Targeting Phagocytes: Learning from *Staphylococcus aureus*

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Learning from *Staphylococcus aureus*

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(met een samenvatting in het Nederlands)

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CHAPTER ONE

GENERAL INTRODUCTION

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Staphylococcus aureus

In 1880, the Scottish surgeon Alexander Ogston (1844-1929) isolated "tufts and chains of round organisms in great numbers" out of pus, a mixture of predominantly dead neutrophils [1]. A few years later, Ogston adapted the nomenclature from micrococci to what we now refer to as *Staphylococcus aureus* [1, 2]. *S. aureus* is a Gram-positive bacterium and a common commensal that colonizes the skin and anterior nares of 20-30% of the general human population [3]. Furthermore, 60% of the population are intermittent carriers of *S. aureus* [4]. Even though extranasal *S. aureus* carriage has been observed, the anterior nares have been suggested to be the main reservoir for *S. aureus* colonization [3, 5]. Studies have additionally shown that this nasal carriage of *S. aureus* can be a potential factor leading to *S. aureus* infection [6]. Although *S. aureus* presents itself as a commensal, *S. aureus* is also a major pathogen and one of the most common causes of infections in humans. However, the basis for *S. aureus* colonization and its relationship to infection are incompletely understood. *S. aureus* can infect almost all host tissues and organs causing a variety of diseases, spanning from superficial skin and soft tissue infections (SSTIs) to more severe and invasive diseases like necrotizing pneumonia, osteomyelitis, endocarditis and bloodstream infections with a poor prognosis and high mortality [7]. Treatment of *S. aureus* infections is composed primarily out of antibiotics [8]. However, the emergence of methicillin resistant *S. aureus* (MRSA) strains has made it increasingly more difficult to treat these infections with antibiotics [8-10]. Methicillin resistance components are encoded on the staphylococcal cassette chromosome *mec* (SCC*mec*) and are transferred and acquired via mobile genetic elements (MGE) [11, 12]. MRSA infections account for 30-50% of hospital acquired *S. aureus* infections in the Western Pacific region, the United States and Latin America, and are a major concern due to the high morbidity and mortality [13, 14]. The prevalence of MRSA in Europe is lower at 20-30% [14], and below 2% in the Netherlands [15]. Although MRSA was initially associated with nosocomial infections, a change in MRSA epidemiology occurred, showing the emergence of MRSA infections in subjects with no prior hospital association [16]. These community-associated MRSA (CA-MRSA) isolates are genetically distinct and can produce additional virulence factors, such as Panton-Valentine leukocidin (PVL), compared to health-care-associated MRSA (HA-MRSA) strains [17, 18]. Treatment and prevention of *S. aureus* infections is further hampered by a lack of advancement in *S. aureus* vaccine development [19]. This is thought to be mainly due to the lack of understanding *S. aureus* pathogenesis in humans,

and the reliance on suboptimal pre-clinical animal models that poorly reflect *S. aureus* infections in humans [20]. As a result, there is a need for novel therapeutics in the fight against *S. aureus*. A better understanding of *S. aureus* pathogenesis and host-factors involved in these processes might reveal potential targets for therapeutics and anti-virulence strategies.

Innate immunity: Phagocytes, CD45 and GPCRs

Upon infection, bacteria are recognized and faced with the host humoral and cellular innate immune response [21]. The complement system, a major effector mechanism in the humoral defence, comprises more than 35 proteins that are present in host plasma and cell surfaces [22, 23]. Activation of the complement system via either the classical, lectin or alternative pathway, results in a cascade of proteolytic events followed by the release of multiple cleaved fragments of complement components [21, 24]. Together with immunoglobulins, these complement components opsonize invading bacteria, aiding the recognition and uptake by phagocytes [25]. Neutrophils are the most abundant at 60% of the total leukocyte population and are the most important phagocytic cells during acute bacterial infections [26]. Patients with complement system or neutrophil deficiencies are susceptible to severe and fatal bacterial infections [26]. In addition, neutrophil function disorders, such as chronic granulomatous disease, have been shown to be predisposing factors associated with certain bacterial infections, in which *S. aureus* being the causative pathogen in many occasions [27, 28]. These findings show the key role that neutrophils play in the containment of *S. aureus* in the early stages of infection [29].

Circulating peripheral blood neutrophils are recruited and are slowed down approaching the site of inflammation or infection by changes on the endothelial surface, such as the expression of P-selectin glycoprotein ligand 1 (PSGL1), induced by resident leukocytes and the endothelium [30, 31]. Neutrophils subsequently extravasate and follow chemokine gradients composed of a wide array of chemoattractants, such as the complement component C5a and the bacterial *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), towards the site of infection [31]. In addition, neutrophils are activated and primed by chemoattractants that interact with G-protein-coupled receptors (GPCRs) present on the neutrophils' surface and via Toll-like receptors (TLRs) [32]. Neutrophils are the first to arrive at the site of infection and rapidly phagocytose bacteria that are opsonized by antibodies or by components of the complement

system [29, 33]. The phagocytic vacuoles containing bacteria subsequently fuse with azurophilic granules that contain antimicrobial proteins such as neutrophil serine proteases, myeloperoxidase, lysozyme and pore-forming peptides [34]. Together with reactive oxygen species (ROS), these antimicrobial proteins kill phagocytosed bacteria. The uptake and intracellular killing of bacteria by neutrophils is accompanied by additional extracellular antimicrobial approaches such as the degranulation of microbicidal proteins and formation of neutrophil extracellular traps (NETs) [33, 35]. In conclusion, neutrophils are well equipped to kill invading bacteria, including staphylococci.

The reaction of leukocytes to stimuli is initiated and controlled via the expression of different receptors. Some receptors are widely expressed on all leukocytes, whereas other receptors are exclusively expressed on leukocyte subsets like neutrophils. CD45 is a highly conserved transmembrane glycoprotein and abundantly expressed on all nucleated hematopoietic cells [36-38]. Also known as Ly-5, leukocyte common antigen or protein tyrosine phosphatase receptor type C (PTPRC), CD45 is as a pan-leukocyte market and is encoded by the gene *PTPRC*. Contrary to the low homology (35%) of the CD45 extracellular domain, the cytoplasmic region is highly conserved (95%) in all mammals [38, 39]. The cytoplasmic region of CD45 contains two protein tyrosine phosphatase domains that are connected to the extracellular region by a single transmembrane region [40]. The ectodomain of CD45 is heavily N-glycosylated and consists of three fibronectin type III repeats and a cysteine-rich region [38, 39]. Several isoforms of human CD45 are expressed via the alternative splicing of exons 4, 5 and 6 [38]. These exons encode amino acid sequences proximal to the cysteine rich region and contain multiple glycosylation sites [39]. The glycosylation pattern of the extracellular domains is dependent on the cell type, developmental stage and activation state, suggesting a role of glycosylation of CD45 in cellular functions [39, 41]. It is known that CD45-deficient mice and humans develop a severe-combined immunodeficiency (SCID) phenotype, suggesting an important role of CD45 in the immune system [42, 43]. The function of CD45 in innate immunity and bacterial infections however has rarely been assessed. It has been suggested that CD45 may modulate chemokine-induced signalling on neutrophils [44-46]. In fact, a study has shown that CD45 modulates chemoattractant-mediated neutrophil response to *S. aureus* infection [47]. Regardless, the role and function of CD45 remains poorly understood.

During the 1960's and 1970's there was a growing notion that phagocytes have the ability to chase, capture and clear invading bacteria by responding to small molecules of bacterial and host origin [48, 49]. Leukocytes sense these small molecules via the expression of a large number of surface receptors. The family of seven-transmembrane (7TM) GPCRs actively participate in host defense and inflammation by modulating the up-regulation of surface receptors, release of enzymes, ROS production, chemotaxis and priming of immune cells [50-52]. Structurally, GPCRs consist of an extracellular N-terminus, seven α-helical transmembrane domains, three intra- and extracellular loops and an intracellular C-terminal tail [53, 54]. In addition, the extracellular N-terminal region contains N-linked glycosylation sites and some possibly sulfated tyrosine residues [55]. GPCRs are receptors for the previously mentioned small molecules, i.e., chemokines and anaphylatoxins [56, 57]. Generally, chemokines can be divided in four subfamilies based on the position of the first two cysteines; C,CC, CXC and CX_3C, followed by an identifying number [58, 59]. The GPCRs that bind these chemokines are named after the interacting chemokine subfamily, followed by the letter R for receptor [58, 59]. Depending on the receptor, the interaction between GPCRs and their ligands can be specific or promiscuous. Additionally, CC- and CXC- chemokine receptors only recognize chemokines within the corresponding chemokine subfamily. For example, CXC chemokine receptor 1 (CXCR1) binds CXCL8, CXCL6 and CXCL7 [56, 57, 60]. CXC chemokine receptor 2 (CXCR2) is even more promiscuous and binds CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8 [60, 61]. The apparent redundancy is suggested to contribute to the fine-tuning of immune responses [62]. In contrast, certain GPCRs like C5aR1 interact exclusively with C5a [63, 64]. C5a is the 74 amino-acid cleaved product of complement C5 and it is a potent anaphylatoxin as well as a potent mediator of chemotaxis [64, 65]. Its receptor C5aR1 is expressed on a wide variety of non-inflammatory and inflammatory cells, and is abundantly expressed in particular on neutrophils [64-66]. The interaction between C5a and C5aR1 results, amongst others, in the recruitment of phagocytes to the site of complement activation [67].

The expression of GPCRs on different cell types is highly variable (Figure 1) [55]. Pathogens have exploited this differential expression of GPCRs to target specific cell types [55]. For example, the malaria parasites *Plasmodium vivax* and *Plasmodium knowlesi* target the Duffy antigen receptor for chemokines (DARC, also known as ACKR1) expressed on erythrocytes (Figure 1) [68, 69].

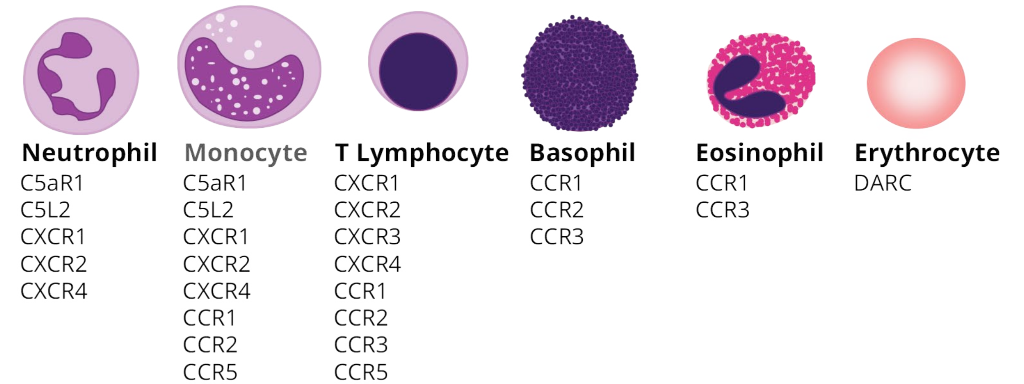

Figure 1: Dominant chemokine and anaphylatoxin receptors expressed on cells of the human myeloid and lymphoid lineage. Illustration of extensively researched chemokine and anaphylatoxin receptors expressed on certain cells of the innate and adaptive immunity. The C5aR1 is expressed only on neutrophils and monocytes, where as other receptors such as CXCR4 are more widely expressed. DARC is exclusively expressed on erythrocytes. Illustration does not cover all chemokine receptors expressed on the respective cell types and is limited to chemokine receptors relevant for the chapters in this thesis.

Moreover, CXCR4 and CCR5 expressed on lymphocytes (Figure 1) serve as co-receptors involved in the entry and infection by the human immunodeficiency virus (HIV) [70, 71]. In the same way, *S. aureus* secretes specific virulence factors that target GPCRs expressed on phagocytes to evade host immune defences [29, 72].

S. aureus immune evasion: CHIPS and leukocidins

The list of described *S. aureus* immune evasion molecules is at 35 and counting. These immune evasion molecules can be divided according to their underlying mechanisms of action: *S. aureus* proteins that block the function of host enzymes or receptors, proteins that possess enzymatic properties, cytotoxic proteins and proteins that modulate host-cell responses [72]. As a result, *S. aureus* is able to defend and counter the attack of the host immune system and interfere with every step of the innate immune response. In fact, *S. aureus* is able to engage at the very beginning by interfering with the extravasation of phagocytes via the secretion of the staphylococcal superantigen-like protein 5 (SSL5) that blocks neutrophil rolling on endothelial cells [73]. Subsequent chemotaxis, priming and activation of neutrophils are hindered by staphylococcal secreted proteins that target specific GPCRs on phagocytes [74-77]. Additionally, *S. aureus* blocks the recognition and opsonization by the humoral innate immune system. Circulating antibodies against *S. aureus* are inactivated by immunoglobulin binding proteins

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such as staphylococcal protein A (SpA), *Staphylococcus aureus* binder of IgG (Sbi) and SSL10, subsequently disrupting the onset of an adequate antibody response and opsonization [78-80]. Opsonization and further activation of the complement system are avoided by the *S. aureus* complement inhibitors such as staphylococcal complement inhibitor (SCIN), Extracellular complement-binding protein (Ecb) and Extracellular fibrinogen-binding protein (Efb) [81, 82]. Other important host-cell receptors involved in *S. aureus* recognition, such as Toll-Like- and Fc-receptors, are also functionally blocked by *S. aureus* [72, 83, 84].

A particular protein, the Chemotaxis Inhibitory Protein of *Staphylococcus* (CHIPS) impairs activation and antagonizes neutrophil and monocyte chemotaxis [77, 85, 86]. CHIPS is a small 14.1-kD protein and binds with high affinity (*K*d=1.1nM) to the N-terminus C5aR1, functionally blocking the interaction with C5a [85, 86]. In addition, the N-terminal domain of CHIPS binds and inhibits the interaction of the formylated peptide fMLP with the receptor FPR1 [86-88]. The interaction of CHIPS with C5aR1 has been extensively studied, and all the residues involved in the interaction between CHIPS and C5aR1 are known. In particular, the amino acid residues 10-24 of C5aR1 are involved in the interaction with CHIPS. Specifically, the 3 aspartic acid residues and the sulfated tyrosines on position 11 and 14 have been shown to be important in this interaction [77, 85, 89]. Furthermore, CHIPS has a 30-fold reduced capacity to inhibit C5a mediated calcium mobilization in murine neutrophils compared to human neutrophils [77]. Due to the knowledge gained and the ability to inhibit the human C5aR1 with such specificity and affinity, it has been proposed that CHIPS might serve as a potential anti-inflammatory drug in diseases in which C5aR1 stimulation plays an important role [77]. However, the human specific nature of CHIPS [77] has made it challenging to assess both its contribution to *S. aureus* pathophysiology, as well as the therapeutic potential *in vivo*.

The plasma membrane of the phagocyte serves as a protective lipid bilayer, separating the intracellular components from the extracellular environment. In addition, the plasma membrane plays an important role in phagocytosis, cell signalling and adhesion [90]. Disrupting membrane integrity can lead to dysfunctional bacterial surveillance and even loss of cellular viability [90]. Notably, the plasma membrane serves as a target for *S. aureus* pore-forming toxins (PFTs) [91]. *S. aureus* produces a group of PFTs that consist of two non-associated monomers, which multimerize upon binding to the target cell's membrane, resulting in a pore that spans the lipid bilayer and induces cell

death. These bi-component PFTs are also called leukocidins and are considered an important group of staphylococcal virulence factors [91, 92]. Among the leukocyte population, phagocytes are considered the major target of leukocidins [93]. Additionally, staphylococcal leukocidins target natural killer cells (NK-cells), dendritic cells, lymphocytes and erythrocytes [94]. Human *S. aureus* isolates secrete up to five different leukocidins that target phagocytes; Panton–Valentine leukocidin (PVL), γ-haemolysin AB (HlgAB) and CB (HlgCB), leukocidin ED (LukED) and leukocidin AB (LukAB, also knowns as LukGH) [92, 94]. In addition, *S. aureus* strains associated with zoonotic infections have been described to secrete leukocidin MF'(LukMF') and leukocidin PQ (LukPQ) [95, 96]. As many *S. aureus* virulence factors, leukocidins display host- and cellular tropism [97]. The human specificity and differences in susceptibility between human and animal leukocytes have hindered the assessment of bi-components leukocidins during infections *in vivo*.

Based on chromatography elution profiles, the individual leukocidin subunits are designated as S(slow)- or F(fast)-migrating component, and each having a molecular weight of approximately 33kD [92]. The leukocidin subunits consist of a cap, rim and a stem domain [92, 98-100]. The current opinion on the sequences of events leading to leukocidin pore-formation starts with the primary interaction of the leukocidin S-component with a specific proteinaceous receptor on the surface of target cells [91, 92, 101-103]. Subsequently, an F-component is recruited which, together with the S-component, hetero-oligomerizes (Figure 2) [98, 99]. Of the leukocidins, LukAB is an exception due to it forming a dimer in solution and binding its target pre-assembled [104]. Upon oligomerization, a conformational change is induced in which the stem domain of the subunits unfolds and penetrates the cell plasma membrane [99]. Structural studies have suggested that these leukocidins form an octameric pore of alternating S- and F-subunits, which is about 1-2 nm in diameter [98, 99]. Even though it has been shown that the F-component may interact independently of a primary bound S-component [105-107], the cellular targets and mechanisms dictating F-component interaction remain unknown. Recently, proteinaceous targets have been identified for all of *S. aureus* leukocidins. The S-components of leukocidins, with the exception of LukAB, target specific GPCRs present on host cells [92]. The human C5aR1 and C5a anaphylatoxin chemotactic receptor 2 (C5aR2/C5L2) were identified as targets for PVL and HlgCB, providing an explanation for the species- and cell tropism of PVL and HlgCB [108, 109].

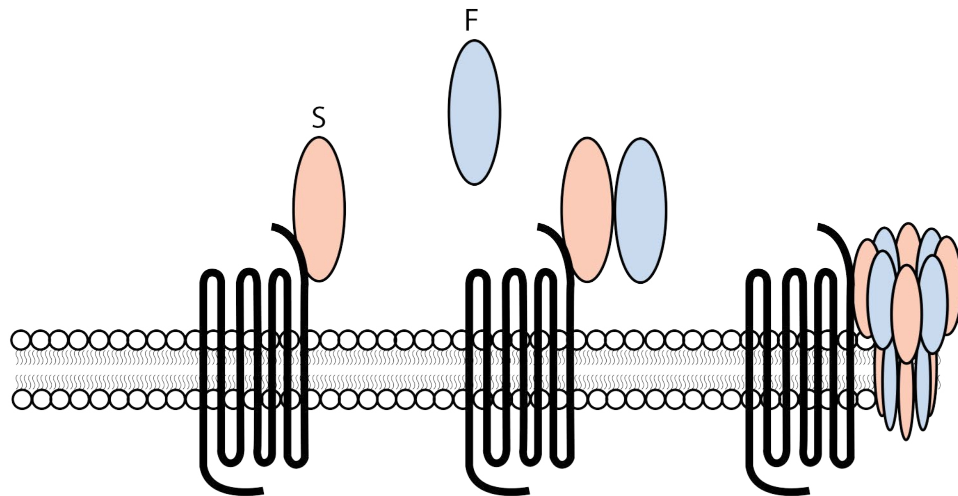

Figure 2: Model for bi-component leukocidin pore formation. Leukocidins consisting out of an S- and F-component induce pore-formation on target cells in a stepwise approach. The primary interaction consists out of the S-component interacting with a specific receptor on the surface of target cells. Subsequently, an F-component is recruited that interacts with the S-component/receptor complex, forming a ring-like octamer of alternating S- and F-components. A conformational change of the S- and F-components induces the inward collapse of the stem region forming a β-barrel pore that spans the cell membrane.

LukED targets leukocytes via CC-chemokine receptor 5 (CCR5), as well as CXCR1 and CXCR2 [92]. HlgAB targets CXCR1, CXCR2 and CCR2 [92]. In addition, HlgAB and LukED target the erythroid receptor DARC [92, 110]. LukAB targets CD11b, a component of the Mac-1 integrin and highly expressed on phagocytes [104, 111]. Even though the discovery of S-component targets has elucidated the interspecies and cell-specific variations in leukocidins susceptibility, the molecular mechanisms and determinants dictating leukocidin cytotoxicity remain incompletely understood. In addition, the apparent redundant deployment of two leukocidins that target the same human C5aR1, and the overlapping cell-tropism between leukocidins remains enigmatic. Whether leukocidins also employ F-components cellular surface targets as part of the initial interaction preceding leukocidin mediated pore-formation is unknown and remains to be established.

The involvement and contribution of PVL and other leukocidins in *S. aureus* pathophysiology is still debated. Epidemiological studies suggested an association of PVL with invasive SSTI and necrotizing pneumonia [112, 113]. However, the lack of an adequate *in vivo* model has hindered the assessment of

human specific leukocidins such as PVL, LukAB, HlgAB and HlgCB [104, 108, 114-117]. All in all, *S. aureus* counterattacks and evades recognition, opsonization, phagocytosis and killing by phagocytes via the secretion of multiple virulence factors that target the host immune system on different levels.

Aim and outline of this thesis

A better understanding and knowledge of the mechanisms of action of the major virulence factors of *S. aureus* not only allows for identification of new targets that could be exploited for anti-virulence strategies to limit *S. aureus* infections, but also for improvement of current *in vivo* infection models and offer key clues for drug and vaccine development. However, most of the *S. aureus* virulence factors are human specific and have a lower to no affinity to the homologous proteins in other animal species [29, 72]. The human specificity of *S. aureus* virulence factors complicates the assessment on how these virulence factors contribute to *S. aureus* pathogenesis and pathophysiology *in vivo*. This thesis describes the discovery of host-specific factors dictating sensitivity to the *S. aureus* C5aR1-interacting leukocidins. In addition, we show the therapeutic potential of a *S. aureus* virulence factor that targets the human C5aR1. **In Chapter 2**, we show the development of a transgenic mouse expressing the human C5aR1 in order to assess the contribution of HlgCB and PVL in *S. aureus* pathophysiology. Furthermore, we show the identification of the first F-component receptor for leukocidins, in which PVL specifically employs human CD45 as a target for LukF-PV. In **Chapter 4** we show the assessment of the *S. aureus* secreted CHIPS as a therapeutic agent in mice using our previously develop transgenic mouse expressing human C5aR1. In addition, we investigate the safety and efficacy of CHIPS in a phase-I human trial. **In Chapter 3** we describe a role for post-translational modifications (PTM) in the interaction between leukocidins and their GPCR targets, dictating leukocidin sensitivity. Finally, the implications of discovering CD45 and PTM in dictating leukocidin susceptibility, as well as using CHIPS as a therapeutic agent *in vivo* are discussed in **Chapter 5**.

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CHAPTER TWO

HUMAN CD45 IS AN F-COMPONENT SPECIFIC RECEPTOR FOR THE STAPHYLOCOCCAL TOXIN PANTON-VALENTINE LEUKOCIDIN

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Abstract

The staphylococcal bi-component leukocidins Panton-Valentine Leukocidin (PVL) and γ-Hemolysin CB (HlgCB) target human phagocytes. Binding of the toxins' S-components to human C5aR1 contributes to cellular tropism and human specificity of PVL and HlgCB. To investigate the role of both leukocidins during infection, we developed a human C5aR1 knock-in (hC5aR1KI) mouse model. HlgCB but, unexpectedly, not PVL contributed to increased bacterial loads in tissues of hC5aR1KI mice. Compared to humans, murine hC5aR1KI neutrophils showed a reduced sensitivity to PVL, which was mediated by the toxin's F-component LukF-PV. By performing a genome-wide CRISPR/Cas9 screen, we identified CD45 as a receptor for LukF-PV. The human-specific interaction between LukF-PV and CD45 provides a molecular explanation for resistance of hC5aR1KI mouse neutrophils to PVL and likely contributes to the lack of a PVL-mediated phenotype during infection in these mice. This study demonstrates an unsuspected role of the F-component in driving the exquisite sensitivity of human phagocytes to PVL.

Introduction

Staphylococcus aureus is a major bacterial pathogen in humans and is responsible for a diverse disease spectrum ranging from superficial skin and soft tissue infections to severe invasive disease. Severe infections with *S. aureus* have a poor prognosis [1]. Treatment is further complicated by the emergence of methicillin-resistant *S. aureus* (MRSA) strains [2] and by a lack of advancements in vaccine development [3]. A better understanding of the host-pathogen interaction during infection with *S. aureus* is essential to develop new therapeutic approaches.

Phagocytes play a pivotal role in the containment of *S. aureus* early after infection [4]. To counteract elimination by phagocytes, *S. aureus* secretes an arsenal of virulence factors. Among these are the leukocidins, a family of bi-component pore-forming toxins that target and kill phagocytes [5, 6]. Human *S. aureus* isolates secrete up to five different leukocidins [7]: Panton-Valentine Leukocidin (PVL, or LukSF-PV), γ-Hemolysin AB and CB (HlgAB, HlgCB), Leukocidin ED (LukED), and Leukocidin AB (LukAB, also known as LukGH). Chromatography elution profiles differentiate the leukocidin protein components into S(slow)- and F(fast)- migrating components, which are, with the exception of LukAB, secreted as inactive monomers [5]. Each canonical leukocidin combination consists of S- and F-components that hetero-oligomerize into an octameric membrane-spanning pore [5, 7]. The leukocidins show structural and functional resemblance to the single-component pore-forming toxin of *S. aureus*, α-Toxin (Hemolysin-α, Hla) [8], but the biological rationale for a bi-component system remains unresolved. Functional interactions by formation of non-canonical combinations of S- and F-components that are active (as for PVL and HlgCB [9-11]) or inactive (as for PVL and LukED [12]) suggest that the contribution of leukocidins to pathogenesis differs when expressed simultaneously. However, the contribution of the leukocidins to infection is incompletely understood [5, 7]. Specificity for human phagocytes and resistance of murine phagocytes towards the majority of leukocidins hinders investigation during infection [7]. Recently, proteinaceous receptors have been identified for all leukocidins [7, 13-20]. These receptors are targeted by the S-components in a species-specific manner. For the S-components of PVL and HlgCB, LukS-PV and HlgC respectively, the human complement receptor C5aR1 (hC5aR1) was identified as the major receptor [14, 16]. The identification of hC5aR1 as shared receptor for LukS-PV and HlgC explains the specificity for human phagocytes since both toxins are incompatible

with the murine C5aR1 orthologue [14, 16]. Although differences exist in the interaction between LukS-PV and HlgC with hC5aR1 [21, 22], the necessity for *S. aureus* to secrete apparently redundant toxins is incompletely appreciated [7]. While receptors have been identified for all leukocidin S-components, it remains to be established if the F-components have host receptors as well. A recent study on the equine specific leukocidin of *S. aureus*, LukPQ, suggests that the F-component might be involved in determining host tropism [17].

Due to the human specificity of both toxins, studies addressing the role of PVL and HlgCB during infection have proven to be challenging in mice [6, 23, 24]. In rabbits, PVL contributes to necrotizing pneumonia [25], osteomyelitis [26], and modestly enhances early stages of bacteraemic spread in a bloodstream infection model [27]. The contribution of PVL to skin infections in rabbits remains controversial [28, 29], although presence of the genes encoding PVL is epidemiologically linked to severe skin and soft tissue infections in humans [30]. More recently, non-obese diabetic (NOD)/severe combined immune deficiency (SCID)/IL2Rγnull (NSG) mice engrafted with primary human hematopoietic cells were found to be more susceptible to skin lesions [31] and pneumonia [32] than control mice in a PVL-dependent manner. These reports have focused on PVL exclusively. To the best of our knowledge, no studies have reported on the role of HlgCB during infection *in vivo [5]*. In order to understand the mechanisms of pore formation during infection and to assess both the contribution of each individual hC5aR1-targeting leukocidin as well as that of the leukocidins as a group, a humanized *in vivo* model is needed.

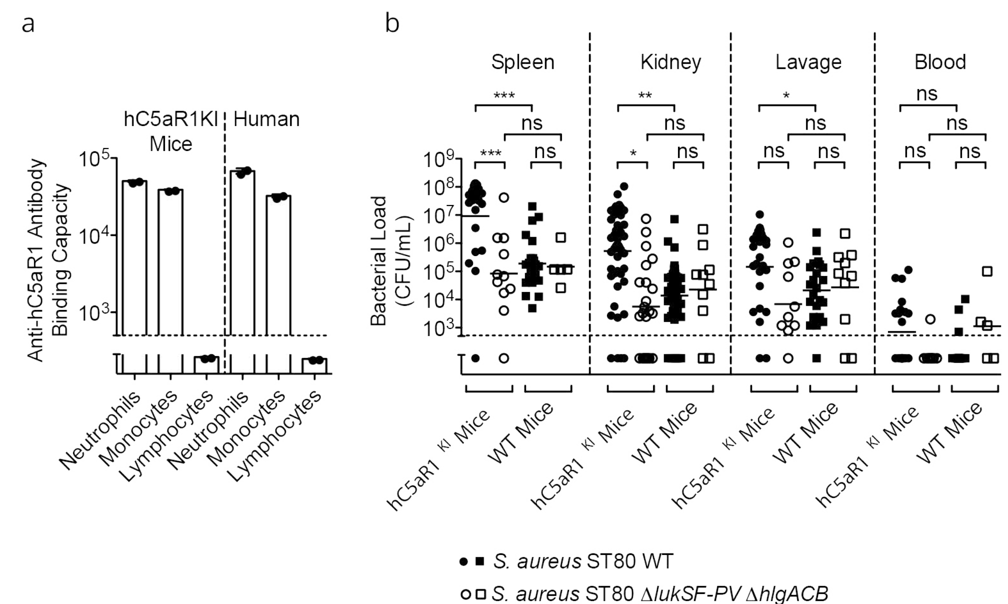
Here, we report on the development of a hC5aR1 knock-in (hC5aR1^{KI}) mouse to investigate the role of PVL and HlgCB during infection with *S. aureus*, and on the subsequent identification of CD45 as a receptor for LukF-PV, the F-component of PVL.

