Bittersweet Memories

Immunity against *Staphylococcus aureus* wall teichoic acid

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Immunity against *Staphylococcus aureus* wall teichoic acid

Bitterzoete herinneringen
Hoe het immuunsysteem reageert op *Staphylococcus aureus* celwand suikers

(met een samenvatting in het Nederlands)

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Disfruta de la vida*

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"Make everything as simple as possible, but not simpler"

ALBERT EINSTEIN

Chapter 1

General introduction

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Staphylococcus aureus: a common foe

Staphylococcus aureus is a Gram-positive bacterium that persistently colonizes 30% of the human population, whereas transient colonization is even more prevalent (1,2). The primary niche for *S. aureus* is the anterior nares of the nose, although *S. aureus* can also be found in the pharynx, on moist skin surface such as the axillae and the groin area, and in the gastrointestinal tract (2,3). In addition to asymptomatic colonization, *S. aureus* is a successful opportunistic pathogen that causes a multitude of infections that vary from superficial, self-limiting skin infections to more invasive clinical manifestations such as endocarditis, pneumonia, osteomyelitis and prosthetic joint infection (4). *S. aureus* is both the leading cause of skin and soft tissue infections (SSTIs) (5), as well as bloodstream infections with a 20% mortality rate (6,7). Up until the 1990s, documented *S. aureus* infections were largely confined to healthcare settings, where hospital-acquired (HA) infections affected vulnerable patients such as immunocompromised or surgical patients. However, community-acquired (CA) *S. aureus* infections also occur in otherwise healthy individuals and have been rapidly increasing since the mid-1990s (5). Especially for CA-infections, the skin represents the primary entry site for *S. aureus* host invasion, and SSTIs represent over half of all *S. aureus*-related hospitalizations in the United States (8). Nasal carriage is an important risk factor for SSTIs and invasive *S. aureus* infection, which is often induced by endogenous strains (1,9,10). Furthermore, the emergence of antibiotic-resistant strains, e.g. methicillin-resistant *S. aureus* (MRSA), is gradually limiting treatment options (11). Consequently, the World Health Organization (WHO) has marked *S. aureus* as a high-priority pathogen in 2017, underscoring the need to develop new antibiotics or alternative therapies (12).

What constitutes protective immunity to *S. aureus*?

Boosting host immune responses to *S. aureus* by vaccination is considered a promising strategy to prevent and combat *S. aureus* infections. In addition, vaccination presents an attractive alternative to antibiotic treatment. However, to this day all vaccine candidates have failed to provide evidence of protection in human clinical trials (13). Reasons for this failure include the inability to translate findings from preclinical animal models to humans, suboptimal vaccine antigens and an incomplete understanding of correlates of protection (14–17). In addition, *S. aureus* produces a wide arsenal of human-specific virulence factors that promote immune evasion and are thought to counter vaccine-induced immunity (18,19). For the development of an effective vaccine, a comprehensive understanding of protective human immunity to *S. aureus* is needed. The majority of the human population has acquired some level of immunity to *S. aureus* due to natural exposure as indicated by the presence of *S. aureus*-specific antibodies and T cells in the circulation (20,21). Although naturally-acquired immunity does not fully protect from reinfection in the case of SSTIs (22), it does significantly decrease the risk of mortality in case of *S. aureus* bacteremia (10). Consequently, mapping the natural immune responses to *S. aureus* may provide critical insights in both host and bacterial components that confer protective immunity to this elusive pathogen.

The exact correlates of protection for *S. aureus* infection are currently unknown. However, the observation that specific immunodeficiencies increase susceptibility to *S. aureus* infections has pinpointed critical players and processes in the anti-*S. aureus* immune response. Neutrophils are considered key effector cells since patients with impaired neutrophil functions frequently suffer from severe *S. aureus* infections (23). During infection, neutrophils extravasate from blood vessels via the process of rolling, adhesion and transmigration through activated endothelial cells, and will continue to migrate to the infection site via chemotaxis (23,24). Following phagocytosis, neutrophils kill engulfed bacteria by the production of reactive oxygen species (ROS) and degranulation of antimicrobial protein (AMP)-containing granules into the phagosome (23). Neutrophils can also kill *S. aureus* in a phagocytosis independent manner via the formation of neutrophil extracellular traps (NETs), which are composed of extruded DNA and associated granular proteins that trap extracellular bacteria to prevent dissemination and promote AMP-mediated killing (24). The adaptive immune system cooperates with the innate immune system to enhance neutrophil function and subsequent killing of *S. aureus* for example through the production of *S. aureus*-specific opsonic antibodies (25). Moreover, T helper cells regulate granulopoiesis, neutrophil effector functions and recruitment to the site of infection by producing pro-inflammatory cytokines IL-17A and IFNγ (26,27). Consequently, patients with impaired T cell responses, for example due to HIV infection or specific Th17 deficiencies, are at risk of *S. aureus* SSTIs (28,29). It is therefore expected that an effective vaccine should not only induce opsonic antibodies, but also T cell responses that cumulatively enhance neutrophil function.

Vaccine development for extracellular bacteria has primarily been focused on the induction of opsonic antibodies that target surface antigens. After binding, these antibodies greatly enhance uptake and killing by phagocytes such as neutrophils, via complement activation and Fc receptor-mediated phagocytosis. Several of our most effective childhood vaccines, e.g. bacterial vaccines that prevent infections by *Streptococcus pneumoniae*, *Haemophilus influenzae* type b and *Neisseria meningitidis*, target surface glycans namely capsular polysaccharides. Unfortunately, the application of capsular polysaccharides as vaccine antigens to prevent *S. aureus* infection has proven unsuccessful. However, other glycosylated structures have not yet been studied in this context and may be interesting vaccine targets since they are surface exposed, naturally immunogenic and confer virulence potential to *S. aureus*.

Here, I will describe the glycosylated structures present in the cell wall of *S. aureus* (shown in **figure 1A**), including peptidoglycan, capsular polysaccharides, glycosylated proteins and teichoic acids, and how the immune system recognizes these bacterial surface carbohydrates. I will then continue to summarize the specific human immune responses to *S. aureus* wall teichoic acid, an abundant cell wall glycopolymer, in the context of skin infection.

S. aureus surface glycans

Peptidoglycan

The Gram-positive bacterial cell wall is characterized by a thick peptidoglycan layer. Peptidoglycan is one of the main determinants for mechanical and structural integrity of the cell wall, allows nutrient influx and acts as a scaffold for cell-wall anchored surface proteins and glycans such as teichoic acids (30). Peptidoglycan is in essence one macromolecule composed of long glycan strands consisting of repeating disaccharides subunits of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc)(30). These glycan polymers are cross-linked by peptide sidechains either directly or indirectly via interpeptide bridges, creating an intricate meshwork that provides structural integrity while still allowing nutrient uptake (31). In S. aureus, peptide sidechains are indirectly linked by characteristic pentaglycine bridges (32).

Capsule

S. aureus can produce capsular polysaccharides (CP), which forms another glycan layer to cover the bacterial surface. CP production increases bacterial virulence, due to its anti-phagocytic properties and enhanced bacterial survival in blood (33). For S. aureus, a total of 11 different capsular serotypes exist, although CP serotype 5 (CP5) and CP8 account for the majority of human isolates (34). CP5 and CP8 are structurally very similar; both consist of polymerized trisaccharides composed of repeating units of D-N-acetyl mannosaminuronic acid (ManNAcA), L-N-acetyl fucosamine (L-FucNAc), and D-N-acetyl fucosamine (D-FucNAc), but differ in the glycosidic linkages between these sugars and the sites of O-acetylation (35). CPs are covalently attached to peptidoglycan MurNAc (36,37).

Glycosylated cell-wall anchored surface proteins

Incorporation of proteins in the Gram-positive cell wall can occur in a covalent and noncovalent manner. Proteins that need to be covalently anchored are characterized by an LPXTG motif at the C-terminus, which is recognized by the conserved enzyme sortase A for attachment to the peptidoglycan pentaglycine bridge (38). *S. aureus* expresses around 24 different LPXTG proteins, although expression varies between and within strains and is influenced by specific growth conditions (39,40). A selection of these proteins is glycosylated, including the family of serine-aspartate dipeptide-repeats (SDR) proteins SdrC, SdrD, SdrE, clumping factor A (ClfA) and clumping factor B (ClfB). SDR proteins are modified with one or two GlcNAc subunits through the dedicated activity of glycosyltransferases SdgA and SdgB (41,42). This protein modification protects SDR proteins from degradation by human neutrophil serine protease cathepsin G and further enhances *S. aureus* pathogenesis during blood stream infections (41,42). Other large glycosylated proteins include the 227 kDa sized serine-rich adhesion for platelets (SraP)

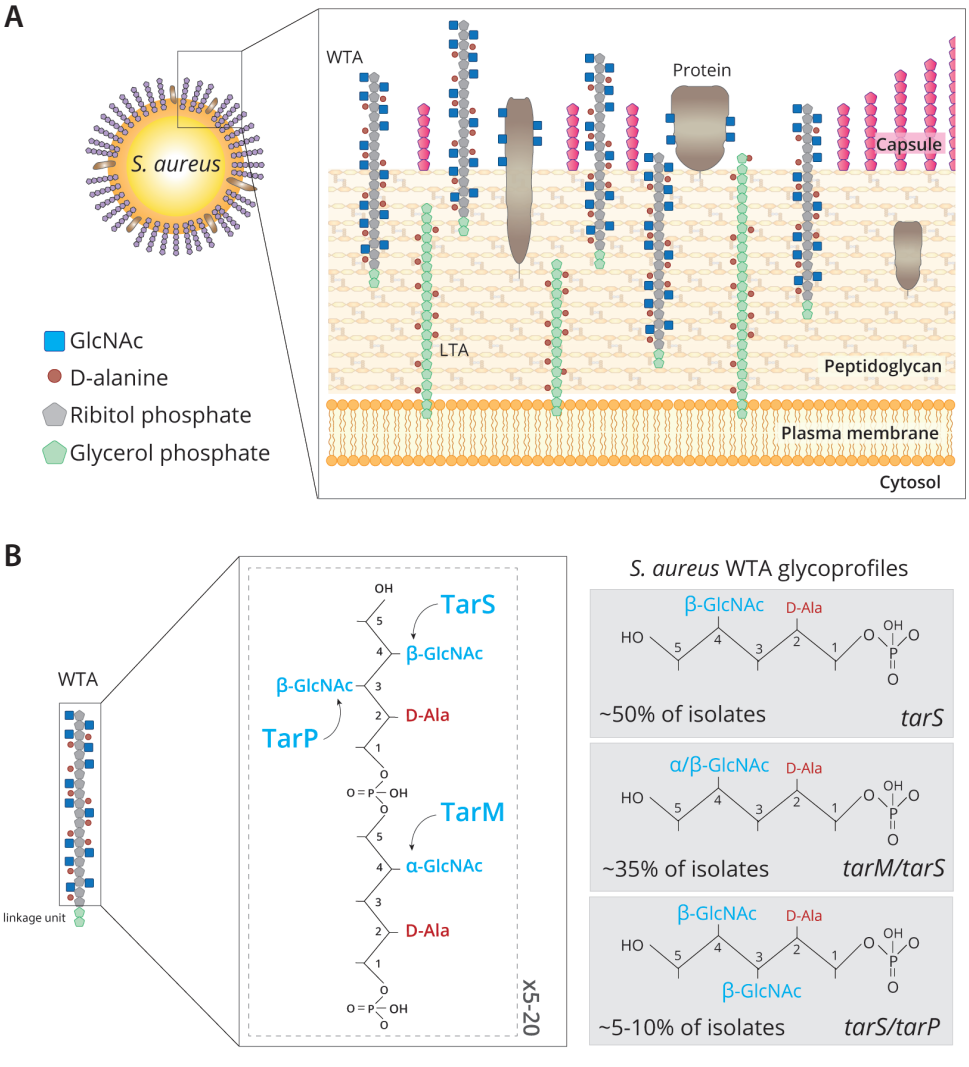


Figure 1. Glycosylated structures present in the *S. aureus* cell wall. (A) Schematic overview of the *S. aureus* cell envelope with incorporated glycans. WTA; wall teichoic acid, LTA; lipoteichoic acid (B) Structure and GlcNAcylation of WTA, by WTA glycosyltransferases TarS, TarP and TarM, with the estimated distribution of WTA glycoprofiles among *S. aureus* isolates.

and 250 kDa plasmin-sensitive protein PIs (43,44). Glycosylation of the C-terminal SDR region of PIs is mediated by SdgA and SdgB similar to SDR proteins, as well as GtfC and GtfD (43). The serine-rich regions of SraP can be modified with GlcNAc by dedicated glycosyltransferases GtfA and GtfB (45). Glycosylation of both SraP and Pls is believed to enhance biofilm formation.

