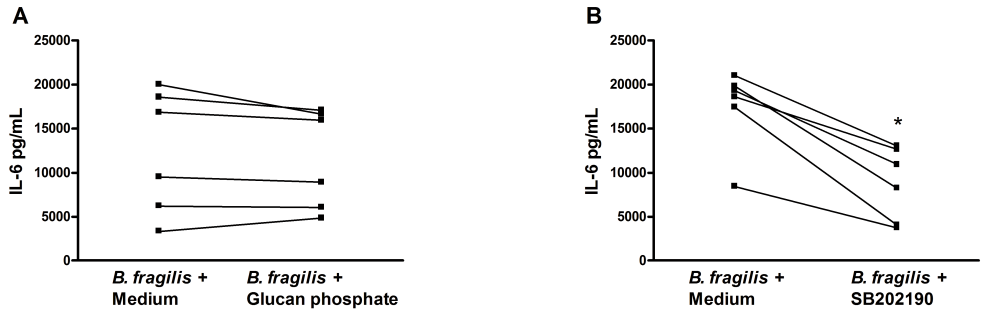

Figure 3. Role of Dectin-1 and NOD-like receptors in immune recognition of *B. fragilis*. (A) PBMCs (5 x 10^6/mL) were pre-incubated for 1 hour with medium or glucan-phosphate (200 µg/mL) followed by 24 hours incubation with heat-killed *B. fragilis* JY 1032005 (1 x 10^7 cfu/mL). Subsequently, levels of IL-6 were measured in supernatants by ELISA (n=6). (B) PBMCs (5 x 10^6/mL) were pre-incubated for 30 min with medium or RICK inhibitor SB202190 (1 µM) and incubated with heat-killed *B. fragilis* JY 1032005 (1 x 10^7 cfu/mL) for 24 hours. Subsequently, levels of IL-6 were measured in supernatants by ELISA (n=6). Wilcoxon signed-rank test; * $P<0.05$. Individual data are presented.

Role of Dectin-1 and NOD-like receptors in immune recognition of *B. fragilis*

TLR2 has been previously demonstrated to interact with Dectin-1 [28, 29] and NOD-like receptors [30] for the induction of cytokines. Production of IL-6 (Figure 3A) in response to *B. fragilis* was not significantly affected by the Dectin-1 inhibitor glucan-phosphate, whereas glucan-phosphate significantly decreased levels of IL-6 (data not shown) in response to *Candida albicans*. To investigate the role of NOD-like receptors, we stimulated PBMCs with heat-killed *B. fragilis* in the presence of the RICK inhibitor SB202190, which is an adaptor molecule mediating NOD1/2-dependent signals. The RICK inhibitor significantly decreased production of IL-6 (Figure 3B), indicating that NOD-like receptors recognize *B. fragilis* and induce proinflammatory cytokine production.

Impact of Toll-like receptor single-nucleotide polymorphisms on immune recognition of *B. fragilis*

In the cohort of healthy individuals, SNPs of *TLR2*, *TLR1*, *TLR6* and *TLR4* were determined and PBMCs were stimulated with *B. fragilis* to assess IL-6 and IL-1β cytokine production. PBMCs from individuals bearing the *TLR2* SNP R753Q displayed no difference in IL-6 production in response to *B. fragilis* (Figure 4A). However, PBMCs from individuals homozygous for the *TLR1* SNP S248N produced significantly less IL-6 (Figure 4B) compared to those from heterozygous individuals. Similar results were found for the genetically linked *TLR1* SNP I602S (93% linkage between these 2 polymorphisms; data not shown). No significant differences in IL-6 production were present in *B. fragilis* stimulated PBMCs from individuals homozygous for the *TLR6* SNP P249S compared to those from wild-type and heterozygous individuals (Figure 4C). PBMCs from individuals heterozygous for the *TLR4* SNPs D299G and T399I (100% cosegregation) produced similar levels of IL-6 in response to *B. fragilis* (Figure 4D) compared to PBMCs from wild-type individuals. In addition, cytokine induction by *E. coli* LPS stimulated PBMCs did not differ between groups.

In contrast to the IL-6 cytokine production, PBMCs from individuals bearing the *TLR2* SNP R753Q polymorphism displayed significantly increased IL-1β in response to *B. fragilis* compared to those from wild-type individuals (Figure 4E). Similar to the IL-6 results, IL-1β was significantly decreased in PBMCs from individuals homozygous for the *TLR1* SNP S248N compared to heterozygous individuals in response to *B. fragilis* (Figure 4F). No significant differences in IL-1β production were observed in the genotype groups for *TLR6* SNP P249S and *TLR4* SNPs D299G and T399I (Figures 4G, H). These results show that SNPs in *TLR1* and *TLR2*, in contrast to SNPs in *TLR6* and *TLR4*, affect cytokine induction by *B. fragilis*. Furthermore, in contrast to SNPs in *TLR1*, the *TLR2* SNP R753Q leads to higher production of IL-1β.

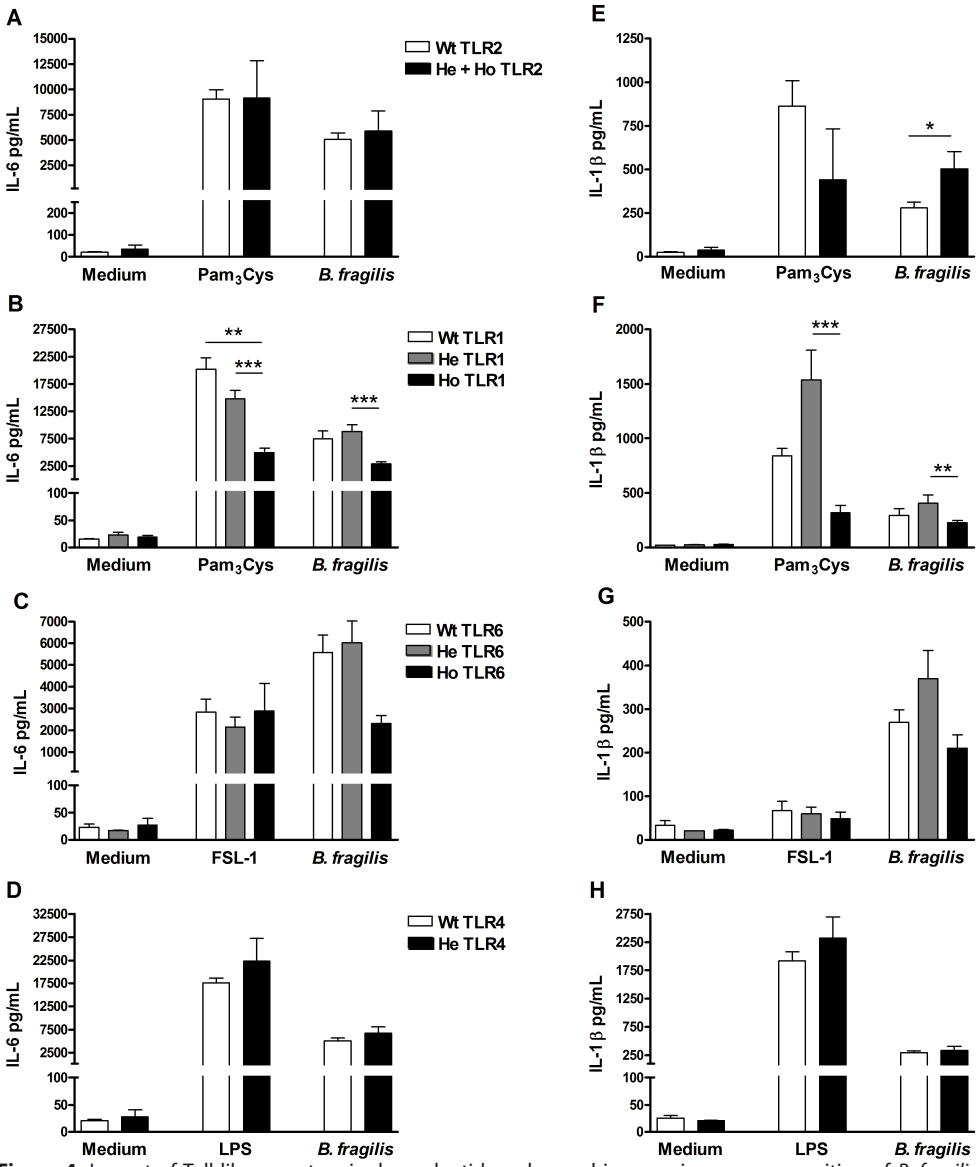

Figure 4. Impact of Toll-like receptor single-nucleotide polymorphisms on immune recognition of *B. fragilis*. (A - H) PBMCs (5 x 10^6/mL) from individuals carrying various TLR SNPs were incubated for 24 hours with *B. fragilis* or various control TLR ligands. Subsequently, levels of IL-6 (left panel) and IL-1β (right panel) were measured in supernatants by ELISA. Wt, wild-type; He, heterozygous; Ho, homozygous. (A and E) PBMCs (5 x 10^6/mL) from individuals not carrying the *TLR2* SNP R753Q (Wt, n=59) and individuals carrying the *TLR2* SNP R753Q (He, n=5 and Ho, n=1) were incubated with medium, Pam_3Cys (10 μg/mL) or heat-killed *B. fragilis* NCTC 10584 (1 x 10^6 cfu/mL). Mann-Whitney U Test * P<0.05. (B and F) PBMCs (5 x 10^6/mL) from individuals not carrying the *TLR1* SNP S248N (Wt, n=3) and individuals carrying the *TLR1* SNP S248N (He, n=29; Ho, n=41) were incubated with medium, Pam_3Cys (10 μg/mL) or heat-killed *B. fragilis* NCTC 10584 (1 x 10^6 cfu/mL). Kruskal-Wallis test; ** P<0.01, *** P<0.001. (C and G) PBMCs (5 x 10^6/mL) from individuals not carrying the *TLR6* SNP P249S (Wt, n=23) and individuals carrying the *TLR6* SNP P249S (He, n=39; Ho, n=11) were incubated with medium, FSL-1 (1 μg/mL) or heat-killed *B. fragilis* NCTC 10584 (1 x 10^6 cfu/mL). (D and H) PBMCs (5 x 10^6/mL) from individuals not carrying the

TLR4 SNPs D299G and T399I (Wt, n=59) and individuals carrying the *TLR4* SNPs D299G and T399I (He, n=5) were incubated with medium, *E. coli* LPS (10 ng/mL) or heat-killed *B. fragilis* NCTC 10584 (1 x 10^6 cfu/mL).

Impact of NOD-like receptor and Dectin-1 single-nucleotide polymorphisms on immune recognition of *B. fragilis*

PBMCs from individuals heterozygous for the *NOD2* SNP 1007finsC produced significantly lower levels of IL-6 (Figure 5A), in response to *B. fragilis* compared to wild-type PBMCs. Muramyl dipeptide (MDP), a specific NOD2 ligand, showed a similar response (Figure 5A). In contrast, *B. fragilis* stimulated PBMCs from individuals heterozygous and homozygous for the *NOD1* SNP ND1 +32656 displayed similar levels of IL-6 (Figure 5B), compared to those from wild-type individuals. Both *NOD2* SNP 1007finsC and *NOD1* SNP ND1 +32656 genotype groups produced IL-1β cytokine profiles comparable to those observed for IL-6 (Figure 5C, D). These results confirm a role for NOD2, but not NOD1, in the immune recognition of *B. fragilis*. Furthermore, no differences in IL-6 and IL-1β production (data not shown) were observed between PBMCs heterozygous for the *Dectin-1* SNP Y238X compared to wild-type PBMCs in response to *B. fragilis*, confirming that Dectin-1 is not involved in immune recognition of *B. fragilis*.

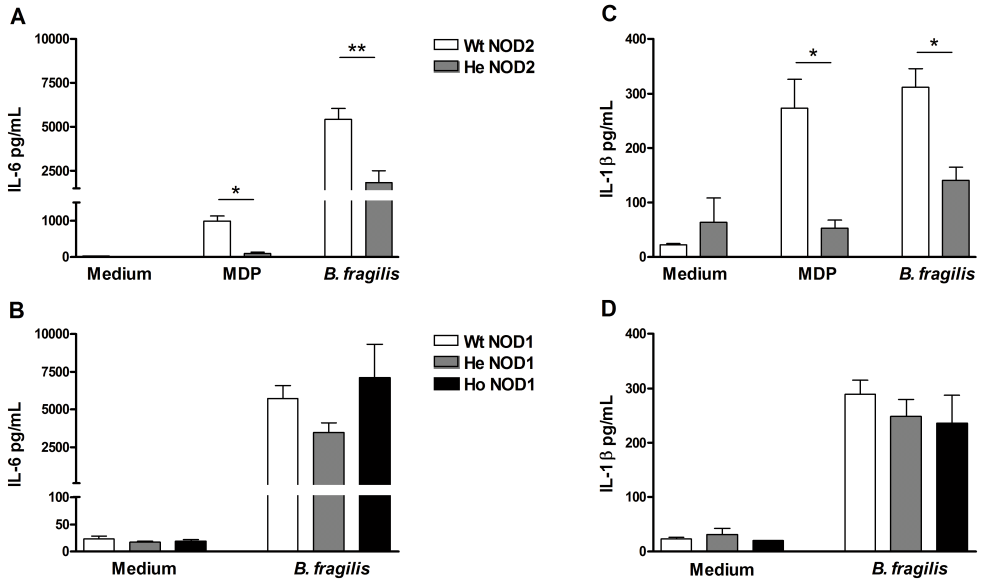

Figure 5. Impact of NOD-like receptor single-nucleotide polymorphisms on immune recognition of *B. fragilis*. (A - D) PBMCs from individuals carrying various NLR SNPs were incubated for 24 hours with *B. fragilis* or various control NLR ligands. Subsequently, levels of IL-6 (left panel) and IL-1β (right panel) were measured in supernatants by ELISA. Wt, wild-type; He, heterozygous; Ho, homozygous. (A and C) PBMCs (5 x 10^6/mL) from individuals not carrying the *NOD2* SNP 1007finsC (Wt, n=60) and individuals carrying the *NOD2* SNP 1007finsC (He and Ho, n=5) were incubated with medium, muramyl dipeptide (MDP; 10 µg/mL) or heat-killed *B. fragilis* NCTC 10584 (1 x 10^6 cfu/mL). Mann-Whitney U test; * $P<0.05$, ** $P<0.01$. (B and D) PBMCs (5 x 10^6/mL) from individuals not carrying the *NOD1* SNP ND1 +32656 (Wt, n=39) and individuals carrying the *NOD1* SNP ND1 +32656 (He, n=20; Ho, n=6) were incubated with medium or heat-killed *B. fragilis* NCTC 10584 (1 x 10^6 cfu/mL).

Discussion

In the present study, we show that *B. fragilis* has potent stimulatory effects on innate immunity, with strong induction of IL-6 and IL-8 production, and a moderate stimulation of IL-1β and TNFα. In contrast, *B. fragilis* poorly induced IL-10, IFNγ, IL-23 and IL-17 cytokine responses in human peripheral blood mononuclear cells. In addition, we demonstrate that TLR2, TLR1 and NOD2, but not TLR4, TLR6, Dectin-1 or NOD1, mediate pattern recognition of *B. fragilis* by human PBMCs.

The strong IL-6 and TNFα cytokine responses induced in PBMCs by whole *B. fragilis* are in line with the findings of Nagy *et al*., who observed significant induction of IL-6 and TNFα in human mononuclear cells stimulated with *B. fragilis* [14]. Other studies exploring the host response to *B. fragilis* have been conducted on specific cell wall components of this bacterium. Mice injected with *B. fragilis* LPS displayed slightly increased blood levels of IL-6, TNFα and IFNγ [31]. *B. fragilis* outer membrane protein A (OmpA), one of its major outer-membrane components, induced the release of IL-1α, TNFα, IFNγ and IL-6 by murine splenocytes [32]. However, most research has focused on *B. fragilis* capsular polysaccharide A (PSA) of *B. fragilis* strain ATCC 25285. In line with our findings showing a poor induction of Th17 responses by *B. fragilis*, PSA suppressed IL-17 production in mice and induced IL-10 and TGF-β2 production by $CD4^+Foxp3^+$ regulatory T cells, resulting in protection against experimental colitis [4, 33] and experimental autoimmune encephalomyelitis [34]. In contrast, IL-17 was a critical component of the response to *B. fragilis* ATCC 25285 or capsular polysaccharide from *Streptococcus pneumoniae* type 1 and blockade of IL-17 inhibited abscess formation in mice [35]. These different observations are likely to be explained by variation in host, body site and experimental setup (in vivo/in vitro).

The poor IL-17 induction by *B. fragilis* may seem unexpected. IL-17 is a potent chemotactic peptide of neutrophils to sites of inflammation [36], and the majority of cells present in mixed infection abscesses in which *B. fragilis* is a participant are neutrophils. A possible explanation for the poor IL-17 induction in our findings is the absence of IL-23 induction by *B. fragilis*, a cytokine necessary for the maintenance of an IL-17 producing Th17 cell population [37]. On the other hand, *B. fragilis* strongly induced IL-8, which is another important chemotactic factor for recruiting neutrophils to sites of infection. The strong induction of IL-8 is likely to explain the ability of *B. fragilis* to induce abscess formation [38]. In contrast, *B. fragilis* enterotoxin (BFT) has previously been shown to induce IL-8 and TGF-β in human colonic epithelial cell lines [39, 40] and, more recently, to induce IL-17 producing Th17 cells in mice [41]. However, BFT has no role in our system, as we used washed heat-killed bacteria in all experiments.

We have assessed the role of major PRRs for the recognition of *B. fragilis* through several complementary approaches. Firstly, using stable CHO cell lines expressing either TLR2 or TLR4, we demonstrate that *B. fragilis* is recognized by TLR2, but not by TLR4. CHO cells constitutively express functional hamster TLR4, but not TLR2 [42], explaining the relatively strong CD25 induction in *E. coli* LPS stimulated CHO cells even without the presence of hTLR4. The conclusions from CHO cells were strengthened by experiments in human primary cells in which TLR2 or TLR4 were blocked by specific inhibitors during *B. fragilis* stimulation. Previous studies, using whole heat-killed *B. fragilis* [15] or *B. fragilis* LPS and PSA [16-18, 43], are in line with our results and may suggest that either the *B. fragilis* LPS or PSA are responsible for its recognition by TLR2. In contrast, 1 study found TLR4, but not TLR2, to be important in immune recognition of *B. fragilis* LPS [44] and reasons for this discrepancy are unclear so far. Through heterodimeric association with either TLR1 or TLR6, TLR2 can recognize distinct substrates [45]. To the best of our knowledge, this is the first study investigating the role of TLR1 and TLR6 in the immune recognition of *B. fragilis*. Blocking TLR1 and TLR6 clearly showed that TLR1, but not TLR6, is involved in immune recognition of *B. fragilis*.

In addition to the effect of TLRs, we have also assessed the role of other PRR families for the recognition of *B. fragilis*. Dectin-1 has been previously shown to collaborate with TLR2 for the induction of cytokine production [28, 29]. However, in line with the absence of known Dectin-1 ligands in the structure of the *Bacteroides* cell wall, blockade of Dectin-1 did not influence cytokine production induced by *B. fragilis*. In contrast, bacterial cell wall contains an important amount of peptidoglycans, components recognized by the NLR receptors NOD1 and NOD2 [46, 47]. Blocking NOD1/NOD2 signaling using the RICK inhibitor SB202190 resulted in a decreased production of proinflammatory cytokines after stimulation of cells with *B. fragilis*. However, SB202190 is a non-specific RICK inhibitor and has been shown to inhibit other targets [48], indicating a possible role for either NOD1 or NOD2 for the recognition of the microorganism.

An additional methodology we employed was to investigate the effect of loss-of-function SNPs in PRR genes on cytokine responses induced by *B. fragilis*. SNPs were selected upon previously published functional effects on protein function and/or gene expression. In other studies, SNPs in *TLR1* (S248N, I602S), *TLR2* (R753Q), *TLR6* (P249S) demonstrated to have a reduced potential to activate NF-κB after stimulation in HEK293T cells [23]. Decreased cytokine responses were observed after PBMC stimulation in the presence of SNPs in *NOD2* (1007finsC) [25] and *Dectin-1* (Y238X) [24], whereas increased cytokine responses were observed in the presence of SNPs in *NOD1* [25]. SNPs in *TLR4* (D299G, T399I) are associated with a blunted response to inhaled LPS [26].

The finding that SNP R753Q in *TLR2* affected cytokine induction by *B. fragilis*, whereas SNPs in *TLR4* did not, confirmed the importance of TLR2 and not TLR4 in *B. fragilis* immune recognition. Interestingly, recognition by TLR2 leads to down regulation of IL-1β induction, suggesting a possible immunomodulatory role, which has been previously reported for TLR2 [49]. Furthermore, these results confirm that heterozygosity for both SNPs D299G and T399I in the *TLR4* gene does not lead to a phenotype different from that of wild-type *TLR4* [27, 50]. Polymorphisms in *TLR1* and *TLR6* confirmed results from blocking experiments and indicated that TLR1, but not TLR6, is important for *B. fragilis* pattern recognition and proinflammatory cytokine production. Furthermore, by investigating the influence of SNPs in NOD-like receptors on cytokine responses, we demonstrate that NOD2, but not NOD1, is important in the pattern recognition of *B. fragilis*, and this in line with earlier observations from our group [15].

In conclusion, in the present study we have systematically assessed major classes of PRRs and cytokines regarding their involvement in the innate immune response against *B. fragilis*. TLR2, TLR1 and NOD2, but not TLR4, TLR6, Dectin-1 and NOD1, are important in the pattern recognition of *B. fragilis* in human PBMCs. This leads to a strong induction of IL-6 and IL-8, the moderate stimulation of IL-1β and TNFα production, but a poor IL-10, IFNγ, IL-23 and IL-17 cytokine response. With the increasing ability of bacteria to resist antibiotic treatment, the need for new targets to control infections caused by *B. fragilis* will arise, and an immunotherapeutic approach has been advocated. This study suggests such targets and future research is needed to explore their role in the management of *B. fragilis* infections.

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Chapter 4

TLR1, *TLR2* and *TLR6* gene polymorphisms are associated with increased susceptibility to complicated skin and skin structure infections

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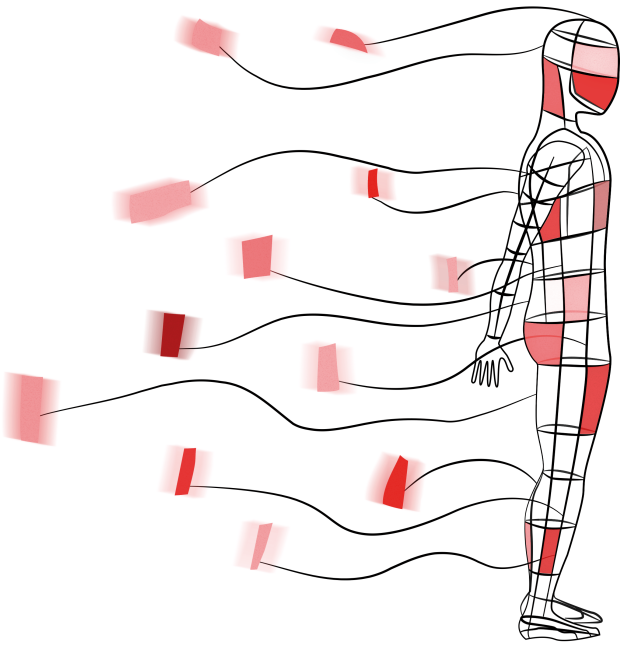

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Abstract

Background. Complicated skin and skin structure infections (cSSSIs) are characterized by infections with either gram-positive and gram-negative aerobic or anaerobic bacteria, as well as a polymicrobial etiology. These invading microorganisms are recognized by pattern-recognition receptors (PRRs) of the innate immune system. This study assessed whether genetic variation in genes encoding PRRs influences the susceptibility to cSSSIs.
Methods. A total of 318 patients with cSSSI and 328 healthy controls were genotyped for 9 nonsynonymous single-nucleotide polymorphisms (SNPs) in PRR genes coding for Toll-like receptors (TLRs) 1, 2, 4, 6; NOD-like receptor 2; and the signaling adaptor molecule TIRAP. Associations between susceptibility to cSSSIs and a SNP were investigated by means of logistic regression models. In an additional cohort of 74 healthy individuals in whom the same SNPs were genotyped, peripheral blood mononuclear cells (PBMCs) were obtained and stimulated with *Staphylococcus aureus*. Interleukin 6 (IL-6) concentrations were determined in supernatants by enzyme-linked immunosorbent assay to determine the correlation between genotypes and levels of IL-6 secretion.
Results. In the genetic association analysis, polymorphisms in *TLR1* (S248N and R80T), *TLR2* (P631H) and *TLR6* (P249S) were associated with an increased susceptibility to cSSSIs. No association with susceptibility to cSSSIs was observed for polymorphisms *TLR2* (R753Q), *TLR4* (D299G and T399I), *NOD2* (P268S) and *TIRAP* (S180L). In the functional analysis, individuals bearing the *TLR1* 248N or 80T allele showed lower IL-6 secretion upon stimulation with *S. aureus*.
Conclusions. Polymorphisms in *TLR1*, *TLR2* and *TLR6* are associated with increased susceptibility to cSSSIs. For TLR1, impaired proinflammatory cytokine production due to the polymorphism is most likely the mechanism mediating this effect.