Results

Human C5aR1 increases bacterial loads during *S. aureus* infection

To investigate the contribution of HlgCB and PVL to infection, we developed a humanized knock-in mouse (hC5aR1^{KI}). Quantification of hC5aR1 expression in hC5aR1^{KI} mice recapitulated expression levels on human leukocytes (Figure 1a) [33]. Compared to wild type (WT) murine phagocytes, hC5aR1^{KI} phagocytes signaled normally in response to murine and human C5a (Supplementary Figure 1).

Next, a wild type (WT) MRSA strain (ST80, harbouring the genes encoding PVL, and HlgAB and HlgCB [34]) was injected intraperitoneally in hC5aR1^{KI} and WT mice (Figure 1b). 24 hours after infection, hC5aR1^{KI} mice displayed 10- to 100-fold higher bacterial loads in spleen and kidneys compared to WT mice infected with the same WT *S. aureus* strain (Figure 1b). The hC5aR1-dependent increase in bacterial burden was also observed in the peritoneal cavity. Bacterial loads in the peripheral blood showed a similar trend. These data demonstrate that hC5aR1 expression on phagocytes results in increased bacterial loads during infection with an *S. aureus* strain producing both PVL and HlgCB.



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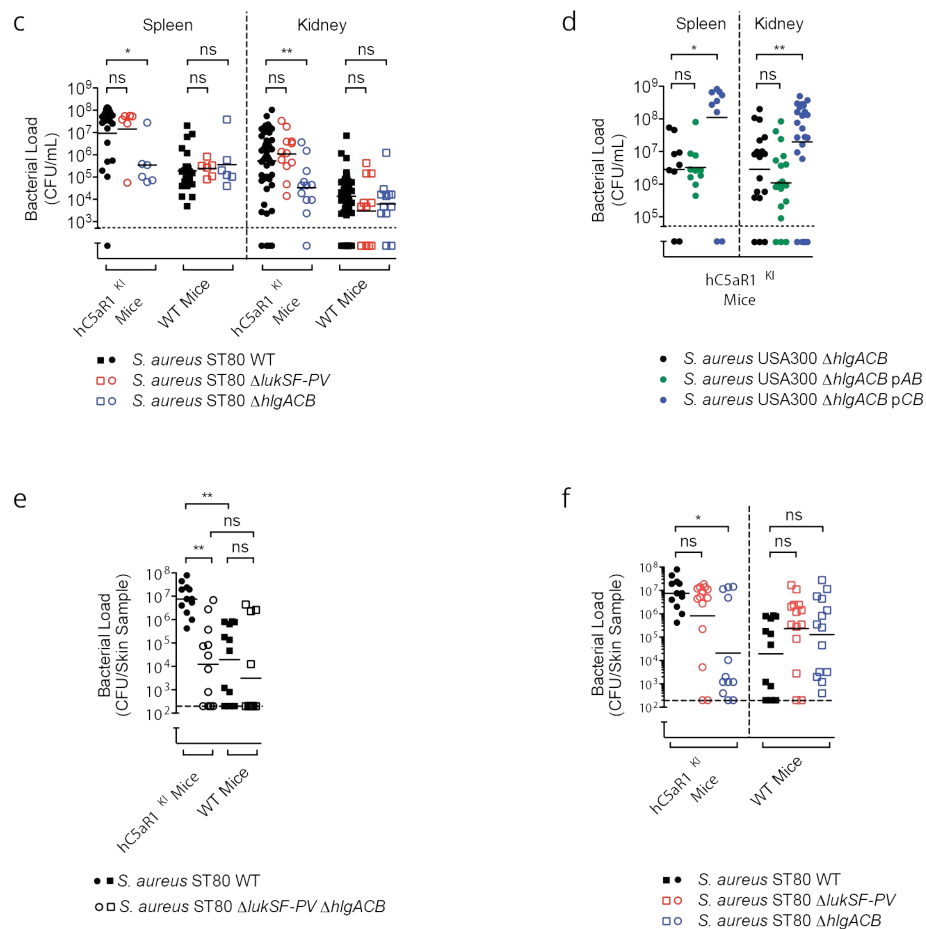


Figure 1. Human C5aR1 and HlgCB but not PVL contribute to increased bacterial loads in hC5aR1KI mice. (a) Expression of hC5aR1 on leukocytes from humans and hC5aR1 knock-in (hC5aR1KI) mice demonstrated as the antibody-binding capacity of an anti-hC5aR1 monoclonal antibody. Mean is shown, with n=2 biologically independent samples. (b) Bacterial loads after intraperitoneal infection with *S. aure*us wild type (WT) or an isogenic Δ*lukSF-PV*Δ*hlgACB* mutant strain in hC5aR1KI and WT mice. Sample sizes for hC5aR1KI and WT mice, respectively: n=25 and n=23 for *S. aureus* WT; n=11 and n=5 for *S. aureus* Δ*lukSF-PV*Δ*hlgACB*. (c) Bacterial loads in spleen and kidneys after infection with *S. aureus* WT ST80 or an isogenic Δ*lukSF-PV* or Δ*hlgACB* mutant strain. Sample sizes for hC5aR1KI and WT mice, respectively: n=25 and n=23 for *S. aureus* WT; n=6 and n=6 for *S. aureus* Δ*lukSF*; n=6 and n=6 for *S. aureus* Δ*hlgACB*. (d) Bacterial loads in spleen and kidneys after infection of hC5aR1KI mice with *S. aureus* Δ*hlgACB* complemented with a plasmid encoding *hlgAB* (p*AB*) or *hlgCB* (p*CB*). Sample sizes: n=9 for *S. aureus* Δ*hlgACB*; n=10 for *S. aureus* Δ*hlgACBpAB*; n=11 for *S. aureus* Δ*hlgACBpCB*. (e) Bacterial loads in the skin recovered after subcutaneous infection with *S. aure*us wild type (WT) or an isogenic Δ*lukSF-PV*Δ*hlgACB* strain. Sample sizes: n=12 for all groups. (f) Bacterial loads in the skin after infection with *S. aureus* WT ST80 or an isogenic Δ*lukSF-PV* or Δ*hlgACB* mutant strain. Sample sizes for hC5aR1KI and WT mice, respectively: n=12 and n=12 for *S. aureus* WT; n=14 and n=14 for *S. aureus* Δ*lukSF*; n=12 and n=12 for *S. aureus* Δ*hlgACB*. For panels (b-f), mice were distributed over independent experiments; solid horizontal lines express the geometric means; horizontal dashed lines indicate the detection threshold. Statistical significance is displayed as * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, ns for statistically not significant, and was calculated using ANOVA analysis of variance with Bonferroni posttest correction for multiple comparison. Exact p-values are provided in Supplementary Table 3. See also Supplementary Figure 1.

Leukocidins promote susceptibility of hC5aR1KI mice to *S. aureus* infection

To investigate if the observed differences between WT and hC5aR1KI mice are due to hC5aR1-targeting leukocidins, we infected hC5aR1KI and WT mice with an isogenic Δ*lukSF-PV* Δ*hlgACB* double mutant *S. aureus* strain. No differences were observed between hC5aR1KI or WT mice in any of the cultured compartments after infection with the double mutant *S. aureus* strain (Figure 1b). However, compared to the WT *S. aureus* strain, infection in hC5aR1KI mice with the double mutant *S. aureus* strain resulted in a 10- to 100-fold reduction in bacterial burdens in spleen and kidneys, with a similar trend in the peritoneal cavity and peripheral blood (Figure 1b). In WT mice, no differences were observed in any of the cultured compartments after infection with the WT or double mutant *S. aureus* strain (Figure 1b). These results thus confirm that the increased susceptibility of hC5aR1KI mice for WT *S. aureus* is mediated by at least one of the two hC5aR1-targeting leukocidins.

HlgCB but not PVL contributes to *S. aureus* pathophysiology in hC5aR1KI mice

To assess the individual involvement of HlgCB and PVL in hC5aR1-dependent *S. aureus* pathogenesis, hC5aR1KI mice were challenged with WT and isogenic single mutant *S. aureus* strains lacking either *hlgACB* (Δ*hlgACB*) or *lukSF-PV* (Δ*lukSF-PV*). Infection of hC5aR1KI mice with Δ*lukSF-PV* or WT bacteria resulted in similar bacterial loads in spleen and kidneys when compared to animals infected with WT bacteria (Figure 1c). However, hC5aR1KI mice infected with *S. aureus* Δ*hlgACB* showed decreased bacterial loads in spleen and kidneys compared to WT bacteria (Figure 1c)**.** These results demonstrate that the increased susceptibility of hC5aR1KI mice to WT *S. aureus* is mediated by *hlgACB*.

The *hlgACB* gene cluster encodes two functional pore-forming toxins (HlgAB and HlgCB), of which HlgCB but not HlgAB targets hC5aR1 [7, 16]. To confirm that the differences in bacterial loads recovered from hC5aR1KI mice infected with WT versus Δ*hlgACB* bacteria are mediated by HlgCB and not HlgAB, hC5aR1KI mice were challenged with Δ*hlgACB* bacteria complemented with a plasmid encoding *hlgAB* or *hlgCB* (Figure 1d). Infection of hC5aR1KI mice with *S. aureus* Δ*hlgACB* reconstituted with *hlgCB* but not *hlgAB* showed an increased number of CFUs in spleen and kidneys (Figure 1d), demonstrating that HlgCB promotes *S. aureus* pathogenicity in hC5aR1KI mice during systemic infection.

To further assess the role of HlgCB and PVL, we infected hC5aR1^KI and WT mice subcutaneously. Similar to the systemic infection model, mice expressing hC5aR1 displayed increased bacterial loads in the skin after infection with an *S. aureus* strain producing both PVL and HlgCB (Figure 1e). Furthermore, susceptibility of hC5aR1^KI mice for WT *S. aureus* was again mediated by at least one of the two hC5aR1-targeting leukocidins since infection with the double mutant completely annuled the phenotype (Figure 1e). Also, infection of hC5aR1^KI mice with *S. aureus* Δ*lukSF-PV* did not affect the bacterial loads while hC5aR1^KI mice infected with *S. aureus* Δ*hlgACB* showed a 100-fold decrease in the bacterial loads recovered from the skin compared to WT bacteria (Figure 1f).

Taken together, our investigations demonstrate a hC5aR1-dependent contribution of HlgCB during infection with *S. aureus*. However, the absence of a role for PVL was unexpected [25-28, 31, 32], and prompted us to investigate whether leukocytes of hC5aR1^KI mice lack another factor that may be involved in the human-specific cytotoxicity of PVL.

PVL and HlgCB differentially target hC5aR1^KI murine neutrophils in an F-component specific manner

Bone-marrow derived hC5aR1^KI murine neutrophils were isolated and compared to human neutrophils for susceptibility to HlgCB and PVL at concentrations for which neutrophils of WT mice are fully resistant [14, 16]. No differences in susceptibility to HlgCB-induced pore formation between human neutrophils and hC5aR1^KI murine neutrophils were observed (Figure 2a). However, hC5aR1^KI murine neutrophils showed a decreased sensitivity to PVL when compared to human neutrophils (Figure 2a). As hC5aR1 expression on hC5aR1^KI murine neutrophils reflected that on human neutrophils (Figure 1a), we questioned if reduced susceptibility of hC5aR1^KI murine neutrophils for PVL was due to a species-specific interaction of the cells with the toxin's S-component or F-component. Non-canonical pairing of the S- and F-components of PVL and HlgCB allows for the formation of functional pores in human phagocytes [9-11]. hC5aR1^KI murine neutrophils were as susceptible towards LukS-PV/HlgB as human neutrophils (Figure 2a), indicating that reduced susceptibility of hC5aR1^KI murine neutrophils to PVL is not due to a compromised interaction of LukS-PV with the cells. Correspondingly, we observed comparable binding of LukS-PV to hC5aR1^KI murine and human neutrophils (Supplementary Figure 2). However, hC5aR1^KI murine neutrophils were less susceptible to HlgC/LukF-PV in comparison to human neutrophils (Figure 2a).

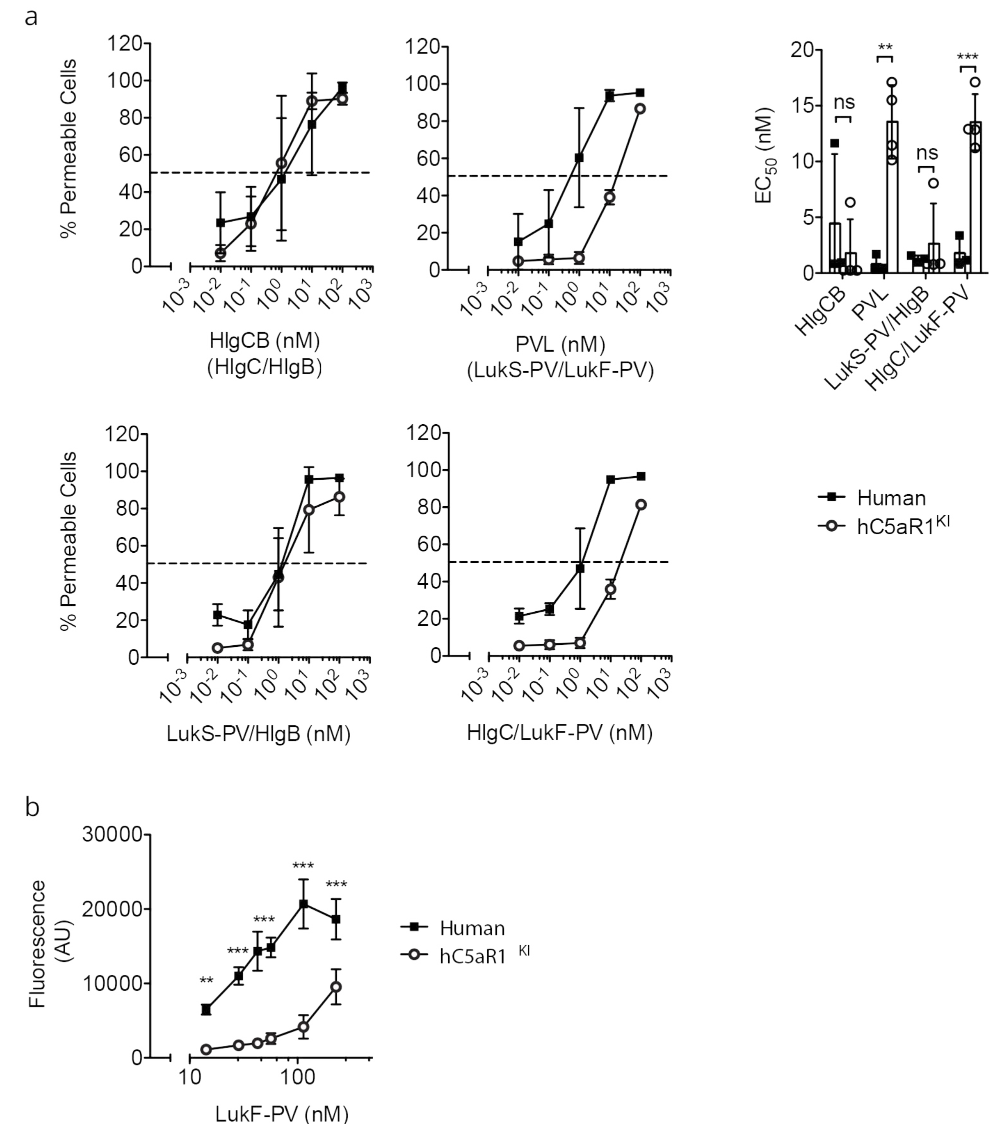


Figure 2. PVL and HlgCB differentially target hC5aR1^KI murine neutrophils in an F-component specific manner. (a) Susceptibility of bone marrow derived neutrophils from hC5aR1 knock-in (hC5aR1^KI) mice (n=4) and human neutrophils isolated from healthy donors (n=3) after exposure to canonical and non-canonical toxin combinations at the indicated concentrations. Cell permeability was determined by flow cytometry using propidium iodide at 30 minutes post-toxin treatment. Dashed horizontal lines indicate the 50% effective concentrations (EC_{50}), also expressed as a separate graph for statistical comparison. For all graphs, mean and s.d. are shown. Statistical significance was calculated using a two-sided Student's *t*-test. (b) Bone marrow derived neutrophils from hC5aR1^KI mice and human neutrophils were treated with LukF-PV at the indicated concentrations. Binding was subsequently determined by flow cytometry. Mean and s.d. are shown, with n=3. Statistical significance was calculated using ANOVA analysis of variance with Bonferroni posttest correction for multiple comparison. For all panels, statistical significance is displayed as ** for $p < 0.01$, *** for $p < 0.001$, and ns for statistically not significant. Exact p-values are provided in Supplementary Table 3. See also Supplementary Figure 2.

This finding indicates that the species-specific phenotype of PVL on hC5aR1[KI] murine neutrophils is mediated by the F-component, LukF-PV. Indeed, reduced binding of LukF-PV to hC5aR1[KI] murine neutrophils was observed when compared to human neutrophils (Figure 2b).

These observations demonstrate that the observed reduced susceptibility of hC5aR1[KI] murine neutrophils towards PVL is mediated by its F-component, and imply the involvement of a host factor displaying human-specific interaction with LukF-PV.

PVL targets CD45

To identify additional host factors involved in PVL-mediated cytotoxicity, a genome-wide CRISPR/Cas9 screen for PVL-resistance was set up in human U937-hC5aR1-SpCas9 cells. Focusing on cell-surface proteins, the gene encoding C5aR1, *c5ar1*, was identified as a top hit, illustrating the validity of the screening method (Figure 3a, Supplementary Tables 1 and 2). The other most enriched gene encoding a predicted surface protein was *ptprc* (Figure 3a, Supplementary Tables 1 and 2). *ptprc* encodes protein tyrosine phosphatase receptor type C (PTPRC), also known as CD45. Expression levels of C5aR1 and CD45 on the surface of U937-hC5aR1-SpCas9 cells (Figure 3b) were in the same order of magnitude as on human neutrophils (Supplementary Figure 3a).

To validate the involvement of CD45 in PVL-susceptibility, single knock-out cells were generated. Mutant cells specifically lacked expression of C5aR1 or CD45 (Figure 3b)**.** Subsequently, cells were challenged with PVL or HlgCB at approximately half maximal effective toxin concentrations (EC_{50}). As expected, C5aR1^{-} CD45^{+} cells were resistant to both PVL and HlgCB (Figure 3c). C5aR1^{+} CD45^{-} cells were resistant to pore formation induced by PVL (Figure 3c). Lactate dehydrogenase (LDH) release confirmed that PVL induces actual cell lysis in a CD45-dependent manner (Supplementary Figure 3b). Notably, absence of CD45 on the cellular surface did not affect susceptibility to HlgCB-toxicity (Figure 3c). These findings show that PVL, but not HlgCB, targets CD45 to induce cell lysis, thereby providing evidence that PVL and HlgCB are functionally different toxins.

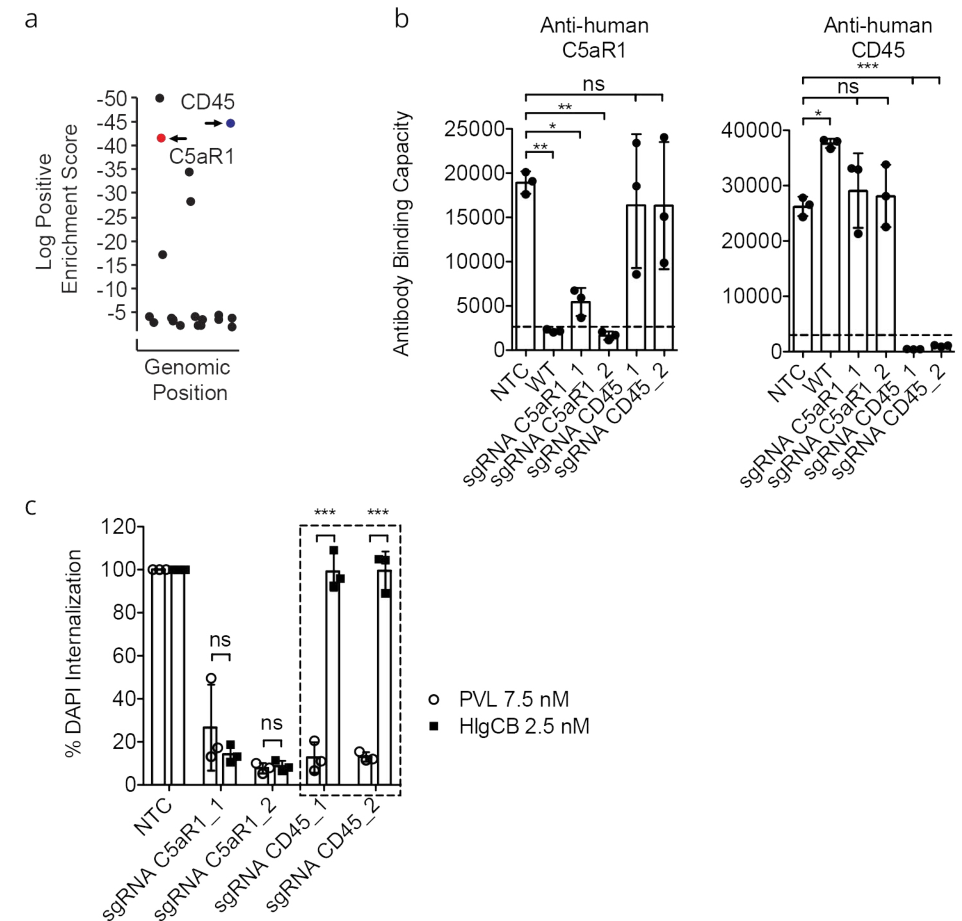
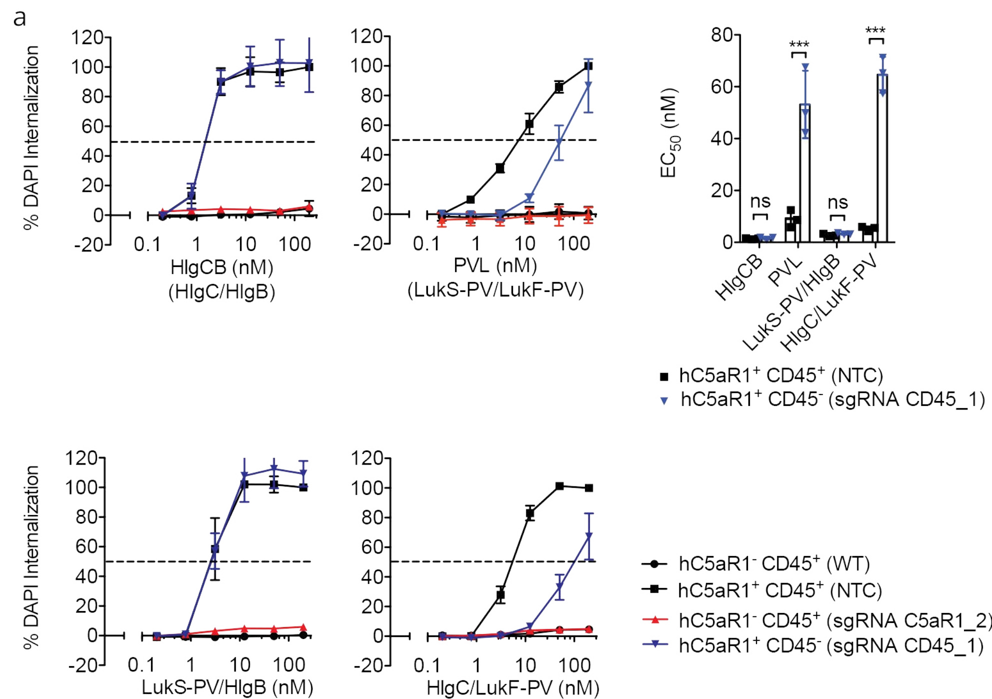


Figure 3. PVL targets CD45. (a) Cellular components crucial for PVL-mediated killing identified by introduction of a genome-wide sgRNA library in U937-hC5aR1-SpCas9 cells coupled to deep sequencing. The top 20 most significantly enriched genes as calculated by the MaGeCK 'positive enrichment score' are visualized, with the two surface proteins C5aR1 and CD45 highlighted. (b) Validation of receptor expression in U937-hC5aR1-SpCas9 cell lines transduced with two different sgRNAs for C5aR1 (hC5aR1^{-} CD45^{+}) or CD45 (hC5aR1^{+} CD45^{-}) in order to generate single gene knock-out cell lines. Receptor expression is demonstrated as the antibody-binding capacity of an anti-hC5aR1 or anti-hCD45 monoclonal antibody. As a control, wild type U937 cells (WT, hC5aR1^{-} CD45^{+}) and U937-hC5aR1-SpCas9 cells transduced with a non-targeting control (NTC, hC5aR1^{+} CD45^{+}) were used. The dashed horizontal line indicates the detection threshold. Mean and s.d. are shown, with n=3. Statistical significance was calculated using ANOVA analysis of variance with Bonferroni posttest correction for multiple comparison. (c) Validation of the genome-wide CRISPR/Cas9 screen for PVL-resistance in U937-hC5aR1-SpCas9 cells. Cells were exposed to PVL (7.5 nM) or HlgCB (2.5 nM). As a readout for cell permeability, internalization of DAPI was tested at 30 minutes post-toxin treatment on a monochromator-based microplate reader and expressed in relation to the maximal area under the curve for U937-hC5aR1-SpCas9 cells transduced with a non-targeting control (NTC). Two guide RNAs were tested for C5aR1 and CD45. Mean and s.d. are shown, with n=3. Statistical significance was calculated using a two-sided Student's *t*-test. For all panels, statistical significance is displayed as * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, ns for statistically not significant. Exact p-values are provided in Supplementary Table 3. See also Supplementary Tables 1 and 2, and Supplementary Figure 3 and 4.

PVL targets CD45 in an F-component specific manner

To further investigate the role of CD45 in cellular susceptibility towards PVL, mutant cells were incubated with different concentrations of PVL and HlgCB. Absence of CD45 resulted in an increased EC_{50} for PVL, but not for HlgCB (Figure 4a). Activation of C5aR1 by its ligand C5a was not affected by knocking-out CD45, indicating that reduced susceptibility of $C5aR1^{+}$ $CD45^{-}$ cells to PVL-toxicity is not due to an interplay between C5aR1 and CD45 (Supplementary Figure 4a). These results indicate that CD45 is directly involved in cell-susceptibility towards PVL but not HlgCB.

To test if resistance of $CD45^{-}$ cells towards PVL but not HlgCB results from a disturbed interaction with the toxin's S-component or F-component, non-canonical toxin combinations of PVL and HlgCB were subsequently tested. A comparable EC_{50} for LukS-PV/HlgB was observed for $CD45^{-}$ cells and $CD45^{+}$ cells (Figure 4a). However, an increase of the EC_{50} in $CD45^{-}$ cells, similar to that for PVL, was observed for HlgC/LukF-PV (Figure 4a). These findings demonstrate that resistance of $CD45^{-}$ cells towards PVL is depending on LukF-PV, suggesting that CD45 acts as a receptor for LukF-PV.