Teichoic acids

Teichoic acids (TAs) are glycopolymers that are characteristic components of the cell envelope of Gram-positive bacteria including *S. aureus*. Despite their universal expression in Gram-positive bacteria, the structural composition and related functionality of TAs can be species, and sometimes even strain-specific. Broadly, two types of TA can be distinguished; lipoteichoic acids (LTAs), which are covalently linked to the plasma membrane and wall teichoic acids (WTAs), which are covalently attached to the peptidoglycan cell wall.

S. aureus LTA consists of 18-50 repeating units of glycerolphosphate (GroP), which is attached to the plasma membrane via a glycolipid anchor (46). The GroP backbone can be decorated at C2 with positively-charged D-alanine residues or, less frequently, α-GlcNAc moieties (47). D-alanylation of LTA is dominant under standard laboratory growth conditions, although high salt conditions may promote LTA glycosylation (48).

WTAs constitute a major component of the *S. aureus* cell wall, comprising up to 60% of the total cell wall biomass under certain growth conditions (35,49). In the majority of *S. aureus* isolates, WTA is composed of a polymerized backbone consisting of 20-40 ribitolphosphate (RboP) subunits. This RboP polymer is covalently attached to peptidoglycan MurNAc, the same position as CP, through a linker unit. It was estimated that every ninth MurNAc unit within individual peptidoglycan strands is linked to a single WTA polymer, generating a sort of sugar veil on the cell surface (50). The negatively-charged poly-RboP chain is decorated with GlcNAc moieties and D-alanine residues, giving this molecule its zwitterionic properties similar to LTA (51). *S. aureus* isolates of the ST395 lineage do not express RboP-WTA, but GroP-WTA with α-N-acetylgalactosamine (α-GalNAc) modifications. This WTA composition resembles that of coagulase-negative staphylococci that generally express GroP-WTA but with glucose modifications (52). In comparison, RboP-WTA is commonly expressed by *Listeria monocytogenes*, *Enterococcus faecalis* and certain strains of *Bacillus subtilis* but instead of GlcNAc, these species decorate their WTA with glucose or GalNAc moieties (53).

In general, TAs display overlapping functions that are critical for *S. aureus* survival through their involvement in different key processes including bacterial cell division, cation homeostasis and protection from environmental stress (51,54). D-alanylation of TAs confers resistance to host antimicrobials found on the skin surface including free fatty acids and cationic antimicrobial peptides (55,56). Moreover, D-alanylated TAs reduce susceptibility to glycopeptide antibiotics such as vancomycin (57). Next to these

overlapping functions of TAs, WTA harbors specific functions that depend on the presence of additional GlcNAc modifications, which will be addressed below in more detail.

WTA biosynthesis and glycosylation

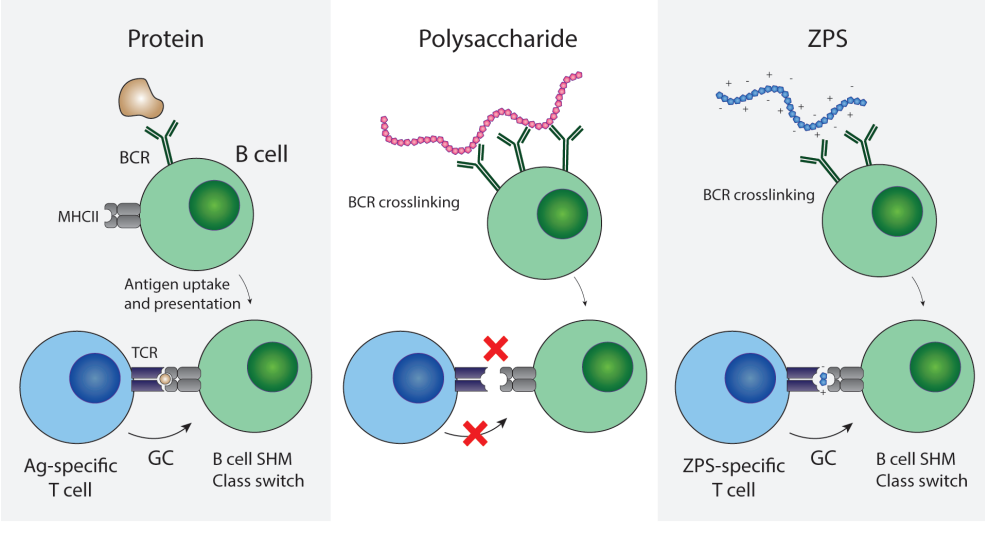
The genes required for WTA biosynthesis are designated as tag (for GroP) and tar (for RboP) genes and encode for enzymes and transport proteins with specific roles in the biosynthesis pathway. This pathway entails the synthesis of sugar building blocks on a carrier molecule (UDP-GlcNAc and GDP-ManNAc), assembly of the poly-RboP chain on a lipid-linked carrier in the plasma membrane, transport across the plasma membrane (flipping) and attachment to peptidoglycan (35,58). Glycosylation of RboP-WTA with GlcNAc occurs after polymer assembly within the plasma membrane, whereas D-alanylation by the DltABCD system occurs after peptidoglycan attachment. While WTA synthesis is believed to be constant, WTA incorporation in peptidoglycan is increased when the Agr sytem is activated which is linked to increased bacterial virulence (59). Furthermore, variation in WTA glycosylation has been genetically and functionally characterized, providing insight for host-pathogen interaction.

Decoration of the RboP-WTA backbone with GlcNAc moieties is crucial for bacterial pathogenesis and survival. On the one hand, glycosylated WTA mediates horizontal gene transfer of pathogenicity islands (52), while on the other hand also preventing bacterial killing by lytic phages (60). Importantly, nasal colonization of *S. aureus* partially depends on WTA (61), through the binding of D-alanylated WTA to the scavenger receptor class F, member 1 (SCARF1, formerly known as SREC-I) expressed by nasal epithelial cells (62). In addition, GlcNAcylated WTA exhibits enhanced binding to nasal epithelial cells although the dedicated receptor has not yet been identified (63).

In *S. aureus*, three distinct glycosyltransferases have been described that confer GlcNAcylation of the RboP-WTA backbone, namely TarS, TarM and TarP (64–66). TarS and TarM both modify WTA at C4 of the RboP subunit with a β-linked GlcNAc (β-GlcNAc) and α-GlcNAc, respectively, whereas TarP modifies the WTA RboP backbone at C3 with β-GlcNAc (67). Therefore, three different WTA glycotypes exist (**figure 1B**). Additional variation in glycosylation results from simultaneously expression of multiple glycosyltransferases, which results in a mixture of different WTA glycotypes or glycoprofile on the *S. aureus* surface.

TarS is encoded in the core genome of *S. aureus*, and can be found in all RboP-WTA producing *S. aureus* strains (53,63). About 35% of human isolates additionally carry the tarM gene. Finally, tarP is present in 5-10% of *S. aureus* isolates, mainly healthcare and livestockassociated MRSA strains (64). Recently, *S. aureus* strains were found that carry all three glycosyltransferase genes, although it remains unclear if all three Tar enzymes can be expressed as well (68). In addition to gene presence, WTA glycosylation can also change as a result of gene regulation. Indeed, alterations in WTA glycosylation can occur

in response to environmental cues. Glycosylation by TarM and TarP is dominant over TarS during in vitro culture due to higher inherent enzymatic activity. However, TarS glycosylation increases at the expense of TarP/TarM modifications during in vivo murine infection and in high salt conditions (69). Moreover, the presence of β-lactam antibiotics promotes β-GlcNAcylation of WTA by TarS, which mediates resistance to β-lactams (66). In contrast, TarM expression is enhanced during oxidative stress, likely due to activation of the two component GraRS regulon (70,71). Insight into WTA glycosylation is of interest since GlcNAcylated WTA is important for host interaction including colonization and immune recognition (62,67).



	Protein	Polysaccharide	ZPS
Antibody response	IgG1, IgG3, IgA	IgM, IgG2	IgM, IgG2, IgG1
Long-lasting B cell memory	+++	+/-	+

Figure 2. Overview of human B cell responses to bacterial proteins and polysaccharides. Within secondary lymphoid organs, B cell binding and uptake of foreign proteins is mediated via the B cell receptor (BCR), after which fragmented peptides are presented on major histocompatibility complex class II (MHCII) molecules. Activated B cells interact with T cells via MHCII and the T cell receptor (TCR). Encounter of an antigen-specific (Ag) T cell promotes the formation of germinal centers (GCs), where activated B cells undergo somatic hypermutation (SHM), and Ig class switching. Activated B cells differentiate into Ig producting plasmablasts and memory B cells. BCR mediated uptake of polysaccharides induces GC-independent B cell responses, in the absence of MHCII presentation. Zwitterionic polysaccharides (ZPS) can be presented on MHCII, inducing both GC-dependent and independent B cell responses.

Immune recognition of bacterial carbohydrates

Since glycosylated cell wall structures are essential for bacterial colonization and virulence including *S. aureus*, the host immune system utilizes both innate and adaptive immunity to recognize these structures. C-type lectins constitute a family of pattern recognition receptors (PRRs) and are dedicated to the innate immune recognition of glycan motifs on both self and non-self glycans including microbes (72). C-type lectins are classified as non-immunoglobulin proteins that bind carbohydrates in a calcium dependent manner, without conformational changes (so called 'lock-and-key' binding mode) (73). Lectins have a carbohydrate recognition domain (CRD), and often exist as multimers which can further oligomerize to increase functional affinity. C-type lectins are present in soluble form and expressed on the surface of immune cells as C-type lectin receptors (CLRs). Soluble C-type lectins including collectins (e.g. mannose binding lectin; MBL) and ficolins can bind glycan antigens on the surface of pathogens thereby activating the complement system via the so-called lectin pathway (73). The complement system is another important part of the innate immune system, and constitutes a family of approximately 30 soluble serum components. Upon activation, these components orchestrate a tightly regulated cascade reaction that leads to enhanced neutrophil chemotaxis (via C5a formation), direct killing of Gram-negative bacteria (via the formation of membrane attack complexes; MAC) and neutrophil-mediated uptake and killing of Gram-positive bacteria (via C3b opsonization) (74). CLRs expressed by innate immune cells, such as neutrophils and DCs, regulate various cellular processes directly via intracellular signaling or indirectly via modulation of other PRRs e.g. Toll-like receptors (TLRs). Upon activation, CLRs mediate phagocytosis, antigen presentation, cytokine production and T cell differentiation (75). Compared to other PRRs however, CLRs have received little attention in the past due to inherent challenges in studying protein-glycan interactions. Novel tools such as synthetic bacterial glycans and recombinant host C-type lectins have been indispensable in the characterization of lectin-mediated host-pathogen interactions (76).