Introduction

Complicated skin and skin structure infections (cSSSIs) involve deeper soft tissue, require substantial surgical intervention, or occur in a compromised host with a comorbid condition which may affect the response to treatment [1]. Common examples of cSSSIs are soft-tissue abscesses, diabetic foot infections, surgical-site and trauma-induced wound infections [1, 2]. Gram-positive and gram-negative aerobic or anaerobic bacteria, alone or as part of a polymicrobial infection, are the causative agents of cSSSIs, with *Staphylococcus aureus* and β-hemolytic streptococci being most frequent [1-3]. The infection location, the clinical situation, and the medical history of the patient are important characteristics that determine the type of pathogens involved [1, 2].

cSSSIs are associated with significant morbidity and mortality leading to prolonged and expensive hospitalizations, with a considerably impact on healthcare resource utilization [4]. cSSSIs often require surgical debridement of the infection in combination with antibiotic therapy [2]. However, the increasing ability of bacteria to resist antibiotic treatment, such as methicillin-resistant *S. aureus* and vancomycin-resistant enterococci, complicates management of these infections [5]. In addition, differences in severity and occurrence of cSSSIs in patients are not solely explained by comorbidities or the virulence of a certain pathogen, as previously healthy young individuals can develop a severe infection caused by components of the commensal flora [6]. For these reasons, there is a need to identify host factors that predispose to development of cSSSIs, to define a predictive profile for persons at risk of developing these infections and potential individualized diagnosis and treatment.

One of the most significant discoveries to help understand the role of the host defense system against invading pathogens is the identification of germ-line-encoded pattern-recognition receptors (PRRs) able to recognize conserved structures of microbial species called pathogen-associated molecular patterns (PAMPs) [7]. Currently, 5 major PRR classes have been described: Toll-like receptors (TLRs), nucleotide-binding domain leucine-rich repeat containing receptors (NLRs), C-type lectins, RNA helicase RIG-I-like receptors and cytoplasmic DNA receptor AIM2-like receptors [8, 9]. Of these PRR families, TLRs were the first to be discovered, and 10 TLRs have since been characterized in humans. TLRs recognize PAMPs by N-terminal leucine-rich repeats followed by a transmembrane region leading to the cytoplasmic Toll/Interleukin-1R homology (TIR) domain, which interacts with several adaptor proteins, such as myeloid differentiation primary response gene 88 (MyD88) and MyD88 adaptor-like/Toll-interleukin 1 receptor domain-containing adaptor protein (Mal/TIRAP), activating transcription factors, resulting in pro- and antiinflammatory cytokine production and generation of an adaptive immune response [8].

Several TLRs have been implicated as important in the host defense against cSSSI pathogens. Gram-positive bacteria such as *S. aureus* contain cell wall components that initiate immune responses through TLR2 [10], most often by the formation of heterodimers with either TLR1 or TLR6 [11, 12]. In contrast, lipopolysaccharide derived from the gram-negative bacterium *Escherichia coli* is recognized by TLR4 [13]. Furthermore, peptidoglycans of both gram-positive and gram-negative bacteria (*S. aureus* and *E. coli*) are recognized by NOD2, a member of the NLR family of cytoplasmic receptors [14].

Genetic variants of TLRs [15-25], NOD2 [26-28] and the signaling adaptor TIRAP [29, 30] have previously been associated with susceptibility to infections. However, none of these studies have systematically investigated the role of these PRRs in the susceptibility to cSSSIs. In this study, we hypothesized that polymorphisms in genes encoding PRRs or their signaling adaptors may influence the host susceptibility to cSSSIs. To test this hypothesis, we determined the prevalence of 7 nonsynonymous single-nucleotide polymorphisms (SNPs) in TLR genes, 1 in the NLR (NOD2) gene and 1 in the gene encoding the signaling adaptor TIRAP, in a cohort of 318 patients with cSSSI and 328 healthy controls. The genetic studies were supported by functional assays investigating the influence of the specific polymorphisms on the capacity to induce interleukin 6 (IL-6) cytokine responses after stimulation of human peripheral blood mononuclear cells (PBMCs).

Materials and methods

Subjects

From a total population of 813 patients with cSSSI included in a large, randomized, multicenter, clinical trial [6], 389 patients gave informed consent to participate in this genetic substudy. Reasons for noninclusion in the genetic substudy were absence of an invitation to participate in the genetic substudy (the major reason) or refusal to participate. Of the 389 patients, 71 were excluded because they were not white (n=2) or not of Eastern European ethnicity (n=69), resulting in 318 white patients with cSSSI from 7 East European countries who were eligible to participate in this study.

Inclusion of patients in the trial was performed from September 2006 to June 2008, in compliance with Food and Drug Administration (FDA) guidance, in which the term "uncomplicated and complicated skin and skin structure infections" was used [31]. The FDA issued a new guidance document in 2010, which used the term "acute bacterial skin and skin structure infections" [32]. We used the term "cSSSI" in this study because not only terminology but also criteria for satisfying the definition of these infections have changed.

Inclusion criteria and definition of cSSSIs (subtype/diagnosis: major abscess, diabetic

foot infection, wound infection, or infected ischemic ulcer) are described in detail in the trial report [6]. A total of 89% of patients with cSSSI had community-acquired infections, and the remaining 11% had nosocomial infections. Seventy percent of the patients with cSSSI had polymicrobial infections. In 97% of the infections, at least 1 gram-positive microorganism was isolated. The prevalence of the predominant pathogens, mainly *S. aureus*, β-haemolytic *Streptococcus* group A-G and *Enterococcus faecalis*, causing these infections in patients with cSSSI are displayed in Table 1.

A total of 328 Eastern European, white, healthy controls recruited from the community, the majority of whom where males, were selected to geographically match the patients with cSSSI. In addition, 74 Dutch, white, healthy individuals were recruited from the community to correlate their genotype with IL-6 secretion upon stimulation of PBMCs. All subjects gave informed consent for genetic analysis.

Table 1. Prevalence of baseline causative organisms isolated from patients with complicated skin and skin structure infections.

Causative organism	Percentage of all causative organisms
Staphylococcus aureus	31.5
Enterococcus faecalis	12.1
Streptococcus pyogenes	6.9
Streptococcus agalactiae	5.8
Streptococcus equisimilis	3.9
Escherichia coli	12.1
Enterobacter cloacae	4.3
Bacteroides fragilis	3.9
Acinetobacter baumannii	3.2
Other gram-positive organisms	3.6
Other gram-negative organisms	12.7

Data are limited to organisms isolated ≥3% of all causative organisms.

Genotyping

DNA from patients with cSSSI was extracted from blood collected in PAXgene DNA tubes (Qiagen, Hildesheim, Germany) according to the PLUS XL manual kit (LGC genomics GmbH, Berlin, Germany). DNA from healthy controls was isolated using the Gentra Pure Gene blood kit (Qiagen), according to the manufacturer's protocol. Nonsynonymous SNPs of the analyzed PRR genes were selected from the National Center for Biotechnology Information SNP database if they had previously described associations with human disease or known functional effects on gene expression or protein function. In total, 9 nonsynonymous missense SNPs in genes *TLR1*, *TLR2*, *TLR4*, *TLR6*, *NOD2*, and *TIRAP* were genotyped (Table

2). Gene fragments were amplified by commercially available TaqMan SNP genotyping assays according to manufacturer's protocol on a 7300 real-time polymerase chain reaction system (Applied Biosystems, Foster City, CA, USA). Quality control was performed by the incorporation of positive and negative controls and duplication of random samples across different plates.

Table 2. Genotyped nonsynonymous missense single-nucleotide polymorphisms (SNPs) in genes coding for pattern-recognition receptors and the signaling adaptor TIRAP.

Gene	SNP ID	Mutation	Nucleotide change[#]	Amino acid change[#]	Taqman Assay ID
TLR1	rs4833095	Missense	C > T	S248N	C__44103606_10
TLR1	rs5743611	Missense	G > C	R80T	C__27855269_10
TLR2	rs5743704	Missense	C > A	P631H	C__27855269_10
TLR2	rs5743708	Missense	G > A	R753Q	C__27860663_10
TLR4	rs4986790	Missense	A > G	D299G	C__11722238_20
TLR4	rs4986791	Missense	C > T	T399I	C__11722237_20
TLR6	rs5743810	Missense	C > T	P249S	C___1180648_20
NOD2	rs2066842	Missense	C > T	P268S	C__11717470_20
TIRAP	rs8177374	Missense	C > T	S180L	C__25983622_10

[#] The first nucleotide (and corresponding amino acid) is the ancestral and therefore is considered the wild-type allele. ID, identification number.

In vitro PBMC stimulation and cytokine detection assays

Venous blood was obtained from 74 healthy controls. PBMCs were isolated and stimulated as described earlier [33], with minor modifications. In brief, PBMCs were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Next, PBMCs were washed twice with phosphate-buffered saline (pH=7.4), and concentrations were adjusted to 5 x 10^6 cells/mL for experiments in Roswell Park Memorial Institute 1640 Dutch Modified culture medium (supplemented with 2 mM L-glutamine, 1 mM pyruvate and 50 µg/mL gentamicin; GIBCO Invitrogen, Carlsbad, CA, USA).

PBMCs (5 x 10^5 cells) were added to 96-well round-bottom plates (Greiner, Nurnberg, Germany) and stimulated with either 1 x 10^6 colony forming units/mL of heat-killed *S. aureus* ATCC 29213 or specific TLR ligands (10 µg/mL Pam_3Cys or 1 µg/mL FSL-1; EMC Microcollections, Tübingen, Germany) in a final volume of 200 µL. PBMCs were incubated for 24 hours at 37°C and 5% CO_2 to induce IL-6 production, after which the plates were centrifuged and supernatants were collected. All supernatants were stored at -20°C. IL-6 concentrations were measured in supernatants by a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's instructions (Sanquin Reagents, Amsterdam, The Netherlands).

Statistical analysis

Chi-square analysis of deviation from Hardy-Weinberg equilibrium (HWE) was performed for all 9 SNPs separately in healthy controls, using a web-based HWE calculator [34]. The association between susceptibility to cSSSIs and a SNP were investigated by means of univariate logistic regression models, using IBM SPSS 18 software (IBM, Armonk, NY, USA). SNPs were evaluated using dominant model analysis (in which heterozygotic and homozygotic individuals for the allelic variant are combined and compared to wild-type individuals) and recessive model analysis (in which wild-type and heterozygotic individuals are combined and compared to homozygotic individuals for the allelic variant). No correction for multiple testing was performed. Pairwise linkage disequilibrium (LD), D' and r^2, were calculated using IBM SPSS 18 software. Multivariate logistic regression analyses were performed assuming a dominant model. Model reduction was done via forward and backward model selection methods, and in the most complex model all 9 genotyped SNPs were considered. P-values <0.05 were considered to be statistically significant. Odds ratios (ORs) and 95% confidence intervals (CIs) were reported for these tests of association.

All statistical analyses and graphs of the IL-6 concentrations in the in vitro PBMC stimulation assays were performed and created, respectively, using GraphPad Prism 5.00 software (GraphPad Software, La Jolla, CA, USA). Results were stratified by genotype, and differences in IL-6 secretion between genotypes were analyzed by Mann-Whitney U tests.

Results

Univariate logistic regression of single polymorphisms in PRR and signaling adaptor TIRAP genes in patients with cSSSI and healthy controls

Polymorphisms in genes of *TLR1*, *TLR2*, *TLR4*, *TLR6*, *NOD2* and *TIRAP* were genotyped in 318 patients with cSSSI and 328 healthy controls for the analyses of genetic association with susceptibility to cSSSIs. In the dominant model, genotyping revealed an association between susceptibility to cSSSIs and 3 TLR polymorphisms: *TLR1* S248N (P=0.03; OR=1.78 [95% CI, 1.04-3.05]), *TLR2* P631H (P=0.04; OR=1.86 [95% CI, 1.02-3.40]), *TLR6* P249S (P=0.03; OR=1.41 [95% CI, 1.03-1.93]), in patients with cSSSI, compared to healthy controls (Table 3). Polymorphism *TLR1* R80T was borderline insignificant (P=0.06; OR=1.41 [95% CI, 0.98-2.03]). In contrast to healthy controls, patients with cSSSI were more often heterozygous or homozygous for the allelic variant of these SNPs. No association with susceptibility to cSSSIs was observed in individuals carrying polymorphisms in *TLR2* R753Q, *TLR4* D299G and T399I, *NOD2* P268S and *TIRAP* S180L (Table 3). In addition, recessive model analysis confirmed the association between susceptibility to cSSSIs and *TLR1* S248N (P=0.01; OR=1.54 [95% CI, 1.13-2.11]) and showed borderline insignificance for *TLR6* P249S (P=0.06;

OR=1.60 [95% CI, 0.98-2.61]) whereas *TLR2* P631H, *TLR1* R80T, *TLR2* R753Q, *TLR4* D299G and T399I, *NOD2* P268S and *TIRAP* S180L did not influence susceptibility to cSSSIs (data not shown).

All genotyped SNPs were in HWE for the healthy controls (data not shown). LD analyses indicated that polymorphisms *TLR1* S248N and *TLR1* R80T (P<0.0001; r^2=0.27; D'=0.53),

Table 3. Distribution of pattern-recognition receptor and signaling adaptor TIRAP genotypes in 318 patients with complicated skin and skin structure infections (cSSSIs) and 328 healthy controls.

Polymorphism	Wild-type, No. (%)	Heterozygous, No. (%)	Homozygous, No. (%)	P-value[#]	Odds ratio (95% CI)[#]
TLR1 S248N	CC	CT	TT	0.03	1.78 (1.04-3.05)
Patients with cSSSI	23 (7.2)	120 (37.7)	175 (55.0)		
Healthy controls	40 (12.2)	143 (43.6)	145 (44.2)		
TLR1 R80T	GG	GC	CC	0.06	1.41 (0.98-2.03)
Patients with cSSSI	231 (72.6)	81 (25.5)	6 (1.9)		
Healthy controls	259 (79.0)	63 (19.2)	6 (1.8)		
TLR2 P631H	CC	CA	AA	0.04	1.86 (1.02-3.40)
Patients with cSSSI	287 (90.3)	31 (9.7)	0 (0.0)		
Healthy controls	310 (94.5)	18 (5.5)	0 (0.0)		
TLR2 R753Q	GG	GA	AA	0.76	0.89 (0.42-1.90)
Patients with cSSSI	305 (95.9)	13 (4.1)	0 (0.0)		
Healthy controls	313 (95.4)	15 (4.6)	0 (0.0)		
TLR4 D299G	AA	AG	GG	0.37	0.81 (0.51-1.29)
Patients with cSSSI	280 (88.3)	35 (11.0)	2 (0.6)		
Healthy controls	282 (86.0)	43 (13.1)	3 (0.9)		
TLR4 T399I	CC	CT	TT	0.37	0.81 (0.51-1.29)
Patients with cSSSI	280 (88.3)	35 (11.0)	2 (0.6)		
Healthy controls	282 (86.0)	43 (13.1)	3 (0.9)		
TLR6 P249S	CC	CT	TT	0.03	1.41 (1.03-1.93)
Patients with cSSSI	130 (40.9)	144 (45.3)	44 (13.8)		
Healthy controls	162 (49.4)	136 (41.5)	30 (9.1)		
NOD2 P268S	CC	CT	TT	0.87	0.98 (0.71-1.33)
Patients with cSSSI	179 (56.3)	107 (33.6)	32 (10.1)		
Healthy controls	182 (55.7)	119 (36.4)	26 (8.0)		
TIRAP S180L	CC	CT	TT	0.43	1.16 (0.80-1.68)
Patients with cSSSI	240 (75.7)	66 (20.8)	11 (3.5)		
Healthy controls	257 (78.4)	65 (19.8)	6 (1.8)		

[#] Data are based on a dominant model analysis. CI, confidence interval.

TLR1 S248N and *TLR6* P249S (P<0.0001; r^2=0.47; D'=0.59), *TLR1* R80T and *TLR6* P249S (P<0.0001; r^2=0.26; D'=0.48) were not independent from another, although the strength of their correlation was observed to be low. In contrast, LD analyses for *TLR4* D299G and *TLR4* T399I revealed these SNPs were perfectly correlated (P<0.0001; r^2=1.00; D'=1.00).

Multivariate logistic regression of polymorphisms in PRR and signaling adaptor TIRAP genes in patients with cSSSI and healthy controls

All 9 genotyped SNPs were considered for inclusion as explanatory variables in the multivariate logistic regression model with cSSSI status as main outcome. On the basis of results from univariate analyses, a dominant model analysis was performed. Forward and backward selection procedures resulted in the same final model, with SNPs *TLR1* R80T, *TLR2* P631H and *TLR6* P249S best explaining the susceptibility to cSSSIs. The SNPs *TLR1* R80T (P=0.01; OR=1.60 [95% CI, 1.10-2.34]), *TLR2* P631H (P=0.03; OR=1.94 [95% CI, 1.06-3.56]) and *TLR6* P249S (P=0.01; OR=1.55 [95% CI, 1.12-2.15]) independently influenced susceptibility to cSSSIs. Incorporation of the remaining 6 SNPs in the analysis did not improve the prediction of cSSSI status (P>0.3).

Functional effects of *TLR1* S248N and R80T, *TLR2* P631H, and *TLR6* P249S polymorphisms on cytokine production

Functional consequences of the TLR polymorphisms associated with susceptibility to cSSSIs were studied by stimulation of PBMCs obtained from an additional cohort of 74 healthy volunteers. PBMCs were stimulated with *S. aureus*, the pathogen most frequently causing cSSSIs, or with the following specific TLR ligands: Pam_3Cys for TLR1 and TLR2 and FSL-1 for TLR6. IL-6 secretion was assessed in cell culture supernatants after 24 hours of stimulation and was stratified based on the particular TLR genotype. Unstimulated PBMCs did not induce detectable IL-6 cytokine responses (data not shown). Homozygosity (TT; n=41) for the *TLR1* S248N polymorphism was associated with dramatically lower IL-6 cytokine responses, compared to wild-type (CC; n=3) and heterozygous (CT; n=29) genotypes after *S. aureus* and Pam_3Cys stimulation (Figure 1A and 1E, respectively). Also, heterozygosity (GC; n=13) for the *TLR1* R80T polymorphism resulted in significantly lower IL-6 cytokine responses, compared to the wild-type (GG; n=58) genotype after *S. aureus* but not Pam_3Cys stimulation (Figure 1B and 1F, respectively). This decreased IL-6 cytokine response after *S. aureus* stimulation was not observed in individuals homozygous for the variant allele (CC; n=2), most likely because of its low prevalence.

No significant differences in IL-6 response could be detected between the wild-type (CC; n=71) and heterozygous (CA; n=2) genotypes of the *TLR2* P631H polymorphism (Figure 1C and 1G), again most likely because of the low prevalence of the allelic variant among the study participants. No homozygotes (AA; n=0) for *TLR2* P631H were present among

the 74 individuals. A decreasing trend in IL-6 response was observed in PBMCs stratified for the *TLR6* P249S polymorphism (CC; n=22, CT; n=39, TT; n=11) after *S. aureus* but not FSL-1 stimulation, although results were not statistically significant (Figure 1D and 1H).

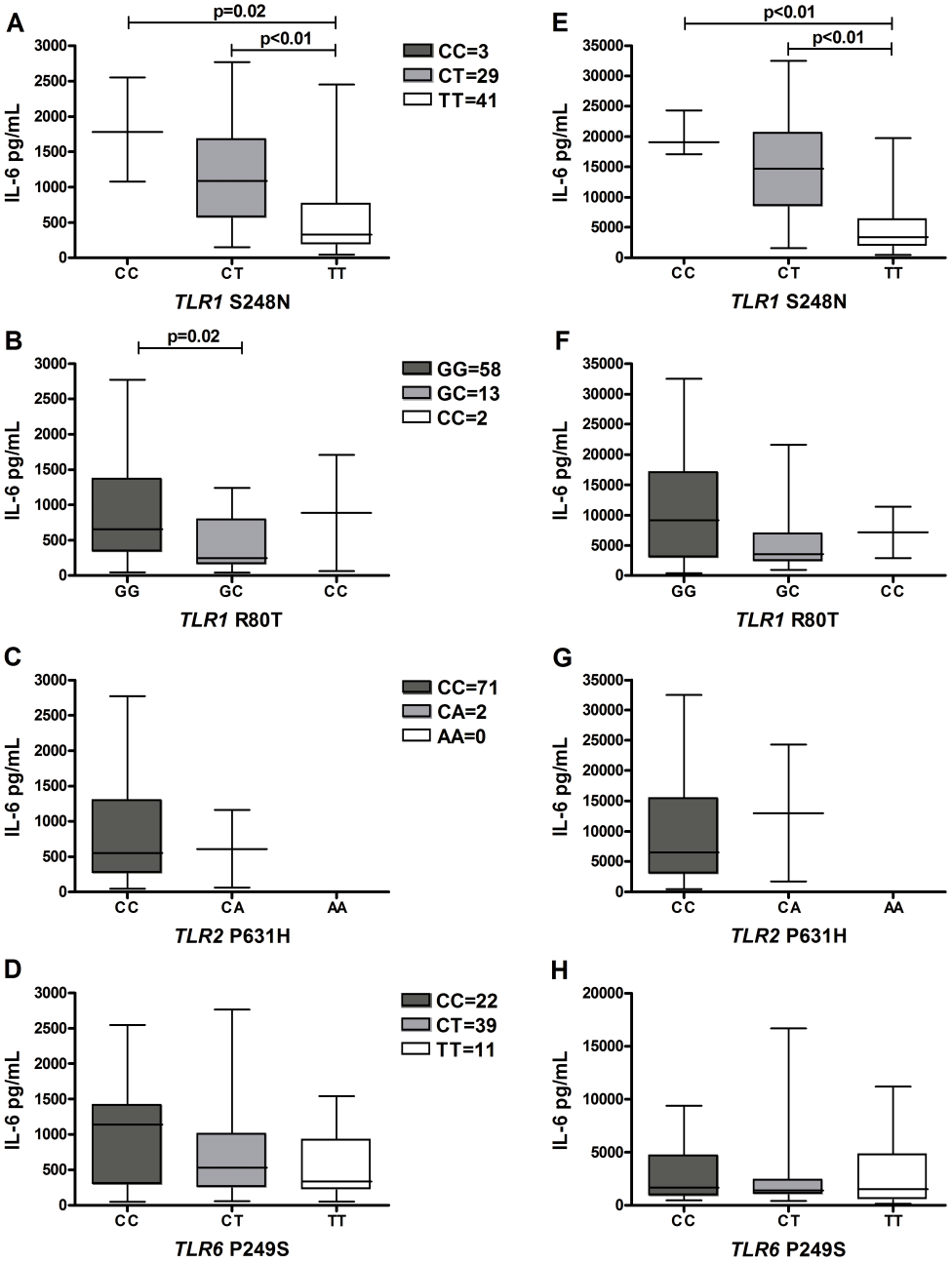

Figure 1. Correlation of complicated skin and skin structure-associated pattern-recognition receptor genotypes with interleukin 6 (IL-6) levels after 24 hours of peripheral blood mononuclear cell stimulation. *Left*, stimulation with heat-killed *Staphylococcus aureus* (1x10^6 colony-forming units/mL). *Right*, stimulation with specific Toll-like receptor (TLR) agonists. Specific TLR agonists were Pam_3Cys (10 µg/mL) for TLR1 and TLR2 and FSL-1 (1 µg/mL) for TLR6 stimulation. Results are stratified by genotype (A, E) *TLR1* S248N, (B, F) *TLR1* R80T, (C, G) *TLR2* P631H, (D, H) *TLR6* P249S. Dark grey represents individuals homozygous for the wild-type allele, light grey individuals heterozygous for the allelic variant, and white individuals homozygous for the allelic variant. Data were analyzed by Mann-Whitney U tests. Boxes represents the median and interquartile ranges; whiskers represent minimal and maximal values.

Discussion

This study demonstrates that polymorphisms in PRRs genes *TLR1*, *TLR2* and *TLR6* are associated with an increased susceptibility to cSSSIs. In the univariate logistic regression analysis, 3 polymorphisms were found to be associated with an increased susceptibility to cSSSIs: *TLR1* S248N, *TLR2* P631H and *TLR6* P249S, with borderline insignificance for *TLR1* polymorphism R80T. When studying the predictive power of a combination of SNPs by means of a multivariate logistic regression analysis, *TLR1* polymorphism S248N is not included in the model. The combination of polymorphisms *TLR1* R80T, *TLR2* P631H, and *TLR6* P249S better explains the susceptibility to cSSSIs than models including *TLR1* polymorphism S248N. In addition, PBMCs of individuals carrying the *TLR1* allele 248N or 80T produced significantly less IL-6 in response to the cSSSI pathogen *S. aureus*. No association was observed with susceptibility to cSSSIs for polymorphisms in *TLR2* R753Q, *TLR4* D299G and T399I, *NOD2* P268S, and *TIRAP* S180L.

The patients included in this study had predominantly gram-positive bacterial infections caused by *Staphylococcus*, *Streptococcus*, and *Enterococcus* species [6]. It is important to observe that TLR1, TLR2, and TLR6 are the main innate immune receptors for recognition of gram-positive bacteria. For instance TLR2, as a homodimer, or in heterodimeric combination with either TLR1 or TLR6, recognizes cell wall components of *S. aureus* [11, 12].

All these cSSSI-associated polymorphisms have been previously linked to infectious disease. The *TLR1* polymorphism S248N has been demonstrated to result in increased susceptibility to gram-positive infection in sepsis [25], candidemia [21] and leprosy [22]. The other *TLR1* polymorphism R80T is associated with pancolitis [35], invasive aspergillosis after hematopoietic stem cell transplantation [18], and candidemia [21]. Furthermore, the *TLR2* polymorphism P631H is significantly overrepresented in patients with tuberculosis, compared with contact controls [36], whereas the *TLR6* polymorphism P249S is associated with malaria [37] and invasive aspergillosis [18].

In addition to the genetic association between PRR polymorphisms and cSSSIs, we also studied the functional consequences of these polymorphisms. Presence of the *TLR1* 248N or 80T allele results in significantly lower IL-6 secretion, and a similar trend was observed for the *TLR6* 249S allele. IL-6 blockade by tocilizumab has been recently related to staphylococcal infections [38], illustrating the potential relevance of the effects of *TLR1* SNPs on IL-6 production. No differences in IL-6 response were detected in groups stratified for the *TLR2* polymorphism P631H, most likely because of the rare occurrence of heterozygotes and the absence of homozygotes for the allelic variant.