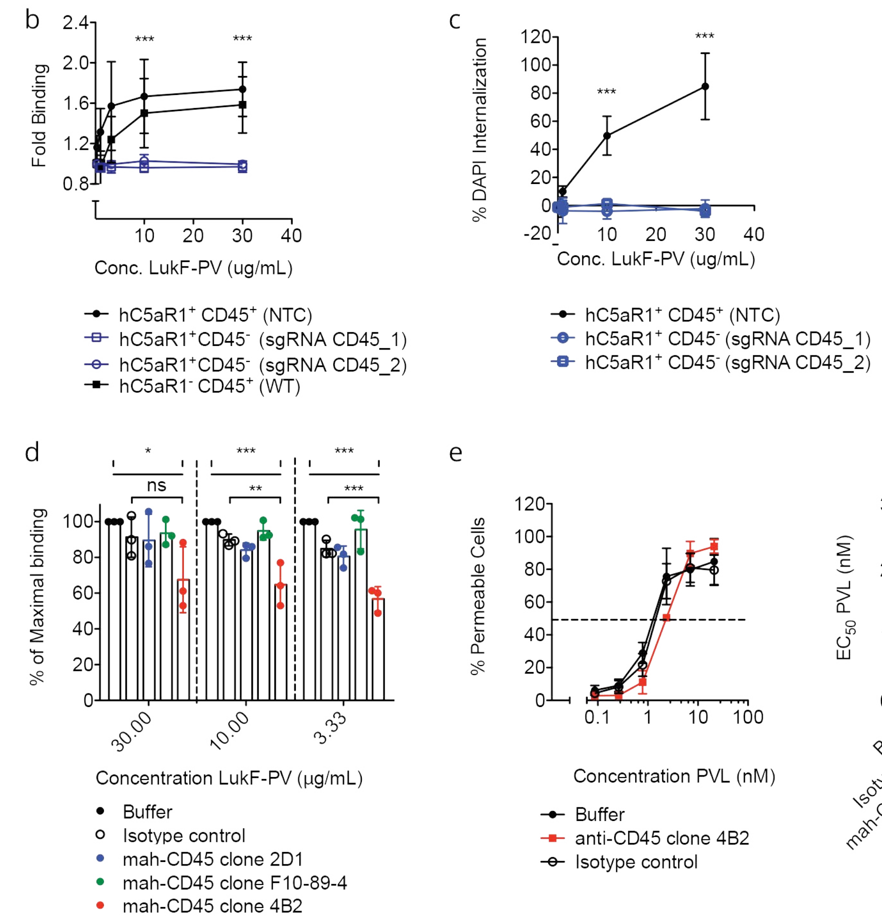


Figure 4. PVL targets CD45 in an F-component specific manner. (a) Susceptibility of wild type U937 cells (WT, $hC5aR1^{-}$ $CD45^{+}$), U937-hC5aR1-SpCas9 cells transduced with a non-targeting control (NTC, $hC5aR1^{+}$ $CD45^{+}$), a sgRNA for *c5ar1* ($hC5aR1^{-}$ $CD45^{+}$), or a sgRNA for *cd45* ($hC5aR1^{+}$ $CD45^{-}$), to canonical and non-canonical toxin combinations. As a readout for cell permeability, internalization of DAPI was tested at 30 minutes post-toxin treatment on a monochromator-based microplate reader and expressed in relation to the maximal area under the curve for NTC U937-hC5aR1-SpCas9 cells. (b) Binding of LukF-PV to cells as detected by flow cytometry, expressed as the fold increased binding related to background binding. In addition to WT U937 and NTC U937-hC5aR1-SpCas9 cells, two U937-hC5aR1-SpCas9 cell lines transduced with a vector containing a sgRNA for *cd45* (both $hC5aR1^{+}$ $CD45^{-}$) were tested. (c) Pore formation of cells after pre-incubation with LukF-PV, followed by a washing step and subsequent addition of LukS-PV (0.64 µg/mL). Internalization of DAPI was tested at 30 minutes after addition of LukS-PV on a monochromator-based microplate reader and expressed in relation to the maximal area under the curve for NTC U937-hC5aR1-SpCas9. (d) Binding of LukF-PV to human neutrophils after pre-incubation with monoclonal anti-CD45 antibodies (10 µg/mL). Binding is expressed in relation to buffer-only treated cells, as detected by flow cytometry. (e) Pore formation of human neutrophils after pre-incubation with monoclonal antibodies (10 µg/mL). Cell permeability was determined by flow cytometry using DAPI at 30 minutes post-toxin treatment. For panels (a) and (e), the dashed horizontal lines indicate the EC_{50}, also expressed as a separate graph for statistical comparison. For all panels, mean and s.d. are shown with n=3. Statistical significance was calculated using a two-sided Student's *t*-test for panel (a), and using ANOVA analysis of variance with Bonferroni posttest correction for multiple comparison for panels (b-e). Statistical significance is displayed as * for p <0.05, ** for p < 0.01, *** for p < 0.001, and ns for statistically not significant. Exact p-values are provided in Supplementary Table 3. See also Supplementary Figure 4b.

To study the interaction between CD45 and LukF-PV, we tested binding of LukF-PV to the cell surface. In CD45$^-$ cells, binding of LukF-PV was reduced compared to CD45$^+$ cells irrespective of co-expression of C5aR1 (Figure 4b, Supplementary Figure 4b, c). Despite non-specific background binding in CD45$^-$ cells, CD45-dependent binding sites of LukF-PV could be saturated, indicating a specific interaction between CD45 and LukF-PV (Figure 4b, Supplementary Figure 4b, c). Pre-incubation of cells with LukF-PV followed by a washing step and subsequent addition of LukS-PV showed a CD45-dependent susceptibility of cells to pore formation (Figure 4c), demonstrating that CD45-dependent binding of LukF-PV specifically contributes to pore formation.

If CD45 is a receptor for LukF-PV, CD45 neutralization could interfere with binding of LukF-PV and PVL-cytotoxicity. Pre-treatment of human neutrophils with a monoclonal antibody against CD45 (clone 4B2) reduced binding of LukF-PV (Figure 4d). A small shift was observed for the EC_{50} of PVL in cells pre-treated with the antibody against CD45 (Figure 4e, Supplementary Figure 4d), providing further evidence for the interaction between LukF-PV and CD45.

CD45 is a receptor for LukF-PV

CD45 has multiple isoforms due to alternative splicing of exons encoding the distal part of the extracellular domain (Supplementary Figure 5a) [35]. To investigate if the isotype affects susceptibility towards PVL, the PVL-CD45 interaction was further investigated by expression of the shortest and longest CD45 isoforms (R0 and RABC, respectively) in C5aR1$^+$ CD45$^-$ U937 cells (Figure 5a, Supplementary Figure 5). Expression of both CD45 isoforms restored PVL-susceptibility, indicating that the distal extracellular domain of the receptor is not required for interaction with LukF-PV (Figure 5a). To confirm that LukF-PV directly binds to CD45, surface plasmon resonance (SPR) was performed using recombinant CD45 or C5aR1$^+$ U937 cells expressing human CD45. LukF-PV bound recombinant receptor and cells with a K_d of 1.2 μM and 1.4 μM, respectively (Table 1).

Thus, these data not only identify CD45 as a receptor for PVL, but also highlight CD45 as an F-component-specific leukocidin receptor.

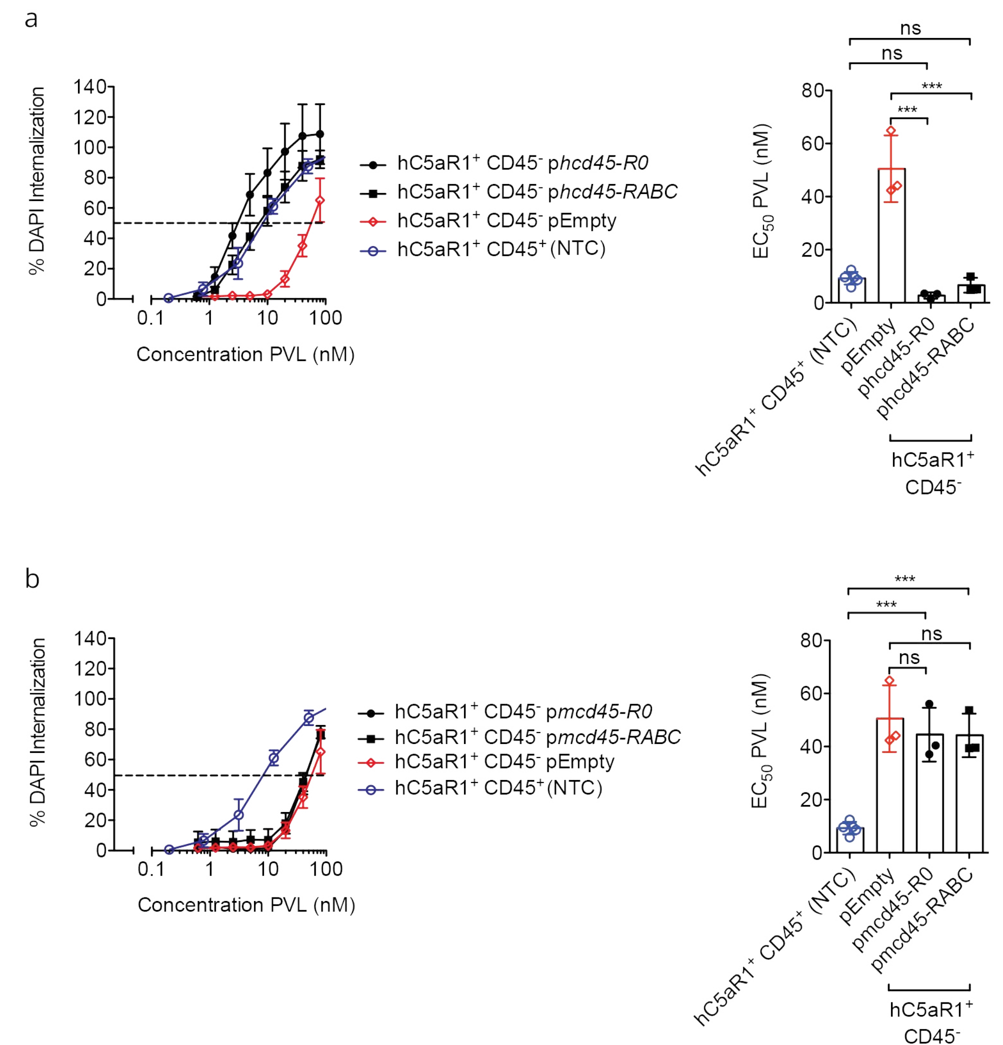


Figure 5. PVL targets CD45 in a human-specific manner. Susceptibility to PVL of U937-hC5aR1-SpCas9 cells transduced with a sgRNA targeting *cd45* (hC5aR1$^+$ CD45$^-$), and subsequently transduced with a plasmid containing either (a) human CD45 isoform R0 (p*hCD45-R0*) or RABC (p*hCD45-RABC*), (b) murine CD45 isoform R0 (p*mCD45-R0*) or RABC (p*mCD45-RABC*), or an empty plasmid (pEmpty). As a readout for cell permeability, internalization of DAPI was tested at 30 minutes post-toxin treatment on a monochromator-based microplate reader and expressed in relation to the maximal area under the curve for U937-hC5aR1-SpCas9 cells transduced with a non-targeting control (NTC, hC5aR1$^+$ CD45$^+$). Dashed horizontal lines indicate the 50% effective concentration (EC_{50}), also expressed as a separate graph for statistical comparison. For all panels, mean and s.d. are shown with n=3. Statistical significance is displayed as *** for $p < 0.001$, ns for statistically not significant, and was calculated using ANOVA analysis of variance with Bonferroni posttest correction for multiple comparison. Exact p-values are provided in Supplementary Table 3. See also Supplementary Figure 5 and 6.

Species	Recombinant CD45	U937 cells CD45+
Human	1.2 μM ± 0.2	1.4 μM ± 0.4
Murine	14.9 μM ± 1.9	14.2 μM ± 4.0

Table 1. Surface plasmon resonance analysis of LukF-PV and CD45 from human and mouse. Affinity (K_d) was measured for recombinant human and murine CD45-R0, and for U937 cells expressing human or murine CD45 isotype R0 (U937-hC5aR1-SpCas9 cells transduced with a sgRNA targeting *cd45* (hC5aR1$^+$ CD45$^-$), and subsequently transduced with a plasmid containing either human CD45 isoform R0 or murine CD45 isoform R0). Mean affinities and s.e.m. are shown, with n=3.

PVL targets CD45 in a human-specific manner

Since neutrophils express CD45 [35] but hC5aR1^{KI} murine neutrophils showed a reduced F-component dependent sensitivity towards PVL, we hypothesized that LukF-PV interacts with CD45 in a species-specific manner and expressed the murine CD45 isoforms R0 or RABC in C5aR1$^+$ CD45$^-$ U937 cells (Figure 5b, Supplementary Figure 5). Neither murine CD45 isoforms were capable of restoring PVL-susceptibility (Figure 5b). Next, we expressed human CD45 in hC5aR1^{KI} murine macrophages. Expression of human CD45 in hC5aR1^{KI} murine macrophages enhanced susceptibility to PVL-induced pore formation (Supplementary Figure 6), mirroring the phenotype observed in U937 cells (Figure 5a). SPR using recombinant murine CD45 or C5aR1$^+$ U937 cells expressing murine CD45 revealed a K_d for binding of LukF-PV of 14.9 μM and 14.2 μM, respectively (Table 1). Thus, the affinity of LukF-PV for murine CD45 is ±10 fold lower compared to the human receptor.

These results provide a molecular explanation for the species-specific interaction between LukF-PV and its receptor CD45. Incompatibility of PVL with murine CD45 likely explains the LukF-PV dependent reduced sensitivity towards PVL that was observed in hC5aR1^{KI} murine neutrophils.

Discussion

The S-components of PVL and HlgCB target C5aR1 in a human-specific manner 14, 16]. Human-specificity has hindered *in vivo* studies towards the role of these leukocidins. We developed a hC5aR1^{KI} mouse to investigate the role of PVL and HlgCB during infection with *S. aureus*. While HlgCB contributed to increased bacterial loads in hC5aR^{KI} mice, no contribution of PVL was observed. The unexpected lack of a PVL-dependent phenotype during infection in these mice urged us to screen for additional host factors targeted by PVL. We show that PVL targets CD45 in a human-specific and LukF-PV dependent manner, thereby demonstrating that LukF-PV and CD45 are specifically involved in pore formation. CD45 is expressed on all nucleated hematopoietic cells and is an abundant cell surface protein [35]. Our data indicate that reduced susceptibility of C5aR1$^+$ CD45$^-$ cells to PVL-toxicity is not due to an affected signaling of C5aR1, but by a decreased binding of LukF-PV to CD45$^-$ cells.

The current model for leukocidin-targeting of host cells proposes initial S-component binding followed by subsequent recruitment of the F-component [5]. We demonstrate that specific binding of LukF-PV is CD45-dependent and independent of the S-component, indicating that the established model needs to be revised. Identification of a receptor for the F-component supports our understanding of the biological importance of two-component pore-forming systems. By targeting CD45 via its F-component in addition to C5aR1 via its S-component, PVL deploys a two-step control mechanism over phagocyte-tropism and host species specificity. Furthermore, identification of CD45 as a receptor for LukF-PV supports the notion that PVL and HlgCB are non-redundant toxins on both a molecular and a functional level. Future investigations will have to identify putative receptors for F-components of other leukocidins.

The effects of CD45-expression on susceptibility of C5aR1$^+$ cells to PVL-pore formation are moderate in terms of EC_{50}. This moderate effect is reflected in the micromolar affinity of LukF-PV for CD45, which is significantly lower than the affinity of LukS-PV for C5aR1 [14]. Murine CD45 could not restore susceptibility of C5aR1$^+$ CD45$^-$ cells to PVL and the affinity of LukF-PV to murine CD45 is lower than the affinity to human CD45, suggesting a critical threshold allowing engagement of the toxin-receptor complex during pore formation. The extracellular domain shared by all CD45 isoforms is heavily glycosylated and contains a cysteine rich

region and three fibronectin type III repeats (Supplementary Figure 5a) [35]. Although the overall organization of the extracellular domain is conserved, it is only 39% homologous between humans and mice (Supplementary Figure 5c) [35]. Incompatibility of LukF-PV with murine CD45 may be dictated by multiple residues or posttranslational modifications (Supplementary Figure 5d).

Specificity of LukF-PV for human CD45 offers a molecular explanation for the observed F-component dependent resistance of hC5aR1^{KI} mouse neutrophils to PVL *in vitro*, which is supported by enhanced susceptibility of hC5aR1^{KI} mouse macrophages expressing human CD45. The relative resistance of hC5aR1^{KI} murine neutrophils to PVL likely contributes to the unexpected lack of a PVL-mediated phenotype during infection with *S. aureus* in these mice. As a result, functional interactions between PVL and HlgCB during infection in hC5aR1^{KI} mice could not be investigated. Future options to assess the contribution of the leukocidins as a group are engineering of advanced genetically-modified animal models [7], or engrafting mice with primary human hematopoietic cells [31, 32]. Due to the small protective effects of monoclonal antibodies *in vitro* and the heterogeneity associated with human hematopoietic cells engraftment in mice [31, 32], we have been unable to use this strategy *in vivo* to investigate the CD45-LukF-PV interaction during infection.

The mechanisms for predisposition of otherwise healthy individuals to severe infections with *S. aureus* are poorly understood [36, 37]. Human genetic factors might account for an unfavorable outcome [38, 39]. CD45-deficiency was described in patients with SCID [40, 41], and abnormal splicing of CD45 frequently occurs [42]. Variations in the CD45 gene are probable candidates to explore for genetic predisposition to severe infections.

By taking advantage of the conserved susceptibility of hC5aR1^{KI} mouse neutrophils to HlgCB, we show that HlgCB contributes to increased bacterial loads by employing hC5aR1. Our data support the notion that leukocidins play an essential role in the pathogenicity of *S. aureus [15, 16, 20, 27]*. Strategies aimed at protecting phagocytes from cytotoxicity by blocking the interaction between toxin and receptor offer avenues for therapeutic intervention. Receptor competition by means of monoclonal antibodies or small molecule receptor antagonists confers protection against toxin-mediated pore formation *in vitro [18, 20, 21]*. The establishment of the role of hC5aR1 during infection and the identification of CD45 as a receptor for LukF-PV provide a rationale to further investigate the leukocidin receptors as candidate drug targets for severe *S. aureus* infections.

MATERIALS AND METHODS

Construction and generation of hC5aR1^{KI} mice

hC5aR1^{KI} mice were generated as previously described[43] at the Institut Clinique de la souris (Ilkirch-Graffenstaden, France) using standard knock-in techniques. Briefly, the targeting vector comprised a 4.5-kilobase region of mouse C57BL/6 genomic DNA upstream the C5aR gene (gene ID ENSMUSG00000049130) exon 2 (and ending with the murine ATG), exon2 from hC5aR1 (gene ID ENSMUST00000050770) encoding the full length hC5aR1 from amino-acid 2 to the stop codon of the protein in frame with the ATG from the murine C5aR1 gene and a 3.5 kilobase region of the mouse C57BL/6 C5aR1 3'UTR region. The obtained vector was electroporated into C57BL/6N embryonic stem (ES) cells. ES cells containing the correctly targeted hC5aR1 as verified by Southern blot were analysed by karyotyping before injection into blastocytes. Following verification of germline transmission, the LoxP-flanked neomycin selection cassette was deleted using deleter mice as previously described [44]. Mice homozygous for the hC5aR1 were generated and validated by genotyping PCR.

Construction of CRISPR/Cas9 library

A genome-scale sgRNA library was designed, consisting of ±260,000 sgRNAs targeting every unique Refseq annotated (hg19) protein coding isoform with up to 12 sgRNAs, plus 7,700 non-target control sequences (NTC). Where possible, the earliest possible coding exon of each transcript variant was targeted. All sgRNAs were designed to target the spCas9 recognition sequence (N)20NGG and must have passed the following off-targeting criteria: 1) the 11bp-seed may not have an exact match to any other region in the human genome, and 2) if there is an exact off-target seed match, the remained of the sgRNA-sequence must have at least 7 mismatches with the potential off-target site. We selected up to 12 sgRNAs/transcript for which the sequences are presented in Supplementary Table 2.

The designed 20 nucleotides target specific sgRNA sequences were flanked by overhangs compatible with Gibson Assembly and synthesized as a pool on microarray surfaces (CustomArray, Inc.). The synthesised sgRNA template sequences were of the format: 5'-GGAGAACCACCTTGTTGG-(N)20-GTTTAAGAGCTATGCTGGAAAC-3'. Template pools were PCR amplified by using Phusion Flash High-Fidelity PCR Master Mix (ThermoFisher Scientific) according to the manufacturers protocol with 1 ng/µL sgRNA template DNA, 1 µM forward primer (5'-GGAGAACCACCTTGTTGG-3'), 1 µM reverse primer (5'-GTTTCCAGCATAGCTCTTAAAC-3') and the following cycle numbers: 1x (98ºC for 3 min), 15x (98ºC for 1 seconds, 55ºC for 15 seconds, 72ºC for 20 seconds) and 1x (72ºC for 5 min). PCR products were purified using Minelute columns (Qiagen). The library vector sgLenti (MP-783) was prepared by restriction digest with AarI (Thermo-Fischer) at 37ºC overnight, followed by extraction from 1% agarose gel of the digested band and purification via NucleoSpin columns (Macherey-Nagel). Using Gibson Assembly Master Mix (NEB), 1000 ng digested sgLenti and 100 ng amplified sgRNA library insert were assembled in a total reaction volume of 200 µL. The reaction was purified using P-30 buffer exchange columns (Biorad) that were equilibrated 5x with H2O and the total eluted volume was transformed into three vials of Electromax DH5α (ThermoFisher). Bacteria were recovered, cultured overnight in 500 mL LB (100 µg/mL ampicillin) and used for Maxiprep (Qiagen). In parallel, a fraction of the transformation reaction was plated and used to determine the total number of transformed clones. The library cloning coverage (number of bacteria colonies per sgRNA plasmid) was determined to be >100x to ensure even representation of the sgRNA sequences.

Cell lines and constructs

U937 human monocytic cells were obtained from ATCC (American Type Culture Collection), cultured in RPMI supplemented with penicillin/streptomycin and 10% fetal calf serum, and tested for mycoplasma contamination. U937 cells were not authenticated. To sensitize the cells to PVL and HlgCB, human C5aR1 (CD88; NM_001736) was first stably expressed in U937 cells using a lentiviral expression system (U937-hC5aR1 cells). We cloned the human C5aR1 cDNA in a dual promoter lentiviral vector (BIC-PGK-Zeo-T2a-mAmetrine; RP172), derived from no.2025.pCCLsin.PPT.pA.CTE.4x-scrT.eGFP.mCMV.hPGK.NG-FR.pre (kindly provided by Dr Luigi Naldini, San Raffaele Scientific Institute, Milan, Italy) as described elsewhere [45]. This lentiviral vector contains a human EF1A promoter to facilitate potent expression of the downstream cloned gene and expresses the fluorescent protein mAmetrine and selection marker ZeoR from a different promoter (PGK). Virus was produced in 24-well plates using standard lentiviral production protocols and the third-generation packaging vectors pMD2G-VSVg, pRSV-REV, and pMDL/RRE. Briefly, 0.25 µg lentiviral vector and 0.25 µg packaging vectors were cotransfected in 293T cells by using 1.5 µl Mirus LT1 tranfection reagent (Sopachem, Ochten, The Netherlands). After 72 hours, 100 µl unconcentrated viral supernatant adjusted to 8 mg/mL polybrene was used to infect ~50,000 U937 cells by spin infection at 1,000g for 2 hours at 33°C.

U937-hC5aR1 expressing cells were selected by culturing in 400 µg/mL Zeocin. To allow screening in U937-C5aR1 cells by using the genome-wide sgRNA library described below, the pSicoR-CRISPR-PuroR vector[46] was altered to replace the PuroR for BlastR and remove the U6 promoter. This vector (pSicoR-SpCas9-BlastR; RP-613) expresses a human codon-optimized nuclear-localized *S. pyogenes* Cas9 gene in the absence of a U6-promoter-sgRNA cassette. U937-hC5aR1 cells were transduced with the pSicoR-SpCas9-BlastR vector and selected to purify with 20 µg/mL blasticidin, to generate U937-hC5aR1-SpCas9 cells.

U937-hC5aR1-SpCas9 cells were transduced by sgRNA-expressing vectors to generate knock-out cell lines to allow for genome-wide CRISPR/Cas9 library screening (described below) or to generate single-gene knock-out cell lines. For this, a lentiviral vector was generated consisting of the pSicoR vector expressing PuroR-T2A-mCherry expressed from the EF1A promoter and a U6 promoter driving expression of a crRNA/sgRNA sequence (sgLenti, MP-783). To generate knock-out cells for C5aR1 or CD45, the following crRNA sequences were cloned in sgLenti: C5aR1_1 TCATCATAGTGCCCATAATC; C5aR1_2 GATGGCATTGATGGTCCGCT; CD45_1 TCACACTTATACTCATGTTC; CD45_2 ATTCTGTGTATCACAAGTAA. Upon virus production as described above, U937-hC5aR1-SpCas9 cells were transduced with the sgRNA-expression viruses and selected to purity by puromycin treatment (2 µg/mL) to enrich for CD45$^-$ cells.

To allow cDNA rescue experiments, we cloned an anti-CD45 sgRNA (GAAACTTGCTGAACACCCGC) in the pSicoR-CRISPR-PuroR vector [46] which co-expresses SpCas9 and puroR. Upon knock-out of CD45 from U937-C5aR1 cells, cDNA expression vectors were introduced to express the human and mouse CD45-R0 and CD45-RABC genes. For this, the coding regions of human CD45-R0 (NM_080921.3), human CD45-RABC (NM_002838.4), and mouse CD45-R0 (NM_011210.3) were amplified from cDNA vectors purchased from Sino Biologicals Inc. and cloned in a dual promoter lentiviral vector derived from no.2025.pCCLsin.PPT.pA.CTE.4x-scrT.eGFP.mCMV.hPGK.NGFR.pre (kindly provided by Dr Luigi Naldini, San Raffaele Scientific Institute, Milan, Italy). This vector was altered to express the BlastR gene downstream of the PGK promoter and the CD45 genes downstream of the EF1A promoter (RP-138). To prevent targeting of the human CD45 isoforms by the anti-hCD45 sgRNA present in the U937-hC5aR1-SpCas9 CD45-knockout cells, silent mutations were engineered in sgRNA-target sequence in the coding region of both hCD45-R0 and hCD45-RABC.

The ABC region of mCD45-RABC (NM_001111316) was ordered as human codon-optimized sequence as a gBlock (Integrated DNA Technologies) and cloned in between the codons coding for amino acids 30 and 31 in the mCD45-R0 vector described above by means of overlapping extension PCR. Human and mouse CD45-R0 and CD45-RABC were transduced in U937-hC5aR1-SpCas9 CD45-knockout cells and selected to purity by blasticidin selection.

hC5aR1^{KI} bone marrow-derived macrophages (BMDMs) were immortalized as previously described by transducing primary bone marrow cells with J2 virus at day 3 post-isolation [47].

Genome-wide CRISPR/Cas9 library screen with PVL in U937-hC5aR1 cells

600*10^6 U937-hC5aR1-SpCas9 cells were transduced with the genome-wide sgRNA-expression library by spin-infection at 1,000*g* for 90 min at 33°C in the presence of 4 µg/mL polybrene. Approximately 15% of the cells were transduced resulting in a ~350-fold overrepresentation of the library. Transduced cells were selected to purity with 2.0 µg/mL puromycin initiated at two days post transduction. Twelve days post transduction, 2*10^8 cells were incubated with 31 nM PVL for 30 min at 37°C, which resulted in depletion of >99.5% of the cells. Cells were washed to remove the toxin and allowed to recover in complete RPMI for 15 days to enrich for viable cells. In parallel, an untreated control sample of sgRNA-tranduced cells was maintained at high complexity (>2*10^8 cells) throughout this time period. Genomic DNA was isolated from 5*10^7 outgrowing cells and 1*10^8 untreated control cells by standard phenol–chloroform extraction. sgRNA inserts were subsequently PCR amplified for 16 cycles with primers 5'- GGCTTGGATTTCTATAACTTCGTATAGCA-3' and 5'-CGGGGACTGTGGGCGATGTG-3' using the Titanium Taq PCR kit (Clontech). The PCR products were pooled and amplified using primers containing Illumina adapter sequences and a unique index for 15 cycles using forward primer 5'- AATGATACGGCGACCACCGAGATCCACAAAAGG-AAACTCACCCTAAC-3' and reverse primer 5' CAAGCAGAAGACGGCATACGAGAT-AGTCTCGTGACTGGAGTTCAGACGTG-3' (RO-1479) for the treated sample or 5'- CAAGCAGAAGACGGCATACGAGATTGTCAGGTGACTGGAGTTCAGACGTG-3' (RO-1478) for the untreated control sample. The 344-bp PCR products were purified from 2% agarose gel using a PCR purification kit (Qiagen) and the DNA yield and quality was assessed by Bioanalyzer and Qubit analysis. PCR products were subsequently pooled in equimolar ratios and subjected to deep-

sequencing using the Illumina NextSeq500 platform. Sequences were aligned to the sgRNA library by using Bowtie2 (PMID: 22388286) and the counts per sgRNA were calculated. We used the MaGeCk package (PMID: 25476604) (available from https://sourceforge.net/projects/mageck/) as a computational tool to identify genes significantly enriched in the screens by comparing sgRNA read counts of control cells versus PVL-incubated cells. The genes, including significance for enrichment as calculated by the MaGeCK 'positive enrichment score', are presented in Supplementary Table 1.

Complementation of hC5aR1^{KI} murine macrophages

Primary hC5aR1^{KI} bone marrow cells were transduced with lentiviruses at day 3 post-isolation. Lentiviruses were added onto hC5aR1^{KI} bone marrow cells (2*10^{6} cells per well of a 6 wells plate) at a multiplicity of infection of 100:1 as determined by titration on 293T human embryonic kidney cells. Transduction was promoted by a 2,000 *g* spinoculation during 2 hours at room temperature. After 6 hours of incubation at 37°C, 2 mL of fresh medium were added. Cells were carefully washed the next day and further incubated for 3 days before analysis. Adherent macrophages were collected by washing the plate once with PBS and incubating it with Versene (ThermoFisher Scientific) for 5 min at room temperature.