Adaptive immune recognition of bacterial surface antigens is mediated by antibodies, or immunoglobulins (Ig), which are abundantly present in blood. In humans, five antibody classes exist, namely IgG, IgA, IgM, IgD and IgE, with different effector functions. IgG can be further subdivided in four subclasses; IgG1, IgG2, IgG3 and IgG4 (77). Antibodies are produced by activated antigen-specific B cells or plasmablasts upon antigen encounter, and the type of antigen determines the quality of this response (**figure 2**). Microbial proteinaceous antigens mainly induce IgG1 and IgG3 responses, which are the preferred antibody subclasses to promote through vaccination due to its high affinity, Fc receptor-mediated cellular responses and capability to potently activate complement (77). However, antibody responses to bacterial carbohydrates are dominated by IgM and IgG2, which is indicative of T cell-independent B cell responses since most carbohydrate antigens cannot be presented on MHC-II (78). IgM and IgG2 are generally considered inferior compared to IgG1 for host defense due to their lower affinity, reduced longevity and capacity to trigger cellular responses (77). For soluble IgM, which exists in pentameric and hexameric forms, lower affinity interactions are compensated for by high avidity

interactions (79). Consequently, IgM binding to surface glycans plays an important role in battling invasive bacterial infections through its highly efficient activation of the complement system (80,81). Furthermore, IgM harbors broad reactivity and can bind various microbial glycans, possibly providing a broad first line of defense when pathogens invade the body (82). In addition to IgM and IgG2, T cell-dependent B cell responses have also been reported for specific bacterial carbohydrates, which induce IgG1 via affinity maturation and class switching (83). Specifically, glycan-dependent MHC-II presentation depends on zwitterionic properties of the polysaccharides, including teichoic acids and capsular polysaccharides from *S. pneumoniae*, *Bacillus subtilis* and *S. aureus* (78). The antibody responses to non-zwitterionic polysaccharides can be improved by covalently linking carbohydrates to a carrier protein, a strategy used in glycoconjugate vaccines, thus employing T cell help and forming durable memory B cell responses (84).

Immunogenicity of *S. aureus* cell wall carbohydrates

Innate immune recognition of *S. aureus* peptidoglycan and LTA is mediated by intracellular NOD-like receptors and surface expressed Toll-like receptor 2 (TLR2) respectively, expressed by epithelial and various phagocytic cells (85). However, no interactions between CLRs and *S. aureus* cell wall carbohydrates have been identified so far, expect for WTA which will be discussed later on.

Peptidoglycan may be targeted by antibodies and complement to induce opsonophagocytosis, but is shielded from recognition by decoration with proteins and wall teichoic acids (86,87). The glycan epitopes of SDR proteins are very immunogenic and induce highly opsonic antibodies (41). The same applies for the capsular polysaccharides of *S. aureus*. Analogous to the successful application of capsular polysaccharides in bacterial vaccines, *S. aureus* CP5 and CP8 showed promise as vaccine antigens in preclinical studies as part of both single and multicomponent vaccines. Unfortunately, CP-based vaccines failed to confer protection from *S. aureus* infection in numerous clinical trials (88–91). A possible explanation is the fact that CP production is heterogeneous, growth-phase dependent and not always advantageous for bacterial survival (92). Furthermore, CP production is completely absent or defective in a selection of prevalent S. aureus human isolates such as the pathogenic USA300 lineage, common causes of CA-SSTIs in the USA (93). Hence, these strains would escape opsonization by CP-based vaccines.

Both C-type lectin and antibody responses to *S. aureus* WTA have been reported, and will be discussed in the next section. Importantly, the innate and adaptive immune recognition of WTA depend on specific GlcNAc modifications. Hence, different WTA glycoprofiles could support bacterial immune evasion, whereas other favor bacterial recognition and clearance.

S. aureus vs Skin: an important immunological barrier organ

"I've got you under my skin", a famous song by Cole Porter from 1936 and sung again by Frank Sinatra in 1956. A way to express affection to another, but it also indirectly refers to one of the most important functions of skin, namely the formation of a physical barrier to external hazards, which thus provides protection to the host. The skin forms the biggest organ of the human body that besides its barrier function also helps regulate body temperature, prevents dehydration, protects from damaging UV radiation and acts as sensory organ (94). The skin surface forms a hostile environment for microbes due to the low pH and temperature, limited nutrient availability and presence of free fatty acids and antimicrobial peptides. Regardless, skin is colonized by communities of well-adapted microbes, collectively referred to as skin microbiota (95). In addition to being a physical barrier, skin is an immunocompetent organ with a variety of immune cells that provide a first line of defense against microbial pathogens when the physical barrier is breached, while also maintaining tolerance to harmless skin commensals and prevention excessive inflammation (96).

Human skin consists of two different compartments, the epidermis and the dermis, where different immune cells reside. The epidermis can be further dissected in four layers or strata, i.e. stratum corneum, stratum granulosum, stratum spinosum and stratum basale. Keratinocytes are the main cell type found in the epidermis (over 90%) that exhibit a high turnover, and their differentiation and maturation state matches the specific stratum. In the stratum basale, proliferating undifferentiated keratinocytes form a single layer. Upon differentiation and transition to the upper epidermal layers, keratinocytes start producing keratin proteins and lipids. In the stratum corneum, the main barrier function is maintained by corneocytes; dead flattened keratinocytes. Other immune cells in the epidermis include Langerhans cells (3-5%) and more scarce $CD4^+$ and $CD8^+$ T cells (<1%) (97,98). In contrast to the epidermis, the dermis is composed of collagen and elastin fibers, and is home to a wider range of immune cells including dermal dendritic cells (dDCs), macrophages, mast cells, $CD4^+$, $CD8^+$, and γδ-T cells (99,100). Since the dermis is heavily vascularized, circulating immune cells can migrate into the skin from the circulation, e.g. in case of infection.

As mentioned before, *S. aureus* is the leading cause of SSTIs worldwide (5), reflecting that the skin forms the primary entry site for *S. aureus* to invade the host. For effective design of immune-based therapies against *S. aureus*, it is essential to study human immune responses to *S. aureus* skin infection and how these contribute to protective immunity. Here, I will summarize the main skin-resident immune cells, and known interaction and responses to *S. aureus* WTA (**figure 3**).

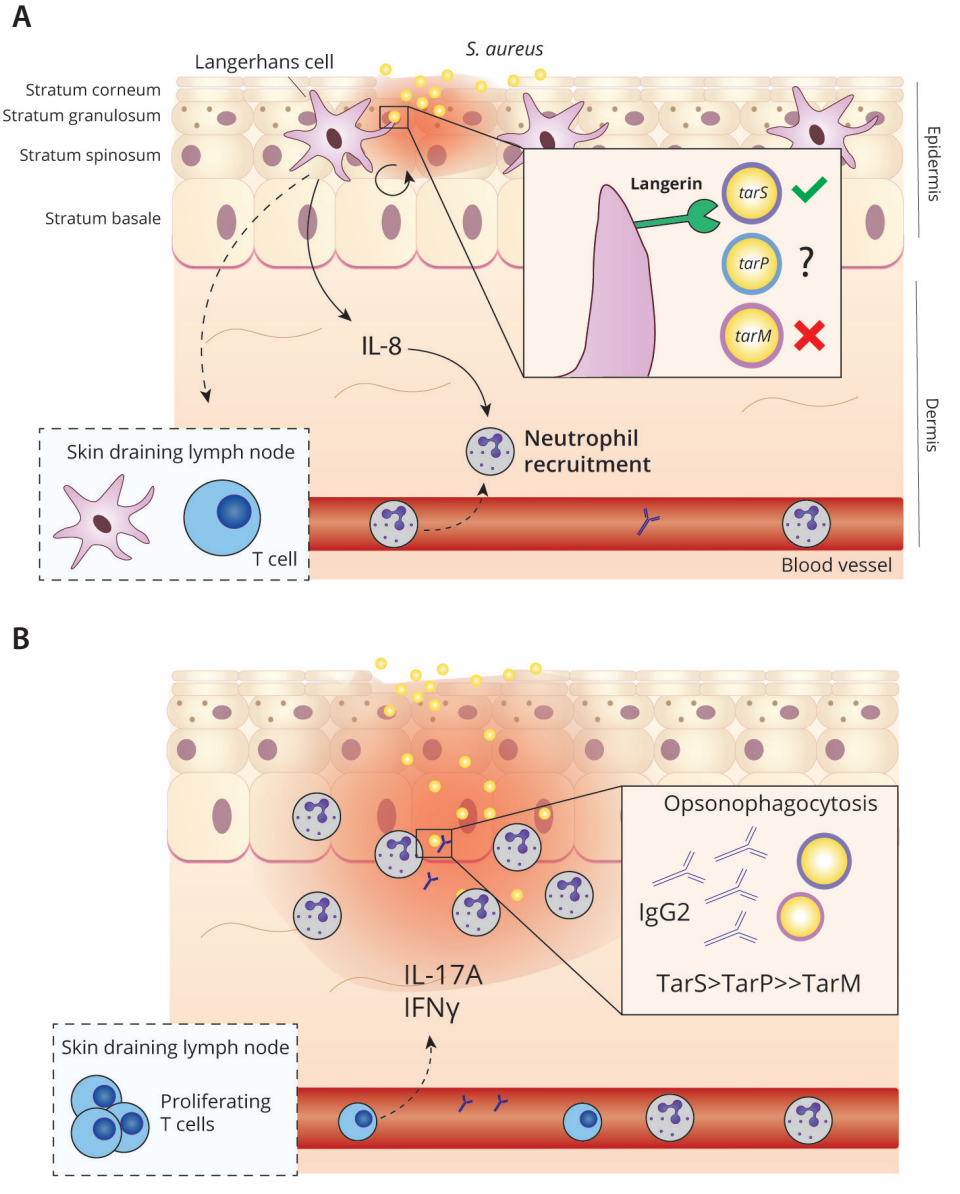


Figure 3. Immune responses to *S. aureus* WTA during skin infection. (A) During early stages of *S. aureus* skin invasion, LCs sense β-GlcNAc WTA producing *S. aureus* (modified by TarS) via the CLR Langerin. Activated LCs produce pro-inflammatory cytokines including IL-8 and migrate out of the epidermis to activate T cells in the skin draining lymph nodes. (B) Later in infection, *S. aureus* specific T cells will proliferate, migrate to the site of infection and produce IL-17A and IFNγ. *S. aureus* is opsonized by WTA-specific IgG2 antibodies that increase neutrophil-mediated opsonophagocytosis and killing of *S. aureus*.

Immune responses to *S. aureus* WTA during skin infection

Keratinocytes

Besides being responsible for maintaining the physical barrier, keratinocytes are also considered unprofessional immune cells that express different PRRs such as TLRs, NOD-like receptors and CLRs (101). Moreover, keratinocytes can present foreign peptide antigens on MHC-II thereby promoting local memory Th1 responses (102). Upon activation, keratinocytes produce pro-inflammatory cytokines including IL-1 and different antimicrobial peptides (AMPs). *S. aureus* has evolved mechanisms to circumvent AMP-mediated damage, such as D-alanylation of WTA resulting in repulsion of cationic AMPs (56). To this date, no specific interactions between *S. aureus* WTA and keratinocytes have been identified. However, keratinocytes do play a central role in orchestrating innate immune defenses against *S. aureus* skin infection (101).

Langerhans cells

Langerhans cells (LCs) are professional antigen-presenting cells (APCs) that reside in the epidermis, but can also be found in epithelial surfaces of mucosal tissues. Unique features of human LCs include the expression of the CLR langerin (CD207) and the formation of cytosolic tennis racket-shaped organelles called Birbeck granules (103,104). Langerin is a trimeric type II transmembrane receptor that binds sulfated, mannosylated glycans and β-glucans in a calcium-dependent manner (105,106). Subsequently, langerin expression enables LCs to sense various pathogens including HIV, *Mycobacterium leprae*, *Yersinia pestis* and *Candida albicans* (107–110). Thus, LCs often act as the first responders to pathogen skin invasion and are key in early initiation of pro-inflammatory immune responses.

Langerin binding results in receptor-mediated endocytosis of cargo and transport to the Birbeck granules, which are likely required for antigen processing (111,112). Interestingly, inhibition or lack of langerin expression abolishes the formation of Birbeck granules, although no apparent pathologies were noted in a langerin-deficient individual (113,114). Other directly downstream effects of langerin binding are currently unknown, as the short intracellular domain does not contain classical signaling motifs. Activated LCs will migrate out of the epidermis to the dermis and skin-draining lymph nodes, where they can present foreign antigens to both naïve and memory $CD4^+$ T cells in a MHC-II dependent manner as well as $CD8^+$ T cells via MHC-I cross-presentation (115,116). Next to host defense responses, LCs also have an immunomodulatory role by inducing regulatory T cells thereby promoting tolerance to skin commensals (117). Whether LC activation results in pro-inflammatory or tolerogenic responses may be context- and stimulus-dependent (117,118). In addition to acquisition of antigen in situ, LCs can also protrude the stratum corneum with their dendrites and directly sample antigen from the external environment to induce antigen-specific immunity (119,120).

