Skin immunity in protection against *Staphylococcus aureus* infections

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CHAPTER 1

General Introduction

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C-Type Lectin Receptors in Host Defense Against Bacterial Pathogens

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Staphylococcus aureus

Staphylococcus aureus is a Gram-positive bacterium, and one of the most common causes of skin and soft tissue infections (SSTI) worldwide [1]. *S. aureus* is part of the human microbiome, permanently colonizing the skin and nares of up to 30% of the population. In addition, it is estimated that up to 60% of the population can be classified as transient *S. aureus* carriers [2–4]. The transition from commensal towards life-threatening pathogen is likely multifactorial, involving disruption of the host-microbiota homeostasis, immune status of the patient, as well as the intrinsic virulence potential of the *S. aureus* strain, which enables colonization and survival on surfaces and within tissues of the human body [5]. Many important components that promote *S. aureus* survival are incorporated in the bacterial cell envelope, which determines cell shape, shields bacteria from the harsh environment and modulates bacterial immune recognition and responses.

As for all Gram-positive bacteria, the cell envelope of *S. aureus* is composed of the cytoplasmic membrane, which is covered by a thick layer of peptidoglycan (PG) as the main structural component of the cell wall. PG is composed of polymerized *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) residues and the attached stem peptides are cross-linked through a unique pentaglycine bridge. This creates a dense, yet flexible, structure [6,7] that allows the bacterium to withstand high internal turgor pressure and provides mechanical protection [8]. In addition, PG is also used as a scaffold to anchor a select set of proteins that contain a signature LPXTG amino acid motif and glycopolymers called wall teichoic acid (WTAs). WTAs serve a critical role both in bacterial virulence and physiology [9–11]. The majority of *S. aureus* strains express WTA composed of a poly-ribitol phosphate (RboP) backbone decorated with positively-charged D-alanine and GlcNAc residues [12]. The exception is the ST395 lineage, which produces WTA composed of poly-glycerol phosphate (GroP) decorated with α-*N*-acetyl-D-galactosamine residues (GalNAc) [13]. ST395 is genetically distant from other *S. aureus* lineages and its WTA shares structural similarities with coagulase-negative staphylococci (CoNS) [14]. The impact of different WTA glycosylation patterns for detection of *S. aureus* will be further described in the following paragraphs.

Skin as first line of defense

The skin is the most extensive outer surface of the human body and constitutes a harsh, nutrient-poor, acidic and dry landscape. Its outermost viable layer, i.e. the epidermis, consists of overlapping strata of keratinocytes. The apical site of the epidermis is covered by the stratum corneum, which consists of terminally differentiated keratinocytes called corneocytes that are chemically cross-linked to fortify the skin barrier. Below the epidermis,

the dermis is predominantly composed of fibroblasts that form a loose, irrorated and innervated connective matrix. Skin appendages, such as sweat glands and hair follicles, interrupt the continuity of the stratum corneum and extend into the dermis. These appendages exert protective and homeostatic functions and are unevenly distributed across the skin depending on body location, further increasing the heterogeneity of the skin landscape.

The skin, especially the epidermis is exposed to the external non-sterile environment through colonization with a heterogeneous community of microbes–the skin microbiota. The composition of the skin microbiota differs widely between different parts of the body; in fact, depending on the pH, humidity, as well as salt and oxygen content, each area of the epidermis constitutes a multitude of ecological niches in a single host [15,16]. Skin is not only a habitat for beneficial microorganisms but also a common entry point for pathogens; bacteria, fungi, viruses and parasites can enter the body when the cutaneous barrier is breached, for example by minor abrasions, an injury, or a surgical incision. A subsequent skin infection can lead to diseases of various severities, ranging from localized and uncomplicated to systemic and life-threatening disease. Overall, SSTIs represent a heterogeneous group of clinical conditions affecting the skin and underlying tissues. Despite the high burden of skin infections in the population, the molecular mechanisms mediating the entry of most pathogens through the skin and the immune defense mechanisms to clear these pathogens are still poorly understood.

Virulence factors associated with skin infections

S. aureus produces a plethora of virulence factors, which are either secreted or surface expressed. The virulence factors that are implicated in in skin infections or pathologies will be discussed here.

A well-studied group of virulence factors are the superantigens (SAgs), which share structural 3D homology and cause excessive T cells stimulation by aspecifically crosslinking T cell receptors (TCRs) and major histocompatibility complex (MHC) class II receptors on antigen-presenting cells (APCs), resulting in excessive secretion of proinflammatory cytokines [17]. Among the 20 different SAgs produced by *S. aureus*, some have been associated with progression of autoinflammatory skin pathologies [17]. Atopic dermatitis (AD), a common inflammatory skin disease, is characterized by high *S. aureus* burden. Skin-homing regulatory T cells (Treg) may display Th2 phenotype upon stimulation with Staphylococcal enterotoxin B (SEB), aggravating skin inflammation [18]. Moreover, *S. aureus* isolates recovered from skin of steroid-resistant AD and a severe form of AD called eczema herpeticum produce a broader spectrum of SAgs, such as toxic shock syndrome toxin-1

(TSST-1), in comparison to isolates from other AD patients [19,20]. However, SAgs are not only produced by *in vitro* cultured *S. aureus* isolates but are actually detected directly in skin from AD patients in contrast to healthy subjects [21]. Although molecular mechanism action of SAgs in the pathogenesis of skin diseases is not fully understood, both SEB and TSST-1 have been proven to interact with keratinocytes, resulting in transcriptional changes involved in signaling pathways involved in cytokine production [22].

In addition to SAgs, *S. aureus* can produce a broad spectrum of toxins, of which several are important for skin colonization and infection [23]. Especially α-toxin (Hla), which lyses eukaryotic cells through β-barrel membrane pores, is implicated in the development of skin dermonecrosis by promoting lesion formation and impairment of bacterial clearance [24–26]. To assemble on the cell surface, Hla targets the A Disintegrin And Metalloproteinase domain-containing protein 10 (ADAM10) receptor, which is highly expressed on the epithelial cells including keratinocytes [27,28]. At lower concentrations, Hla can cause ADAM10 activation-dependent E-cadherin cleavage, which has been shown to play an important role in *S. aureus*-mediated skin and pulmonary pathologies in mice [29–33]. Panton-Valentine Leukocidin (PVL), which belongs to the family of leukocidins, is also involved in skin infections. PVL is a β-barrel toxin that binds to the complement receptors C5aR and C5L2 expressed on the surface of neutrophils [34]. Although PVL-producing *S. aureus* strains have been associated with skin inflammation in animal and human skin explant models, its molecular mechanism during the skin infection is not fully understood yet [35,36]. Another family of toxins are the Phenol-soluble modulins (PSMs), which can be divided into α- or β-PSMs. In contrast to Hla and PVL, PSMs mediate lysis independent of receptor binding [37] and exert their lytic activity after uptake of bacteria by neutrophils [38]. Especially α-PSMs have been shown to be important virulence factors in both murine and rabbit skin infection models, since *S. aureus* strains lacking α-PSMs showed reduced ability to cause abscess formation [39,40].

Of the surface-expressed proteins, clumping factor A and B (ClfA/B) are important for *S. aureus* skin colonization since they mediate bacterial attachment to specific host proteins. ClfA can bind fibrinogen, which contributes to high bacterial burden in skin abscesses [41]. ClfB was initially identified as a crucial factor in nasal colonization via binding to the cytokeratin 10 and loricrin, components of cornified squamous cells in nares [4,42]. However, loricrin can be expressed by other types of cornified epithelial cells as well, such as terminally differentiated keratinocytes, thus resulting in a contribution of ClfB to skin abscess formation [43]. In addition, ClfB was identified as an important factor mediating *S. aureus* adhesion to corneocytes in the atopic dermatitis (AD) patients [44]. Although ClfA and ClfB do not mediate cell lysis their impact on bacterial adherence strongly influences *S. aureus* colonization and persistence at the site of infection.

Skin antigen-presenting cells and detection of *S. aureus*

Both the epidermal and dermal compartments of the skin are populated with a distinct and diverse repertoire of immune cells that constantly interact with microbes and their products. APCs within these skin layers APCs continuously sample the tissues through the expression of pattern-recognition receptors (PRRs; discussed in more detail below) and represent an important cellular link between innate and adaptive immunity, including T cell polarization, antibody production as well as immunological memory formation [45,46]. After antigen uptake, APCs mature and upregulate surface molecules such as costimulatory molecules and MHC class II, allowing them to prime naïve antigen-specific T cells after migration to lymph nodes or directly activate memory T cells present in the tissues [47]. In addition, resident memory T cells can be activated directly within the skin after pathogen re-encounter. Locally, APC activation results in cytokine production, which is important to attract other immune cells such as neutrophils from the circulation to the site of infection and clear invading microbes [48,49].

Although bacteria can be found throughout the entire healthy skin section, the first contact with the immune cells often occurs in the epidermis where Langerhans cells (LCs), a subtype of APCs, reside [50]. LCs form an important first line of defense against pathogens [51,52]. Although most studies focus on skin LCs, their localization is not restricted to the skin; they are also present in mucosal tissues and other epithelial linings, for example foreskin, cervical mucosal tissue, tonsils, tongue and the upper respiratory tract [53,54]. In addition to uptake of antigens within tissues, LCs can also sample the extracellular environment by extending their dendrites through tight junctions between keratinocytes [55]. This sampling behavior contributes to production of systemic antibody formation and can induce inflammatory signals, such as TNFα and IL-1β production [55,56]. In the dermis, a wider range of immune cells is present, including dendritic cells (DCs), $CD4^+$ and $CD8^+$ T cells, gamma delta T cells and monocytes. DCs are another subset of professional APCs that can be divided into different subtypes based on the expression of their surface markers. DCs have been shown to play an important role in *S. aureus* recognition followed by induction of adaptive responses. Immature monocyte-derived DCs (iDCs) are capable of phagocytosing both *S. aureus* and *S. epidermidis* and induce specific T cell proliferation. However, levels of T cell responses are higher for *S. aureus* strains producing SAgs since activate T cell non-specifically [57]. At the same time the SAg SEB is able to influence T cell polarization towards a Th2 phenotype via altering DC responses [58,59]. This type of response is observed in the initial phase of AD. Moreover, *S. aureus* toxin LukAB can mediate DCs lysis, thereby interrupting naïve CD4+ T cells priming resulting in low activation and proliferation [60]. These few examples show how antigen recognition influences subsequent adaptive responses.

To efficiently detect invading microbes, APCs express a wide range of innate PRRs that sense specific bacterial constituents or microbe-associated molecular patterns (MAMPs). Among these PRRs, four different families are currently distinguished [61]. Nucleotide-binding oligomerization domain-like receptors and retinoic acid-inducible gene-I-like receptors (RLRs) are the cytoplasmic sensors of a cell, whereas Toll-like receptors (TLRs) are expressed in endosomes and on the cell surface [61]. The fourth family is comprised by the C-type lectin receptors (CLRs) that consists of transmembrane and soluble extracellular proteins [45]. Especially TLRs and CLRs have been identified as important sensors by which APCs recognize *S. aureus*.

TLR2 is a key receptor for staphylococcal recognition through the interaction with bacterial lipoproteins [62]. Sensing of *S. aureus* lipoproteins depends on PSMs, which acts as surfactant proteins on the bacterial membrane to promote lipoprotein shedding and subsequent TLR2 activation [63]. TLR2 can form heterodimers with TLR1 and TLR6, enabling recognition of a wide range of bacterial lipoproteins and other antigens [64]. Apart from the surface-expressed TLRs, cytosolic TLRs also contribute to *S. aureus* detection. After *S. aureus* phagocytosis by DCs, TLR9 binds bacterial DNA and induces interferon I production [65]. Interestingly,

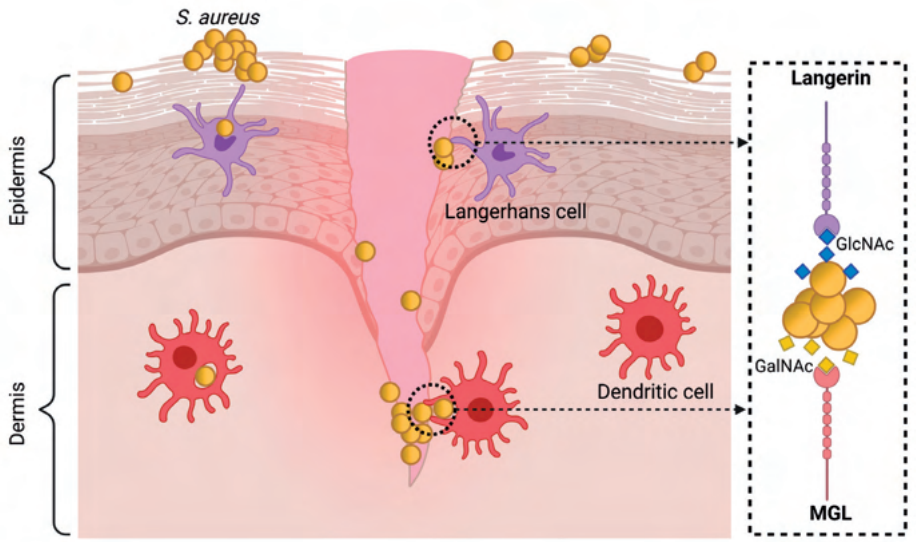

Figure 1. The interaction of C-Type Lectin Receptors (CLRs) expressed by Antigen Presenting Cells (APCs) in the skin with *S. aureus* carbohydrates. Langerhans cells present in the epidermis express Langerin which recognizes β-*N*-acetyl-D-glucosamine (GlcNAc) residues on *S. aureus* Wall Teichoic Acid (WTA). In the dermis dendritic cells express Macrophage Galactose-Type C-Type Lectin receptor (MGL) which interacts with α-*N*-acetyl-D-galactosamine (GalNAc) present on WTA of the *S. aureus* lineage: ST395.

activation of TLR9 has negative consequences in terms of clearing of *S. aureus* from lungs in the murine pulmonary infection model [65].

CLRs are specialized in the recognition of exposed sugar residues or sugar motifs present on self as well as non-self structures [48,49]. Recognition of specific glycans occurs through one or more carbohydrate recognition domains (CRDs) in a Ca^{2+}-dependent manner. CLRs expressed on APCs can be divided based on their topology as type I and type II transmembrane proteins; type I receptors are characterized by the N-terminus pointing out of the cell and multiple CRDs, whereas type II receptors have their N-terminus directed toward the cytoplasm and an extracellular C-terminus that contains a single CRD [66]. Receptors of both groups have a stalk region, a transmembrane region, and an intracellular domain with or without a signaling motif. Within type I and type II receptors, CLRs can additionally be categorized based on conserved amino acid motifs in their CRDs that determine their glycan specificity and Ca^{2+} coordination. CLRs with an EPN (Glu-Pro-Asn) amino acid motif in their CRD, such as DC-SIGN (CD209), langerin (CD207) and mannose receptor (MR, CD206), preferentially bind glycans with equatorial 3- and 4-hydroxyl groups such as mannose, fucose, and GlcNAc residues. On the other hand, CLRs with a QPD (Gln-Pro-Asp) motif preferentially bind glycans with axial 4-hydroxyl groups such as galactose and GalNAc terminated glycans [67]. In humans, most CLRs possess the EPN motif, with the exception of Macrophage Galactose-type C-type lectin (MGL, CD301), which possesses the QPD motif [68]. Despite the shared structural features of their CRDs, CLRs display considerable variation in overall structure, cellular expression profiles and signal transduction cascades. These differences have important consequences for the specific contributions of CLRs to antimicrobial immunity, since they strongly affect ligand specificity and the induced immune responses, which can either support host defense or allow immune escape. An example of such interaction between *S. aureus* and a CLR on DCs has been previously described by van Dalen et al. [69]. Langerin on LCs senses a β-GlcNAc modification on RboP-WTA, triggering immune responses, such as production of proinflammatory cytokines that are important for induction of Th17 responses [69]. Since *S. aureus* expresses additional glycosylated structures, it is likely that additional interactions with CLRs on different APC subsets will be identified, providing insight in *S. aureus* host defense mechanisms.

Adaptive immunity in the skin

Adaptive immune responses in the skin are orchestrated by B and T lymphocytes. Experimental evidence suggests that healthy human skin contains on average 1 million T cells per cm^2, coming to a total of approximately 2×10^{10} T cells [70]. This is roughly twice the amount of total T cells in the circulation [70]. All these T cells are memory T cells that, after the T cell contraction phase and re-establishment of skin homeostasis, remain in

the skin. Among the memory T cells, different types are distinguished, of which tissue-resident memory T cells (Trm) are the most abundant [71]. Trm cells are a subset of T cells characterized by the life-long retention at barrier tissues, including skin. They are important players in facilitating local adaptive responses. Upon antigen reencounter, Trm proliferate in the tissue of residency thus enriching the already existing pool of local memory T cells [72,73]. Trm were initially thought to reside permanently within tissues, however more recent data have shown that they recirculate and remigrate [74]. This is aligned with the finding that Trm responses initiated at the site of infection, such as barrier tissues, can cause both Trm expansion and contribution to already circulating memory T cells which can be described as 'outside-in' immune recall [75]. However, within the circulation, Trm represent only a minor percentage of the total memory T cell pool [74].

Skin Trm (Tsrm) are characterized by the expression of the skin-homing marker Cutaneous Lymphocyte-Associated (CLA) antigen, the tissue-retention marker CD69, T cell memory marker CD45RO, and part of them express the integrin CD103 [71,74]. Skin Trm cells comprise several subsets such as $CD4^+$ Th1, $CD4^+$ Th2, $CD4^+$ Th17, $CD8^+$ Tc1, $CD8^+$ Tc17 and regulatory T cells (Treg). Both tissue-resident and circulating T cells are important players in adaptive

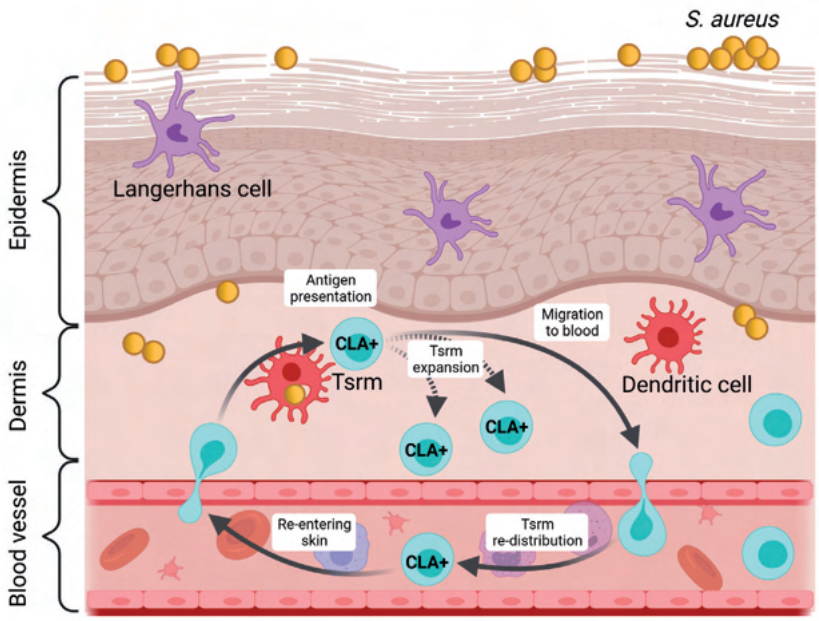

Figure 2. The recirculation of skin-resident T cells (Tsrm) between skin and blood. Upon *S. aureus* recognition and uptake, dendritic cells present antigen to *S. aureus*-specific T cells expressing the skin-tropic marker Cutaneous Lymphocyte-Associated antigen (CLA). Antigen presentation causes Tsrm expansion and their migration to the bloodstream where they redistribute within the body and re-enter the skin again.

responses against *S. aureus* [76]. They are essential for the maturation and class switch of antibodies and neutrophil recruitment through the production of IL-17A [77,78]. These aspects have been shown to be essential in the *S. aureus* infection clearance [79–81]. Moreover, IL-17A plays important role in the protection after vaccination as deduced from experiments in mice [82,83]. Importance of $CD4^+$ T cells in the protection against *S. aureus* will be discussed in detail in further chapters.

Aim and the outline of this thesis

Defense against *S. aureus* infection requires the contribution of both innate and adaptive arms of the immune system. Understanding which bacterial molecules are important for the *S. aureus* recognition by DCs could represent an important factor in vaccine design, especially in the context of formation of T cell memory responses. In the first part of my thesis I described a new interaction between CLRs and *S. aureus* and how this impacted proinflammatory responses. Next, I investigated preexisting memory T cell responses towards *S. aureus* in the skin and blood of healthy individuals.

In **Chapter 2**, I describe the impact of WTA glycosylation on the recognition of *S. aureus* isolates from the ST395 lineage by monocyte-derived dendritic cells (moDCs). I discovered that the CLR Macrophage Galactose-Type C-Type Lectin receptor (MGL) detects these particular isolates through interaction with the GalNAc-attached glycan moiety attached to GroP-WTA. Since similar WTA structures are supposedly expressed by coagulase-negative staphylococci (CoNS), I compared different *S. aureus* strains and CoNS for their interaction with recombinant and cell surface-expressed MGL. Cellular responses of primary moDCs, such as activation markers and cytokine production, were also analyzed. These results indicated that *S. aureus* ST395 induces MGL-dependent production of proinflammatory cytokines by moDCs.

Chapter 3 describes the adaptive responses towards *S. aureus* in the skin of healthy subjects. *S. aureus*-specific skin-resident memory T cells (Tsrm) were identified in cell suspensions prepared from healthy human skin by measuring cell proliferation and cytokine production (IL-17A, IL-22, GM-CSF, IFNγ and TNFβ) in response to heat-killed *S. aureus*, but not *S. epidermidis*. In addition, proinflammatory cytokine responses in the skin biopsies infected with *S. aureus* were observed after 24 and 72 h post infection. This study is the first evidence of the presence of *S. aureus*-specific $CD4^+$ Tsrm in the skin.

Chapter 4 is dedicated to identification of *S. aureus*-specific $CD4^+$ T cells in the blood of healthy subjects. Different approaches to identify these specific T cells were compared and optimized. Finally, we used an assay based on activation induced markers (AIM) and single-

cell sorted cells expressing CD137 and OX40 on their surface after incubation with heat-killed *S. aureus* for further analysis by mRNA-sequencing. Preliminary results show presence of seven different clusters in analyzed *S. aureus*-specific $CD4^+$ T cells, among which cytotoxic (CTL) and regulatory (Treg) $CD4^+$ T cells could be distinguished. In addition, comparison of expression profiles of skin-tropic cells expressing Cutaneous Lymphocyte-Associated (CLA) antigen versus CLA^-, non-skin homing cells, revealed high frequency of regulatory T cells in the CLA^+ population. Obtained data on the quality of pre-existing anti-*S. aureus* $CD4^+$ T cells could contribute to better understanding of $CD4^+$ T cell responses during infection and vaccination.

Chapter 5 summarizes and discusses the obtained results in the context of timely *S. aureus* literature on the role of skin immunity in fighting *S. aureus* infections. Moreover, vaccination as a preventive strategy for *S. aureus* infections will be discussed.

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CHAPTER 2

The C-Type Lectin Receptor MGL senses *N*-acetylgalactosamine on the unique *Staphylococcus aureus* ST395 Wall Teichoic Acid

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Abstract

Staphylococcus aureus is a common skin commensal but is also associated with various skin and soft tissue pathologies. Upon invasion, *S. aureus* is detected by resident innate immune cells through pattern-recognition receptors (PRRs), although a comprehensive understanding of the specific molecular interactions is lacking. Recently, we demonstrated that the PRR langerin (CD207) on epidermal Langerhans cells senses the conserved β-1,4 linked *N*-acetylglucosamine (GlcNAc) modification on *S. aureus* wall teichoic acid (WTA), thereby increasing skin inflammation. Interestingly, the *S. aureus* ST395 lineage as well as certain species of coagulase-negative staphylococci (CoNS) produce a structurally different WTA molecule, consisting of poly-glycerolphosphate with α-O-*N*-acetylgalactosamine (GalNAc) residues, which are attached by the glycosyltransferase TagN. Here, we demonstrate that *S. aureus* ST395 strains interact with the human Macrophage Galactose-type lectin (MGL; CD301) receptor, which is expressed by dendritic cells and macrophages in the dermis. MGL bound *S. aureus* ST395 in a *tagN*- and GalNAc-dependent manner but did not interact with different *tagN*-positive CoNS species. However, heterologous expression of *S. lugdunensis tagN* in *S. aureus* conferred phage infection and MGL binding, confirming the role of this CoNS enzyme as GalNAc-transferase. Functionally, the detection of GalNAc on *S. aureus* ST395 WTA by human monocyte-derived dendritic cells significantly enhanced cytokine production. Together, our findings highlight differential recognition of *S. aureus* glycoprofiles by specific human innate receptors, which may affect downstream adaptive immune responses and pathogen clearance.

Introduction

Staphylococcus aureus is a common member of the human microbiome and colonizes up to 30% of the population, where it mostly resides in the nares and on the skin [1–3]. *S. aureus* is a leading cause of surgical site infections and skin infections as well as health-care associated pneumonias (Pozzi et al., 2017). Treatment of infections is hampered by the continuous emergence of antimicrobial resistance, most prominently methicillin-resistant *S. aureus* and vancomycin-resistant *S. aureus* [4,5]. Understanding the molecular mechanisms underlying different *S. aureus* infections will support the development of new treatment strategies including vaccines.

Components of the bacterial cell envelope are critical for *S. aureus* host-pathogen interaction, both at the level of colonization but also during systemic infection by evading host immune responses [6]. One of the most abundant and exposed structures on the Gram-positive cell wall is wall teichoic acid (WTA). WTA is a glycopolymer that is covalently bound to peptidoglycan. WTA is critical for *S. aureus* physiology and infection biology through its role in cation sequestration, horizontal gene transfer by bacteriophages and adherence function to human nasal epithelial cells [7–9]. In the majority of *S. aureus* strains, WTA is composed of a poly-ribitolphosphate (RboP) backbone decorated with positively-charged D-alanine and *N*-acetyl-D-glucosamine (GlcNAc) residues. Synthesis of the WTA RboP backbone and its modification is orchestrated by tar genes. *tarM*, *tarS* and *tarP* encode specific glycosyltransferases that catalyze the attachment of GlcNAc residues. TarM adds α-GlcNAc residues at C4 hydroxyl groups of RboP, whereas TarS and TarP modify RboP with β-GlcNAc residues at C4 or C3 hydroxyl groups, respectively [10–12]. The WTA α- and β-GlcNAc modifications impact interactions of *S. aureus* with both innate and adaptive immune components, including mannose binding lectin (MBL), langerin, and antibodies [12–16].

Not all *S. aureus* strains express structurally identical WTA. In contrast to the common RboP-GlcNAc WTA, *S. aureus* isolates of the ST395 lineage produce WTA composed of a poly-glycerolphosphate (GroP) backbone decorated with α-N-acetyl-D-galactosamine (α-GalNAc) residues, which are attached by glycosyltransferase TagN [9,17]. The synthesis of this structurally different WTA impacts recognition and horizontal gene transfer by phages [9,17]. Interestingly, GroP-GalNAc WTA is also produced by several coagulase negative staphylococci (CoNS), which are also common inhabitants of skin but are generally less associate with skin pathologies compared to *S. aureus* [9,17,18].

C-type lectin receptors (CLRs) are a family of pattern-recognition receptors (PRR) that are dedicated to sense both self and non-self glycan structures through their characteristic carbohydrate recognition domains (CRDs) [19]. CLRs have a particular expression pattern on subsets of immune cells. We recently identified that the CLR langerin (CD207), which

is exclusively expressed on Langerhans cells (LCs) in the skin epidermis, interacts with *S. aureus* through WTA β-1,4-GlcNAc, which affects LC responses and skin inflammation in mice [16]. In contrast, *S. aureus* ST395 does not interact with langerin [16]. However, both dermal DCs and dermal macrophages express the trimeric CLR Macrophage Galactose-type Lectin (MGL; CD301), which recognizes terminal GalNAc residues as a result of a Gln-Pro-Asp motif in its CRD [20]. GalNAc is incorporated into, among others, pathogen-produced lipo-oligosaccharides from *Campylobacter jejuni* and *Neisseria gonorrhoeae* [21,22], and confers binding to MGL in a Ca^{2+}-dependent manner, inducing uptake and cellular responses [23]. We therefore hypothesized that *S. aureus* ST395 might also be recognized by MGL via α-GalNAc modifications on WTA and may impact downstream immune responses.

Using recombinant MGL constructs, we demonstrate that human MGL and mouse MGL2 interact with *S. aureus* ST395 WTA in a α-GalNAc- and *tagN*-dependent manner. Interestingly, *tagN*-encoding CoNS did not interact with MGL, although heterologous expression in a *tagN*-deficient *S. aureus* background proves their function as GalNAc transferases. Importantly, loss of *tagN* in *S. aureus* ST395 attenuates production of specific cytokines by human monocyte-derived dendritic cells (moDCs).