Isolation of human and murine leukocytes

Cells were isolated as described elsewhere [14, 16]. Briefly, bone marrow of mice was harvested and immune cells collected. Human leukocytes were isolated by Ficoll/Histopaque centrifugation. When required, hypotonic lysis of residual erythrocytes was performed by a 30 second incubation in sterile water followed by addition of a large volume of PBS. All *in vitro* experiments with cells were performed with RPMI (Invitrogen) supplemented with 0.05% human serum albumin (HSA) (Sanquin) unless specified otherwise.

Bacterial strains and culture conditions

S. aureus strains used for this study are as follows. *S. aureus* strain USA300 clone SF8300 is a minimally passed representative PVL-positive community-associated MRSA isolate from the United States [27], of which the isogenic Δ*hlgACB* mutant and complemented strains were described elsewhere [16, 48]. *S. aureus* strain ST80 is a European community-associated MRSA isolate [49], of which the isogenic Δ*lukSF-PV* mutant was previously reported on [48]. The ST80 isogenic

Δ*hlgACB* mutant and Δ*lukSF-PV* Δ*hlgACB* double mutant strains were generated as described elsewhere [16]. All strains were cultured in brain heart infusion (BHI). For in vivo experiments, mid-exponential subcultures were washed extensively in PBS.

Recombinant protein production and purification

LukS-PV, LukF-PV, HlgC, and HlgB used for this study were cloned and expressed as described elsewhere [14, 16, 48]. FITC-labelled LukS-PV and LukF-PV, used for binding studies in murine and human neutrophils, were previously described [50, 51]. For binding studies in U937 cells, random Alexa Fluor™ 647 (AF-647) labelled LukF-PV was used. AF-647 NHS Ester (Succinimidyl Ester) was acquired from Molecular Probes/ThermoFisher Scientific. The reactive dyes were dissolved in DMSO to a concentration of 10 mg/mL. An amount of 100 μg purified toxin was labelled with 10 μg reactive dye in a total volume of 100 μl PBS containing 0.1 M sodium carbonate pH 8.4 for 90 minutes at room temperature protected from light. Subsequently, the labelled protein was separated from free non-reacted dye using a protein desalting spin column. Protein concentration was determined with Nanodrop One (Thermo Fisher) and labelling verified by SDS-PAGE and fluorescence imaging.

***In vivo* infection**

hC5aR1^{KI} or C57BL6/N (WT, Charles River) mice, aged between 6 and 12 weeks and matched for weight and sex, were injected intraperitoneally (IP) with 5*10^{7} CFU. Mice were sacrificed 24h post-infection and the relevant compartments harvested as described elsewhere [16]. Briefly, peripheral blood, peritoneal lavage fluid, and homogenized organs were serially diluted and plated on Tryptic Soy Agar (TSA) for incubation at 37°C overnight, followed by CFU counting. For the skin infection model, mice were shaved on their back and hair removal cream was applied for 1 minute before being extensively washed. Three days later, mice were injected subcutaneously on both sides of their back with 2*10^{6} CFU in 100 μl PBS. At day 5 post-inoculation, mice were euthanized and the skin lesion and the underneath tissue were collected in 1 mL PBS. Tissue was grinded using the Precellys homogenizer (Bertin Instruments). The homogenate was diluted and plated on blood agar plates (BioMérieux) using the easySpiral Dilute (Interscience). CFU were automatically counted in a blinded manner using the Scan300 counter with a manual correction to remove non-haemolytic CFU. Sample sizes were determined following previous [16, 18] and preliminary experiments showing sufficient power.

Calcium mobilization assays

Murine neutrophils or immortalized murine hC5aR1^{KI} bone marrow-derived macrophages (BMDMs) were loaded with Fluo-4-AM (Molecular Probes / Thermo Fisher) for 30 minutes at 37°C in the dark following manufacturer's instructions. Cells were then washed twice in HBSS supplemented with 2.5 mM probenicid, 0.1% (w/v) bovine serum albumin, Hepes 25 mM. Cells were incubated for 5 minutes at 37°C before FACS analysis (Accuri C6). Cells were analysed for 20 seconds to obtain the baseline fluorescence before addition of murine C5a (Prospec) or human C5a (Prospec, or Peprotech), after which acquisition was further continued.

U937 cells were loaded with 2 mM Fluo-3AM (Molecular Probes / Thermo Fisher) in RPMI/HSA for 20 minutes at room temperature under constant agitation, washed with buffer, and suspended to 10^6 cell/mL in RPMI/HSA. Each sample of cells was first measured for approximately 10 seconds to determine the basal fluorescence level. Next, a titrated range of C5a (Sigma) was added and rapidly placed back in the sample holder to continue the measurement. Cells were analysed by flow cytometry, gated on forward and side scatter to exclude dead cells and debris.

Cell permeability assays

All human and murine primary cells were pre-stained before toxin treatment. Human PBMC were stained for CD14 expression (clone M5E2, BD-Bioscience). Bone marrow derived murine cells were stained in the presence of Fcy-receptor block (TruStain fcX, BioLegend) with Ly6G (clone 1A8, BD) and Ly6C (clone AL-21, BD) antibodies during 20 minutes at 4°C. Cells were washed once in PBS 3% FCS and exposed to recombinant proteins in 100 µL of PBS with 3% fetal calf serum at room temperature. Cells were subsequently analyzed by flow cytometry using propidium iodide (PI) at 10 µg/ml. For competition experiments, human neutrophils were pre-incubated with 10 µg/mL mouse anti-human CD45 (clone 4B2, obtained from the American Tissue Culture Collection) or isotype control during 15 minutes at room temperature. 30 minutes after subsequent addition of the toxin, cells were analyzed by flow cytometry using intracellular staining by 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI, Moleculair Probes/Thermo Fisher).

U937 cells were exposed to canonical or non-canonical recombinant proteins and measured for 30 min at 37°C in a monochromator-based microplate reader (FLUOstar Omega, BMG Labtech) using using 2,5 µg/mL DAPI. As PVL and HlgCB are two-component toxins, equimolar concentrations of polyhistidine-tagged LukS-PV, LukF-PV, HlgC and HlgB were used. Pore formation was defined as a collective positive DAPI signal and the area under the curve was calculated for comparison.

Binding assays

Binding of proteins was measured by incubating cells with directly labelled proteins during 30 minutes at room temperature. After washing, binding was detected by flow cytometry. For competition experiments, human neutrophils were pre-incubated with 10 µg/mL of different mouse anti-human CD45 monoclonal antibodies (clone 4B2, obtained from the American Tissue Culture Collection; clone 2D1, BD Biosciences; clone F10-89-4, BioRad) or isotype control during 15 minutes at room temperature.

Lysis assays

U937 cells were exposed to PVL for 1 hour followed by a short spin down. Supernatants were collected and the presence of lactate dehydrogenase (LDH) was detected using the Cytotox-96 Non-Radioactive Cytotoxicity Assay (Promega), following the manufacturer's instruction. OD was measured at 490 nm on a microplate reader (BioRad). Results were normalized to the manufacturer's Lysis solution (9% v/v Triton X-100).

Determination of receptor expression levels

Receptor expression levels were determined as described elsewhere [16]. For quantification, single cell suspensions were stained with mouse anti-human C5aR1 (clone S5/1, AbD Serotec), mouse anti-human CD45 (clone 2D1, BD Biosciences), or isotype controls, followed by FITC-conjugated goat-anti mouse antibody (Dako). Antibody binding was quantified by calibration to defined antibody binding capacity unites, usint QIFIkit (Dako). For validation of receptor expression after complementation, cells were incubated with PE-labeled mouse anti-human C5aR1 (clone S5/1, AbD Serotec), APC-labeled mouse anti-human CD45 (clone 2D1, BD Biosciences), or Cy-Chrome-labeled rat anti-mouse CD45 (clone 30-F11, BD Biosciences). Samples were subsequently measured using flow cytometry.

Surface plasmon resonance analyses

Recombinant human and mouse CD45 isoform R0 protein (R&D Systems) was purchased and immobilised onto a series S CM5 chip using a Biacore S200 system (GE) using methods previously described [13]. Whole U937 cells expressing human or murine CD45 isoform R0 (U937-hC5aR1-SpCas9 cells transduced with a sgRNA targeting *cd45* (hC5aR1^{+} CD45^{-}), and subsequently transduced with a plasmid containing either human CD45 isoform R0 (p*hCD45-R0*) or murine CD45 isoform R0 (p*mCD45-R0*)), were fixed using 4% formaldehyde and washed three times with PBS and resuspended at 10^{7} cells/mL. Cells were immobilised onto a Series S C1 sensor chips using the C1 wizard methodology on the Biacore T200 control system as previously described [52]. Cells were flowed at 5 μL per minute for 900 seconds to load the chip to saturation. Whole U937 cells not expressing CD45 (U937-hC5aR1-SpCas9 cells transduced with a sgRNA targeting *cd45* (hC5aR1^{+} CD45^{-})) were loaded using the same methodology onto flow cell one to allow for double reference subtraction. For both the whole cell and recombinant protein assays, LukF-PV was flowed over the immobilised CD45 in two concentration ranges (1.6 nM to 1.0 μM and 16.0 nM to 10.0 μM). Data for mouse CD45 were obtained from only the higher concentration series.

Statistical analyses

Calculations of the area under the curves, calculations of half-maximal effective lytic concentrations using linear regression analyses, and all statistical analyses were performed using Prism 7.0 (GraphPad Software). Flow cytometric analyses were performed with FlowJo (Tree Star Software). Statistical significance was calculated using ANOVA analysis of variance and Student's *t*-test with posttest correction for multiple comparison, where appropriate. Exact p-values are provided in Supplementary Table 3.

Ethics statement

Human leukocytes were isolated after informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. In The Netherlands, approval was obtained from the medical ethics committee of the UMC Utrecht, the Netherlands (protocol METC 07-125/C). In France, blood was obtained from healthy donors from the Etablissement Français du Sang Auvergne Rhône Alpes, France under the convention EFS 16-2066. Ethical approval was obtained from the Comité de Protection des Personnes Sud Méditerranée I.

All experiments involving animals were reviewed and approved by the animal ethics committees of Lyon, France (CECCAPP, protocol number ENS2012_033, ENS2013_033, ENS2014_035, ENS2017_022).

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and the supplementary information, or from the corresponding authors upon request. Relevant accession codes are provided within the specific Methods sections.

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Author Contributions

Conceptualization: A.T., M.G., B.B., F.V., T.H., A.S. Methodology: A.T., M.G., R.L., P.H., K.K., C.D., M.J., T.H., A.S. Investigation: A.T., M.G., P.A., A.M., J.J., C.H., E.B., C.D., T.H., A.S. Resources: C.H., M.B., C.D., M.J., M.M. Funding: G.L., F.V., J.S., P.H., T.H. Writing: A.T., M.G., T.H., A.S. Supervision: R.L., P.H., T.H., A.S.

Supplementary tables

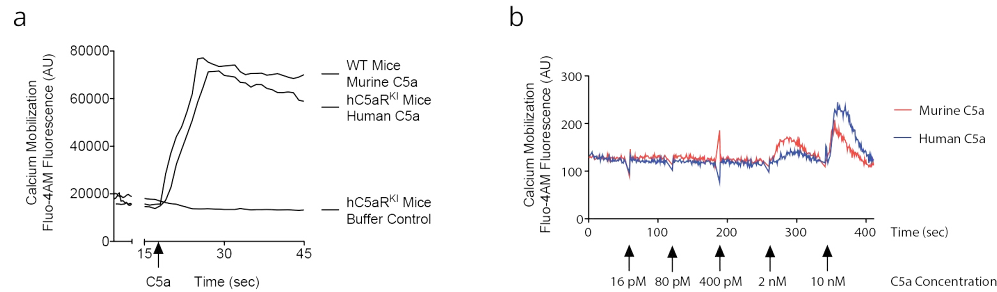
Gene	Score
SLC35B2	2,75E-50
PTPRC	**6,83E-45**
C5AR1	**8,03E-42**
PAPSS1	7,96E-35
TPST2	1,37E-28
SGMS1	1,11E-17
CENPL	5,12E-05
MAD2L1	1,71E-04
HAUS6	1,80E-04
NOP58	2,72E-04
CDK7	3,84E-04
DARS	5,49E-04
RPL7	5,67E-04
ECT2	7,52E-04
EEF1A1	9,94E-04
SCFD1	3,23E-03
BRIX1	4,05E-03
B3GALNT2	7,38E-03
PLK4	7,65E-03
PTP4A2	7,79E-03

Supplementary Table 1 (Related to Figure 3). Screening results for resistance to PVL-toxicity. CRISPR/Cas9 library screen for PVL-resistance set up in U937-hC5aR1 cells, selected after exposure to PVL. The top 20 most significantly enriched genes as calculated by the MaGeCK 'positive enrichment score' are highlighted in grey. Provided as separate document.

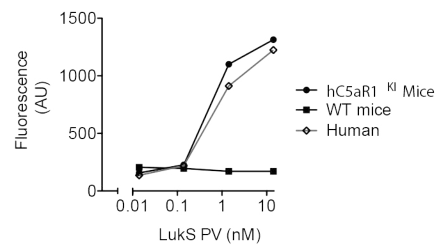
	Index	Comparator	p-Value			
			80 ug/mL	40 ug/mL	20 ug/mL	10 ug/mL
Panel b	NTC	sgRNA CD45_1	<0.0001	<0.0001	<0.0001	<0.0001

Supplementary Table 3 (Related to Figures 1-5). Exact p-values. The exact p-values were calculated by statistical tests as specified in the respective figure legends. Provided as separate document.

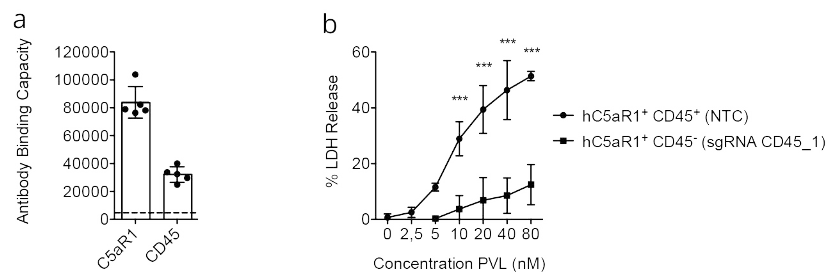
Supplementary figures



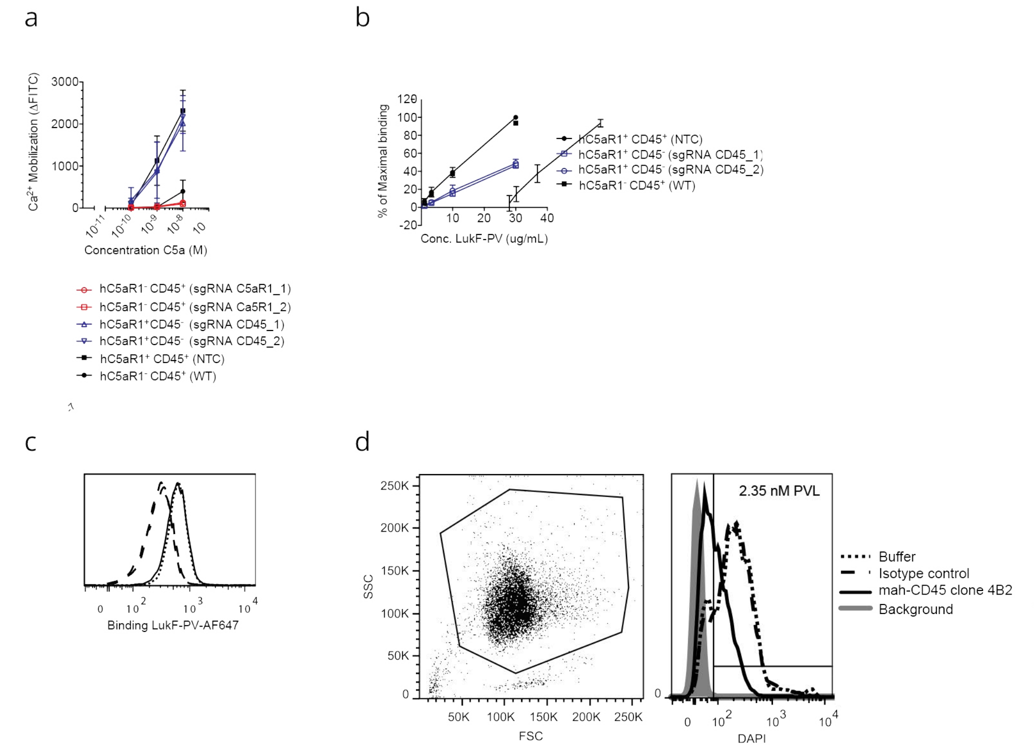
Supplementary Figure 1 (Related to Figure 1). (a) Calcium mobilization in bone marrow derived neutrophils from hC5aR1 knock-in (hC5aR1^{KI}) mice in response to 10^{-8} M human C5a, and wild type (WT) mice in response to 10^{-8} M murine C5a. (b) Calcium mobilization in immortalized bone marrow derived macrophages from hC5aR1 knock-in (hC5aR1^{KI}) mice in response to different concentrations of human or murine C5a. Curves depict representative samples of two independently repeated experiments.



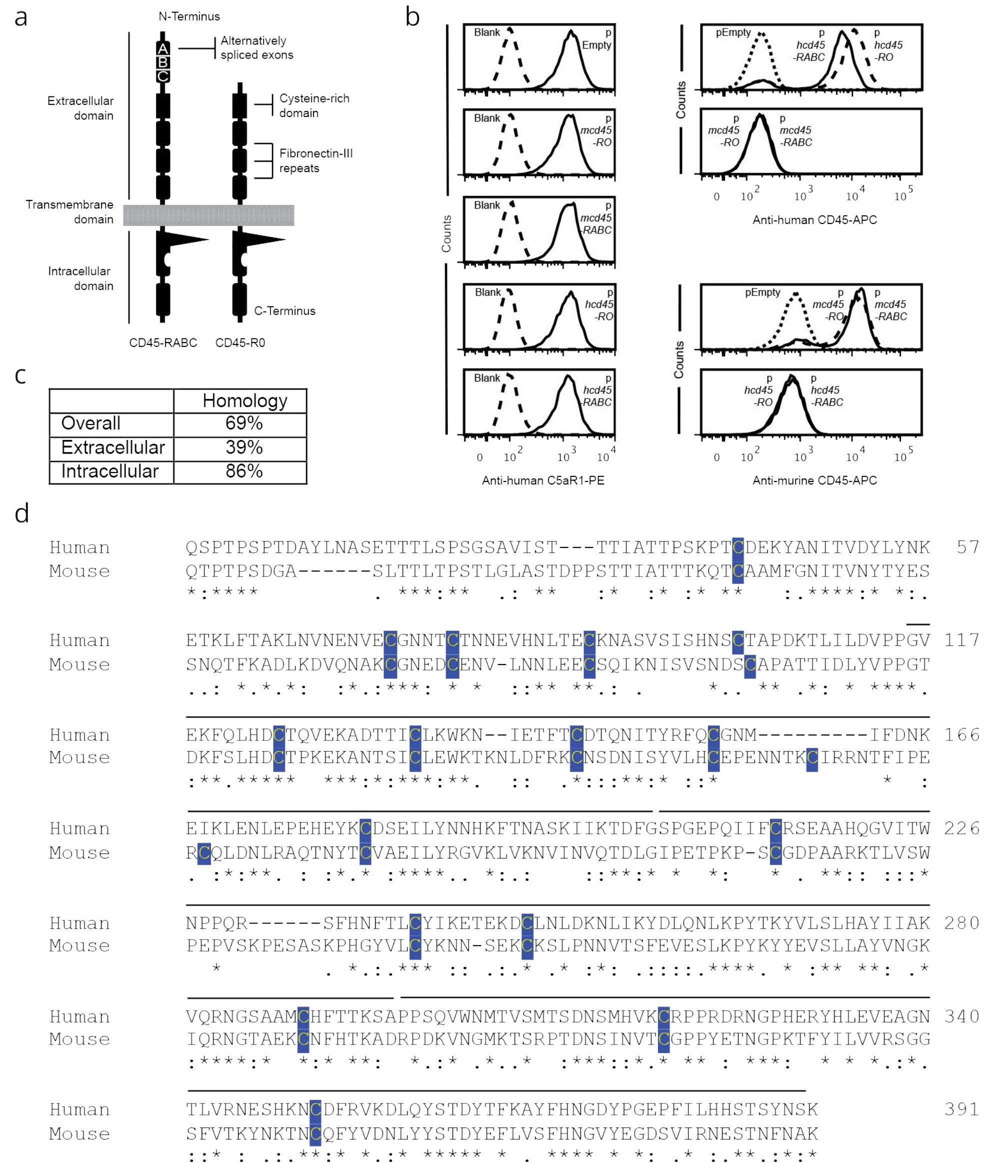
Supplementary Figure 2 (Related to Figure 2). Binding of LukS-PV-FITC to bone marrow derived neutrophils from hC5aR1 knock-in (hC5aR1^{KI}) mice and wild type (WT) mice, and human neutrophils isolated from healthy donors as detected by flow cytometry. Curves depict representative samples of two independently repeated experiments.



Supplementary Figure 3 (Related to Figure 3). (a) Expression levels of hC5aR1 and CD45 on human neutrophils semi-quantitatively demonstrated as the antibody-binding capacity of anti-hC5aR1 and anti-hCD45 monoclonal antibodies. Mean and s.d. are shown, with n=5. (b) Release of lactate dehydrogenase (LDH) in U937-hC5aR1-SpCas9 cells transduced with a non-targeting control (NTC, hC5aR1^{+} CD45^{+}) or a vector containing a gRNA for *cd45* (hC5aR1^{+} CD45^{-}) after exposure to PVL. Mean and s.d. are shown, with n=3. Statistical significance is displayed as *** for p < 0.001, and was calculated using ANOVA analysis of variance with Bonferroni posttest correction for multiple comparison. Exact p-values are provided in Supplementary Table 3.

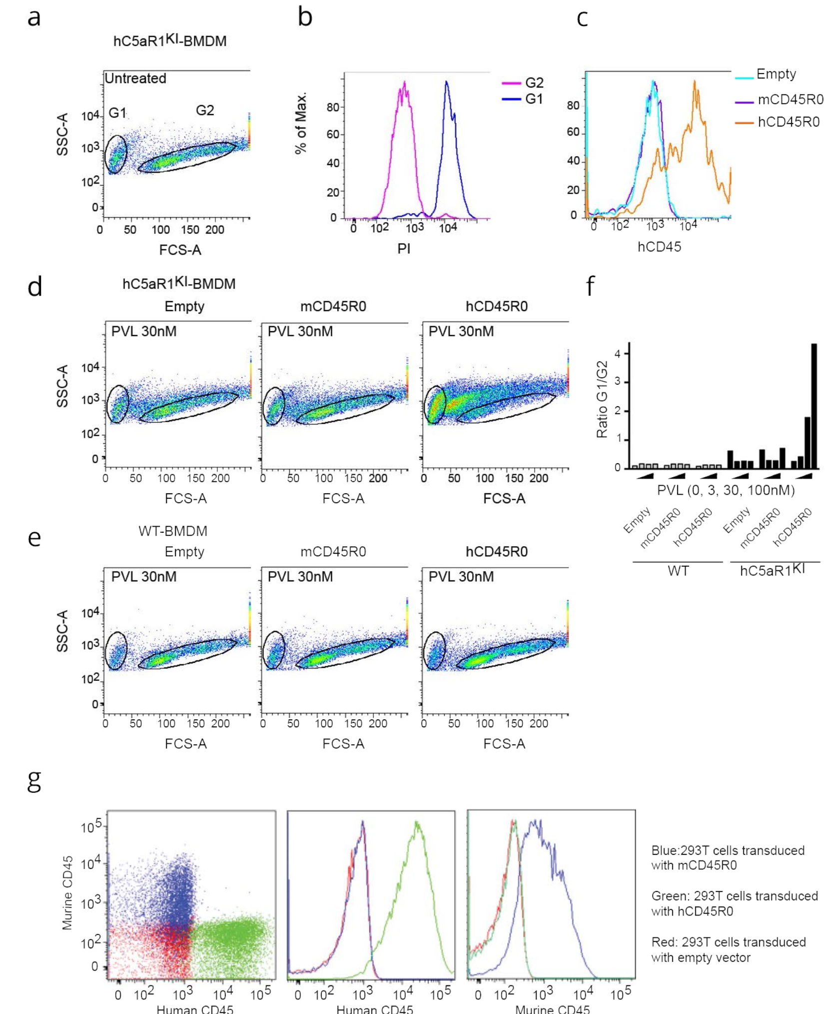


Supplementary Figure 4 (Related to Figures 3 and 4). (a) Calcium mobilization in wild type U937 cells (WT, hC5aR1^{-} CD45^{+}), U937-hC5aR1-SpCas9 cells transduced with a non-targeting control (NTC, hC5aR1^{+} CD45^{+}), two vectors containing a gRNA for *c5aR1* (both hC5aR1^{-} CD45^{+}) or *cd45* (both hC5aR1^{+} CD45^{-}) in response to human C5a. Bars express mean with s.d., with n=3. (b) Binding of LukF-PV-AF647 to cells as detected by flow cytometry. In addition to WT U937 and NTC U937-hC5aR1-SpCas9 cells, two U937-hC5aR1-SpCas9 cells lines transduced with a vector containing a gRNA for *cd45* (both hC5aR1^{+} CD45^{-}) were tested. Abbreviations as specified in panel (a). Bars express mean with s.d., with n=3. (c) Binding of LukF-PV-FITC U937 cells in the presence or absence of CD45-expression. Solid line: wild type U937 cells (WT, hC5aR1^{-} CD45^{+}); dotted line: U937-hC5aR1-SpCas9 cells transduced with a non-targeting control (NTC, hC5aR1^{+} CD45^{+}); dashed lines: U937-hC5aR1-SpCas9 cells transduced with two vectors containing a gRNA for *cd45* (both hC5aR1^{+} CD45^{-}). Histogram depicts a representative sample at 10 µg/mL LukF-PV, of two independently repeated experiments. (d) DAPI internalization 30 minutes after exposure to PVL in human neutrophils pre-treated with 10 µg/mL of the mouse anti-human monoclonal antibody CD45 clone 4B2, or isotype control. Histogram depicts a representative sample at 2.35 nM of PVL, of two independently repeated experiments.



	Homology
Overall	69%
Extracellular	39%
Intracellular	86%

Supplementary Figure 5 (Related to Figure 5). (a) Schematic representation of CD45 isoforms RABC and R0, including the different structural regions in the extracellular domain of the receptor. (b) Expression of hC5aR1 and human or murine CD45 on U937-hC5aR1-SpCas9 cell lines transduced with a sgRNA for CD45 (hC5aR1⁺ CD45⁻), and subsequently transduced with a plasmid containing either human CD45 isoform R0 (p*hCD45-R0*) or RABC (p*hCD45-RABC*), murine CD45 isoform R0 (p*mCD45-R0*) or RABC (p*mCD45-RABC*), or an empty plasmid (pEmpty). Histograms depict representative examples, of two independently repeated experiments. (c) Homology between mouse and human CD45-R0 amino acid sequences, expressed as percentage. (d) Amino acid sequence alignment of the extracellular domain of human and murine CD45 isoform R0 using the Clustal W multiple sequence alignment tool. Cysteines are highlighted in blue, and the fibronectin-III domains are lined.