LC sensing of *S. aureus* WTA

LCs can sense a broad range of viral and fungal pathogens, but the recognition of bacteria has remained elusive due to restricted and limited TLR expression (121). Recent work has shown that LCs are able to sense *S. aureus* and thereby distinguish from other skin commensal bacteria including coagulase-negative staphylococci (122). This distinction relies on the interaction between CLR langerin and TarS-dependent modification of WTA, whereas TarM-modified WTA does not confer langerin binding. It is unknown whether langerin binds to TarP-modified WTA. Langerin binding to *S. aureus* results in LC activation, with increased expression of maturation markers and production of pro-inflammatory cytokines, including IL-8, that enhanced overall skin inflammation (122). Thus, LCs may contribute to the anti-*S. aureus* immune response, via the early detection of *S. aureus* skin invasion.

Macrophage and dermal DC sensing of *S. aureus* WTA

Dermal DCs (dDCs) express a broad repertoire of PRRs, including TLRs and CLRs, to sense and internalize invading pathogens (123). dDCs are involved in orchestrating pro-inflammatory responses locally and are able to migrate to skin draining lymph nodes to activate T helper cells (124). Different DC subsets are present in the dermis, namely conventional DCs (cDCs), which can be further classified in cDC1 and cDC2, plasmacytoid DCs (pDCs) and monocyte-derived DCs (moDCs)(123). While the CLR langerin binds RboP-WTA producing *S. aureus*, the CLR macrophage galactose-type lectin (MGL), expressed by dDCs and macrophages, was found to interact with GroP-WTA producing *S. aureus* (125,126). MGL specifically binds α-GalNAc modifications on GroP-WTA, leading to production of pro-inflammatory cytokines in moDCs (125). In addition, RboP-WTA might also activate dDCs and macrophages since these cells express SCARF-1, the receptor responsible for the interaction of D-alanylated WTA with nasal epithelial cells (127,128).

T cells

More T cells are present in skin than in the circulation. The majority of this population has a memory phenotype and resides in the skin for a long time without recirculating (129,130). Tissue-resident memory T cells provide local protection from reinfection, and can be found in all barrier organs (131). Protective skin-resident memory T cell responses have been identified for skin pathogens *Candida albicans*, *Leishmania major* and Herpes simplex virus (132–136). For *S. aureus*, only circulating memory T cell responses, and not skin resident memory T cells, have been identified and studied thus far and play a major role in the anti-*S. aureus* immune response (21,137). Upon activation, circulating T cells can migrate to the skin via the expression of the skin-homing marker cutaneous lymphocyte antigen (CLA) (138). While Th1 cells are considered important against invasive *S. aureus* infections (139), Th17 cells contribute to overall tissue immunity by production of IL-

17, a key cytokine that promotes the production of AMPs by keratinocytes and enhances neutrophil production, functioning and recruitment to the site of infection (26,140). WTA can induce specific T cell responses, since its characteristic zwitterionic properties enable MHC-II presentation by APCs (141). Similarly, WTA-specific T cells have been identified in healthy individuals but it is not clear if and how these cells contribute to protective immunity (21). In mice, WTA-specific CD4+ T cells mediate abscess formation and bacterial clearance during *S. aureus* skin infection (142).

Opsonins for *S. aureus* WTA

Although not present in skin under homeostatic conditions, circulating humoral immune components and neutrophils also participate in the anti-*S. aureus* immune response during skin infection. Moreover, the combination of neutrophils, opsonizing antibodies and complement within infected skin mediates phagocytosis and killing of *S. aureus* in situ, partially by targeting WTA (143). The complement system can also be activated through the lectin pathway after binding of soluble lectins MBL and ficolin to α-GlcNAc-WTA and β-GlcNAc-WTA. MBL binding to WTA may be of greater relevance during early childhood and becomes of less importance once WTA-specific antibodies are produced (144,145).

A reported 70% of the human antibody repertoire directed against *S. aureus* surface antigens is specific for WTA (146). Despite the possibility of MHCII presentation, WTA-specific antibodies are mainly IgG2. Antibodies for β-1,4-GlcNAc-modified WTA are most abundant and show cross-reactivity for β-1,3-GlcNAc-modified WTA (147). Antibody binding to WTA potently induces opsonophagocytosis and killing of S. aureus (148–150). IgM responses to WTA have not been studied to date. Zwitterionic WTA also induces high affinity IgG1 antibodies, although levels are much less abundant than IgG2 (146,151). It is currently not known how and if differences in WTA specific antibody responses are associated with resistance to *S. aureus* infections.

Outline of this thesis

This thesis describes novel insights in the human immune response to *S. aureus* WTA in the context of skin infection. **Chapter 2** expands on previous findings that S. aureus interacts with LCs through langerin and describes the interaction between langerin and β-GlcNAc WTA expressing *S. aureus*. Using synthetic WTA oligomers as a novel tool to study host lectin responses to *S. aureus* WTA, we confirm that human langerin interacts with β-GlcNAc modifications on WTA, mediated by both TarS and TarP. In **Chapter 3**, we highlight the use of fully synthetic WTA oligomers as powerful tools to perform in-depth structure binding analysis of glycan-specific monoclonal antibodies (mAbs). We show that WTA-mAb binding and subsequent complement activation are not only affected by anomeric GlcNAc configuration but also by the position of the GlcNAc moiety on the poly-RboP chain. **Chapter 4** provides an extensive overview of the antibody repertoire

to the different WTA-GlcNAc modifications in healthy donors. Moreover, this chapter shows how these antibody profiles are affected in ICU patients with culture-confirmed invasive *S. aureus* infection. Furthermore, for the first time we report WTA-specific IgM responses in these two human cohorts. **Chapter 5** describes the identification of *S. aureus*-specific tissue-resident memory T cells in healthy human skin that proliferate rapidly upon incubation with *S. aureus* and produce pro-inflammatory cytokines like IL-17A, IL-22 and IFNγ. Consequently, these cells may contribute to protective immunity during skin infection. Finally, **chapter 6** summarizes and discusses these findings, while providing insight on how immunity to WTA contributes to protection from *S. aureus*infections.

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2

"Doubt can only be removed by action"

JOHANN WOLFGANG VON GOETHE

Chapter 2

Impact of glycan linkage to *Staphylococcus aureus* wall teichoic acid on langerin recognition and Langerhans cell activation

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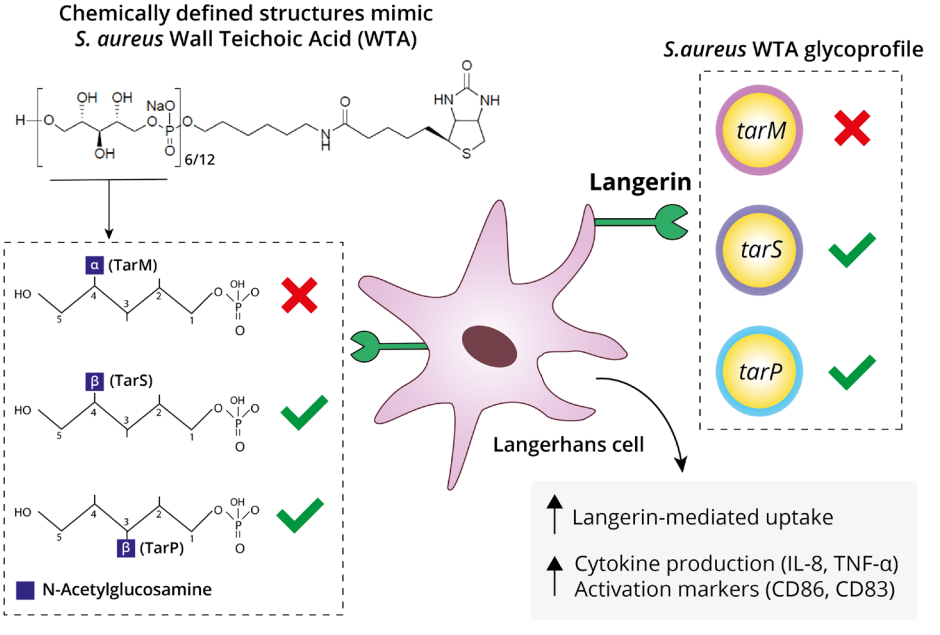
Abstract

Staphylococcus aureus is the leading cause of skin and soft tissue infections. It remains incompletely understood how skin-resident immune cells respond to invading *S. aureus* and contribute to an effective immune response. Langerhans cells (LCs), the only professional antigen-presenting cell type in the epidermis, sense *S. aureus* through their pattern-recognition receptor langerin, triggering a pro-inflammatory response. Langerin recognizes the β-1,4-linked N-acetylglucosamine (β1,4-GlcNAc) but not α-1,4-linked GlcNAc (α1,4-GlcNAc), which are added by dedicated glycosyltransferases TarS and TarM, respectively, on the cell wall glycopolymer Wall Teichoic Acid (WTA). Recently, an alternative WTA glycosyltransferase, TarP, was identified, which also modifies WTA with β-GlcNAc but at the C-3 position (β1,3-GlcNAc) of the WTA ribitol phosphate (RboP) subunit. Here, we aimed to unravel the impact of β-GlcNAc linkage position for langerin binding and LC activation. Using genetically-modified *S. aureus* strains, we observed that langerin similarly recognized bacteria that produce either TarS- or TarP-modified WTA, yet tarP-expressing *S. aureus* induced increased cytokine production and maturation of in vitro-generated LCs compared to tarS-expressing *S. aureus*. Chemically-synthesized WTA molecules, representative of the different *S. aureus* WTA glycosylation patterns, were used to identify langerin-WTA binding requirements. We established that β-GlcNAc is sufficient to confer langerin binding, thereby presenting synthetic WTA molecules as a novel glycobiology tool for structure-binding studies and for elucidating *S. aureus* molecular pathogenesis. Overall, our data suggest that LCs are able to sense all β-GlcNAc-WTA producing *S. aureus* strains, likely performing an important role as first responders upon *S. aureus* skin invasion.



Introduction

Staphylococcus aureus is a Gram-positive bacterium that transiently colonizes an estimated 20% of the human population at different sites of the body, including the nasopharynx, skin and gastrointestinal tract (1). The skin is a common entry site for *S. aureus*, making it the leading cause of skin and soft tissue infections (SSTIs)(2). Consequently, efficient and rapid recognition of invading *S. aureus* by resident skin immune cells is critical for local eradication. When local immune defense fails, bacteria can disseminate into deeper tissues or even cause systemic infections, which are associated with high overall disease burden and mortality. The high recurrence of *S. aureus* SSTIs indicates that protective immune memory is defective, the reasons for that remain elusive. Indeed, there are no clear correlates of protection known for *S. aureus*, which has been a challenging aspect for vaccine development (3). A complete understanding of the local skin immune response to *S. aureus* may identify factors that protect the host from (re-)infection, thereby providing critical insight for the development of a future *S. aureus* vaccine.

The skin contains a large arsenal of immune cells, which reside in different compartments within the skin. Langerhans cells (LCs), a highly specialized macrophage subset with dendritic cell-like functions, are the main antigen-presenting cells within the epidermis (4). Human LCs appear to have an important dual role in maintaining skin homeostasis, by balancing both tolerogenic responses towards skin commensals as well as pro-inflammatory responses to invading pathogens (5-10). However, the ability of LCs to recognize and respond to invading bacteria remains elusive due to their restricted expression of Toll-like receptors (11,12). C-type lectin receptors (CLRs) constitute a family of pattern-recognition receptors (PRRs), which are dedicated to the recognition of glycans (13). A signature CLR of LCs is langerin (CD207) (14). Langerin is a trimeric type II transmembrane receptor with specificity for sulfated and mannosylated glycans as well as β-glucans, which are recognized in a calcium-dependent manner (15-17). The direct downstream effects of receptor activation remain to be determined, since langerin only contains a short cytoplasmic tail without classical signaling motifs (14). It is generally assumed that langerin-bound cargo is endocytosed and processed for antigen presentation to CD4 T cells via major histocompatibility complex class II (MHC-II) (18-20).