Material and methods

Bacterial strains

Bacteria (Supplementary Table S1) were grown either on Todd Hewitt (Oxoid) agar or in Todd Hewitt broth (THB) supplemented with chloramphenicol (Sigma-Aldrich) at a concentration 10 µg/ml when required. For all experiments, bacteria were grown overnight, subcultured the next day in fresh THB and grown to exponential phase (optical density at 600 nm (OD_{600}) = 0.6) for use in experiments.

Molecular cloning

TagN was amplified using primer pair N474-slug-bam (up) (5´-ATCGGATCCAAAGGAGGTATTATAATGGCATTAAAGAAATTTATAATTAATCA-3´) and N474-slug-Eco (dn) (5´-GAGAGAATTCCTATTTAAGTAGCTTATAAAATTCATTA-3´) and genomic DNA of *S. lugdunensis* HKU09-01 as template. The amplicon was cloned into shuttle-vector pRB474 (Bruckner, 1992) via the BamHI and EcoRI restriction sides.

SaPI transfer assay

Lysate of SaPIbov1 (φ187) bearing a tetracyclin resistance marker was generated as previously described [17]. In brief, overnight culture of PS187 SaPIbov1::tet (final OD 0.1) of was incubated with ϕ187 (final concentration of 107 plaque forming units (PFU)/mL) in a final volume of 10 mL TSB for 30 min at 37°C and subsequently at 30°C until visible bacterial

lysis. The obtained lysate was centrifuged and filtered (pore size 0.2-0.45 µm). SaPI transfer was performed by mixing 100 µL of SaPI lysate with 200 µL of stationary bacteria (OD = 0.5) and subsequent incubation for 15 min at 37°C. The mixture was centrifuged for 3 min at 10,000 g and plated on TSA plates supplemented with 3 µg/mL tetracycline. Plates were incubated overnight at 37°C and transductants were enumerated.

WTA isolation and analysis by PAGE

WTA was isolated as previously described [9]. Briefly, overnight culture of *S. aureus* PS187 was grown in BM (0.5 % w/v yeast extract; 1% w/v Soy peptone; 0.5% NaCl; 0.1 % K_2HPO_3) supplemented with 0.25% w/v glucose was harvested by centrifugation and washed using ammonium acetate buffer (AAB, 20 mM, pH 4.8). Bacterial cells were opened using a Euler cell mill (2.5 mL AAB/ 4.5 glass beads/ 1 g cell pellet). The obtained lysate was digested overnight with RNAse and DNAse at 37°C, subsequently treated by ultrasonification, and incubated with 2% SDS for 1 hour at 60°C. Purified peptidoglycan was washed extensively with AAB. WTA was released by 5% TCA treatment for 4 h at 60°C. The supernatant was neutralized using NaOH and dialyzed against ddH_2O.

PAGE analysis of WTA occurred as previously described [10]. WTA samples (400 nmol phosphate) were applied to a polyacrylamide gel (26%) and separated electrophoretically for 13h at 25 mA. WTA bands were visualized using Alcian blue (0.005%) in staining solution (40% ethanol, 5% acetic acid).

Lectin binding assay

Bacteria were harvested by centrifugation (4,000xg, 10 min) and re-suspended to OD_{600} of 0.4 in TSM buffer (20 mM Tris (Roche), 150 mM NaCl (Sigma-Aldrich), 2 mM $CaCl_2 \cdot 2\ H_2O$ (Merck), 2 mM $MgCl_2 \cdot 6\ H_2O$ (Merck), pH 7.0) with 0.1% bovine serum albumin (BSA, Merck). Bacteria were incubated with 5 µg/ml of recombinant human MGL-his (R&D Systems), 10 µg/ml of recombinant mouse MGL2-his (R&D Systems), 4 µg/ml of SBA-FITC (soy bean agglutinin, Vector Laboratories) or 2 µg/ml of sWGA-FITC (succinylated wheat germ agglutinin, Vector Laboratories). Binding of recombinant human MGL and murine MGL2 was detected using anti-hisTag FITC-conjugated antibodies (LifeSpan BioSciences). For blocking, we used soluble N-acetyl-D-galactosamine (Fluka, Sigma-Aldrich) or glucose (Merck) at 50 mM. Samples were analyzed using flow cytometry (FacsVerse, BD Biosciences).

Isolation of human monocytes and differentiation to immature dendritic cells

Buffy coats from healthy anonymous donors were purchased from Sanquin Amsterdam and obtained according to the good clinical practice in accordance with the declaration of Helsinki. Donors have given their written consent to the study. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats using Ficoll-Paque PLUS (GE

Healthcare) density gradient and monocytes were obtained as described in [24]. Briefly, harvested PBMCs were washed twice with RPMI 1640 (Lonza) supplemented with 5% fetal bovine serum (FBS, Biowest). Monocytes were further isolated from the PBMC fraction using density gradient of 60%, 47.5% and 34% Percoll (Sigma-Aldrich) in RPMI 1640 + 10% FBS. Harvested monocytes were washed three times with RPMI 1640 + 5% FBS and incubated at the concentration $0.5x10^6$ cells/ml with differentiation medium consisting of RPMI 1640 supplemented with 10% HyClone FBS (GE Healthcare), 800 IU/ml GM-CSF (Bio Connect), 250 IU/ml IL-4 (Thermo Fisher Scientific), 100 IU/ml penicillin-streptomycin, 2.4 mM L-glutamine for 5 to 7 days to obtain immature DCs.

Binding of FITC labeled bacteria to moDCs

To perform bacteria binding assays, *S. aureus* strains were labelled with FITC (fluorescein isothiocyanate, Sigma-Aldrich). Five ml of bacterial culture in exponential phase were pelleted and re-suspended in cold PBS with 0.1% BSA. Bacteria were incubated with 0.5 mg/ml FITC for 30 minutes on ice protected from light. Bacteria were washed three times with cold PBS + 0.1% BSA supplemented with 1% ammonia and re-suspended in TSM + 0.1% BSA at OD_{600} of 0.4.

Immature moDCs were harvested by centrifugation and re-suspended in TSM + 0.1% BSA (1x106 cells/ml). Cells were incubated with bacteria at 1:2, 1:5, 1:10 and 1:20 cell to-bacteria ratios in a 96-well round bottom plate for 30 minutes in 4°C protected from light. For blocking, cells were pre-incubated for 15 min at room temperature with 1 mM EGTA (Brunschwig Chemie), 50 mM GalNAc (Fluka, Sigma-Aldrich), or 50 mM glucose (Merck). Next, cells were incubated with bacteria at 1:10 cell-to-bacteria ratio for 30 minutes at 4°C, protected from light. Samples were washed with TSM + 1% BSA, fixed using 1% formaldehyde in PBS and analyzed using flow cytometry. Microscopy pictures were prepared using 1:50 cell to bacteria ratio suspensions. Cells were attached to the glass slides using a Shandon Cytospin 3 centrifuge. Cellular membranes were stained using WGA-Alexa Fluor 647 (Thermo Fisher Scientific), cell nucleus with DAPI (Sigma-Aldrich). Samples were fixed with 1% formaldehyde (Merck) in PBS (Lonza) and cover slides were attached with mounting medium. Samples were analysed using confocal laser scanning microscopy (SP5, Leica).

Stimulation of moDCs with gamma-irradiated bacteria

S. aureus strains at exponential growth phase were washed with PBS and re-suspended in PBS with addition of glycerol. Gamma-irradiation of bacteria was performed by Synergy Health Ede B.V., a STERIS company (Ede, The Netherlands) and loss of viability was verified by culture. Concentrations of all bacterial suspensions were measured using MACSQuant Analyzer 10.

Immature moDCs were harvested, washed and re-suspended in RPMI + 5% FBS. Before use, cells were stained for expression of MGL and maturation markers using MGL-PE, CD80-PE, CD83-APC, CD86-APC (all SONY Biotechnology), CD40-FITC and HLA-DR-APC (both BD Biosciences) antibodies and their corresponding isotype controls (BD Biosciences), diluted according to the manufacturers' instructions. Samples were analyzed using flow cytometry. Bacteria were diluted in RPMI + 5% FBS and mixed with 0.5x105 immature moDCs in 1:2, 1:5 and 1:10 cell-to-bacteria ratios. Suspensions were incubated in Corning 96-well Round Bottom Ultra-Low Attachment plates (Sigma-Aldrich) for 16 h at 37°C with 5% CO_2. For blocking, moDCs were incubated for 16 h with *S. aureus* PS187 WT at 1:2, 1:5 and 1:10 cell-to-bacteria ratios in the presence of anti-MGL blocking antibodies (ASGPR/MGL, clone 125A10.03, Dendritics) or isotype control antibodies (produced and purified in-house) at the concentration 10 µg/ml. Supernatants were collected after centrifugation and cells from 1:10 cell-to-bacteria conditions were stained as described previously. IL-8, IL-12 and TNFα concentrations of the collected supernatants were analyzed by Luminex assay.

Statistical analysis

Data obtained from flow cytometry was analyzed using FlowJo 10 (FlowJo LLC). Statistical analysis of data was performed using GraphPad Prism 7.02 (GraphPad Software). One-way ANOVA followed by Dunnett's or Tukey's test or two-way ANOVA followed by Tukey's test were performed. Only significant differences between samples ($p < 0.05$) were indicated on graphs.

Results

Human MGL interacts with *S. aureus* ST395 strains in a *tagN*-dependent manner

Human MGL is the only CLR family member with specificity for α-GalNAc [25]. Since the *S. aureus* ST395 lineage produces GalNAc-decorated WTA, we investigated whether *S. aureus* ST395 was recognized by human MGL. Using flow cytometry, we tested multiple *S. aureus* isolates from the ST395 lineage for binding to recombinant soluble his-tagged MGL. All strains of this lineage bound MGL, whereas no interaction was observed with USA300 and Newman strains (non-ST395 strains), which both express RboP-GlcNAc WTA (Figure 1A). Interestingly, the levels of MGL binding varied for different ST395 strains (Figure 1A), likely reflecting different expression levels of the MGL ligand. Addition of soluble GalNAc prevented interaction of MGL with ST395 strain PS187, whereas similar levels of glucose did not affect binding (Figure 1B), indicating that binding occurs through the MGL CRD. To confirm that the interaction between *S. aureus* ST395 and MGL depends on WTA GalNAc, we assessed binding of MGL to wildtype (WT) PS187 and the isogenic mutant strain GN1, which lacks the C-terminal glycosyltransferase domain of *tagN* and is consequently deficient for WTA α-GalNAc [17]. MGL binding was lost in the *tagN*-deficient mutant and could be restored by complementation with plasmid-expressed full-length *tagN* (Figure 1C), confirming that WTA α-GalNAc of *S. aureus* ST395 is the ligand of MGL.

We have previously observed that langerin shows a certain level of species specificity, i.e. mouse langerin does not interact with *S. aureus* [16]. Therefore, we investigated interaction of PS187 with mouse homologue MGL2 [26,27]. Like human MGL, mouse MGL2 interacted with PS187, could be blocked with GalNAc and interaction was lost upon deletion of *tagN* (Figure 1D, E), suggesting that the interaction is, at least partially, conserved across species.

S. lugdunensis tagN encodes a GalNAc-transferase that produces a MGL ligand

Similar to *S. aureus* ST395 WTA, certain CoNS species express GroP-type WTA. In addition, several CoNS species express homologues of the *tagN* gene, suggesting that CoNS may decorate WTA in a similar fashion as *S. aureus* ST395 strains [17]. Indeed, complementation of PS187 GN1 with a *tagN* homolog from Staphylococcus *carnosus* restores GalNAc glycosylation and phage susceptibility [17]. Similarly, we were able to confer susceptibility to phage ϕ187 by complementing the GN1 mutant, for which no transductants were obtained, with *tagN* from *Staphylococcus lugdunensis* (Figure 2A). In addition, the WTA migration of this complemented strain was similar to that of WTA from PS187 WT (Figure 2B). Importantly, heterologous expression of *S. lugdunensis tagN* in PS187 GN1 also restored binding to MGL (Figure 2C). In contrast, none of the CoNS species that contain a *tagN* homolog interacted with MGL (Figure 2D), despite reactivity with the GalNAc-specific plant lectin SBA for

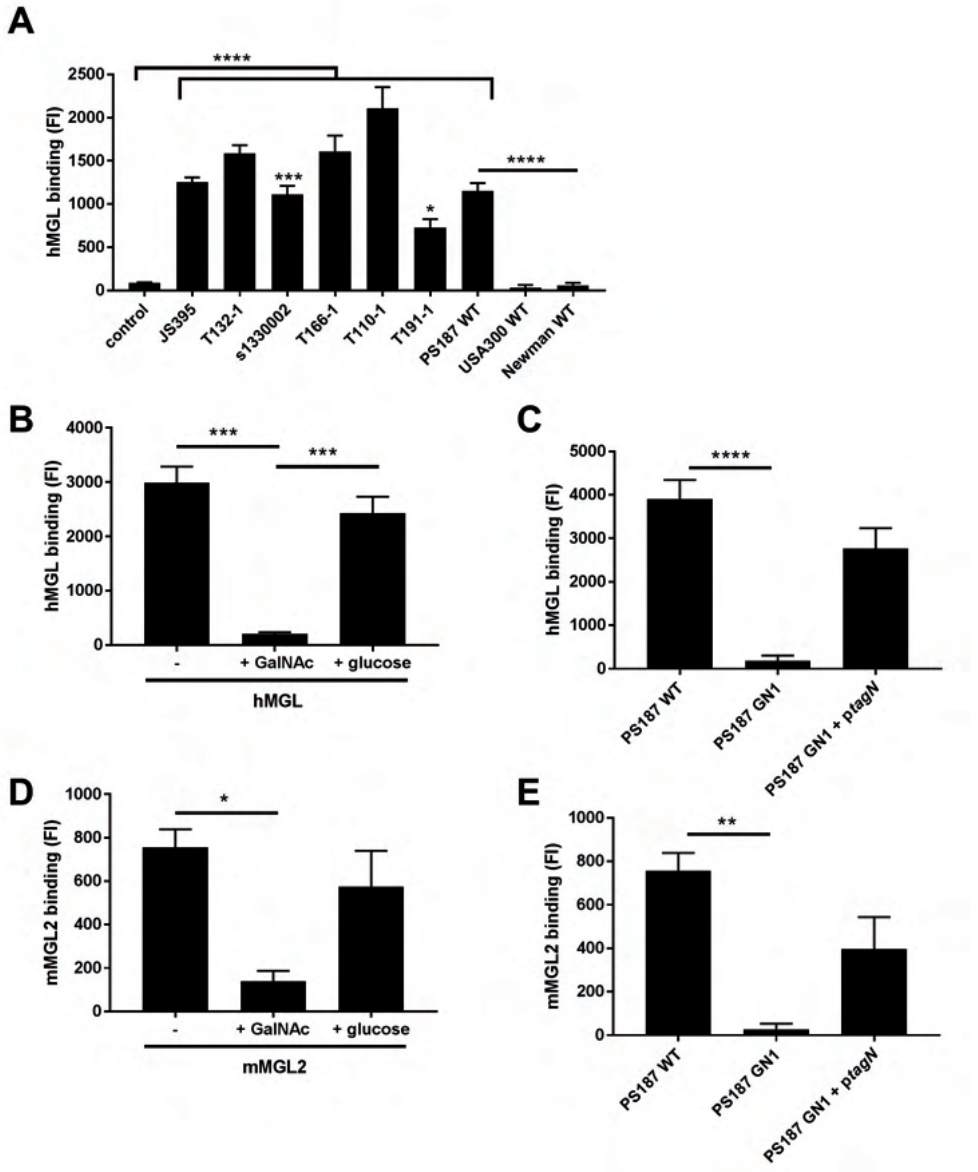

Figure 1. Human and mouse MGL interact with *S. aureus* ST395 strains in a *tagN*-dependent manner. (A) hMGL binding to different *S. aureus* ST395 lineage strains, USA300 WT and Newman WT detected by anti-hisTag-FITC antibody. Control represents *S. aureus* PS187 WT incubated with secondary detection antibody. **(B, D)** Interaction between **(B)** hMGL or **(D)** mMGL2 to *S. aureus* PS187 WT in the absence or presence of GalNAc (50 mM) or glucose (50 mM). **(C, E)** Binding of **(C)** hMGL or **(E)** mMGL2 to PS187 WT, GN1, GN1+p*tagN* and two non-ST395 strains. Means of geometric mean fluorescence intensity ± SEM from three independent experiments are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$.

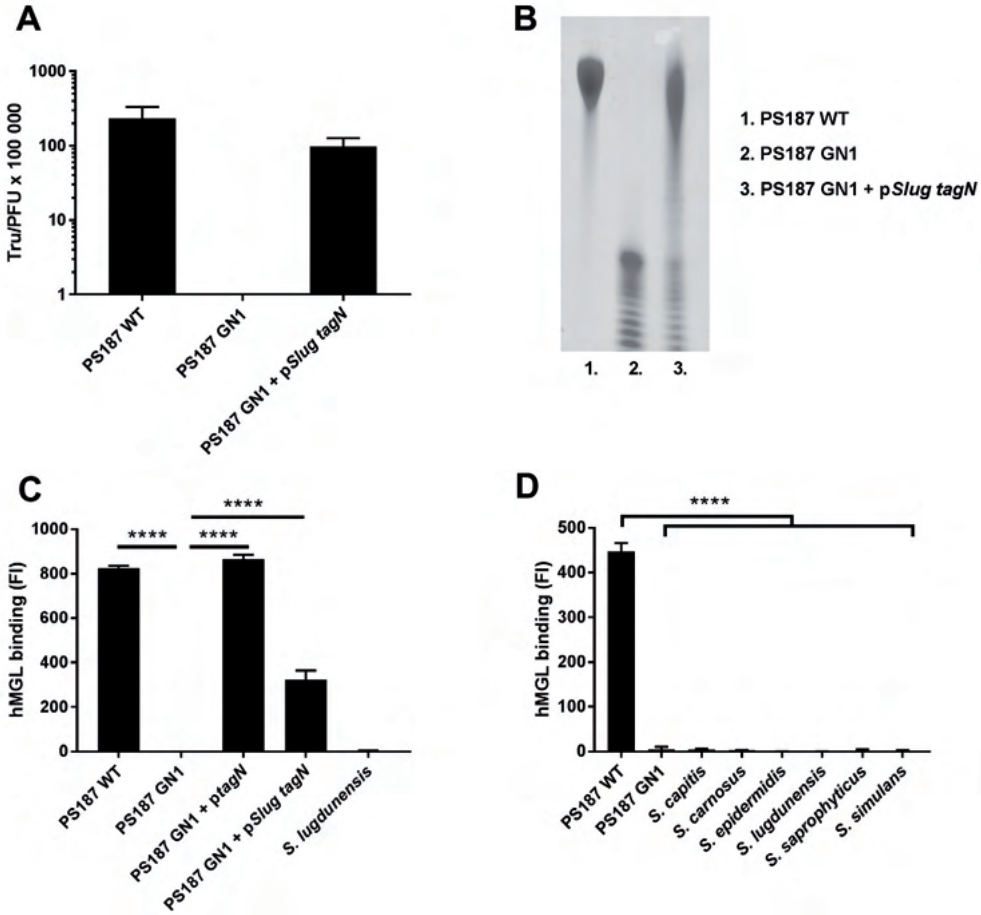

Figure 2 *S. lugdunensis tagN* encodes a GalNAc-transferase that produces a MGL ligand. (A) Transfer of SaPI BovI via phage ϕ187 into PS187 WT, GN1 mutant and GN1 complemented with *tagN* from *S. lugdunensis* (p*Slug tagN*). Values are displayed as transductants per Plaque Forming Units (TrU/PFU). In case of GN1 no transductants were obtained. **(B)** PAGE analysis of WTA from *S. aureus* PS187 WT, GN1 mutant and GN1 complemented with *tagN* from *S. lugdunensis* (p*Slug tagN*). **(C)** Binding of hMGL to *S. aureus* PS187 WT, GN1 mutant and GN1 complemented with either PS187 *tagN* (p*tagN*) or p*Slug tagN*. **(D)** Interaction of different CoNS species with hMGL. Means of geometric mean fluorescence intensity ± SEM from three independent experiments are shown. **** $p < 0.0001$.

S. carnosus, *S.* capitis and *S.* saprophyticus (Supplementary Figure S1). These data suggest that *S. lugdunensis tagN* encodes a GalNAc transferase. However, it is likely not or only lowly expressed in *S. lugdunensis* in our culture conditions.

S. aureus PS187 interacts with and activates human monocyte-derived dendritic cells

MGL is expressed on a range of immune cells including human dendritic cells (DCs) and macrophages residing in skin and lymph nodes, blood CD1c$^+$ DCs and immature monocyte-

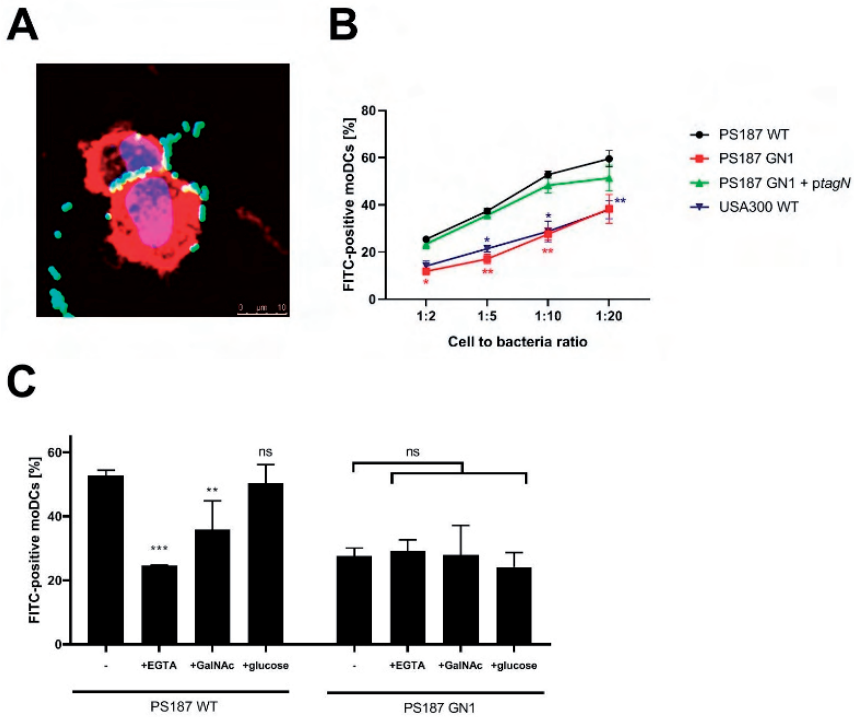

Figure 3. WTA-GalNAc contributes to interaction between human moDCs and *S. aureus* PS187. (A) Binding of FITC-labeled *S. aureus* PS187 WT (green) to immature moDCs (membrane in red, nucleus in blue). Cytospin samples were prepared from cell suspensions incubated with bacteria in 1:50 ratio for 30 min. **(B)** Binding of FITC-labeled *S. aureus* strains to moDCs at different cell-to-bacteria ratios after 30 minutes of incubation. Data are presented as mean ± SEM of percentage of FITC-positive moDCs (n = 3). Significance shown as compared to binding of PS187 WT to moDCs within certain ratio in red for dGN1 mutant and in blue for UAS300 WT. **(C)** Binding of FITC-labeled *S. aureus* PS187 WT and GN1 to moDCs in 1:10 cell-to-bacteria ratio after 30 minutes in the absence or presence of 1 mM EGTA, 50 mM GalNAc or 50 mM glucose (control). Data are presented as a mean ± SEM of percentage of FITC-positive moDCs (n = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

derived dendritic cells (moDCs) [28–30]. To investigate the interaction of MGL with *S. aureus* ST395 strains in a more biologically relevant system, we used a cell-based assay with human immature moDCs. FITC-labeled *S. aureus* PS187 WT bound readily and in a ratio-dependent manner to moDCs (Figure 3A, B). Interestingly, binding was reduced for the *tagN*-deficient mutant and USA300 strains, which both do not express GalNAc on their surface (Figure 3B). Binding to moDCs was restored to WT levels in the *tagN*-complemented strain (Figure 3B). Complementary, we assessed the effect of different blocking agents, i.e. EGTA, GalNAc and glucose (as a control; Figure 3C). Binding of PS187 WT, but not of the GN1 mutant, was reduced upon co-incubation of EGTA and GalNAc, but not glucose (Figure 3C). These data demonstrate that the PS187-moDC interaction is partially preventable by addition of GalNAc or calcium scavenging, which is in line with a possible role for MGL.

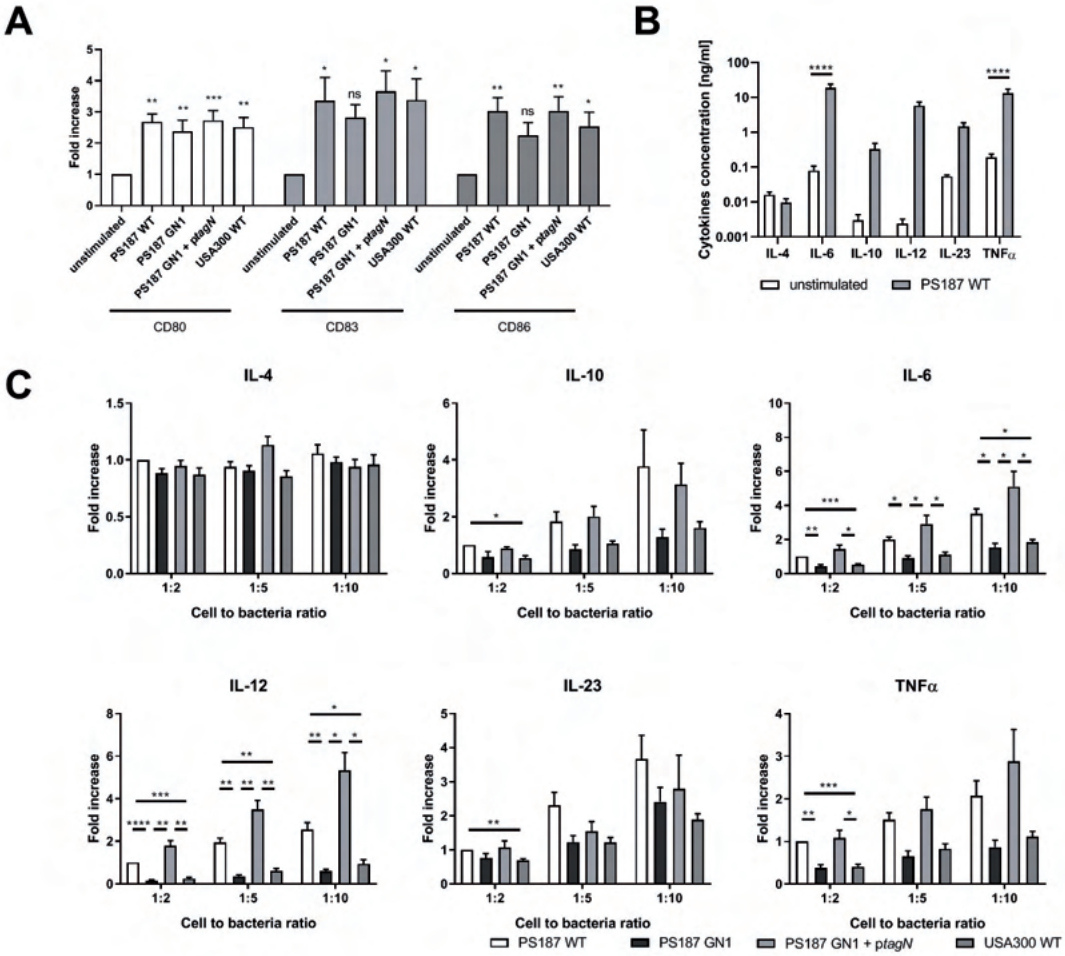

Figure 4. Human moDCs are activated by *S. aureus* PS187 and cytokine production is affected by WTA-GalNAc. (A) Relative expression of surface maturation markers on moDCs after stimulation with gamma-irradiated *S. aureus* strains at cell-to-bacteria ratio of 1:10 for 16h. Data are presented as fold change ± SEM relative to unstimulated control (n = 3 donors). **(B)** Cytokine expression by moDCs after 16 h of incubation without or with gamma-irradiated *S. aureus* PS187 WT in 1:10 cell-to-bacteria ratio. Data are presented as mean of cytokine concentration ± SEM (n = 6 donors). **(C)** Cytokine expression by moDC after incubation with gamma-irradiated *S. aureus* strains in 1:2, 1:5 and 1:10 cell-to-bacteria ratio for 16h. Data are presented as mean of fold increase over PS187 WT 1:2 ± SEM (n = 6 donors). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$.

Loss of interaction with MGL may affect immune activation of moDCs, such as expression of co-stimulatory molecules or cytokine production, resulting in different immunological responses. We therefore investigated moDCs maturation and cytokine production after stimulation with gamma-irradiated *S. aureus* PS187 WT, GN1, *tagN*-complemented GN1 or USA300 WT for 16 h. moDCs upregulated maturation markers CD80, CD83, CD86 and CD40, indicating that all *S. aureus* strains activate moDCs (Figure 4A, Supplementary Figure S2).