Supplementary Figure 6 (Related to Figure 5). Expression of human CD45 renders hC5aR1^{KI} bone marrow-derived macrophages (BMDMs) susceptible to PVL. (a-f) Primary BMDMs from WT or hC5aR1^{KI} mice were transduced at day 4 post-isolation with lentiviruses encoding murine CD45 isoform R0 (mCD45R0), human CD45 isoform R0 (hCD45R0), or a control lentivirus (Empty). 5 days post-transduction, cells were collected. (a) Analysis by flow cytometry (SSC: side scatter; FSC: forward scatter) demonstrated (b) a population of dead cells in gate 1 (G1) staining positive for propidium iodide (PI) and a population of live cells in gate 2 (G2) negative for PI staining. (c) Expression of human CD45 in G2 was assessed by flow cytometry. (d) hC5aR1^{KI} or (e) WT BMDMs transduced with the indicated lentiviruses were treated with PVL (30 nM) for 30 min at room temperature before analysis. (f) The ratio of the number of events in G1 over the number of events in G2 was quantified by flow cytometry in the indicated BMDMs transduced with the indicated lentiviruses and treated with increasing concentrations of PVL (no toxin, 3 nM, 30 nM, 100 nM). As a reference, human monocyte-derived macrophages are fully susceptible to PVL at 3 nM (Perret M et al., Cell Micro 2012). (g) Ectopic expression of murine CD45 in BMDMs could not be visualized due to high endogenous expression of murine CD45. As a control for ectopic expression of murine CD45 in BMDMs, expression of murine CD45 was verified on 293T human embryonic kidney cells transduced in a similar manner as BMDMs. One experiment representative of two independent experiments is

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CHAPTER

THREE

HOST-RECEPTOR POST-TRANSLATIONAL MODIFICATIONS AND STAPHYLOCOCCAL LEUKOCIDIN TOXICITY

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Abstract

Staphylococcal bi-component pore-forming toxins, better known as leukocidins, target and lyse human phagocytes in a receptor-specific manner. S-components of the leukocidins Panton–Valentine leukocidin (PVL), γ-haemolysin AB (HlgAB) and CB (HlgCB) and leukocidin ED (LukED) specifically employ receptors that belong to the class of G-protein coupled receptors (GPCRs). Although these receptors share a common structural architecture, little is known about the conserved characteristics of the interaction between leukocidins and GPCRs. In this study, we investigated host cellular pathways contributing to susceptibility towards *S. aureus* leukocidin cytotoxicity. We performed a genome-wide CRISPR-Cas9 screen for toxin-resistance in U937 cells sensitized to leukocidins by ectopic expression of different GPCRs. Our genome-wide screening identifies post-translational modification pathways involved in the sulfation and sialylation of cell surface-expressed molecules. Subsequent validation experiments show that these post-translational modification pathways affect the interaction between the leukocidin S-components and their respective GPCRs. This study identifies a conserved role for sialylation, and a divergent role for sulfation in the interaction between different leukocidins and their respective host-counterpart receptors. Leukocidin receptors may serve as targets for anti-staphylococcal interventions and understanding toxin-receptor interactions will facilitate the development of novel therapeutics. Variations in the genes encoding post-translational modification pathways may provide insight into observed differences in susceptibility of humans to infections with *S. aureus*.

Introduction

Staphylococcus aureus is a commensal Gram-positive bacterium that colonizes the skin and anterior nares of 20-30% of the general human population [1]. *S. aureus* causes a variety of diseases, ranging from superficial skin and soft tissue infections to severe invasive infections with a poor prognoses and high mortality [2, 3]. Upon infection, *S. aureus* is faced with the host humoral and cellular innate immune response [4]. *S. aureus*, in return, secretes an arsenal of virulence factors to circumvent host defences and avoid killing by phagocytes [5, 6]. One important group of *S. aureus* virulence factors, the leukocidins, specifically target and lyse host phagocytes [7-10].

S. aureus leukocidins are bi-component beta-barrel pore-forming toxins [10, 11]. Human *S. aureus* isolates secrete up to five leukocidins; Panton–Valentine leukocidin (PVL), γ-haemolysin AB (HlgAB) and CB (HlgCB), leukocidin ED (LukED) and leukocidin AB (LukAB, also knowns as LukGH) [12]. Based on chromatography elution profiles, the individual leukocidin subunits are designated S(slow)- or F(fast)-migrating components [10]. Proteinaceous targets have been identified for all *S. aureus* leukocidins. The S-component of the leukocidins, with the exception of LukAB, target specific G-protein coupled receptors (GPCRs) present on host cells [10]. The C5a anaphylatoxin chemotactic receptor 1 (C5aR1, also known as CD88) and C5a anaphylatoxin chemotactic receptor 2 (C5aR2, also known as C5L2) were identified as targets for PVL and HlgCB. LukED targets leukocytes via CC-chemokine receptor 5 (CCR5), as well as CXC chemokine receptor 1 (CXCR1) and CXC chemokine receptor 2 (CXCR2) [10]. HlgAB targets CXCR1, CXCR2 and CCR2 [10]. In addition, HlgAB and LukED both target the Duffy antigen receptor for chemokines (DARC, also known as ACKR1), an atypical chemokine receptor expressed on erythrocytes [10, 13]. Although these receptors share a common structural seven-transmembrane spanning architecture, little is known about the conserved or divergent characteristics of the interaction between leukocidins and GPCRs. The apparent redundant deployment of two leukocidins that target the same human C5aR1, and the overlapping cell-tropism between leukocidins remains enigmatic. Furthermore, additional host cell processes such as intracellular pathways and molecular determinants involved in leukocidin-receptor interactions are incompletely understood.

The binding of LukS-PV to the N-terminus of C5aR1 was previously shown to be mediated by sulfation of the receptor [14]. In addition, LukED and HlgAB toxicity of DARC expressing cells is suggested to be sulfation dependent [15], indicating that receptor sulfation may play a conserved role in *S. aureus* leukocidin susceptibility. In this study, we developed a genome-wide CRISPR-Cas9 based approach to screen for host factors involved in PVL and HlgCB mediated cytotoxicity. We identify intracellular post-translational modifications (PTM) that dictate susceptibility to PVL and HlgCB on C5aR1 expressing cells. However, when assessing the role of the same PTM in CXCR2 expressing cells, we identified a conserved role for sialylation but a divergent role for GPCR sulfation influencing susceptibility to HlgAB and LukED. These findings further substantiate the complexity underlying the interaction between *S. aureus* bi-components toxins and their target cells.

Results

CRISPR-Cas9 screen identifies PTM pathways.

In order to identify possible host cellular pathways involved in PVL- and HlgCB-mediated susceptibility of human phagocytes, a genome-wide CRISPR/Cas9 screen for both PVL- and HlgCB resistance was set up in human U937 promyelocytic cells. These cells were made sensitive to PVL- and HlgCB mediated pore-formation by overexpressing hC5aR1 (U937-hC5aR1), and subsequently a human codon-optimized nuclear-localized *S. pyogenes* Cas9 gene was introduced (U937-hC5aR1-SpCas9). Cellular components involved in PVL and HlgCB toxicity were detected via the introduction of a genome-wide sgRNA library coupled to deep sequencing to identify genes inactivated in cells surviving toxin treatment. *C5AR1* was identified as a top hit in both the HlgCB- and PVL-resistance screen, validating the screening method (Figure 1).

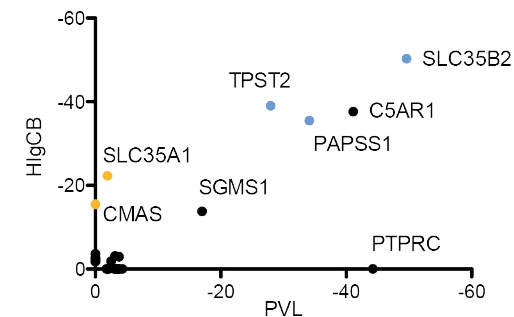

Figure 1: CRISPR-screen reveals intracellular proteins involved in PVL and HlgCB toxicity. Cellular components that are involved in PVL- and HlgCB-mediated killing identified by the introduction of a genome-wide sgRNA library in U937-hC5aR1-SpCas9 cells coupled to deep sequencing. Most significantly enriched genes, as calculated by the MaGeCK 'positive enrichement score', are visualized for HlgCB and PVL. Graph depicts proteins involved in the tyrosine sulfation pathway (blue) and proteins involved in the sialylation pathway (orange).

The screenings for both PVL and HlgCB-resistance revealed the involvement of genes encoding the Solute Carrier Family 35 Member B2 (*SLC35B2)*, 3'-Phosphoadenosine 5'-Phosphosulfate Synthase 1 *(PAPSS1)* and Tyrosylprotein Sulfotransferase 2 (*TPST2)* (Figure 1). In addition, the screening for HlgCB-resistance indicated the involvement of genes encoding the Solute Carrier Family 35 Member A1 *(SLC35A1)* and Cytidine Monophosphate N-Acetylneuraminic Acid Synthetase (*CMAS)* (Figure 1). Finally, the alpha hemolysin (Hla) determinant sphingomyelin synthase 1 (SGMS1) [16] (Figure 1) was identified. *SLC35B2*,

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PAPSS1 and *TPST2* encode proteins involved in the tyrosine sulfation pathway, a post-translational modification (PTM) process in which a sulfate group is enzymatically added to a tyrosine residue. Moreover, *SLC35A1* and *CMAS* encode proteins involved in the sialylation pathway, a PTM process implicated in the attachment of sialic acid to other molecules. In the current study we focused on genes involved in intracellular pathways as our screening consistently suggested a role for PTMs in dictating sensitivity towards PVL and HlgCB.

Sulfation of C5aR1 facilitates PVL and HlgCB cytotoxicity.

To validate the involvement of *SLC35B2*, *PAPSS1* and *TPST2* in PVL and HlgCB cytotoxicity, single knock-out cells were generated in the U937-hC5aR1-SpCas9 cells. Knocking-out *SLC35B2*, *PAPSS1* (Data not shown) and *TPST2* (Data not shown) all resulted in a decrease of tyrosine sulfation, as determined by flow cytometry using anti-sulfotyrosine antibodies that detect general cellular tyrosine sulfation (Figure 2a). Lack of tyrosine sulfation did not affect overall C5aR1 expression, as determined using a sulfation-independent C5aR1 antibody (Figure 2a). Subsequently, knock-out cells lacking SLC35B2, PAPSS1 or TPST2 were challenged with half-maximum effective concentration (EC$_{50}$) of PVL or HlgCB. As expected, C5aR1^{+}SLC35B2^{-}, C5aR1^{+}PAPSS1^{-} and C5aR1^{+}TPST2^{-} cells were less susceptible to pore formation induced by both PVL and HlgCB (Figure 2b). C5aR1 has sulfated tyrosine residues at position 11 and 14 that have been previously described to be involved in the interaction with a staphylococcal C5aR1 inhibitor [17]. Binding of LukS-PV to an N-terminal C5aR1-peptide was also previously shown to be mediated by sulfation of the peptide [14]. We hypothesized that knocking-out SLC35B2, PAPSS1 and TPST2 in U937-C5aR1 cells affects C5aR1 sulfation, and binding of LukS-PV. To this end, we determined C5aR1 sulfation with an antibody detecting C5aR1 in a sulfation-dependent manner. Knocking-out either SLC35B2, PAPSS1 (Data not shown) or TPST2 (Data not shown) reduced hC5aR1 sulfation (Figure 2a). Subsequently we assessed binding of LukS-PV, but also HlgC, to these cells. Although overall expression of C5aR1 is normal (Figure 2a), no binding of LukS-PV or HlgC was detected on C5aR1^{+}SLC35B2^{-}, C5aR1^{+}PAPSS1^{-} or C5aR1^{+}TPST2^{-} cells (Figure 2c). Thus, these results show cellular susceptibility towards PVL and HlgCB is mediated by C5aR1-sulfation dependent binding of LukS-PV and HlgC.

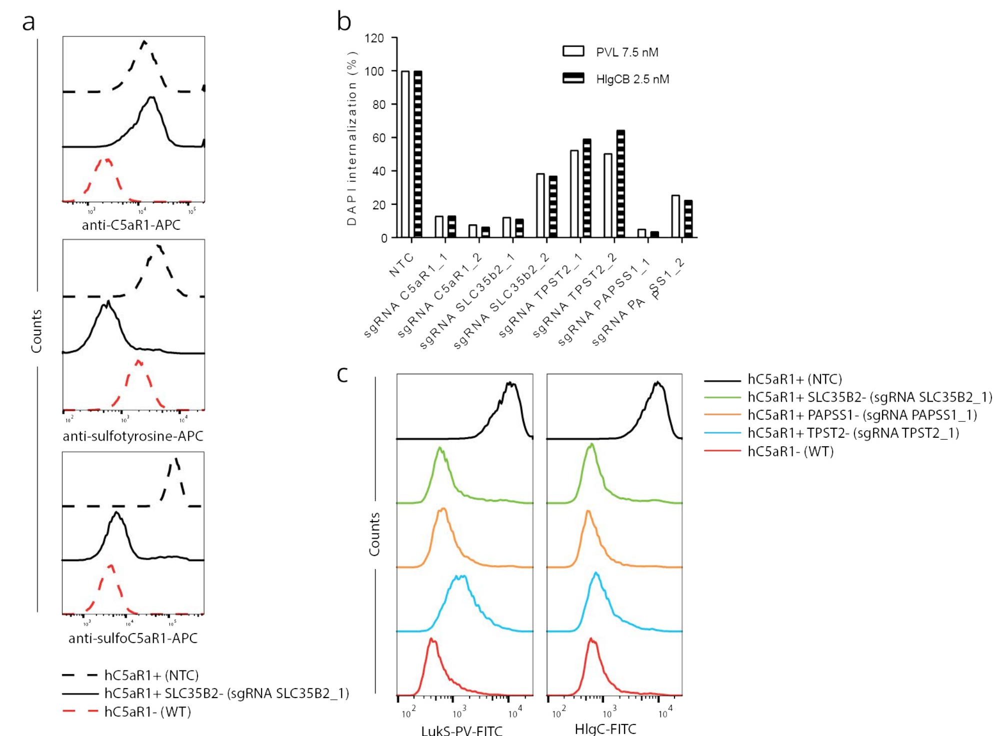

Figure 2: Sulfation of C5aR1 facilitates PVL and HlgCB cytotoxicity. (a) Expression of hC5aR1, sulfotyrosine and sulfated hC5aR1 on U937-hC5aR1-SpCas9 cell lines transduced with sgRNA for SLC35b2 (hC5aR1+ SLC35b2_1-), non-targeting control sgRNA (NTC) and wild type U937. SLC35b2 histogram depicts representative cellular- and C5aR1 sulfation levels as cells transduced with sgRNA for TPST2 and PAPSS1 (data not shown). (b) Validation of the genome-wide CRISPR-Cas9 screen for PVL and HlgCB resistance in U937-hC5aR1-SpCas9 cells. Cells were exposed to PVL (7.5nM) or HlgCB (2.5nM). As a readout for cell permeability, internalization of DAPI was tested at 30 minutes post-toxin treatment on a monochromator-based microplate reader and expressed in relation to U937-hC5aR1-SpCas9 cells transduced with a NTC. Two guide RNAs were tested for C5aR1, SLC35b2, TPST2 and PAPSS1. Chart is representative for multiple experiments. (c) Binding of 3 µg/mL His-tagged LukS-PV and HlgC to the different C5aR1-expressing cells, followed by anti-His-FITC antibodies and detected by flow cytometry. The depicted histograms are representative of 2 experiments.

Sialylation of C5aR1 facilitates PVL and HlgCB cytotoxicity.

In addition to genes involved in the sulfation pathway, our screening for HlgCB-resistance acquired genes involved in the sialylation process. In order to validate these findings, *SLC35A1* and *CMAS* single knock-out cells were generated in U937-hC5aR1-SpCas9 cells. The detectable difference between non-sialylated Lewis^{X} (CD15) [18] and sialylated Lewis^{X} (CD15s) expression using an anti-Sialyl-Lewis^{X} antibody was applied as a readout to assess overall cellular sialylation. C5aR1^{+}SLC35A1^{-} and C5aR1^{+}CMAS^{-} cells showed no binding of anti-Sialyl-Lewis^{X}

antibodies (Figure 3a), confirming that these cells lack sialic acid modification. Subsequent toxin challenge confirmed that C5aR1⁺SLC35A1⁻ and C5aR1⁺CMAS⁻ cells showed reduced susceptibility to pore formation induced by an EC_{50} of HlgCB (Figure 3b). As *SLC35A1* was also identified as one of the top 20 hits in our PVL-resistance screen, we challenged C5aR1⁺SLC35A1⁻ and C5aR1⁺CMAS⁻ cells with an EC_{50} of PVL. As expected, knocking-out *SLC35A1* resulted in a decreased susceptibility to PVL (Figure 3b). In addition, C5aR1⁺CMAS⁻ cells were less susceptible to PVL mediated pore formation (Figure 3b). As shown in figure 2c, a decrease in susceptibility of hC5aR1 expressing cells to PVL and HlgCB was attributed to impaired C5aR1 sulfation. We hypothesized that reduced susceptibility of C5aR1⁺SLC35A1⁻ and C5aR1⁺CMAS⁻ cells to HlgCB and PVL is also due to an impaired interaction of the toxin's S-component with hC5aR1, and determined the binding of HlgC and LukS-PV to C5aR1⁺SLC35A1⁻ and C5aR1⁺CMAS⁻ cells. As expected, both LukS-PV and HlgC showed a similar reduced binding to C5aR1⁺SLC35A1⁻ and C5aR1⁺CMAS⁻ cells (Figure 3d, LukS-PV binding not shown). In conclusion, these data show that sialylation of the hC5aR1 is a molecular determinant dictating susceptibly to PVL and HlgCB by modulating S-component interaction. To further assess the extent of the role of SLC35B2, PAPSS1, TPST2, SLC35A1 and CMAS in cellular susceptibility to PVL and HlgCB, mutant cells were incubated with different concentrations of PVL and HlgCB. The absence of cellular sulfation or sialylation resulted in an increased EC_{50} for both PVL and HlgCB (Figure 3d). These findings suggest that the sialylation and the sulfation of the hC5aR1 are not essential for PVL and HlgCB cytotoxicity, but rather enhance the interaction and sensitivity to these toxins.

Sialyation, but not sulfation, enhances lukED and HlgACB cytotoxicity

We hypothesized that PTMs of host receptors not only play a role in the interaction of C5aR1-interacting toxins, but also in the interaction of LukED and HlgAB with their GPCR targets. To study the role of sulfation and sialylation in LukED- and HlgAB-receptor interaction and susceptibility, we generated knock-outs in cells ectopically expressing their shared cell surface target CXCR2. As representatives of the sialylation and sulfation PTM pathways, *SLC35B2* and *SLC35A1* were selected for the subsequent generation of single knock-out cell lines. All cell constructs expressed comparable levels of CXCR2 (Figure 4a). Accordingly, CXCR2⁺SLC35B2⁻ cells lacked detectable levels of sulfation and CXCR2⁺SLC35A1⁻ cells were devoid of sialylation (Figure 4a). Next, CXCR2⁺SLC35A1⁻ and CXCR2⁺SLC35B2⁻ cells were challenged with different concentrations of HlgAB and LukED. CXCR2⁺SLC35A1⁻ cells are less sensitive to both HlgAB and LukED (Figure 4c).

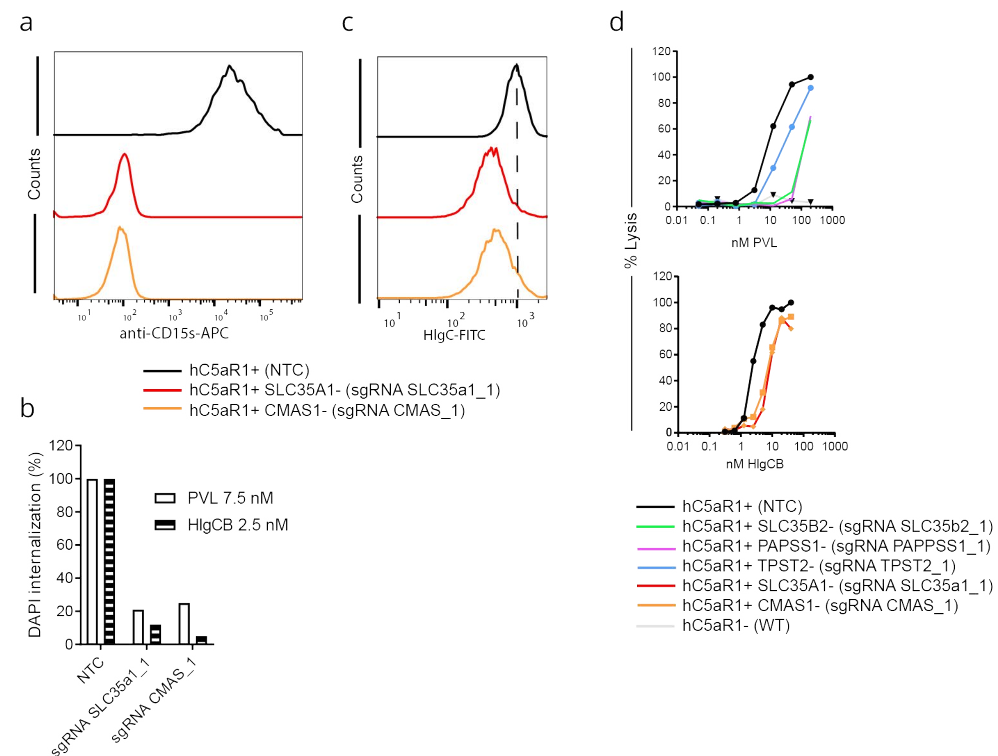

Figure 3: Sialylation of C5aR1 facilitates PVL and HlgCB cytotoxicity. a, Expression CD15s on U937-hC5aR1-SpCas9 cell lines transduced with sgRNA for SLC35A1 (hC5aR1+ SLC35a1_1-), non-targeting control sgRNA (NTC), and CMAS (hC5aR1+ CMAS_1-). b, Validation of the genome-wide CRISPR-Cas9 screen for PVL resistance in U937-hC5aR1-SpCas9 cells. Cells were exposed to PVL (7.5nM) or HlgCB (2.5nM). As a readout for cell permeability, internalization of DAPI was tested at 30 minutes post-toxin treatment on a monochromator-based microplate reader and expressed in relation to U937-hC5aR1-SpCas9 cells transduced with a NTC. c, Binding of HglCb to cells as detected by flow cytometry, shown as a histogram representative of 2 experiments. d, U937-C5aR1-SpCas9 cells were exposed to various concentrations of PVL and HlgCB. As a readout for cell permeability, internalization of DAPI was tested at 30 minutes post-toxin treatment on a monochromator-based microplate reader and expressed in relation to the maximal area under the curve for U937-hCXCR2-SpCas9 cells transduced with a NTC.

Surprisingly however, CXCR2⁺SLC35B2⁻ cells were as susceptible to HlgAB and LukED, compared to the positive control (Figure 4b).Thus, these results show that sialylation in CXCR2⁺ cells enhances the interaction of HlgAB and LukED with CXCR2. Contrary to what expected however, sulfation of CXCR2 does not play a role in HlgAB or LukED mediated pore formation, highlighting a divergent role of sulfation in dictating toxin susceptibility between GPCRs.

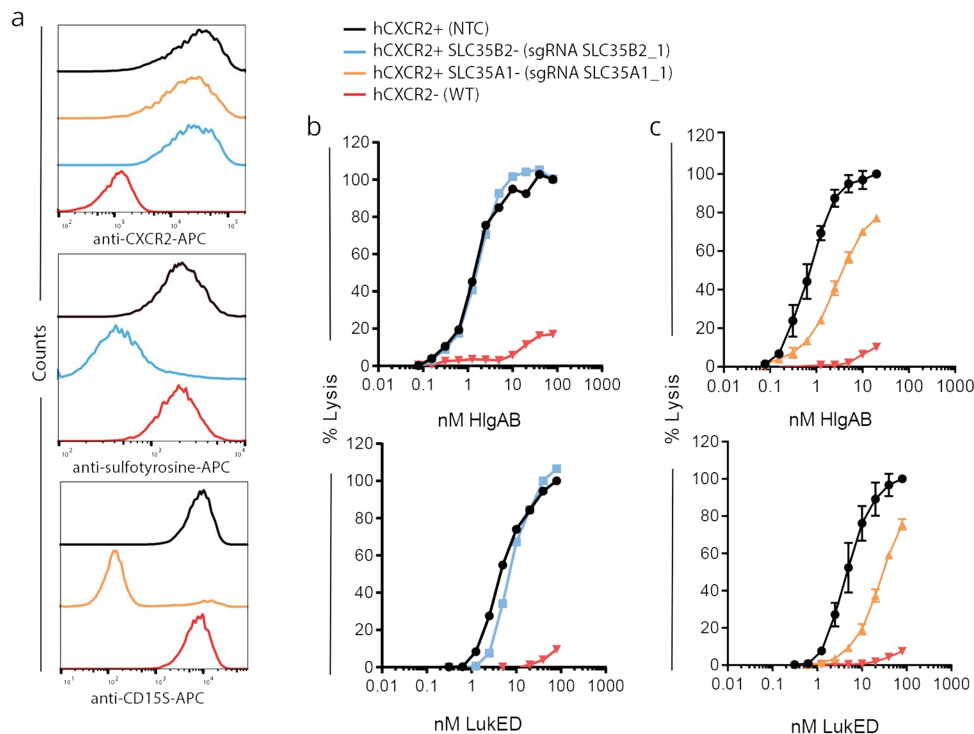

Figure 4: Conserved role for sialylation, but not sulfation, in LukED and HlgAB cytotoxicity of CXCR2. (a) Expression of hCXCR2, sulfotyrosine and CD15S on U937-hCXCR2-SpCas9 cell lines transduced with sgRNA for SLC35b2 (hC5aR1+ SLC35b2_1-), SLC35a1 (hC5aR1+ SLC35a1_1-), non-targeting control sgRNA (NTC) and wild type U937. (b, c) U937-hCXCR2-SpCas9 cells were exposed to various concentrations of LukED and HlgAB. As a readout for cell permeability, internalization of DAPI was tested at 30 minutes post-toxin treatment on a monochromator-based microplate reader and expressed in relation to the maximal area under the curve for U937-hCXCR2-SpCas9 cells transduced with a NTC.

Discussion

We used a genome-wide CRISPR-Cas9 based approach to screen for intracellular pathways involved in susceptibility towards GPCR-targeting leukocidins. Here, we identify two intracellular PTM pathways affecting susceptibility of cells to PVL and HlgCB. PTM of proteins is essential in many cellular processes [19]. These post-translational enzymatic reactions modify specific protein backbones and sidechains, modulating protein function [19].The PTM of GPCRs is important for regulating structure, function and association with their natural ligands [20-23]. However, PTM moieties on GPCRs have also been suggested to play a role in the interaction with different pathogens [24, 25]. Both PVL- and HlgCB-resistance screening revealed multiple genes encoding proteins involved in the tyrosine sulfation pathway, a PTM of secreted and transmembrane proteins performed by all eukaryotic cells [26, 27]. Some chemokine receptors bear N-terminal sulfated tyrosine positions that facilitate the initial interaction with chemokines [27]. It has been previously proposed that the sulfation of N-terminal tyrosines mediates initial binding of LukS-PV to C5aR1 [14]. This was observed using N-terminal C5aR1 sulfopeptides and it was not clear to what extent C5aR1 sulfation affects binding of LukS-PV to the full receptor, and subsequent PVL-mediated pore formation on target cells. We show that the disruption of sulfation of C5aR1 results in a reduced S-component binding to C5aR1 expressing U937 cells. C5aR1 expressing cells that were disrupted of sulfation show an increased EC50 towards PVL and HlgCB. This suggests that sulfation of C5aR1 facilitates the interaction between the S-components and C5aR1 *in vitro*.

Besides PVL and HlgCB, *S. aureus* secretes two other leukocidins that target specific GPCRs on leukocytes (HlgAB, LukED) [10]. Although we hypothesized that sulfation of N-terminal tyrosine on GPCRs defines a conserved host interaction site for staphylococcal leukocidins, knocking-out sulfation in CXCR2 expressing cells did not affect LukED or HlgAB cytotoxicity. CXCR2 contains two extracellular N-terminal tyrosines, however it is unclear whether these are actually sulfated [28]. The N-terminus of CXCR2 lacks tyrosine sulfation determinants, such as acidic sulfation motifs mediating TPST activity (Figure 5), and therefore could be sulfated with less efficiency or not modified at all [28, 29]. DARC, the erythrocyte receptor for leukocidins and a close homologue of CXCR2, has been shown to interact with both HlgAB and LukED in a N-terminal sulfated tyrosine dependent manner [15]. This suggests a divergent role for GPCR sulfation in leukocidin activity, in which LukE and HlgA interact sulfation dependent in a receptor specific manner.

```
C5aR1
mdsfnyttpd yghyddkdtl dlntpvdkts ntlrvpdila lvifavvflv gvlgnalvvw

CCR2
mlstsrsrfi rntnesgeev ttffdydyga pchkfdvkqi gaqllpplys lvfifgfvgn

CCR5
mdyqvsspiy dinyytsepc qkinvkqiaa rllpplyslv fifgfvgnml vililinckr

CXCR1
msnitdpqmw dfddlnftgm ppadedyspc xletetlnky vviiayalvf llsllgnslv

CXCR2
medfnmesds fedfwkgedl snysysstlp pflldaapce pesleinkyf vviiyalvfl

DARC
mgnclhrael spstenssql dfedvwnssy gvndsfpdgd yganleaaap chscnlldds
```

Figure 5: N-terminus tyrosine sulfation on GPRs. Illustration of the 1-60 amino acids of C5aR1, CCR2, CCR5, CXCR1, CXCR2 and DARC. The amino acids in the transmembrane region of the corresponding GPCR are highlighted in grey, the tyrosines are depicted in red, acidic residues adjacent to a tyrosine are illustrated in blue, and sulfated tyrosines are highlighted in green.

N-terminal tyrosine sulfation has been described for the other GPCRs targeted by one or more leukocidins (CXCR1, CCR2 and CCR5) [21, 25, 28, 30]. However, it remains to be established whether tyrosine sulfation of these GPCRs also mediates leukocidin susceptibility. Tyrosine sulfation is heterogeneous and tissue specific [27, 31] resulting in variable sulfation profiles possibly contributing to the host and cellular tropism of leukocidins. It remains to be established whether there is variability in sulfation of GPCRs on phagocytes during different stages of activation or infection, and possibly contributing to the interaction of leukocidins with their respective receptor.