Recent work demonstrated that langerin allows human LCs to discriminate *S. aureus* from other staphylococci through a specific interaction with glycosylated Wall Teichoic Acid (WTA) (21). WTA is a major component of the Gram-positive bacterial cell wall and a well-known immunogenic antigen for opsonic antibodies targeting *S. aureus* (22-24). *S. aureus* WTA consists of a polymerized ribitol phosphate (RboP) backbone that can be co-decorated with positively-charged D-alanine and N-acetylglucosamine (GlcNAc) residues. D-alanylation of WTA is highly regulated and impacts bacterial surface charge, thereby providing protection from host cationic antimicrobial peptides (AMPs) and the lipopeptide antibiotic daptomycin (25-28). WTA glycosylation can be mediated by different glycosyltransferases, resulting in distinct WTA glycoforms. Three different WTA

glycoforms have been identified in *S. aureus*, which differ in the configuration and position of GlcNAc linkage. Langerin binding to *S. aureus* is conferred by β-1,4-GlcNAc modified WTA, which requires the glycosyltransferase TarS that is present in nearly all S. aureus strains (29,30). Approximately 30% of *S. aureus* strains derived from nasal isolates co-express *tarM*, which encodes a glycosyltransferase that modifies WTA with α-1,4-GlcNAc (29,31). Although α-1,4-GlcNAc did not confer langerin binding, it attenuated langerin binding to β-1,4-GlcNAc WTA, likely as a result of substitution or steric hindrance. This suggests that *S. aureus* clones co-expressing *tarM*/*tarS* can alter WTA glycosylation to evade innate immune activation by LCs (21). Interaction between β-1,4-GlcNAc expressing *S. aureus* and langerin increased pro-inflammatory cytokine production by in vitro-generated LCs and in the skin of human langerin-transgenic mice after epicutaneous infection, suggesting a contribution to anti-bacterial host defense (21). Overall, WTA glycosylation impacts the ability of LCs to sense invading *S. aureus* and mount a local immune response (21).

In addition to TarM and TarS, a third glycosyltransferase, TarP, has recently been identified (32). TarP modifies the WTA backbone with β-linked GlcNAc residues similar to TarS but at the C3 position of RboP instead of C4. *TarP* is always co-expressed with *tarS* and is associated with, but not limited to, healthcare-associated and livestock-associated MRSA strains belonging to clonal complexes 5 and 398 (32,33). TarP can functionally replace TarS with regard to β-lactam resistance and phage susceptibility via the decoration of WTA with β-GlcNAc moieties (30,32). However, whether the same applies to immune recognition remains to be fully elucidated. For example, TarP-modified WTA displayed attenuated immunogenicity in mice compared to TarS-modified WTA and co-modification of WTA by TarP may lower *S. aureus* antibody recognition despite the presence of antibodies to both WTA glycoforms in serum from healthy individuals (24,33).

In this study, we assessed the impact of TarP-mediated WTA glycosylation on langerin recognition and responses, i.e. antigen uptake and cytokine production, of in vitro-generated LCs. We describe that langerin-mediated recognition and uptake of *S. aureus* is similar for strains expressing β-1,3 GlcNAc WTA or β-1,4 GlcNAc WTA. Despite similar recognition and uptake, LC cytokine production was more pronounced upon interaction with *tarP*-expressing bacteria compared to *tarS*-expressing bacteria. Finally, employing synthetic WTA molecules with specific GlcNAc modifications (34), we demonstrate that β-GlcNAc WTA is sufficient but not exclusively required for *S. aureus* binding to langerin-expressing cells. Overall, we provide evidence that LCs are able to sense and respond to all *S. aureus* strains that produce β-GlcNAc-modified WTA. Furthermore, the use of chemically synthesized WTA structures provides a valuable toolbox to study the interaction between host immune molecules such as CLRs and *S. aureus* WTA in more detail.

Results

TarP and TarS both confer binding of human langerin to *S. aureus*

TarP can replace several key functions of TarS, including resistance to β-lactam antibiotics and susceptibility to siphophage infection (32). In contrast, decoration of WTA with β-1,3-GlcNAc in addition to or instead of β-1,4-GlcNAc may impact immune detection by antibodies (24,32). We recently identified that β-1,4-GlcNAc WTA is specifically detected by the human innate receptor langerin (21). To assess whether human langerin was also able to detect *tarP*-expressing *S. aureus* strains, we employed a FITC-labeled recombinant construct of the extracellular carbohydrate domain (ECD) of human langerin (langerin-FITC) (35). Using *S. aureus* strain N315 that naturally expresses both *tarS* and *tarP* (32), we observed that langerin binding was significantly impaired upon deletion of both glycosyltransferases (ΔtarPS), but not in either of the single mutant strains (**Fig. 1A**). Subsequent complementation of the ΔtarPS double mutant with a plasmid containing either tarS or tarP restored the binding to recombinant langerin-FITC (**Fig. 1A**). This observation in differential langerin binding amongst the N315 mutant panel persisted over a 100-fold concentration range of langerin-FITC, although at higher

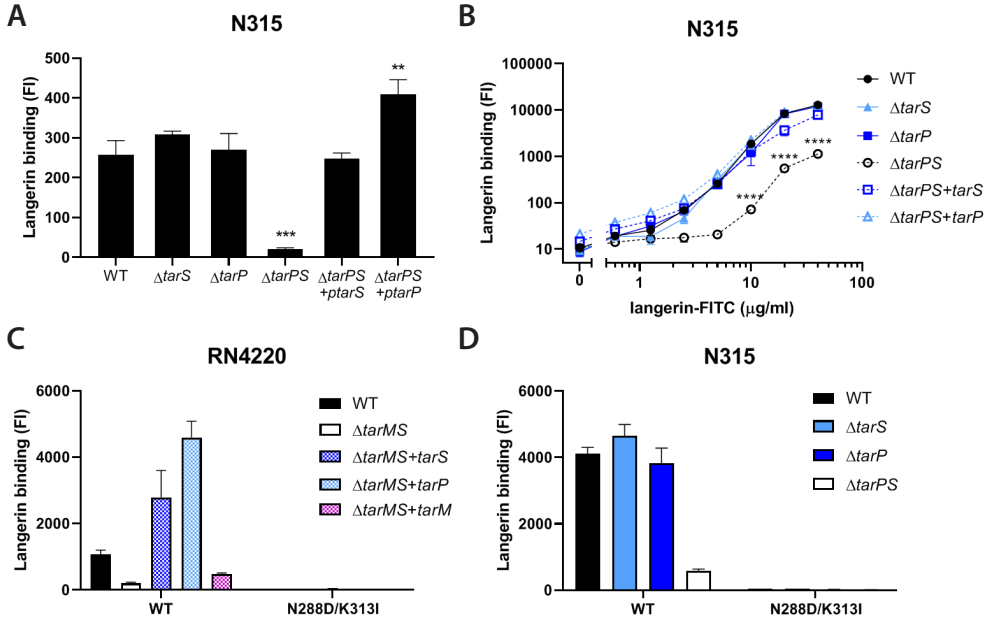


Figure 1. WTA β-GlcNAcylation by TarS and TarP confers langerin binding to *S. aureus*. Binding of recombinant human langerin-FITC A) to N315 WT, Δ*tarS*, Δ*tarP*, Δ*tarPS*, Δ*tarPS* +p*tarS* and Δ*tarPS* +p*tarP* at a fixed concentration of 5 μg/ml, B) to the indicated N315 strain panel using a concentration range of langerin-FITC (0.6-40 μg/ml). C, D) Binding of FITC-labeled recombinant human langerin wildtype and N288D/K313I double SNP variant (10 μg/ml) to C) RN4220 WT, Δ*tarMS*, Δ*tarMS* +p*tarS*, Δ*tarMS* +p*tarP* and Δ*tarMS* +p*tarM* and D) the N315 mutant panel (mentioned above). Data is depicted as geometric mean fluorescence intensity (FI) + standard error of mean (SEM) of biological triplicates. **p < 0.01, ***p < 0.001, ****p < 0.0001.

concentrations langerin-FITC also showed significant binding to the ΔtarPS strain (**Fig. 1B**). Binding to the N315 ΔtarPS strain was also dependent on the langerin carbohydrate recognition domain (CRD) since the interaction could be blocked by addition of mannan (**Supporting Fig. 1B**). Similar binding experiments were additionally performed in *S. aureus* strain RN4220, which naturally co-expresses *tarS* and *tarM*, but not *tarP*. As previously reported (21), langerin binding to RN4220 wild-type was significantly reduced in the ΔtarMS double mutant (**Fig. 1C**). Binding could be restored by complemention with either *tarS* or *tarP* but not *tarM* (**Fig. 1C**). For the N315 and RN4220 strain panels, expression of the correct WTA glycoform was confirmed through binding of specific mAbs (**Supporting Fig. 1A**;(24)). Overall, langerin binds to TarP-modified WTA independent of strain background.

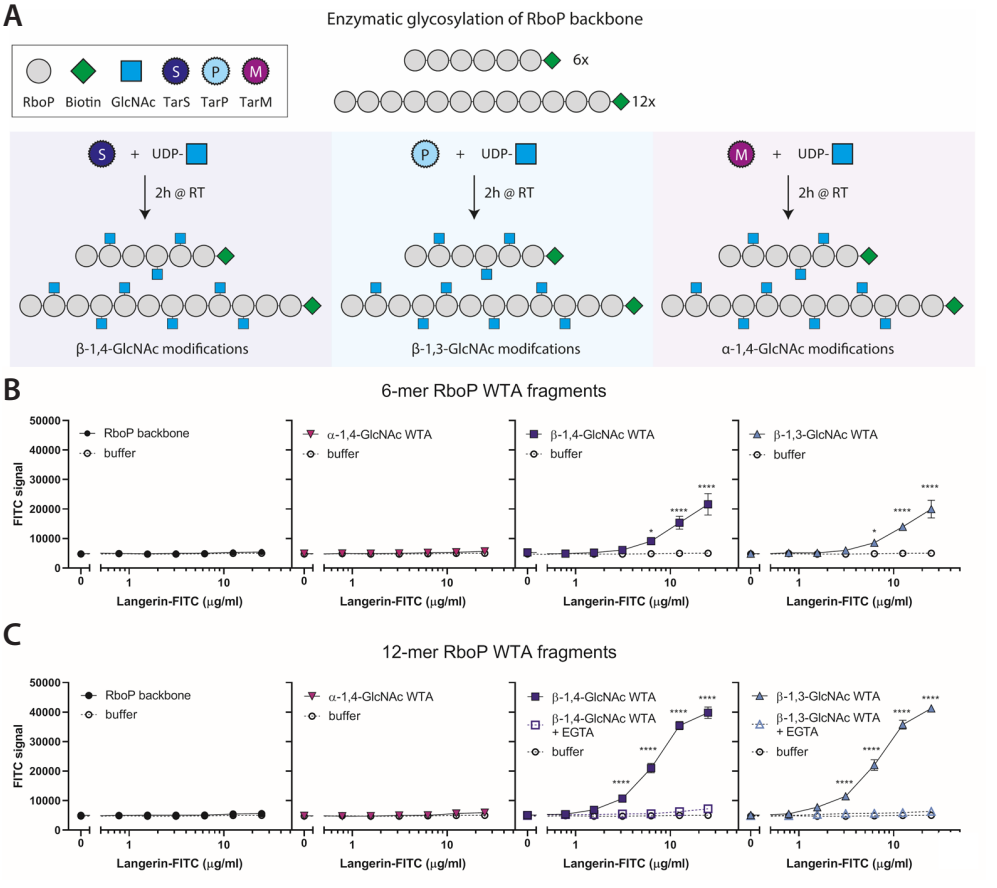


Figure 2. β-GlcNAc-modified WTA is sufficient to confer langerin binding. A) Schematic overview of the synthetic WTA structures and in vitro glycosylation by recombinant TarS, TarP or TarM. B) Binding of recombinant human langerin-FITC (0.4-25 μg/ml) to RboP hexamers alone (RboP backbone) or after in vitro glycosylation by TarS, TarP or TarM. C) Binding of recombinant human langerin-FITC (0.4-25 μg/ml) to RboP dodecamers alone (RboP backbone) or after in vitro glycosylation similar to RboP hexamers. Binding to β-GlcNAc WTA was assessed in the absence and presence of EGTA (10 mM). Data for panel B and C is shown as fluorescence signal + SEM of three independent experiments, and was compared with the negative control (buffer). *p < 0.05, ****p < 0.0001.