We observed little effect on expression of HLA-DR except with PS187 WT (Supplementary Figure S2). However, there was no difference in the induction of moDC maturation by the different *S. aureus* strains (Figure 4A, Supplementary Figure S2). We also analyzed moDC cytokine production. *S. aureus* PS187 WT induced expression of IL-6, IL-12p70, IL23p19, IL-10 and TNFα, but not IL-4 when incubated with moDCs (Figure 4B). Interestingly, at a cell-to-bacteria ratio of 1:2, cytokine production was significantly lower when strains did not produce GalNAcylated WTA, i.e. PS187 GN1 and USA300 WT (Figure 4C). At higher ratios, this difference was robust for IL-6 and IL12p70 and trends remained for IL-10, IL23p19 and TNFα (Figure 4C). Cytokine production by moDCs was restored to PS187 WT levels in cells stimulated with the *tagN*-complemented strain (Figure 4C). Overall, these data indicate that the production of select pro-inflammatory cytokines, i.e. IL-6 and IL12p70, by moDCs is enhanced by recognition of the α-GalNAc modifications present on *S. aureus* PS187 WTA.

To determine whether differences in cytokine production are not just WTA GalNAc-dependent but also MGL-dependent, we attempted to block the interaction using a commercially available anti-MGL blocking antibody. These experiments are technically complicated by the presence of Protein A (SpA) and Sbi on the *S. aureus* surface, as these proteins bind IgG Fc, thereby possibly increasing DC interaction by binding to the blocking antibody. moDC cytokine production in response to PS187 WT was not affected by the presence of either the blocking antibody or the isotype control antibody compared to bacteria alone (Supplementary Figure S3). We confirmed that incubation of the antibodies with moDCs by itself did also not significantly affect cytokine production (Supplementary Figure S4). Therefore, we are currently unable to prove that moDC cytokine production in response to *S. aureus* PS187 occurs through MGL.

Discussion

Here, we show the molecular interaction between WTA of *S. aureus* ST395 and MGL, an innate receptor of the CLR family. This interaction is dependent on α-GalNAc modifications of *S. aureus* WTA and contributes to increased cytokine production in MGL-expressing moDCs. Although Winstel et al. showed the importance of *S. aureus* GalNAc glycosylation for phage infection [17], there was no previous indication for interaction with human receptors. Since the ST395 lineage is present in nasal and blood culture isolates [31], interaction with MGL may be biologically relevant in context of recognition and clearance by the immune system.

This is the first identification of MGL interaction with a Gram-positive bacterium. Previous studies have identified MGL ligands on the surface of Gram-negative pathogens, including *C. jejuni*, *N. gonorrhoeae* and *Escherichia coli* strain R1 [21,22,32]. For *E. coli* strain R1 soluble

LOS was identified as a ligand for recombinant human MGL, although no functional consequences were assessed [32]. For *C. jejuni*, MGL ligands are GalNAc residues incorporated in LOS and N-glycosylated proteins [21]. For *N. gonorrhoeae*, the ligand is a terminal GalNAc residue on the lipo-oligosaccharide (LOS) of phenotype C strains, which influenced binding to moDCs and subsequent T helper differentiation[22]. Similarly, our data show that loss of GalNAc on the *S. aureus* surface decreases binding to moDCs, which corresponds with assays using recombinant MGL. Importantly, binding of GN1 to moDCs could be restored by *tagN* complementation, suggesting that one of the involved receptors is MGL. Experiments using the calcium chelator EGTA and GalNAc monosaccharide also decreased moDC binding, although GalNAc had only a modest inhibiting effect. This may implicate the involvement of other calcium-dependent, GalNAc-independent receptors in the interaction between *S. aureus* PS187 and DCs.

Presence of the GalNAc-WTA epitope also affected DC cytokine production, especially increasing production of IL-6 and IL-12p70 across the tested range of bacteria-to-cell ratios. This is in contrast to observations with *C. jejuni*, where absence of the MGL ligand on glycosylated proteins actually increased DC cytokine production, suggesting that MGL triggering dampened immune activation. These contrasting outcomes suggest that the context in which MGL is engaged influences how MGL affects DC responses. Indeed, previous reports have indicated that MGL triggering alone does not induce high cytokine secretion by CD1c^{+} DCs but requires co-stimulation with Toll-like receptor ligands to differentially affect IL-8, IL-10 and TNFα production [30,33]. Since Toll-like receptor ligands differ between Gram-positive and Gram-negative bacteria, this may explain the different effects on DC cytokine production that we observe here. Alternatively, we can speculate that observed differences in DC cytokine production are not completely MGL-dependent. It cannot be excluded that additional receptors were triggered in the absence of WTA-GalNAc as a result of newly exposed structures on the *S. aureus* surface. Additionally, other receptors may be more important for induction of cytokines, which is also implied by the experiments using anti-MGL blocking antibodies, which did not affect cytokine production.

It has been well established that MGL binds to galactose- and GalNAc-rich terminal motifs in a Ca^{2+}-dependent manner [25,34]. These modifications are often found in the extracellular matrix of host tissues. Interaction of DCs with extracellular matrix via MGL inhibits their migration from dermis to the lymph nodes. Therefore, the presence of MGL-expressing DCs in the skin might be explained by the abundance of GalNAc epitopes in dermal tissues [35]. Recognition of GalNAc-decorated bacteria, such as *S. aureus* PS187, by dermal DCs can disrupt the interaction with the extracellular matrix, which consequently would allow DCs to migrate to the lymph nodes to present antigen. In addition, the observation that the detection of GalNAc-epitopes enhances cytokine production additionally suggests that

this interaction is an immune defense strategy and likely not a part of the immune evasion repertoire of *S. aureus*.

When dermal DCs encounter pathogens and migrate to the lymph nodes to present antigens, the final step is to develop an adaptive immune response to eradicate these pathogens. Previous studies have demonstrated high levels of IgG antibodies against WTA GlcNAc modifications in human serum, indicating the importance of this epitope in adaptive immunity. Specifically, the anti-WTA β-1,4-GlcNAc antibodies induce complement activation and opsonophagocytosis of *S. aureus* strains expressing a RboP-GlcNAc WTA [15]. Since *S. aureus* ST395 expresses an alternative WTA structure with a different glycosylation pattern, it will be of interest to study whether antibody responses are mounted against this specific WTA glycoepitope and whether these antibodies facilitate *S. aureus* phagocytosis and killing.

Surprisingly, our data did not show any binding of MGL to CoN*S*. This could simply reflect lack of *tagN* gene expression under the tested growth conditions. Alternatively, it may indicate that additional factors such as epitope density, capsule expression or overall accessibility prevent MGL interaction, which are potentially different in *S. lugdunensis* compared to *S. aureus*. The observation that heterologous expression under a constitutive promoter confers both susceptibility to phage ϕ187 as well as MGL binding does not exclude either possibilities, but does confirm that *tagN* from *S. lugdunensis* encodes an α-GalNAc transferase. The observed discrepancy between SBA and MGL binding to several CoNS suggests that, despite high homology between *S. aureus* and CoNS *tagN* genes, the pattern or specificity of WTA GalNAc decoration may be slightly different, thereby preventing interaction for MGL.

In summary, we have demonstrated that *S. aureus* ST395 lineages engage the receptor MGL and induce maturation and cytokine production of human DCs, which is partially dependent on expression of WTA-GalNAc. Together with the previous findings that RboP-GlcNAc interacts with langerin, these findings create an overall view that the *S. aureus* WTA glycosylation profile dictates the interaction with specific innate immune receptors on antigen-presenting cells, which may have important consequences for immune defense and pathogen clearance.

Acknowledgments

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Author Contributions

M.E.M., R.v.D., A.P. and N.M.v.*S.* planned the experiments. M.E.M. and D.G. performed the experiments and analyzed the data. M.E.M. performed statistical analysis. G.X. analyzed *S. lugdunensis* genome, D.G. did molecular cloning, SaPI transfer assay and WTA isolation and Page analysis, A.P. provided the bacterial strains. M.E.M. and N.M.v.*S.* wrote the manuscript. All authors revised and approved the manuscript.

Competing interests

M.E.M is a Ph.D. fellow and is enrolled in the Infection and Immunity Ph.D. program, part of the Graduate school of Life Sciences at the University of Utrecht and participated in a post graduate studentship program at GSK.

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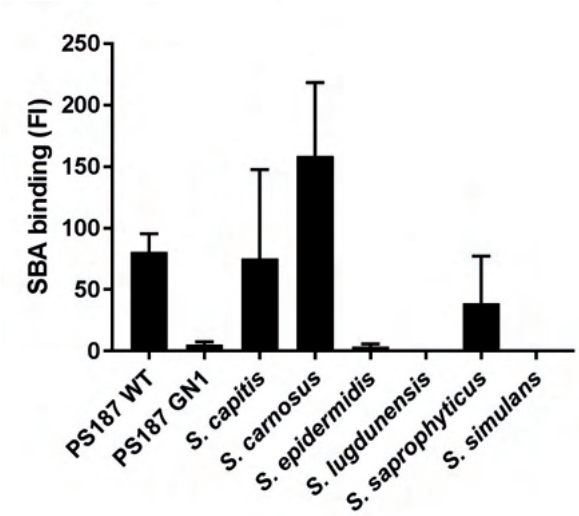
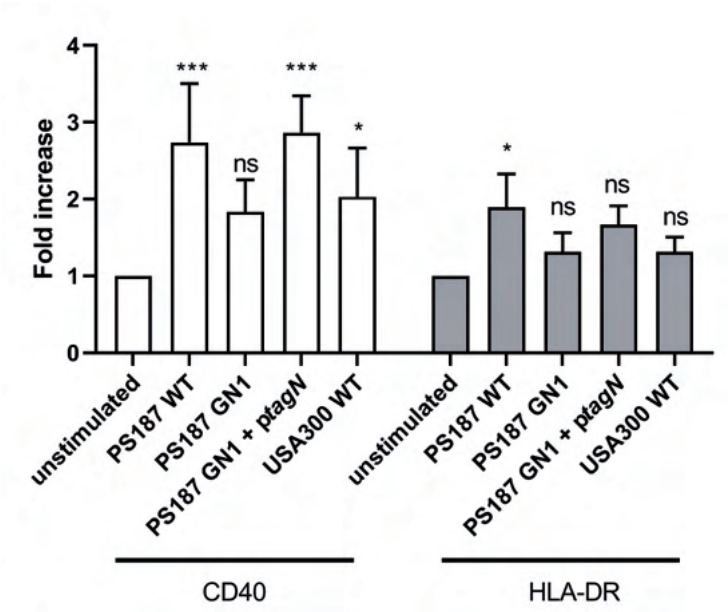
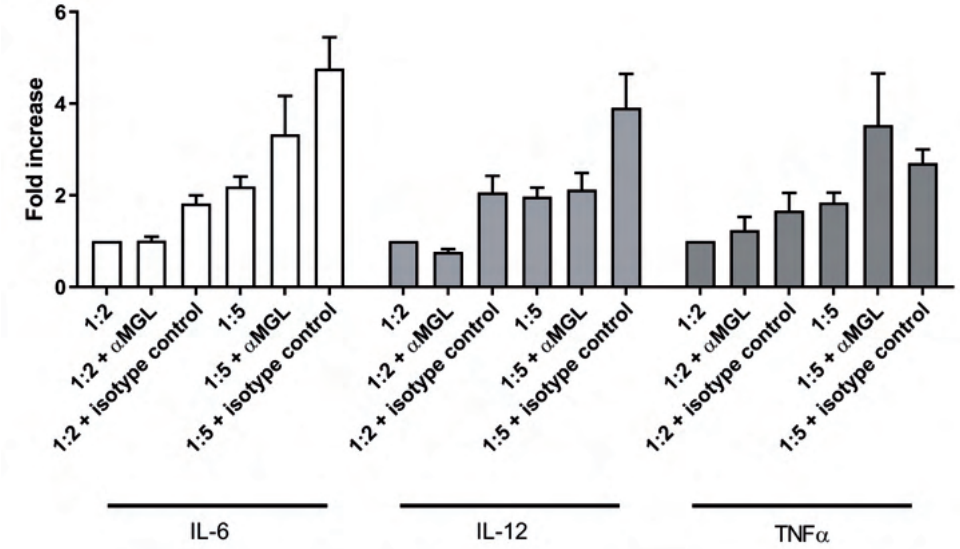
Supplementary Figures



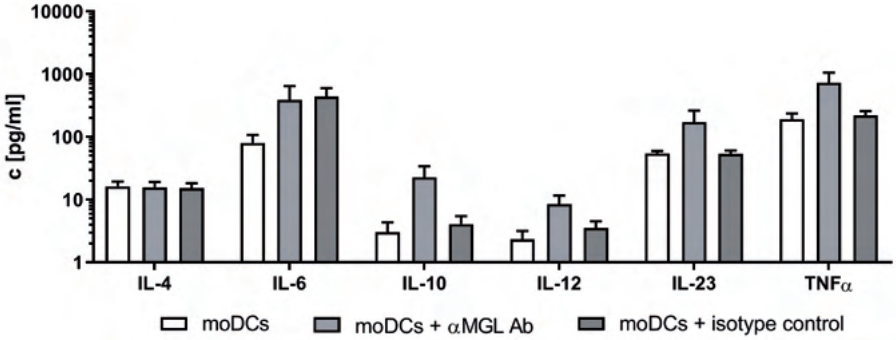
Supplementary Figure S1. Binding of FITC-labeled soy bean agglutinin (SBA) to *S. aureus* PS187 WT, GN1 mutant and coagulase negative staphylococci. Bars represent mean of fluorescence intensity ± SEM from three independent experiments.



Supplementary Figure S2. Relative expression of surface maturation markers CD40 and HLA-DR on human moDCs 16 h after stimulation with gamma-irradiated *S. aureus* strains in 1:10 cell-to-bacteria ratio. Data are presented as fold change in fluorescence intensity ± SEM relative to unstimulated control.



Supplementary Figure S3. Production of IL-6, IL-12p70 and TNFα by human moDCs 16 h after stimulation with gamma-irradiated *S. aureus* PS187 WT in the absence or presence of anti-MGL blocking antibody (αMGL) or isotype control antibody. Data are presented as fold increase over 1:2 cell-to-bacteria ratio for each cytokine. Mean ± SEM from three independent experiments using three different donors are shown.



Supplementary Figure S4. Production of IL-4, IL-6, IL-10, IL-12p70, IL-23p19 and TNFα by human moDCs after 16 h incubation in the absence or presence of anti-MGL blocking antibody (αMGL) or isotype control antibody. None of the cytokines is significantly affected by presence of the antibodies.

Supplementary Table 1. Bacterial strains used in this study

Strain	Source
S. aureus Newman wild type (ST254, CC8)	ATCC, Cat#13420
S. aureus USA300 wild type (NRS384, ST8, CC8)	NARSA strain collection
S. aureus PS187 wild type (ST395, CC395)	ATCC, Cat#15564
S. aureus PS187 GN1	(Winstel *et al.*, 2014)
S. aureus PS187 GN1 + pRB *tagN*	(Winstel *et al.*, 2014)
S. aureus PS187 GN1 + pRB *S. lugdunensis tagN*	This study
S. aureus JS395 wild type (ST395, CC395)	(Francois *et al.*, 2007)
S. aureus T132-1 wild type (ST395, CC395)	(Holtfreter *et al.*, 2007)
S. aureus s1330002 wild type (ST395, CC395)	(Holtfreter *et al.*, 2007)
S. aureus T166-1 wild type (ST395, CC395)	(Holtfreter *et al.*, 2007)
S. aureus T110-1 wild type (ST395, CC395)	(Holtfreter *et al.*, 2007)
S. aureus T191-1 wild type (ST395, CC395)	(Holtfreter *et al.*, 2007)
S. capitis ATCC27840 wild type	ATCC, Cat#27840
S. carnosus TM300 wild type	(Rosenstein *et al.*, 2009)
S. epidermidis 1457 wild type	(Mack *et al.*, 1992)
S. lugdunensis SL13 wild type	(Chassain *et al.*, 2012)
S. lugdunensis HKU09-01	(Tse *et al.*, 2010)
S. saprophyticus ATCC 35552 wild type	ATCC, Cat#35552
S. simulans ATCC 27848 wild type	ATCC, Cat#27848
S. aureus PS187 SaPIbovI (tst::tet)	(Winstel *et al.*, 2014)

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CHAPTER 3

Staphylococcus aureus-specific tissue-resident memory CD4$^+$ T cells are abundant in healthy human skin

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Abstract

The skin is an immunocompetent tissue that harbors several kinds of immune cells and a plethora of commensal microbes constituting the skin microbiome. *Staphylococcus aureus* is a prominent skin pathogen that colonizes a large proportion of the human population. We currently have an incomplete understanding of the correlates of protection against *S. aureus* infection, however genetic and experimental evidence has shown that $CD4^+$ T cells play a key role in orchestrating a protective anti-*S. aureus* immune response. A high *S. aureus*-specific memory $CD4^+$ T cell response has been reported in the blood of healthy subjects. Since T cells are more abundant in the skin than in blood, we hypothesized that *S. aureus*-specific $CD4^+$ T cells could be present in the skin of healthy individuals. Indeed, we observed proliferation of tissue-resident memory $CD4^+$ T cells and production of IL-17A, IL-22, IFN-γ and TNF-β by cells isolated from abdominal skin explants in response to heat-killed *S. aureus*. Remarkably, these cytokines were produced also during an *ex vivo* epicutaneous *S. aureus* infection of human skin explants. These findings highlight the importance of tissue-resident memory $CD4^+$ T cells present at barrier sites such as the skin, a primary entry site for *S. aureus*. Further phenotypical and functional characterization of these cells will ultimately aid in the development of novel vaccine strategies against this elusive pathogen.

Introduction

The skin provides a physical and immunological barrier for invading pathogens, while also maintaining symbiotic interactions with skin commensals. There are numerous specialized immune cells present in the skin that maintain skin homeostasis and act as the first line of defense against pathogens. It has been estimated that human skin contains roughly twice as many memory T cells than blood [1]. Different memory T cell subsets can be phenotypically identified in human skin based on the presence of surface markers and the capacity to emigrate and enter the circulation. Tissue resident memory T (Trm) cells are a subset of memory T cells phenotypically and functionally distinct from their circulating counterparts [1,2]. In particular, human skin-resident memory T (Tsrm) cells can be identified through the surface expression of the skin-homing marker cutaneous lymphocyte-associated antigen (CLA), the memory T cell marker CD45RO and the tissue-retention marker CD69. CLA binds selectively and avidly to the vascular lectin E-selectin while CD69 prevents sphingosine-1-phosphate receptor 1 mediated egress from tissues into the circulation [3]. Skin-resident T cell memory has been observed in response to *Candida albicans*, *Leishmania major*, Herpes simplex virus as well as commensal bacteria. Most importantly, Tsrm cells contribute to localized protection against re-infection with cutaneous pathogens [4–9]. In addition, Trm cell development has been tracked in mice following vaccination and was positively correlated with vaccination efficacy [10–13], making Trm cells a promising target for vaccination [14–19].

The Gram-positive bacterium *Staphylococcus aureus* is the leading cause of skin and soft tissue infections globally [20]. In addition, the rapid emergence of antibiotic resistance has highlighted the need for alternative treatments such as vaccination to combat *S. aureus* infections. However, to design an efficacious vaccine, it is important to have a complete understanding of the correlates of protection against this pathogen, which is currently lacking [21,22].

Based on data from mouse and human studies, there is a general consensus that CD4+ T cells, and in particular Th17 and Th1 subsets, contribute to protective immunity against *S. aureus* infection [23–26]. Furthermore, healthy individuals have a considerable number of circulating memory CD4+ T cells specific for *S. aureus*, likely due to repeated encounters over time with this skin pathobiont [27,28]. However to our knowledge, the existence of *S. aureus*-specific tissue resident memory CD4+ T cells in healthy human skin has not yet been addressed.

Using human skin explants, which represents a valuable model to study skin-resident immune responses of human skin to microbes [29], we here show that *S. aureus*-specific CD4+ Tsrm cells are commonly found in the skin of healthy individuals. This finding uncovers CD4+ Tsrm cells as previously neglected cellular players in the cutaneous human immune

response to *S. aureus* and thus may aid in the development of novel vaccine strategies against *S. aureus* SSTIs.

Materials and Methods

Preparation of single cell suspensions from human skin explants

Fresh human skin explants (16 cm^2) derived from abdominoplasty surgical waste of healthy women (age 40 ± 11, body mass index 25 ± 3) were purchased from Biopredic (France). Explants were shipped at 4°C and received within 48h following the surgery. Upon arrival, samples were immediately processed as shown in Figure 1A. In short, adipose tissue was removed with dissection scissors followed by additional scraping with a disposable scalpel (Swann-Morton, Sheffield). Skin was cut in 1 cm^2 pieces, washed repeatedly with PBS and incubated for 1h at RT in RPMI-1640 (Invitrogen) containing 1 mg/ml collagenase type 1 (Life technologies). Next, skin pieces were extensively minced with disposable scalpels and incubated overnight at 37°C at 5% CO_2 in a 6-well plate with 1 mg/ml collagenase type 1 (Life technologies) and 20 µg/ml DNAse (Sigma) in 5 ml c-RPMI (RPMI-1640 containing penicillin-streptomycin-glutamine, sodium pyruvate, minimum essential medium non-essential amino acids (all from Gibco), and 10% heat-inactivated FBS (Hyclone). The next day, the skin cell suspension was pipetted vigorously, pooled and filtered sequentially through a 100 µm and a 40 µm cell strainers (Corning). Skin debris was further removed by Ficoll-Paque Premium (GE Healthcare) gradient separation. The viability (83% ± 6.5) and the cell yield (0.63 ± 0.33 $x10^6$ cells/cm^2 skin) of the obtained single cell suspensions were assessed with a Vi-CELL XR cell counter (Beckman Coulter).

Heat-killed (HK) microbes

Methicillin resistant *S. aureus* USA300 LAC strain and the coagulase negative staphylococci *S. epidermidis* 1457 strain and *S. lugdunensis* SL13 strain were grown to mid-exponential phase (OD_{600} 0.6) [30,31]. Next, bacteria were washed with PBS to remove secreted proteins, resuspended in sterile PBS, plated on Tryptic Soy Agar (TSA) for CFU counts and inactivated in a dry block heater at 90°C for 45 minutes. After inactivation bacteria were washed three times with PBS and protein content was measured using the Pierce BCA Protein Assay kit (Thermo Scientific). Samples concentrations were adjusted to 25 µg/ml, which corresponds to approximately $1x10^8$ CFU/ml (Brown et al. 2015). Bacterial killing was verified by plating the HK bacteria for 5 days on TSA. Heat-killed (HK) bacteria were aliquoted and stored at -20°C. HK *S. epidermidis* (FDA strain PCI 1200), HK *C. albicans* (ATCC 10231), and HK *E. coli* (O111:B4) were purchased from Invivogen.

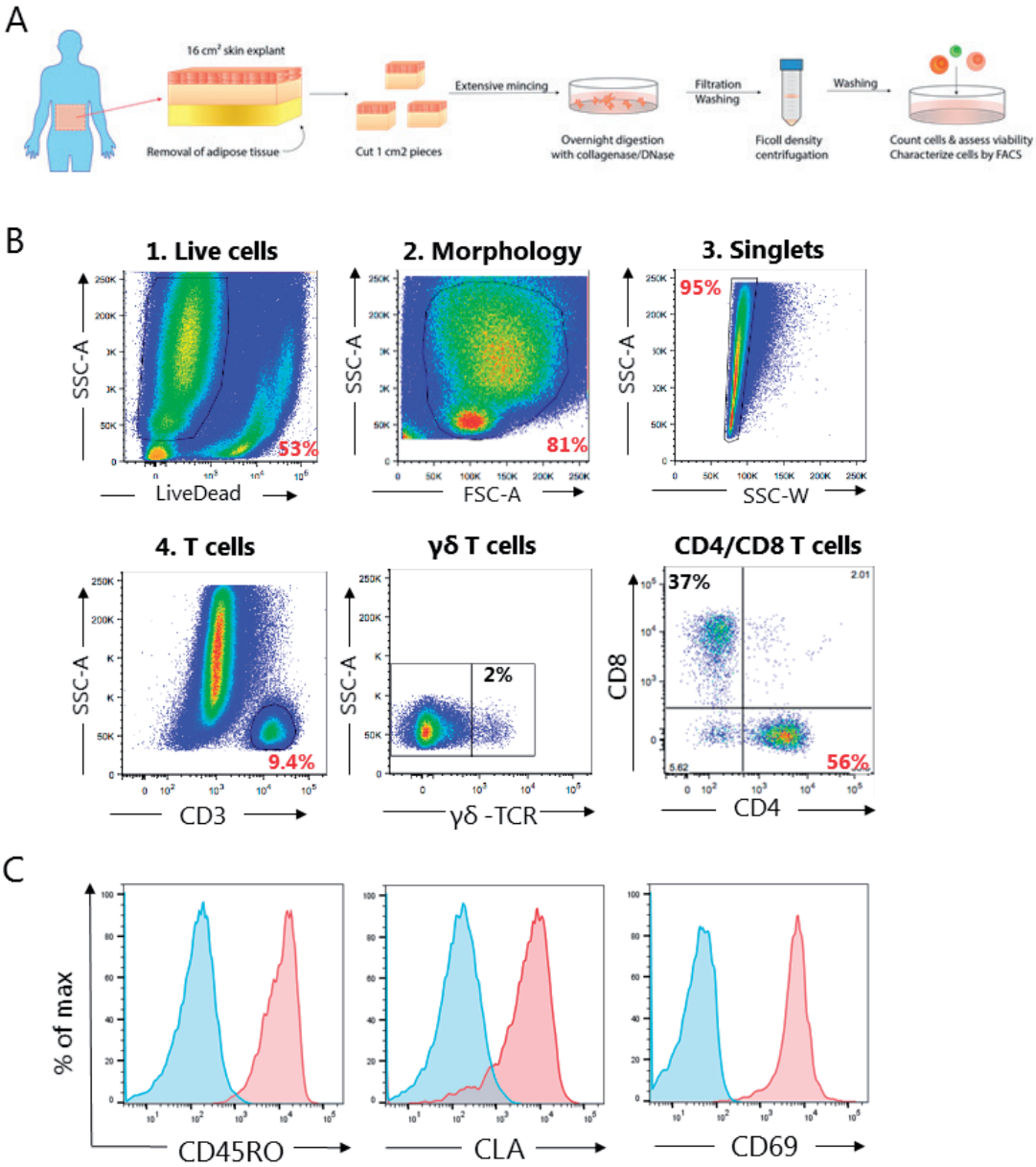


Figure 1. Characterization of T cell subsets in cell suspensions obtained from human skin explants (A) Schematic overview of the protocol used to obtain single cell suspensions from human skin explants. **(B)** Gating strategy to analyze T cell subsets, γδ-, CD4+ and CD8+ T cells, in single cell suspensions. **(C)** Representative histograms (in red) showing the surface expression for skin resident memory T cells markers, CD45RO (memory), CLA (skin homing), and CD69 (tissue retention) on live CD4+ cells. Blue histograms represent unstained cells.

$CD4^+$ T cell proliferation and cytokine production in response to HK microbes by Click-iT EdU/V-PLEX assay

Single cell suspensions obtained from the skin explants were seeded at 500,000 live cells/well for all conditions but CD3/CD28 (for which half the number of cells were plated) in a final volume of 200 µl c-RPMI in round-bottom 96 wells plates (Corning). Cells were rested for at least 24h at 37°C with 5% CO_2 to restore surface marker expression and reduce cellular stress due to the isolation procedure. Cell culture medium was replaced with: 1) fresh medium alone (no stimulation, negative control) or containing: 2) 10^6 CFU HK microbes corresponding to a multiplicity of infection of 2; 3) Tetanus toxoid (5 µg/ml, Novartis); 4) anti-CD28 (2 µg/ml, clone CD28.2, BD Bioscience, cat # 555725) added to anti-CD3 (1 µg/ml, clone OKT3, BD Bioscience, cat # 566685) coated wells (CD3/CD28, polyclonal stimulation, positive control). After three days of culture the thymidine analogue EdU (10 µM) was added to the cultures for the last 16 hours. At day four, cell culture supernatants were collected and stored at -20°C for cytokine analysis while $CD4^+$ T cell proliferation was assessed by Click-iT EdU assay (Click-iT Plus EdU Alexa Fluor 488 Flow cytometry assay kit, Invitrogen), as recently described (Clemente et al., manuscript in preparation). Cytokines were measured using the 27-V-PLEX human kit (MesoScale Discovery) following manufacturer's instructions. Plates were analyzed by a MESO Quickplex SQ 120 reader and cytokine concentrations were determined using MSD discovery workbench 4.0. Values below or above the detection limits were given the value of ½ LLOD (Lower Limit Of Detection) or 2xULOD (Upper Limit Of Detection), respectively. Cytokines that were consistently above or below the detection limits, or showed no differences across all stimuli were excluded from further analysis.

Flow cytometry

For the phenotypic characterization of T cell subsets in the single skin cell suspensions, cells were stained with Live/Dead Near-IR Dead cell stain kit (Invitrogen) for 20 minutes at room temperature (RT), washed and blocked with 2% rabbit serum in PBS on ice for 20 minutes. Next, cells were stained for CD4, CD8, γδ-TCR, CD45RO, CLA and CD69 for 20 minutes at 4°C, washed with PBS, and fixed with Cytofix (BD Bioscience).