In a recent paper by Ben-Ali *et al.*, functional characterization of genetic variants in the TLR1-2-6 family were studied by transfection of various TLR constructs in HEK 293T cells containing a NF-κB luciferase reporter construct. Transfection of alleles *TLR1* 248N or 80T, *TLR2* 631H and *TLR6* 249S all led to decreased NF-κB activation, compared to their ancestral variant, supporting our findings on the functional consequences of these polymorphisms. In addition, computational PolyPhen algorithm studies to predict the impact of nonsynonymous variants suggested that the *TLR2* polymorphism P631H is a damaging mutation, whereas the *TLR1* polymorphisms S248N and R80T, as well as the *TLR6* polymorphism P249S, were classified as benign for protein function [39].

This study showed that polymorphisms *TLR1* S248N and *TLR1* R80T, *TLR1* S248N and *TLR6* P249S, and *TLR1* R80T and *TLR6* P249S were in LD, although the strength of their correlation was observed to be low. In contrast, the *TLR1* polymorphism S248N is in strong linkage with the nonsynonymous *TLR1* polymorphism I602S (rs5743618; not studied). This polymorphism is associated with impaired trafficking of the receptor to the cell surface, leading to decreased cell membrane expression of TLR1 [25]. These findings may imply that the polymorphism I602S is causing the functional effects observed.

Presence of the *TLR2* 631H allele has been shown to decrease internalization of the TLR2 complex from the plasma membrane after recognition of its ligand [36]. Although internalization of the wild-type TLR2 complex is not necessary for intracellular signaling [40], one can expect that inhibition of internalization by this polymorphism still could modulate functional responses as described by reduced NF-κB activation [39].

Although the *TLR2* R753Q, *TLR4* D299G and T399I, *NOD2* P268S, and *TIRAP* S180L polymorphisms were previously associated with infectious disease, no correlation between these polymorphisms and susceptibility to cSSSIs was observed here. The absence of an effect of these polymorphisms could in part be due to the type of pathogens present in patients with cSSSI, of which the majority are gram-positive bacteria.

To our knowledge this is the first study investigating the role of SNPs in PRR genes in disease susceptibility to cSSSIs. Unfortunately, a confirmation cohort was not available and future studies should validate the findings reported here.

In conclusion, this study demonstrates that 4 polymorphisms in genes *TLR1*, *TLR2* and *TLR6* are associated with an increased susceptibility to cSSSIs. These infections are predominantly caused by gram-positive bacteria, in which specifically these TLRs are of major importance for innate immune recognition. Functional characterization showed impaired TLR function and decreased cytokine responses. Although gram-positive bacterial infection and impaired TLR function strengthen the genetic associations observed, further studies are warranted to elucidate whether characterization of a predictive profile using TLR polymorphisms in *TLR1*, *TLR2* and *TLR6* can play a beneficial role for individuals at risk of developing cSSSIs.

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Chapter 5

Genetic variation in *TLR10*, an inhibitory Toll-like receptor, influences susceptibility to complicated skin and skin structure infections

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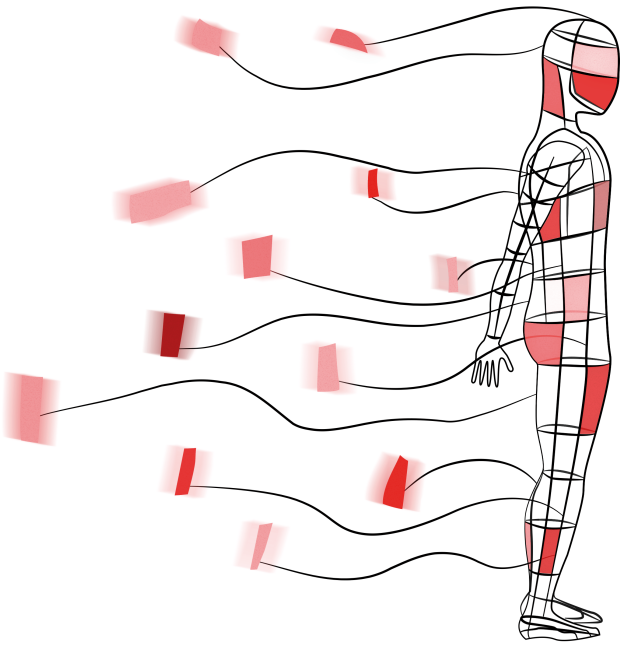

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Abstract

Background. Toll-like receptors (TLRs) play a central role in the innate immune response to complicated skin and skin structure infections (cSSSIs), with TLR10 being the first family member known to have an inhibitory function. This study assessed the role of TLR10 in recognition of cSSSI-related pathogens and whether genetic variation in *TLR10* influences susceptibility to cSSSIs.

Methods. Human peripheral blood mononuclear cells (PBMCs) preincubated with anti-TLR10 antibody and HEK-293 cells overexpressing TLRs were exposed to cSSSI pathogens, and cytokine secretion was determined by enzyme-linked immunosorbent assay. A total of 318 patients with cSSSI and 328 healthy controls were genotyped for 4 nonsynonymous single-nucleotide polymorphisms in *TLR10*, and functional consequences of the *TLR10* SNPs were assessed via in vitro stimulation assays.

Results. PBMC stimulation with cSSSI pathogens in the presence of TLR10 neutralizing antibody significantly increased interleukin 6 (IL-6) secretion. Overexpression of TLR10 completely abrogated TLR2-induced IL-8 secretion of HEK-293 cells in response to cSSSI pathogens. Three polymorphisms in *TLR10*, I775L, I369L and N241H, were associated with reduced susceptibility to cSSSIs. The presence of *TLR10* alleles 775L, 369L or 241H increased IL-6 secretion by PBMCs in response to cSSSI pathogens.

Conclusions. TLR10 is a modulatory receptor of innate immune responses to cSSSI-related pathogens, and genetic variants in *TLR10* are associated with protection against cSSSIs.

Introduction

Complicated skin and skin structure infections (cSSSIs) were termed by the Food and Drug Administration as infections involving the deeper soft tissue that require significant surgical intervention, such as major abscesses, diabetic foot infections, surgical-site and trauma-induced wound infections, and infections in individuals with a significant underlying disease state that complicates the response to treatment. Also included are infections located in an anatomical site in which the chance of involvement of anaerobic or gram-negative pathogens is high [1, 2]. Gram-positive bacteria such as *Staphylococcus aureus* (*S. aureus*) and β-hemolytic streptococci are the predominant causative organisms, but presence of gram-negative bacteria (e.g. *Escherichia coli*) and anaerobes (e.g. *Bacteroides fragilis*), as well as a polymicrobial etiology, is frequent [2-4].

Cells of the innate immune system recognize pathogen-associated molecular patterns (PAMPs) on the surface of invading microorganisms through distinct families of pattern-recognition receptors (PRRs). Toll-like receptors (TLRs) are the best-documented family of PRRs and 10 members have been characterized in humans [5]. In general, binding of ligands to TLRs results in interaction with downstream adaptor proteins and activation of transcription factors, followed by the production of inflammatory cytokines and chemokines and induction of an immune response.

TLRs are essential in the host defense against cSSSI pathogens. Cell wall components of gram-positive bacteria such as *S. aureus* are ligands for TLR2, in combination with TLR1 and TLR6 [6]. Malfunction of TLRs may lead to an inappropriate overactivation or underactivation of the immune response, resulting in the development of cSSSIs. Our group has recently observed that genetic variants in *TLR1*, *TLR2* and *TLR6* were associated with an increased susceptibility to cSSSIs. In addition, functional studies of these genetic variants showed an effect on interleukin 6 (IL-6) secretion by peripheral blood mononuclear cells (PBMCs) after exposure to *S. aureus* [7]. *TLR1*, *TLR2*, and *TLR6* form a cluster on chromosome 4, together with *TLR10*.

First described by Chuang and Ulevitch in 2001, TLR10 was found to be closely related to TLR1, TLR6, and to a lesser extent, TLR2 (50%, 49%, and 30% amino acid identity, respectively) [8]. TLR10 was predominantly expressed in lymphoid tissues including spleen, lymph node, thymus and tonsil specimens [8]. Expression of TLR10 has mainly been observed in immune cells, such as B cells, dendritic cells, monocytes, neutrophils, and eosinophils [9-13], but also in nonimmune cells such as trophoblasts [14]. TLR10, similar to TLR1 and TLR6, forms heterodimers with TLR2 [9, 15, 16] but differs in its lack of downstream signaling [17]. Recent studies suggest TLR10 is an unusual PRR, with mainly

inhibitory functions on TLR2-driven immune responses [17-20], whereas its involvement in the recognition of cSSSI pathogens is unknown.

Genetic variation in the *TLR10* gene has previously been associated with inflammatory diseases [21-23] and cancer [24-26], while its role in susceptibility to cSSSIs and consequences to cSSSI pathogen-induced immune responses remain to be elucidated.

In this study, we hypothesized that TLR10 influences recognition of cSSSI pathogens and that genetic variation in *TLR10* may result in an altered immune response to cSSSI pathogens, hence influencing susceptibility to infection.

Materials and methods

Subjects and blood samples

A total of 646 white East European individuals were included in our association study, among whom were 318 patients with cSSSI from a randomized, multicenter clinical trial [27] (subtype/diagnosis: major abscess, diabetic foot infection, wound infection or infected ischemic ulcer) and 328 healthy controls recruited from the community. Detailed information on inclusion criteria in the study cohort are described elsewhere [7]. In addition, 74 white, healthy Dutch individuals were recruited from the community to correlate their genotype with IL-6 secretion upon stimulation of PBMCs. All subjects gave informed consent for genetic analysis. PBMCs were isolated from buffy coats of healthy individuals for experiments regarding antibody blocking of TLR10.

In vitro PBMC stimulation assays

PBMC isolation and stimulation assays were performed as described earlier [28], with minor modifications. PBMCs were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) from venous blood. Next, PBMCs were washed twice with phosphate-buffered saline (pH=7.4) and resuspended in Roswell Park Memorial Institute 1640 Dutch modified culture medium (supplemented with 2 mM L-glutamine, 1 mM pyruvate and 50 µg/mL gentamicin; GIBCO Invitrogen, Carlsbad, CA, USA) for experiments.

PBMCs (5 x 10^5 cells) were added to 96-well round-bottom plates (Greiner, Nurnberg, Germany) in the presence of either 1 x 10^6 colony-forming units/mL of heat-killed *S. aureus* ATCC 29213, *Bacteroides fragilis* NCTC 10584 or ATCC 25285, *Escherichia coli* ATCC 25922 or 10 µg/mL TLR2 ligand Pam_3Cys (EMC Microcollections, Tübingen, Germany) in a final volume of 200 µL. After incubation for 24 hours at 37°C and 5% CO_2, supernatants were collected and stored at -20°C. For the TLR10 blocking experiments, PBMCs were preincubated for 1

hour with 10 µg/mL anti-TLR10 antibody (Novus Biologicals, Cambridge, United Kingdom) or IgG1κ isotype control (R&D Systems, Minneapolis, MN, USA) before exposure to cSSSI pathogens or Pam_3Cys.

Transfection and stimulation of human embryonic kidney (HEK)-293 cells

Transfection of HEK-293 cells with human *TLR10*, *TLR2* or both was performed as previously described [18, 20]. HEK-293 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 7.5% fetal bovine serum (Hyclone, Thermo Scientific, Logan, UT, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen), at 37°C and 5% CO_2. The plasmid-selecting agents G418 (1 mg/mL; Sigma-Aldrich, St Louis, MO, USA) for *TLR2*, blasticidin (5 µg/mL; Invivogen) for *TLR10* and a combination of both for *TLR2/10* were added to the culture medium to ensure presence of these specific TLRs in HEK-293 cells. Correct TLR expression was confirmed by reverse transcription-polymerase chain reaction (PCR) and flowcytometry. When 80% confluency was reached, the HEK-293 cells were passaged and used in stimulation experiments. Nontransfected, *TLR10*-transfected, *TLR2*-transfected, and *TLR2/10*-transfected HEK-293 cells (1×10^6) were added to 96-well flat-bottom plates (Greiner) in the presence of either 1×10^7 cfu/mL of heat-killed *S. aureus* ATCC 29213, *B. fragilis* ATCC 25285, *E. coli* ATCC 25922, or TLR ligands Pam_3Cys (TLR2; 1 µg/mL; EMC Microcollections) and lipopolysaccharide (LPS; TLR4; 10 ng/mL; Sigma-Aldrich) in a final volume of 200 µL. After incubation for 24 hours at 37°C and 5% CO_2, supernatants were collected and stored at -20°C.

Cytokine detection assays

IL-6 secretion was selected as the primary read-out cytokine after PBMCs stimulation because it is crucial in the response against cSSSI pathogens, as blockage of IL-6 by tocilizumab has been recently related to staphylococcal infections [29]. In supernatants from the HEK-293 cells IL-8 was determined. IL-6 secretion and IL-8 secretion were determined in supernatants by commercially available enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer's protocol (Sanquin Reagents, Amsterdam, The Netherlands).

Genotyping of *TLR10* polymorphisms

Genomic DNA from patients with cSSSI was isolated using the PLUS XL manual kit (LGC genomics GmbH, Berlin, Germany). Genomic DNA from healthy controls was isolated using the Gentra Pure Gene Blood kit (Qiagen). Four nonsynonymous *TLR10* single-nucleotide polymorphisms (SNPs; Table 1), with previously described associations with human diseases and a minor allele frequency of at least 5% among different populations, were selected from the National Center for Biotechnology Information SNP database. *TLR10* gene fragments were amplified using commercially available TaqMan SNP

genotyping assays according to manufacturer's protocol on a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Quality control of the assay was warranted by incorporating positive and negative controls and duplicating random samples across different plates. Correct assessment of *TLR10* SNPs by Taqman SNP genotyping assays was validated by whole-exome sequencing on a selection of the cohort of healthy, white, Dutch individuals.

Table 1. Genotyped nonsynonymous single-nucleotide polymorphisms (SNPs) in *TLR10*.

Gene	SNP ID	Mutation	Nucleotide change[#]	Amino acid change[#]	Taqman Assay ID
TLR10	rs4129009	Missense	A > C	I775L	C____309084_10
TLR10	rs11096955	Missense	A > C	I369L	C____309086_10
TLR10	rs11096957	Missense	A > C	N241H	C____309088_10
TLR10	rs11466653	Missense	T > C	M326T	C__25643406_10

[#] The first nucleotide (and corresponding amino acid) is the ancestral and therefore is considered the wild-type allele. ID, identification number.

Statistical analysis

Chi-square analysis of deviation from Hardy-Weinberg equilibrium (HWE) were performed for all 4 *TLR10* SNPs in healthy controls, using a web-based HWE calculator [30]. The association between susceptibility to cSSSIs and a SNP were investigated by means of univariate logistic regression models with IBM SPSS 18 software (IBM, Armonk, NY, USA). SNPs were evaluated using a dominant model analyses and P-values of <0.05 were considered to be statistically significant. Odds ratios including 95% confidence intervals were reported for these tests of association. Pairwise linkage disequilibrium (LD), D' and r^2, were calculated using IBM SPSS 18 software.

The statistical analyses and graphical presentation of the cytokine concentrations in the PBMC and HEK-293 cells stimulation assays were performed using GraphPad Prism 5.00 software (GraphPad Software, La Jolla, CA, USA).

Results

Blocking TLR10 increases IL-6 secretion by human PBMCs after exposure to cSSSI pathogens

To determine the biological function of TLR10 in the innate immune response to cSSSI pathogens, human PBMCs were preincubated with TLR10 neutralizing antibody or isotype control followed by stimulation with heat-killed *S. aureus*, *B. fragilis*, *E. coli*, or TLR1/2 ligand Pam_3Cys (Figure 1). Blocking of TLR10 significantly increased IL-6 secretion after exposure to Pam_3Cys (P=0.0078), *S. aureus* (P=0.0195), and *B. fragilis* (P=0.0039) and approached

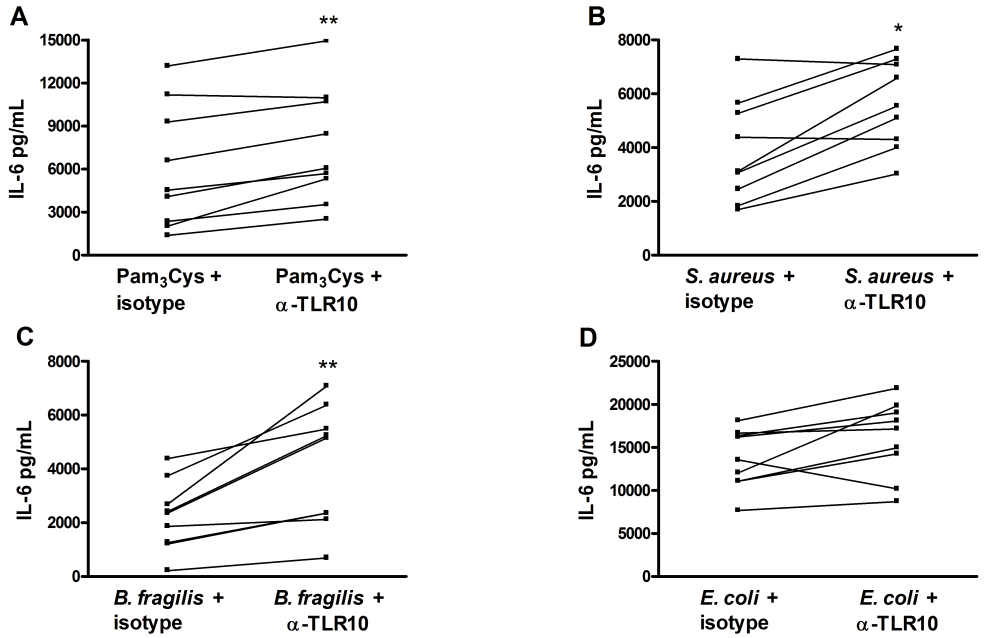

Figure 1. Blocking Toll-like receptor 10 (TLR10) increases interleukin 6 (IL-6) secretion in human peripheral blood mononuclear cells (PBMCs) after exposure to common complicated skin and skin structure (cSSSI) pathogens. PBMCs (5 x 10^6/mL) were preincubated for 1 hour with 10 µg/mL anti-TLR10 (α-TLR10) antibody or isotype control, followed by incubation for 24 hours with 10 µg/mL Pam_3Cys (A), 1 x 10^6 colony-forming units/mL heat-killed cSSSI pathogens *Staphylococus aureus* ATCC 29213 (B), *Bacteroides fragilis* ATCC 25285 (C), *Escherichia coli* ATCC 25922 (D). IL-6 secretion was determined in supernatants by an enzyme-linked immunosorbent assay. Data are presented of 9 different buffy-coats performed in 3 separate experiments. *P<0.05, and **P<0.01, by the Wilcoxon signed-rank test.

significance after *E. coli* stimulation (P=0.0547). No differences were observed for PBMCs exposed to TLR4 ligand LPS in the presence or absence of TLR10 neutralizing antibody (Supplementary figure 1).

Overexpression of TLR10 abrogates TLR2-mediated cytokine secretion in HEK-293 cells after exposure to cSSSI pathogens

To investigate how TLR10 exerts its inhibitory effect on the recognition of cSSSI pathogens, HEK-293 cells were transfected with human *TLR2*, *TLR10* or both *TLR2* and *TLR10*. All combinations were cultured for 24 hours in the presence of the PRR ligands Pam_3Cys (TLR1/2) and LPS (TLR4) and cSSSI pathogens *S. aureus*, *B. fragilis* and *E. coli*. Thereafter, IL-8 secretion was determined in supernatant by ELISA (Figure 2). Unstimulated cells did not induce significant levels of IL-8. Nontransfected HEK-293 cells or transfected with only *TLR10* were unable to induce significant IL-8 secretion in response to any of the stimuli (Figure 2). HEK-293 cells transfected with *TLR2* alone did not respond to LPS but demonstrated a strong induction of IL-8 secretion in the presence Pam_3Cys, as well as cSSSI

pathogens *S. aureus*, *B. fragilis* and *E. coli* (Figure 2). In contrast, the presence of *TLR2* in combination with *TLR10* resulted in a significant reduction of IL-8 secretion after exposure to Pam_3Cys (P=0.0022) and cSSSI pathogens *S. aureus* (P=0.0050), *B. fragilis* (P=0.0022) and *E. coli* (P=0.0022) in HEK-293 cells (Figure 2).

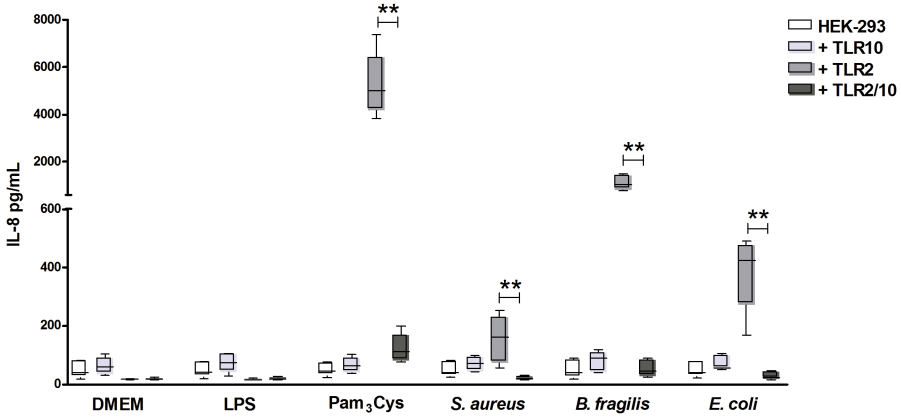

Figure 2. Overexpression of Toll-like receptor 10 (TLR10) abrogates TLR2-mediated cytokine secretion in human embryonic kidney (HEK)-293 cells after exposure to common complicated skin and skin structure infection (cSSSI) pathogens. HEK-293 cells transfected with *TLR10*, *TLR2*, or both were stimulated for 24 hours with TLR ligands lipopolysaccharide (LPS; 10 ng/mL), Pam_3Cys (1 µg/mL) or 1 x 10^7 colony-forming units/mL of the heat-killed cSSSI pathogens *Staphylococcus aureus* ATCC 29213, *Bacteroides fragilis* ATCC 25285, or *Escherichia coli* ATCC 25922. Interleukin 8 (IL-8) secretion was determined in supernatants by an enzyme-linked immunosorbent assay. Boxes represent median values and interquartile ranges; whiskers represent minimum and maximum values from 6 separate experiments. **P<0.01, by Mann-Whitney U test.

Table 2. Distribution of *TLR10* genotypes in 318 patients with complicated skin and skin structure infection (cSSSI) and 328 healthy controls.

Polymorphism	Wild-type, No. (%)	Heterozygous, No. (%)	Homozygous, No. (%)	P-value[#]	Odds ratio (95% CI)[#]
TLR10 I775L	AA	AC	CC	0.002	0.603 (0.438-0.831)
Patients with cSSSI	214 (67.3)	89 (28.0)	15 (4.7)		
Healthy controls	180 (55.4)	119 (36.6)	26 (8.0)		
TLR10 I369L	AA	AC	CC	0.019	0.676 (0.487-0.937)
Patients with cSSSI	123 (38.7)	145 (45.6)	50 (15.7)		
Healthy controls	98 (29.9)	156 (47.6)	74 (22.6)		
TLR10 N241H	AA	AC	CC	0.019	0.676 (0.487-0.937)
Patients with cSSSI	123 (38.7)	145 (45.6)	50 (15.7)		
Healthy controls	98 (29.9)	156 (47.6)	74 (22.6)		
TLR10 M326T	TT	TC	CC	0.174	0.667 (0.370-1.200)
Patients with cSSSI	298 (93.7)	20 (6.3)	0 (0.0)		
Healthy controls	298 (90.9)	29 (8.8)	1 (0.3)		

[#] Data are based on a dominant model analysis. CI, confidence interval.

Genetic variation in *TLR10* is associated with reduced susceptibility to cSSSIs

Data from 318 patients with cSSSI and 328 healthy controls were included to assess the role of *TLR10* polymorphisms I775L, I369L, N241H, and M326T in susceptibility to cSSSIs (Table 2). All *TLR10* polymorphisms were in HWE for the healthy controls. LD analyses revealed that I369L and N241H were in complete linkage (P<0.0001, r^2=1.00, D'=1.00), whereas moderate LD was observed for I775L with I369L and N241H (P<0.0001, r^2=0.64, D'=0.82) and weak LD was observed for M326T with I775L (P<0.001, r^2=0.01, D'=0.47) and M326T with I369L and N241H (P<0.0001, r^2=0.25, D'=0.52).

Univariate logistic regression analysis indicated that 3 *TLR10* polymorphisms were associated with susceptibility to cSSSIs (Table 2): I775L (P=0.002) and I369L and N241H (P=0.019). For all 3 *TLR10* polymorphisms, patients with cSSSI were significantly less often heterozygous and homozygous for the allelic variant, compared with the healthy controls. The *TLR10* polymorphism M326T was not associated with susceptibility to cSSSIs (P=0.167).