While it was apparent that langerin binding to *S. aureus* required either TarP or TarS, it was not clear whether the receptor bound the two different modifications in a similar way. Previously, we showed that langerin binding to *S. aureus* was abrogated when a naturally-occurring double SNP was introduced into the human langerin ECD (36). Using these same langerin SNP constructs, we observed a similar loss of binding to *tarP*-expressing *S. aureus* (**Fig. 1C, D**). These data suggest that the WTA β-1,3-GlcNAc moiety created by TarP is similarly dependent on these two residues in the CRD of langerin compared to the β-1,4-GlcNAc moiety on WTA generated by TarS.

WTA β-GlcNAc is sufficient to confer langerin binding

TarP-expressing *S. aureus* can bind langerin in a similar way to *S. aureus* expressing *tarS*. However, we also observed significant residual binding in the Δ*tarPS* background at higher langerin concentrations (**Fig. 1B**). We therefore asked whether WTA-β-GlcNAc is sufficient to confer binding to *S. aureus* or whether additional bacterial co-factors are required. The isolation of WTA from the bacterial cell wall is challenging; the procedure is labor intensive but moreover, the instability and variation in isolated WTA creates difficulties for assay reproducibility. Therefore, we used our previously developed system (24), where chemically-synthesized WTA backbone fragments of defined length are glycosylated by specific recombinant Tar enzymes in vitro (**Fig. 2A**). With this robust system, we have previously studied the interaction of specific WTA glycoforms and antibodies in a reproducible and low background manner (24). In this study, we used both hexameric and dodecameric RboP backbones to assess the influence of WTA chain length on langerin binding. Differently glycosylated biotinylated WTA structures were coated on streptavidin-coated ELISA plates and incubated with a concentration range of recombinant langerin-FITC. Only wells coated with β-1,4-GlcNAc- and β-1,3-GlcNAc-glycosylated WTA structures mediated concentration-dependent binding to langerin and no binding was observed to the RboP backbone or α-1,4-GlcNAc-glycosylated WTA (**Fig. 2B, C**). In addition, langerin binding was increased when the WTA backbone was extended from 6- to 12-RboP units (**Fig. 2B, C**). Interaction between recombinant langerin-FITC and synthetic WTA was completely abolished in the presence of EGTA (**Fig. 2C**), which scavenges calcium ions required for receptor binding. Langerin binding likely requires more than two β-GlcNAc residues, since we could not detect binding to a fully synthetic WTA molecule consisting of hexameric RboP backbone and β-1,4-GlcNAc coupled to the third and terminal RboP subunit (**Supporting Fig 2A, B**). In contrast, monoclonal antibodies specific for either α-GlcNAc-WTA or β-GlcNAc-WTA were able to bind the fully synthetic WTA structures (**Supporting Fig 2C**). This does not only indicate that fully-synthetic structures were coated correctly to the wells but also underlines the differences in minimal binding requirements to glycosylated WTA between antibodies and langerin. Overall, these data confirm that β-GlcNAc WTA is sufficient to confer interaction with langerin and does not require the presence of D-alanine residues on WTA nor additional bacterial factors.

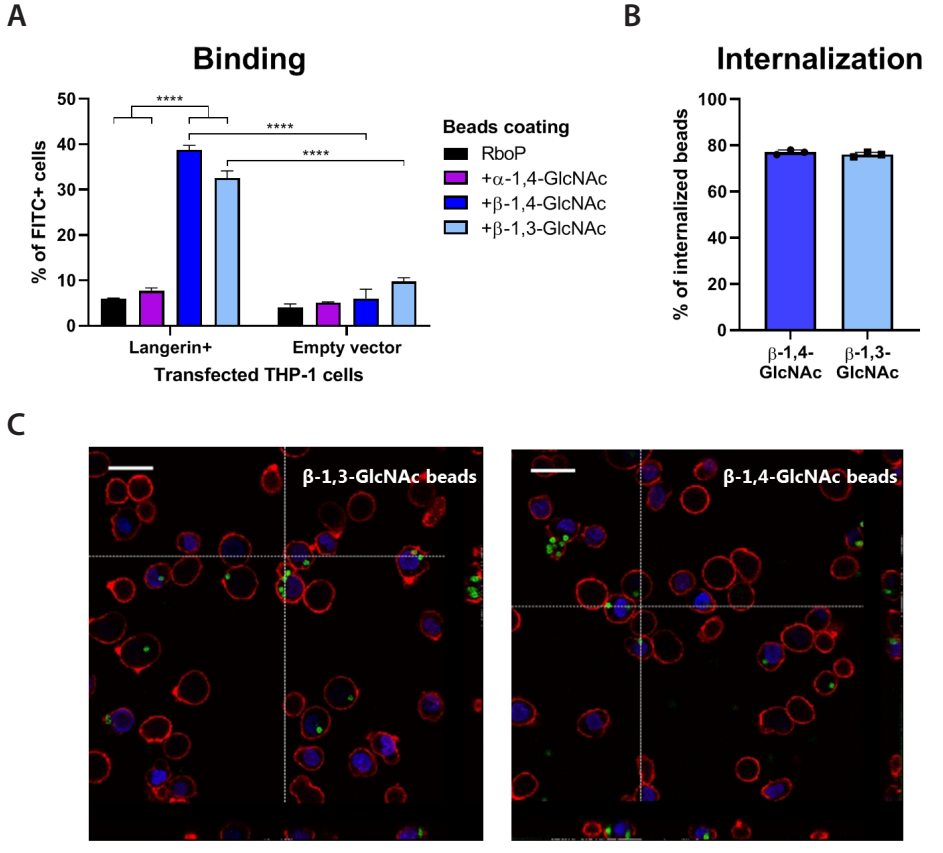


Figure 3. Binding and internalization of β-GlcNAc-WTA-coated beads by langerin-expressing THP-1 cells. A) Binding of FITC-labeled beads, coated with unglycosylated or in vitro glycosylated RboP hexamers, to THP-1 cells transfected with human langerin or empty vector at a bead-to-cell ratio of 1. Adherence is represented by % of FITC+ cells. B) Proportion of adherent β-GlcNAc WTA beads that is internalized by Langerin+ THP-1 cells. C) Confocal microscopy images (40X) of β-GlcNAc WTA beads (FITC-labeled: green) bound to and internalized by Langerin+THP-1 cells (WGA-Alexa 647: red, DAPI: blue). Vertical lines correspond to cross section of z-stack on the right, horizontal lines to cross section below, scale bars correspond to 25 μm. For panels A and B, graphs represent mean + SEM of biological triplicates, ****p < 0.001

We also assessed binding of beads, coated with the differently glycosylated WTA oligomers, to surface-expressed langerin on transfected THP-1 cells. FITC-labeled beads were coated with synthetic glycosylated WTA hexamers, and coating was verified by binding of monoclonal antibodies specific for either α-GlcNAc or β-GlcNAc WTA (**Supporting Fig. 3**). We observed strong binding of β-GlcNAc WTA beads, modified by either TarS or TarP, to langerin-expressing THP-1 cells but not empty vector control cells (**Fig. 3A**). In addition to binding, Langerin+ THP-1 cells internalized the majority of adhered beads as assessed by flow cytometry (**Fig. 3B**) and confocal microscopy (**Fig. 3C**). No apparent differences in receptor binding or cellular uptake were observed for TarS- and TarP-modified WTA beads in this system, suggesting that both modifications confer a similar function with regard to langerin interaction.

The expression of β-GlcNAc WTA contributes significantly to the interaction between *S. aureus* and LCs

We have recently shown that langerin significantly contributes to the interaction between *S. aureus* and primary human LCs (21). In addition, in vitro-generated muLCs were used as a LC cell model to demonstrate the impact of langerin recognition on activation of APCs (21). Here, we again used muLCs to study the binding of surface-expressed langerin to β-GlcNAc WTA modifications mediated by TarS or TarP. In line with the THP-1 binding experiments, muLCs also specifically bound to β-GlcNAc WTA beads, irrespective of linkage to C3 (TarP) or C4 (TarS) (**Fig. 4A**). At a bead-to-cell ratio of 10, beads decorated with β-1,3-GlcNAc WTA adhered significantly better compared to beads decorated with β-1,4-GlcNAc WTA (**Fig. 4A**). This observed binding was mediated by the presence of langerin, as we were able to block the binding of muLCs to β-GlcNAc WTA beads by addition of mannan, a ligand for langerin, or specific langerin-blocking monoclonal antibodies (**Fig. 4B**). These data show that β-GlcNAcylated WTA is sufficient to confer binding to muLCs and does not require bacterial co-factors.

Next, we assessed whether β-GlcNAc WTA was necessary for *S. aureus* binding to muLCs. For these experiments we used the RN4220 Δ*tarMS* background where *tarM*, *tarS* and *tarP* are individually and constitutively expressed from a complementation plasmid. We observed an approximately 3-fold higher binding to muLCs by *S. aureus* strains expressing β-GlcNAc WTA compared to α-GlcNAc-WTA producing *S. aureus* (**Fig. 4C**). However, even in the absence of β-GlcNAc WTA, *S. aureus* was able to adhere to muLCs. Furthermore, binding of β-GlcNAc WTA producing *S. aureus*, but not α-GlcNAc producing *S. aureus*, to muLCs was significantly blocked by addition of mannan (**Fig. 4C**). These results indicate that the interaction between Langerin and β-GlcNAc WTA is an important determinant, although not exclusively required, for *S. aureus* binding to LCs.

To assess the downstream effects of langerin-mediated binding of *S. aureus* to muLCs, and potential differences herein between β-1,4-GlcNAc-WTA versus β-1,3-GlcNAc-WTA producing *S. aureus*, we stimulated muLCs for 24 hours with gamma-irradiated RN4220 Δ*tarMS*, complemented with either plasmid-expressed *tarS*, *tarP* or *tarM*. Surface expression of activation markers CD86 and CD83 increased in a dose-dependent manner in response to all three strains. Expression of CD86 and CD83 was highest in response to *tarP*-complemented *S. aureus* and differed significantly from *tarS*-complemented S. aureus (**Fig. 4D**). The production of IL-8 and TNF-α showed a similar pattern, where all three strains induced a dose-dependent cytokine response with highest cytokine levels in response to *tarP*-complemented *S. aureus* (**Fig. 4E**). In line with previous results, *tarM*-complemented *S. aureus* showed the lowest activation of muLCs, both in surface expression of CD86 and CD83, as well as cytokine production. This data suggests that besides the known effect between α-GlcNAc-WTA and β-GlcNAc-WTA, there could be additional differences in langerin-mediated LC activation between β-1,3-GlcNAc-WTA and β-1,4-GlcNAc-WTA.