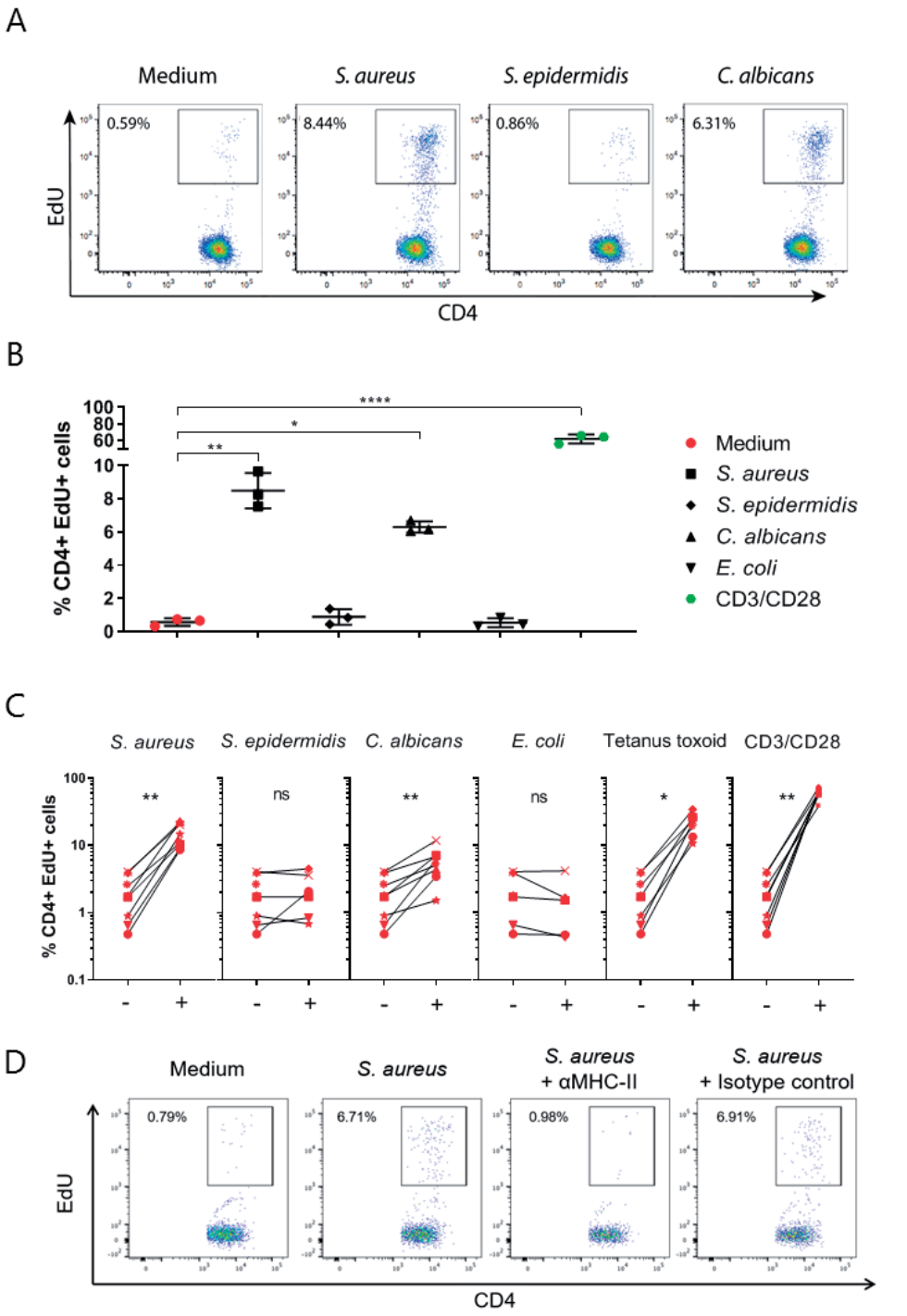
For T cell proliferation experiments, after surface staining with CD4, CD8, CD45RO and CLA and fixation, cells were permeabilized with PBS 1% BSA,0.5% saponin for 30 minutes at 4°C, washed with PBS 1% BSA, 0.5% saponin followed by the Click-iT reaction (Click-iT Plus EdU Alexa Fluor 488 Flow cytometry assay kit, Invitrogen). After 30 minutes at RT, cells were washed with PBS 1% BSA, 0.5% saponin and stained for CD3 for 15 minutes at RT. After two washes, the cells were analyzed on a BD LSR II flow cytometer, and data was analyzed using FlowJo 10 (TreeStars). All antibodies used in this study are shown in Supplementary Table 1.

Cytokine production in response to *in vitro* epicutaneous *S. aureus* infection of human skin explants

Infection of human skin explants was performed according to a previously described protocol (Olaniyi et al., 2018). In short, after removal of adipose tissue, the skin sheet was pinned in a dissection board and stripped 30 times with a hypoallergenic tape (Transpore, 3M). Eight mm punch biopsies were collected using disposable biopsy punches (Kai Medical). The punches were washed with culture medium (Advanced DMEM; Gibco) once, followed by two washes with PBS to remove antibiotics. Next, the punches were placed in 12-well transwell plates with 0.4 µm pore size (Corning), containing 1 ml of culture medium. Finally, the punches were infected in duplicate with USA300 LAC strain ($5x10^6$ CFU in 1 µl PBS) and cultured at air-liquid interface for 2, 24 or 72 hours at 37°C, 5% CO_2. At each indicated time point, culture supernatants were collected, filtered and stored at -20°C for cytokine analysis that was performed using the 27-V-PLEX human kit (MesoScale Discovery).

Statistical analysis

GraphPad Prism 8.0.1 was used to perform statistical analysis. Data were analyzed using one-way ANOVA with Dunnett's test, paired Wilcoxon test or paired t-test, as indicated. Significant differences ($p<0.05$) are shown.


A
Medium
S. aureus
S. epidermidis
C. albicans
0.59%
8.44%
0.86%
6.31%
EdU
CD4
B
% CD4+ EdU+ cells
Medium
S. aureus
S. epidermidis
C. albicans
E. coli
CD3/CD28
C
S. aureus
S. epidermidis
C. albicans
E. coli
Tetanus toxoid
CD3/CD28
% CD4+ EdU+ cells
ns
D
Medium
S. aureus
S. aureus + αMHC-II
S. aureus + Isotype control
0.79%
6.71%
0.98%
6.91%
EdU
CD4

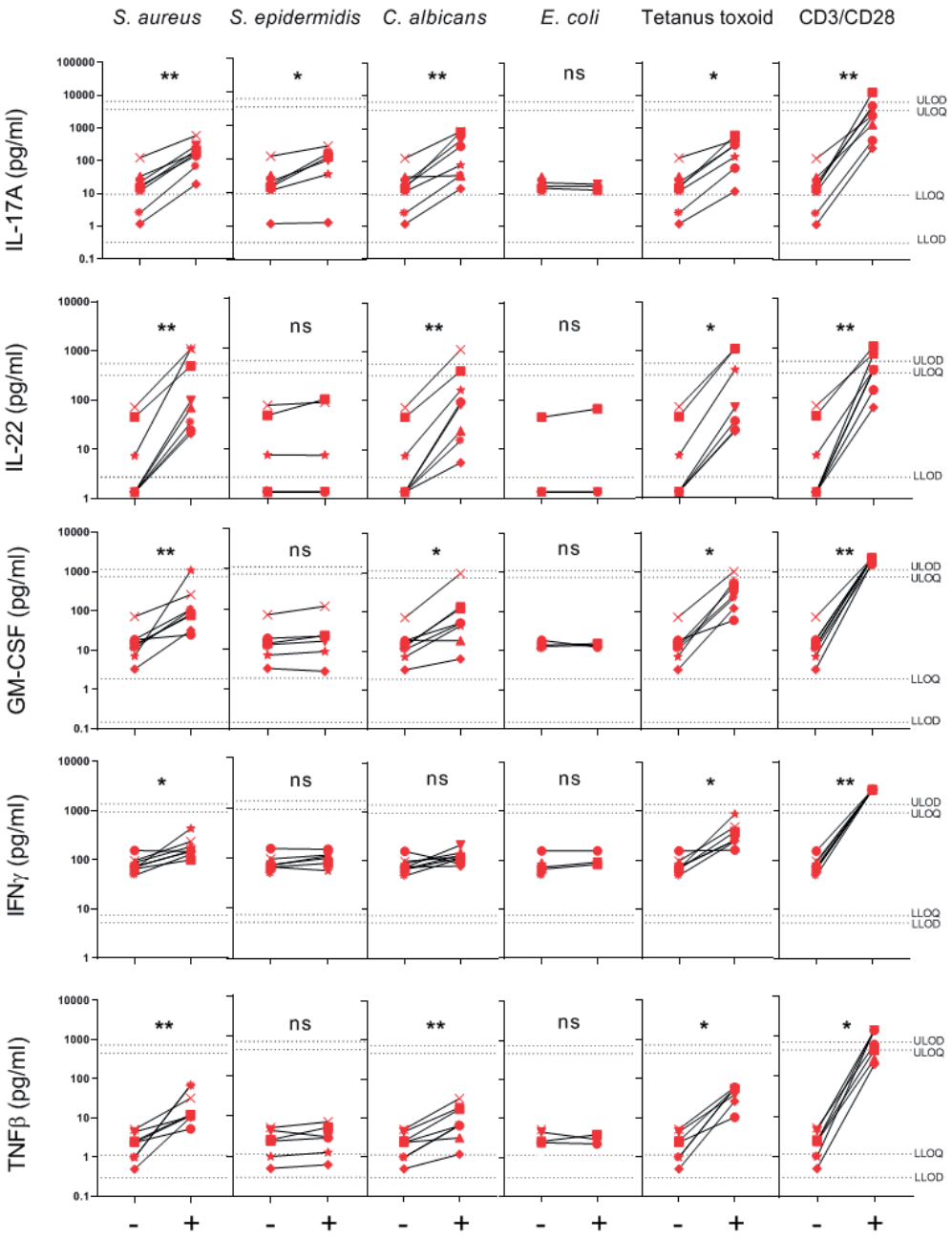
< Figure 2. Tissue-resident memory CD4+ T cells present in the skin of healthy subjects proliferate in response to *S. aureus*. (A) Representative dot-plots showing CD4+EdU+ cells in cell cultures, obtained from a skin explant of an healthy subject, stimulated for 4 days with heat-killed (HK): *S. aureus* USA300 LAC strain, *S. epidermidis* PCI 1200 strain, *C. albicans*, or left unstimulated (medium). **(B)** Reproducibility of the Click-iT EdU assay. Representative results showing the percentages of CD4+ EdU+ T cells obtained from triplicate skin cell cultures from the healthy subject shown in panel A in response to different stimuli. *p< 0.05, **p<0.01, ****p<0.0001 as assessed by one-way ANOVA. **(C)** Proliferation of CD4+ Tsrm cells from skin explants of 8 healthy subjects in response to different HK microbes, Tetanus toxoid, anti-CD3/anti-CD28 antibodies or medium alone. Average percentages of CD4+EdU+ cells of each of the 8 subjects analyzed, in triplicate, are shown by an identifying symbol. Per donor, each stimulated group, indicated by a +, was compared to the non-stimulated group (medium), indicated by a -, by paired Wilcoxon test, *p<0.05, **p<0.01. **(D)** Representative dot plots showing proliferating CD4+ Tsrm cells (CD4+EdU+) after 4-day culture in medium alone (negative control) or with HK *S. aureus* alone or in combination with MHC class-II blocking antibodies or isotype control antibodies.

Results

Tissue-resident memory CD4+ T cells present in the skin of healthy subjects proliferate in response to *S. aureus*

To investigate if healthy human skin contains CD4+ Tsrm cells specific for *S. aureus,* we stimulated single cell suspensions prepared from skin explants from 8 healthy donors with heat-killed (HK) *S. aureus*. The vast majority (>90%) of the isolated CD4+ T cells had a Tsrm phenotype based on the surface expression of the memory marker CD45RO, the skin-tropic marker CLA and the tissue-retention marker CD69 (Figure 1C). After 4 day stimulation with HK microbes, we identified CD4+ T cells that have neo-synthesized DNA by the flow cytometry-based Click-iT EdU proliferation assay (CD4+EdU+ cells, Fig. 2A, B). These cells were CD4+ Tsrm based on their expression of CD45RO and CLA (Supplementary Fig. 1). As shown in Figure 2C, the analysis of 8 healthy subjects showed a statistically significant CD4+ Tsrm cell proliferation in response to HK *S. aureus* but not to HK *S. epidermidis*, which is a major component of the human skin microbiome [32]. In agreement with previous studies, we observed specific CD4+ Tsrm cell proliferation in response to *C. albicans* (Fig. 2C) [6], while no proliferation was observed in response to *E. coli*, which is not part of the skin microbiome (Fig. 2C). Interestingly, a strong proliferation of CD4+ Tsrm cells was also induced by the recall antigen Tetanus toxoid (Fig. 2C) that commonly induces a strong T cell response in human blood [27,33]. Polyclonal T cell stimulation with anti-CD3/CD28 antibodies, which was used as positive control, induced the strongest CD4+ Tsrm cell proliferation in all donors as expected (Fig. 2C).

Heat-inactivated intact bacteria have been described to be devoid of superantigens, which stimulate T cells in a non-specific manner [25,34]. To further prove that the observed CD4+ Tsrm cell proliferation was antigen-specific, we added MHC class-II blocking antibodies or isotype control to the skin cell cultures. Indeed, in the presence of MHC-II blocking antibodies, CD4+


S. aureus
S. epidermidis
C. albicans
E. coli
Tetanus toxoid
CD3/CD28
IL-17A (pg/ml)
IL-22 (pg/ml)
GM-CSF (pg/ml)
IFNγ (pg/ml)
TNFβ (pg/ml)
ULOD
ULOQ
LLOQ
LLOD
ns
-
+

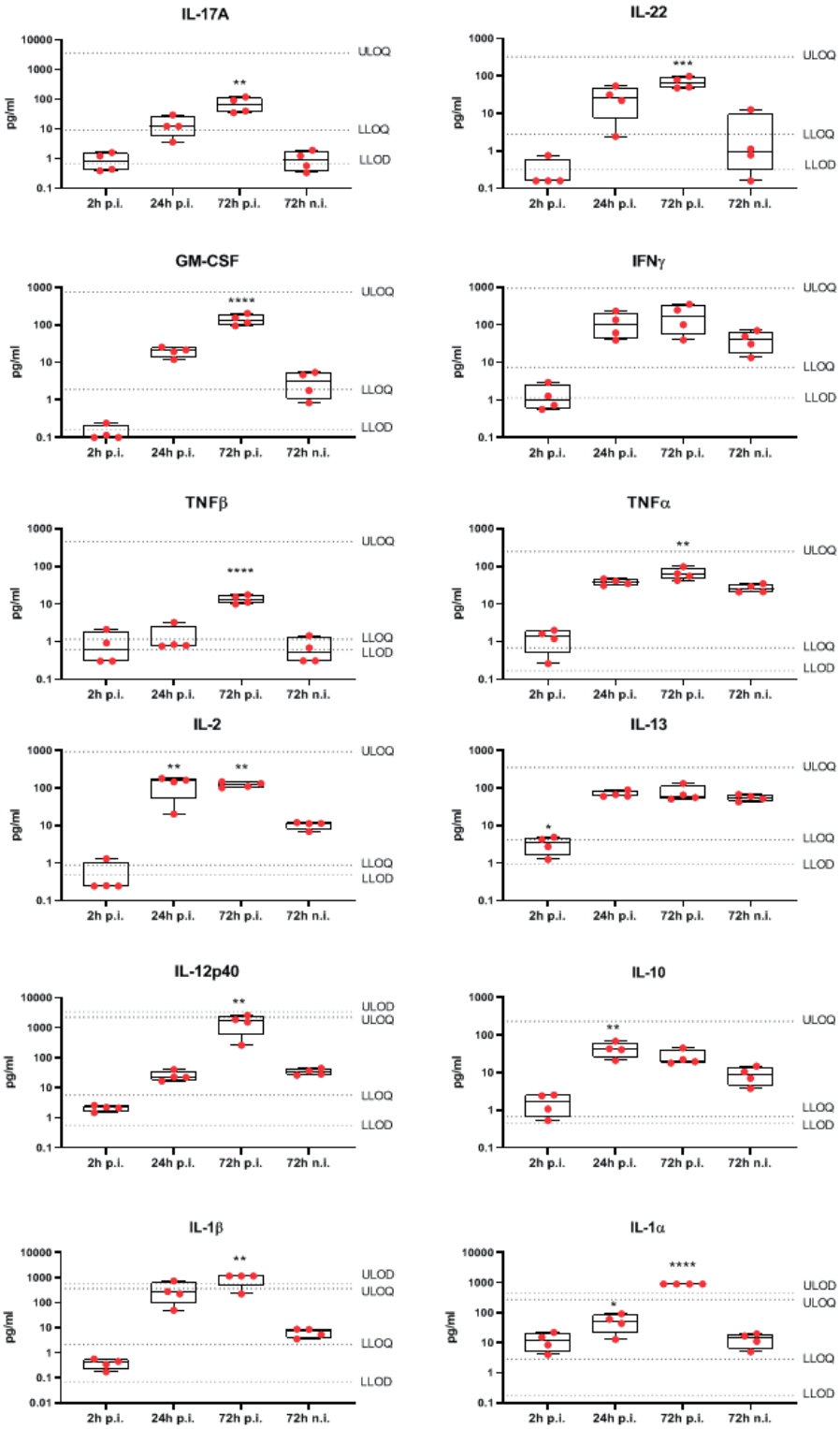
< Figure 3. Cytokines secreted in the supernatants of skin cell cultures stimulated with different HK microbes Supernatants were collected from samples used to determine CD4+ Tsrm cell proliferation by the Click-iT EdU assay. Cytokines were quantified by V-PLEX assays. Cytokine production in response to the different stimuli is shown for 8 donors (indicated by individual symbols), except for Tetanus toxoid (7), *S. epidermidis* (6) and *E. coli* (3). Per donor, each stimulated group, indicated by a +, was compared to the non-stimulated group (medium), indicated by a -, by paired Wilcoxon test, *p< 0.05, **p<0.01. LLOD: Lower limit of detection, ULOD: upper limit of detection, LLOQ: lower limit of quantification, ULOQ: upper limit of quantification

T cell proliferation in response to HK *S. aureus* was abolished while the isotype control had no effect (Fig. 2D). In addition no CD4+ T cell proliferation was detected by Click-iT EdU assay upon stimulation of peripheral blood mononuclear cells (PBMCs) of some healthy subjects with HK *S. aureus*, while a strong proliferation was observed in response to the staphylococcal T cell superantigen SEB, as expected (Supplementary Fig. 2A).

To further assess the staphylococcal species-specificity on CD4+ Tsrm cell proliferation, we analyzed the proliferative response to the coagulase-negative *S. lugdunensis*, which is also a skin commensal [32]. Analysis of cells obtained from explants from 5 healthy subjects showed no proliferation in response to either *S. lugdunensis* SL13 strain or *S. epidermidis* 1457 strain while proliferation to *S. aureus* USA300 LAC strain was confirmed (Supplementary Fig 2B). Taken together, these findings support the presence of *S. aureus*-specific CD4+ tissue-resident memory T cells in healthy human skin.

Cells isolated from the skin of healthy subjects produce pro-inflammatory cytokines in response to *S. aureus*

To further investigate the response of healthy human skin to *S. aureus*, we analyzed the cytokine profile in the supernatants of *S. aureus*-specific CD4+ Tsrm cells analyzed by Click-iT EdU assay (Fig. 2B), collected after 4 days of stimulation, by 27-V-PLEX. As shown in Figure 3, significant increases in production of IL-17A (141.20 vs 12.34 pg/ml), IL-22 (131.67 vs 4.22 pg/ml), IFN-γ (176.61 vs 78.82 pg/ml), GM-CSF (138.73 vs 13.34 pg/ml), and TNF-β (18.48 vs 1.84 pg/ml) were observed in response to HK *S. aureus* stimulation while HK *S. epidermidis* induced a significant increase in IL-17A production only (48.29 vs 12.34 pg/ml). Interestingly, *C. albicans,* as well as Tetanus toxoid and the polyclonal stimulation with anti-CD3/CD28 antibodies induced the same pattern of cytokines as *S. aureus* while in response to *E. coli* none of the analyzed cytokines was induced. Remarkably, the production of IL-17A, IL-22, IFN-γ, GM-CSF and TNF-β in response to HK *S. aureus* stimulation were substantially decreased by MHC class II blocking antibodies, indicating that CD4+ Tsrm cells were a major source of these cytokines (data not shown). In addition we obtained direct evidence of IL-17A and IL-22 production by CD4+EdU+ Tsrm cells by intracellular cytokine staining (data not shown).


IL-17A
IL-22
GM-CSF
IFNγ
TNFβ
TNFα
IL-2
IL-13
IL-12p40
IL-10
IL-1β
IL-1α
pg/ml
ULOQ
LLOQ
LLOD
ULOD
2h p.i.
24h p.i.
72h p.i.
72h n.i.

< Figure 4. Cytokines produced by human skin from healthy subjects in response to *ex vivo* epicutaneous *S. aureus* USA300 LAC infection. Eight mm biopsy punches, prepared from tape stripped human skin explants from 4 donors, were put in transwells at air-liquid interface and infected epicutaneously with 10^7 CFU *S. aureus* USA300 LAC strain in duplicate, or were left non-infected (n.i.) for 2, 24 or 72 hours when culture medium was collected. Cytokine concentrations were assessed by V-PLEX assay. Box-and-whiskers extend from the 25th to 75th percentiles and the line inside the box represents the median. LLOD: Lower limit of detection, ULOD: upper limit of detection, LLOQ: lower limit of quantification, ULOQ: upper limit of quantification. Differences between cytokine concentrations measured at 2, 24, 72 hpi were compared with non-infected control at 72h using an one-way ANOVA, *p< 0.05, **p<0.01, ***p<0.001, ****p<0.0001

Ex vivo epicutaneous *S. aureus* infection of healthy human skin induces pro-inflammatory cytokines

To understand the local immune response to *S. aureus* within healthy human skin, we used an *ex vivo* epicutaneous infection model [35]. Skin explants were tape-stripped to remove the *stratum corneum,* followed by topical application of 10^7 CFU *S. aureus* USA300 LAC strain. Cytokines were quantified in the skin explants culture media by 27-V-PLEX at different time-points post infection (p.i.). At 24 hours p.i. only IL-10, IL-1α and IL-2 were produced at significantly higher levels compared to the non-infected control while at 72h p.i. also IL-17A, IL-22, IFN-γ, IL-1α, IL-1β , GM-CSF, IL-12p40, TNF-α and TNF-β levels were increased (Figure 4). These data show that, while at an initial stage of *S. aureus* infection the cytokine response of the skin is limited, it becomes strongly proinflammatory at later stages of infection. Furthermore, the cytokines induced by stimulation of cells extracted from the skin with HK *S. aureus*, namely IL-17A, IL-22, GM-CSF, IFN-γ and TNF-β, were induced also by epicutaneous infection of human skin with live *S. aureus*, strengthening the value of this *in vitro* model.

Discussion

Here, we show that *S. aureus*-specific CD4+ tissue-resident memory T cells are abundant in the skin of healthy subjects. In particular, by the use of a novel assay, Click-iT EdU/V-PLEX, which allows simultaneous detection of CD4+ T cell proliferation and cytokine quantification in cell culture supernatants, we revealed that stimulation of cells isolated from abdominal skin explants with HK *S. aureus* USA300 LAC strain induced: 1) The proliferation of CD4+ T cells that were identified as skin-resident memory T (Tsrm) cells based on the expression of the CLA skin-homing, CD45RO memory, and CD69 tissue-retention markers. 2) The secretion of proinflammatory cytokines, namely IL-17A, IL-22, GM-CSF, IFN-γ and TNF-β. Remarkably, neither proliferation of CD4+ Tsrm cells nor secretion of proinflammatory cytokines except for IL-17A was observed in response to the common skin commensal *S. epidermidis*.

Mechanisms enabling the host to mount protective immune responses against pathogens while establishing a privileged relationship with commensal bacteria have been intensively studied but still remain largely unknown. One important feature of commensal-specific immunity is its uncoupling from inflammation and the maintenance of tissue homeostasis [8]. Cytokines play a key role not only in the promotion of skin inflammation but also in skin homeostasis. Cytokine analysis of culture medium of both isolated skin cells stimulated with HK *S. aureus* and human skin explants infected *ex vivo* with *S. aureus* showed the production of cytokines involved in skin inflammation and tissue repair. Genetic evidence has highlighted a key role for IL-17-mediated immunity in protection against *S. aureus* skin infection, but not invasive staphylococcal disease, similarly to what observed for *C. albicans* infections [36,37]. IL-17 enhances the recruitment of neutrophils, which can kill *S. aureus*, to the site of infection, and stimulates the production of antimicrobial peptides (AMPs) that can be directly bactericidal [38–40]. IL-22 promotion of skin inflammation is well established [41], however a role of IL-22 in skin homeostasis has also emerged. In particular, IL-22 has been shown to induce the proliferation of keratinocytes and AMPs production [42,43]. In addition, IL-22 induces MHC class II expression on keratinocytes thereby promoting the selective accumulation of commensal-induced IFN-γ producing $CD4^+$ T cells within murine skin [44]. TNF-β induces angiogenesis, thereby contributing to wound repair [45,46]. Interestingly, cytokine analysis of human skin explants infected epicutaneously with *S. aureus* revealed also a significant production of IL-1α and IL-1β (Fig. 4). These results are in agreement with previous studies showing that these cytokines were produced by murine keratinocytes after an epicutaneous *S. aureus* challenge [47,48]. The lack of IL-1 production by isolated human skin cells stimulated with HK *S. aureus* could be due to the lack of secreted bacterial proteins, including alpha-toxin that has a prominent role in the induction of IL-1 production by keratinocytes [49].

The Click-iT EdU/V-PLEX assay does not allow the identification of the cellular source of the detected cytokines. However, since we observed that the polyclonal T cell stimulation with anti-CD3/CD28 antibodies induced the same cytokine profile as HK *S. aureus* stimulation, and MHC class II blocking antibodies substantially decreased the production of these cytokines in response to HK *S. aureus* stimulation (data not shown) it seems likely that $CD4^+$ Tsrm cells are a major source of the observed cytokines [50], although cytokine production by non-classical T cells cannot be ruled out [51–53]. In addition we obtained direct evidence of IL-17A and IL-22 production by $CD4^+EdU^+$ Tsrm cells by intracellular cytokine staining (data not shown). It should be noted that studies, performed both in human and murine skin, suggest that Trm cells accumulate in the skin as a function of the number of infectious and inflammatory events over time. Indeed, laboratory mice, like newborn, but not adult, humans lack effector-differentiated and peripherally distributed memory T cells, including Tsrm cells [54,55]. In mice, γδ T cells have been identified as key IL-17 producers upon *S. aureus* infection [52]. However, it has been shown that following infection of laboratory

mice with *C. albicans*, while the initial IL-17-producing cells were γδ T cells, at later times the majority of *C. albicans*-reactive IL-17-producing T cells were CD4+ Tsrm cells. Importantly, IL-17-producing CD4+ Trm cells that responded to *C. albicans* were identified in normal human skin [4]. Similarly, since humans, unlike laboratory mice, naturally encounter *S. aureus* repeatedly over time, we hypothesize that CD4+ Tsrm cells are the primary source of IL-17 produced in response to this bacterium in human skin. Indeed, comparable levels of IL-17A were produced in response to HK *S. aureus* or *C. albicans* in our experiments. On the other hand, *S. epidermidis* colonization of mouse skin has been shown to induce IL-17A-producing CD8+ T cells restricted to non-classical MHC class I molecules and characterized by immunoregulatory and tissue-repair signatures, which home to the epidermis [9]. These cells could be the source of IL-17A produced in response to HK *S. epidermidis* in our experiments, although further research is needed to address this point.

A major difference between *S. aureus* and *S. epidermidis* is the secretion of numerous virulence factors including proteases and toxins such as alpha-toxin, that can damage the skin epithelial integrity [35,56]. Our results suggest that in order to halt *S. aureus* invasion, the cutaneous immunity deploys CD4+ Tsrm cells that secrete several cytokines with proven anti-*S. aureus* and tissue-repair activities. Induction of such a mild anti-bacterial immune response might be a strategy to limit local infection and prevent systemic spread, promoting a long-lasting equilibrium between this pathobiont and the host. Interestingly, this could be achieved through alpha-toxin that has been shown to modulate mouse CD4+ T cell differentiation limiting Th1 while promoting Th17 responses [57]. However, once the skin is breached, the local immunity is dampened or the bacterial load is exceedingly high, this local response is no longer sufficient to control *S. aureus* [37]. Indeed, the importance of antibodies and Th1 cells in controlling systemic *S. aureus* infections has been highlighted [25,58].

Interestingly, a recent paper showed that neonatal mouse skin colonization with *S. epidermidis* facilitated immune tolerance to this bacterium via the induction of regulatory T (Treg) cells [49]. This was not the case for *S. aureus* that, through alpha-toxin mediated IL-1β production by myeloid cells, limited the development of *S. aureus*-specific Treg cells thus enhancing skin inflammation upon later-life exposure to *S. aureus* [49]. Similarly, we cannot rule out the presence of *S. epidermidis*-specific Treg cells, which are known to proliferate under homeostatic conditions, in our experiments [6].