Sialic acid moieties on surface of host cells have been described to be involved in the attachment and invasions of various pathogens [32]. The sialic acid-binding adhesin (SabA) secreted by *Helicobacter pylori* mediates adherence specifically to the Lewis blood group antigens Sialyl-Lewis^x[33]. In fact, Sialyl-Lewis^x has been described as a PTM mediating affinity between many bacterial toxins and their targets. The *E. coli* subtilase cytotoxin (SubAB), *S. enterica* typhoid toxin and *S. pneumoniae* cytolysin all interact in an Sialyl-Lewis^x dependent manner [32]. A previous study has shown that the pre-incubation with the ganglioside GM1, a sialic acid-containing oligosaccharide, disrupted HlgCB pore formation[34], indicating a role of sialylated lipids in HlgCB toxicity. We confirmed these findings as our HlgCB-, but also PVL-resistance screen identified *CMAS* and *SLC35A1*, genes encoding proteins in the sialylation pathway, to be involved in leukocidin cytotoxicity. Knocking out either *CMAS* or *SLC35A1* resulted in improper binding of HlgC and LukS-PV to target cells. This indicates that sialic acid modification of cellular targets might have a conserved role for bacterial toxins in mediating cellular susceptibility, including *S. aureus* leukocidins. However, it remains to be investigated whether sialylation dependent leukocidin susceptibility is limited to the sialylation of their respective GPCRs, or other cell surface moieties. Knocking-out individual genes encoding proteins involved in the sialylation (*CMAS* or *SLC35A1*) or sulfation pathway (*SLC35B2*, *PAPSS1* or *TPST2*) resulted in the disruption of the sialylation and sulfation PTM in U937-cell lines. This suggests that these proteins exist as part of a non-redundant sequential sulfation or sialylation processes. The mechanisms for predisposition of otherwise healthy individuals to severe infections with *S. aureus* are poorly understood [35, 36]. Human genetic factors concerning PTMs might account for an unfavorable outcome [37, 38]. Variations in the genes encoding PTM pathways may provide insight into observed differences in susceptibility of humans to infections with *S. aureus.*

Materials and methods

Cell lines and constructs

U937 human monocytic cells were obtained from ATCC (American Type Culture Collection) and cultured in RPMI supplemented with penicillin/streptomycin and 10% fetal calf serum. First, human C5aR1 (CD88; NM_001736) and CXCR2 (CD182; NM_001168298.1) were stably expressed in U937 cells using a lentiviral expression system (U937-hC5aR1, U937-hCXCR2 cells). Briefly, We cloned the human C5aR1 and CXCR2 cDNA in a dual promoter lentiviral vector (BIC-PGK-Zeo-T2a-mAmetrine; RP172), derived from no.2025.pCCLsin.PPT.pA.CTE.4x-scrT.eGFP.mCMV.hPGK.NG-FR.pre as described elsewhere [39]. The transfection of 293T cells with the hC5aR1 and hCXCR2 lentiviral expression systems, and subsequent transduction of U937-cells were performed as previously described [40]. To allow screening, a codon-optimized nuclear-localized *S. pyogenes* Cas9 gene was subsequently transduced in U937-hC5aR1 as described elsewhere [40]. The genome-scale sgRNA CRISPR-Cas9 library was designed as previously described [40]. To generate *SLC35B2*, *PAPSS1*, *TPST2*, *SLC35A1* and *CMAS* knock-out cells, crRNA sequences were cloned in sgLenti (Supplementary Table 1). U937-hC5aR1-SpCas9 and U937-hCXCR2-SpCas9 cells were transduced with the sgRNA-expression viruses and selected to purity by puromycin treatment (2 µg/mL) to enrich for knocked out cells.

Genome-wide CRISPR/Cas9 library screen with PVL in U937-hC5aR1 cells

Genome-wide CRISPR-Cas9 screen for PVL and HlgCB resistance was performed as described previously [40].Briefly, cells transduced with CRISPR-library were selected to purity with 2.0 µg/mL puromycin initiated at two days post transduction. Twelve days post transduction, $2*10^8$ cells were incubated with 15 nM PVL or 15 nM HlgCB for 30 min at 37°C, which resulted in depletion of >99.5% of the cells. Cells were washed to remove the toxin and allowed to recover in complete RPMI for 15 days to enrich for viable cells. Genomic DNA was isolated and sgRNA inserts were subsequently PCR amplified for 16 cycles with primers 5'-GGCTTGGATTTCTATAACTTCGTATAGCA-3' and 5'-CGGGGACTGTGGGCGATGTG-3' using the Titanium Taq PCR kit (Clontech). The PCR products were pooled and amplified using primers containing Illumina adapter sequences and a unique index. PCR products were subsequently pooled in equimolar ratios and subjected to deep-sequencing using the Illumina NextSeq500 platform. Sequences were aligned to the sgRNA library by using Bowtie2 (PMID: 22388286) and the counts

per sgRNA were calculated. We used the MaGeCk package (PMID: 25476604) (available from https://sourceforge.net/projects/mageck/) as a computational tool to identify genes significantly enriched in the screens by comparing sgRNA read counts of control cells versus PVL- and HlgCB incubated cells.

Toxin production and cell permeability assays

LukS-PV, LukF-PV, HlgC, HlgA, HlgB, LukE and LukD used during this study were cloned and expressed as described elsewhere [14, 41, 42]. U937 cells were exposed to canonical recombinant proteins and measured for 30 min at 37°C in a monochromator-based microplate reader (FLUOstar Omega, BMG Labtech) in the presence of 2,5 µg/mL 4',6-diamidino-2-phenylindole (DAPI, Moleculair Probes/Thermo Fisher) to determine pore-formation. As PVL, HlgCB, HlgAB and LukED are two-component toxins, equimolar concentrations of polyhistidine-tagged S- and F-components were used. Pore formation was defined as a collective positive DAPI signal and the area under the curve was calculated for comparison.

Binding assays

Binding of proteins was measured by incubating cells with single 10µg/ml his-tagged S-components on ice, followed by anti-his-FITC (LifeSpan BioSciences).

Determination of receptor expression levels

Receptor expression levels were determined as described elsewhere [42]. Single cell suspensions were stained with mouse anti-human C5aR1 (Sulfation independent clone S5/1, AbD Serotec), mouse anti-human C5aR1 (Sulfation dependent clone 347214, RD) mouse anti-human CXCR2 (clone 6D499, Abnova), mouse-anti-sulfotyrosine (Clone Sulfo-1C-A2, EMD Millipore), mouse-anti-hCD15s (Clone CLSLEX1, BD Pharmingen), or isotype controls, followed by FITC-conjugated goat-anti mouse antibody (Dako) or APC-conjugated goat-anti mouse antibody (Jackson Immunoresearch). For validation of receptor expression after complementation, cells were incubated with PE-labeled mouse anti-human C5aR1 (clone S5/1, AbD Serotec), PE-labeled mouse anti-human CXCR2 (Clone 48311, R&D), Samples were subsequently measured using flow cytometry.

Statistical analyses

Calculations of the area under the curves, calculations of half-maximal effective lytic concentrations using linear regression analyses, and all statistical analyses were performed using Prism 7.02 (GraphPad Software). Flow cytometric analyses were performed with FlowJo (Tree Star Software). Statistical significance was calculated using Student's *t*-test with posttest correction for multiple comparison, where appropriate.

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CHAPTER FOUR

TARGETING THE HUMAN C5A-RECEPTOR: FROM VIRULENCE TO THERAPY

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Abstract

Staphylococcus aureus secretes an arsenal of virulence factors that target the human immune system. Some of these virulence factors interact with specific receptors present on human neutrophils. The secreted Chemotaxis Inhibitory Protein of *Staphylococcus aureus* (CHIPS), binds to the human complement component 5a receptor (C5aR), blocking the interaction with C5a and impeding neutrophil migration towards the site of infection. We hypothesized that CHIPS could be exploited to treat C5aR1 mediated diseases in humans. We show that administration of CHIPS in human C5aR1 knock-in mice successfully dampens C5a mediated neutrophil migration induced by a type 3 hypersensitivity reaction. We initiated a small phase-I trial to evaluate the potential use of CHIPS in human subjects. Even though CHIPS toxicology studies in animal models were successful, healthy human volunteers showed adverse effects directly after CHIPS administration. Subjects showed mild leukocytopenia and increased C-reactive protein concentrations, suggesting an inflammatory response. This is believed to be related to the presence of circulating anti-CHIPS antibodies in humans. The use of staphylococcal proteins, or other bacterial proteins, as therapeutics or immune-modulators in humans is possibly hampered by pre-existing circulating antibodies. Our data show the therapeutic potential of CHIPS as an anti-inflammatory agent in C5aR mediated diseases. Despite the unexpected safety issues in human subjects that temper the use of CHIPS in its current form as a therapeutic candidate, valuable pharmacokinetic and biological information was acquired from this pilot study.

Introduction

The complement system is a major effector mechanism in the humoral immune defense [1, 2]. The complement system is comprised out of more than 35 proteins that are present in host plasma and on cell surfaces. After activation of the complement system via either the classical, lectin or alternative pathway, a cascade of proteolytic events results in the release of multiple cleaved fragments of complement components [3, 4]. The complement component C5a, a 74 amino-acid cleaved product of complement C5, is a powerful anaphylatoxin as well as a potent mediator of chemotaxis that interacts with the C5a-receptor 1(C5aR1). This receptor belong to the family of seven-transmembrane G-protein coupled receptors (GPCR), that is expressed on a wide variety of inflammatory cells [5-7]. The interaction between C5a and C5aR1 results in the recruitment of phagocytes to the site of complement activation, linking the humoral immunity with the cellular innate responses [8].

It has become clear that C5aR1 is also involved in a variety of other inflammatory processes. Upregulation of C5aR1 on internal organs during the onset of sepsis, together with the excessive release of C5a, was proposed to lead to multi organ failure and death in rats [9, 10]. Blockade of C5aR1 with polyclonal anti-C5aR1 antibodies was protective and increased survival in animal sepsis model [9]. C5a and C5aR1 have also been described to be involved in disease processes such as ischemia-reperfusion injury, rheumatoid arthritis, asthma, immune complex diseases, neurodegeneration and Alzheimer’s disease [11-14]. Targeting of the C5aR1 has also shown to be beneficial in some of these disease processes in animals [15-19]. Furthermore, inhibiting the proteolytic cleavage of C5 to C5a with Eculizumab, a monoclonal anti-C5 antibody, is used for the treatment of paroxysmal nocturnal haemoglobinuria, further emphasizing the relevance of the C5aR1 as a therapeutic target [20-22].

Staphylococcus aureus is a common commensal as well as a human pathogen that causes a variety of diseases, ranging from superficial skin and soft tissue infections to severe invasive infections with a poor prognoses and high mortality [23]. Upon infection, *S. aureus* is faced with the host humoral and cellular innate immune responses resulting in the activation of phagocytes to contain the infection [4, 24]. In return however, *S. aureus* has an arsenal of secreted virulence factors to evade the immune system [24, 25]. One of these virulence

factors is the Chemotaxis Inhibitory Protein of *Staphylococcus aureus* (CHIPS). CHIPS binds to the N-terminus of human C5aR1 with high affinity (K_d=1.1nM) and functionally blocks the interaction with C5a, thus preventing activation and antagonizing chemotaxis [26-28]. These properties of CHIPS to inhibit the human C5aR1 with high specificity and affinity makes it a promising candidate as an anti-inflammatory drug in diseases in which C5aR1 stimulation plays an important role. Previous studies have shown that the antagonistic activity of CHIPS on mouse C5aR1 is 30-fold lower compared to human C5aR1 expressing cells [26]. This human specificity of CHIPS has hampered the assessment of CHIPS *in vivo* during inflammation and infection.

Here, we report the application of a transgenic human C5aR1 knock-in mouse (hC5aR1^{KI}) to assess CHIPS as a potential anti-inflammatory compound in C5aR1-mediated diseases. Furthermore, we investigate the safety and efficacy of CHIPS in a phase-I, randomized, double blind, placebo-controlled study.

Results and discussion

CHIPS binds hC5aR1^{KI} murine neutrophils and inhibits stimulation by murine C5a

In order to validate the suitability of our hC5aR1^{KI} mouse [29] as a model to evaluate CHIPS *in vivo*, we first assessed the activity of CHIPS on hC5aR1^{KI} murine neutrophils. To this end, the binding of CHIPS on bone-marrow derived hC5aR1^{KI} murine neutrophils was determined and compared to human neutrophils isolated from peripheral blood. We confirmed that CHIPS binds to hC5aR1^{KI} murine neutrophils at comparable levels as human neutrophils (Figure 1a). To further assess the activity of CHIPS, inhibition of hC5aR1 was assessed on human and hC5aR1^{KI} murine neutrophils. Wild-type (*wt*) murine neutrophils respond normally to mC5a but CHIPS is ineffective in inhibiting mC5a-mediated Ca-mobilization on these mC5aR expressing cells (Figure 1b). Correspondingly, CHIPS inhibition of mC5a mediated Ca-mobilization of bone-marrow derived hC5aR1^{KI} neutrophils reflected that of human neutrophils isolated from peripheral blood (Figure 1b). Hereby, we confirm the binding and inhibition of hC5aR1^{KI} murine neutrophils by CHIPS, proving that our hC5aR1^{KI} mouse is a suitable model to assess CHIPS activity *in vivo*.

CHIPS inhibits C5aR mediated neutrophil migration in vivo

To assess the in vivo therapeutic potency of CHIPS, the immune complex-mediated Arthus reaction model [30, 31] was used in hC5aR1KI mice. The resulting inflammatory response and neutrophil recruitment in the Arthus reaction is mainly C5a mediated. By simultaneously administering ovalbumin intravenous (i.v.) and rabbit anti-ovalbumin IgG intraperitoneal (i.p.), an immune complex mediated type 3 hypersensitivity reaction is induced that leads to the generation of C5a [30, 31]. An Arthus reaction was successfully induced in hC5aR1KI mice as reflected by the influx of neutrophils to the peritoneal cavity (Figure 2a). Administration of CHIPS reduced the number of neutrophils recovered from the peritoneal cavity of hC5aR1KI mice (Figure 2a). Some mice that received CHIPS showed suboptimal inhibition of neutrophil migration, whereas a single mouse showed no evident decrease in neutrophils recovered compared to untreated mice (Figure 2a).

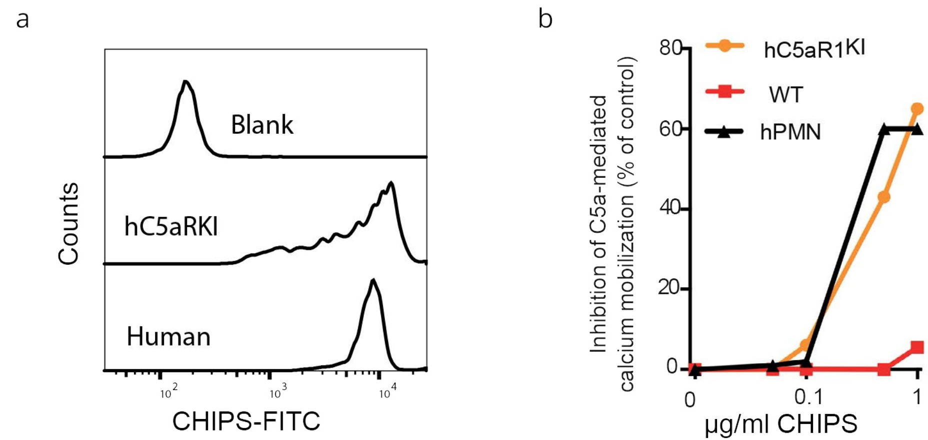

Figure 1: CHIPS binds and inhibits hC5aR[KI] murine neutrophils comparable to human neutrophils. Quantification of hC5aR1 expression in hC5aR1[KI] mice showed similar expression levels compared to human leukocytes[29]. Furthermore, hC5aR1[KI] murine neutrophils responded normally to both murine C5a (mC5a) and human C5a as measured by calcium mobilization[29]. (a) hC5aR[KI] bone marrow neutrophils and human blood neutrophils were isolated and incubated with 3µg/ml his-tagged CHIPS followed by anti-his-FITC antibodies. Cells were analysed by flow cytometry and the FITC fluorescent signal depicted as histograms. (b) As our hC5aR1[KI] murine model generates mC5a, the assessment of CHIPS inhibition was performed by mC5a stimulation. Bone marrow neutrophils of hC5aR[KI] , wild-type mice and human neutrophils were pre-incubated with CHIPS at the indicated concentration and subsequently stimulated with murine C5a (10-8M). The basal fluorescence level was first measured for each sample before the addition of murine C5a. The C5a-mediated calcium influx was analysed by flow cytometry using FLuo-4AM. The average FLuo-4AM fluorescent signal was used to calculate CHIPS mediated inhibition of C5a responses. One experiment representative of two independent experiments is shown.

As *S. aureus* also colonizes rabbits [32], it is possible that the rabbit anti-ovalbumin IgG fraction used to induce formation of immune complexes also contains specific antibodies against CHIPS with potentially neutralizing capacities. To this end, we determined the presence of anti-CHIPS antibodies in the rabbit anti-ovalbumin IgG used. Although the rabbit IgG fraction did contain very low levels of anti-CHIPS antibodies (Figure 2b), the presence of these anti-CHIPS antibodies only slightly neutralized CHIPS *in vitro* and evidently did not neutralize CHIPS *in vivo* (Figure 2c, a). Taken together, our investigations demonstrate the therapeutic potential of CHIPS by inhibiting C5a-mediated neutrophil migration *in vivo* in hC5aR1KI mice after inducing an Arthus reaction.

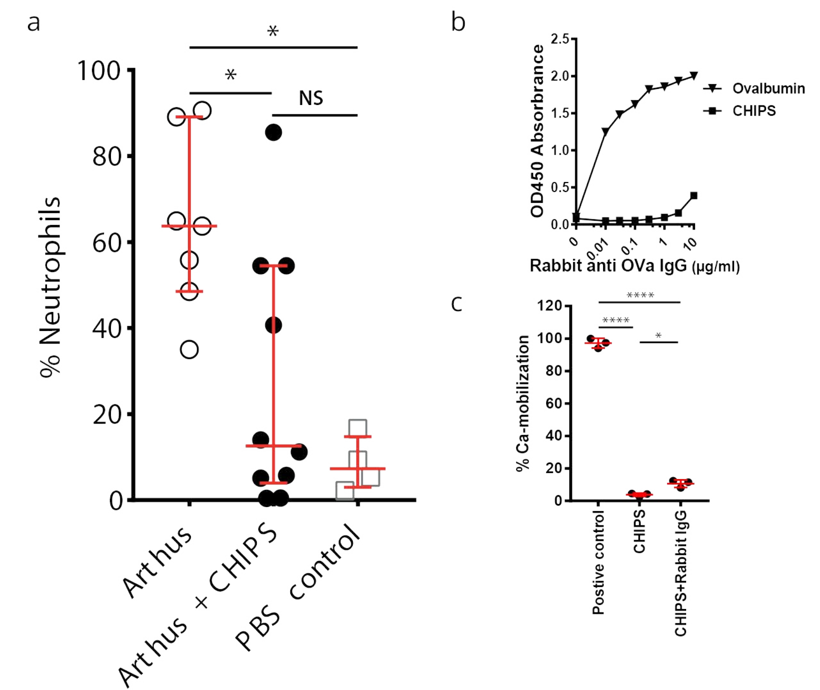

Figure 2: CHIPS inhibits neutrophil migration in vivo. (a) 60µg CHIPS (n=10) was injected i.p., and together with ovalbumin i.v. in hC5aRKI mice 30 minutes prior to inducing the Arthus reaction. Samples were compared to mice that did not receive CHIPS (n=7). Control mice (n=4) received PBS i.v. and i.p.. Peritoneal cavity lavage was performed 6-hours post Arthus induction. Percentage neutrophil influx was analysed by flow cytometry by gating on CD45+GR-1+F4/80- population and depicted as percentage of total leukocytes (CD45+) retrieved after peritoneal lavage. All groups consisted evenly out of female and male mice. Combined data from 2 independent experiments shown. (b) the presence of anti-OVA and anti-CHIPS antibodies in the rabbit anti-OVA IgG fraction was determined by ELISA. (c) To detect neutralizing anti-CHIPS antibodies in the rabbit anti-OVA IgG, CHIPS (500ng/ml) was incubated with 10µg/ml rabbit anti-OVA IgG or PBS. Subsequently, Fluo-4AM labelled human PMNs were incubated with CHIPS/Rabbit IgG or CHIPS/PBS and challenged with human C5a. Ca-mobilization was determined via flow cytometry and normalized to human PMNs that did not receive CHIPS. Significance was calculated using ANOVA, and when needed, followed by Kruskal-Wallis post-test for multiple comparison and displayed as *P<0.05,****P<0.0001 and NS for not significant.

CHIPS in human volunteers

S. aureus is commonly present as a commensal bacterium in humans and the chp gene is present in the majority of *S. aureus* strains. Consequently most, if not all humans, carry pre-existing anti-CHIPS antibodies [33-35]. These anti-CHIPS antibodies present in human sera have been shown to interfere with CHIPS function *in vitro* [33]. As a consequence, the presence of anti-CHIPS antibodies could neutralize CHIPS or induce an antibody-mediated immune reaction *in vivo*, hampering CHIPS function. To limit these undesired effects *in vivo*, only subjects with low anti-CHIPS titers were included in the study (antibody titer ≤ 3.92, as part of the exclusion criteria). To this end, we determined anti-CHIPS antibody titers in study subjects prior to receiving CHIPS. As expected, the 6 trail subjects have pre-existing anti-CHIPS antibodies (Figure 3a). To have an indication how subject titers relate to the general population, anti-CHIPS IgG titers were determined in sera collected from 168 human volunteers. As expected, anti-CHIPS IgG is detected in all 168 volunteers, resembling a Gaussian distribution [33] (Figure 3a). Accordingly, anti-CHIPS IgG titers from subjects were within the normal range of tested sera, representative of the anti-CHIPS IgG titers of the general population (Figure 3a). The anti-CHIPS antibody titers in subjects were considered low enough to not affect the safety assessment of CHIPS.

To further assess the safety of CHIPS, pre-clinical safety experiments were conducted in non-human subjects, prior to administration in humans. In all the animal toxicology studies, we did not observe any CHIPS-related toxicologically significant changes in clinical observations, body weight, food consumption, haematology, coagulation, blood chemistry parameters, ophthalmoscopy, electrocardiograms, macroscopic or microscopic pathology or behaviour (full pre-clinical assessment disclosed in Supplementary Text 1). Notably, a transient decrease in mean arterial blood pressure (40%) was observed in beagles receiving a high dose of 20 mg kg^{-1} CHIPS (Supplementary Text 1). However, mean arterial blood pressure returned to normal within five minutes post dosing. In all, these results suggest that side effects induced by CHIPS are unlikely to be observed in human subjects. As a result, the safety of CHIPS was subsequently studied in a set of six human subjects during a phase-1 clinical study.

Based on the toxicology studies, the administration of a single low dose of 0.1 mg kg^{-1} CHIPS was considered safe and administered in four human subjects. First, we determined the presence of CHIPS in sera of the volunteers during different

time-points post-CHIPS administration. In only two out of four subjects that received the CHIPS protein, subjects #104 and #105, CHIPS could be detected 15 min post-i.v. injection with a gradual decline after 1 hour (Figure 3b). CHIPS was not detected in the sera of subjects #103 and #106 (Figure 3b). These observed differences in the detection of CHIPS in blood of the subjects seems to correlate with their initial level of anti-CHIPS antibodies.

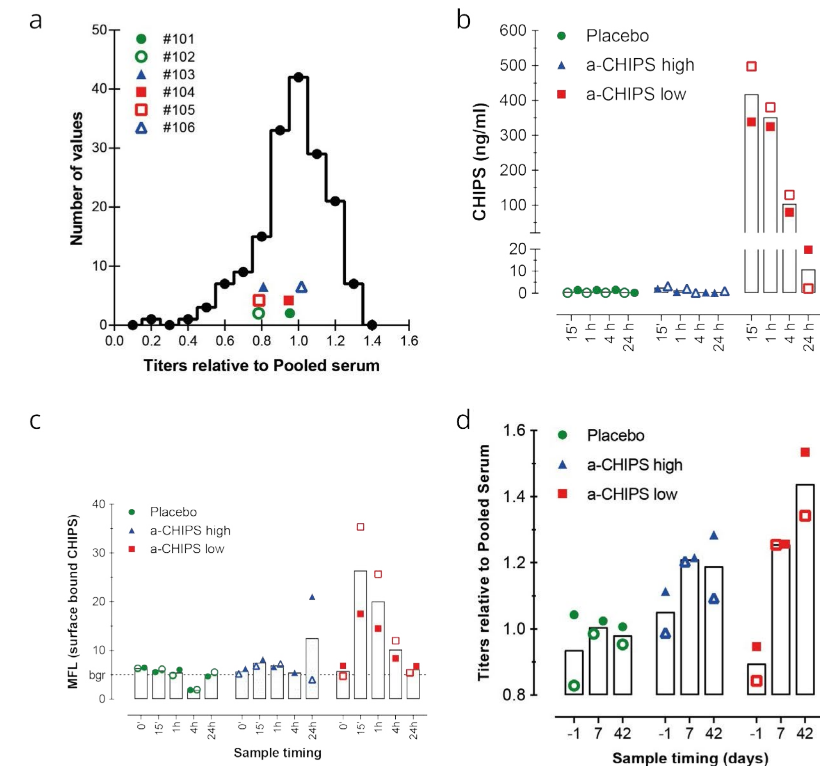

Figure 3: CHIPS and anti-CHIPS antibodies in humans. (a) Frequency distribution of IgG anti-CHIPS titer in healthy human donors (n=168). The titer was defined as the log dilution that gives an absorbance of OD0.300 after subtraction of background value. Titers were depicted relative to the mean human pooled serum (HPS) titer (3.75). Anti-CHIPS antibody titer of the 6 subjects before study entry are depicted in the same graph as comparison. The ■ represents subjects that had low anti-CHIPS antibodies (anti-CHIPS low), ▲ represents subjects with high anti-CHIPS antibodies (anti-CHIPS high) and the ● represents subjects in the placebo group. Open and closed symbols differentiate between receivers in each group. (b) Pharmacodynamics of CHIPS detected in the sera of the volunteers. CHIPS was measured by a specific capture ELISA at various time points after intravenous injection of CHIPS. (c) CHIPS is recovered on the surface of peripheral blood neutrophils. At various time points after i.v. injection, the presence of CHIPS bound to the surface of neutrophils was detected with rabbit-anti-CHIPS antibodies. Values are expressed as mean fluorescence (MFL) of gated neutrophils in EDTA whole blood samples. Background MFL value for the secondary FITC labelled conjugated was 6. (d) Immunogenicity of CHIPS in healthy human subjects. Specific IgG titer towards CHIPS were determined in all subjects before trial start, 7 and 42 days after trial closing and are depicted relative to HPS.

We hypothesized that the higher anti-CHIPS antibody titers hamper the detection of CHIPS by ELISA. Possibly, the epitope recognized by the capture monoclonal anti-CHIPS antibody is occupied by anti-CHIPS antibodies of the subjects. Consequently, we divided the four volunteers in two separate groups based on their anti-CHIPS antibody titer; anti-CHIPS low (subjects #104 and #105) and anti-CHIPS High (subjects #103 and #106). The measured CHIPS serum concentration in subjects #104 and #105 are also potentially an underestimation due to the interference of pre-existing anti-CHIPS antibodies. In addition, for subjects #104 and #105 that had detectable levels of CHIPS 15 min post i.v. injection, CHIPS concentrations dropped a 2-log fold over the course of 24 hours (Figure 3b). These data show that CHIPS is taken up systemic within 15 min and cleared after 24 hours post i.v. administration. We calculated a predicted half-life of CHIPS to be at least 1.5 hours in humans. CHIPS binds the C5aR1 on human neutrophils with high affinity *ex vivo* [26]. However, *in vivo* binding of CHIPS could be hampered by circulating antibodies. In order to assess if CHIPS interacts with its therapeutic target, we determined the binding of CHIPS *in vivo* on neutrophils of the subjects. The amount of CHIPS present on the surface of neutrophils was determined at various time points post-CHIPS administration using a rabbit-anti-CHIPS antibody [36]. Notably, the binding of CHIPS on the surface of neutrophils was only detected in subjects with a low anti-CHIPS antibody titer (subjects #104 and #105) (Figure 3c). It is possible that the anti-CHIPS antibodies present in serum also interfere with the direct detection of CHIPS on neutrophils. Therefore, the lack of a direct detection cannot exclude the absence nor presence of CHIPS bound to the receptor in the individuals with high anti-CHIPS antibody titers. All in all, we show that CHIPS binds circulating human blood neutrophils, confirming the interaction with target cells *in vivo*.