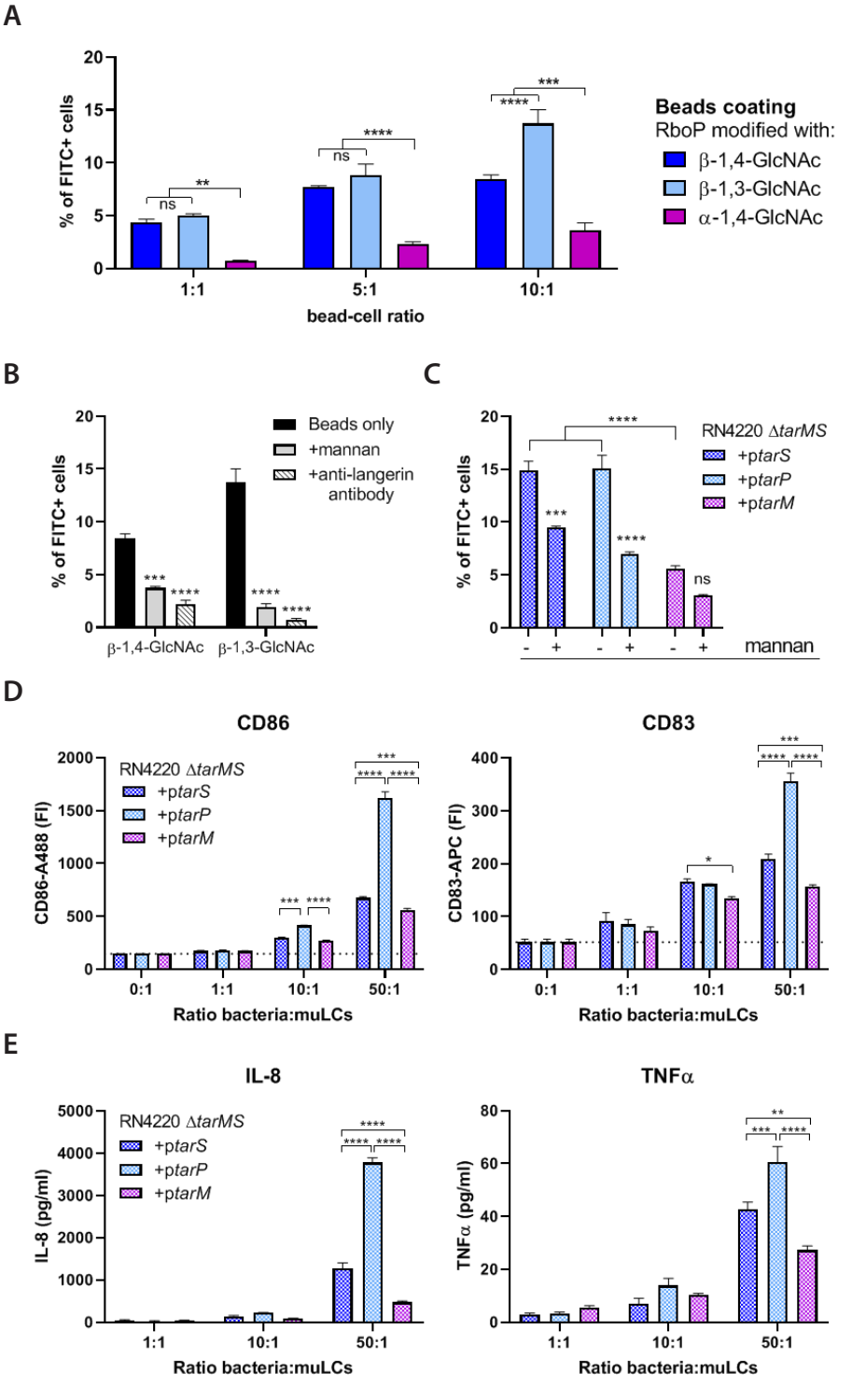

A
% of FITC+ cells
bead-cell ratio
1:1
5:1
10:1
Beads coating
RboP modified with:
β-1,4-GlcNAc
β-1,3-GlcNAc
α-1,4-GlcNAc
B
Beads only
+mannan
+anti-langerin antibody
β-1,4-GlcNAc
β-1,3-GlcNAc
C
RN4220 ΔtarMS
+ptarS
+ptarP
+ptarM
mannan
D
CD86
CD83
CD86-A488 (FI)
CD83-APC (FI)
Ratio bacteria:muLCs
0:1
1:1
10:1
50:1
E
IL-8
TNFα
IL-8 (pg/ml)
TNFα (pg/ml)

Figure 4. *S. aureus* WTA glycoform affects binding to and activation of in vitro-generated LCs. A) Binding of FITC-labeled beads, coated with in vitro glycosylated RboP dodecamers, to muLCs at bead-to-cell ratios of 1, 5, and 10. Bead adherence is displayed as % of FITC+ cells. B) Binding of FITC-labeled beads coated with TarS- or TarP-modified RboP dodecamers to muLCs at a bead-to-cell ratio of 10 in the absence (similar to A) or presence of mannan (20 μg/ml) or anti-langerin blocking antibody (20 μg/ml). C) Binding of FITC-labeled RN4220 Δ*tarMS* complemented with plasmid-expressed *tarS, tarP* or *tarM* to muLCs at a bacteria-to-cell ratio of 1. Bacterial binding is represented by % of FITC+ cells D) Surface expression of activation marker CD86 and maturation marker CD83 by muLCs after 24h stimulation with γ-irradiated RN4220 Δ*tarMS* complemented with plasmid-expressed *tarS, tarP* or *tarM*, at bacteria-to-cell ratios of 1, 10, and 50. E) Concentration of IL-8 and TNFα in the supernatant of muLCs described in D. The data for all panels represent mean + SEM of biological triplicates. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Discussion

LCs are among the first responders upon invasion of *S. aureus* into the skin, contributing to early initiation of pro-inflammatory responses and recruitment of neutrophils. At the molecular level, langerin is an important sensor of specific *S. aureus* cell wall constituents, i.e. β-GlcNAcylated WTA, which can be mediated by the housekeeping glycosyltransferase TarS and the accessory enzyme TarP (21,32). Using a combination of recombinant langerin and langerin-transfected cell lines, genetically-modified *S. aureus* strains and in vitro generated LCs, we demonstrate that the interaction between langerin and *tarP*-expressing *S. aureus* results in similar binding but quantitatively-different immunological responses. Moreover, comparing the binding of beads coated with synthetic glycosylated WTA oligomers and *S. aureus* modified strains emphasized that the interaction between LCs and *S. aureus* is largely, but not solely, dependent on the expression of β-GlcNAc WTA.

Binding of recombinant langerin to *S. aureus* was abrogated in bacteria that lack WTA glycosyltransferases, i.e. N315Δ*tarPS* and RN4220Δ*tarMS* bacteria. However, at higher concentrations, residual langerin binding to these WTA-deglycosylated strains was still observed, suggesting the presence of a second, currently unidentified minor ligand for langerin on the *S. aureus* surface. This observed binding was specific, as the binding was saturable and was inhibited by addition of mannan (**Supporting Fig. 1B**). *S. aureus* expresses a wide variety of surface proteins that contribute to skin colonization and infection (37). Interestingly, some of these proteins, such as the serine-aspartate repeat (SDR) proteins and SraP, are heavily glycosylated (38-40), thereby representing potential targets for langerin in addition to β-GlcNAc WTA.

The toolbox of synthetic WTA fragments allowed us to gain more insights into the binding requirements of langerin to glycosylated WTA. Following current consensus, the WTA backbone consists of up to 40 repeating units of RboP that can be co-decorated with D-alanine and GlcNAc residues (41). The synthetic RboP polymers used here are only modified with GlcNAc and do not contain D-alanine residues. Consequently, we conclude that D-alanylation of WTA is dispensable for langerin binding in our assays,

although we cannot rule out that the interaction would be affected by the presence of D-alanine. Also, when expressed by *S. aureus*, the absence or presence of D-alanine does not seem to impact langerin binding (**Supporting Fig. 1C**). In addition, we observed a strong impact of GlcNAc abundance on langerin binding; doubling the length of the synthetic WTA backbone enhanced langerin binding, which is most likely explained by an increased number of GlcNAc moieties following in vitro glycosylation. Furthermore, we did not observe langerin binding to fully defined WTA structures, which only contained two β-GlcNAc modifications (**Supporting Fig. 2B**). This could be due to a limited sensitivity of our assay. Alternatively, it may indicate that langerin requires more than two β-GlcNAc moieties or differently spaced β-GlcNAc moieties to interact. In contrast, two GlcNAc moieties are sufficient for antibodies to interact with WTA (**Supporting Fig. 2C**). Currently, not much is known about the regulation of WTA biosynthesis and glycosylation, although both the length of the WTA backbone as well as the expression of glycosyltransferases are believed to be affected by environmental cues. In the skin, activation of the Agr regulon results in increased WTA expression on the surface (42). Additionally, TarS-mediated WTA glycosylation increases under infection conditions at the expense of TarM- or TarP-mediated glycosylation, which dominate WTA glycosylation under in vitro growth conditions (32,43,44). Consequently, more β-1,4-GlcNAc moieties are produced in vivo (43), which would greatly enhance receptor avidity of langerin and impact its function (15).

TarP can replace TarS in several key processes, including β-lactam resistance (30,32). However, whether the same applies to immune recognition still remains to be fully clarified. In mice, TarP-modified WTA appeared less immunogenic as compared to TarS-modified WTA (32). Previous work has shown the existence of cross-reactive human antibodies to both β-GlcNAc epitopes, while other antibodies seem to be more exclusively directed towards β-1,4-GlcNAc (24). Until now, no studies have assessed the potential discrimination between *tarS*- and *tarP*-expressing *S. aureus* strains by innate immune cells. From our cell-based assays, β-1,3-GlcNAc-modified WTA has a similar ability to bind langerin compared to β-1,4-GlcNAc-modified WTA. However, LC activation as detected by cytokine production appears to be higher in response to *tarP*- versus *tarS*-expressing *S. aureus* strains. This observed difference in LC activation between TarS- and TarP-modified WTA could only be observed at higher bacteria to cell ratios. This raises the question whether these observations represent physiologically relevant conditions, although the ratios tested here seem possible given the estimated density of LCs in human skin (45). Nonetheless, this finding potentially underlines an important difference in the stimulatory capacity of both modifications, where β-1,3-GlcNAc is more immunostimulatory for innate responses, whereas β-1,4-GlcNAc is dominant for adaptive antibody recognition. One explanation for this could be the difference in glycosylation between both glycosyltransferases. TarP modifies the RboP backbone with GlcNAc moieties at a higher efficiency than TarS, which could subsequently enhance receptor clustering and internalization by LCs. Moreover, glycosylation by TarS or TarP differentially affects D-alanylation of WTA, resulting in overall charge differences (32). As a consequence, TarP-mediated glycosylation might

negatively affect antigen-presentation by APCs as a result of decreased zwitterionic charge properties. As a result, T cell responses and T cell-dependent B cell responses to TarP-modified WTA may be hampered. Furthermore, T cell-independent B cell responses to TarP-modified WTA could be affected as well, via decreased crosslinking of the B cell receptor. However, more research is needed to support this hypothesis, and the synthesis of WTA oligomers with added D-alanine modifications will serve as an excellent tool to study this.

Our results underline the ability of muLCs to detect and internalize *S. aureus* that express β-GlcNAc on their surface. In line with previous work, we observed that langerin recognition increased surface expression of activation markers CD86 and CD83 and enhanced the production of pro-inflammatory cytokines such as IL-8, which would generally serve to recruit neutrophils to the site of infection to promote rapid eradication of invading *S. aureus* (21). An epicutaneous infection of human langerin transgenic mice with *tarS*-expressing *S. aureus* revealed an increased skin inflammatory response compared to wild-type mice, however no significant reduction in bacterial load could be observed (21). Whether and how the interaction between human langerin and WTA would contribute to LC-mediated immunity against *S. aureus* still remains to be elucidated. Besides processes such as antigen uptake and presentation to $CD4^+$ T cells, little is known about direct downstream responses of langerin (18-20,46). Moreover, a lack of robust models, including limited access to human skin explants, differences in langerin ligand specificity (17) and immune cell subsets in commonly used experimental animals (47), represent significant challenges to study immature LC function. The synthetic WTA oligomers used here could represent a robust tool to specifically study downstream effects of langerin receptor binding, and could even be used in combination with appropriate TLR stimulation to unravel LC responses in response to specific langerin-TLR triggers (48).

Overall, langerin senses all β-GlcNAc WTA-producing *S. aureus* strains, which contributes to but is not exclusively required for recognition by LCs. In addition, we suspect the existence of a second langerin ligand on the surface of *S. aureus*. It is currently difficult to dissect the functional consequences of LCs responses in more relevant biological systems. In addition, we also lack knowledge on in vivo expression of WTA glycosyltransferases, the resulting WTA glycoform and the spatial distribution across the bacterial cell wall, which all impact interaction and responses triggered by CLRs such as langerin. Future research will need to elucidate the impact of the *S. aureus* WTA glycoform on the ability of LCs in situ to sense invading *S. aureus* in the skin, a frequent point of entry, and whether this interaction aids in prevention of bacterial dissemination by mounting an effective local response.