The immune response of the skin to *S. aureus* has been intensively investigated in a number of elegant mouse studies [19]. However, the anatomical and immunological differences between mouse and human skin together with the different composition and exposure to skin microbiome limit the translational value of the results obtained in mice [29,59]. As a more biologically relevant model, we used human skin explants generated as surgical waste from cosmetic surgery performed on the abdomen. Of note, although *Staphylococcus aureus*

colonization is most consistently identified in humans in the anterior nares, colonization has also been reported at other body sites including axilla, inguinal and rectal areas [60]. In addition, *S. aureus* was cultured from 30% of abdominal skin swabs from healthy subjects [61]. Since variability was reported among different skin sites, as a consequence differences in the relative abundance in CD4$^+$ Trm cells specific for *S. aureus* at different locations can be envisaged. Nevertheless, studies have shown that following skin infection, Tsrm cells can migrate out of the skin and populate distant skin sites thus forming global skin immunity [16,62]. Interestingly, *S. aureus*-specific CD4$^+$ Trm cells have been identified in gut tissue of healthy individuals. These cells showed increased IL-17A and reduced IFN-γ production as compared to cells with similar reactivity present in the circulation [63], similarly to what we report here for *S. aureus*-specific CD4$^+$ Tsrm cells. Indeed, this seems to be a common characteristic of barrier-protective Trm cells. In addition, since some inborn errors of IL-17 immunity predispose not only to skin but also to lung *S. aureus* infection [37], the presence of *S. aureus*-specific CD4$^+$ Tsrm cells in the lungs and their phenotype should be assessed.

While numerous efforts have been made towards designing a vaccine against *S. aureus*, unfortunately to this date none have been successful [64]. Perhaps the most fundamental reason explaining the past failures of *S. aureus* vaccines is the lack of a complete understanding of protective immunity. Our results enforce the conclusion that since the contribution of local immune memory within tissues is becoming evident, it should be evaluated in vaccination efficacy studies [50]. Since a sizable percentage of people experiencing *S. aureus* SSTI has recurrent infections [22,65], it will be very informative to analyze the CD4$^+$ Tsrm response to *S. aureus* in this population. In addition, it would be interesting to investigate the *S. aureus*-specific CD4$^+$ Tsrm response in patients with atopic dermatitis, a chronic and relapsing inflammatory skin disorder associated with skin barrier impairment and the predominant *S. aureus* colonization [66].

In summary, we describe a skin-resident memory CD4$^+$ T cell population within healthy human skin that is specific for the human skin pathogen *S. aureus*. While further research is needed to better characterize the phenotype, the antigen-specificity and the protective potential of these cells, this finding highlights that skin-resident memory CD4$^+$ T cells could be a powerful and exploitable arm of adaptive immunity against this elusive pathobiont.

Conflict of interest

AH, MEM and ARC are Ph.D. fellows and are enrolled in the Infection and Immunity Ph.D. program, part of the graduate school of Life Sciences at Utrecht University and participated in a post graduate studentship program at GSK. ST, FB and ES are employees of the GSK group of companies. FB hold shares in the GSK group of companies. FB holds pending and issued patents (WO/2019/158537, WO/2015/144691, WO/2015/144653, WO/2015/144655, WO/2014/033190, WO/2014/033191, WO/2014/033192, WO/2014/033193, WO/2013/030378, WO/2010/119343) on *S. aureus* vaccine formulations.

Author contributions

AH, MEM, ES were involved in designing the study. AH, MEM, BC and ARC performed experiments. ST and BC set up the Click-iT EdU assay. ST provided valuable technical support with flow cytometry. AH, MEM, BC and ES analyzed the data. AH, MEM and ES wrote the paper. FB and ES supervised the project. All authors critically revised the manuscript and approved it before submission.

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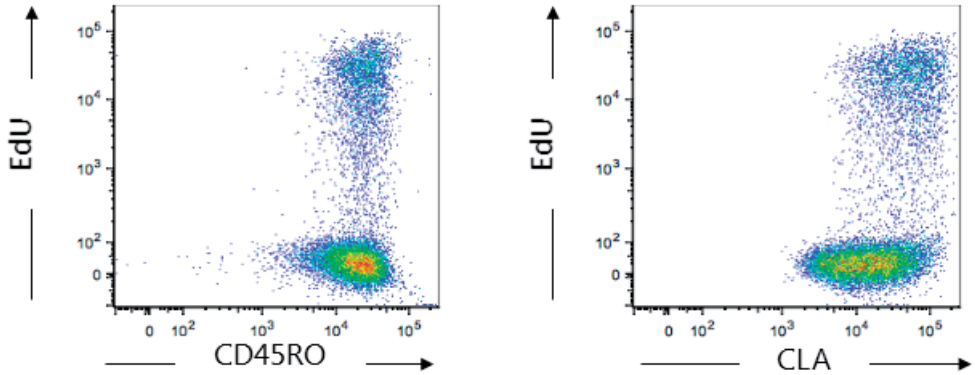
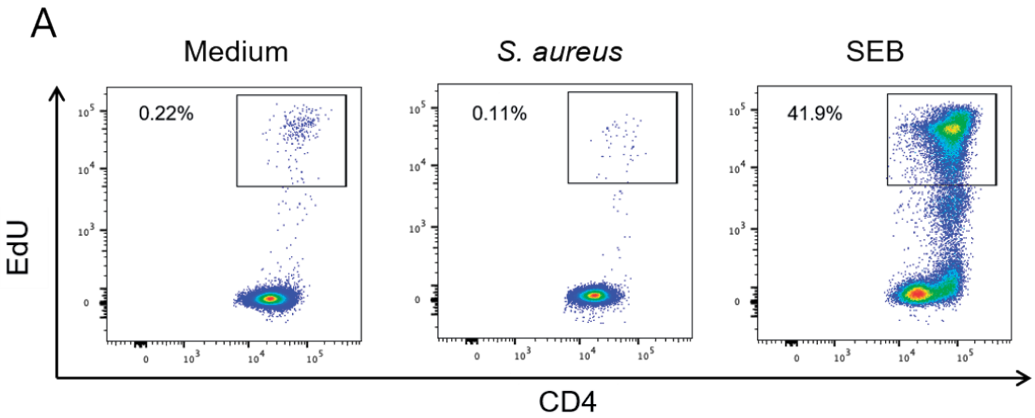
Supplementary material

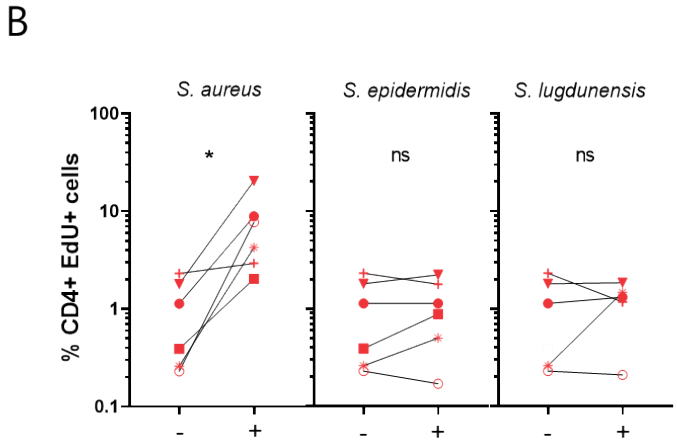
Supplementary Table 1. List of antibodies used in this study

Antibody	Supplier	Clone	Catalogue number
anti-human CD3-PE-CF594	BD biosciences	UCHT1	562280
anti-human CD4-BV421	BD biosciences	RPA-T4	562424
anti-human CD8-APC	Biolegend	RPA-T8	301014
anti-human-γδ-TCR-PECy7	Biolegend	B1	331222
anti-human CLA-BV605	BD biosciences	HECA-452	563960
anti-human CD45RO-BV650	BD biosciences	UCHL1	563750
anti-human CD69-BUV737	BD biosciences	FN50	564439
anti-human MHC class II HLA-DR, DP, DQ blocking antibody	BD biosciences	Tu39	555556
mouse IgG2a, κ Isotype Control for anti-MHC class II blocking antibody	BD biosciences	G155-178	554645



Supplementary figure 1. Click-iT EdU assay tracks *S. aureus*-specific CD4⁺ Tsrm cell proliferation. Representative dot plots showing surface expression of CD45RO and CLA on proliferating CD4⁺ T cells (CD4⁺EdU⁺), gated on live CD3⁺CD4⁺CD8⁻ cells.





Supplementary figure 2. Specificity of CD4+ Tsrm cell proliferation in response to *S. aureus*. **(A)** Representative dot-plots showing CD4+EdU+ cells in PBMCs stimulated for 4 days with heat-killed (HK) *S. aureus*, the T cell superantigen *S. aureus* enterotoxin B (SEB, 1 µg/ml Sigma-Aldrich), or left unstimulated (medium). **(B)** CD4+ Tsrm proliferation in response to different HK staphylococcal species: *S. aureus* USA300 LAC, *S. epidermidis* 1457 strain, and *S. lugdunensis* SL13 strain. Per donor, each stimulated group, indicated by a +, was compared to the non-stimulated group (medium), indicated by a -, by paired Wilcoxon test, *p<0.05.

CHAPTER 4

Characterization of *Staphylococcus aureus*-specific CD4$^+$ T cells in the blood of healthy subjects by single cell mRNA sequencing

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Manuscript in preparation

Abstract

Staphylococcus aureus is found as a skin commensal in approximately 30% of healthy subjects at any given time. Therefore, colonized subjects develop *S. aureus*-specific $CD4^+$ memory T cells that have been found both in the blood and skin. Genetic and experimental evidence has highlighted the requirement for IL-17/Th17 $CD4^+$ T cells in protection from *S. aureus* skin and soft tissue infections, whereas Th1 type of responses are important for systemic infections. Investigation of the cellular responses induced by *S. aureus* is important for understanding how to achieve long-term protection. The identification of antigen-specific $CD4^+$ T cells in the blood can be challenging due to their low frequency. Therefore, an appropriate tool for detection has to be applied. In this study we implemented an activation induced marker (AIM) assay to screen PBMCs from 16 healthy subjects for *S. aureus*-specific $CD4^+$ T cells. Antigen-specific $CD4^+$ T cells were identified based on the upregulation of the surface expressed activation markers, CD137 and OX40, upon stimulation with heat-killed *S. aureus*. $CD4^+CD137^+OX40^+$ T cells from 2 subjects were FACS-sorted and analysed by single cell mRNA sequencing. Based on the transcriptomes of sorted cells we found clusters of cells characterised by the expression of genes associated with regulatory and cytotoxic T cells. In our previous study we identified *S. aureus*-specific tissue-resident memory $CD4^+$ T cells (Trm) in the skin of healthy subjects, characterized by the expression of the skin-homing marker CLA. Since Tsrm have been shown to egress from the skin subsequently becoming circulating CLA^+ T cells, we used index sorting to identify skin-tropic cells and analysed transcriptional differences between CLA^+ and CLA- cells among sorted $CD4^+$ T cells. In summary, this study provides preliminary data on $CD4^+$ T cell responses induced by commensal exposure to *S. aureus*.

Introduction

Staphylococcus aureus is a Gram-positive bacterium that persistently colonizes the skin and nares of approximately 30% of the population, however it is hypothesised that the percentage of asymptomatic persistent and transient carriers can reach up to 60% at any given time [1]. As a consequence, healthy subjects develop *S. aureus*-specific CD4$^+$ memory T cells that have been found both in the blood [2,3] and in the skin [15]. Remarkably, genetic and experimental evidence has highlighted the importance of CD4$^+$ T cells and IL-17 response for protection from *S. aureus* infections [4,5]. In particular, the protective role of Th17 and Th1 cells has been shown for local infections, such as skin and soft tissue infections (SSTIs), or systemic *S. aureus* infections, respectively [6–10]. Therefore, the characterization of CD4$^+$ T cells induced by natural exposure to *S. aureus* is of paramount importance since this pre-existing immunity determines the response to *S. aureus* infections as well as to vaccination against *S. aureus*.

Unfortunately, due to their low frequency, identification of antigen-specific CD4$^+$ T cells is challenging. Detection of these cells can be achieved by either direct or indirect assays. An example of a commonly applied direct assay is the use of peptide-MHC class II multimers (*e.g.* tetramers, dextramers) to detect antigen-specific T cells. However, this approach has several limitations: first, it implies the knowledge of targeted peptides, second, the high diversity of HLA class II molecules and their promiscuous peptide binding properties preclude full characterization of the repertoire of antigen-specific CD4$^+$ T cells. Alternatively, indirect assays identify antigen-specific cells based on functional responses induced by TCR triggering upon *in vitro* stimulation with the antigen of interest, which can be broadly divided into cell activation, proliferation and polarization. Proliferation assays require several days of stimulation and thus can introduce a bias towards antigen-specific T cells with the highest proliferative capacity. Another commonly used assay: Intracellular Cytokine Staining (ICS), allows for the detection of multiple cytokines simultaneously in the cell cytoplasm upon short stimulation. ICS can be used together with staining for phenotypic T cell markers allowing for an extensive characterization of T cells that specifically respond to the antigen of interest. However, ICS only permits the detection of a limited number of cytokines at a time and due to the assay sensitivity may not always allow to detect cytokines produced at very low concentrations. In addition, since ICS requires cell fixation, it is not compatible with downstream sample processing such as mRNA sequencing. Activation Induced Marker assays (AIMs) allow the identification of antigen-specific T cells based on the expression/upregulation of activation markers, such as CD40L, CD137 or OX40, on the surface of T cells [11]. The main advantages of AIMs are the relatively short stimulation time, the independence of cytokine production, and the fact that cells remain alive. Since not all antigen-specific T cells produce cytokines or proliferate upon stimulation, AIMs potentially allow to track a wider and more representative panel of antigen-specific T cells as compared

to the previously described assays. AIMs have been recently used to successfully identify virus-specific T cells in COVID-19 infected patients [12,13].

So far identification of *S. aureus*-specific CD4$^+$ T cells has been performed based on their proliferative capacity and/or cytokine production after stimulation with heat-killed bacteria or selected staphylococcal antigens [2,3,6,14]. In the present study we identified *S. aureus*-specific CD4$^+$ T cells in the PBMCs of 15 out of 16 healthy subjects tested using AIM. *S. aureus*-specific CD4$^+$ T cells from 2 positive donors were single-cell sorted and analysed by mRNA sequencing (scRNAseq). Preliminary analysis of the transcriptomes of single cells identified *S. aureus*-specific CD4$^+$ cytotoxic (CTLs) and regulatory T cells (Tregs). Previously, we identified *S. aureus*-specific tissue-resident memory T (Tsrm) cells in the skin of healthy subjects [15]. Based on a recent study showing that Tsrm cells can exit the skin and be found in the circulation as Cutaneous Lymphocyte-Associated antigen (CLA)-positive cells [16] we used index sorting to identify CLA$^+$ cells among sorted *S. aureus*-specific CD4$^+$ T cells. This could therefore give information about the response of CD4$^+$ Tsrm cells present in the skin. In addition, we performed ICS and CFSE proliferation assays to compare the capability of different assays to capture the complexity of the CD4$^+$ T cell response induced by *S. aureus*.

Materials and Methods

Preparation of heat-killed *S. aureus*

Heat-killed *S. aureus* (HKSA) preparation was performed as previously described [15]. Briefly, methicillin-resistant *S. aureus* USA300 LAC strain was grown to exponential phase (optical density $(OD)_{600}$ 0.6). To remove secreted proteins, bacteria were washed in sterile PBS followed by inactivation in a dry block heater at 90°C for 45 min. Next, bacteria were washed three times with PBS and bacterial killing was verified by plating bacteria on blood agar plates. Protein concentration in the HKSA sample was measured by the Pierce™ BCA Protein Assay according to the manufacturer's instructions (Thermo Scientific). Colony forming unit (CFU) counts were then estimated using the previously described protein to CFU ratio: 25 µg/ml corresponds to $\sim 1 \times 10^8$ CFU/ml [6].

CD4$^+$ and CD4- T cells preparation

Peripheral blood mononuclear cells (PBMCs) from healthy subjects were purchased from Caprion (Belgium) and obtained according to good clinical practice in accordance with the declaration of Helsinki. Donors have given their written consent to participate in this study. Cells were thawed in prewarmed PBS without Ca^{2+} or Mg^{2+} (Gibco) containing 2.5 mM EDTA (Sigma) and 20 µg/ml Dnase I (Sigma), washed and counted using the Vi-CELL XR cell counter (Beckman Coulter). Cells were resuspended in separation buffer (PBS 2 mM EDTA, 0.5% bovine serum albumin (BSA; Sigma)), and the isolation of CD4$^+$ cells was performed

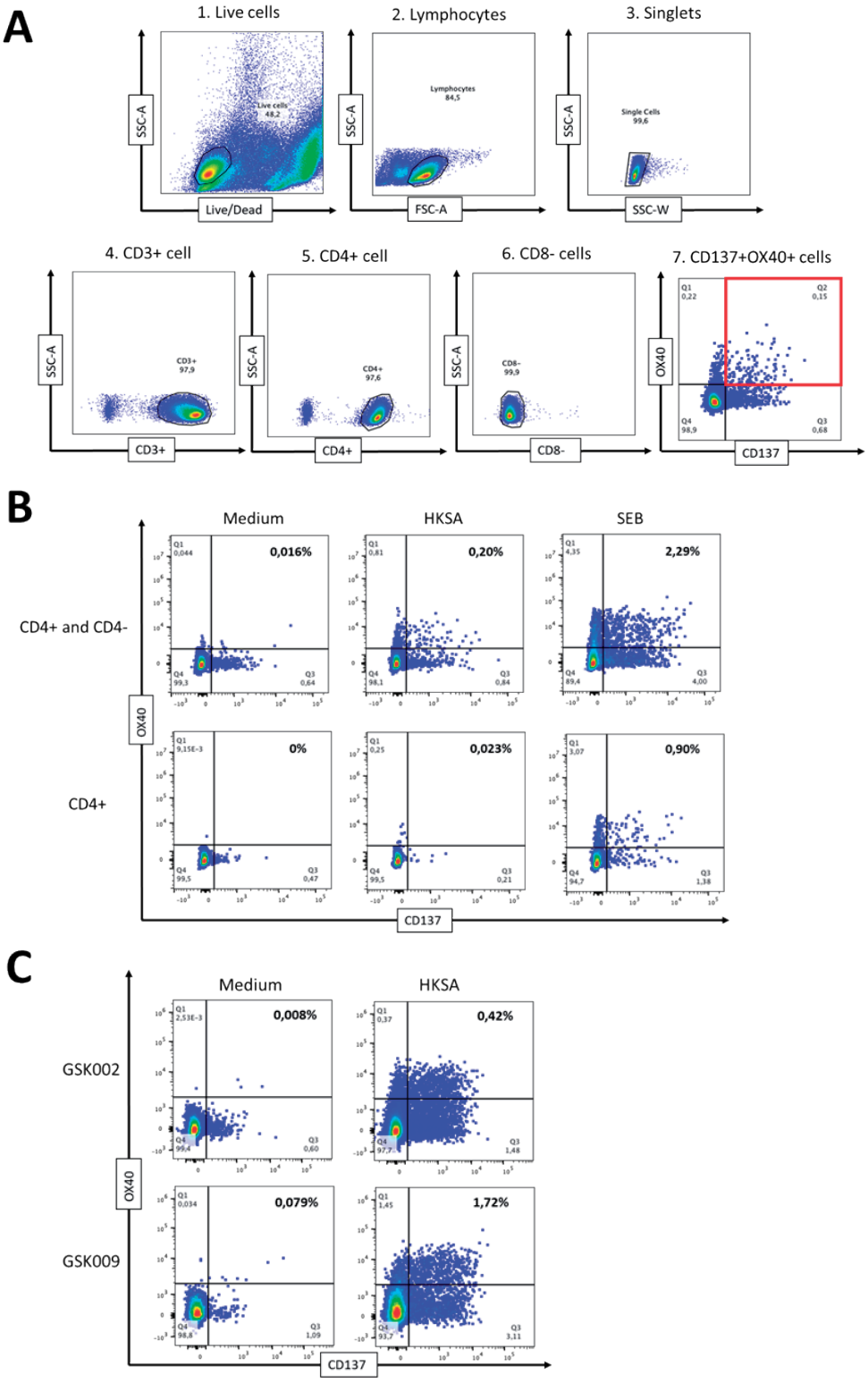
according to the instructions for the MACS CD4$^+$ negative isolation kit (Miltenyi Biotec). Both CD4$^+$ and CD4- fractions were collected, washed and resuspended at a concentration of 10^6 cells/ml in c-RPMI (RPMI-1640 supplemented with 1% minimum essential medium non-essential amino acids, 1% penicillin-streptomycin-glutamine, 1% sodium pyruvate (all from Gibco), 5% foetal bovine serum (Hyclone)). The CD4- fraction containing antigen-presenting cells (APC) was irradiated with 30 Gray as described by Brown *et al* [6].

Antigen Induced Marker assay (AIM) and single cell sorting.

CD4$^+$ and CD4- fractions were mixed at 1:1 ratio in 200 µl (2×10^5 cells in total) and were added pipetted in to a 96-well round bottom plate (Corning). Heat-killed *S. aureus* USA300 LAC was pre-diluted in c-RPMI and 50 µl of the pre-diluted solution was added to wells reaching a final concentration in well of 2.5×10^6 CFU/ml As negative and positive control, c-RPMI and Staphylococcal enterotoxin B (SEB; final concentration 1 µg/ml) were used, respectively. Cells were stimulated for 24 h at 37°C, 5% CO_2. Before staining for surface markers, cells were washed twice with PBS and stained with Live/Dead Near-IR Dead cell stain kit (Invitrogen) for 20 min at room temperature (RT). Samples were washed, blocked with 2% rabbit serum in PBS on ice for 20 min followed by addition of anti-CD3, -CD4, -CD8, -CD137, -OX40 and -CLA antibody mix and 20 min incubation on ice (full list of antibodies in Supplementary Table 1). Cells were washed twice and multiple wells from the same conditions were pooled into polypropylene FACS tubes (Corning) for single cell sorting using BD FACS-Aria Fusion. Cells were selected and sorted when they displayed upregulation for both CD137 and OX40 (Figure 1A) into 384-well plates containing lysis buffer (Single Cell Technologies, Utrecht, The Netherlands). We applied flow cytometric index sorting in order to correlate phenotypic and molecular profiles at the single cell level. Plates were stored at -80°C until shipment on dry ice to Single Cell Technologies (Utrecht, The Netherlands), where sample work up, mRNA sequencing and data analysis was performed. Cells were sequenced using SORT-seq platform described in detail by Muraro *et al.* [17].

Intracellular Cytokine Staining (ICS)

CD4$^+$ and CD4- fractions prepared as described above were mixed 1:1 in 200 µl (2×10^5 cells total) and added to 96-well round bottom plate (Corning). To change culture medium cells were pelleted and resuspended in 100 µl c-RPMI with 1% FBS. Stimuli were diluted in c-RPMI containing 2 µg/ml of co-stimulatory molecules anti-CD28 antibody and anti-CD49d antibodies (BD Bioscience) and 100 µl was added to the cells. The stimulation panel and the final concentrations of stimuli were identical to those used in the AIM. Cells were stimulated for 2 h at 37°C, 5% CO_2. Next, Brefeldin A (Sigma) was added at a final concentration of 5 µg/ml and cells were left for another 16 h at 37°C, 5% CO_2. In the morning, cells were washed twice with PBS, stained with Live/Dead Near-IR Dead cell stain kit (Invitrogen) for 20 min at room temperature (RT). Next, samples were washed with PBS, fixed and permeabilised using Cytofix/Cytoperm solution (BD Biosciences) for 20 min on


A
1. Live cells
2. Lymphocytes
3. Singlets
SSC-A
Live/Dead
FSC-A
SSC-W
4. CD3+ cell
5. CD4+ cell
6. CD8- cells
7. CD137+OX40+ cells
CD3+
CD4+
CD8-
OX40
CD137
B
Medium
HKSA
SEB
CD4+ and CD4-
CD4+
0,016%
0,20%
2,29%
0%
0,023%
0,90%
C
GSK002
GSK009
0,008%
0,42%
0,079%
1,72%

< Figure 1. Gating strategy to sort *S. aureus*-specific CD4⁺ T cells using AIM assay. (A) CD3⁺CD4⁺CD8- cells expressing CD137⁺OX40⁺, highlighted in the red square gate, were single-cell sorted for scRNA-seq analysis after 24-hour stimulation with the indicated stimuli. Data for one donor, representative of 16 donors; **(B)** Upregulation of CD137 and OX40 on CD4⁺ T cells stimulated with heat-killed *S. aureus* (HKSA) is APC-dependent. CD4⁺ T cells were stimulated with HKSA or SEB in the presence or absence of the g-irradiated CD4- fraction containing APCs. SEB acts as an oligoclonal stimulus for T cells and is therefore partially independently of APCs. Data for one donor, representative of 3 donors tested; **(C)** Frequency of CD137⁺OX40⁺ cells within CD4⁺ T cell subset upon stimulation with HKSA (Supplementary Table 2) for donors GSK002 and GSK009, who were selected for scRNA-seq analysis.

ice, followed by two washing steps with 1X Perm/Wash buffer (BD Biosciences). Next, cells were incubated for 30 minutes on ice with 2% rabbit serum followed by the addition of anti-CD3, CD4, CD8, CD40L, IL-2, IL-17, TNF-α, IFN-γ, CD137, OX40 and CLA antibody mix and 30 min incubation on ice (full list of antibodies in Supplementary Table 1). Cells were washed twice, and cytokine production was assessed using BD LSRFortessa. Live cells were gated based on their morphology, then single CD3⁺CD4⁺CD8- cells were selected and cytokine production in CD40L⁺ cells was evaluated.

CFSE proliferation assay

After isolation, CD4⁺ cells were washed with PBS and resuspended in 1 ml of sterile PBS at a concentration between 2-30 × 10^6 cells/ml. One ml of 2 µM Carboxy Fluorescein Succinimidyl Ester (CFSE) in PBS was added to the cells and suspension was gently vortexed and kept in the dark at room temperature (RT) for 8 minutes. Then, 2 ml of FBS were added to cells that were incubated at RT for 2 minutes. Cells were washed three times with PBS and resuspended in c-RPMI with 5% FBS and incubated for 30 min at 37°C, 5% CO_2. Cell counts and viability were assessed using Vi-CELL XR cell counter (Beckman Coulter). Cells were diluted to 1 × 10^6 viable cells/ml. Next, CD4⁺ were mixed with the irradiated CD4- fraction at a ratio of 1:1. The total cell suspension (200 µl; 2 × 10^5 cells total)) was seeded in wells of a 96-well round bottom plate (Corning) and stimuli were added in 50 µl of c-RPMI, 5% FBS. Stimulation panel and the final stimuli concentrations were similar as described in the AIM assay. Cells were incubated for 7 days at 37°C, 5% CO_2. Next, samples were washed twice with PBS, stained with Live/Dead Near-IR Dead cell stain kit (Invitrogen) for 20 min at room temperature (RT). After washing with PBS, samples were incubated for 20 minutes on ice with 2% rabbit serum followed by incubation with antibodies specific for CD3, CD4, CD8, CD137, OX40 and CLA (full list of antibodies in Supplementary Table 1) for 20 min on ice. Samples were washed with PBS, fixed with Cytofix (BD Bioscience) and cell proliferation was assessed using BD LSRFortessa. Live cells were gated based on their morphology, then single CD3⁺CD4⁺CD8- cells were selected and level of CFSE staining was assessed.

Software and data analysis

Samples acquired using flow cytometer were analysed with FlowJo 10.6.1 (TreeStars). Single-cell mRNA sequencing data was analysed by Single Cell Discoveries using Seurat plugin for R. Additional sequencing analysis was performed in-house using BBrowser (BioTuring). Reactome database was used to analyse metabolic pathways in the sequenced cells.

Results

S. aureus-specific CD4$^+$T cells present in the blood of healthy subjects can be identified by Activation Induced Marker assay (AIM)

To identify *S. aureus*-specific CD4$^+$ T cells in the blood of healthy subjects, PBMCs from 16 donors were screened using AIM. Briefly, equal numbers of CD4$^+$ and CD4- fractions obtained from PBMCs were co-cultured and stimulated with heat-killed *S. aureus* for 24 h after which T cells were stained with a specific antibody panel. *S. aureus*-specific CD4$^+$ T cells were detected through the upregulation of both CD137 and OX40 (Figure 1A for gating strategy), two activation markers belonging to the TNFR family. Overall, the frequency of double-positive cells varied between 0.12%–1.72% of CD4$^+$ T cells and *S. aureus*-specific CD4$^+$ T cells were detected in 15 out of the 16 donors (Supplementary Table 2).