All tested subject had pre-existing anti-CHIPS antibodies. As a specific antibody response is mediated against CHIPS, it is likely that a re-challenge with CHIPS will lead to an increase in antibody titers. To determine the immunogenicity of CHIPS, anti-CHIPS serum titers were measured during different time points pre- and post-CHIPS administration. An increase in anti-CHIPS titer was observed in individuals receiving CHIPS that had a low anti-CHIPS antibody titer (subjects #104 and #105) pre-CHIPS administration (Figure 3d).The rapid boost of circulating IgG titers by the staphylococcal protein CHIPS in humans indicates high immunogenicity and pre-existing memory, supporting a concept of expected exposure to secreted staphylococcal proteins starting at early age [34, 35, 37].

CHIPS induced adverse effects in humans

The administration of CHIPS in human subjects was tolerated by two subjects (subjects #103 and #104), moderately tolerated in subject #105 but subject #106 (subject with a high anti-CHIPS antibody titer) developed serious symptoms directly after the CHIPS infusion, which was diagnosed as an anaphylactic reaction (Supplementary Text 2). No adverse events were reported in subjects receiving placebo. To determine whether the subjects developed a CHIPS-mediated inflammation response, white blood cell count (WBC) and C-reactive protein concentration (CRP) were measured pre- and post-dosing. CHIPS induced a transient leukocytopenia in the subjects receiving CHIPS that resolved within two days (Figure 4a). Within the group of subjects that received CHIPS there was a mild increase in CRP (average of 42 mg ml-1) at day 2 post CHIPS dose compared to controls. CRP levels returned to normal when subjects were screened during follow up at day 15 (Figure 4b). This indicates that there was indeed an inflammation response upon CHIPS administration.

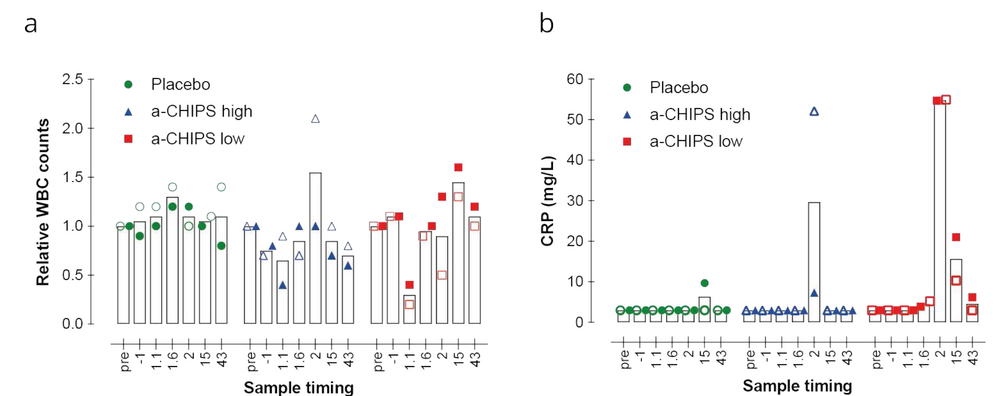

Figure 4: CHIPS induces leukocytopenia and increased CRP levels in humans. Levels of circulating (a) peripheral white blood cells and (b) serum inflammation marker CRP. At various time points after intravenous injection of CHIPS, WBC counts and CRP measurements were performed. (1.1 and 1.6 indicate 1 day and 1 or 6 hours respectively). Data for WBC are expressed relative to the value at T = 0 and data for CRP are expressed in mg/mL. The ■ represents subjects that had low anti-CHIPS antibodies (anti-CHIPS low), ▲ represents subjects with high anti-CHIPS antibodies (anti-CHIPS high) and the ● represents subjects in the placebo group. Open and closed symbols differentiate between receivers in each group.

Circulating immune complexes and increased serum tryptase

Mast cells play a central role in anaphylaxis and other allergic conditions. Immune complexes can activate mast cells by FcR crosslinking and through activation of complement and the generation of C5a [38]. Circulating immune complexes (CIC) induce the abundant secretion of the serine proteinase tryptase by mast cells, which can be used as an indicator of anaphylaxis. Since all subjects had pre-existing anti-CHIPS antibodies, we evaluated whether intravenous administration of CHIPS leads to the formation of CIC. Circulating immune complexes were detected in the subjects receiving intravenous CHIPS (Figure 5a). Subject #106, who suffered an anaphylactic reaction following the administration of CHIPS, showed the highest CIC levels, contrary to subjects #104 and #105 who remained at baseline. CIC were also detected in subject #103, who has the highest anti-CHIPS antibody titer but reported only minor adverse effects. No CIC were detected in subjects that received the placebo.

Subsequently, we measured the serum tryptase levels in the subjects. An increase in serum tryptase concentration was detected in all subjects except subject #103, that reached a maximum at approximately 10 minutes post dose and continued to drop to baseline levels after 24h (Figure 5b). Notably, subject #106 had the highest levels of tryptase, which correlates with the high levels of CIC measured. These data suggest that CHIPS administration in subjects with high circulating anti-CHIPS titers results in an inflammatory response and adverse effects. Due to these effects, the study was stopped and no further administrations of CHIPS was undertaken. Earlier studies showed that CHIPS does not bind other cells than those expressing the C5aR and there is no evidence of direct cell activation by CHIPS [27, 28, 33]. The development of a second-generation CHIPS protein with a preserved activity but with reduced immunogenic properties could make a promising new candidate anti-inflammatory drug. Mapping of the epitopes for human IgG within the CHIPS protein will be an important first step in this development [39]. We previously identified several unique conformational epitopes on CHIPS using affinity purified human IgG from a preparation for intravenous use [39]. Despite developing a version of CHIPS with low interaction with pre-existing anti-CHIPS antibodies, the high immunogenicity of CHIPS will limit it suitable for therapies requiring a single administration. Despite the neutralizing effect of anti-CHIPS antibodies, we were able to detect significant serum concentrations of the CHIPS protein. The observation of the inhibition of this activity by anti-CHIPS antibodies together with the induction of an anaphylactic response shows that CHIPS in its current form is not suitable for use as an anti-inflammatory agent. Nevertheless, future molecules based on the CHIPS protein could very well be potential new candidates. Knowledge of the exact mechanisms of action and the active sites within the CHIPS molecule can lead to the development of small molecule anti-inflammatory drugs based on the CHIPS mechanism of action.

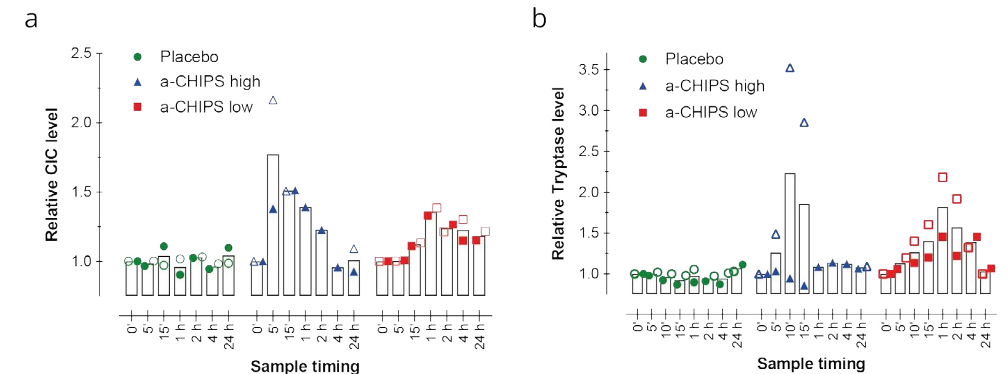

Figure 5: Circulating immune complexes and increase serum tryptase. Adverse effects of CHIPS as measured by levels of a) Circulating Immune Complexes (CIC), and b) mast cell marker tryptase. At various time points after intravenous injection of CHIPS, specific assays were performed for both markers. The ■ represents subjects that had low anti-CHIPS antibodies (anti-CHIPS low), ▲ represents subjects with high anti-CHIPS antibodies (anti-CHIPS high) and the ● represents subjects in the placebo group. Open and closed symbols differentiate between receivers in each group.

04

Materials and Methods

Ethics statement

The RCT study protocol (JPD-003/002/NL) and amendments were approved by an independent ethics committee. The study was performed in compliance with the 'Declaration of Helsinki' (Scotland, October 2000) and OECD Principles of Good Laboratory Practice and applicable regulatory regulations. For neutrophil isolation approval was obtained from the medical ethics committee of the University Medical Center Utrecht (METC-protocol 07-125/C approved March 01, 2010; Utrecht, The Netherlands). The use of animals was approved by the National Ethical Committee for Animal Experiments and performed according to the guidelines of the Central Animal Facility of the Utrecht University (Project# AVD115002016565).

Isolation of Rabbit anti-ovalbumin IgG

IgG was purified from Rabbit anti-Chicken-Egg Albumin, delipidized whole antiserum (Sigma-Aldrich) using multiple runs over a 1 ml Protein-A HiTrap column (GE Healthcare Life Sciences) on an ÄKTA FPLC (GE Healthcare Life Sciences). Rabbit IgG was eluted from the column with 0.1 M citric acid, pH 3.0 and collected fractions were neutralized with 1M Tris-HCl, pooled and dialyzed against PBS. Protein concentration was determined at 280 nm using a molar extinction coefficient of 1.35 for Rabbit IgG.

Peritoneal Arthus reaction and neutrophil migration

Human C5aR1^{KI} mice were generated and characterized as described elsewhere [29, 40]. The Arthus reaction was initiated upon i.v. injection in hC5aR1^{KI} mice (male and female) of 100 µl of OVA (20 mg kg^{-1} of body weight; Sigma-Aldrich) immediately followed by an i.p. injection of 800 µg of rabbit anti-OVA IgG (Sigma) in 500 µl PBS. For mice in the CHIPS group, 60 µg CHIPS was administered i.p. 30 minutes prior to initiation of the Arthus reaction and simultaneously with OVA i.v.. For the control group, PBS was administered i.v. and i.p.. Mice were euthanized by CO_2 suffocation 6-hours after the onset of the peritoneal Arthus reaction and the peritoneal cavity washed with two times 5 ml of ice-cold RPMI 0.1% HSA/5mM EDTA. Peritoneal fluid was recovered and centrifuged at 1200 rpm for 10 min to collect the exudate cells. Cell pellets were resuspended in 500 µl buffer and counted with trypan blue in a TC20 automated cell counter (BioRad). Cells were stained in the presence of a Fcy-receptor blocker, with anti-mouse CD45-APC (clone 30-F11, BD Biosciences), anti-mouse Gr1-PE (1A8,

BD Biosciences), anti-mouse F4/80 FITC (BM8, eBioscience), anti-human C5aR-FITC (clone S5/1, SeroTec), isotype rat-IgG2a-FITC (R&D) and rat-IgG2b-PE (BD Biosciences). Samples were analyzed by flow cytometry. Collected peritoneal cells were washed with PBS and the cell number adjusted to 5×10^6 cell ml^{-1}. Cytospin slides were prepared with 50 µl 5×10^4 cell suspension and stained with DiffQuick. The percentage of neutrophils was determined by flow cytometry analysis and confirmed by the number of neutrophils based on morphology following DiffQuick staining. Mouse neutrophils were isolated from bone-marrow as described elsewhere [41, 42]. Briefly, bone marrow cells were collected by flushing the femurs and tibias with 10 ml of cold HBSS + 15 mM EDTA + 30 mM Hepes + 0.1 % HSA. A two-layer Percoll density gradient (2 ml each in PBS) composed of 81% and 62.5% was used to enrich neutrophils from the total leucocyte population. Interphase between 62.5% and 81% was collected. Cells were washed once with buffer and resuspended in PRMI1640 with 0.1% HSA. Staining of bone marrow cells was performed as described above.

Preclinical assessment of CHIPS toxicity in animal models

Conventional pre-clinical toxicology studies were preformed to investigate the safety of intravenous CHIPS. These included; (I)The effects of CHIPS on various cardiovascular and respiratory parameters in one group of three anesthetized beagle dogs. The dogs were administered CHIPS in incremental doses of 0.2, 2.0 and 20 mg kg^{-1} , infused intravenously over one minute at approximately 30 minute intervals. (II) Behavioral ('Irwin') test in mice: CHIPS was administered as a single intravenous injection to male ICR CD-1 mice (three per group) at doses of 7.5, 25 and 75 mg kg^{-1} in order to assess effects on general behavior. An additional group received an equivalent volume (10 ml kg^{-1}) of vehicle (0.9% w/v sterile saline). (III) Acute intravenous toxicity study in rats: Intravenous administration of 96.1 mg·kg^{-1} CHIPS as a single dose (the maximum practically achievable due to volume considerations) to five male and five female rats. (IV) Acute intravenous toxicity in mice: Intravenous administration of 96.1 mg kg^{-1} CHIPS as a single dose to five male and five female mice. (V) Seven day intravenous bolus preliminary toxicity study in rats (24 males and 24 females, maximum dose 10 mg kg^{-1}). (VI) Seven day intravenous bolus toxicity study in rats (76 males and 76 females, maximum dose 10 mg kg^{-1}). (VII) Seven day intravenous bolus dose range finding study in dogs (two males and two females, maximum dose 20 mg kg^{-1}). (VIII) Seven day intravenous bolus toxicity study in the dogs (12 males and 12 females, maximum dose 20 mg kg^{-1}).

Inclusion of human volunteers

Full description of study population, including number of subjects, inclusion, exclusion and removal criteria are described in supplementary Protocol No.: JPD-003/002/NL. Briefly, inclusion criteria for healthy volunteers were as follows: (I) Adult males within an (II) age range 18-50 and (III) a body mass index (BMI) of 18-30 kg m^{-2}. Medical screening was divided in two parts. Subjects were screened for anti-CHIPS antibody titers. Only subjects with a low titer (equal or lower to 3.92, defined as the log of the serum dilution that gives an absorbance value of 0.300 in the ELISA) were screened for the second part within three weeks before dosing and include: medical history, physical examination, measurement of blood pressure, heart rate, respiration and temperature, alcohol breath test, blood and urine tests, electrocardiogram (ECG) and drug screening.

Admission and follow up

Full description of the admission and follow up, treatments and stopping rules are described in (supplementary Protocol No.: JPD-003/002/NL). Briefly, six selected subjects (four receiving CHIPS and two controls) were admitted to the Clinical Pharmacology Unit (Kendle, Utrecht, The Netherlands) on the day before dosing. Baseline measurements, including blood samples for safety, urinalysis, interim medical history, physical examination, vital signs and ECG were done. On the day of dosing CHIPS (0.1 mg kg^{-1} administered as a single dose of sterile frozen isotonic saline solution containing CHIPS at a concentration of 5 mg ml^{-1}) or placebo (0.9% NaCl) was administered by intravenous infusions over five minutes. Subjects were connected to a telemetry system for cardiac monitoring from 30 minutes before dosing until four hours after start of dosing. The blood pressure of subjects was measured continuously using a Finapres from five minutes before dosing until 30 minutes after start dosing. Vital signs were measured and ECG's were made at certain time points during the admission period. For safety, clinical status and laboratory values (haematology, biochemistry, coagulation and urinalysis) of all subjects were monitored. Adverse events were documented and characterized according to their severity and relationship to CHIPS or placebo. The subjects were discharged at 24 hours after dosing. Two weeks after dosing subjects returned to the Unit for a visit to evaluate vital signs, ECG, blood and urine and anti-CHIPS antibody level. A follow up visit was scheduled six weeks after dosing.

Cloning and expression of CHIPS

CHIPS was cloned and expressed as described earlier [26, 36]. Briefly, the CHIPS gene (*chp*; GenBank: AF285146.1), without the signal sequence, was cloned into the pRSET vector directly downstream the enterokinase cleavage site and before the EcoRI restriction site by overlap extension PCR. Bacteria were lysed with CelLytic B Bacterial Cell Lysis/Extraction Reagent (Sigma) and lysozyme according to the manufacturer's description. The histidine-tagged protein was purified using a nickel column (HiTrap Chelating HP, 5ml, Amersham Biosciences) following the manufacturer's instructions and cleaved afterwards with enterokinase (Invitrogen). Samples were checked for purity and presence of protein using 15% SDS-PAGE (Polyacrylamide gel electrophoresis, Mini Protean 3 System, Bio-Rad) and Coomassie Brilliant Blue (Merck) staining.

Purification of CHIPS for intravenous use

Full-length CHIPS was expressed in *E. coli* containing the coding sequence of CHIPS directly downstream to PelB coding sequence in a growth media consisting of soya peptone and yeast extract in eight liter fermentation media. CHIPS was isolated both from the growth media and the cells by a two stage cation exchange purification process followed by a desalting step. The bacterial cell pellet was resuspended in phosphate buffer (30 mM; pH 7.0), containing NaCl (10 mM), DTT (10 mM) and frozen. This was subsequently thawed at 37°C, incubated on ice and sonicated. After centrifugation at 15,000 rpm, an amber colored 'cell' supernatant was recovered. The supernatant was diluted four-fold with 30 mM phosphate buffer and passed over a Source S-30 column. The material was eluted with a phosphate buffer salt gradient and fractions containing CHIPS were combined and purified further by using a polishing column with a shallow salt gradient. Fractions containing CHIPS with purity greater than 97% (by HPLC) were combined and passed through a Sephadex G 25 desalting column to remove phosphate and excess of sodium chloride. Endotoxin was removed by gently shaking over resin (Biorad) and the preparation was sterilized through ultra-filtration. We confirmed the purity by HPLC-MS on a Microbondapac CN-RP column with a mobile gradient phase consisting of water-TFA to Methanol-TFA. The end product was diluted with sterile saline to the desired concentration and stored at -20ºC.

Isolation of human PMN

Blood obtained from healthy volunteers was collected into tubes containing sodium heparin (Greiner Bio-One) as anticoagulant. Heparinized blood was diluted 1/1 (v/v) with PBS and layered onto a gradient of 10 ml Ficoll (Amersham Biosciences, Uppsala, Sweden) and 12 ml Histopaque (density 1.119 g ml^{-1}; Sigma-Aldrich, St. Louis,MO). After centrifugation (320 g, for 20 min at 22°C), the neutrophils were collected from the Histopaque phase and washed with cold RPMI 1640 medium containing 25 mM HEPES buffer, L-glutamine (Invitrogen Life Technologies) and 0.05% HSA (Sanquin, Amsterdam, the Netherlands). The remaining erythrocytes were lysed for 30 s with ice-cold water, after which concentrated PBS (10 x PBS) was added to restore isotonicity. After washing, cells were counted and resuspended in RPMI-1640/0.05% HSA at 10^7 neutrophils ml^{-1}.

Determining Circulating Immune Complexes, C-Reactive Protein and serum tryptase

CIC were determined by 2 different ELISA's from Quidel (San Diego, CA): the CIC-C1q enzyme immunoassay is based on the principle that complement fixing IC will bind to immobilized human C1q purified protein; the CIC-Raji Cell Replacement enzyme immunoassay measures IC containing C3 activation fragments by using a mAb that specifically binds the iC3b, C3dg and C3d activation fragments of C3 in a manner which is analogous to the classical Raji cell CR2 binding reaction. The data of both assays were combined and results expressed relative to the value at time point 0. CRP levels were determined by the diagnostic department according to standard protocols. Serum derived tryptase (both α-and β-form) was measured on the UniCAP®-100 using the ImmunoCAPTM technology (Pharmacia Diagnostics, Woerden, The Netherlands). The normal geometric mean for serum tryptase in healthy controls is 5.6 µg l^{-1}. Results were expressed relative to the value at time point 0.

ELISA for anti-CHIPS antibodies and CHIPS levels

Rabbits were immunized with recombinant CHIPS using Freund's Complete Adjuvants and boosted with Freund's incomplete adjuvants. Bleedings were checked for reactivity with CHIPS by ELISA as described for human anti-CHIPS antibodies (see below). From the final bleeding, IgG was purified by standard Protein-G (Pharmacia) affinity chromatography according to the manufacturer's instructions. For the anti-CHIPS ELISA, microtiter plates (Greiner) were coated with 50 µL CHIPS per well at 1 µg ml^{-1} in PBS overnight at 4°C. All wash steps were performed thrice with PBS-0.05%Tween-20 and subsequent incubations were done for one hour at 37°C. Plates were blocked with PBS-0.05%Tween-20 4% BSA, washed and incubated with sera or antibodies diluted in PBS-0.05%Tween-20 1% BSA. Bound antibodies were detected with species-specific goat anti-IgG conjugated with peroxidase (all from Southern, Birmingham, USA) and TMB as substrate. The reaction was stopped with H_2SO_4 and the absorbance measured at 450nm in a BioRad ELISA-reader. For the capture ELISA, microtiter plates were coated with 50 µL α-CHIPS mAb 2G8 at 3 µg mL^{-1} in PBS overnight at 4°C. Plates were blocked with PBS-0.05%Tween-20 4% BSA, washed and incubated with diluted samples and a two-fold dilution range of CHIPS as standard in PBS-0.05%Tween-20 4% BSA. Subsequently, plates were incubated with 0.33 µg mL^{-1} rabbit α-CHIPS IgG and 1:5000 diluted peroxidase-conjugated goat anti-rabbit IgG (Southern). Bound antibodies were quantified with TMB as substrate, the reaction stopped with 1 N H_2SO_4 and OD was measured at 450 nm on a BioRad ELISA reader.

Statistical analysis

Calculations of statistical analyses were performed using Prism 7.0 (GraphPad Software). Flow cytometric analyses were performed with FlowJo (Tree Star Software). Significance was calculated using analysis of variance (ANOVA) followed by Kruskal-Wallis as post-test correction for multiple comparison. All statistical methods with regards to the human trails are described in the supplementary (Protocol No.: JPD-003/002/NL.)

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Supplementary Text 1: Pre-clinical assessment of CHIPS as a therapeutic agent

CHIPS successfully dampened the C5aR dependent Arthus reaction in a mouse model expressing the hC5aR. To allow testing of CHIPS in human volunteers, pre-clinical safety experiments in non-human subjects were required . In none of the toxicology animal studies did administration of CHIPS cause any CHIPS related toxicologically significant changes in clinical observations, body weight, food consumption, haematology, coagulation, blood chemistry parameters, ophthalmoscopy, electrocardiograms, macroscopic or microscopic pathology or behavior. The effects of CHIPS on various cardiovascular and respiratory parameters in anesthetized beagle dogs was examined. In the dogs receiving low dose CHIPS (0.02 and 2 mg•kg-1) there was no evidence of cardiovascular or respiratory effects when compared to infusion of vehicle (isotonic saline). Following intravenous administration of 20 mg•kg-1 CHIPS a transient decrease in mean arterial blood pressure (40%) was recorded approximately one minute after start of administration. Mean arterial blood pressure levels returned to pre-dose levels within approximately five minutes following the start of dosing. The effect on blood pressure coincided with transient, inconsistent changes in heart rate. One dog was administered a repeat intravenous dose of CHIPS (20 mg•kg-1) approximately 30 minutes following the first administration of CHIPS. Transient effects on cardiorespiratory parameters similar to those recorded following the first dose were not apparent after the repeat administration of CHIPS. However, the second administration produced a prolonged reduction in mean arterial blood pressure, reaching a maximum of 18% at approximately 30 minutes following the second administration. In this animal only, twelve minutes following the repeated administration of CHIPS a generalized skin reaction appeared consistent with some form of mild allergic reaction. The results of this study suggested that cardiorespiratory effects are unlikely to be observed in the human subjects in the used dose range (0.1 mg•kg-1). Furthermore, any effects that might occur were expected to be transient and reversible.

CHAPTER FIVE

SUMMARIZING DISCUSSION: TARGETING PHAGOCYTES

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Studying *S. aureus* leukocidins: a challenging endeavour

S. aureus is a major bacterial pathogen in humans that, combined with the acquisition of antibiotic resistance, is of serious concern to public health [1-4]. Neutrophils are the first to arrive at the site of infection and subsequently phagocytose and kill *S. aureus* [5-7]. Neutrophils play a crucial role in the containment and clearance of *S. aureus* [5-7]. It is therefore not surprising that many *S. aureus* secreted proteins play a role in circumventing phagocytosis by targeting neutrophils. It has been evident for more than a century that *S. aureus* secretes a particular substance(s) that interacts with and kills leukocytes [8-10]. It took almost seven decades to attribute this leukocidal activity to cytolytic toxins secreted by *S. aureus* [11-13]. *S. aureus* produces β-barrel pore forming toxins, such as the single-component α-Hemolysin (Hla) and multiple bi-component toxins that target the cell membrane resulting in the lysis of host immune cells [14, 15]. It is now clear that all human *S. aureus* isolates are able to produce potent bi-component toxins, better known now as leukocidins, that target and lyse phagocytes. In addition, clinical and epidemiological studies suggest the involvement of the bi-component toxin PVL as an important factor contributing to the epidemic spread and increased virulence of CA-MRSA strains [16, 17]. However, the mechanisms of pore formation and the contribution of PVL and other leukocidins to *S. aureus* pathophysiology are incompletely understood. It was long assumed that leukocidins interact with lipid constituents on target cells [14, 18, 19]. However, lipids alone could not explain the apparent host- and cell-tropism of leukocidins, indicating that there is something more involved in leukocidin targeting of phagocytes [8, 14, 20-22]. The first major breakthrough came in 2010 when a specific proteinaceous receptor, the transmembrane metalloprotease ADAM10, was identified for Hla [23]. This catalysed the identification of specific receptors for all leukocidins [24-29], clarifying the observed species- and cell-specific toxicity of *S. aureus* leukocidins. Murine infection models, to an extent, have proven useful in understanding the role of these leukocidins in *S. aureus* pathogenesis [30, 31]. The leukocidin LukED targets cells of the adaptive immunity via CCR5 and is compatible with murine CCR5 [29]. This made it possible to show the contribution of LukED as it kills cells of the adaptive immune system in mice *in vivo* [29]. In addition, LukED targets neutrophils, monocytes and NK-cells via CXCR1 and CXCR2, which also promote *S. aureus* pathogenesis in mice *in vivo* [27]. Another leukocidin, HlgAB, targets CCR2, CXCR1 and CXCR2. HlgAB interacts with murine inflammatory macrophages via CCR2, as shown in a murine peritonitis model [28]. However,

due to incompatibility between HlgAB and murine CXCR2, current mouse models are not suitable to fully comprehend the extent of HlgAB contribution to *S. aureus* pathogenesis *in vivo* [28]. Other Leukocidins that exert a narrow host-specificity, such as the PFTs HlgCB and PVL that interact with human C5aR1 [24, 26], have been difficult to study *in vivo*. Murine models have failed to demonstrate a role for PVL in *S. aureus* pathogenesis *in vivo*, probably as murine neutrophils are resistant to PVL [32-34]. In contrast, rabbit neutrophils are as susceptible to PVL toxicity as human neutrophils *ex vivo* [35]. Therefore, it was believed that rabbits might serve as a better animal model to study the role of PVL in *S. aureus* pathogenesis *in vivo*. Studies using rabbits as an animal model have shown a modest PVL-mediated affect in skin infections [36], necrotizing pneumonia [35] and early stages of bacteremia [37]. However, most rabbits are likely to have been previously exposed to *S. aureus* [38], resulting in the presence of anti-bodies against *S. aureus* that could influence the adequate assessment of leukocidins and other *S. aureus* virulence factors. The lack of an adequate *in vivo* model additionally hinders the assessment of the apparent redundant deployment of two leukocidins that target the same human C5aR1. The overlapping cell-tropism between leukocidins also remains enigmatic. All in all, the human specific nature of leukocidins has hindered the full assessment and contribution of leukocidins in *S. aureus* pathogenesis *in vivo* [39]. In addition, animals such as mice have been used as preclinical models in *S. aureus* vaccine and drug development, possibly resulting in the neglection of potential relevant targets. The development of humanized mice, either via the engraftment of human haematopoietic stem cells [40] or the transgenic expression of human proteins [41], offers an alternative to investigate human tropic factors such as leukocidins *in vivo*.

Targeting neutrophils via C5aR1 *in vivo*

In **Chapter 2** we describe the development of a transgenic mouse expressing the human C5aR1. This permitted us to study the role of both HlgCB and PVL during infection *in vivo*. HlgCB is present in 99% of *S. aureus* strains and is present in the *S. aureus* core genome [17, 42]. In contrast, PVL is located on the prophage locus ϕSa2 [43-45] and only present in 2-3% of *S. aureus* isolates [17]. However, more than 90% of *S. aureus* strains that cause necrotizing pneumonia carry PVL [16], suggestive of a causative link. A previous study using a rabbit model of necrotizing pneumonia has shown a PVL mediated phenotype *in vivo* [35], however, the epidemiological correlation between PVL and necrotizing pneumonia remains weak and controversial [46]. We first opted for a systemic infection model in our hC5aR1^{KI} mice, as studies previously addressing leukocidins all showed a role for leukocidins during systemic infection [27-29]. We show that HlgCB contributes to increased bacterial loads in multiple organs beyond the primary infection site in hC5aR1^{KI} mice. The increase in bacterial loads could be due to clearance failure at the primary site of infection. However, a possible active role of HlgCB in the dissemination of *S. aureus* cannot be ruled out. Additional experiment would be necessary, specifically assessing the possible role of HlgCB in the escape of *S. aureus* from the peritoneal cavity into the bloodstream. Even though both HlgCB and PVL target human C5aR1 *in vitro*, only a HlgCB-dependent phenotype was observed in our intraperitoneal injection model in hC5aR1^{KI} mice. CA-MRSA and PVL have been discussed and epidemiologically linked to the severity of SSTIs [46]. This prompted us to additionally assess the role of HlgCB and PVL during skin infections in our hC5aR^{KI} mouse. A HlgCB-mediated increase in bacterial loads was observed, suggesting a role of HlgCB in skin infections. However, no PVL mediated phenotype was observed in our skin infection model. This was contrary to previous findings that showed a PVL-mediated increase in skin lesion size when injected subcutaneously in (NOD)/severe combined immune deficiency (SCID)/IL2rγ^{null} (NSG) mice engrafted with human CD34^{+} umbilical cord blood cells [47]. Surprisingly, they did not observe an increase in CFU compared to the PVL^{-} *S. aureus* strains used in their study. A lack of PVL contribution in both systemic and skin infection in our hC5aR1^{KI} mice was attributed to an improper cellular susceptibility, as murine hC5aR^{KI} neutrophils were more resistant to PVL compared to HlgCB. Subsequent hybrid pairing of S-and F-subunits of HlgCB and PVL showed a LukF-PV mediated modulation of hC5aR^{KI} murine neutrophils, but not human neutrophils, to PVL sensitivity. These findings were suggestive of a yet uncharacterized target employed by LukF-PV to engage human target cells.