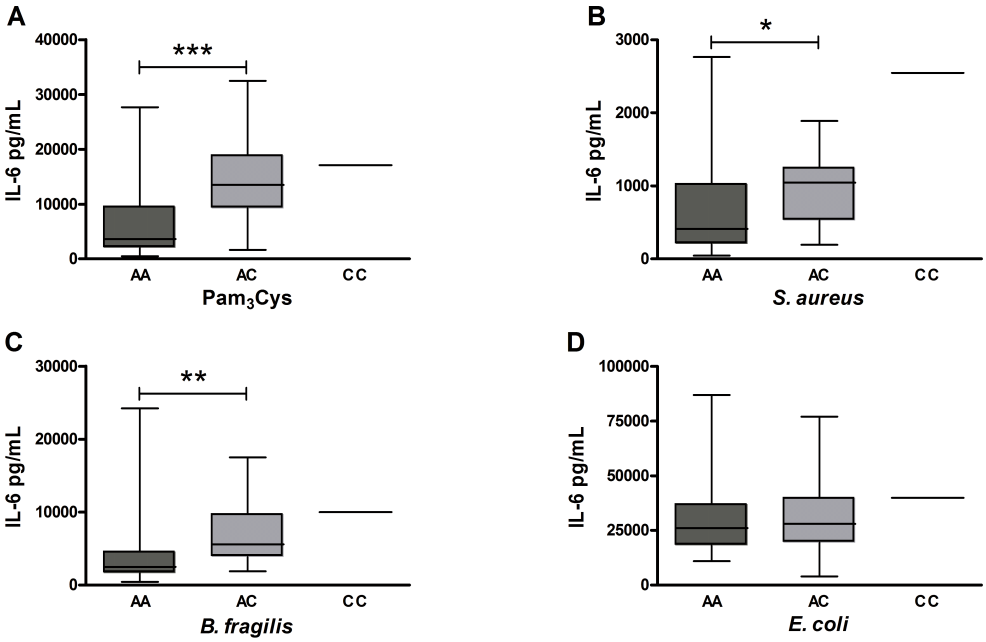

Figure 3. Correlation of the *TLR10* I775L genotype with interleukin 6 (IL-6) secretion by human peripheral blood mononuclear cells (PBMCs) after exposure for 24 hours to common complicated skin and skin structure infection (cSSSI) pathogens. PBMCs were exposed to 10 µg/mL Pam_3Cys (A), 1 x 10^6 colony-forming units/mL of the heat-killed cSSSI pathogens *Staphylococcus aureus* ATCC 29213 (B), *Bacteroides fragilis* NCTC 10584 (C), or *Escherichia coli* ATCC 25922 (D). Boxes represent the median values and interquartile ranges; whiskers represent minimum and maximum values. IL-6 secretion was determined in supernatants by an enzyme-linked immunosorbent assay, and results were stratified for the *TLR10* I775L genotype. Dark grey represents individuals with the AA genotype (n=47), light grey represents individuals with the AC genotype (n=22), and white represents individuals with the CC genotype (n=1). *P<0.05, **P<0.01, and ***P<0.001, by the Mann-Whitney U test.

TLR10 polymorphisms modulate immune response to cSSSI pathogens

To study the functional consequences of *TLR10* polymorphisms, PBMCs were obtained from an additional cohort of 74 healthy volunteers. PBMCs were exposed to cSSSI pathogens *S. aureus*, *B. fragilis*, and *E. coli* and the PRR ligands Pam_3Cys (TLR1/2) and LPS (TLR4). After 24 hours, IL-6 secretion was measured by ELISA and stratified by *TLR10* genotype (Figure 3-5). Unstimulated PBMCs did not induce detectable IL-6 secretion (data not shown). No differences were observed between genotypes for all *TLR10* polymorphisms after PBMC exposure to LPS (Supplementary figure 2). Heterozygosity for *TLR10* polymorphism I775L (AC; n=22) significantly increased IL-6 secretion, compared with the wild-type genotype (AA; n=47), after PBMC exposure to Pam_3Cys, *S. aureus*, and *B. fragilis*, and a similar trend was observed for *E. coli* stimulation. Homozygosity for *TLR10* polymorphism I775L (CC; n=1) induced even higher IL-6 secretion compared with the wild-type and heterozygous genotype after exposure to Pam_3Cys, *S. aureus*, *B. fragilis*, and *E. coli*, but only 1 volunteer carried this genotype (Figure 3).

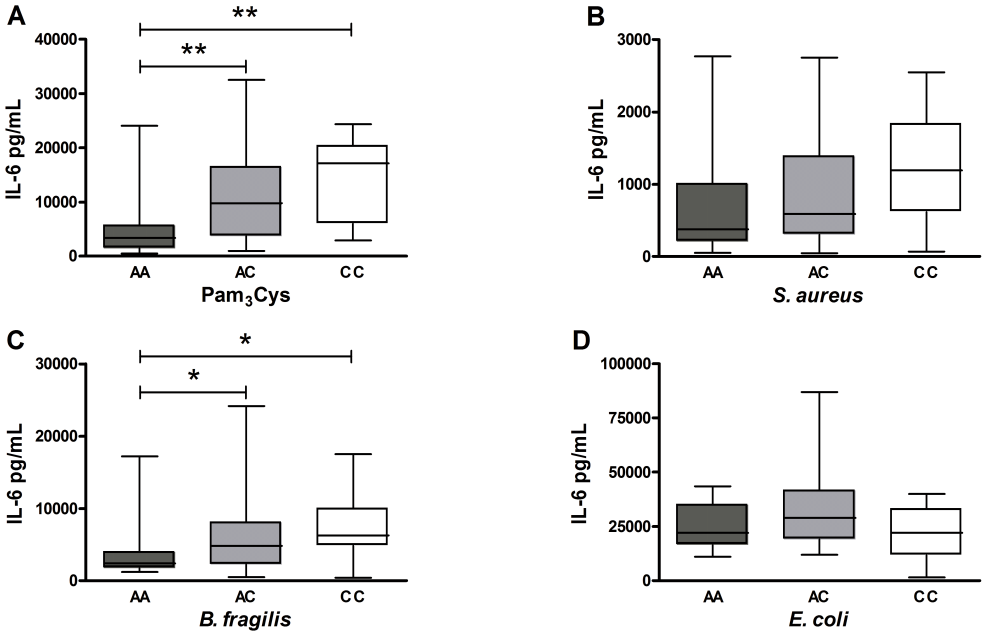

Figure 4. Correlation of the *TLR10* I369L/N241H genotype with interleukin 6 (IL-6) secretion by human peripheral blood mononuclear cells (PBMCs) after exposure for 24 hours to common complicated skin and skin structure infection (cSSSI) pathogens. PBMCs were exposed to 10 µg/mL Pam_3Cys (A), 1 x 10^6 colony-forming units/mL of the heat-killed cSSSI pathogens *Staphylococcus aureus* ATCC 29213 (B), *Bacteroides fragilis* NCTC 10584 (C), or *Escherichia coli* ATCC 25922 (D). Boxes represent the median values and interquartile ranges; whiskers represent minimum and maximum values. IL-6 secretion was determined in supernatants by an enzyme-linked immunosorbent assay, and results were stratified for the *TLR10* I369L/N241H genotype. Dark grey represents individuals with the AA genotype (n=26), light grey represents individuals with the AC genotype (n=38), and white represents individuals with the CC genotype (n=9). *P<0.05, **P<0.01, ***P<0.001, by Mann-Whitney U test.

The *TLR10* polymorphisms I369L and N241H were also in complete linkage for the cohort of 74 healthy volunteers, and therefore results were combined in Figure 4. Homozygosity (CC/CC; n=9) and heterozygosity (AC/AC; n=38) for *TLR10* polymorphism I369L/N241H significantly increased IL-6 secretion, compared with the wild-type genotype (AA/AA; n=26) after exposure to Pam_3Cys and *B. fragilis*. A similar increasing trend was observed for *S. aureus* and *E. coli* stimulation (Figure 4).

No differences in IL-6 secretion were detected between heterozygous and wild-type genotypes for *TLR10* polymorphism M326T after PBMC exposure to Pam_3Cys, *S. aureus*, *B. fragilis* and *E. coli*. In addition, no homozygotes for *TLR10* polymorphism M326T were present among the 74 individuals (Figure 5). Secretion of IL-1β and IL-8 by PBMCs showed similar patterns as IL-6 for all *TLR10* polymorphisms (Supplementary figures 3 and 4).

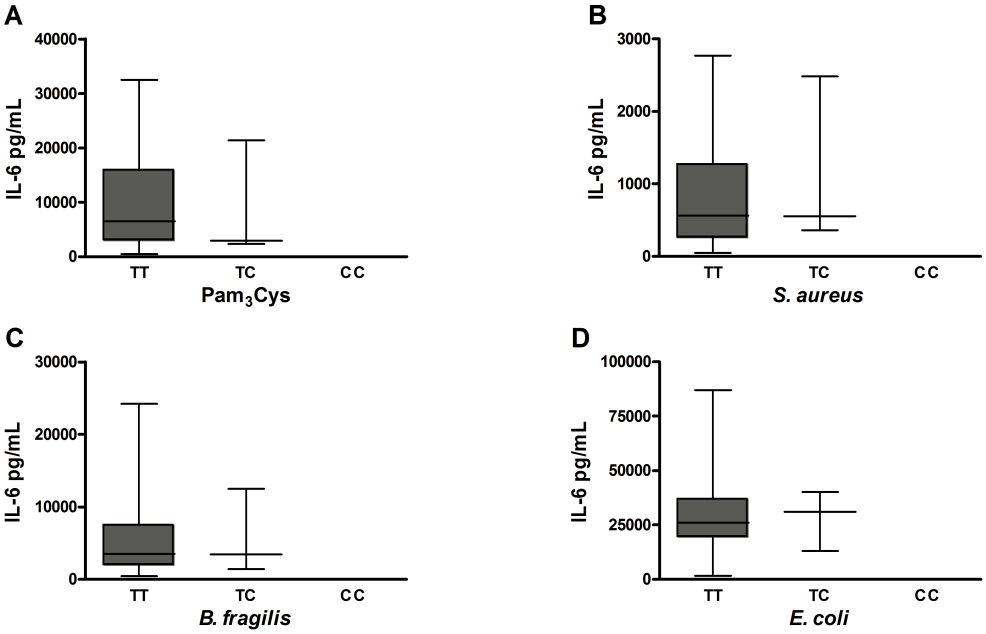

Figure 5. Correlation of the *TLR10* M326T genotype with interleukin 6 (IL-6) secretion by human peripheral blood mononuclear cells (PBMCs) after exposure for 24 hours to common complicated skin and skin structure infection (cSSSI) pathogens. PBMCs were exposed to 10 μg/mL Pam_3Cys (A), 1 x 10^6 colony-forming units/mL of the heat-killed cSSSI pathogens *Staphylococcus aureus* ATCC 29213 (B), *Bacteroides fragilis* NCTC 10584 (C), or *Escherichia coli* ATCC 25922 (D). Boxes represent the median values and interquartile ranges; whiskers represent minimum and maximum values. IL-6 secretion was determined in supernatants by an enzyme-linked immunosorbent assay, and results were stratified for the *TLR10* M326T genotype. Dark grey represents individuals with the TT genotype (n=70), light grey represents individuals with the TC genotype (n=3). Data were analyzed by Mann-Whitney U test.

Discussion

The present study demonstrates that TLR10 is an inhibitory receptor of TLR2-driven immune activation by the common cSSSI pathogens *S. aureus*, *E. coli*, and *B. fragilis*. Genetic variants I775L, I369L, and N241H in *TLR10* were significantly associated with a reduced susceptibility to cSSSIs, and the presence of the *TLR10* alleles 775L, 369L or 241H increased cytokine secretion in response to cSSSI pathogens.

Since its discovery, much effort has been put in delineating the ligand and function of TLR10. Coimmunoprecipitation and molecular modeling studies have repeatedly suggested TLR10's ability to form homodimers and heterodimers with TLR2, but also combinations with TLR1 and TLR6 have been described [9, 15-17]. Downstream, TLR10 was found to associate with MyD88 [9], but it failed to activate typical TLR signaling pathways via nuclear factor-κB [17]. Molecular modeling of the TLR10/2 complex suggested that Pam_3Cys might be the ligand [16]. To study the involvement of TLR10 in the recognition of cSSSI pathogens, we first neutralized the function of TLR10 on human PBMCs following exposure to cSSSI pathogens or Pam_3Cys and observed increased IL-6 secretion. Second, we overexpressed TLR2, TLR10, or both in HEK-293 cells, to model the responses of primary immune cells. TLR10 abrogated the TLR2-mediated cytokine secretion after exposure to cSSSI pathogens. Similar effects for TLR10 were observed after exposure to TLR1/2 ligand Pam_3Cys, TLR6/2 ligand FSL-1 and pathogens *Borrelia burgdorferi* and *Yersinia pestis* [18, 20]. These results were further underscored by in vivo experiments using human TLR10 transgenic (TLR10tg) mice, as mice do not have a functional TLR10 [17]. TLR10tg mice injected intraperitoneally with Pam_3Cys produced lower cytokine levels than their wild-type counterparts, confirming the suppressive effect of TLR10 [20].

In contrast to the inhibitory function reported here, 2 recent studies have suggested that TLR10 acts as a stimulatory receptor in the innate immune recognition of *Listeria monocytogenes* and influenza virus [31, 32]. Possible reasons for the dissimilarity might lay in different engagement of the TLR10 receptor by these pathogens, or differences in the experimental setup. Both studies mainly base these conclusions on experiments involving small interfering RNA (siRNA), using epithelial and macrophage cell lines, which hampers a direct comparison with our findings. Inhibition of TLR10 expression in primary human cells using a siRNA approach has previously been shown to have varying efficiency, but this approach also revealed upregulation of IL-6 secretion by TLR10-silenced macrophages, confirming our results [20].

All 4 studied *TLR10* polymorphisms have previously been associated with other diseases. *TLR10* polymorphisms I775L, I369L, and N241H all influenced susceptibility to prostate

cancer [24, 25] and Crohn's disease [23]. In addition, *TLR10* polymorphism I775L was associated with susceptibility to asthma [21, 22]. *TLR10* polymorphism I369L was also associated with susceptibility to Ménière's disease [33] and sarcoidosis [34], whereas *TLR10* polymorphism M326T was only found to be associated with small tumor size of papillary thyroid carcinoma [26]. We found *TLR10* polymorphisms I775L, I369L, and N241H but not M326T to be significantly associated with susceptibility to cSSSIs. For all 3 associated *TLR10* polymorphisms, healthy controls were more often heterozygous and homozygous for the allelic variant (775L, 369L or 241H), compared with patients with cSSSI, indicating a reduced risk of cSSSIs. Similar findings were reported in most other *TLR10* association studies, and the type of disease possibly explains findings in which this was not the case.

Genetic variation in the *TLR10* gene may modulate the balance between the proinflammatory and antiinflammatory responses and as such explain the influence on susceptibility to cSSSIs. The presence of the *TLR10* alleles 775L, I369L or 241H increased IL-6 secretion after exposure to the cSSSI pathogens *S. aureus*, *E. coli*, and *B. fragilis*. In line with our results, recent publications indicate the same genetic variants increased pro-inflammatory cytokine secretion in response to TLR1/2 ligand Pam_3Cys and pathogens *B. burgdorferi* (Lyme disease) and *Y. pestis* (plague) [18-20].

In conclusion, the present study demonstrates that TLR10 has a suppressive function on TLR2-driven immune activation by cSSSI-related pathogens. Furthermore, genetic variation in the *TLR10* gene influences the susceptibility to cSSSIs and results in an impaired TLR10 function and enhanced immune response to cSSSI pathogens. Further studies are warranted to decipher the exact mechanism by which TLR10 induces its inhibitory effect and to elucidate the potential of these *TLR10* polymorphisms in risk assessment, individual diagnosis, and treatment for patients with cSSSI.

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We thank all patients, investigators, and study centers, for participating in the RELIEF clinical trial; Johanna Beekman (Bayer Healthcare, Berlin, Germany), for collection, preparation, and storage of patient blood samples for genotypic analysis; Liesbeth Bruckers (CenStat, Hasselt University, Hasselt, Belgium) and Rob ter Horst (Department of Internal Medicine, Radboud University Medical Center, Nijmegen, The Netherlands), for assistance with statistical analysis.

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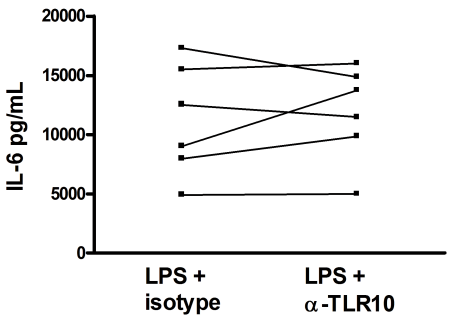
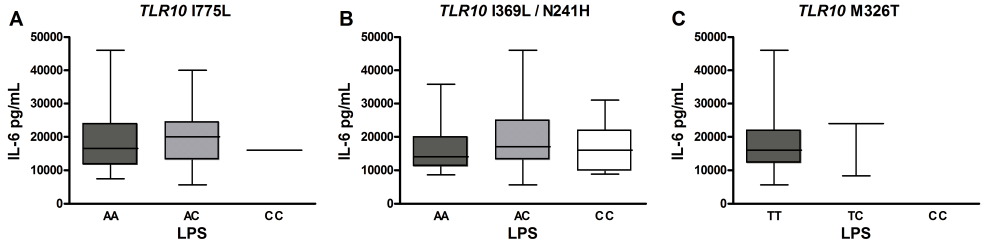
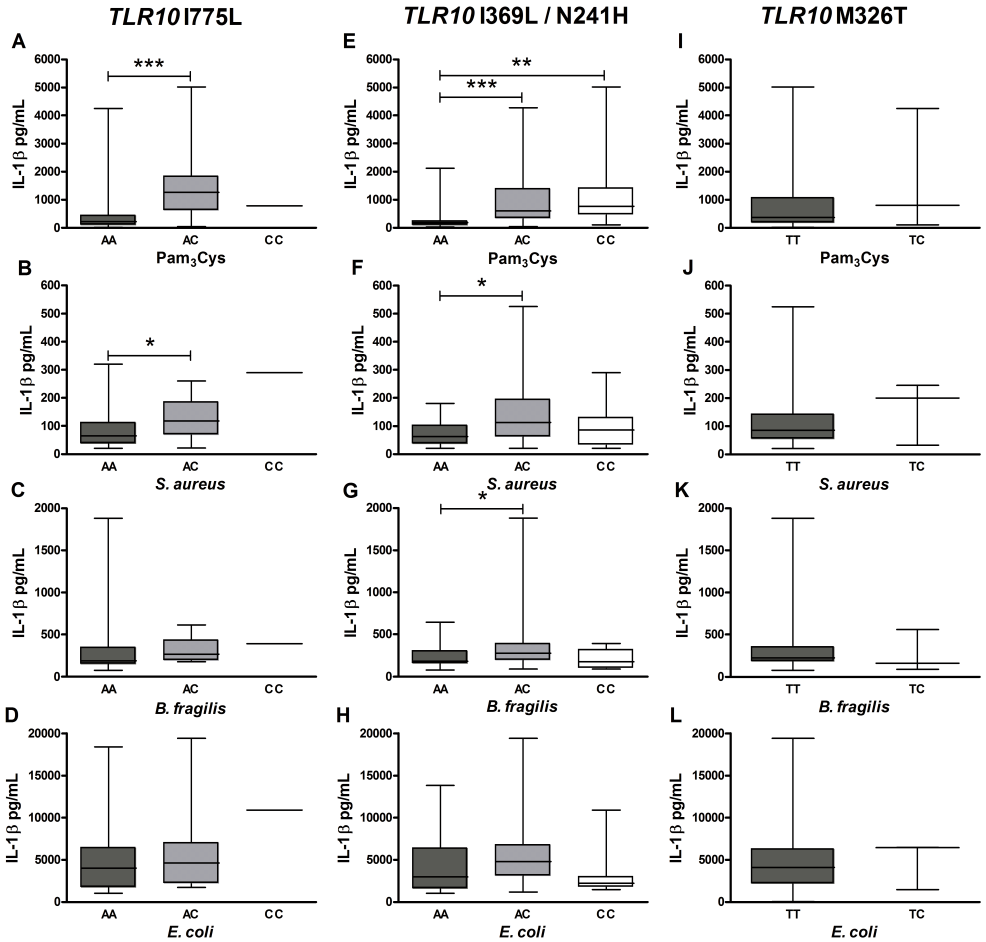
Supplementary figures



Supplementary figure 1. Blocking Toll-like receptor 10 (TLR10) does not influence interleukin 6 (IL-6) secretion in human peripheral blood mononuclear cells (PBMCs) after exposure to *Escherichia coli* lipopolysaccharide (LPS). PBMCs (5 x 10^6/mL) were preincubated for 1 hour with 10 µg/mL anti-TLR10 (α-TLR10) antibody or isotype control, followed by incubation for 24 hours with *E. coli* LPS (10 ng/mL). IL-6 secretion was determined in supernatants by an enzyme-linked immunosorbent assay. Data are presented of 6 different buffy coats performed in 3 separate experiments. Data were analyzed by Wilcoxon signed-rank test.

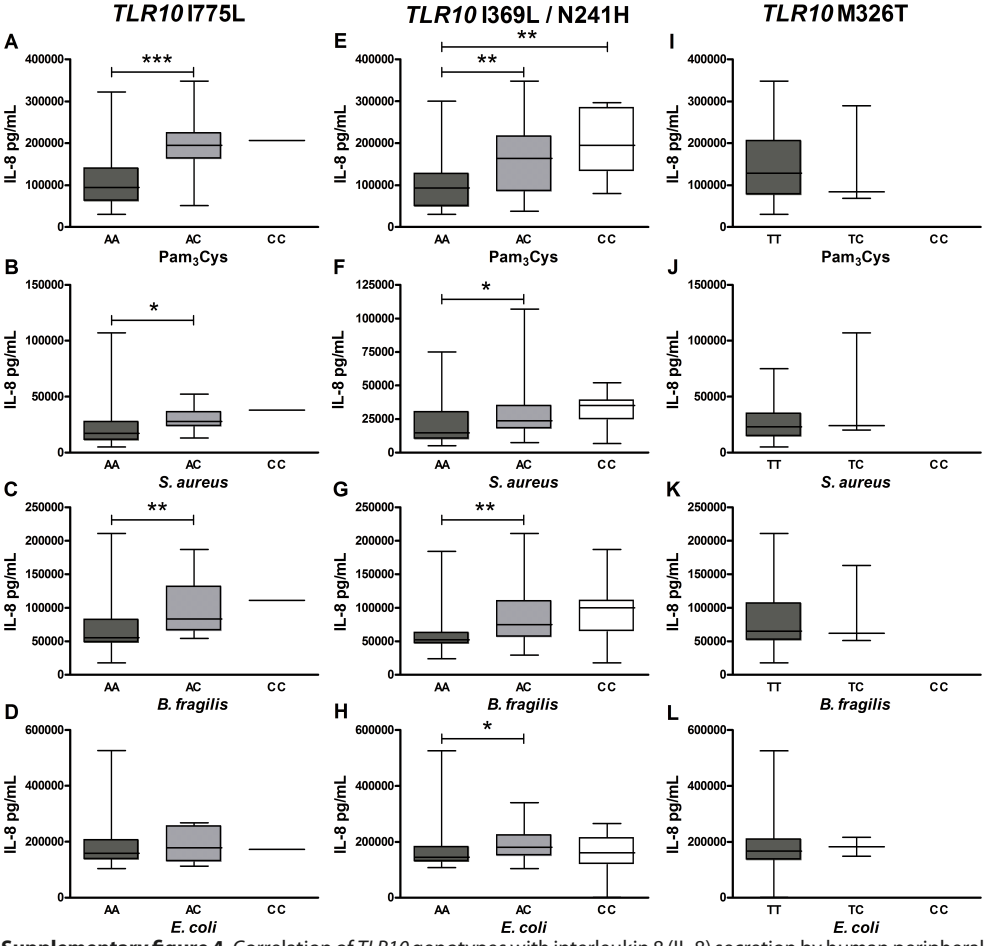


Supplementary figure 2. Correlation of *TLR10* genotypes with interleukin 6 (IL-6) secretion by human peripheral blood mononuclear cells (PBMCs) after exposure for 24 hours to *Escherichia coli* lipopolysaccharide (LPS; 10 ng/mL). Boxes represent the median values and interquartile ranges; whiskers represent minimum and maximum values. IL-6 secretion was determined in supernatants by enzyme-linked immunosorbent assay and the results were stratified for *TLR10* genotype; *TLR10* I775L, AA (n=47), AC (n=22), and CC (n=1) (A); *TLR10* N241H/I369L, AA (n=26), AC (n=38) and CC (n=9) (B); *TLR10* M326T, TT (n=70), TC (n=3) (C). Data were analyzed by Mann-Whitney U test.



Supplementary figure 3. Correlation of *TLR10* genotypes with interleukin 1β (IL-1β) secretion by human peripheral blood mononuclear cells (PBMCs) after exposure for 24 hours to common complicated skin and skin

structure (cSSSI) pathogens. PBMCs were exposed to 10 µg/mL Pam_3Cys (A, E, I), 1 x 10^6 colony forming units/mL of the heat-killed cSSSI pathogens *Staphylococcus aureus* ATCC 29213 (B, F, J), *Bacteroides fragilis* NCTC 10584 (C, G, K), or *Escherichia coli* ATCC 25922 (D, H, L). Boxes represent the median values and interquartile ranges; whiskers represent minimum and maximum values. IL-1β secretion was determined in supernatants by an enzyme-linked immunosorbent assay and results were stratified for *TLR10* genotype; *TLR10* I775L, AA (n=47), AC (n=22) and CC (n=1) (A-D); *TLR10* I369L/N241H, AA (n=26), AC (n=38) and CC (n=9) (E-H); *TLR10* M326T, TT (n=70), TC (n=3) (I-L). *P<0.05, **P<0.01, ***P<0.001, by Mann-Whitney U test.