CD137 and OX40 are receptors for CD137L and OX40L, respectively, which are expressed on the surface of APCs [18]. It is generally accepted that upregulation of CD137 and OX40 requires antigen presentation by APCs. To verify that the upregulation of CD137 and OX40 depended on antigen presentation by APCs, we stimulated CD4$^+$ T cells in the presence or absence of the γ-irradiated CD4- fraction containing APCs. In the absence of the CD4- cell fraction, HKSA exposure did not induce upregulation of CD137 and OX40 (Figure 1B). As expected also SEB, an oligoclonal stimulus that crosslinks Vb6 chain of TCR on T cells and MHC class II molecules on APCs, induced only a marginal CD137$^+$OX40$^+$ upregulation in the absence of the CD4- cell fraction (Figure 1B). Based on the frequency of CD137$^+$OX40$^+$ cells and their viability after stimulation, we selected two donors, GSK002 and GSK009 (Supplementary Table 2), from which *S. aureus*-specific CD4$^+$CD137$^+$OX40$^+$ T cells were single-cell sorted for further analysis by scRNA-seq (Figure 1C).

Single-cell mRNA sequencing analysis identified *S. aureus*-specific CD4$^+$ cytotoxic (CTL) and regulatory (Treg) T cells

After discarding low quality cells from our scRNA-seq dataset, the data was normalized for sequencing depth per cell and log-transformed. Next, we looked for the most variable genes (Supplementary figure 1 A, B). Based on the outcome of differential expression analysis, cells from both donors were divided into seven different clusters represented on the tSNE plot shown in Figure 2A. A total of 745 CD4$^+$ T cells were included in the final analysis, of which 338 and 407 cells belonged to donor GSK002 and GSK009, respectively (Figures 2B). Genes characterised by the highest fold change (log2FC) in each cluster were associated either as signature markers of T cell subsets or with biological processes (Supplementary figure 1). Out of seven clusters, clusters 1 and 4 were found to be most distinctive in terms of expression profiles (Figure 3 A, B) and they were analysed in more detail.

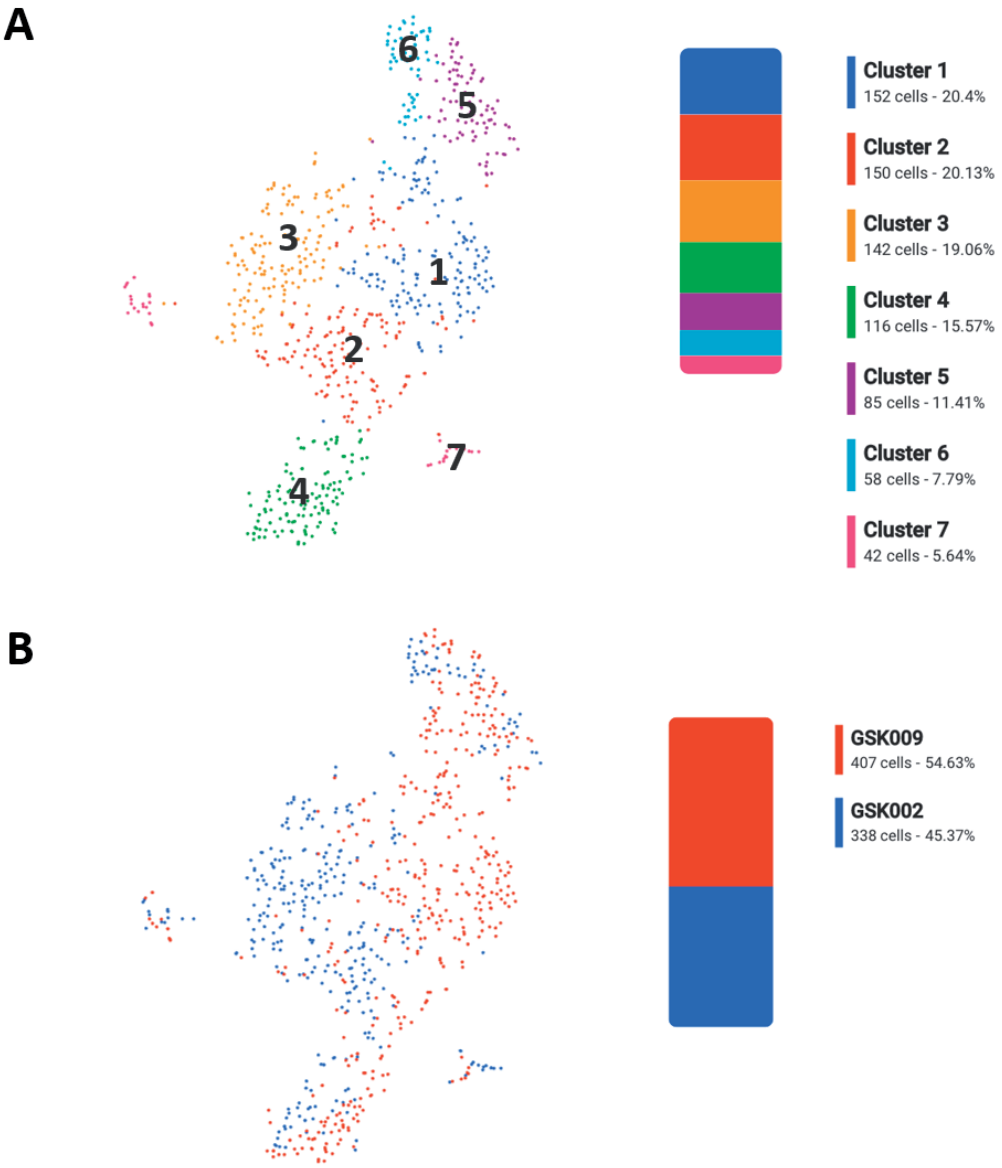


Figure 2. Clustering analysis of sc RNA-seq data. (A) mRNA sequencing of 745 single *S. aureus*-specific CD4$^+$ T cells, which were sorted based on CD137$^+$OX40$^+$ upregulation, from 2 donors, GSK002 (338 cells) and GSK009 (407 cells). *S. aureus*-specific CD4$^+$ T cells were distributed over seven clusters represented on the tSNE plot. The number and proportion of cells contributing to each cluster is reported on the right; **(B)** Contribution of cells from GSK002 (blue) and GSK009 (red) to each cluster in the tSNE plot. Numbers of cells and proportion for each donor are represented on the right**.**

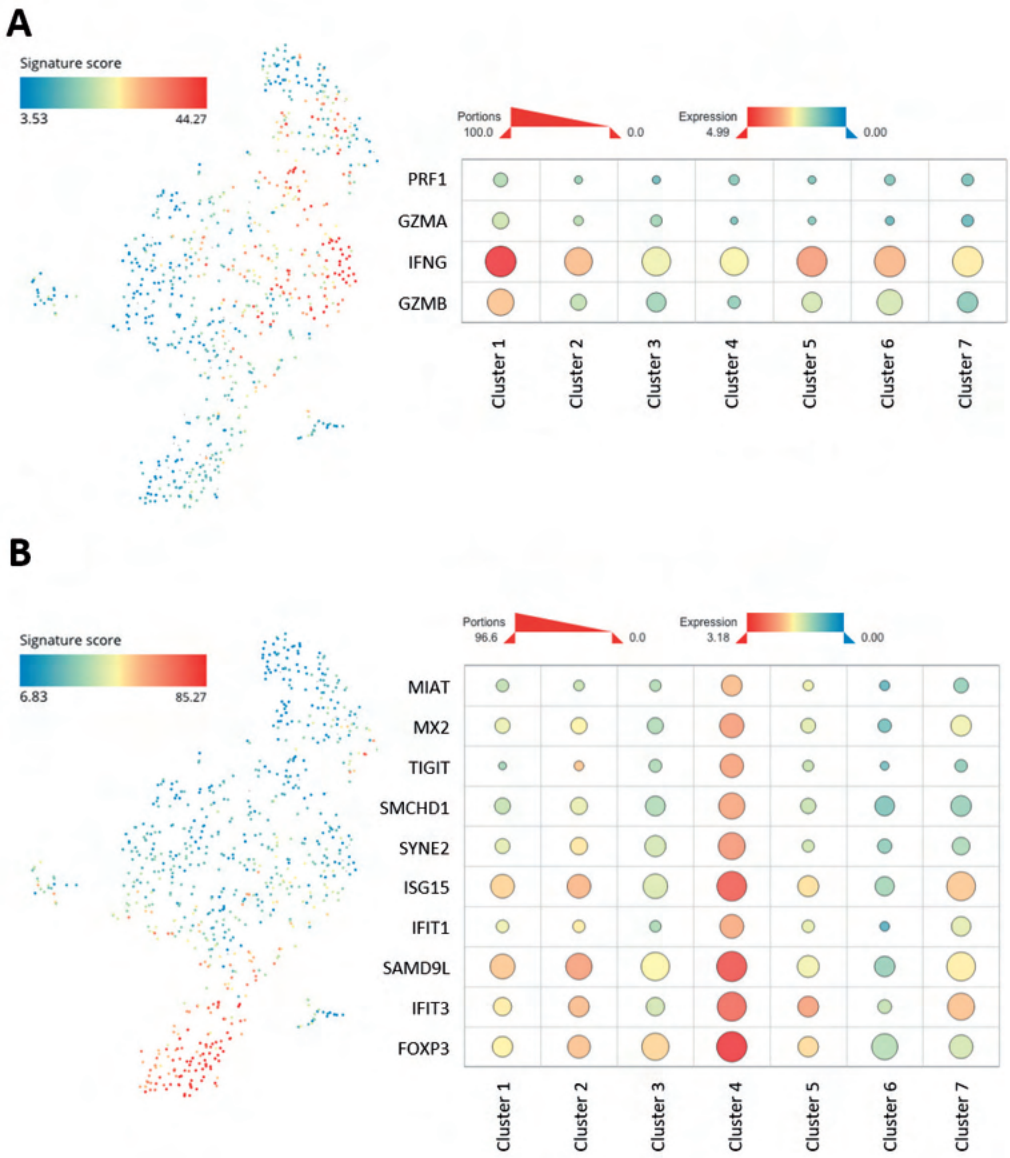


Figure 3. Detailed analysis of altered mRNA transcripts in clusters 1 and 4. On the left tSNE plots representing in blue upregulated genes in Cluster 1 **(A)** and Cluster 4 **(B)**. On the right, heatmaps representing the average level of gene expression in each cluster. Colour indicates level of expression whereas size of a dot percentage of cells expressing the gene. Each row of the heatmap represents a specific gene as indicated by the name to the left of the row. Upregulation of genes that correspond to cytotoxic cells (CTL) or regulatory T cells (Treg) in cells forming Cluster 1 **(A)** or Cluster 4 **(B)** is shown as average for all cells in all clusters.

Cells in cluster 1 showed upregulation of transcripts for granzyme A (GZMA), granzyme B (GZMB), perforin (PRF-1) and IFN-γ (IFNG), which are the typically expressed by cytotoxic T lymphocytes (CTLs) (Figure 3A). Classically, human CTLs are $CD8^+$, however, more recent studies have demonstrated the presence of $CD4^+$ CTLs in cancers and viral infections [19]. Although no $CD4^+$ CTL exclusive markers have been identified so far, markers used for the identification of $CD8^+$ CTL have been successfully used to identify $CD4^+$ CTL [20]. In a recent study from Hashimoto *et al.*, $CD4^+$ CTL were identified in the blood of supercentenarians using scRNA-seq based on the upregulation of genes for cytotoxic molecules such as granzyme family genes or perforin [20]. Interestingly, cluster 1 was only observed in GSK009 (Figure 2B, 3A), showing donor-to-donor variability in terms of adaptive immune responses toward *S. aureus*. Analysis of more donors will be performed to address this important point.

Cells in the cluster 4 were characterized by the upregulation of several Treg-specific genes, including FOXP3, the master transcription factor driving the development of Tregs (Figure 3B) [21]. Another gene highly upregulated in cluster 4 and characteristic for a Treg subclass is TIGIT, a co-inhibitory molecule that binds to CD155 on dendritic cells [22,23]. Binding of TIGIT to CD155 suppresses IL-12 while simultaneously inducing IL-10 production, a cytokine mediating anti-inflammatory responses [22]. Cells from cluster 4 also showed upregulation of transcripts for proteins belonging to the Interferon Induced proteins with Tetratricopeptide repeats (IFIT) family, namely IFIT1, IFIT3 and Interferon-stimulated gene 15 (ISG15) (Figure 3B) which are classically upregulated during viral infections [24]. Treg were observed in the samples from both donors, indicating that this regulatory response could be a common feature of *S. aureus* exposure in the human population.

CLA^+ *S. aureus*-specific $CD4^+$ T cells are enriched in Treg transcripts compared to CLA^- *S. aureus*-specific $CD4^+$ T cells

One of the aims of this study was to determine the correlation between the occurrence *S. aureus*-specific $CD4^+$ T cells in the circulation of healthy subjects and their skin-homing capacity. For this reason, we performed index sorting, which allowed us to link the phenotype of each sorted cells including expression of the skin-homing marker CLA to their transcriptional profile [16,25]. In total we analysed 349 cells with CLA^+ and 548 cells with CLA^- phenotype and observed contribution of both groups to all clusters. Next, we performed differential expression analysis of gene transcripts to identify differences between CLA^+ and CLA^-cells. Compared to the CLA^- population, the CLA^+ population showed increased expression of FOXP3, IFIT3, ISG15, CD74, STAT1 and RGS1 (Figure 4), genes characteristic for Tregs subset (cluster 4). CD74 transcript has been previously detected in human $CD4^+$ Tregs cells using scRNA-seq [26], which is in line with our study. In addition, among CLA^+ cells showed higher transcript levels of Signal transducer and activator of transcription 1-alpha/beta (STAT1) compared to CLA^- cells. High levels of STAT-1 were observed in $CD4^+$ Treg cells of systemic lupus erythematosus patients. Upregulation of

Upregulated	Downregulated
CD74	GZMB
FOXP3	PNP
NEAT1	SATB1
SAMD9L	MIR155HG
MX1	NPM1
RGS1	HSP90AB1
OAS1	PHB
EPSTI1	RPS17
ISG15	SNHG16
RNF213	BHLHE40
MALAT1	RAN
STAT1	RPL29

Figure 4. Table representing up- and downregulated transcripts in the CLA$^+$ cluster compared to CLA- T cells. Upon index sorting we correlated surface expression of CLA with sequenced cells. Next, differential expression analysis between CLA$^+$ and CLA$^-$ cells was performed showing differences in the gene expression between these two populations.

Regulator of G Protein Signalling 1 (RGS1) is attributed to mouse Tregs, but not naïve CD4$^+$ T cells phenotype [27]. Interestingly, in a study comparing transcriptome of Trm and circulating T cells, downregulation of RGS1 was identified as a core signature of tissue residency [28]. However, it is important to mention that this marker was identified in Trm directly isolated from the tissue, and not circulating Trm as we analysed here. Therefore, it is probable that cells switch the transcriptional profile of RGS1 upon migration back to the tissue [28]. A similar explanation may apply to BHLHE40, which is increased in lung CD8$^+$ Trm compared to circulating CD8$^+$ T cells [29], but in our study shows downregulation in CLA$^+$ cells compared to CLA$^-$ cells (Figure 4). Overall, transcriptional changes in these tissue-residency markers could potentially orchestrate migration of CLA$^+$ cells from and to the skin. Although the majority of the genes that were differentially expressed by CLA$^+$ versus CLA$^-$ *S. aureus*-specific T cells are associated with Tregs, we can only conclude that CLA$^+$ cells enrich this cluster the most. Simultaneously, CLA$^+$ are also present in other clusters, however, at the lower frequency than CLA$^-$.

scRNA-seq signatures correlate with proliferation and cytokine production observed in the cellular assays

We assessed cytokine production and cell proliferation of CD4$^+$ T cells upon stimulation with HKSA using PBMCs from the same 2 donors that were analysed by scRNA-seq. To determine proliferative capacity, CD4$^+$ T cells were loaded with CFSE, which fluorescently labels cell cytoplasm and is diluted after each cell division. Consequently, daughter cells become progressively less fluorescent ($CFSE_{low}$) upon each cell division. CD4$^+$ T cells from both donors

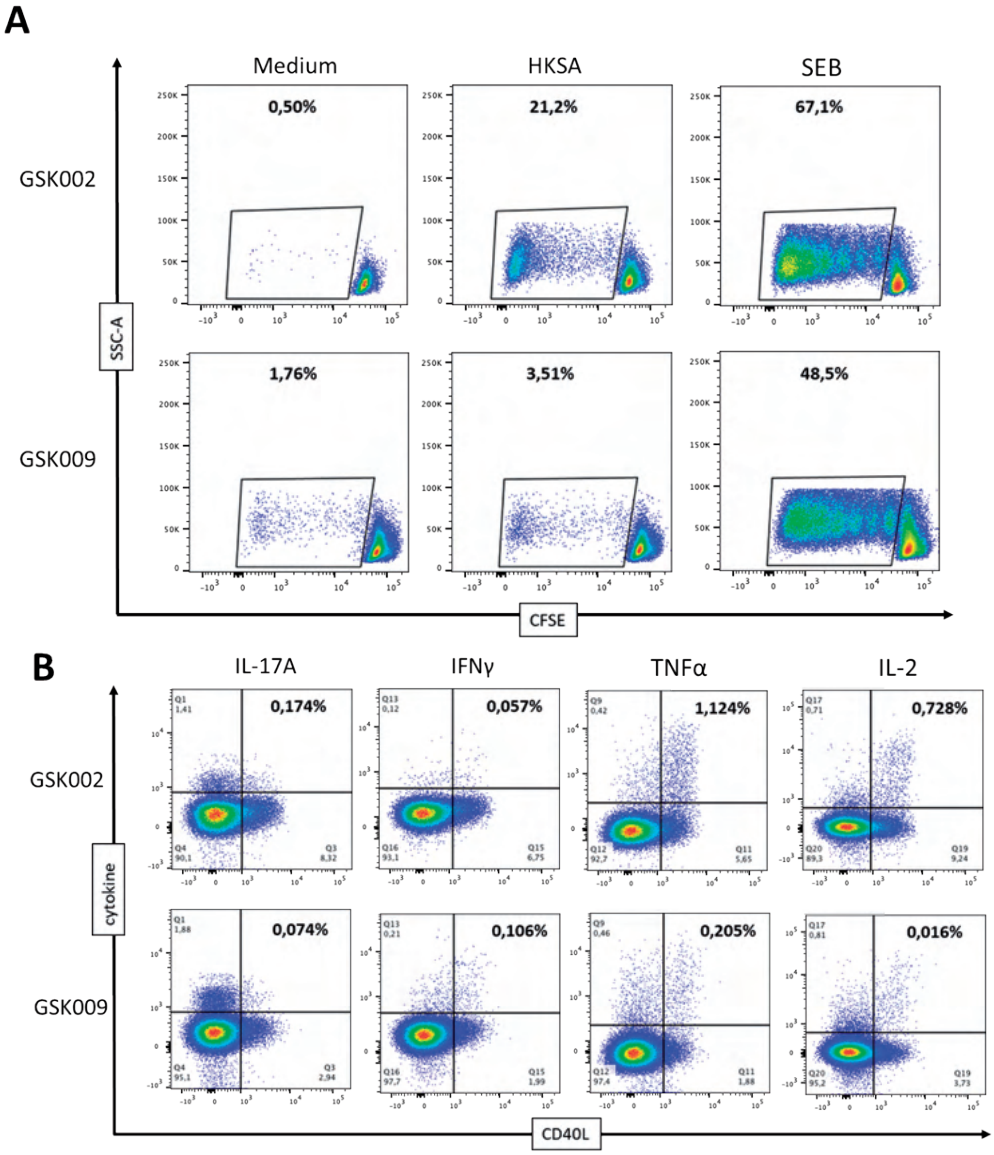


Figure 5. CFSE proliferation assay of *S. aureus*-specific T cells from the two donors analysed by sc mRNAseq. (A) CFSE-loaded CD4$^+$ T cells in co-culture with γ-irradiated CD4- fraction (containing APCs) were stimulated with HKSA or SEB or left unstimulated (medium) for 7 days. Cells were stained for surface expression of CD3, CD4 and CD8 to gate on CD4$^+$ T cells and analysis of CFSE expression. Cells were analysed by FACS as described in Materials and Methods; **(B)** Intracellular cytokine staining of *S. aureus*-specific CD4$^+$ T cells from donors GSK002 and GSK009. Cells were stimulated with HKSA or a positive control SEB (data not shown) or left unstimulated (medium) for 2 h, before addition of brefeldin and further stimulation for additional 16 h. The percentages of CD4$^+$CD40L$^+$cytokine$^+$ cells reported are medium-subtracted values**.**

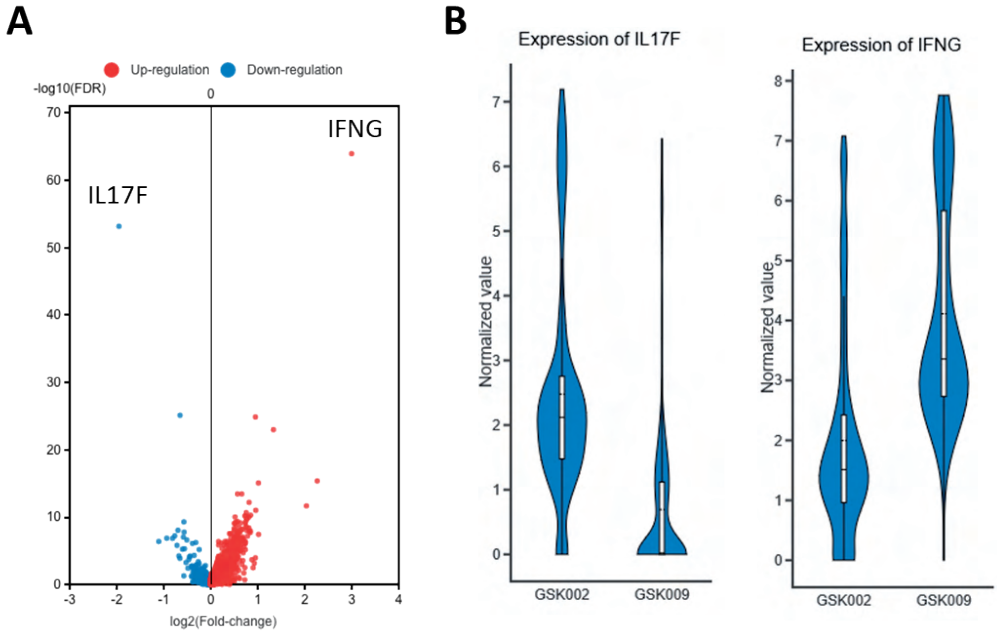


Figure 6. (A) Volcano and **(B)** violin plots showing differential gene expression between cells from donors GSK009 and GSK002. **(A)** IL17F and IFNG appeared as two genes with the highest dissimilarity index. **(B)** IL17F is almost exclusively expressed by donor GSK002, whereas transcript for IFNG are present in both donors, but strongly upregulated in donor GSK009.

proliferated in response to HKSA, although with different intensity. Surprisingly, donor GSK002 showed a stronger proliferation in response to stimulation with HKSA as compared to donor GSK009 (Figure 5A). This is opposite from the observations with these donors in the AIM assay (Figure 1C, Supplementary table 1). As expected, CD4$^+$ T cells from both donors proliferated in response to SEB showing that PBMC samples were generally of good quality (Figure 5A). In addition, we analysed transcripts using Reactome database and observed that antigen-specific CD4$^+$ T cells from both donors could be generally characterized by a high activation state due to upregulation of genes characteristic for cell cycle (data not shown). To gain additional insight for the *S. aureus*-specific CD4$^+$ T cell response, we also performed an ICS assay. Donor GSK002 displayed a higher frequency of IL-2-producing cells, a cytokine that drives T cell proliferation, compared to donor GSK009 (Figure 5B). This finding correlates with the higher number of proliferating cells in this donor (Figure 5A). Donor GSK009, which contained the CTL cluster (Figure 2A, B), displayed a higher frequency of IFN-γ-producing cells (Figure 5B), in agreement with upregulation of the IFNG gene (Figure 3A). Donor GSK 002 and GSK009 differed in the frequency of TNFα-producing T cells (Figure 5B), however, no correlation with sequencing data was observed. IL-17A-producing T cells could be detected at the low frequencies for both donors (Figure 5B). Performing differential expression analysis on cells from these two donors, IL17F and IFNG genes were

identified as being significantly different between donors (Figure 6A, B). Again, donor GSK009 showed higher expression of IFNG in contrary to donor GSK002, but in contrast almost completely lacked production of IL17F (Figure 6). Unfortunately, IL-17F was not included in our ICS assay, so these results need further verification.

Discussion

S. aureus is a leading cause of skin and soft tissue infections worldwide. *S. aureus* infections are multifactorial, involving various components of the immune system and despite many efforts, no efficacious vaccine has been developed so far [30]. For this reason, gaining an understanding into the processes underlying *S. aureus* infection and generation of immune memory towards this pathogen are crucial to design an effective treatment. One of the important steps to achieving this goal is the qualitative analysis of human adaptive immune responses elicited after exposure to *S. aureus*. Here we demonstrate the identification and characterization of *S. aureus*-specific $CD4^+$ T cells from healthy donors using AIM followed by scRNA-seq. This novel approach allowed us to identify rare populations of $CD4^+$ T cells such as CTLs and Tregs.

In humans, the majority of CTLs are represented by $CD8^+$ T cells and their functions are mainly associated with killing of cancerous and virus-infected cells. Upon antigen recognition via MHC class I, activated $CD8^+$ T cells mediate killing of target cells via one of three pathways: cytokine production, mainly IFNγ and TNFα, release of cytotoxic granules or directly via Fas/FasL interaction. In contrast, functions of $CD4^+$ CTL have yet to be fully described. For a long time, $CD4^+$ CTL were believed to be an artefact of prolonged *in vitro* culture. However, more recent studies identified this T cell subset in blood of healthy subjects and patients with chronic viral infections as well [19,20]. Although $CD4^+$ CTL can be generated *in vivo* during various inflammatory conditions, some studies suggest that they primarily differentiate from $CD4^+$ Th1 subsets after long-term exposure to low doses of antigen, as in chronic viral infections [19,31]. It is well known that *S. aureus* colonizes skin and nares of many healthy subjects, often without overt signs of infection. As a result, staphylococcal antigens are likely constantly sampled and presented by APCs as evidenced by the near universal presence of anti-*S. aureus* antibodies in the circulation of healthy individuals. Possibly, this frequent or even continuous immune expose may induce the generation of $CD4^+$ CTL. Another possible explanation could come from influenza virus infection studies where, during the chronic phase of the infection, impaired antiviral $CD8^+$ CTL activity results in compensation by $CD4^+$CTL [32]. Indeed, *S. aureus* is able to lyse $CD8^+$ CTL through the production of staphylococcal α-toxin (Hla) [33]. Therefore, it may be possible that absence of $CD8^+$ drives the formation of $CD4^+$ CTL during *S. aureus* infection [33]. An unwanted effect however could be that $CD4^+$ CTL kill antigen-loaded APCs through MHC class II-

mediated antigen recognition, thereby disrupting antigen presentation and generation of memory T cells. Overall, this study provides the first evidence of *S. aureus*-specific CD4$^+$ CTLs in the circulation of healthy donors. The elucidation of their function requires additional studies.

Tregs are another subset of T cells identified in this study. Tregs are critical players in adaptive immunity, responsible for controlling excessive immune responses, thereby preventing immunopathology [34]. Through the secretion of immunosuppressive cytokines, such as IL-10 and TGF-β, and direct cell-to-cell interactions, they regulate the differentiation and proliferation of other T cell subsets [34], whereas impaired Treg functions have been associated with excessive inflammatory processes [35]. Hence our body is broadly colonized by different commensal bacteria presence of Tregs at the barrier sites is necessary to maintain tolerance towards them [36]. Indeed, accumulation of commensal-specific Tregs in the neonatal skin upon bacterial colonization correlates with generation of immune tolerance [37]. On the other hand, too much inhibition of proinflammatory responses during infection can result in inefficient bacterial clearance. Generation of Tregs has been associated with several *S. aureus* virulence factors. Upon *in vitro* stimulation of human PBMCs with *S. aureus* protein A (SpA) production of IL-2, G-CSF and IL-10, cytokines associated with Tregs development, was detected and eventually generation of Tregs [38,39]. Moreover, when stimulating with whole bacteria, the induction of Tregs was enhanced suggesting that more *S. aureus* factors could be associated with this process [38]. Secreted bacterial molecules may also contribute to the generation of Tregs during infection. Indeed, several studies using mouse models have associated phenol soluble modulins (PSMs) with the generation of tolerogenic DCs displaying reduced antigen uptake and increased IL-10 production, thus resulting in generation of Tregs while inhibiting Th1 differentiation [40–42]. In contrast, a study from Tebartz *et al.* attributed tolerogenic responses during chronic *S. aureus* infections mostly to DCs as opposed to Tregs [43]. The majority of previously-described studies were performed in mice during infection or using human PBMCs stimulated *in vitro*. Therefore, it is unclear how these results translate to the human situation especially in healthy donors. However, by detecting Tregs in samples included in this study, we can speculate on how the generation of Tregs may support the long-term colonization of nares and skin by *S. aureus*, the default life-style of this bacterium, without causing inflammation. This hypothesis is well aligned with a persistence mechanism of several *Leishmania* species, a protozoan parasite commonly colonizing and infecting skin. In patients with cutaneous leishmaniasis persistence of this pathogen in the skin after lesions have healed is related to presence of Treg accompanied with impaired proinflammatory responses [44,45]. High numbers of Tregs accumulate in the skin lesions of patients with *Leishmania* infections and presumably dampen effector T cell responses, permitting protozoan survival [45,46]. In case of infection however, the induction of tolerogenic DCs and subsequent generation of Tregs will promote anti-inflammatory responses and may allow bacterial dissemination. Interestingly, Tregs were detected in

both analysed donors, whereas CD4$^+$ CTL were only present in the samples acquired from donor GSK009. This is a clear example of donor-to-donor variability in the immune response towards *S. aureus* and could be a relevant observation in the context of epidemiological studies. In fact, differences in the adaptive memory responses towards *S. aureus* could be an explanation for divergent clinical presentations often observed with individuals harbouring *S. aureus*.