The identification of the first leukocidin F-component target

It was long speculated whether leukocidins also employ F-components cellular surface targets as part of the initial interaction preceding pore-formation. HlgB, for example, can bind independent of HlgA to human erythrocytes [48], suggesting that a possible primary interaction of the S-component is not necessary in HlgAB mediated pore formation. In addition, a recent study identified an equine specific leukocidin, LukPQ, that showed an F-component mediated host-specific interaction when non-canonically paired with LukED [49]. Until recently, the existence of F-component targets and the role of F-components in

leukocidin pore formation was enigmatic. Using a genome-wide CRIPSR based approach described in **Chapter 2**, we identified CD45 as the extracellular target for LukF-PV. We confirmed that PVL specifically employs human CD45, and not murine CD45, as the target for LukF-PV. Even though the human CD45 specific interaction of LukF-PV likely explains the lack of a PVL mediated phenotype in our hC5aR^{KI} mouse model, the question arises if and how this additional LukF-PV binding to human CD45 prerequisite affects *S. aureus* pathophysiology.

Not all human *S. aureus* isolates carry the *pvl* gene. However, *pvl* carrying MRSA strains are suggested to be more virulent and have been associated with the development of severe necrotizing pneumonia [16, 50]. *S. aureus* induced necrotizing pneumonia is suggested to be PVL, as well as neutrophil mediated [35]. Nonetheless, the mechanisms involved in the onset of necrotizing pneumonia and the inducement of tissue necrosis are incompletely understood. It is possible that *S. aureus* gains access to the alveoli and induces the activation of neutrophils and the release pro-inflammatory mediators, resulting in the recruitment of neutrophils to the infected lung tissue [35, 51-53]. Neutrophils, as they express C5aR1, are subsequently lysed by PVL, releasing proteases and ROS into the surrounding environment, inducing tissue damage of alveolar epithelial and endothelial barriers [52]. C5aR1 was initially thought to be expressed exclusively on cells of myeloid origin. However, studies have shown the expression of C5aR1 on cells of solid organs such as vascular smooth muscle, lung bronchial and alveolar epithelial cells [54]. These cells of non-myeloid origin lack CD45 expression. It is not clear if PVL directly interacts or lyse lung cells via C5aR1 [55, 56]. However, as neutrophils are the primary target, *S. aureus* can designate the pan-leukocyte marker CD45, followed by C5aR1 as a phagocytic marker, forming part of a two-step-control mechanism. This enables *S. aureus* to differentiate between the potentially non-threatening C5aR1 expressing cells and neutrophils. In this simple, but yet effective way of selecting CD45$^+$C5aR1$^+$ cells, *S. aureus* can specifically target neutrophils in an infection environment. The targeting and lysing of neutrophils via a two-step-control mechanism by selecting human CD45 and human C5aR1 expressing cells might also results in the impaired clearance by neutrophils. This could result in unchecked bacterial growth and subsequent tissue damage by other *S. aureus* secreted cytotoxins.

LukS-PV binds human neutrophils and monocytes, but not lymphocytes [21, 24, 57]. LukS-PV, as a single subunit, is non-toxic and able to functionally inhibit C5a mediated activation of human neutrophils *in vitro* [24]. It is not clear whether single PVL subunits contribute to the pathogenesis of *S. aureus*. The pan-leukocyte marker CD45 is a highly conserved transmembrane glycoprotein and abundantly expressed on all nucleated hematopoietic cells [58-60]. As CD45 plays a role in T-cell development, signalling and function [60, 61], it is possible that *S. aureus* might modulate lymphocytes signalling via the binding of LukF-PV to CD45. Furthermore, the function of CD45 in innate immunity and bacterial infections has rarely been assessed and is unknown. LukF-PV might serve as a tool to elucidate the function of CD45 in innate immune responses *in vitro*. However, assessing the contribution of human CD45 in PVL mediated *S. aureus* pathogenesis, or as a single LukF-PV subunit *in vivo,* would be challenging. The generation of a double hC5aR1/hCD45 knock-in mouse as a murine model might elucidate the importance of the PVL-CD45 interaction in infection. However, the process is laborious and the lack of an existing human CD45 knock-in mouse model impedes the development of a double hC5aR1/hCD45 knock-in mouse via backcross. This is not a surprise, as the gene encoding CD45 (*PTPRC*) is highly complex with 34 exons spanning over 110 kb on the genome, making it not amenable for CRISPR-mediated knock-in without a preliminary characterization of the exons involved in the LukF-hCD45 interaction. In addition, the expression of human CD45 may trigger a deleterious phenotype as CD45 is a phosphatase expressed on all nucleated cells of myeloid origin. Nevertheless, the identification of the F-component receptor for PVL has consequences for our current understanding of leukocidin pore-formation.

In **Chapter 2**, we applied non-conventional hybrid pairing of S- and F-subunits of PVL and HlgCB to induce functional pore-formation on target cells *in vitro*. Non-cognate pairing of other leukocidin S- and F-subunits have also been described *in vitro* [39, 49, 62, 63]. *In vivo* studies have shown that non-cognate pairing of PVL and LukED is likely possible, but results in attenuated virulence of *S. aureus* [63]. As many clinical *S. aureus* isolates secrete all five bi-component toxins, non-cognate pairing of leukocidins could results in 13 different toxin complexes [39]. The discovery of an F-components target, and possibly the existence of other F-components targets, might suggest a far more complex range of cytotoxic activities by employing non-cognate pairing, and possibly contributing to *S. aureus* pathogenesis. However, it remains to be resolved whether non-cognate pairing between leukocidins could also enhance *S. aureus* virulence *in vivo*.

It was long questioned why *S. aureus* would secrete multiple bi-component toxins that lyse phagocytes, a seemingly redundant strategy deployed by *S. aureus* to evade the host innate immune system. The first hints indicating that the secretion of multiple leukocidins is more than just simple redundancy came with the discovery of CCR5 as one of the receptors for LukED and therefore LukED could also lyse cells of the adaptive immunity [29]. The discovery of other specific leukocidin GPCR targets further showed that leukocidins are not just a redundant feature, but possess the ability to select and drive host- and cell-specific cytotoxicity. However, that could not explain the apparent redundant deployment of two leukocidins that target the same human C5aR1. HlgCB and PVL both target neutrophils via C5aR1. On a molecular level however, the interaction between HlgCB and PVL with C5aR1 differs. While PVL exclusively interacts with the human and rabbit C5aR1, HlgCB interacts with multiple mammalian orthologues of C5aR1 and can additionally employ the human C3a receptor (hC3aR) as a target[26]. Nonetheless, the interaction between HlgCB and hC3aR is inefficient [26]. In **Chapter 2** we show that the F-components of HlgCB and PVL interact with different targets. LukF-PV interacts with human CD45. It is possible that HlgB also employs a specific cellular surface target, however, this was not picked up in our HlgCB screen. Even though the target of HlgB remains unknown, our studies show an additional non-redundant interaction between the C5aR1 interacting leukocidins. The CRISPR-Cas9 -based approach applied in **Chapter 2 and 3** could serve as a mean to elucidate the F-component targets and possibly other host-dependent factors for all leukocidins by using a similar genome-wide CRISPR-Cas9 library in cell-lines expressing the corresponding GPCR target. In **Chapter 3** we show a conserved , as well as a divergent role for host GPCR PTM in leukocidin susceptibility, further emphasizing the non-redundant role of leukocidins.

Intracellular pathways dictating leukocidin susceptibility

Genes encoding intracellular proteins involved in post-translational modification (PTM) pathways were additionally identified in both our PVL- and HlgCB-susceptibility screen. PTM of proteins is essential in many cellular processes [64]. In fact, PTM of GPCRs is important for regulating structure, function and association with natural ligands [65-68]. However, PTM moieties on GPCRs have also been suggested to be involved in mediating the interaction with different human pathogens [69, 70]. The sialic acid-binding adhesin (SabA) secreted by *Helicobacter pylori* mediates adherence specifically to the Lewis blood group antigens Sialyl-Lewis^{x} [71]. In addition, Sialyl-Lewis^{x} has been described as a PTM mediating affinity between many bacterial toxins and their targets. The *E. coli* subtilase cytotoxin (SubAB), *S. enterica* typhoid toxin and *S. pneumoniae* cytolysin all interact in a Sialyl-Lewis^{x} dependent manner [72]. In **Chapter 3** we show the identification of sialylation as a host-dependent-factor contributing to PVL, HlgCB, LukED and HlgAB mediated pore formation. However, our findings suggest that the sialylation of C5aR1 and CXCR2 are not essential for PVL, HlgCB, HlgAB and LukED, but rather enhance the interaction and sensitivity to these toxins. The binding of LukS-PV to the N-terminus of C5aR1 was previously shown to be mediated by sulfation of the receptor [24]. Our findings suggest that the sulfation of the hC5aR1 is also not essential for PVL and HlgCB cytotoxicity, but rather enhance the interaction and sensitivity to these toxins. However, sulfation of CXCR2 does not play a role in HlgAB or LukED mediated pore formation, highlighting a divergent role of sulfation in dictating cellular susceptibility between GPCRs to leukocidins.

Tyrosine sulfation is catalysed by tyrosylprotein sulfotranseferase (TPST) through the transfer of an active sulfate group from the sulfate donor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to tyrosine residues [73, 74]. Tyrosylprotein Sulfotransferase 2 (TPST2) and 3'-phosphoadenosine 5'-phosphosulfate synthetase 1 (PAPSS1) were picked-up in our screening and are involved in the sulfation of C5aR1, enhancing the interaction with PVL and HlgCB. However, there is variation in expression levels of TPST amongst cell types [75-77]. Tyrosine sulfation is heterogeneous and tissue specific [73, 77] resulting in variable sulfation profiles possibly contributing to the host and cellular tropism of leukocidins. It remains to be established whether there is variability in sulfation of GPCRs on phagocytes during different stages of activation or infection, and possibly contributing to the interaction of leukocidins with their respective receptor. Co-evolution of leukocidins with their host receptor could have led to the selection of active sulfated GPCRs by leukocidins, as sulfated GPCRs are more prone to be involved in chemokine and anaphylatoxin interaction and immune response. Nevertheless, the process of tyrosine sulfation is incompletely understood. Variations in the genes encoding PTM pathways may provide insight into observed differences in susceptibility of humans to infections with *S. aureus.*

Challenging the model for bi-component pore-formation

In 2007, a model for leukocidin function on target cells was proposed. With the exception of elucidating specific proteinaceous targets for all leukocidin S-subunits, the model for leukocidin pore-formation remained unchallenged. However, the findings described in **Chapter 2** suggest a different approach to leukocidin pore-formation and cell specificity for PVL. The previous model (Figure 1a) suggested that leukocidins are secreted as water-soluble monomeric subunits by *S. aureus*. In the case of PVL, LukS-PV recognizes hC5aR1 on the surface of target cells. This interaction between LukS-PV and human C5aR1 is subsequently followed by the recruitment of the LukF-PV, resulting in oligomerization of alternating S- and F-components. This model suggests a stepwise process involving the primary interaction of LukS-PV, as previous studies were unable to detect LukF-PV binding in the absence of a primary bound LukS-PV [21]. However, the identification of human CD45 as target for LukF-PV challenges this previous model for leukocidin pore-formation (Figure 1a and b). LukF-PV can bind independent of LukS-PV to human CD45 expressing cells, indicating that the primary interaction of LukS-PV in not necessary for the recruitment of LukF-PV. LukS-PV and LukF-PV can interact independent of each other. However, contrary to C5aR1, the expression of CD45 is not essential for PVL cytotoxicity, but rather enhance the interaction and sensitivity to C5aR1 expressing cells. Studies suggest that the PVL induced octameric pore consists of a LukS-PV/LukF-PV ratio of 1:1, in which each LukS-PV monomer binds one C5aR1 [78-80]. It is unknown whether each LukF-PV is also bound to CD45.

Exploiting S. aureus virulence factors as therapeutic agents

S. aureus has evolved to secrete molecules that target and evade the innate immune system. Some of these evasion molecules have anti-inflammatory properties and could serve as therapeutics in diseases in which abnormal immune activation plays a role. Studies have proposed and discussed the therapeutic potential of staphylococcal virulence factors before. For example, the *S. aureus* secreted formyl peptide receptor-like 1 inhibitor (FLIPr) and its homologue FLIPr-like inhibit Fc-receptors for IgG (FcγR) [81] could serve as a therapeutic agent as FcγRs play a role in immune complex based inflammation and disease [82]. In addition, the staphylococcal Superantigen-like protein 7 (SSL7) inhibits the cleavage of complement C5 and could serve as a novel C5 inhibitor [83, 84].

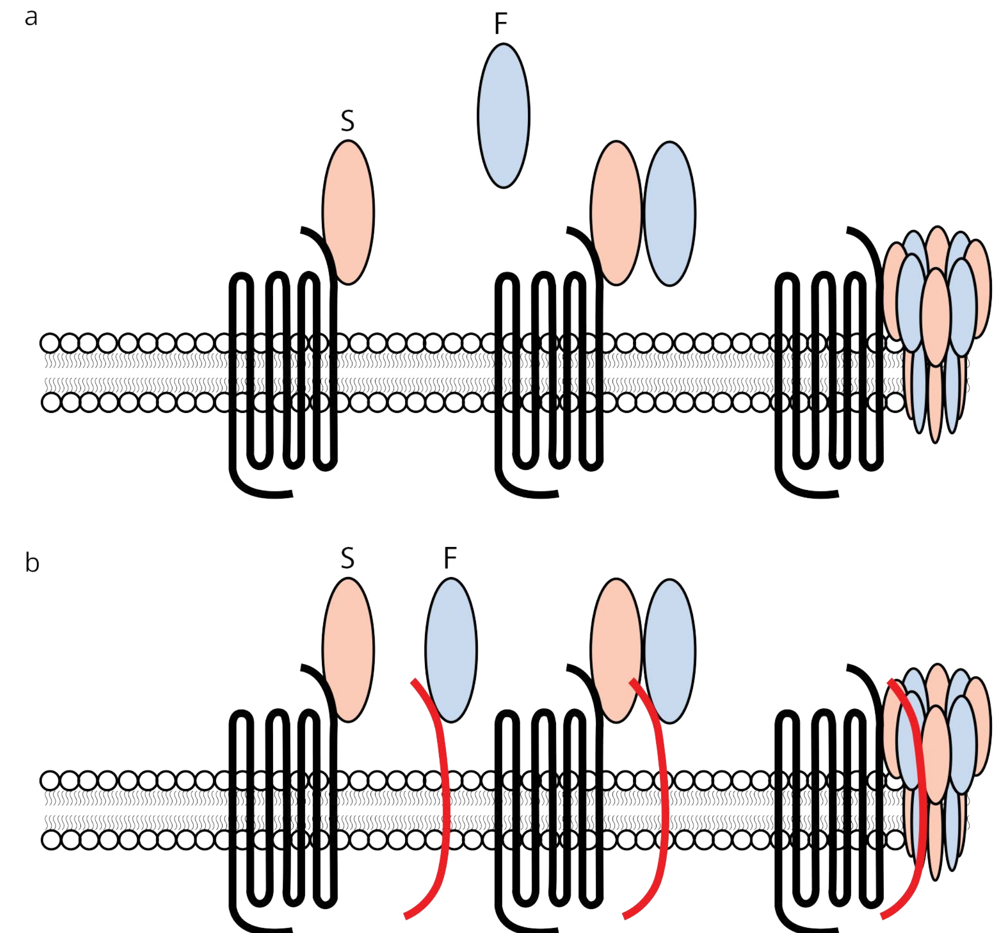

Figure 1: Previous and proposed model for PVL pore-formation. (a) PVL consisting out of a S- and F-component, LukS-PV and LukF-PV respectively, induce pore-formation on hC5aR1 cells in a stepwise approach. The primary interaction consists out of the S-component interacting with hC5aR1 on the surface of target cells. Subsequently, an F-component is recruited that interacts with the S-component/receptor complex, forming an ring-like octamer of alternating S- and F-components. A conformational change of the S- and F-components induces the inward collapse of the stem region forming a β-barrel pore that spans the cell membrane. (b) PVL consisting out of a S- and F-component, LukS-PV and LukF-PV respectively. Two separate interactions occur and consists of LukS-PV interacting with hC5aR1, and LukF-PV interacting with hCD45 on the surface of target cells. The interaction of LukS-PV and LukF-PV results in a complex inducing the formation of a ring-like octamer of alternating S- and F-components. A conformational change of the S- and F-components induces the inward collapse of the stem region forming a β-barrel pore that spans the cell membrane.

GPCRs play a central role in inflammatory diseases and therefor are interesting targets for therapeutics [85, 86]. In particular, C5a and C5aR1 have been described to be involved in disease processes such as ischemia-reperfusion injury, rheumatoid arthritis, asthma, immune complex diseases, neurodegeneration and Alzheimer's disease [87-90]. In **Chapter 4** we use the immune complex-mediated Arthus reaction model that results in an inflammatory response and neutrophil recruitment that is mainly C5a mediated. We show that CHIPS can inhibit hC5aR1 mediated neutrophil migration hC5aR*KI* mice *in vivo*. These findings demonstrate the therapeutic potential of CHIPS as a C5aR1 inhibitor *in vivo*. However, subsequent administration of CHIPS in human subjects led to immune complex formation with circulating anti-CHIPS antibodies and induced adverse effects. The observation of the inhibition of the activity of CHIPS by anti-CHIPS antibodies together with the induction of an anaphylactic response shows that CHIPS in its current form is not suitable for use as an anti-inflammatory agent. Humans do not only carry pre-existing CHIPS antibodies, but also antibodies against many staphylococcal secreted factors [91-94]. The use of staphylococcal proteins, or even bacterial proteins in general, as therapeutic agents could result in immune complex formation and therefor unsuitable as therapeutic agents. Nevertheless, future development of small molecule anti-inflammatory drugs based on the CHIPS , or other staphylococcal proteins, mechanism of action could very well be potential new candidates.

Concluding remarks

We develop a hC5aR1^{KI} mouse to show the role of HlgCB in *S. aureus* pathogenesis *in vivo*. In addition, we identify CD45 as a novel F-component receptor for PVL and how LukF-PV also plays a role in driving the species- and cell-specificity of PVL. The discovery of an F-component target demonstrates how *S. aureus* effectively targets human neutrophils by deploying a two step-control-mechanism. We utilize the hC5aR1^{KI} mouse to show the therapeutic potential of CHIPS, an *S. aureus* secreted protein that targets the human C5aR1 expressed on neutrophils. Understanding and knowledge of the mechanisms of action of PVL and other human specific factors could not only identify new targets that could be exploited for anti-virulence strategies to limit *S. aureus* infections, but also improve current *in vivo* infection models.

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CHAPTER SIX

NEDERLANDSE SAMENVATTING
DANKWOORD
CURRICULUM VITAE
LIST OF PUBLICATIONS

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Nederlandse Samenvatting

De mens draagt bacteriën overal op en in het lichaam. De meeste bacteriën zijn onschuldig. Dit komt doordat deze bacteriën in de gaten worden gehouden door het immuunsysteem. Het immuunsysteem grijpt in en speelt een belangrijke rol in de afweer tegen ziekteverwekkende bacteriën. Witte bloedcellen vormen een belangrijk onderdeel van deze afweer en kunnen worden onderverdeeld in verschillende type witte bloedcellen met elk een specifieke functie. Neutrofiele granulocyten (in dit proefschrift ook wel als neutrofielen, PMN of fagocyten aangeduid) zijn de meest voorkomende witte bloedcellen en vormen ongeveer 60% van de totale witte bloedcellen in het bloed. Neutrofielen zijn essentieel in de eerste afweer tegen bacteriën en andere ontstekingsreacties. Het opzoeken en herkennen van bacteriën gebeurt via verschillende receptoren die op de buitenkant van witte bloedcellen en dus ook op neutrofielen aanwezig zijn. Sommige receptoren komen voor op verschillende type witte bloedcellen, maar sommige receptoren zijn heel specifiek aanwezig op alleen neutrofielen. Receptoren ontvangen signalen vanuit de omgeving afkomstig van o.a. de bacterie zelf en van de mens. Door die signalen weten de neutrofielen waar de infectie zich bevindt en verplaatsen ze zich naar de plek van de infectie om daar de bacteriën op de eten (fagocyteren). Zo worden onder andere ziekmakende bacteriën onschadelijk gemaakt.

Een van de meest voorkomende verwekkers van bacteriële infecties is *Staphylococcus aureus* (in het kort *S. aureus*). *S. aureus* kan verschillende soorten infecties veroorzaken, van vrij onschuldige huidinfecties tot ernstige infecties met fatale gevolgen. Bacteriën kunnen ongevoelig (resistent) worden voor antibiotica. De komst van methicilline-resistente *S. aureus* (MRSA) varianten, die resistent zijn voor veelgebruikte antibiotica, is een gevaar voor de volksgezondheid. Deze antibioticaresistente *S. aureus* varianten veroorzaken doorgaans ook ernstigere infecties. Het is dus noodzakelijk dat wij nieuwe behandelmethoden en effectieve vaccins ontwikkelen tegen *S. aureus*. Wat *S. aureus* heel bijzonder maakt, is dat deze bacterie heel veel verschillende moleculen kan produceren die zorgen dat *S. aureus* het immuunsysteem kan ontwijken. Omdat neutrofielen zo een belangrijke rol spelen in de afweer tegen bacteriën, is het niet verrassend dat veel van deze “immune evasion” moleculen gericht zijn tegen neutrofielen.

In **hoofdstuk 1** vertellen wij uitgebreid over *S. aureus* en geven wij een paar voorbeelden van de targets van *S. aureus* moleculen, in dit geval de receptoren op neutrofielen, die *S. aureus* gebruikt om de werking van neutrofielen te belemmeren of zelfs de neutrofielen te doden. Onderzoek naar de processen en het begrijpen van hoe deze moleculen te werk gaan is essentieel om therapieën en vaccines te ontwikkelen tegen *S. aureus*. Een belangrijke groep van moleculen die betrokken is bij het ontwijken van het humaan (menselijk) immuunsysteem zijn de bi-component toxines (ook wel leukocidins genoemd). *S. aureus* leukocidins zijn toxines die heel specifiek witte bloedcellen, waaronder ook neutrofielen, onschadelijk maken. Zoals de naam al aangeeft, bestaan deze bi-component leukocidins uit twee componenten, die samen gaten kunnen maken in witte bloedcellen. Deze toxines kunnen heel gericht o.a. neutrofielen aangrijpen door receptoren te misbruiken die specifiek op neutrofielen aanwezig zijn. In **hoofdstuk 1** beschrijven wij een model van hoe men voorheen dacht hoe deze leukocidins gaten maken in witte bloedcellen. Het bewijzen van dit model is lastig aangezien deze leukocidins specifiek humane receptoren aangrijpen. Hierdoor kon men heel lang niet de toegevoegde waarde van leukocidins bestuderen in levende organismen (*in vivo*) zoals muizen. Het direct testen op mensen is onethisch en gevaarlijk. Wel zijn er alternatieven om dit humaan-specifieke probleem op te lossen.

In **hoofdstuk 2** beschrijven wij hoe wij een humaan transgene muis (het inbrengen van menselijk erfelijk materiaal in een muis) hebben ontwikkeld. Dit houdt in dat deze muis humane receptoren bevat. Het ontwikkelen van een transgene muis heeft het mogelijk gemaakt om te bestuderen wat voor effect bepaalde leukocidins hebben *in vivo*. Behalve erachter komen wat voor rol leukocidins spelen in de eerste stadia van een *S. aureus* infectie, bewijzen wij voor het eerst dat beide componenten van deze bi-component toxines een rol spelen in het aangrijpen en onschadelijk maken van neutrofielen. Voorheen dacht men dat alleen één component een rol speelde bij dit proces. Als gevolg, verandert dit ons begrip van hoe leukocidins te werk gaan en verklaart het een aantal over de jaren heen onopgeloste vraagstukken. In **hoofdstuk 5** dragen wij een nieuw model voor van hoe leukocidins witte bloedcellen onschadelijk maken. De nieuwe bevindingen beschreven in **hoofdstuk 2** laten zien hoe meerdere receptoren aan de buitenkant van witte bloedcellen, specifiek neutrofielen, een rol spelen in de interactie met *S. aureus* leukocidins. Met deze kennis kunnen wij betere diermodellen ontwikkelen om zowel de wijze waarop *S. aureus* infectie

ontstaat te bestuderen, als nieuwe behandelingsmethoden voor *S. aureus* te ontwikkelen.

In **hoofdstuk 3** wordt het iets complexer, waarbij wij laten zien dat zelfs processen binnen witte bloedcellen van invloed zijn op wat er aan de buitenkant gebeurt. Sterker nog, wij beschrijven de specifieke rol van twee intracellulaire (in de cel) processen die invloed hebben op de interactie met *S. aureus* leukocidins. Daarnaast laten wij in **hoofdstukken 1, 2 en 3** goed zien hoe *S. aureus* zich heeft aangepast aan het humane immuunsysteem om deze zo goed en efficiënt mogelijk te ontwijken. Sommige moleculen die door *S. aureus* zijn uitgescheiden zijn bestemd voor het onderdrukken van een adequate immuunreactie. Deze moleculen werken zodanig efficiënt dat wij ons hebben afgevraagd of deze moleculen ook gebruikt kunnen worden als therapie in ziektes waarbij een ontregelde immuunreactie een rol speelt. Omdat dit molecuul ook alleen op humane cellen werkt, was het bewijzen van onze onderzoeksvraag een uitdaging. In **hoofdstuk 4** laten wij, door gebruik te maken van een transgene muis, zien dat een *S. aureus* molecuul daadwerkelijk gebruikt kan worden als therapeutisch middel. Aan de hand van deze bevinding hebben wij besloten om dit molecuul in mensen te injecteren om zo de veiligheid te bestuderen. Helaas reageert het menselijk immuunsysteem zodanig fel op dit molecuul, dat het niet verstandig is om te gebruiken als een behandelmethode in mensen.

In **hoofdstuk 5** plaatsen wij alle bevindingen van dit proefschrift in context. Behalve een nieuw model voor de werking van leukocidins, bespreken wij hoe processen binnen een cel effecten hebben op hoe efficiënt leukocidins werken. Dit allemaal met betrekking tot het humane afweersysteem. De resultaten beschreven in dit proefschrift kunnen gebruikt worden om betere proefdiermodellen en vaccines te ontwikkelen tegen *S. aureus*.

Dankwoord

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Curriculum Vitae

Angelino Tromp was born on April 16th, 1988 in the Hague, the Netherlands. At the age of one year and 8 months he moved to Curaçao, a former Dutch Caribbean colony. There, he graduated from high school, Radulphus College, in 2007. In that same year he moved to the Netherlands and started his bachelor's studies in Life Sciences and majored in Microbiology with a minor in Drug Design at the HU University of Applied Sciences Utrecht. During his bachelor's he performed a clinical and a research internship at the VU University Medical Center under the supervision of Prof. dr. Paul Savelkoul, dr. Rogier Schade and dr. Marre van den Brand at the department of Medical Microbiology and Infection prevention. After graduating in 2011, he continued with the master's Biomedical Sciences at the VU University in Amsterdam. As part of the master's program, he specialized in the field of immunology and infectious diseases. He did his first research internship in the lab of Prof. dr. Ferry Ossendorp, under the supervision of dr. Rodney Rosalia and dr. Luis Cruz in the department of Immunohematology and Blood Transfusion at Leiden University Medical Center. Angelino later performed his second research internship in the lab of Prof. dr. Jos van Strijp, under the supervision of Prof. dr Suzan Rooijakkers and dr. Daphne Stapels in the department of Medical Microbiology at the University Medical Center Utrecht. He graduated in 2015, upon which he continued as a PhD-candidate in the lab of Prof. dr. Jos van Strijp. During his PhD he focussed on human specific staphylococcal virulence factors and was supervised by dr. Pieter-Jan Haas and dr. András Spaan. The results of his research projects have been described in this thesis and published in a peer-reviewed scientific journals.

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List of publications

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