Supplementary figure 4. Correlation of *TLR10* genotypes with interleukin 8 (IL-8) secretion by human peripheral blood mononuclear cells (PBMCs) after exposure for 24 hours to common complicated skin and skin structure (cSSSI) pathogens. PBMCs were exposed to 10 µg/mL Pam_3Cys (A, E, I), 1 x 10^6 colony forming units/mL of the heat-killed cSSSI pathogens *Staphylococcus aureus* ATCC 29213 (B, F, J), *Bacteroides fragilis* NCTC 10584 (C, G, K), or *Escherichia coli* ATCC 25922 (D, H, L). Boxes represent the median values and interquartile ranges; whiskers represent minimum and maximum values. IL-8 secretion was determined in supernatants by an enzyme-linked immunosorbent assay and results were stratified for *TLR10* genotype; *TLR10* I775L, AA (n=47), AC (n=22) and CC (n=1) (A-D); *TLR10* I369L / N241H, AA (n=26), AC (n=38) and CC (n=9) (E-H); *TLR10* M326T, TT (n=70), TC (n=3) (I-L). *P<0.05, **P<0.01, ***P<0.001, by Mann-Whitney U test.

Chapter 6

Polymorphisms in cytokine genes *IL6*, *TNF*, *IL10*, *IL17A* and *IFNG* influence susceptibility to complicated skin and skin structure infections

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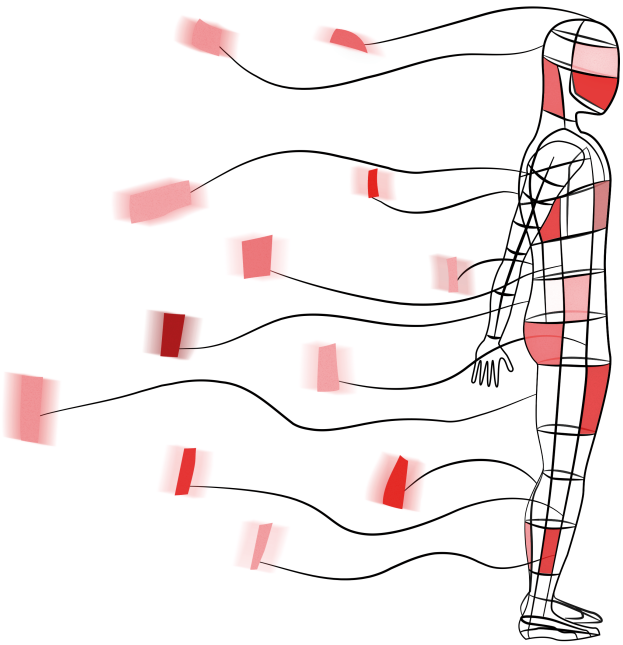

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Abstract

Complicated skin and skin structure infections (cSSSIs) are caused by gram-positive and gram-negative, aerobic and anaerobic pathogens, with a polymicrobial etiology being frequent. Recognition of invading pathogens by the immune system results in the production of pro- and anti-inflammatory cytokines, which are extremely important for intercellular communication and control of infection. This study assessed whether genetic variation in genes encoding cytokines influences the susceptibility to cSSSIs. For the association study, 318 patients with cSSSI and 328 healthy controls were genotyped for single-nucleotide polymorphisms (SNPs) in cytokine genes *IL1A*, *IL1B*, *IL1RN*, *TNF*, *IL10*, *IL17A*, *IL17F* and *IFNG*. For immunological validation, peripheral blood mononuclear cells (PBMCs) from 74 healthy individuals, genotyped for SNPs of interest, were stimulated with *Staphylococcus aureus* or *Escherichia coli* and corresponding cytokine levels were determined by enzyme-linked immunosorbent assay (ELISA). Polymorphisms *IL6* rs1800797, *TNF* rs1800629, *IL10* rs1800871, *IL17A* rs8193036 and *IFNG* rs2069705 influenced susceptibility to cSSSIs. No differences in cytokine responses, stratified for genotype, were detected after PBMC stimulation. No association with cSSSIs was observed for polymorphisms *IL1A* rs17561 and rs1800587, *IL1B* rs16944 and rs1143627, *IL1RN* rs4251961, *TNF* rs361525, *IL10* rs1800896, *IL17A* rs2275913 and *IL17F* rs763780. In conclusion, polymorphisms in *IL6*, *TNF*, *IL10*, *IL17A* and *IFNG* are associated with susceptibility to cSSSIs.

Introduction

Complicated skin and skin structure infections (cSSSIs) include infections involving the deeper soft tissue, requiring substantial surgical intervention, or occur in individuals with a significant underlying disease state that may affect the response to treatment [1]. Superficial infections located in an anatomical site in which the chance of involvement of anaerobic or gram-negative pathogens is high should also be considered cSSSIs. Predominant types of cSSSIs are major abscesses, diabetic foot infections, surgical-site and trauma-induced wound infections [1, 2]. Causative organisms, gram-positive or gram-negative, aerobic or anaerobic, alone or as part of a polymicrobial infection, vary depending on the clinical situation, location of the infection and medical background of the patient [1-3].

cSSSIs frequently lead to hospitalization and are associated with considerable morbidity, mortality and healthcare resource utilization [4]. The management of cSSSIs often requires a combination of surgical debridement and antibiotic therapy [2]. Due to increasing antimicrobial resistance, the latter is becoming a clinical challenge [5]. In addition, differences in prevalence and severity of cSSSIs cannot be solely explained by the virulence of a pathogen or co-morbidities of the patient, as even healthy individuals can develop severe skin and soft tissue infections due to organisms of the commensal microflora [6]. Hence, there is a need to identify host characteristics that predispose to the development of cSSSIs, in order to delineate a predictive profile for individuals at risk and possible individualized diagnosis and treatment for cSSSIs.

When a pathogen enters normally sterile parts of the body, cells of the innate immune system recognize pathogen-associated molecular patterns (PAMPs) on the surface of these invading microorganisms through pattern-recognition receptors (PRRs). Recognition by these receptors leads to activation of internal signaling pathways, resulting in the induction of pro- and anti-inflammatory cytokines, and finally the generation of an adaptive immune response [7]. Cytokines, small proteins that are produced by a variety of cells and which exert their effect through binding to specific cellular membrane receptors, are extremely important for intercellular communication and control of infection. Each pathogen induces the production of a variety of pro- and anti-inflammatory cytokines by immune cells. For example, gram-positive bacteria induce stronger stimulation of IFNγ, TNFα and IL-1β than gram-negative bacteria, which instead induce more IL-6 and IL-10 [8]. In addition to the beneficial effects on the activation of the host defense, the overproduction of cytokines can result in tissue damage that can have damaging effects to the host.

Genetic variation in cytokine genes is known to affect susceptibility to a variety of infectious diseases, such as bacterial sepsis [9, 10], mycobacterial infection [11, 12] and malaria [13], while their role in susceptibility to cSSSIs remains to be elucidated. In the present study, we hypothesized that polymorphisms in genes encoding cytokines may influence the host susceptibility to cSSSIs. To test this hypothesis, we analyzed the distribution of single-nucleotide polymorphisms (SNPs) in genes coding for cytokines in a cohort of patients with cSSSI and compared them with healthy controls. In order to determine the mechanistic effects for the observed genetic associations, functional assays were performed investigating the consequences of specific cytokine polymorphisms on the ability of human peripheral blood mononuclear cells (PBMCs) to produce cytokines after stimulation with cSSSI pathogens.

Materials and methods

Subjects

A total of 646 Eastern European individuals were included in the association study; 318 patients with cSSSI (subtype/diagnosis: major abscess, diabetic foot infection, wound infection or infected ischemic ulcer) from a randomized, multicentre clinical trial [6] and 328 healthy controls recruited from the community. Inclusion criteria in the study cohort are described elsewhere [14]. Eighty-nine percent of patients with suffered from community-acquired infections and the remaining 11% had nosocomial infections. Seventy percent of the patients with cSSSI had polymicrobial infections. In 97% of the infections, at least 1 gram-positive microorganism was isolated. The most prevalent pathogens were *Staphylococcus aureus*, β-hemolytic *Streptococcus* group A-G, *Enterococcus faecalis* and *Escherichia coli* [14]. An additional cohort of 74 European healthy individuals was recruited from the community to correlate the genotype with cytokine secretion upon stimulation of PBMCs. All subjects gave informed consent for genetic analysis.

Genotyping

DNA from patients with cSSSI was extracted from blood collected in PAXgene DNA tubes (Qiagen, Hildesheim, Germany) according to the PLUS XL manual kit (LGC genomics GmbH, Berlin, Germany). DNA from healthy individuals was isolated using the Gentra Pure Gene Blood kit (Qiagen), according to the manufacturer's protocol. SNPs of the analyzed cytokine genes were selected from the National Center for Biotechnology Information SNP database (http://www.ncbi.nlm.nih.gov/snp/) upon previously described associations with human diseases, located in either the exonic or 5' gene region, with a minor allele frequency of at least 5% among different populations. In total, 15 SNPs in *IL1A*, *IL1B*, *IL1RN*, *TNF*, *IL10*, *IL17A*, *IL17F* and *IFNG* were selected and genotyped (Table 1). Gene fragments were amplified by commercially available TaqMan SNP genotyping assays according to

manufacturer's protocol on a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Quality control was performed by the incorporation of positive and negative controls and duplication of random samples across different plates.

Table 1. Genotyped single-nucleotide polymorphisms (SNPs) in genes coding for cytokines.

Gene	SNP ID	Mutation	Nucleotide change[#]	Amino acid change[#]	Taqman Assay ID
IL1A	rs17561	Missense	G > T	A114S	C___9546471_10
IL1A	rs1800587	5' UTR	C > T	-	C___9546481_20
IL1B	rs16944	5' near gene	A > G	-	C___1839943_10
IL1B	rs1143627	5' near gene	C > T	-	C___1839944_10
IL1RN	rs4251961	5' near gene	T > C	-	C__32060323_10
IL6	rs1800796	5' near gene	G > C	-	C__11326893_10
IL6	rs1800797	5' near gene	G > A	-	C___1839695_20
TNF	rs361525	5' near gene	G > A	-	C___2215707_10
TNF	rs1800629	5' near gene	G > A	-	C___7514879_10
IL10	rs1800871	5' near gene	C > T	-	C___1747362_10
IL10	rs1800896	5' near gene	A > G	-	C___1747360_10
IL17A	rs2275913	5' near gene	A > G	-	C___1747360_10
IL17A	rs8193036	5' near gene	T > C	-	C___1799585_10
IL17F	rs763780	Missense	T > C	H161R	C___2234166_10
IFNG	rs2069705	5' near gene	C > T	-	C__15944115_20

[#] The first nucleotide (and corresponding amino acid) is the ancestral and therefore is considered the wild-type allele. ID, identification number.

In vitro PBMC stimulation and cytokine detection assays

PBMCs were isolated and stimulated as described previously [15], with minor modifications. In brief, PBMCs were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Next, PBMCs were washed and resuspended in Roswell Park Memorial Institute 1640 Dutch Modified culture medium (supplemented with 2 mM L-glutamine, 1 mM pyruvate and 50 µg/mL gentamicin; GIBCO Invitrogen, Carlsbad, CA, USA). 5 x 10^5 PBMCs were added to 96-well round-bottom plates (Greiner, Nurnberg, Germany) and stimulated with heat-killed 1 x 10^6 colony-forming units (cfu)/mL *S. aureus* ATCC 29213 or *E. coli* ATCC 25922 in a final volume of 200 µL. PBMCs were incubated at 37°C and 5% CO_2 for 24 hours to induce IL-6, TNFα and IL-10 or 7 days for IFNγ or IL-17 production, after which supernatants were collected. Cytokine concentrations were measured in supernatants by commercially available enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer's instructions (IL-6, IL-10 and IFNγ, Sanquin Reagents, Amsterdam, The Netherlands; TNFα and IL-17, R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Chi-square analyses of deviation from Hardy-Weinberg equilibrium (HWE) were performed for all 15 SNPs in healthy controls using a web-based HWE calculator [16]. SNPs deviating from HWE were excluded for further analysis. The association between susceptibility to cSSSIs and a SNP were investigated by means of univariate logistic regression models with IBM SPSS 18 software (IBM, Armonk, NY, USA). SNPs were evaluated using dominant (heterozygotic and homozygotic individuals for the allelic variant were combined and compared to wild-type individuals) and recessive (wild-type and heterozygotic individuals were combined and compared to homozygotic individuals for the allelic variant) model analyses. Pairwise linkage disequilibrium (LD), D' and r^2, were calculated using IBM SPSS 18 software (IBM). Multivariate logistic regression analyses were performed assuming a dominant or recessive model. Model reduction was done via forward and backward model selection methods. P-values below 0.05 were considered to be statistically significant, and odds ratios including 95% confidence intervals were reported.

All statistical analyses and graphical presentation of the cytokine concentrations in the in vitro PBMC stimulation assays were performed using GraphPad Prism 5.00 software (GraphPad Software, La Jolla, CA, USA). The results were stratified by cytokine genotype and differences in cytokine secretion between genotypes were analyzed by Mann-Whitney U tests.

Results

Univariate logistic regression of SNPs in cytokine genes in patients with cSSSI versus healthy controls

Genetic variation in *IL1A*, *IL1B*, *IL1RN*, *IL6*, *TNF*, *IL10*, *IL17A*, *IL17F* and *IFNG* was investigated in 318 patients with cSSSI and 328 healthy controls to assess their role in the susceptibility to cSSSIs. All genotyped polymorphisms were in HWE (data not shown) except for *IL6* rs1800796, which led to the exclusion of this polymorphism for further analyses.

Dominant model analyses resulted in a significantly different distribution for cytokine polymorphisms *IL6* rs1800797 (P=0.001), *TNF* rs1800629 (P=0.013), *IL10* rs1800871 (P=0.012), *IL17A* rs8193036 (P=0.001) in patients with cSSSI versus healthy controls (Table 2). Patients were significantly more often heterozygous and homozygous for the allelic variant of *IL6* rs1800797, *TNF* rs1800629 and *IL17A* rs8193036 than healthy controls. In contrast, patients with cSSSI were significantly less often heterozygous and homozygous for the allelic variant of *IL10* rs1800871 than healthy controls.

Recessive model analyses confirmed the association between *IL6* rs1800797 and

susceptibility to cSSSIs (P=0.009; OR=1.773 [95% CI, 1.152-2.727]), whereas *TNF* rs1800629, *IL10* rs1800871, *IL17A* rs8193036 did not influence susceptibility in this model. In addition, recessive model analysis identified *IFNG* rs2069705 to be associated with susceptibility to cSSSIs (p=0.003, OR=0.619 [95% CI, 0.452-0.847]). Patients were significantly less often homozygous for the allelic variant of *IFNG* rs2069705 than healthy controls.

No significant association with susceptibility to cSSSIs was observed for both dominant and recessive model analysis in individuals carrying polymorphisms in *IL1A* rs17561 and rs1800587, *IL1B* rs16944 and rs1143627, *IL1RN* rs4251961, *TNF* rs361525, *IL10* rs1800896, *IL17A* rs2275913 and *IL17F* rs763780.

Analyses of LD revealed that cSSSI-associated polymorphisms *IL6* rs1800797 and *TNF* rs1800629 were not independent, but their correlation was weak: P<0.05, r^2=0.09, D'=0.13. In addition, cSSSI-associated polymorphism *IL10* rs1800871 correlated with *IL10* rs1800896 (P<0.0001, r^2=0.51, D'=0.75). cSSSI-associated polymorphism *IL17A* rs8193036 weakly correlated with *IL17A* rs2275913 (P<0.0001, r^2=0.34, D'=0.41) and *IL17F* rs763780 (P<0.01, r^2=0.11, D'=0.17).

Multivariate logistic regression of SNPs in cytokine genes in patients with cSSSI versus healthy controls

All genotyped polymorphisms, except for *IL6* rs1800796, which failed HWE, were considered for inclusion as explanatory variables in the multivariate logistic regression model with outcome cSSSI status.

In the dominant model analysis, the final model consisted of *IL6* rs1800797, *TNF* rs1800629, *IL10* rs1800871, *IL17A* rs8193036 best explaining susceptibility to cSSSIs. Polymorphisms *IL6* rs1800797 (P=0.002, OR=1.679 [95% CI, 1.211-2.327]), *TNF* rs1800629 (P=0.027, OR=1.512 [95% CI, 1.049-2.179]), *IL10* rs1800871 (P=0.014, OR=0.671 [95% CI, 0.488-0.922]) and *IL17A* rs8193036 (P=0.002, OR=1.674 [95% CI, 1.209-2.317]) independently influenced susceptibility to cSSSIs.

In the recessive model analysis, the final model consisted of *IL6* rs1800797 and *IFNG* rs2069705 best explaining susceptibility to cSSSIs. Polymorphisms *IL6* rs1800797 (P=0.009, OR=1.777 [95% CI, 1.152-2.742]) and *IFNG* rs2069705 (P=0.003, OR=0.621 [95% CI, 0.452-0.853]) were independently associated with susceptibility to cSSSIs.

Table 2. Distribution of cytokine genotypes in 318 patients with complicated skin and skin structure infections (cSSSI) and 328 healthy controls.

Polymorphism	Wild-type, No. (%)	Heterozygous, No. (%)	Homozygous, No. (%)	P-value[#]	Odds ratio (95% CI)[#]
IL1A rs17561	GG	GT	TT	0.518	0.903 (0.663-1.230)
Patients with cSSSI	170 (53.5)	118 (37.1)	30 (9.4)		
Healthy controls	167 (50.9)	132 (40.2)	29 (8.8)		
IL1A rs1800587	CC	CT	TT	0.565	0.913 (0.670-1.244)
Patients with cSSSI	172 (54.1)	116 (36.5)	30 (9.4)		
Healthy controls	170 (51.8)	129 (39.3)	29 (8.8)		
IL1B rs16944	AA	AG	GG	0.789	0.938 (0.589-1.495)
Patients with cSSSI	41 (12.9)	132 (41.5)	145 (45.6)		
Healthy controls	40 (12.2)	155 (47.3)	133 (40.5)		
IL1B rs1143627	CC	CT	TT	0.981	0.994 (0.618-1.601)
Patients with cSSSI	38 (11.9)	135 (42.5)	145 (45.6)		
Healthy controls	39 (11.9)	157 (47.9)	132 (40.2)		
IL1RN rs4251961	TT	TC	CC	0.631	0.927 (0.680-1.264)
Patients with cSSSI	147 (46.2)	144 (45.3)	27 (8.5)		
Healthy controls	145 (44.3)	147 (45.0)	35 (10.7)		
IL6 rs1800796*	GG	GC	CC		
Patients with cSSSI	278 (87.4)	39 (12.3)	1 (0.3)		
Healthy controls	281 (85.7)	38 (11.6)	9 (2.7)		
IL6 rs1800797	GG	GA	AA	0.001	1.791 (1.300-2.466)
Patients with cSSSI	103 (32.4)	152 (47.8)	63 (19.8)		
Healthy controls	151 (46.2)	136 (41.6)	40 (12.2)		
TNF rs361525	GG	GA	AA	0.661	1.163 (0.593-2.279)
Patients with cSSSI	299 (94.0)	18 (5.7)	1 (0.3)		
Healthy controls	311 (94.8)	17 (5.2)	0 (0.0)		
TNF rs1800629	GG	GA	AA	0.013	1.570 (1.099-2.244)
Patients with cSSSI	223 (70.1)	88 (27.7)	7 (2.2)		
Healthy controls	258 (78.7)	66 (20.1)	4 (1.2)		
IL10 rs1800871	CC	CT	TT	0.012	0.671 (0.492-0.915)
Patients with cSSSI	177 (55.7)	124 (39.0)	17 (5.3)		
Healthy controls	150 (45.7)	152 (46.3)	26 (7.9)		
IL10 rs1800896	AA	AG	GG	0.345	0.851 (0.610-1.189)
Patients with cSSSI	104 (32.7)	152 (47.8)	62 (19.5)		
Healthy controls	96 (29.3)	178 (54.3)	54 (16.5)		

IL17A rs2275913	AA	AG	GG	0.886	1.032 (0.672-1.584)
Patients with cSSSI	48 (15.1)	139 (43.8)	130 (41.0)		
Healthy controls	51 (15.5)	138 (42.1)	139 (42.4)		
IL17A rs8193036	TT	TC	CC	0.001	1.727 (1.256-2.375)
Patients with cSSSI	171 (53.8)	125 (39.3)	22 (6.9)		
Healthy controls	219 (66.8)	94 (28.7)	15 (4.6)		
IL17F rs763780	TT	TC	CC	0.869	1.048 (0.600-1.831)
Patients with cSSSI	292 (91.8)	26 (8.2)	0 (0.0)		
Healthy controls	300 (91.5)	28 (8.5)	0 (0.0)		
IFNG rs2069705	CC	CT	TT	0.183	0.729 (0.457-1.162)
Patients with cSSSI	46 (14.5)	155 (48.7)	117 (36.8)		
Healthy controls	36 (11.0)	133 (40.5)	159 (48.5)		

[#] Data are based on a dominant model analysis. CI, confidence interval. * Significant deviation from Hardy-Weinberg Equilibrium (HWE) in controls was observed for IL6 rs1800796. All other genotyped SNPs were in HWE.

Functional consequences of *IL6* rs1800797, *TNF* rs1800629, *IL10* rs1800871, *IL17A* rs8193036 and *IFNG* rs2069705 on cytokine production

The effect of cytokine polymorphisms, associated with susceptibility to cSSSIs, on cytokine production was studied in an additional cohort of 74 healthy volunteers. PBMCs were isolated and stimulated with *S. aureus* and *E. coli*, prominent pathogens of cSSSIs, after which cytokines were measured by ELISA in cell culture supernatants. Cytokine responses were displayed stratified by genotype of interest (Figure 1). Unstimulated PBMCs did not induce detectable cytokine responses (data not shown). *S. aureus* proved to be a poor inducer of TNFα, IL-10 and IFNγ (Figure 1B,C,E), whereas *E. coli* did not induce IL-17 production (Figure 1I). With cytokine levels for most individuals around the threshold of detection for these stimulations, we were unable to draw conclusions solely on these data (Figure 1B,C,E,I).

No significant differences in cytokine responses between the allelic variants after both *S. aureus* and *E. coli* stimulation could be detected for all 5 SNPs associated with susceptibility to cSSSIs (Figure 1A,D,F,G,H,J).

Discussion

This study identifies 5 polymorphisms in cytokine genes: *IL6* rs1800797, *TNF* rs1800629, *IL10* rs1800871, *IL17A* rs8193036 and *IFNG* rs2069705 to be associated with the susceptibility to cSSSIs. Although clearly induced after in vitro PBMC stimulation with prevalent cSSSI pathogens *S. aureus* or *E. coli*, the corresponding cytokine levels were not modulated by the presence of these cSSSI-associated polymorphisms.

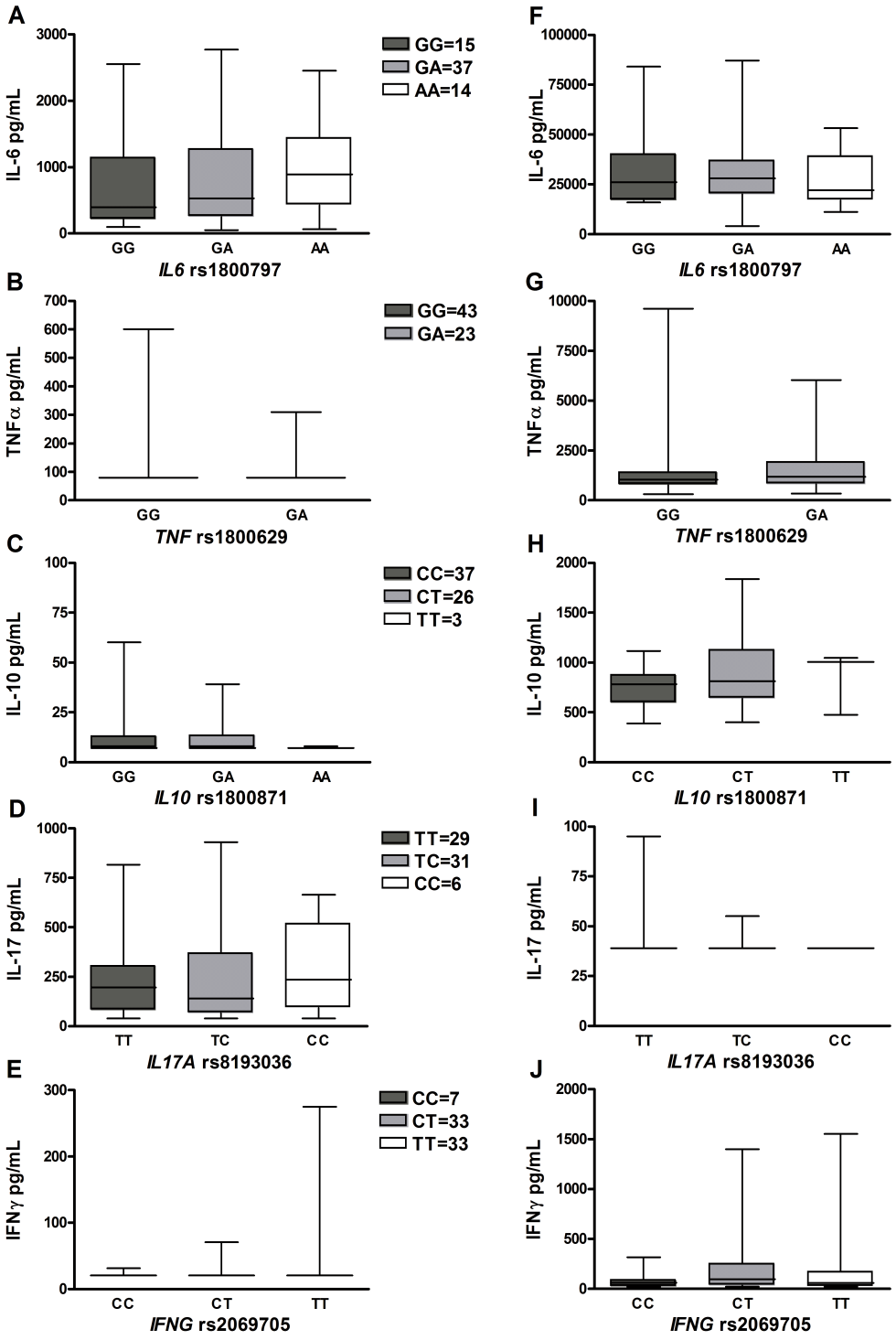
A
IL-6 pg/mL
3000
2000
1000
0
GG=15
GA=37
AA=14
GG
GA
AA
IL6 rs1800797
F
IL-6 pg/mL
100000
75000
50000
25000
0
GG
GA
AA
IL6 rs1800797
B
TNFα pg/mL
700
600
500
400
300
200
100
0
GG=43
GA=23
GG
GA
TNF rs1800629
G
TNFα pg/mL
10000
7500
5000
2500
0
GG
GA
TNF rs1800629
C
IL-10 pg/mL
100
75
50
25
0
CC=37
CT=26
TT=3
GG
GA
AA
IL10 rs1800871
H
IL-10 pg/mL
2000
1500
1000
500
CC
CT
TT
IL10 rs1800871
D
IL-17 pg/mL
1000
750
500
250
0
TT=29
TC=31
CC=6
TT
TC
CC
IL17A rs8193036
I
IL-17 pg/mL
100
75
50
25
0
TT
TC
CC
IL17A rs8193036
E
IFNγ pg/mL
300
200
100
0
CC=7
CT=33
TT=33
CC
CT
TT
IFNG rs2069705
J
IFNγ pg/mL
2000
1500
1000
500
0
CC
CT
TT
IFNG rs2069705

Figure 1. Correlation of complicated skin and skin structure infection-associated cytokine genotypes with corresponding cytokine measurements after 24 hours of peripheral blood mononuclear cell stimulation. *Left panel*, heat-killed *Staphylococcus aureus* (1 x 10^6 colony-forming units [cfu]/mL); *Right panel*, *Escherichia coli* (1 x 10^6 cfu/mL). Corresponding cytokine measurements were stratified by genotype: IL-6 for *IL6* rs1800797 (A, F), TNFα for *TNF* rs1800629 (B, G), IL-10 for *IL10* rs1800871 (C, H), IL-17 for *IL17* rs8193036 (D, I) and IFNγ for *IFNG* rs2069705 (E, J). Dark grey represents individuals homozygous for the wild-type allele, light grey represents individuals heterozygous for the allelic variant and white represents individuals homozygous for the allelic variant. Data were analyzed by Mann-Whitney U tests and presented in a box-and-whisker plot; the box represents the median with the interquartile range; the whiskers represent the minimal and maximal value.