Additional focus of our study was to understand whether we can identify transcriptional markers of skin-poised CD4$^+$ T cells. Since access to human skin is often limited, characterization of circulating Tsrm could provide relevant information about the properties of T cells relevant for the skin without the need of a tissue biopsy. Trsm represent the first line of defence against re-infections, as widely described for viruses like herpes simplex virus (HSV), for which the level of protection correlates directly with the Trm density in the skin [47,48]. Therefore, their extensive characterisation could provide more understanding about cues directing Tsrm to the skin and would be especially of interest in the context of anti-staphylococcal immunity. Although our analysis did not prove that CLA$^+$ cells cluster separately, we could observe that they are strongly enriched for Tregs markers. This can be supported by the previous finding that majority of circulating Tregs express on their surface skin-homing receptors [49]. However, CLA$^+$ population did not exclusively express Treg markers, which could suggest that they consist of cells at multiple differentiation steps. Considering that *S. aureus* exhibits a commensal phenotype without causing inflammation, induction of tolerogenic responses can be beneficial for the host to prevent excessive inflammation. On the other hand, lack of effector cells could lead to slower bacterial clearance when infection does occur. As discussed above, equilibrium between Tregs and effector T cells is important to maintain skin homeostasis. It would be of interest to investigate differences between CLA$^+$ and CLA$^-$ in additional donors to identify genes that could serve as markers for skin-homing cells in the future. The current dataset suggests that skin-homing CD4$^+$ T cells detected in the circulation are not characterised by just one type of response. It is possible that when CLA$^+$ CD4$^+$ T cells home to skin upon recall, *e.g.* during skin infection, they acquire more specific effector functions in situ, such as IL-17 production. Indeed, our results together with previous data obtained by other groups suggest that there is a plasticity between Tregs and Th17 cells induced by *S. aureus* [2,50], highlighting the added value of performing scRNAseq analysis. The equilibrium between these two cell subsets has been shown to be regulated by pro- and anti-inflammatory cytokines, namely IL-1b and IL-2 [50,51] and is accompanied by metabolic pathways shift, with a prominent role of *de novo* fatty acid (FFA) synthesis in controlling the fate between Th17 and Tregs [52]. Remarkably, enhanced extracellular FFA uptake was demonstrated in human CD8$^+$ Trm cells in normal and psoriatic skin, suggesting that FFA have a critical role in the maintenance and persistence in tissue where they mediate protective immunity function [53].

Although our analysis allowed us to identify rare populations of T cells, they were classified based on their activation state, rather than belonging to a certain subclass, like Th1 or Th17 subset. It is in line with recently published study from Cano-Gamez *et al.* showing that CD4$^+$ T cells are homogenous population with cells at multiple interrelated transcriptional states [26]. Cells progress from naïve to memory phenotype to eventually reach effector functions and express cytotoxic molecules such as GZMA and GZMB [26]. This can suggest that observed CD4$^+$ CTL cluster can be the final step in the cell differentiation and other clusters are intermediate steps. However, to fully understand activation state of the remaining clusters trajectory analysis on multiple donors must be performed. Interestingly, Tregs were described as the most distinct cluster branching out from other subsets [26]

An interesting point of this study is the correlation between ICS and CFSE-proliferation assays and scRNA-seq results. We observed that results obtained in both cellular assays generally corresponded to the transcriptional expression profile of both donors. This suggests that selection of antigen-specific cells using AIM assay can identify both cytokine producing and proliferating cells. This is of interest since it allows for a broader inclusion of donors in contrast to the CFSE proliferation or ICS assay, where donors are discarded when CD4$^+$ T cells do not proliferate or lack the production of cytokines in response to *S. aureus* stimulation. Our results show that AIM avoids these false-negative results by capturing all subsets of antigen-specific cells as could be observed in the data from mRNA sequencing. In addition, AIM paired with scRNA-seq allows in-depth characterization of T cell subsets without a need of extensive flow-cytometry staining panels or prolonged culturing times. An important point to make is selection of CD4$^+$ T cell activation markers. Although CD137, OX40 and CD40L are commonly used for selection of activated cells, as described extensively by Elias *et al.*, their expression does not necessarily overlap [11]. Therefore, the selection of the staining panel can determine the frequency and quality of detected cells.

Summarizing, our novel approach allowed us to identify *S. aureus*-specific CD4$^+$ T cells in an unbiased way. We provide an extensive comparison of techniques used for identification of *S. aureus*-specific CD4$^+$ T cells including the first-time approach of in-depth AIM/scRNA-seq characterization of *S. aureus*-specific CD4$^+$ T cells. Although it is difficult to draw definitive conclusions based on the data generated from just two donors, the described results provide an illustration on the multitude of T cell responses initiated when the human immune system encounters *S. aureus*. Next step will be the analysis of *S. aureus*-specific CD4$^+$ T cells from more donors. It is important to understand what the anti-staphylococcal T cell responses in the broader populations are and whether we can recover Tregs and CTLs in more donors. In addition, collection of the bigger dataset might allow identification of skin-tropic CD4$^+$ T cell markers.

Conflict of interest

MEM is Ph.D. fellows enrolled in the Infection and Immunity Ph.D. program of the graduate school of Life Sciences at Utrecht University and participated in a post graduate studentship program at GSK. ST, CS, MB, FB and ES are employees of the GSK group of companies. FB hold shares in the GSK group of companies. FB holds pending and issued patents (WO/2019/158537, WO/2015/144691, WO/2015/144653, WO/2015/144655, WO/2014/033190, WO/2014/033191, WO/2014/033192, WO/2014/033193, WO/2013/030378, WO/2010/119343) on *S. aureus* vaccine formulations.

Author contributions

MEM and ES were involved in designing the study. MEM performed experiments. ST and CS performed sorting and provided valuable technical support with flow cytometry. MEM and ES analysed the data. MEM wrote the manuscript. MB, FB and ES supervised the project. All authors critically revised the manuscript and approved it before submission.

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Supplementary data

Supplementary Table 1. List of antibodies used in this study

Antibody	Supplier	Clone	Cat nr
anti-human CLA-BV605	BD biosciences	HECA-452	563960
anti-human CD4-BV421	BD biosciences	RPA-T4	562424
anti-human CD8-BUV805	BD biosciences	RPA-T8	749366
anti-human CD3-PE-CF594	BD biosciences	UCHT1	562280
anti-human OX40-PE-Cy7	Biolegend	Ber-ACT35	350012
anti-human CD137-APC	Biolegend	4B4-1	309810

CD137+OX40+

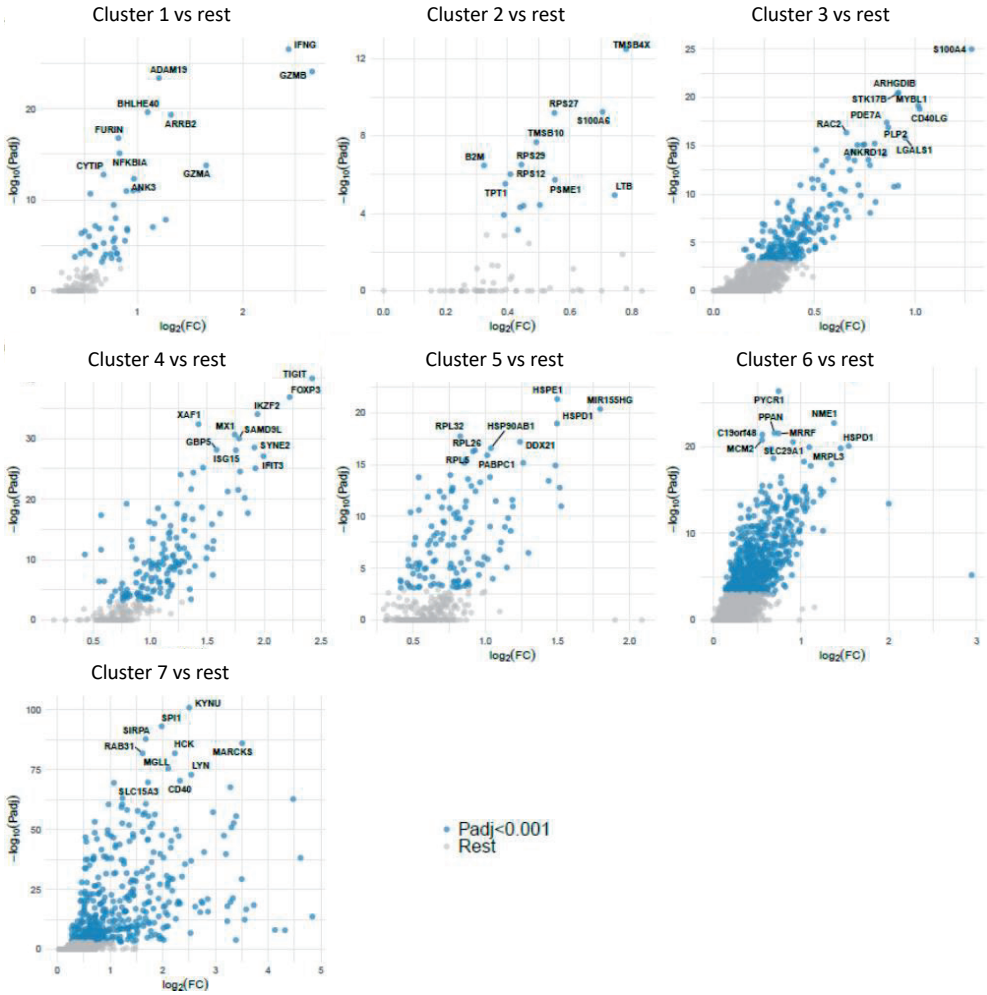
	Frequency of CD137+OX40+			
Donor	Medium	HKSA	SEB	
GSK009	0,08%	1,72%	10,90%	
GSK004	0,03%	1,25%	17,80%	
GSK001	0,08%	1,02%	10,30%	
GSK005	0,00%	0,80%	3,27%	High responders
GSK015	0,06%	0,73%	4,56%	
GSK016	0,00%	0,53%	3,45%	
GSK010	0,02%	0,49%	3,13%	
GSK002	0,01%	0,42%	3,37%	
GSK012	0,00%	0,38%	3,03%	
GSK006	0,00%	0,29%	0,92%	Medium responders
GSK014	0,00%	0,22%	2,46%	
GSK003	0,00%	0,18%	1,07%	
GSK013	0,00%	0,15%	1,19%	Low responders
GSK007	0,01%	0,15%	2,02%	
GSK011	0,00%	0,12%	1,63%	
GSK008	0,00%	0,02%	0,68%	Non responder

Supplementary table 2. Results of screening of PBMCs from 16 healthy subjects for the presence of *S. aureus*-specific CD4+ T cells. Based on the frequency of CD137+OX40+ CD4+ T cells acquired in the AIM assay donors were classified as high, medium, low or non-responders. Only one donor out of the 16 tested did not respond upon stimulation with HKSA. Based on the percentage of CD137+OX40+ cells and their numbers after flow cytometry acquisition Donors GSK002 and GSK009 highlighted in red were selected for the further scRNA-seq analysis.

A

Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Cluster 7
IFNG	TMSB4X	S100A4	TIGIT	NPM1	FABP5	KYNU
GZMB	S100A6	ARHGDIB	FOXP3	HSPE1	PYCR1	SPI1
ADAM19	RPS27	STK17B	IKZF2	MIR155HG	NME1	SIRPA
BHLHE40	TMSB10	MYBL1	XAF1	HSPD1	PPAN	MARCKS
ARRB2	RPS29	CD40LG	MX1	RPL32	MRRF	HCK
FURIN	B2M	PDE7A	SAMD9L	DDX21	C19orf48	RAB31
NFKBIA	RPS12	PLP2	SYNE2	HSP90AB1	MCM2	MGLL
GZMA	PSME1	RAC2	GBP5	RPL26	SLC29A1	LYN
CYTIP	TPT1	LGALS1	ISG15	RPL5	HSPD1	CD40
ANK3	LTB	ANKRD12	IFIT3	PABPC1	MRPL3	SLC15A3

B



< Supplementary figure 1. Results of the differential expression analysis on seven clusters obtained from the scRNA-seq of 745 single CD4+CD137+OX40+ cells sorted from 2 donors, GSK002 (338 cells) and GSK009 (407 cells). For each cluster top 10 genes with the highest log2FC are represented in a **(A)** table or on **(B)** a volcano plot.

CHAPTER 5

Summarizing discussion

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C-Type Lectin Receptors in Host Defense Against Bacterial Pathogens

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Every year, both community-acquired (CA) and healthcare-acquired (HA) strains of methicillin-resistant *S. aureus* (MRSA) are responsible for millions of infections worldwide. In the United States alone, costs associated with treatment of MRSA infections is estimated at $34 526 per patient [1]. Costs even reach up to $13.8 billion annually when infections caused by both methicillin-resistant and methicillin-susceptible *S. aureus* strains are considered [1,2]. Skin and soft-tissue infections (SSTIs) represent the most common *S. aureus* infections. They vary from relatively mild disease manifestations, such as cellulitis and abscesses, to severe and life-threatening diseases, like surgical site infections. Moreover, untreated or inadequate treatment of SSTIs can lead to development of bloodstream infections including sepsis and infective endocarditis [3]. Since the effectiveness of antibiotics to treat *S. aureus* SSTIs is decreasing due to a rise in antimicrobial resistance, the development of new solutions to treat *S. aureus* infections, such as vaccines or antibody treatment, are urgently needed. Many attempts to design a preventive therapy against *S. aureus* have been made, however none of them was successful so far. Two types of approaches can be distinguished: passive immunization using *S. aureus*-targeting antibodies or classical active vaccination with recombinant *S. aureus* proteins and conjugate capsular polysaccharides (CP) to elicit protective (antibody) responses in the host. The reason for the trial failures is multifactorial, including antigen selection and the use of animal models that are not fully translatable to human staphylococcal infection. Although anti-staphylococcal antibodies are present in the serum of healthy individuals and trial vaccinees showed partial protection against *S. aureus* infections, it has now been demonstrated that antibody responses alone are not sufficient to protect against *S. aureus* infections [4–6]. Therefore, vaccines should target different components of the immune system simultaneously.

Selection of the right vaccine antigens is particularly difficult for *S. aureus* as it produces many virulence factors. Due to the strain-to-strain variability and external environment affecting bacterial protein expression, vaccine formulation aims to include bacterial molecules that are highly conserved across *S. aureus* lineages under different circumstances. The attempted strategies have included targeting bacterial toxins to reduce tissue damage and surface-expressed molecules with the aim to promote opsonophagocytosis. The tested *S. aureus* vaccine antigens include staphylococcal α-toxin (Hla), clumping factor A and B (ClfA/B), Panton-Valentine Leukocidin (PVL) and CP5/CP8 [7].

Selection of the model to investigate *S. aureus* infections is crucial to understand what protective responses are elicited by the bacterium *in vivo*. Although *S. aureus* can colonize and infect multiple species it has co-evolved with the human host and expresses several human-specific virulence factors [8]. Therefore, studies using mouse models are not always translatable to the human infection status. Indeed, a recent study from Montgomery *et al.* showed that the genetic background of mouse, in particular its MHC haplotype, can influence the susceptibility to *S. aureus* infection [9,10]. Consequently, selection of a

particular mouse strain can strongly affect outcome of the experiments [9]. Not only genetic differences but also discrepancies between human and mouse anatomy, *e.g.* skin, limit the translatability of results. For example, skin anatomy and immune composition is strikingly different between humans and mice [11], underlying the importance to develop human skin models to study *S. aureus* infections and vaccination [12]. Especially use of novel technologies for vaccine delivery, such as dermal microneedle patches directly targeting skin-resident immune cells, require the development of suitable three-dimensional models. Microneedles application could target select cell population, such as dermal dendritic cells (DCs) and Langerhans cells (LCs) by engaging different receptors in antigen recognition, thereby affecting outcome of vaccination compared to the clinical data obtained so far. Therefore, reinventing vaccination strategy could help to elicit protective responses at the *S. aureus* site of entry – the skin.

S. aureus commonly resides on the skin of healthy individuals, suggesting a balanced interaction between *S. aureus* and the host's local immune defenses. Investigation of the local immune responses is important aspect in understanding the mechanisms underlying *S. aureus* colonization and infection. In this thesis, I focused on several aspects of interplay between *S. aureus* and the immune system: from the first step that starts the immune cascade – recognition by tissue-resident APCs (**Chapter 2**) through the presence and responses of *S. aureus*-specific T cells (**Chapters 3 and 4**). Both parts are strictly connected since the uptake of *S. aureus* by APCs directs the activation and responses of T cells through antigen presentation and cytokine production. Skin resident memory T (Tsrm) cells provide long-lasting protection against various pathogens. Therefore, harnessing their potential is an important aspect in a novel vaccine design to prevent SSTIs and possibly *S. aureus* infections in general. In this final chapter, I will discuss how we can benefit from the new insights in *S. aureus*-host interaction presented in this thesis with a focus on eliciting tissue-resident immune responses.

C-type Lectin Receptors in the recognition and protection against *S. aureus* infections

Glycosylation is the most common form of post-translational modifications that is present in all types of eukaryotic cells [13]. For a long time, it was believed that glycosylation was restricted to eukaryotes. However, it is now firmly established that glycosylation occurs throughout all kingdoms of life, including bacteria. Bacteria can glycosylate different structures of their cell envelope, including proteins and polymeric structures such as wall teichoic acid (WTAs) for *S. aureus* [14,15]. Given the abundance of glycosylation in nature and especially on the surface of (bacterial) cells, it is not surprising that species have evolved receptors recognizing these glycan modifications or patterns [16]. C-Type Lectin

Receptors (CLRs) are a family of pattern-recognition receptors (PRRs) involved in sensing of both self and non-self sugar residues or motifs [17]. Furthermore, CLRs are implicated in the protection against viral, fungal and bacterial pathogens through the initiation or modulation of immune responses [18–21]. CLRs are mainly expressed by APCs, which are crucial sentinel cells for the recognition of microbes both in the circulation but mostly at the barrier tissues, such as the skin, which is a common site of entry for several important pathogens. Previously discovered by van Dalen *et al.,* the interaction between Langerin and β-GlcNAc residues on the *S. aureus* WTA was the first evidence that CLRs are involved in anti-staphylococcal immunity [22]. Langerin is a CLR exclusively expressed on LCs, a subset of macrophages present in the skin epidermis and to some extend mucosal membranes [23]. *S. aureus*-Langerin interaction resulted in production of TNF-α, IL-8, IL-6, IL-12p70 and IL-23 by LCs [22], thus suggesting this molecular interaction could polarize T cells towards Th17, which is important for *S. aureus* defense .

In Chapter 2, I described the interaction between *S. aureus* lineage ST395, using strain PS187 as representative of this lineage, and the Macrophage Galactose-Type C-Type Lectin (MGL/CD301) receptor. Unlike WTA of most *S. aureus* strains composed of poly-ribitol-phosphate (RboP), ST395 represents a unique lineage characterized by production of structurally-different WTA composed of poly-glycerol-phosphate (GroP) [24]. This type of backbone is commonly expressed by coagulase-negative staphylococci (CoNS), including *S. epidermidis* and *S. lugdunensis*, which are dominant members of a healthy skin microbiota. MGL is naturally expressed on human dendritic cells (DCs) and macrophages residing in skin and lymph nodes, blood CD1c + DCs, and immature monocyte-derived DCs (moDCs) [25–27]. This receptor had previously been described as the receptor for the Gram-negative pathogens *Campylobacter jejuni* and *Neisseria gonorrhoeae* through interaction with specific glycosylated structures [28,29]. Using recombinant and cell surface-expressed MGL, I demonstrated that MGL specifically interacts with α-GalNAc residues on PS187 WTA. Engagement of MGL by *S. aureus* PS187 resulted in cytokine production and upregulation of surface activation markers by monocyte-derived DCs (moDCs), particularly IL-6 and IL-12p70, which was reduced when strains lacked α-GalNAc on their WTA. Surprisingly, MGL did not interact with different CoNS, despite the presence of the *tagN* gene, which is responsible for WTA α-GalNAc glycosylation [24]. Possibly our culture conditions are not suitable to induce expression of *tagN* by CoNS resulting in lack of the interaction. In general, it is thought that CoNS induce rather tolerogenic than proinflammatory responses [30]. Therefore, reduction of WTA glycosylation in CoNS may minimize immune recognition and unwanted inflammation. In contrast, the induced pro-inflammatory response following *S. aureus*-MGL interaction suggests the importance of WTA glycosylation for anti-bacterial immunity. However, involvement of MGL is likely restricted to the *S. aureus* ST395 lineage, which accounts only for only 5% of all *S. aureus* isolates [31]. Since both MGL and Langerin are expressed by different cell subsets present in distinct skin compartments, it can be

hypothesized that regardless of the glycosylation profile of infecting *S. aureus* isolate, early sensing and immune activation will occur through lectin-dependent recognition.

Challenges and opportunities in identifying new interactions between CLRs and *S. aureus*

APCs guard our body and continuously sample the environment for the presence of microbe-associated molecular patterns (MAMPs). Upon antigen uptake and processing, APCs present antigens to naïve T cells in the lymph nodes, which eventually leads to their differentiation into memory T cells that both home back to the tissues and circulate in the blood. Hence, APCs are an essential step towards generation of the adaptive immunity. Many studies, including ours, showed that lectin involvement in recognition of bacteria can modulate proinflammatory responses, thereby impacting subsequent instruction of adaptive responses. Therefore, it is not only important that invading bacteria such as *S. aureus* are recognized but also how. Applications of CLR-bacterial interactions include the development of targeting agents for vaccine delivery to specific CLRs on APC subsets, that can help boost an effective adaptive immune and memory response. Glycans conjugated to carrier proteins are effective and well-studied vaccine components [32]. However, no direct effect on lectin interaction in the ensuing vaccine response has been investigated so far. In this regard, the infectious diseases field should take advantage of progress in the area of cancer vaccinology, where studies have shown the benefit of *in vivo* targeting of cancer antigens to APCs to enhance anti-tumor immunity [33]. Understanding of all possible interactions between CLRs and *S. aureus* WTA glycosylation can help design a vaccine that would deliver antigen(s) to APCs in a specific manner and thus allowing us to better direct its mode of action. New comprehensive strategies are required to identify additional WTA-interacting CLRs for *S. aureus* but also other CLR-microbe interactions.

One of the most common methods to screen for these new interactions between CLRs and bacterial glycans is through the application of soluble recombinant lectins. This represents a cost-effective method based on simple binding assays allowing to detect interaction with intact bacteria or bacterial carbohydrates. Lectins of interest can be produced in various expression systems allowing modifications and addition of tags for ease of detection. Although this technique allows to identify the interactions rapidly, it also carries some risks for false positive or false negative results [34]. For example, in the study van Dalen et al [34], no interaction between recombinantly expressed Langerin variant N288D/K313I and *S. aureus* was observed, whereas the same variant expressed on the cell surface still conferred an interaction with the bacterium. This is presumably related to the fact that CLRs often oligomerize on the cellular surface, increasing the avidity of the interaction. In contrast, recombinant lectins are often expressed as extracellular portion with different tags such as the Fc fragment of the IgG antibody, which results in the expression of soluble CLR-fusion proteins dimers (although this was not the case in [34]). However, the dimeric presentation

does not reflect the natural arrangement of several CLRs, such as MGL, langerin and DC-SIGN, as trimers or tetramers on the cell surface [35–37]. Moreover, many bacteria, including *S. aureus*, express Fc-binding proteins, which results in high non-specific background binding [38]. Another approach to increase lectin avidity is use of biotin as a carrier protein for DC-SIGN molecules forming artificial tetrameric lectins with enhanced functionality [39].

Alternatively, instead of using intact bacteria in the binding assay, CLR constructs are used to screen glycan arrays, which display isolated bacterial glycans [40]. This approach may benefit from multivalent glycan display, increasing avidity of the interaction and allowing identification of low affinity interactions. However, isolation of glycans from bacteria can be challenging due to heterogeneity and the presence of labile groups or modifications that can be lost during sample preparation [41,42]. This has sparked the development of synthetic carbohydrate chemistry, which allows the generation of libraries consisting of stable and well-defined glycan structures [43–46]. Although this approach provides an opportunity to screen multiple CLR ligands simultaneously, array-attached glycans may not display the natural conformation occurring on the bacterial surface. Complementary use of all mentioned tools is therefore essential to identify new interactions between bacteria and host CLR.

Although theoretical prediction of the interaction between bacterium and CLR based on the genome sequences and already described glycosylation pathways seems possible, it does not take gene expression into account. An example of this is described in Chapter 2, where we observed that MGL did not interact with CoNS despite the presence of *tagN*. Specific nutrients or environmental conditions could regulate bacterial metabolism, thereby affecting gene expression [47]. Consequently, *in vitro* culturing conditions may not resemble the required conditions, resulting in absence of gene expression and glycosylation. Similarly, *in vitro* findings may not reflect the *in vivo* situation. For example, it was demonstrated that *S. aureus* WTA -GlcNAc glycosylation, which is dominant under standard growth conditions for *tarM*/*tarS* expressing strains, is shifted towards β-GlcNAc glycosylation when grown under stress conditions and in in vivo infection models [48,49]. Finally, in addition to intrinsic regulation, glycosylation may also be altered through external mechanism for example, when *S. aureus* is present in complex microbiota environment. Competing species produce different enzymes including glycan-hydrolyzing enzymes. It has been well established that such enzymes can cleave off glycans from host proteins, thereby exposing potential adherence sites [50,51]. However, a similar glycan modification could occur on the surface of co-colonizing bacteria. As a result, isolates directly obtained from tissues may not be able to interact with CLRs that were identified as interacting receptors using *in vitro* growth conditions.

To fully understood whether bacterial glycosylation helps in host defense or is part of bacterial immune evasion mechanisms, appropriate *in vivo* infection models need to be used or established. This is challenging in the case of CLRs, since CLR glycan interaction displays a certain degree of species specificity as recently demonstrated for langerin [52]. A possible solution is the use of CLR-human transgenic mice [22,53]. In addition, development of human three-dimensional immune competent infection models could overcome the issues related to animal studies [12].

CLRs in vaccination

To optimize immune responses to specific antigens, it could be beneficial to design a vaccine that delivers the antigen to a specific subclass of APCs by targeting specific CLRs. In addition, to improve antigen uptake and immunogenicity, CLR-targeting vaccines may include an additional PRR ligand to allow simultaneous engagement of multiple PRRs. Indeed, a DC-SIGN and TLR7 targeting vaccine improved antigen presentation by the DCs [54]. Using an MGL-targeting vaccine to deliver *S. aureus* antigens could be an interesting concept that would involve a subset of cells that are one of the first points of contact for *S. aureus* – dermal DCs. Since dermal DCs naturally express MGL, targeting them in the skin could be performed using novel technologies such as microneedle patches, that will be discussed in more detail below. Considering that both MGL and Langerin are expressed by skin-resident APCs, it would be interesting to consider multivalent vaccines that target both receptors simultaneously. However, considering that DCs and LCs elicit different type of T cell responses, evaluation of protection post vaccination upon targeting with the same antigen of interest either LCs or DCs has to be done [55]. So far, antigen-loaded liposomes decorated with Langerin-targeting glycans have been successfully used to exclusively target LCs [56,57]. LC-targeting vaccines may be applied through topical application, since LCs dendrites are able to reach the most superficial parts of the epidermis and elicit humoral responses through this sampling route [58,59]. Again, the model in which such vaccines would be tested is critical to translate the outcome. Indeed, in a recent mouse study that analyzed immune protection against *S. aureus* skin infection, dermal DCs, but not LCs were pinpointed to be crucial to elicit immune responses [60,61]. However, these studies did not consider the fact that mouse langerin does not recognize *S. aureus* [22], which may have caused the lack of identifying the contribution of LCs. Development of lectin-targeting molecules to deliver vaccines straight into APCs is a very interesting and novel concept, that obviously can be implemented for many infectious diseases.