These 5 cSSSI-associated polymorphisms have previously been correlated with other infectious diseases. The *IL6* polymorphism rs1800797 influenced susceptibility to leprosy [11]. The *TNF* polymorphism rs1800629 has previously been associated with susceptibility to sepsis [9, 10], malaria [13], and tuberculosis [17]. Furthermore, *IL10* polymorphism rs1800871 is associated with susceptibility to leprosy [11], whereas *IFNG* polymorphism rs2069705 was involved in susceptibility to sepsis [18] and tuberculosis [12]. The *IL17* polymorphism rs8193036 was approaching significance for susceptibility to gram-positive infection in sepsis [19], but has been associated with inflammatory disorders like asthma [20] and Behcet's disease [21]. However, in other studies on infectious diseases no association was found for some of these polymorphisms [22-25].

To determine whether the observed genetic associations also resulted in mechanistic effects, the genetic studies were supplemented by functional assays investigating the consequences of cSSSI-associated cytokine polymorphisms. The gram-positive bacteria *S. aureus* and gram-negative *E. coli*, prevalent pathogens of cSSSIs, were chosen to ensure potent induction of cytokines of interest by PBMCs. No significant differences in corresponding cytokine responses were detected between the allelic variants of all 5 cSSSI-associated polymorphisms after stimulation of PBMCs. Functional consequences of cSSSI-associated polymorphisms have previously been studied. Firstly, the A allele of *IL6* polymorphism rs1800797 resulted in significantly up-regulated baseline expression levels compared to the G allele in HEK and HeLa cell lines using a luciferase reporter assay [11]. Also, serum IL-6 levels were found to be higher in the presence the AA genotype of *IL6* polymorphism rs1800797 [26]. We did not observe differences in baseline IL-6 secretion by unstimulated PBMCs possibly due to IL-6 levels not reaching the detection limit. Secondly, the precise effect of *TNF* polymorphism rs1800629 on gene expression and TNFα production remains controversial. Recently, a large meta-analyses reported the absence of an association between *TNF* polymorphism rs1800629 and TNFα production [27], confirming our findings described here. Thirdly, we observed no differences in IL-10 production by PBMCs after stimulation with *S. aureus* and *E. coli*, whereas PBMCs from individuals carrying the T allele of *IL10* polymorphism rs1800871 were shown to produce lower levels of IL-10 after stimulation with *Mycobacterium leprae* antigens compared to

non-carriers [28]. Fourthly, identical to our results after PBMC stimulation, the different genotypes of polymorphism *IL17A* rs8193036 were found to have similar levels of IL-17A in the serum [29]. Finally, 1 study reported similar serum IFNγ levels for the different genotypes of *IFNG* polymorphism rs2069705 [26].

Polymorphisms in *IL1A* rs17561 and rs1800587, *IL1B* rs16944 and rs1143627, *IL1RN* rs4251961, *TNF* rs361525, *IL10* rs1800896, *IL17A* rs2275913 and *IL17F* rs763780 did not influence susceptibility to cSSSIs. The presence of these polymorphisms did not affect levels of corresponding cytokines after in vitro PBMC stimulation (data not shown).

To our knowledge, this is the first study investigating the role of genetic variation in genes encoding cytokines and susceptibility to cSSSIs. This study demonstrates that 5 polymorphisms in cytokine genes, *IL6*, *TNF*, *IL10*, *IL17* and *IFNG*, modulate susceptibility to cSSSIs. Although these genes are involved in the immune response against cSSSI pathogens, indicated by the production of their corresponding cytokines after in vitro stimulation, polymorphisms in these genes did not influence the cytokine production capacity. In contrast, we have recently described genetic variants in Toll-like receptors which dramatically alter IL-6 cytokine responses by PBMCs after stimulation for 24 hours with *S. aureus* [14]. As the cytokine polymorphisms are located in the promoter region, and as such could influence gene expression levels, it was surprising to find no differences in secreted cytokine levels. We cannot exclude the fact that the kinetics of cytokine secretion were different in the presence of the cSSSI-associated polymorphisms, because levels were only measured after 24 hours. Furthermore, measurement of total concentration does not allow for conclusions to be drawn on altered biological function of the protein due to the polymorphism. Though this effect is unlikely the result of promoter polymorphisms, they could be in strong linkage with an exonic polymorphism which causes the altered biological function.

Unfortunately, we did not have access to a confirmation cohort and the data on predisposing factors in the healthy control cohort, which may act as confounders in the associations between polymorphisms in cytokine genes and susceptibility to cSSSIs, were not available. We did not correlate polymorphisms with specific infection types or specific pathogens, because this would result in the analysis of small subgroups with possibly insufficient power to detect differences. Future studies are warranted to determine how these cytokine polymorphisms in *IL6*, *TNF*, *IL10*, *IL17* and *IFNG* influence susceptibility to cSSSIs and whether they can play a role in the characterization of a predictive profile, individualized diagnosis and treatment for individuals at risk of developing cSSSIs.

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Part 2

The identification of pathogens in complicated skin and skin structure infections

Chapter 7

Direct molecular versus culture-based assessment of gram-positive cocci in biopsies of patients with major abscesses and diabetic foot infections

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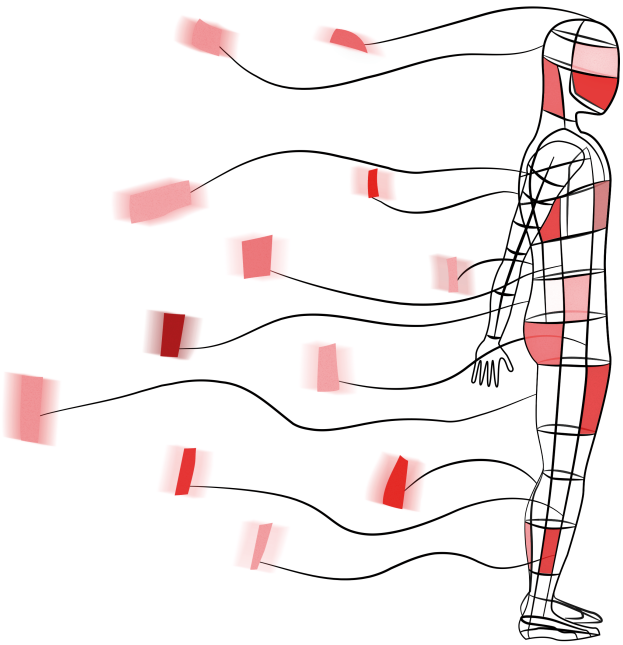

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Abstract

Major abscesses and diabetic foot infections (DFIs) are predominant subtypes of complicated skin and skin structure infections (cSSSIs), and are mainly caused by *Staphylococcus aureus* and β-hemolytic streptococci. This study evaluates the potential benefit of direct pathogen-specific real-time polymerase chain reaction (PCR) assays in the identification of causative organisms of cSSSIs. One-hundred fifty major abscess and 128 DFI biopsy samples were collected and microbial DNA was extracted by using the Universal Microbe Detection kit for tissue samples. Pathogen-specific PCRs were developed for *S. aureus* and its virulence factor Panton-Valentine leukocidin (PVL), *Streptococcus pyogenes*, *S. agalactiae*, *S. dysgalactiae*, and the *S. anginosus* group. Identification by pathogen-specific PCRs was compared to routine culture and both methods were considered as the gold standard for determination of the sensitivity and specificity of each assay. Direct real-time PCR assays of biopsy samples resulted in a 34% higher detection of *S. aureus*, 37% higher detection of *S. pyogenes*, 18% higher detection of *S. agalactiae*, 4% higher detection of *S. dysgalactiae* subspecies *equisimilis*, and 7% higher detection of the *S. anginosus* group, compared to routine bacterial culture. The presence of PVL was mainly confined to *S. aureus* isolated from major abscess but not DFI biopsy samples. In conclusion, our pathogen-specific real-time PCR assays had a higher yield than culture methods and could be an additional method for the detection of relevant causative pathogens in biopsies.

Introduction

Major abscesses and diabetic foot infections (DFIs) are the predominant subtypes of a spectrum of infections termed complicated skin and skin structure infections (cSSSIs). The Food and Drug Administration (FDA) defined cSSSIs as infections of the deeper soft tissues, involving surgical intervention or a significant underlying disease state that complicates the response to treatment. Superficial infections located in an anatomical site in which the chance of involvement of anaerobic or gram-negative pathogens is high should also be considered as cSSSIs [1, 2]. cSSSIs are associated with significant morbidity and mortality, as well as prolonged and expensive hospitalizations [3]. The management of cSSSIs involves surgical debridement of the infection, combined with antibiotic therapy [4].

Gram-positive cocci, in particular *Staphylococcus aureus* and β-hemolytic streptococci, are the leading causative organisms of cSSSIs [2, 4]. In a recent multicenter randomized clinical trial, 65% of the cultured isolates consisted of gram-positive cocci (of which *S. aureus* 33% and β-hemolytic streptococci 15%), whereas gram-negative bacilli (28%) and anaerobes (7%) were found to a lesser extent [5], but geographical differences exist in the type and amount of species isolated [6].

Correct and rapid identification of pathogens is crucial for clinical decision-making and optimal antibiotic therapy. Up to now, routine bacteriological assessment of biopsies from cSSSIs relies upon culture, which, in order to be successful, requires viable pathogens in tissue and the use of suitable culture conditions for growth. Difficult to culture pathogens, those present in low numbers or that died before/during sampling of the infected tissue make detection by culture complicated and time-consuming. This may result in low sensitivity and underestimated bacterial prevalence. Several molecular assays, such as pathogen-specific, broad-range and multiplex polymerase chain reaction (PCR) assays, either directly on clinical samples or cultured isolates, have been developed in recent years to improve bacterial detection [7, 8]. This study evaluates the potential benefit of direct, pathogen-specific real-time PCR assays on clinical samples in the identification of the causative organisms of cSSSIs.

Biopsy samples of 150 major abscesses and 128 DFIs were collected during a multicenter clinical trial involving patients with cSSSIs [5]. Detection of the pathogens *S. aureus* and *Streptococcus* species by real-time PCR directly on DNA isolated from these clinical cSSSI samples was compared to routine cultures.

Materials and methods

Definitions

cSSSIs were characterized as infections of bacterial origin that required hospitalization, initial parenteral therapy for ≥48 hours, and which met at least 1 of the following criteria: deep soft tissue involvement; significant surgical intervention, including drainage and/or debridement; and association with an underlying comorbid condition. Major abscesses were defined as collections of pus associated with extensive cellulitis, requiring surgical intervention followed by antibiotic therapy. DFIs were characterized as infections occurring below the ankle in patients with confirmed diabetes [5].

Collection of major abscess and DFI biopsy samples

From a population of 813 cSSSI patients included in a large randomized, multicenter clinical trial [5], performed from September 2006 to June 2008, 389 patients gave informed consent to participate in this substudy. Of these, the first visit, prestudy treatment biopsy samples were selected resulting in the inclusion of 150 major abscess and 128 DFIs samples from 225 cSSSI patients. Samples were collected via biopsy of tissue or bone, curettage of the wound, or aspiration of purulent discharge. After collection, samples were directly stored in preservation medium (BBL Port-A-Cul Transport vial, Becton Dickinson, Franklin Lakes, NJ, USA) and transported within 72 hours to the central laboratory (Eurofins Medinet SAS, Plaisir, France) [5]. On arrival, samples were split into 2, 1 part for immediate culture and identification by using standard clinical laboratory procedures, and the other part was stored at -80°C for subsequent DNA isolation and PCR analysis.

Bacterial DNA extraction from biopsy samples

Pathogen DNA was manually extracted by using the MolYsis Universal Microbial Detection kit for tissue samples (Molzym, Bremen, Germany), following the manufacturer's instructions. Briefly, selective lysis of human cells is performed, followed by degradation of human DNA. After washing the pellet, a second round of DNA extraction is performed to release DNA from bacterial and fungal cells. This approach enables an increased sensitivity and specificity for pathogen DNA, since interfering non-target human DNA is no longer present [9]. Pathogen DNA extraction was carried out in a laminar flow cabinet to prevent contamination. Prior to further handling, the DNA samples were stored at -20°C.

Real-time PCR primer and probe design

To detect *S. agalactiae*, both subspecies of *S. dysgalactiae*, and *S. pyogenes* the *recA* gene was chosen as the target, based on the results from previous studies [10, 11]. Partial *recA* sequences of clinically relevant *Streptococcus* species were extracted from GenBank (accession numbers EU156792-EU156872), an alignment was generated in MEGA v6 [12],

and primer and probe sets were developed to make sure that there was no overlap with other *Streptococcus* species. The specificity of the primers and probes were in silico tested by a BLAST search in GenBank.

A previously described multiplex real-time PCR assay was used to detect the *S. aureus*-specific fragment *Sa442* and the Panton-Valentine leukocidin gene [13]. The duplex real-time PCRs were performed in reaction volumes of 20 µL consisting of 10 µL 2× LC480 Probe Master Mix (Roche Diagnostics), 0.1 µL of each primer and 0.04 µL of both probes (100 pmol/µL; Eurogentec, Brussels, Belgium), 1.72 µL ddH2O and 8 µL sample-DNA. The PCRs were performed with the following settings: Initial denaturation step for 10 min at 95°C, 50 cycles of 1 s at 95°C, 12 s at 60°C followed by measuring the fluorescence signal, and a cooldown step for 30 s at 40°C.

For detection of members of the *S. anginosus* group (*S. anginosus*, *S. constellatus*, and *S. intermedius*, also termed the *S. milleri* group), a set of primers was developed based on an alignment made in MEGA v6 of the *recN* sequences EU917226–EU917315 [12, 14]. The PCRs were performed in 10 µL reaction volumes, containing 5 µL 2× LC480 Probe Master Mix (Roche Diagnostics, Almere, the Netherlands), 0.5 µL of each primer (10 pmol/µL; Eurogentec), 0.5 µL SYTO82 (40 µM; Molecular Probes, Eugene, OR, USA), 2.5 µL ddH2O, and 1 µL sample DNA. The following PCR program was used: initial denaturation for 10 min at 95°C, 35 cycles 5 s at 95°C, 5 s at 60°C, 10 s at 72°C, followed by a melting curve analysis at 65°C to 95°C. Fluorescence was measured after each extension step.

The primer and probe sequences of all assays are provided in Table 1.

Table 1. Primer and probe sequences.

Specificity	Forward primer	Probe	Reverse primer
*S. aureus**	5′-ACGACTARATAA ACGCTCATTCG-3′	5′-HEX-TGAAATCTCATTA CGTTGCATCGGA-BHQ1-3′	5′-GACGGCTTTTAC ATACAGAACACA-3′
PVL*	5′-AAAAAGGCTCAG GAGATACAAGTG-3′	5′-Cy5-TGGCAGAAATATGG ATGTTACTCATGC-BHQ2-3′	5′-TGCCATAGTGTG TTGTTCTTCTAGT-3′
S. pyogenes	5′-TTGGAAACGAT AGCTAATACCG-3′	5′-HEX-CGCATGTTAGTAAT TTAAAAGGGGCA-BHQ1-3′	5′-CGCAGGTCCA TCTCATAGTG-3′
S. agalactiae	5′-TTGGAAACGAT AGCTAATACCG-3′	5′-FAM-TGTTAGTTATTTAAA AGGAGCAATTGC-BHQ1-3′	5′-CGCAGGTCCA TCTCATAGTG-3′
S. dysgalactiae equisimilis	5′-TTGGAAACGAT AGCTAATACCG-3′	5′-HEX-CCCATGTTAAACAT TTAAAAGGTGCA-BHQ1-3′	5′-CGCAGGTCCA TCTCATAGTG-3′
S. dysgalactiae dysgalactiae	5′-TTGGAAACGAT AGCTAATACCG-3′	5′-FAM-AATGGAGGACCC ATGTCTTTCATTT-BHQ1-3′	5′-CGCAGGTCCA TCTCATAGTG-3′
S. anginosus group	5′-TGGACAGCAT TTGGTGGATA-3′	n/a	5′-GCTTACGCAAC TGACGATACTG-3′

* This assay was previously published by Hopman *et al.* [13].

Real-time PCR assays and analysis

The analytic specificity was tested by applying the newly developed *Streptococcus* assays on a set of 62 clinically relevant *Streptococcus* reference strains. The sensitivity of the *Streptococcus* assays was performed by applying 2-step dilution series of the relevant type strains, starting with an input ranging from 0.01 ng to 4.9 fg per reaction. The equivalent of genomic copies per reaction was calculated based on the genome sizes for each of the *Streptococcus* species [15, 16].

Statistical analysis

McNemar's test was performed to study differences between real-time PCR and culture-based assessment of pathogenic presence, for major abscesses and DFIs separately. The results for major abscesses and DFIs were combined for the determination of the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Both culture and real-time PCR were considered as the gold standard.

Results

Validation of species-specific *Streptococcus* real-time PCR assays and analysis

The *S. agalactiae* real-time PCR assay was found to be positive for the included strains, however, atypical amplification curves were observed for 2 *S. anginosus* strains. The *S. pyogenes* assay, included in the duplex with *S. agalactiae*, was found to be exclusively positive for strains that belong to this species. The *S. dysgalactiae* duplex real-time PCR assay was positive for the included subspecies *dysgalactiae* and *equisimilis*. However, the assay for subspecies *equisimilis* showed an atypical amplification curve for 1 strain of *S. constellatus* and *S. cristatus*.

The lower limit of detection was found to be at the 9th dilution step for the targets *S. agalactiae*, both subspecies of *S. dysgalactiae* and *S. pyogenes*, equivalent of ~39 fg DNA per reaction, equal to ~17 genomic copies per reaction. For the intercalating dye-based assay for *S. anginosus*, *S. constellatus* and *S. intermedius* assay, the lower limit of detection was found to be at the 8th dilution step, equal to 78 fg or ~37 genomic copies per reaction.

Real-time PCR versus culture-based assessment of *S. aureus* prevalence in major abscesses and DFIs

The first visit biopsy samples of 150 major abscesses and 128 DFIs were collected, and the identification of *S. aureus* was performed by real-time PCR and culture. Of the 150 major abscess samples, 81% were positive for *S. aureus* by real-time PCR, whereas 44% were culture-positive for *S. aureus*. None of the culture-positive samples were real-time PCR-negative (Table 2). Of the 128 DFI samples, 88% were positive for *S. aureus* by real-time

PCR, whereas 57% were culture-positive for *S. aureus*. One culture-positive sample was found to be real-time PCR-negative (Table 2). Subsequent real-time PCR determination of the cultured strain from the culture-positive, real-time PCR-negative biopsy sample confirmed the presence of *S. aureus*. Statistical analysis using McNemar's test showed a significant difference between *S. aureus* real-time PCR and culture-based assessment for major abscesses (P<0.001) and DFIs (P<0.001). The sensitivity, specificity, PPV, and NPV for the *S. aureus* real-time PCR and culture are shown in Table 3.

Table 2. Real-time polymerase chain reaction (real-time PCR) versus culture-based assessment of gram-positive coccal prevalence in major abscesses and diabetic foot infections.

Major abscesses				**Diabetic foot infections**			
	Real-time PCR-positive	Real-time PCR-negative	Total		Real-time PCR-positive	Real-time PCR-negative	Total
S. aureus							
Culture-positive	66	0	66	Culture-positive	72	1	73
Culture-negative	55	29	84	Culture-negative	41	14	55
Total	121	29	150	Total	113	15	128
S. pyogenes							
Culture-positive	23	0	23	Culture-positive	1	0	1
Culture-negative	104	23	127	Culture-negative	18	109	127
Total	127	23	150	Total	19	109	128
S. agalactiae							
Culture-positive	5	3	8	Culture-positive	18	10	28
Culture-negative	42	100	142	Culture-negative	21	79	100
Total	47	103	150	Total	39	89	128
S. dysgalactiae* subspecies *equisimilis							
Culture-positive	3	1	4	Culture-positive	16	1	17
Culture-negative	0	146	146	Culture-negative	12	99	111
Total	3	147	150	Total	28	100	128
***S. anginosus* group**							
Culture-positive	16	7	23	Culture-positive	6	1	7
Culture-negative	13	114	127	Culture-negative	13	108	121
Total	29	121	150	Total	19	109	128

Table 3. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of real-time polymerase chain reaction (real-time PCR) and culture-based assessment of gram-positive coccal prevalence in major abscesses and diabetic foot infections.

Species	Reference	Assay	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
S. aureus	Culture	Real-time PCR	99	31	59	98
	Real-time PCR	Culture	59	98	99	31
S. pyogenes	Culture	Real-time PCR	100	86	16	100
	Real-time PCR	Culture	16	100	100	86
S. agalactiae	Culture	Real-time PCR	64	74	27	93
	Real-time PCR	Culture	27	93	64	74
S. dysgalactiae equisimilis	Culture	Real-time PCR	91	95	61	99
	Real-time PCR	Culture	61	99	91	95
S. anginosus group	Culture	Real-time PCR	73	90	46	97
	Real-time PCR	Culture	46	97	73	90

Real-time PCR-based assessment of PVL versus *S. aureus* prevalence in major abscesses and DFIs

The presence of *S. aureus* virulence factor PVL was determined by real-time PCR and correlated to *S. aureus* real-time PCR-positive biopsies. Of interest, the majority of *S. aureus* real-time PCR-positive major abscess samples were also PVL real-time PCR-positive (89%). In contrast, a minority of *S. aureus* real-time PCR-positive DFI samples were PVL real-time PCR-positive (14%). In both groups of major abscesses and DFIs, 2 samples were PVL real-time PCR-positive but not *S. aureus* real-time PCR-positive (Table 4).

Table 4. Real-time polymerase chain reaction (real-time PCR)-based assessment of Panton-Valentine leukocidin (PVL) versus *Staphylococcus aureus* prevalence in major abscesses and diabetic foot infections.

Major abscesses				Diabetic foot infections			
	S. aureus real-time PCR-positive	*S. aureus* real-time PCR-negative	Total		*S. aureus* real-time PCR-positive	*S. aureus* real-time PCR-negative	Total
PVL real-time PCR-positive	108	2	110	PVL real-time PCR-positive	16	2	18
PVL real-time PCR-negative	13	27	40	PVL real-time PCR-negative	97	13	110
Total	121	29	150	Total	113	15	128

Real-time PCR versus culture-based assessment of *S. pyogenes* prevalence in major abscesses and DFIs

Of the 150 major abscess samples, 85% were positive for *S. pyogenes* by real-time PCR, whereas 15% was culture-positive for *S. pyogenes*. None of the culture-positive samples were real-time PCR-negative (Table 2). Of the 128 DFI samples, 15% were positive for *S. pyogenes* by real-time PCR, whereas 1% were culture-positive for *S. pyogenes*. The only

culture-positive sample was also real-time PCR-positive (Table 2). Statistical analysis showed a significant difference between *S. pyogenes* real-time PCR and culture-based assessment for major abscesses (P<0.001) and DFIs (P<0.001). The sensitivity, specificity, PPV, and NPV for the *S. pyogenes* real-time PCR and culture are shown in Table 3.

Real-time PCR versus culture-based assessment of *S. agalactiae* prevalence in major abscesses and DFIs

Of the 150 major abscess samples, 31% were positive for *S. agalactiae* by real-time PCR, whereas 5% were culture-positive for *S. agalactiae*. Three culture-positive samples were real-time PCR-negative (Table 2). Of the 128 DFI samples, 30% were positive for *S. agalactiae* by real-time PCR, whereas 22% were culture-positive for *S. agalactiae*. Ten culture-positive samples were real-time PCR-negative (Table 2). Subsequent real-time PCR determination of the cultured strains from the 13 culture-positive, real-time PCR-negative biopsy samples, confirmed presence of *S. agalactiae* of 4 strains. The other 9 strains were negative for *S. agalactiae* real-time PCR. Statistical analysis showed a significant difference between *S. agalactiae* real-time PCR and culture-based assessment for major abscesses (P<0.001) but not DFIs (P>0.05). The sensitivity, specificity, PPV, and NPV for the *S. agalactiae* real-time PCR and culture are shown in Table 3.

Real-time PCR versus culture-based assessment of *S. dysgalactiae* prevalence in major abscesses and DFIs

Of the 150 major abscess samples, 2% were positive for *S. dysgalactiae* subspecies *equisimilis* by real-time PCR, whereas 3% were culture-positive for *S. dysgalactiae* subspecies *equisimilis*. One of the culture-positive samples was real-time PCR-negative (Table 2). Of the 128 DFI samples, 22% were positive for *S. dysgalactiae* subspecies *equisimilis* by real-time PCR, whereas 13% were culture-positive for *S. dysgalactiae* subspecies *equisimilis*. One DFI sample was found to be culture-positive samples but real-time PCR-negative (Table 2). Subsequent real-time PCR determination of the cultured strains from the 2 culture-positive, real-time PCR-negative biopsy samples confirmed the presence of *S. dysgalactiae* subspecies *equisimilis* of 1 strain. The other strain was negative for *S. dysgalactiae* subspecies *equisimilis* real-time PCR. Statistical analysis showed a significant difference between *S. dysgalactiae* subspecies *equisimilis* real-time PCR and culture-based assessment for DFIs (P<0.01) but not major abscesses (P>0.05). The sensitivity, specificity, PPV, and NPV for the *S. dysgalactiae* subspecies *equisimilis* real-time PCR and culture are shown in Table 3.

None of the 150 major abscess samples were real-time PCR or culture-positive for *S. dysgalactiae* subspecies *dysgalactiae*. Of the 128 DFI samples, only 1 sample was positive for *S. dysgalactiae* subspecies *dysgalactiae* by real-time PCR, but culture-negative, and,

also, 1 sample was culture-positive for *S. dysgalactiae* subspecies *dysgalactiae* but real-time PCR-negative. Subsequent real-time PCR determination of the cultured strain from the culture-positive, real-time PCR-negative biopsy sample was negative for *S. dysgalactiae* subspecies *dysgalactiae* but positive for *S. dysgalactiae* subspecies *equisimilis*.

Real-time PCR versus culture-based assessment of *S. anginosus* group prevalence in major abscesses and DFIs

S. anginosus, *S. constellatus*, and *S. intermedius* together constitute the *S. anginosus* group. Of the 150 major abscess samples, 19% were positive for members of the *S. anginosus* group by real-time PCR, whereas 15% were culture-positive for members of the *S. anginosus* group. Seven culture-positive samples were real-time PCR-negative (Table 2). Of the 128 DFI samples, 15% were positive for members of the *S. anginosus* group by real-time PCR, whereas 6% were culture-positive for members of the *S. anginosus* group. One culture-positive sample was real-time PCR-negative (Table 2). Subsequent real-time PCR determination of the cultured strains from the 8 culture-positive, real-time PCR-negative biopsy samples confirmed identification of *S. anginosus* group of 6 strains. Of the 2 other strains, 1 was negative for *S. anginosus* group real-time PCR and the other strain was not viable. Statistical analysis showed a significant difference between *S. anginosus* group real-time PCR and culture-based assessment, for DFIs ($P<0.01$) but not major abscesses ($P>0.05$). The sensitivity, specificity, PPV, and NPV for the *S. anginosus* group real-time PCR and culture are shown in Table 3.

Discussion

This study is the first to demonstrate that the use of direct real-time PCR versus culture-based assessment for the determination of pathogens in clinical biopsy samples of patients with major abscesses and DFIs resulted in an increased detection of all studied cSSSI pathogens: *S. aureus*, *S. pyogenes*, *S. agalactiae*, *S. dysgalactiae*, and *S. anginosus* group.

The current routine practice for detection and identification of bacterial pathogens in cSSSI is culture of biopsy samples collected from the site of infection. Development of direct real-time PCR assays on bacterial DNA isolated from biopsy samples resulted in a higher detection of 34% for *S. aureus*, 37% for *S. pyogenes*, 18% for *S. agalactiae*, 4% for *S. dysgalactiae* subspecies *equisimilis*, and 7% for the *S. anginosus* group compared to standard cultures. No differences were observed for *S. dysgalactiae* subspecies *dysgalactiae*, which could be due to its low prevalence in human infections [17]. Significant differences were found between real-time PCR and culture-assesment for *S. aureus* (major abscesses and DFIs), *S. pyogenes* (major abscesses and DFIs), *S. agalactiae* (major

abscesses), *S. dysgalactiae* subspecies *equisimilis* (DFIs), and *S. anginosus* group (DFIs). Possible explanations for the real-time PCR-positive, culture-negative biopsy samples are: culture is less sensitive than real-time PCR for the detection of organisms, real-time PCR detects nonviable organisms in contrast to culture, splitting of the biopsy sample into 2 parts for culture and real-time PCR, contamination of the real-time PCR reagents, cross-reactivity of the real-time PCR assay with DNA from other organisms or human origin not controlled for during the development of the assay inducing false-positives.

In contrast, our real-time PCR assays did not identify some pathogens that were grown in culture. Possible explanations for the culture-positive, real-time PCR-negative biopsy samples are: real-time PCR is less sensitive than culture for the detection of organisms, incorrect determination during culture, low prevalence of bacterial DNA in the biopsy sample, splitting of the biopsy sample into 2 parts for culture and real-time PCR, low DNA yield or poor quality DNA after extraction or the presence of factors that inhibit the real-time PCR assay, poor target specificity, and/or competition between primers and probes within the developed real-time PCR not controlled for during the development of the assay.

Other studies evaluating the potential of direct real-time PCR in the detection of the organisms studied here frequently found comparable or increased detection rates compared to culture methods. The detection of (methicillin-resistant) *S. aureus* by real-time PCR was similar to culture in samples from skin and soft tissue infections and osteoarticular infections [18, 19], and increased in a screening for MRSA colonization [20]. In addition, the detection of *S. pyogenes* by real-time PCR was similar to culture in throat swabs from suspected pharyngitis [21, 22]. Furthermore, similar and increased detection rates of *S. agalactiae* were found in screening samples of vaginal and neonatal colonization [23-25], and increased detection was observed by real-time PCR for *S. agalactiae* in cerebrospinal fluid and blood from patients suspected of meningitis and sepsis [26].

Overall, combined detection by either culture and/or real-time PCR resulted in 85% of samples positive for *S. aureus*, 68% positive for *S. pyogenes*, 36% positive for *S. agalactiae*, 12% positive for *S. dysgalactiae* subspecies *equisimilis*, 0.7% positive for *S. dysgalactiae* subspecies *dysgalactiae*, and 20% positive for *S. anginosus* group. This again underlines the importance of gram-positive cocci in cSSSIs. Interestingly, there are differences in the species present in major abscesses and DFIs. *S. pyogenes* was mainly detected in biopsies from major abscesses, whereas *S. dysgalactiae* subspecies *equisimilis* was mainly found in biopsies from diabetic foot infections. Other identified pathogens were similarly present in both major abscesses and DFIs. In addition, a clear distinction was observed between major abscesses and DFIs for PVL positive *S. aureus*. 73% of the major abscesses, but

only 14% of the DFIs, were positive for PVL. PVL is a bi-component pore-forming toxin, encoded by the *lukS-PV* and *lukF-PV* genes, and is associated with lysis of leukocytes [27]. A recent meta-analysis confirmed the association between PVL and *S. aureus* skin and skin structure infections, which was not present for *S. aureus* invasive infections, such as pneumonia, musculoskeletal infections, and bacteremia [28].

In an attempt to evaluate the reliability of the real-time PCR and culture, we have calculated the sensitivity, specificity, PPV, and NPV. Both techniques were separately considered as the gold standard, as it is unknown which technique represents the "true" presence of pathogens in the biopsy samples.

In conclusion, current routine bacteriological assessment of biopsies by culture is time-consuming, requires viable pathogens, culture conditions suitable for growth, and results in lower detection sensitivity and may underestimate bacterial prevalence. However, given the fast and superior detection of cSSSI pathogens by real-time PCR, our study indicates that molecular analysis can be an additional method for the detection of bacteria in clinical samples.

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Chapter 8

Bacteroides fragilis in biopsies of patients with major abscesses and diabetic foot infections: direct molecular versus culture-based detection

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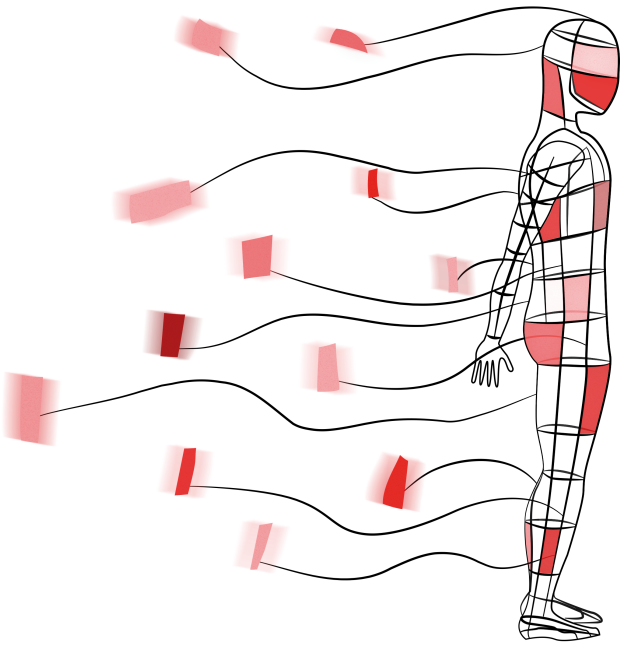

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Abstract

Direct determination by pathogen-specific real-time PCR assay for *B. fragilis* was compared to culture in major abscess and DFI biopsy samples. Real-time PCR resulted in an increased detection rate of 12% for *B. fragilis*, and could improve the detection of *B. fragilis* in clinical samples.

Major abscesses and diabetic foot infections (DFIs) are prevalent subtypes of a spectrum of clinical conditions termed complicated skin and skin structure infections (cSSSIs) [1, 2]. The microbiology of cSSSIs is diverse, as demonstrated during a recent clinical trial in patients with cSSSIs, 65% of the isolates contained gram-positive cocci, while 28% contained gram-negative bacilli. Anaerobes (7%) were found to a lesser extent and consisted mainly of the species *Bacteroides fragilis* (5.2%) [3].

B. fragilis is a gram-negative, obligate anaerobic rod present within the colon as part of the commensal microflora [4]. Both beneficial and harmful features have been described, depending on presence of virulence factors. *B. fragilis* strains producing polysaccharide A (PSA), exhibit immunomodulatory properties in the colon and have been shown to protect against the development of colitis in animal models [5]. In contrast, enterotoxigenic *B. fragilis* strains produce *B. fragilis* toxin (BFT) which is associated with diarrhoea in humans and led to development of colitis and colon cancer in animal models [6].

For accurate clinical decision-making and optimal antibiotic therapy, correct and fast determination of pathogens and their susceptibility is crucial. In routine practice, determination of *Bacteroides* species relies on culture of the clinical sample, during which they are easily overlooked, e.g. pathogens died before/during sampling and handling of the specimen, are present in mixed cultures, growth requires specific anaerobic conditions and requires longer periods of time. Different molecular assays, characterized by high sensitivity and fast turnaround times, have been developed in recent years to improve bacteriological detection [7, 8]. In this study, we evaluated the potential benefit of direct detection on clinical cSSSI samples by pathogen-specific real-time PCR assays for *B. fragilis* (and presence of enterotoxigenic *B. fragilis*).

A total of 150 major abscess and 128 DFI, first visit, pre-study treatment samples of 225 cSSSI patients were collected during a clinical trial [3]. Definitions and inclusion criteria of the study cohort are described elsewhere [3]. Samples were collected via biopsy of tissue or bone, curettage of the wound or aspiration of purulent discharge. After collection, samples were directly stored in preservation medium (BBL Port-A-Cul Transport vial, Becton Dickinson, Franklin Lakes, NJ, USA) and transported within 72 hours to the central laboratory (Eurofins medinet SAS, Plaisir, France). On arrival, samples were split in 2, 1 part for immediate culture and identification using standard clinical laboratory procedures, and another part was stored at -80°C for molecular analysis. Culture was performed according to CLSI guidelines and is described elsewhere [3].

Pathogen DNA was manually extracted by using the MolYsis Universal Microbial Detection kit for tissue samples (Molzym, Bremen, Germany) following the manufacturer's

instructions, to improve sensitivity and specificity for pathogen DNA since interfering human DNA is removed [9]. Pathogen DNA extraction was carried out in a laminar flow cabinet to prevent contamination. Primers and hydrolysis probe (Table 1) to detect *B. fragilis* were designed for this study based on the *recA* gene using Primer3 [10]. To differentiate the 3 BFT-types a melting-curve real-time PCR approach was designed, based on a previously described gene locus [11] , with specific melting-curve peaks at 74°C, 76°C and 78°C for BFT-type 1, 2 and 3, respectively [12, 13]. Real-time PCR reactions were performed as recently described by Stappers *et al.* [14].

Table 1. Primer and probe sequences.

Specificity	Forward primer	Probe	Reverse primer
B. fragilis	5′-GCTTTTGACC GCTTCTATGC-3′	5′-FAM-CCTGTTCATCT CGCAACCCGA-BHQ1-3′	5′-CAATTTCCAA CGCCTGTTCT-3′
Enterotoxigenic *B. fragilis*	5′-CAAGGACAAG AAGTGCTGGA-3′	n/a	5′-TCGCATTCAG GAAGATTCAA-3′

In silico analysis revealed a high-specificity for *B. fragilis* with no cross-specificity for other *Bacteroides* species. Of 29 well-characterized *Bacteroides* clinical isolates, including *B. fragilis* type-strain ATCC 25285, only the 11 *B. fragilis* isolates were positive for the *B. fragilis* real-time PCR. The BFT melting-curve PCR correctly identified 6 BFT-1, 5 BFT-2 and 1 BFT-3 of 12 sequencing confirmed BFT-positive *B. fragilis* strains. The limit-of-detection (LoD) of both assays was determined by 2-fold DNA dilution series. Based on the average *B. fragilis* genome size of 5.3Mbp [15] the LoD was determined as being 156 and 625 genomic copies per reaction for the *B. fragilis* PCR and BFT melting-curve PCR, respectively.

Of the 278 major abscess and DFI samples, 60 were positive for *B. fragilis* by real-time PCR, compared to 26 *B. fragilis* positive culture samples. Real-time PCR detected *B. fragilis* in 36 culture negative samples, but did not detect *B. fragilis* in 2 culture positive samples (Table 2). Subsequent 16S rRNA sequencing of the 2 cultured strains from the culture positive, real-time PCR negative biopsy samples identified them as being *Escherichia coli* and *Streptococcus agalactiae*. Of all 60 real-time PCR positive samples for *B. fragilis*, only 2 were positive for BFT real-time PCR. Both samples were of DFI origin and contained BFT-2 (data not shown).

Direct real-time PCR on DNA isolated from major abscess and DFI samples, resulted in an increased detection rate of 12% for *B. fragilis*, compared to culture. Statistical analysis using McNemar's test confirmed a significant difference between *B. fragilis* real-time PCR and culture-based assessment for both major abscesses ($P<0.001$) and DFIs ($P<0.05$).

Table 2. Real-time polymerase chain reaction (real-time PCR) versus culture-based assessment of *Bacteroides fragilis* prevalence in major abscesses and diabetic foot infections.

Major abscesses				**Diabetic foot infections**			
	Real-time PCR-positive	Real-time PCR-negative	Total		Real-time PCR-positive	Real-time PCR-negative	Total
B. fragilis							
Culture-positive	19	2	21	Culture-positive	5	0	5
Culture-negative	28	101	129	Culture-negative	8	115	123
Total	47	103	150	Total	13	115	128

Species	**Reference**	**Assay**	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
B. fragilis	Culture	Real-time PCR	92	86	40	99
	Real-time PCR	Culture	40	99	92	86

PPV, positive predictive value; NPV, negative predictive value.

The added value of direct real-time PCR for the detection of *B. fragilis* has been studied previously in different clinical samples [16-19]. Increased detection of *B. fragilis* has been reported using direct conventional and real-time PCR in intra-abdominal and pelvic samples [16], real-time PCR in postoperative or traumatic wound infections [17], whereas detection was similar using conventional [18] or nested PCR [19] in various clinical samples.

Both culture and real-time PCR were considered as gold standard for identification of *B. fragilis*. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) are shown in Table 2. Considering culture as a gold standard resulted in a high NPV and a poor PPV for our real-time PCR assay. Possible reasons for this discrepancy are real-time PCR is probably more sensitive than culture, real-time PCR may detect nonviable *B. fragilis*, contamination or cross-reactivity of the real-time PCR assay, or splitting of the biopsy sample for culture and real-time PCR, resulting in loss of the organism in 1 of the aliquots.

According to the BFT melting-curve PCR, *B. fragilis* enterotoxin was not frequent in our cSSSI samples. Other studies have demonstrated 6% - 33% of extraintestinal isolates positive for *B. fragilis* enterotoxin [6].

In conclusion, this study demonstrated that the use of pathogen-specific real-time PCR assays for *B. fragilis*, directly on clinical samples from patients with major abscesses and DFIs, resulted in an increased detection of *B. fragilis* compared to culture and could be an additional method for detection in clinical samples. In the future, the high NPV of our real-time PCR could be helpful in ruling out the presence of *B. fragilis*, the most frequent and virulent species of gram-negative anaerobic pathogens in cSSSI. When combined with

PCR approaches that simultaneously detect (functional) antibiotic resistance genes this diagnostic strategy would support the clinical decision to add or delete antibiotics with an effective anaerobic spectrum.

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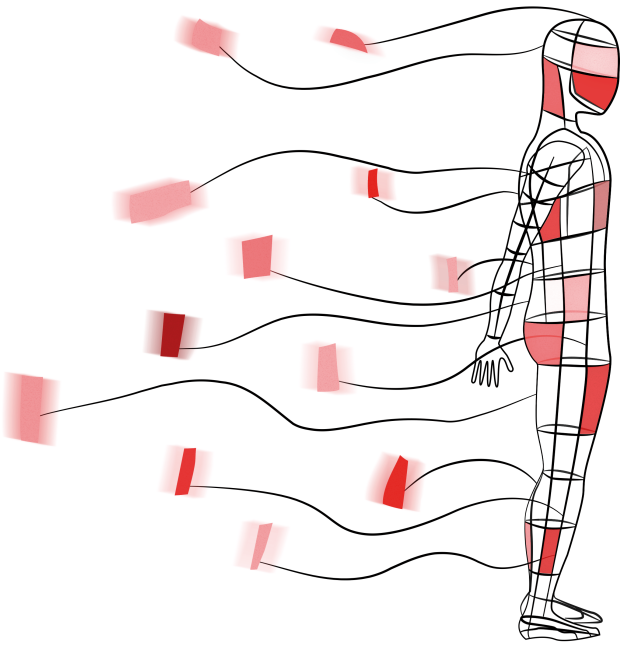
Chapter 9

Summary and conclusions



Summary and discussion

The skin is our first line of defence against pathogens. Breaching this barrier may result in the invasion of microorganisms and the development of complicated skin and skin structure infections (cSSSIs), depending on the immune response of the host and the virulence of the pathogen. This thesis describes several studies investigating the innate immune response, as well as the presence of relevant pathogens and specific virulence factors for the development of cSSSIs.

The host innate immune diversity and response in cSSSIs

In the first part of this thesis we focused on the role and diversity of the innate immune system of the host in cSSSIs. Cells of the innate immune system express pattern-recognition receptors (PRRs), which recognize conserved pathogen-associated molecular patterns, and result in production of cytokines and activation of the immune response [1]. In **Chapter 2**, we review the different PRR families: Toll-like receptors, C-type lectin receptors, NOD-like receptors, RIG-I-like receptors and AIM2-like receptors and describe their signalling pathways and functions in response to infection. In addition, we reviewed how genetic variation in genes encoding these receptors influences their function and susceptibility to infectious disease, emphasizing the fact that infections are strongly influenced by the genetic make-up of each individual [2].

Each microbe activates a distinct set of PRRs, resulting in tailored immune responses for each pathogen. *Bacteroides fragilis*, although a commensal to the human intestinal microflora, is the most frequently isolated anaerobic microorganism in skin and soft tissue infections [3]. Depending on its location in the body and virulence factors, both protective and detrimental effects have been described. One the one hand, *B. fragilis* capsular polysaccharide A (PSA) exhibits immunomodulatory properties in the colon and is associated with protection against colitis in animal models. On the other hand, extraintestinal presence of *B. fragilis* PSA upon breach of the mucosal or skin barrier is associated with abscess formation [4]. Furthermore, enterotoxigenic *B. fragilis* strains expressing *B. fragilis* toxin (BFT) induce diarrhoea in humans and led to the development of colitis and colon cancer in animal models [5]. In **Chapter 3**, we investigated the PRRs involved in the recognition of *B. fragilis* and characterized its induced cytokine profile in peripheral blood mononuclear cells (PBMCs). Using a set of complementary approaches, including cell lines expressing specific PRRs, as well as antibody-blockage and genetic variants of PRRs in PBMCs, we were able to demonstrate that TLR2, TLR1 and NOD2, but not TLR4, TLR6, Dectin-1 or NOD1, mediate pattern recognition of *B. fragilis*. This was supported a previously described role for TLR2 and NOD2 in the recognition of *B. fragilis* [6-10], but contrasted with 1 study indicating TLR4 as a PRR for *B. fragilis* LPS [11]. TLR1

is through heterodimeric association with TLR2 involved in the recognition of various ligands of microbial origin, but the role for TLR1 in recognition of *B. fragilis* was described for the first time in our study. In addition, we demonstrated that *B. fragilis* is a strong inducer of IL-6 and IL-8 production, a moderate inducer of IL-1β and TNFα and a poor inducer of IL-10, IFNγ, IL-23 and IL-17 cytokine responses in human PBMCs. Another study confirmed the production of IL-6 and TNFα upon stimulation of human mononuclear cells with *B. fragilis* [12], whereas others focused on specific cell wall components in the murine setting [10, 13-17].

Besides gram-negative microorganisms such as *B. fragilis*, the vast majority of SSSIs are caused by gram-positive bacteria, mainly *Staphylococcus aureus* and *Streptococcus* species [18]. Based on the findings in Chapter 3 and the previously described role of TLRs and NLRs in recognition of these gram-positive bacteria [19-21], we aimed to investigate whether genetic variation in genes encoding these PRRs would affect the susceptibility to cSSSIs. Presence of genetic variants was determined in a cohort of patients with the important cSSSI subtypes: major abscess, diabetic foot infection, wound infection or infected ischemic ulcers and compared to healthy controls [22]. In **Chapter 4**, we demonstrated that genetic variants in *TLR1* (S248N and R80T), *TLR2* (P631H) and *TLR6* (P249S) were associated with an increased susceptibility to cSSSIs. In contrast to healthy controls, patients with cSSSI were more often heterozygous or homozygous for the allelic variant. The fact that these variants in *TLR1*, *TLR2* and *TLR6* have also been linked to other infectious diseases [23-29] and that specifically these receptors are of major importance for innate immune recognition of gram-positive bacteria [19-21] further underlines their significance in the innate immune response to cSSSIs. Moreover, presence of allelic variants (248N and 80T) in *TLR1* is associated with decreased activation of transcription factor NF-κB [30] and we demonstrated that these variants were associated with decreased cytokine responses of PBMCs after stimulation with *S. aureus* or specific TLR agonists, linking the impaired TLR function with the increased risk of the development of cSSSIs.

TLR1, *TLR2* and *TLR6* are located in a cluster on chromosome 4, together with *TLR10*. In **Chapter 5**, we investigated the role of the innate immune response to cSSSI pathogens and the effect of genetic variation in *TLR10* on the susceptibility to cSSSIs. In contrast to the other members of the TLR2 gene cluster, TLR10 was demonstrated to function as an inhibitory receptor in the innate immune response to cSSSIs. Using a cell line which over-expressed TLR2, TLR10 or both TLR2 and TLR10, we demonstrated that TLR10 inhibited the TLR2-mediated cytokine secretion after exposure to cSSSI pathogens *S. aureus*, *B. fragilis* and *Escherichia coli*. Neutralization the function of TLR10 on human PBMCs, using TLR10 antibodies, resulted in increased cytokine responses following exposure to cSSSI pathogens. Genetic variants I775L, I369L and N241H in *TLR10* were associated

with a reduced susceptibility to cSSSIs, as patients with cSSSI were significantly less often heterozygous and homozygous for the allelic variants compared to the healthy controls. Moreover, presence of these allelic variants in *TLR10* was associated with increased cytokine responses of PBMCs after stimulation with cSSSI pathogens or specific TLR agonists. These findings support the concept that TLR10 functions as a anti-inflammatory PRR [31-34], although 2 contrasting studies suggested a pro-inflammatory effect of this receptor [35, 36].