CD4+ memory T cells in defense against *S. aureus*

Chapters 3 and 4 are dedicated to the presence and activation of *S. aureus*-specific memory T cells in the skin and the circulation, respectively. Chapter 3 provides the first experimental

evidence for the presence of *S. aureus*-specific CD4+ skin-resident memory T cells (Tsrm) in healthy human skin. Resident immune cells isolated from skin explants were stimulated for 4-days with heat-killed microbes. On day 4, proliferation of Tsrm and cytokine production in the supernatants was assessed. Cells that proliferated were characterized by the expression of a T cell memory marker CD45RO and the skin-homing marker cutaneous lymphocyte-associated antigen (CLA). Stimulation of skin-isolated cell suspensions with heat-killed *S. aureus* induced IL-17A, IL-22, GM-CSF, IFNγ and TNFβ production, whereas *S. epidermidis* only induced IL-17A production. In contrast to the cytokine production by skin cell suspensions, epicutaneous infection of whole skin explants with live *S. aureus* also induced IL-1 in addition to the other cytokines already mentioned. The lack of keratinocytes, which are prominent IL-1 producing skin cells, in addition to the use heat-killed bacteria, which lack expression of the IL-1 inducing staphylococcal α-toxin (Hla) [62], in the experimental set up using skin cell suspensions is a likely reason for the disparity in IL-1 production.

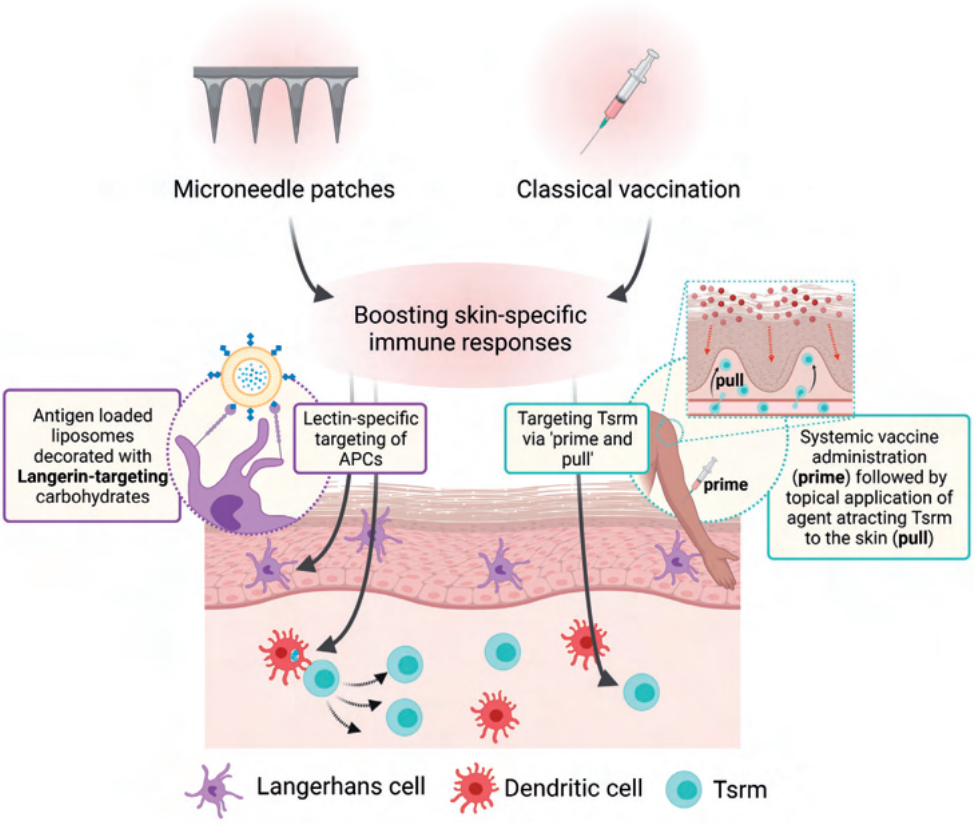
In Chapter 4, I studied *S. aureus*-specific CD4+ T cells in the circulation of healthy subjects with additional focus on skin-tropic cells. Although tissue-resident memory T cells can reside in the skin for long periods of time, it has recently been shown that they can recirculate and migrate back to the skin from blood [63], which allows development of methods to identify skin immune responses using cells from the circulation. Here, I focused on the identification and subsequent isolation of *S. aureus*-specific CD4+ T cells in the blood followed by single cell mRNA sequencing (scRNA-seq) to unravel the transcriptome of these cells. Such an assay would also be highly valuable in the context of clinical trial samples. To obtain *S. aureus*-specific CD4+ T cells, I screened peripheral blood mononuclear cells (PBMCs) from 16 healthy subjects for the presence of *S. aureus*-specific CD4+ T cells, using an activation induced marker (AIM) assay. AIM is a technique that allows the identification of antigen-specific T cells in blood based on the upregulation of specific surface activation markers when stimulated with the antigen of interest. Suspensions of previously isolated CD4+ and irradiated CD4- fraction of cells containing APCs were exposed to heat-killed *S. aureus* and incubated for 24 hours after which expression of CD137 and OX40 was measured. Short stimulation time allows to minimize unspecific cell activation that can be caused by the bystander effect occurring during the longer incubation times like when using classical proliferation assays. In addition, AIM assay allows to detect all antigen-specific cells regardless their cytokine production or proliferative capacity. Based on the outcome, donors were classified into high or low responders including one non-responder. Double-positive CD137+OX40+ cells from two high responders were single cell sorted and scRNA-seq analysis was performed. Data analysis showed presence of seven different clusters among which regulatory (Treg) and cytotoxic (CTL) clusters were the most distinctive in terms of gene expression profiles. Tregs were characterized by the upregulation of gene encoding forkhead box P3 (Foxp3) transcriptional factor. On the other hand, CTL could be distinguished by the upregulation of genes encoding granzyme A, granzyme B and

IFNγ. Using Index sorting we identified CLA+ cells among sequenced cells and differential expression analysis between CLA+ and CLA- cells was performed. Analysis revealed that skin-tropic *S. aureus*-specific CD4+ T cells can be mainly characterized by the expression of genes associated with Tregs. Although combination of AIM with scRNA-seq allowed to identify and characterize rare populations of *S. aureus*-specific CD4+ T cells these results are preliminary, and more donors will be included in the follow-up study.

CD4+ T cells have been previously described as important players in protective immunity against *S. aureus* [64]. Data from *S. aureus* mouse infection models have highlighted the importance of Th1-type responses for protection from systemic infections and Th17 for defense against localized infection, such as SSTIs [4,65–68]. The observed cytokine profile produced by *S. aureus* stimulated skin-resident cells Tsrm described in Chapter 3 aligns with results from studies on mouse skin infections [66,67]. Although *S. aureus*-specific memory T cells were previously identified in the blood of healthy human and vaccinated subjects, no link with their skin-homing capacity has been made so far [4,69,70]. In Chapter 4, we focused our analysis on the transcriptional profile of *S. aureus*-specific CD4+ T cells with skin homing markers from the circulation using single cell sequencing. Single-cell analysis of this rare cell population is a rapidly expanding field that has been successfully used for in-depth characterization of antigen-specific T cells. This approach could potentially unravel all type of processes that are occurring during cell stimulation, such as cytokine production or activation of certain metabolic pathways, which may help to unravel the profile of *S. aureus*-reactive T cells. Moreover, in the light of studies on Tsrm recirculation, it is of interest to understand the differences between skin-homing and non-skin-homing CD4+ T cells. Since skin is an important point of entry for *S. aureus*, it is not surprising that *S. aureus*-specific Tsrm were detected in all skin specimens tested in Chapter 3. What is of interest to point out though, is that we used skin explants from the abdomen, which is not described as a preferred *S. aureus* biological niche. Therefore, the identification of *S. aureus*-specific T cells in these samples is in line with the Tsrm recirculation model described by Klicznik *et al* and supports experimental design proposed in the Chapter 4.

New approaches to elicit T cell responses through a *S. aureus* vaccine

Both Th1 and Th17 are needed to generate broad protection against *S. aureus* infections. However, information gained from clinical trials shows that it is not always possible to predict the type of the responses that are induced by vaccination. The clinical trial described by Levy *et al.* evaluated a vaccine candidate containing CPS5/8 conjugated to tetanus toxoid (TT), Hla and ClfA [71]. Further analysis of the vaccinees' blood revealed that vaccination with Hla and ClfA induced only expansion of Th1 but not Th17 cells [70], although these vaccine components are potent Th17 activator *in vitro*. This study shows that vaccination with Hla induces very low proliferative and cytokine production responses of Th17 cells among vaccinees for unknown reasons [70,72].



New approaches to elicit immune responses in the skin upon vaccination. Using microneedle patches or classical intradermal vaccination, skin-specific immune responses towards *S. aureus* can be boosted. Antigen delivery using liposomes decorated with lectin-specific carbohydrates, e.g. Langerin-targeting glycans, could allow direct delivery of an antigen of interest to specific populations of antigen presenting cells (APCs) expressing the targeted lectin. Alternatively, 'prime and pull' techniques allow for the targeting of skin-resident T cells (Tsrm) though systemic administration of a vaccine, known as 'prime', followed by the topical application of an agent that would attract vaccine-induced T cells to the skin where they become Tsrn, known as 'pull'.

An important point to make is that none of the *S. aureus* vaccines designed so far targeted directly cell subsets present in the first point of contact with *S. aureus* – the skin. Chapter 3 points out that the skin is an important reservoir of *S. aureus*-specific memory T cells. Moreover, Tsrm have been identified as important players in cutaneous immune defense for example against *Candida albicans* skin infections [73]. However, tissue-resident T cells are not only playing a crucial role at the skin, but also other barrier tissues, such as respiratory tract. Furthermore, these tissue-resident Tm have been shown to orchestrate neutrophil recruitment via IL-17A production [74,75] and speed up epithelial tissue responses, which can be crucial for barrier tissue immunity [75]. Consequently, inducing Tsrm specific for *S. aureus* in the skin, the portal of entry, seems to be logical approach for vaccine design. Interestingly,

Tsrm already accumulate over time in the skin as a result of natural exposure but also maintain their diversity and protective functions, such as cytokine production, despite their reduced diversity and functionality in the blood [76]. Especially IFNγ and IL-17 production after stimulation with *S. aureus* and *C. albicans* by blood CD4+ T cells was significantly impaired in the comparison with the skin derived cells [76]. This finding emphasizes the fact that induction of Tsrm through vaccination may induce long-lasting immunity that is not necessarily maintained or observed in the circulation. However, this would require different vaccine design in order to elicit Tsrm responses.

Given the suggested importance of Tsrm in defense against *S. aureus*, vaccination should not just generate specific opsonic antibody responses, but also contribute to the formation of tissue-resident *S. aureus* specific memory T cells. The question is how this can be achieved. Several studies point out that the site of vaccination plays a major role in this process. Intradermal versus intramuscular vaccination against *Leishmania major* has shown that intradermal delivery of the vaccine provides both systemic and local CD4+ Tsrm cell responses, which was not the case for the intramuscular vaccination [77]. Similar finding comes from a mouse melanoma model, where dermal but not intraperitoneal administration route elicited protective CD8+ Tsrm responses in the skin [78]. Where Tsrm are generated upon infection of vaccination is still debated. A study by Liu *et al.* showed that T cell imprinting after vaccination with vaccinia virus (VV) occurs in the local lymph nodes (LN) and generates both tissue-homing and circulating antigen-specific T cells [79]. However, after the VV infection is eliminated, the majority of remaining memory T cells is specific to the route of injection [79]. In contrast, other studies have shown that generation of tissue-homing T cells is orchestrated by DCs from the tissue of interest [80–82]. Despite the mechanism of action, the route of administration obviously plays an important role in establishing specific tissue-resident T cells. This is especially interesting in the context of previously discussed targeting of local dermal DCs and LCs by vaccines, since CLR-specific antigen delivery may support the generation of *S. aureus*-specific Tsrm after intradermal vaccination. Such APC targeting may also be combined in the so-called 'prime and pull' technique, where systemic administration of the antigen is accompanied by local antigen application to enhance cell retention at the tissue of interest. Classical 'prime and pull' without specific APC antigen delivery has been successfully applied for the HSV infection model [83,84].

Final remarks

Results presented in my PhD thesis highlight the importance of both innate and adaptive responses in fighting *S. aureus* infections and inducing these responses through vaccination. Staphylococcal SSTIs are multifactorial diseases that due to their complexity

need a comprehensive treatment, such as a vaccine. Identification of the new interactions between *S. aureus* and host expressed molecules, such as CLRs, can provide more insight in the mechanism of bacterial recognition and how it affects adaptive immunity. Based on the results of my research in this final discussion, I have suggested strategies to induce these responses through a different vaccination design and administration. Results from the multiple failed trials have shown that effective immunity against *S. aureus* cannot be harnessed using classical approaches due to its versatile immune evasion mechanisms. Hereby presented CLR-targeting delivery of antigen and Tsrm inducing vaccination could be combined as a one powerful tool to combat *S. aureus* skin infections.

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CHAPTER 6

Nederlandse samenvatting

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Nederlandse Samenvatting

De Gram-positieve bacterie *Staphylococcus aureus* (*S. aureus*) is een frequent voorkomende bacterie binnen het menselijke microbioom; ongeveer 30% van de gezonde bevolking draagt de bacterie in de neusholte en op de huid. *S. aureus* is een belangrijke oorzaak van operatiewondinfecties en huidinfecties, maar ook pneumonie. De behandeling van *S. aureus* infecties wordt belemmerd door de voortdurende toename van antibioticumresistentie, met name methicilline-resistente *S. aureus* en vancomycine-resistente *S. aureus*. Inzicht in de moleculaire mechanismen die bijdragen aan het onstaan van *S. aureus* infecties zal de ontwikkeling van nieuwe behandelingsstrategieën, waaronder vaccins, ondersteunen.

De huid is het grootste orgaan van het menselijk lichaam. De buitenste laag bestaat uit de hoornlaag, een nutriënt-arm, zuur en droog oppervlak die de huid tegen allerlei niet-steriele omgevingsinvloeden beschermt. Op de hoornlaag bevindt zich ook een heterogene gemeenschap van vele miljoenen micro-organismen – de huidmicrobiota. De huid is niet alleen een habitat voor nuttige micro-organismen, maar ook een toegangspoort voor ziekteverwekkers; bacteriën, schimmels, virussen en parasieten kunnen het lichaam binnendringen als de huidbarrière wordt doorbroken, bijvoorbeeld door kleine schaafwonden, een verwonding of een chirurgische incisie. Een daaropvolgende infectie kan leiden tot ziekten van verschillende ernst, variërend van gelokaliseerde en ongecompliceerde tot systemische en levensbedreigende ziekten. Infecties van de huid en de weke delen (HWDI's) beschrijft een heterogene groep klinische aandoeningen die de huid en onderliggende weefsels aantasten. *S. aureus* is de meest frequente veroorzaker van HWDI's. SSTIs variëert van relatief milde ziekteverschijnselen, zoals cellulitis en impetigo, tot ernstige en levensbedreigende ziekten, zoals operatiewondinfecties. Bovendien kan onbehandelde of inadequate behandeling van SSTI's leiden tot de ontwikkeling van bloedbaaninfecties, waaronder sepsis en endocarditis. Ondanks dat huidinfecties in de bevolking veel voorkomen, is er nog veel onduidelijk over de moleculaire mechanismen die gepaard gaan met het binnendringen van pathogenen in de huid en de immuun afweermechanismen om deze pathogenen te elimineren.

Afweer tegen *S. aureus*-infectie vereist de bijdrage van zowel het aangeboren als het adaptieve immuunsysteem. Meer onderzoek naar welke bacteriële moleculen belangrijk zijn voor de herkenning van *S. aureus* door dendritische cellen (DC's) zou ons vooruit kunnen helpen met vaccins ontwerpen, vooral in de context van T-geheugencel immuun repons. In het eerste deel van mijn proefschrift beschrijf ik een nieuwe interactie tussen C-Type lectinereceptoren (CLR's) op DC's en *S. aureus*. Vervolgens onderzocht ik het voorkomen en

de respons van *S. aureus*-specifieke T-geheugencellen in de huid en het bloed van gezonde individuen.

In **Hoofdstuk 2** beschrijf ik de impact van "wall" teichoïnezuur (WTA) glycosylering op de herkenning van *S. aureus* isolaten uit de ST395 lijn door monocyte-gedifferentieerde dendritische cellen (moDCs). Ik ontdekte dat de CLR macrofaag galactose-type C-type lectinereceptor (MGL) deze specifieke isolaten detecteert door interactie met de GalNAc-gebonden suikergroep die aan het WTA molecuul is bevestigd. Aangezien vergelijkbare WTA structuren tot expressie gebracht worden door coagulase-negatieve stafylokokken (CoNS), vergeleek ik de interactie van verschillende *S. aureus* stammen en CoNS soorten met recombinant-geproduceerd MGL en MGL dat op het celoppervlak tot expressie wordt gebracht. Hiernaast werden de cellulaire respons van primaire moDC's, zoals activatie markers en cytokineproductie, na incubatie met MGL-bindende en niet-bindende *S. aureus* varianten geanalyseerd. Deze resultaten toonden aan dat *S. aureus* ST395 via interactie met MGL de productie van pro-inflammatoire cytokinen door moDC's stimuleert.

Hoofdstuk 3 beschrijft de adaptieve immuunrespons tegen *S. aureus* in de huid van gezonde proefpersonen. *S. aureus*-specifieke huid-residente T-geheugencellen (Tsrm) werden geïdentificeerd in celsuspensies van huid van gezonde mensen door proliferatie en cytokine productie te meten (IL-17A, IL-22, GM-CSF, IFNγ en TNFβ) na blootstelling aan hitte-geïnactiveerde *S. aureus*. Eenzelfde populatie werd niet gevonden in respons tot *S. epidermidis*. Hiernaast werd de pro-inflammatoire cytokinerespons in *S. aureus*-geïnfecteerde huidbiopten geïnfecteerd na 24 en 72 uur na infectie gemeten. Dit onderzoek is de eerste studie die de aanwezigheid van *S. aureus*-specifiek CD4+ Tsrm in de huid beschrijft.

Hoofdstuk 4 is gewijd aan de identificatie van *S. aureus*-specifieke CD4+ T-cellen in het bloed van gezonde proefpersonen. We hebben verschillende manieren om deze specifieke T-cellen te identificeren vergeleken en geoptimaliseerd. Uiteindelijk hebben we *S. aureus*-specifieke T cellen "single-cell"-gesorteerd op basis van de activatie-geïnduceerde markers (AIM) CD137 en OX40 na incubatie met hitte-geïnactiveerde *S. aureus*. Deze T cellen zijn verder geanalyseerd met mRNA-sequencing. De voorlopige resultaten laten zien dat er zeven verschillende clusters in geanalyseerde *S. aureus*-specifieke CD4+ T-cellen aanwezig zijn, waaronder cytotoxische (CTL) en regulerende (Treg) CD4+ T-cellen. Ook hebben we geanalyseerd of het deze clusters verschillende aanwezig waren in T cellen die cutaan lymfocyt-geassocieerd (CLA) antigeen tot expressie brengen, een recetor die zorgt voor homing naar huid in vergelijking met CLA-negatieve cellen. Binnen de CLA+ T cellen bevond zich een hogere frequentie Treg cellen. De verkregen data over de kwaliteit van reeds bestaande anti-*S. aureus* CD4+ T-cellen kunnen bijdragen aan een beter begrip van CD4+ T-celresponsen tijdens infectie en vaccinatie.

Hoofdstuk 5 vat alle verkregen resultaten samen en bespreekt deze in de context van relevante en recente literatuur over *S. aureus* en de rol van huidimmuniteit bij het bestrijden van *S. aureus* infecties. Ook worden de mogelijke implicaties voor preventieve strategieën tegen *S. aureus* infecties besproken.

Streszczenie

Gram-dodatnia bakteria *Staphylococcus aureus* (gronkowiec złocisty) jest powszechnym elementem ludzkiego mikrobiomu, występującym u 30% populacji. Zamieszkujący głównie nozdrza i skórę *S. aureus* jest najczęstszą przyczyną infekcji pooperacyjnych oraz infekcji skórnych, jak również zapaleń płuc związanych z pobytem w szpitalu. Leczenie infekcji wywołanych tym gronkowcem jest wyjątkowo trudne ze względu na narastający problem antybiotykooporności bakterii, w szczególności widoczny wśród metycylino- i wankomycyno-opornych szczepów *S. aureus*. Zrozumienie mechanizmów molekularnych leżących u podstaw różnych infekcji powodowanych przez gronkowca złocistego jest kluczowe dla opracowania nowych strategii leczenia, w tym skutecznych szczepionek.

Skóra jest największą, zewnętrzną powierzchnią ludzkiego ciała i stanowi surowe, ubogie w składniki odżywcze, kwaśne i suche środowisko. Naskórek jest szczególnie narażony na działanie czynników zewnętrznych, wliczając w to kolonizację różnorodnymi drobnoustrojami, które tworzą mikrobiom skóry. Skóra jest nie tylko siedliskiem dla komensali, ale także miejscem, przez które patogenne bakterie, grzyby, wirusy i pasożyty mogą stosunkowo łatwo przedostać się sie w głąb organizmu, gdy bariera skórna zostanie naruszona, na przykład podczas otarć, urazów lub nacięć chirurgicznych. Późniejsza infekcja skóry może prowadzić do chorób o różnym nasileniu, od miejscowych i nieniosących powikłań, aż po choroby ogólnoustrojowe i zagrażające życiu. Zakażenia skóry i tkanek miękkich (*ang.* skin and soft tissue infections, SSTIs) stanowią niejednorodną grupę schorzeń obejmujących skórę i leżące poniżej tkanki. SSTIs są bardzo często wywoływane przez *S. aureus*. Zakażenia te dzielą sie na stosunkowo łagodne stany chorobowe, jak zapalenie tkanki łącznej i ropnie, jak i ciężkie i zagrażające życiu choroby, takie jak infekcje pooperacyjne. Ponadto nieleczone lub leczone w sposób nieodpowiedni SSTIs może prowadzić do rozwoju infekcji krwi, w tym posocznicy i infekcyjnego zapalenia wsierdzia. Pomimo dużej częstotliwości infekcji skóry wśród populacji ludzkiej, mechanizmy molekularne towarzyszące wniknięciu patogenów przez skórę oraz mechanizmy obrony immunologicznej w celu usunięcia tych patogenów są nadal słabo poznane.

Obrona przed infekcjami spowodowanymi *S. aureus* wymaga udziału zarówno odporności wrodzonej jak i nabytej. Zrozumienie, które cząsteczki bakteryjne są istotne dla rozpoznania *S. aureus* przez komórki dendrytyczne (*ang.* dendritic cells, DCs) stanowi ważny czynnik w rozwoju szczepionek, zwłaszcza w kontekście tworzenia pamięci immunologicznej z udziałem limfocytów T. W pierwszej części mojej pracy doktorskiej opisałam nową interakcję między receptorem lektynowym typu C (*ang.* C-Type Lectin Receptors, CLRs) a *S. aureus* oraz

wpływ opisanej interakcji na odpowiedzi prozapalne. Następnie, zbadałam swoiste dla *S. aureus* limfocyty T CD4$^+$ pamięci występujące w skórze i krwi zdrowych osób.

W **Rozdziale 2** opisałam wpływ glikozylacji kwasu techojowego ściany komórkowej (*ang.* Wall Teichoic Acid, WTA) na rozpoznawanie szczepów *S. aureus* należących do typu sekwencyjnego (*ang.* sequencing type, ST) ST395 przez komórki dendrytyczne zróżnicowane z monocytów (*ang.* monocyte-derived dendritic cells, moDCs). W mojej pracy pokazałam, że lektyna MGL (*ang.* Macrophage Galactose-Type C-Type Lectin, MGL) wykrywa *S. aureus* ST395 poprzez interakcję N-acetyloglukozaminy (*ang.* N-acetylglucosamine, GalNAc) obecnej na WTA zbudowanym z polifosforanu glicerolu (*ang.* poly-glycerol phosphate, GroP). Z uwagi na fakt, iż podobne struktury WTA są prawdopodobnie obecne u koagulazo-ujemnych gronkowców (*ang.* Coagulase-negative Staphylococci, CoNS), porównałam różne szczepy *S. aureus* i CoNS pod kątem ich interakcji z rekombinowanym i obecnym na powierzchni komórek MGL. Przeanalizowałam również odpowiedzi komórkowe moDCs, takie jak markery aktywacji i wytwarzanie cytokin. Wyniki wykazały, że *S. aureus* ST395 indukuje zależne od MGL wytwarzanie cytokin prozapalnych przez moDCs.

W **Rozdziale 3** omówiłam mechanizm odpowiedzi nabytej przeciwko *S. aureus* zaobserwowany w skórze osób zdrowych. Występujące w skórze limfocyty T pamięci (*ang.* skin-resident memory T cells, Tsrm), charakterystyczne dla *S. aureus,* zostały zidentyfikowane w zawiesinach komórkowych przygotowanych ze zdrowej ludzkiej skóry, poprzez pomiar ich proliferacji i produkcji cytokin (IL-17A, IL-22, GM-CSF, IFNγ i TNFβ) po stymulacji dezaktywowanym *S. aureus*. Odpowiedzi prozapalne zostały zaobserwowane po stymulacji *S. aureus*, ale nie zidentyfikowano ich po stymulacji *S. epidermidis*. Obecność cytokin prozapalnych w supernatantach zakażonych *S. aureus* po wykonanej stymulacji biopsji skóry zaobserwowano po 24 i 72 godzinach po zakażeniu. Przedstawione wyniki są pierwszym dowodem na obecność w skórze CD4$^+$ Tsrm swoistych dla *S. aureus*.

Rozdział 4 został poświęcony identyfikacji swoistych dla *S. aureus* limfocytów T CD4$^+$ we krwi zdrowych dawców. W tym celu porównano i zoptymalizowano różne metody identyfikacji. Następnie, używając metody opartej na pomiarze wzrostu ekspresji markerów komórkowych indukowanych podczas aktywacji limfocytów T (*ang.* Activation Induced Marker, AIM), zidentyfikowano i wykonano sortowanie zaktywowanych komórek charakteryzujących sie wysoką ekspresją markerów CD137 i OX40 na swojej powierzchni, po inkubacji z dezaktywowanym *S. aureus*. W dalszej kolejności, antygenowo swoiste limfocyty T CD4$^+$ zostały posortowane pojedynczo i poddane dalszej analizie z użyciem sekwencjonowania mRNA (*ang.* single-cell mRNA sequencing, scRNAseq). Wstępne wyniki wykazały obecność siedmiu różnych klastrów w analizowanych limfocytach T CD4$^+$ swoistych dla *S. aureus*. Wśród nich wyróżniono limfocyty T CD4$^+$ cytotoksyczne (*ang.* Cytotoxic T lymphocytes, CTLs) i regulatorowe (*ang.* Regulatory T cells, Tregs).

Ponadto, porównano transkryptom komórek migrujących do skóry charakteryzujących sie obecnością na powierzchni antygenu CLA (*ang.* Cutaneous Lymphocyte-Associated antigen, CLA) z komórkami CLA-, niemigrującymi do skóry. Uzyskane wyniki pokazały wysoki procent limfocytów T regulatorowych w populacji CLA^+. Uzyskane dane dotyczące jakości swoistych dla *S. aureus* limfocytow T $CD4^+$ mogą przyczynić się do zrozumienia ich odpowiedzi immunologicznej podczas infekcji oraz po szczepieniu.

W **Rozdziale 5** podsumowano i omówiono uzyskane wyniki w kontekście aktualnej literatury dotyczącej *S. aureus* i roli odporności skóry w zwalczaniu zakażeń wywoływanych *S. aureus*. Ponadto, omówiono rolę szczepień w zapobieganiu zakażeniom wywoływanym przez *S. aureus.*

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Curriculum Vitae

Małgorzata Ewa Mnich was born on the 10th of February 1992 in Cracow, Poland. In 2011 she graduated from Jan III Sobieski high school and began her studies at the Faculty of Biochemistry, Biophysics and Biotechnology at the Jagiellonian University in Cracow. During the course of her studies she joined the Department of Immunology where, under the supervision of Prof. dr. Joanna Cichy and dr. Joanna Skrzeczyńska-Moncznik she performed research in the field of skin immunology. In 2014 and 2016 she obtained a Bachelor's and cum laude Master's Degree respectively, in Biochemistry. During her studies she was awarded a fully-funded Erasmus on two separate occasions which allowed her to pursue internships in the Laboratory of Molecular Immunology at Rega Institute, KU Leuven, Belgium and later in the Bacterial Glycobiology group at the Department of Medical Microbiology at the University Medical Centre, Utrecht, The Netherlands. The latter of which presented her with the opportunity to join van Sorge group as a PhD student. In July 2017 Małgorzata started a doctoral position within the Marie Skłodowska-Curie funded programme DISSection (Doctoral Industrial School on Human Skin models for Staphylococcal infections), a collaboration between UMC Utrecht and GSK Vaccines in Siena, Italy coordinated and supervised by Prof. dr. Jos van Strijp and dr. Fabio Bagnoli. Her research focused on the interplay of dendritic and T cells with *Staphylococcus aureus*. For the first half of her PhD, taking place from July 2017 until December 2018, she carried out her research in the group of Prof. dr. Nina van Sorge group where she investigated the role of lectin receptors in the recognition of *S. aureus* by dendritic cells. From January 2019 until September 2021 she joined Cellular Immunology group in GSK Vaccines Siena where under supervision of dr. Elisabetta Soldaini, she investigated the interaction between human $CD4^+$ T cells and S. aureus with a strong focus on skin immunity. The four-year PhD project resulted in Małgorzata becoming a first author of peer-revied experimental and review scientific publications.

List of publications

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