PRR recognition of a pathogen results in activation of an immune response and production of pro- and anti-inflammatory cytokines [1]. In **Chapter 6**, we investigated the influence of sequence variants in genes encoding cytokines on the susceptibility to cSSSIs, and studied their functional consequences on the innate immune response to these cSSSI pathogens. We demonstrated that genetic variants in *IL6* rs1800797, *TNF* rs1800629 and *IL17A* rs8193036 increased susceptibility to cSSSIs, whereas *IL10* rs1800871 and *IFNG* rs2069705 were associated with decreased susceptibility to cSSSIs, confirming their previously described importance in susceptibility to infectious disease [37-42]. Patients with cSSSI were more often heterozygous or homozygous for the allelic variant of *IL6*, *TNF* and *IL17A* and less often heterozygous and homozygous for the allelic variant of *IL10* and *IFNG*, compared to healthy controls. In contrast to previously described effects for *IL6* rs1800797 [43] and *IL10* rs1800871 [44], we were unable to demonstrate differences in cytokine responses of PBMCs from individuals carrying the associated genetic variants after stimulation with cSSSI pathogens. As the associated genetic variants are located in the promoter region of the gene, and therefore could impact on gene expression levels, we expected to observe differences in cytokine secretion. Possible reasons how the associated genetic variants can still influence susceptibility to cSSSIs are different kinetics of cytokine secretion, or altered biological function of the protein due to the genetic variant itself or linkage with an exonic variant which causing the effect.

Detection of pathogens in cSSSIs

In the second part of this thesis we focussed on the detection of common cSSSI pathogens, comparing routine culture with direct, pathogen-specific real-time PCR assays in clinical samples of patients with diabetic foot infections and major abscesses. In **Chapter 7**, we determined the presence of *S. aureus*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and the *Streptococcus anginosus* group. In comparison to culture, the direct real-time PCR assays resulted in a higher detection of 34% for *S. aureus*, 37% for *S. pyogenes*, 18% for *S. agalactiae*, 4% for *S. dysgalactiae* subspecies *equisimilis* and 7% for the *S. anginosus* group, whereas no differences were observed for *S. dysgalactiae* subspecies *dysgalactiae*. Differences were significant between real-time PCR and culture for in both major abscesses and DFIs for *S. aureus* and *S. pyogenes*, only major abscesses

for *S. agalactiae*, and only diabetic foot infections for *S. dysgalactiae* subspecies *equisimilis* and *S. anginosus* group. In **Chapter 8**, we determined the presence of *B. fragilis* and the direct real-time PCR assay resulted in 12% higher detection compared to culture, which was both significant in major abscesses and diabetic foot infections. These findings confirm for cSSSIs, which previously has been shown for other infections [45], that molecular detection is a fast, simple and sensitive alternative for the detection of pathogens compared to culture methods. In addition, we also determined the presence of *S. aureus* virulence factor Panton-Valentine leukocidin (PVL) and *B. fragilis* toxin (BFT), in Chapter 7 and Chapter 8 respectively. PVL is a pore-forming toxin, secreted by *S. aureus* and is associated with lysis of leukocytes [46]. We demonstrated that 73% of the major abscesses, but only 14% of the diabetic foot infections, were positive for PVL by real-time PCR, which aligns with a previously described association for PVL with *S. aureus* skin and skin structure infections [47]. BFT-producing *B. fragilis* strains have mainly been associated with diarrhoea in humans, but extraintestinal presence of enterotoxigenic *B. fragilis* has been reported [5]. In our study less than 1% of clinical cSSSI samples contained BFT-producing *B. fragilis*, suggesting BFT does not play a role in cSSSIs.

General conclusions and future perspectives

In this thesis we have investigated the contribution of the innate immunity diversity of the host and presence of relevant pathogens in the development of cSSSIs. The advances presented here provide several additional pieces necessary for understanding the puzzle of skin and skin structure infections. On the side of the host, we have demonstrated a role for several PRRs in the recognition of cSSSI pathogens. We have shown the effect of genetic variation in PRRs of innate immune cells, as well as their effector molecules, the cytokines, on susceptibility to cSSSIs. These findings increase our understanding of the biological mechanisms involved in cSSSI pathogenesis. Furthermore, these host factors that predispose to development of cSSSIs, potentially provides opportunities to define a predictive profile for the screening of persons at risk of these infections and individualized diagnosis and treatment. On the side of the pathogen, we have demonstrated that molecular detection is a fast, simple and sensitive method for the detection of prevalent cSSSI pathogens, compared to routine culture methods. The true value of this method and the impact on clinical decision making and disease outcome for the patient remains to be determined, but the increased detection over culture is promising.

On both the side of the host and the pathogen we focussed on molecular methods that either detected a single genetic variant or a single pathogen. With the development and increasing accessibility of techniques such as next-generation sequencing, future studies should aim to comprehensively study the role of the genetic variants in the entire genome

for susceptibility to cSSSIs. On the side of the pathogen, next-generation sequencing would not only allow for the detection of all pathogens, but also the presence of virulence factors and antibiotic resistance genes in 1 assay. The combination of both approaches has the potential to revolutionize our understanding of the pathogenesis of cSSSIs.

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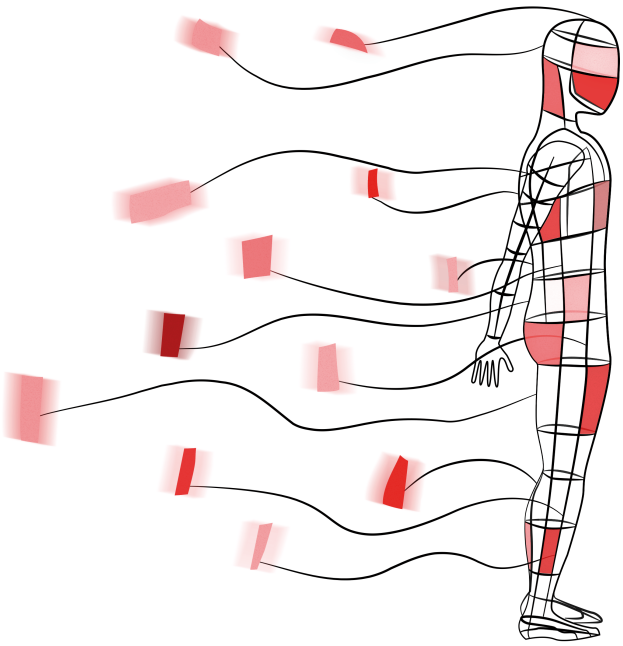
9

Chapter 10

Samenvatting en conclusies



Samenvatting en discussie

De huid is de eerste verdedigingslinie tegen pathogenen. Indien deze barrière doorbroken wordt kan dit leiden tot invasie van micro-organismen en het ontstaan van huid en weke delen infecties. Deze infecties kunnen opgedeeld worden in 2 categorieën: ongecompliceerde (uSSSIs) en gecompliceerde huid en weke delen infecties (cSSSIs). cSSSIs betreft infecties die vaak dieper gelegen zijn en chirurgische behandeling vereisen, of komen voor in een gastheer die aan een aandoening(en) lijdt die de uitkomst van de behandeling kan beïnvloeden. Oppervlakkige infecties in gebieden waar de kans op anaerobe en gram-negatieve pathogenen groot is worden ook beschouwd als cSSSIs. Veel voorkomende cSSSIs zijn: diabetische voetinfecties (DFIs), majeure abcessen van de weke delen en infecties na chirurgie of trauma [1].

De kans op, en ernst van cSSSIs is afhankelijk van de immuunrespons van de gastheer alsmede de virulentie van het pathogeen. In dit proefschrift beschrijven we verschillende studies die aan de ene kant de rol van het aangeboren immuunsysteem en aan de andere kant de aanwezigheid van relevante pathogenen en specifieke virulentiefactoren, in de ontwikkeling van cSSSIs bestuderen.

De aangeboren immuunrespons van de gastheer in cSSSIs

In het eerste deel van dit proefschrift ligt de nadruk op het bestuderen van de rol en diversiteit van het aangeboren immuunsysteem van de gastheer in cSSSIs. Cellen van het aangeboren immuunsysteem bevatten gespecialiseerde pathogeen herkenningsreceptoren (PRRs), waarmee ze pathogeen specifieke moleculaire patronen (PAMPs) kunnen herkennen van binnengedrongen micro-organismen. Deze herkenning leidt tot de productie van signaalstoffen, zogenaamde cytokines, en de activatie van de immuunrespons [2]. **Hoofdstuk 2** geeft een overzicht van de verschillende PRR families: Toll-like receptoren, C-type lectine receptoren, NOD-like receptoren, RIG-I-like receptoren en AIM2-like receptoren en beschrijft de signaalroutes en functies van deze PRRs tijdens infectie. Daarnaast toont het hoe variatie in genen, die coderen voor deze receptoren, de functie en gevoeligheid voor infectieziekten beïnvloeden. Dit hoofdstuk benadrukt het feit dat infectieziekten sterk beïnvloed worden door de genetische opmaak van het individu [3].

Elk micro-organisme activeert een eigen combinatie van PRRs, resulterend in een specifieke immuunrespons voor ieder pathogeen. *Bacteroides fragilis*, een commensaal van de humane darmmicroflora, is het meest frequent geïsoleerde anaerobe micro-organisme in cSSSIs [4]. Afhankelijk van de locatie in het lichaam en de aanwezigheid van virulentiefactoren, zijn zowel beschermende als schadelijke effecten beschreven

voor *B. fragilis*. Aan de ene kant heeft het capsulair polysaccharide A (PSA) van *B. fragilis* immuunmodulerende eigenschappen in het colon en is het geassocieerd met bescherming tegen colitis in diermodellen. Aan de andere kant is aanwezigheid van PSA buiten de darm, na het doorbreken van de mucosa of de huid, geassocieerd met de vorming van abcessen [5]. Daarnaast produceren enterotoxigene *B. fragilis* stammen een toxine (*B. fragilis* toxin; BFT) dat diarree veroorzaakt in mensen en leidt tot colitis en colonkanker in diermodellen [6]. In **Hoofdstuk 3** word onderzocht welke PRRs betrokken zijn bij de herkenning van *B. fragilis* en het door *B. fragilis* geïnduceerde cytokineprofiel in perifeer bloed mononucleaire cellen (PBMCs) gekarakteriseerd. Door gebruik te maken van onder andere cellijnen die specifieke PRRs tot expressie brengen, alsmede blokkade van PRRs met antilichamen en genetische varianten van PRRs, konden wij aantonen dat TLR2, TLR1 en NOD2, maar niet TLR4, TLR6, Dectine-1 en NOD1 betrokken zijn bij de herkenning van *B. fragilis*. Dit werd ondersteund door een eerder beschreven rol voor TLR2 en NOD2 in de herkenning van *B. fragilis* [7-11], maar contrasteerde met een studie waarin TLR4 beschreven wordt als PRR voor *B. fragilis* LPS [12].

Via de vorming van een heterodimeer met TLR2, is TLR1 betrokken bij de herkenning van verschillende microbiële liganden, maar de rol van TLR1 in de herkenning van *B. fragilis* is voor het eerst beschreven in onze studie. Verder hebben we aangetoond dat *B. fragilis* een hoge productie van cytokines IL-6 en IL-8, een gematigde productie van cytokines IL-1β en TNFα en een lage productie van cytokines IL-10, IFNγ, IL-23 en IL-17 induceert in humane PBMCs. Een andere studie bevestigde de productie van IL-6 en TNFα na stimulatie van humane mononucleaire cellen met *B. fragilis* [13], terwijl andere studies zich richtten op specifieke celwand componenten van *B. fragilis* in diermodellen [11, 14-18].

Naast gram-negatieve micro-organismen zoals *B. fragilis*, wordt de meerderheid van de SSSIs veroorzaakt door gram-positieve bacteriën, met name *Staphylococcus aureus* en *Streptococcus* species [19]. Op basis van de bevindingen in Hoofdstuk 3 en de eerder beschreven rol voor TLRs en NLRs in de herkenning van deze gram-positieve bacteriën [20-22], hebben we de relatie tussen genetische variatie in genen die coderen voor deze PRRs en de gevoeligheid voor cSSSIs onderzocht. Aanwezigheid van genetische varianten werd bepaald in een cohort van patiënten met de cSSSI subtypes: majeure abcessen, diabetische voetinfecties, wondinfecties of geïnfecteerde ischemische ulcera en vergeleken met gezonde controles [23]. In **Hoofdstuk 4**, wordt aangetoond dat genetische varianten in *TLR1* (S248N en R80T), *TLR2* (P631H) en *TLR6* (P249S) geassocieerd zijn met een verhoogde gevoeligheid voor cSSSIs. In tegenstelling tot gezonde controles, zijn patiënten met cSSSI vaker heterozygoot of homozygoot voor de allelische variant. Het feit dat deze varianten in *TLR1*, *TLR2* en *TLR6* ook geassocieerd zijn met andere infectieziekten [24-30], en specifiek deze PRRs van groot belang zijn voor de herkenning van gram-positieve bacteriën [20-22],

onderstreept de belangrijke rol van deze receptoren in de aangeboren immuunrespons bij cSSSIs. Bovendien is de aanwezigheid van allelische varianten (248N en 80T) in *TLR1* geassocieerd met een verlaagde activatie van transcriptiefactor NF-κB [31] en hebben wij kunnen aantonen dat aanwezigheid van deze varianten na stimulatie van PBMCs met *S. aureus* en specifieke TLR agonisten leidt tot lagere cytokineresponsen, welke mogelijk de verhoogde gevoeligheid voor cSSSIs verklaren.

TLR1, *TLR2* en *TLR6* liggen in een cluster op chromosoom 4, samen met *TLR10*. In **Hoofdstuk 5** wordt de rol van de aangeboren immuunrespons op cSSSI pathogenen en het effect van genetische variatie in *TLR10* op de gevoeligheid voor cSSSIs onderzocht. In tegenstelling tot de overige TLRs in het TLR2-gencluster, konden wij aantonen dat TLR10 als een remmende receptor functioneert in de aangeboren immuunrespons tegen cSSSIs. Met behulp van een cellijn die TLR2, TLR10, of beide tot overexpressie brengt, hebben we aangetoond dat TLR10 de door TLR2-gemedieerde cytokinesecretie remt na blootstelling aan cSSSI pathogenen *S. aureus*, *B. fragilis* en *Escherichia coli*. Het neutraliseren van de functie van TLR10 in humane PBMCs, met behulp TLR10-antilichamen, resulteerde in verhoogde cytokine responsen na blootstelling aan cSSSI pathogenen. Genetische varianten I775L, I369L en N241H in *TLR10* zijn geassocieerd met een verminderde gevoeligheid voor cSSSIs, wat betekent dat patiënten met cSSSI significant minder vaak heterozygoot en homozygoot zijn voor het allelische varianten vergeleken met gezonde controles. Bovendien konden wij aantonen dat de aanwezigheid van deze allelische varianten in *TLR10* geassocieerd is met verhoogde cytokineresponsen door PBMCs na stimulatie met cSSSI pathogenen of specifieke TLR-agonisten. Deze bevindingen ondersteunen de consensus dat TLR10 functioneert als een anti-inflammatoire PRR [32-35], hoewel 2 contrasterende studies suggereren dat TLR10 een pro-inflammatoire receptor is [36, 37].

PRR herkenning van een pathogeen resulteert in activatie van een immuunrespons en de productie van pro- en anti-inflammatoire cytokines [2]. In **Hoofdstuk 6** wordt het effect van genetische varianten in genen die coderen voor cytokines op de gevoeligheid voor de ontwikkeling van cSSSIs onderzocht, en de functionele gevolgen van deze varianten voor de aangeboren immuunrespons op cSSSI pathogenen. We konden aantonen dat genetische varianten in *IL6* rs1800797, *TNF* rs1800629 en *IL17A* rs8193036 geassocieerd zijn met een verhoogde gevoeligheid voor cSSSIs, terwijl *IL10* rs1800871 en *IFNG* rs2069705 geassocieerd zijn met een verminderde gevoeligheid voor cSSSIs. Dit onderstreept nogmaals de eerder beschreven rol voor deze genetische varianten in de vatbaarheid voor infectieziekten [38-43]. Patiënten met cSSSI waren vaker heterozygoot of homozygoot voor de allelische variant van *IL6*, *TNF* en *IL17A* en minder vaak heterozygoot en homozygoot voor de allelische variant van *IL10* en *IFNG*, vergeleken met gezonde controles. In tegenstelling tot de eerder beschreven effecten voor *IL6* rs1800797 [44] en

IL10 rs1800871 [45], waren wij niet in staat verschillen in cytokineresponsen van PBMCs van individuen met en zonder de geassocieerde genetische varianten aan te tonen na stimulatie met cSSSI pathogenen. Aangezien de geassocieerde genetische varianten gelokaliseerd zijn in het promotorgebied van het gen, en daarom van invloed kunnen zijn op genexpressie, hadden we verwacht verschillen in de cytokinesecretie te observeren. Mogelijk kunnen de geassocieerde genetische varianten de gevoeligheid voor cSSSIs beïnvloeden door een afwijkende kinetiek van cytokinesecretie, of een veranderde biologische functie van het eiwit als gevolg van de genetische variant zelf of gelinkt aan een exon-variant die het effect veroorzaakt.

Detectie van pathogenen in cSSSIs

In het tweede deel van dit proefschrift hebben we ons gericht op de detectie van veel voorkomende cSSSI pathogenen, door routine cultuurtechnieken te vergelijken met directe, pathogeen-specifieke real-time PCR-assays op klinische monsters van patiënten met DFIs en majeure abcessen. In **Hoofdstuk 7**, beschrijft de detectie van *S. aureus*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* en de *Streptococcus anginosus* groep. In vergelijking met de kweek, resulteerde de directe real-time PCR-assays in een hogere detectie van 34% voor *S. aureus*, 37% voor *S. pyogenes*, 18% voor *S. agalactiae*, 4% voor *S. dysgalactiae* subspecies *equisimilis* en 7% voor de *S. anginosus* groep, terwijl geen verschillen werden waargenomen voor *S. dysgalactiae* subspecies *dysgalactiae*. De verschillen tussen de real-time PCR en kweek waren significant voor zowel majeure abcessen en DFIs voor *S. aureus* en *S. pyogenes*, enkel voor majeure abcessen voor *S. agalactiae*, en enkel voor DFIs voor *S. dysgalactiae* subspecies *equisimilis* en de *S. anginosus* groep. In **Hoofdstuk 8**, wordt de aanwezigheid van *B. fragilis* bepaald en de directe real-time PCR resulteerde in een 12% hogere detectie in vergelijking met cultuurtechnieken, wat significant was voor zowel majeure abcessen en DFIs. Deze bevindingen bevestigen voor cSSSIs, wat al eerder aangetoond is voor andere infecties [46], dat moleculaire detectie een snel, eenvoudig en gevoelig alternatief is voor de detectie van pathogenen in vergelijking met cultuurtechnieken. Daarnaast hebben we ook de aanwezigheid van *S. aureus* virulentiefactor Panton-Valentine Leukocidine (PVL) en *B. fragilis* toxine (BFT) bepaald in respectievelijk Hoofdstuk 7 en Hoofdstuk 8. PVL is een poriënvormend toxine wat uitgescheiden wordt door *S. aureus* en gepaard gaat met de lysis van leukocyten [47]. Met behulp van real-time PCR konden we aantonen dat 73% van de majeure abcessen en slechts 14% van de DFIs positief waren voor PVL. Dit komt overeen met een eerder beschreven associatie van PVL met *S. aureus* huid en weke delen infecties [48]. BFT-producerende *B. fragilis* stammen zijn hoofdzakelijk geassocieerd met diarree bij de mens, maar aanwezigheid van enterotoxigene *B. fragilis* buiten de darm is eerder beschreven [6]. Echter, in onze studie bevatte minder dan 1% van de klinische cSSSI monsters BFT-producerende *B. fragilis*, wat suggereert dat BFT geen rol speelt in cSSSIs.

Conclusies en toekomstperspectieven

In dit proefschrift wordt de bijdrage van genetische variatie in de aangeboren immuniteit van de gastheer en de aanwezigheid van pathogenen in de ontwikkeling van cSSSIs onderzocht. De hier gepresenteerde ontwikkelingen bieden een aantal aanvullende stukken die nodig zijn om de ingewikkelde puzzel van de huid en weke infecties op te lossen. Aan de kant van de gastheer hebben we de rol voor enkele PRRs in de herkenning van cSSSI pathogenen aangetoond. Daarnaast hebben we laten zien dat genetische variatie in PRRs van de aangeboren immuniteit, evenals hun effector-moleculen (de cytokines), effect hebben op de gevoeligheid voor cSSSIs. Deze bevindingen versterken onze kennis van de biologische mechanismen die betrokken zijn bij pathogenese van cSSSIs. Bovendien leiden deze factoren van de gastheer tot een predispositie voor cSSSIs, wat mogelijk perspectieven biedt voor de ontwikkeling van een voorspellend profiel. Toepassingen hiervan zijn het screenen van personen die risico lopen op deze infecties, bijvoorbeeld voor een chirurgische ingreep, en voor een geïndividualiseerde diagnose of behandeling. Aan de kant van het pathogeen hebben we aangetoond dat moleculaire detectie, in vergelijking met routine kweekmethoden, een snelle, eenvoudige en gevoelige techniek is voor het detecteren van veel voorkomende cSSSI pathogenen. De werkelijke waarde van deze methode en de invloed op de klinische besluitvorming en de ziekte uitkomst voor de patiënt zal nog moeten blijken, maar de verhoogde detectie ten opzichte van kweektechnieken is veelbelovend.

Zowel in ons onderzoek naar de immuunrespons van de gastheer als het pathogeen hebben wij ons gericht op moleculaire methoden die gericht waren enerzijds op het detecteren van een genetische variant of anderzijds op een specifiek pathogeen. Met de ontwikkeling en de toenemende toegankelijkheid van technieken zoals next-generation sequencing dienen toekomstige studies zich te richten op diepgaande analyses van de rol van genetische varianten in het gehele menselijke genoom op de gevoeligheid voor cSSSIs. Aan de kant van het pathogeen zou next-generation sequencing niet alleen de mogelijkheid bieden tot de detectie van micro-organismen, maar ook de aanwezigheid van virulentiefactoren en antibiotica resistentiegenen; dit alles gecombineerd in één enkel assay. De combinatie van beide benaderingen zal onze kennis van de pathogenese van cSSSIs revolutioneren.

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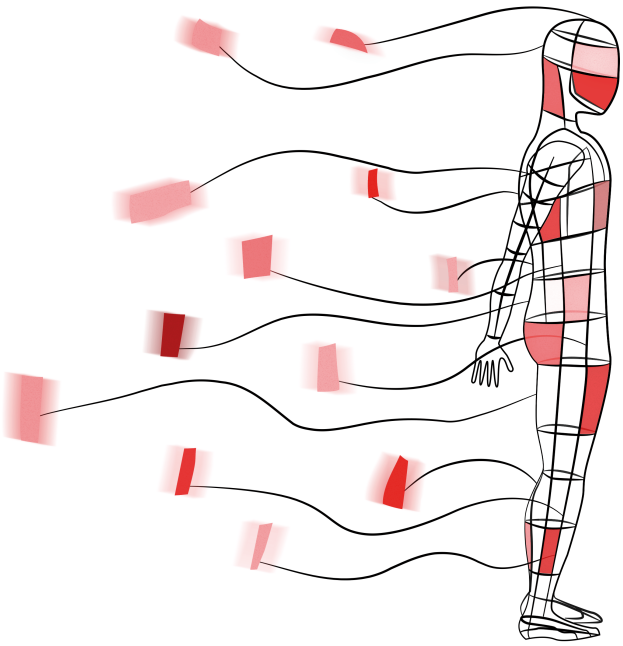
Chapter 11

Acknowledgements

List of publications

Curriculum vitae



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List of publications

1. **Stappers MH**, Hagen F, Reimnitz P, Mouton JW, Meis JF, Gyssens IC. *Bacteroides fragilis* in biopsies of patients with major abscesses and diabetic foot infections: direct molecular versus culture-based detection. Diagn Microbiol Infect Dis, in press.

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6. **Stappers MH**, Thys Y, Oosting M, Plantinga TS, Ioana M, Reimnitz P, Mouton JW, Netea MG, Joosten LA, Gyssens IC. Polymorphisms in cytokine genes *IL6*, *TNF*, *IL10*, *IL17A* and *IFNG* influence susceptibility to complicated skin and skin structure infections. Eur J Clin Microbiol Infect Dis, 2014; 33:2267-2274.

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Curriculum vitae

Mark Stappers was born in Venray on September 19th 1986. He spent his youth in the Limburgian village of Hegelsom and received his VWO-diploma from the Dendron College Horst in 2004. In the same year he started studying Biomedical Sciences at the Radboud University in Nijmegen. During this period he performed 3 internships. During his first internship at the department of Biochemistry of the Radboud University, he studied the role of erythrocyte band-3 protein and binding of anti-band-3 antibodies to these cells during storage under blood bank conditions. His second project, at the department of Rheumatology of the Radboud University focused on the characterization of murine T-helper cell subsets under polarizing conditions. In his last project at the Division of Immunology, Infection and Inflammation of the University of Glasgow he investigated the role of Th17 cells in breach of self-tolerance in murine models for rheumatoid arthritis. Mark obtained his Biomedical Sciences degree in 2010, with a major in Human Pathobiology and minors in Immunology and Auto-Immune Diseases. In October of the same year, he started his PhD research at the department of Internal Medicine of the Radboud University. Under the guidance of prof. dr. Inge Gyssens, prof. dr. Mihai Netea, prof. dr. Leo Joosten and prof. dr. Johan Mouton he investigated the contribution of the innate immunity diversity of the host and presence of relevant pathogens in the development of complicated skin and skin structure infections.This research project was performed in close collaboration with the department of Medical Microbiology and Infectious Diseases of the Canisius-Wilhelmina Hospital, and the department of Immunology - Biochemistry of Hasselt University. The latter from which he received a doctoral grant, providing funding for the final year of his doctorate research. During his PhD project, Mark was given the opportunity to present his work at numerous international scientific conferences. The results from his 4 years of PhD research are described in this thesis. In January 2015, he started as a postdoctoral research fellow, investigating the mechanisms of protective immunity against fungi, at the Aberdeen Fungal Group of the University of Aberdeen.