Conclusion

Here we show that Langerhans cells (LCs), the main antigen-presenting cells in the skin, sense all *S. aureus* strains that express β-GlcNAc WTA, which is conferred by glycosyltransferases TarS as well as the recently described TarP through the C-type lectin receptor langerin. Langerin binding increased bacterial uptake, LC maturation, and the production of pro-inflammatory cytokines such as neutrophil chemoattractant IL-8. Despite similar interaction with langerin, LC activation is more pronounced in response to β1,3-GlcNAc-expressing versus β1,4-GlcNAc-expressing *S. aureus*, suggesting different activation pathways related to specific glycan linkage. Future studies may be able to unravel this linkage-specific activation using chemically-synthesized WTA oligomers, which we demonstrated to be a valuable novel glycobiology tool to study langerin-WTA binding requirements. Furthermore, these stable WTA oligomers may pave the way for future crystallography studies to further characterize WTA-langerin interaction at the atomic level. In summary, our study provides insight into the relevance of unique *S. aureus* WTA glycoforms for immune interactions in specific human tissues. Future studies will undoubtedly benefit from the chemically-synthesized WTA oligomers used here to further our understanding of *S. aureus* molecular pathogenesis.

Methods

Bacterial strains and culture conditions

All plasmids and strains used in this study are listed in Table S1. Bacteria were grown overnight in five ml Todd-Hewitt broth (THB; Oxoid) at 37°C with agitation. Growth medium was supplemented with 10 μg/ml chloramphenicol (Sigma) for plasmid-complemented *S. aureus* strains. Overnight cultures were subcultured the next day in fresh THB and grown to a mid-exponential growth phase, corresponding to an optical density of 0.6-0.7 at 600 nm (OD600).

Generation of complemented N315 Δ*tarPS* strains

Plasmids containing the shuttle vector RB474 with full-length copies of *tarS* or *tarP* as inserts were isolated from complemented RN4220 Δ*tarMS* strains (49), and transformed into *Escherichia coli* DC10B by heat shock. Competent *S. aureus* N315 Δ*tarPS* cells were transformed with pRB474-*tarS* or pRB474-*tarP* (isolated from *E. coli* DC10B) through electroporation with a Bio-Rad Gene Pulser II (100 ohm, 25 μF, 2.5 kV). After recovery, bacteria were plated on Todd-Hewitt agar supplemented with 10 μg/ml chloramphenicol to select plasmid-complemented colonies. The presence of *tarS* or *tarP* was confirmed by PCR analysis, using the primers for TarP (up) 5'-CTTCACGAAAGAGCACTAGAAG-3' and TarP (dn) 5'-TTCCCGGCAAGTTGGTG-3' and for TarS

(up) 5'- GTGAACATATGAGTAGTGCGTA-3' and TarS (dn) 5'-CATAATGTCCTTCGCCAATCAT-3'. The corresponding WTA glycoform of complemented strains was also verified by bacterial staining with WTA-specific Fab fragments, followed by staining with goat F(ab')2 anti-human kappa-Alexa Fluor 647 (5 μg/ml, Southern Biotech) (Supporting figure 1A).

Bacterial binding to recombinant human langerin

Bacteria were grown to mid-exponential phase as described above and collected by centrifugation (10 minutes, 4,000 rpm). Supernatant was discarded and bacteria were resuspended to an OD600 of 0.4, which corresponds to approximately 108 colony forming units (CFU)/ml in TSM buffer (2.4 g/L Tris (Roche), 8.77 g/L NaCl (Sigma Aldrich), 294 mg/L CaCl2.2H20 (Merck), 294 mg/L MgCl2.6H20 (Merck), pH=7.4) containing 0.1% bovine serum albumin (BSA, Merck). Next, bacteria were incubated at 37°C for 30 minutes with FITC-labeled human langerin-extracellular domain (ECD) constructs, referred to as human langerin-FITC, as previously described (21,35). Bacteria were washed once with TSM 0.1% BSA, fixed in 1% formaldehyde in PBS and analyzed by flow cytometry on a FACSverse (BD Biosciences). Per sample, 10,000 gated events were collected and data was analyzed using FlowJo 10 (FlowJo, LLC).

Recombinant expression of monoclonal antibodies and Fab fragments

For monoclonal antibody expression, we cloned the human IgG1 heavy chain (hG) and kappa light chain (hK) constant regions (sequences as present in pFUSE-CHIg-hG1 and pFUSE2-CLIg-hk; Invivogen) in the XbaI-AgeI cloning site of the pcDNA34 vector (Thermo Fisher). VH and VL sequences from monoclonal antibodies specific for α-GlcNAc-WTA (4461), β-GlcNAc-WTA (4497) and β-1,4-GlcNAc-WTA (6292) were derived from patent WO 2014/193722 A1 (50). As the VL of anti-WTA antibody 6292 resulted in precipitation problems, it was adapted towards a Vκ3, leaving the CDR regions (in bold) intact (VL(6292-Vκ3: EIVLTQSPATLSLSPGERATLSCR**ASQGIRNGLG**-WYQQKPGQAPRLLIY**PASTLES**GVPARFSGSGSGTDFTLTISSLEPEDFAVYY-C**LQDHNYPP**TFGQGTKVEIK). The VH and VL sequences, preceded by a Kozak sequence (ACCACC) and the HAVT20 signal peptide (MACPGFLWALVISTCLEFS-MA) were codon optimized for human expression and ordered as gBlocks (17). We cloned VH and VL gBlocks into the pcDNA34 vector, upstream of the IgG1 heavy chain (hG) and kappa light chain (hK) constant regions, respectively, by Gibson assembly (New England Biolabs) according to the manufacturer's instructions. NheI and BsiWI were used as the 3' cloning sites for VH and VL, respectively, in order to preserve the immunoglobulin heavy and kappa light chain amino acid sequence. The constructs were transformed in E. coli TOP10F' by heat shock and clones were verified by PCR and Sanger sequencing (Macrogen). Plasmids were isolated by NucleoBond Xtra Midi kit (Macherey-Nagel) and sterilized using 0.22 μm Spin-X centrifuge columns (Corning). We used EXPI293F cells (Thermo Fisher), grown in EXPI293 Expression medium (Thermo Fisher) at 37°C, 8%

CO2 in culture filter cap conical flasks (Sigma) on a rotation platform (125 rotations/min) for protein production. One day before transfection, cells were diluted to 2x106 cells/ml and 100 mL cell culture was used for transfection the next day. In 10 mL Opti-MEM (Thermo Fisher), 500 µl PEI-max (1 µg/µl; Polysciences) was mixed with DNA (1 µg/ml cells) in a 3:2 ratio of hK and hG vectors. After 20 minutes incubation at room temperature, this DNA/PEI mixture was added dropwise to 100 ml EXPI293F cells. After five days, we verified IgG expression by SDS-PAGE and harvested cell supernatant by centrifugation and subsequent filtration through a 0.45 µM filter. IgG was purified using a HiTrap Protein A column (GE Healthcare) and Äkta Pure (GE Healthcare). Protein was eluted in 0.1 M citric acid, pH 3.0, and neutralized with 1 M Tris, pH 9.0. The IgG fraction was dialyzed overnight against PBS at 4°C. Purified monoclonal antibodies were stored at -20°C. Fab fragments specific for α-GlcNAc-WTA (4461), β-GlcNAc-WTA (4497) and β-1,4-GlcNAc-WTA (6292) were cloned and expressed similar as the full-length monoclonal antibodies, except that the Fab heavy chain ends with 211VEPKSC216. A flexible linker (GGGGS), an LPETG and a 6xHIS tag was added at the C-terminus of each Fab. EXPI293F expression supernatant was dialyzed against 50 mM Tris, 500 mM NaCl; pH8.0, before Fab purification on a HISTrap FF column (GE Healthcare). Fab fragments were dialyzed against 50 mM Tris, 300 mM NaCl; pH8.0 and stored at -20°C.

Production of biotinylated ribitolphosphate (RboP) hexamer (6-) and dodeca (12-) mer

Biotinylated RboP hexamers were synthesized as described previously (24,32). The synthesis of biotinylated RboP dodecamers and chemically-defined glycosylated RboP hexamers will be described in detail elsewhere (S. Ali et al, paper in preparation).

Enzymatic glycosylation of RboP oligomers

Recombinant TarP protein and transformed E. coli TOP10F' strains with pBAD-tarM or pBAD-tarS were kindly provided by Prof. Thilo Stehle (University of Tübingen, Germany) (32,51). Biotinylated RboP oligomers (0.17 mM) were incubated with recombinant glycosyltransferases TarS, TarP or TarM (6.3 µg/ml) for 2 hours at room temperature with UDP-GlcNAc (2 mM, Merck) in glycosylation buffer (15 mM HEPES, 20 mM NaCl, 1 mM EGTA, 0.02% Tween 20, 10 mM MgCl2, 0.1% BSA, pH=7.4). Glycosylated RboP hexamers were coupled to beads by adding 5x107 Dynabeads M280 Streptavidin (Thermo Fisher Scientific) to the individual glycosylation reaction mixtures. After incubation for 15 minutes at room temperature, the coated beads were washed three times with PBS 0.1% BSA 0.05% Tween-20 using a magnetic sample rack and stored at 4°C.

Recombinant Langerin binding to synthetic WTA

Maxisorb plates (Nunc) were coated with 10 µg/ml his-tetrameric-streptavidin-LPETG overnight at 4°C, which was expressed and isolated from a pColdl-Stav-LPETG vector kindly provided by Tsutomu Tanaka (Kobo University, Japan). The plates were washed

three times with TSM 0.05% Tween-20 (TSMT), and subsequently blocked with TSM 1% BSA for 1 hour at 37°C. After three washing steps with TSMT, a 50-fold dilution of the glycosylation mixture described above (corresponding to 0.5 nM RboP 6-mer or 12-mer) was added to the plates and incubated for 1 hour at 37°C. Next, the plates were washed with TSMT, and further incubated with a concentration range of recombinant human langerin-FITC for 30 minutes at 37°C. For blocking experiments, mannan (20 μg/ml) or EGTA (10 mM) were added immediately prior to addition of recombinant human langerin-FITC. Finally, after three washing steps, the plates were analyzed for langerin binding using a Clariostar plate reader (BMG Labtech; excitation 495 nm, emission 535 nm, gain 2,000).

Cell culture and muLC differentiation

MUTZ-3 cells (ACC-295, DSMZ) were provided by Prof. T. de Gruijl (Amsterdam UMC, The Netherlands). Cells were maintained at a cell density of 0.5 - 1x106 cells/ml in 12-well tissue culture plates (Corning) in MEM-alpha (Gibco) with 20% FBS (Hyclone), 1% glutaMAX (Gibco), 10% spent medium from the renal carcinoma cell line 5637 (ACC-35, DSMZ) and 100 U/ml penicillin-streptomycin (Gibco). Cells were routinely cultured at 37°C with 5% CO2. Differentiation of MUTZ-3 cells into MUTZ-3-derived LCs (muLCs) was performed according to described protocols (52,53). In short, MUTZ-3 cells were differentiated in the presence of 100 ng/ml GM-CSF (Genway Biotech), 10 ng/ml TGF-β (R&D systems) and 2.5 ng/ml TNF-α (R&D systems) for 11 days. Twice a week, half of the medium was replaced with fresh medium and double concentration of cytokines. To verify the differentiated muLC phenotype, cells were analyzed by flow cytometry for expression of CD207 (clone DCGM4, Beckman Coulter) and CD1a (clone Hl149, BD Biosciences) as well as the absence of CD34 (clone 581, BD Biosciences). THP-1 cells, transfected with a lentiviral human langerin construct or empty vector, were cultured in RPMI-1640 (Lonza) supplemented with 10% heat-inactivated FBS and 100 U/ml penicillin-streptomycin (Gibco) as described in (21).

Binding and internalization of WTA beads or *S. aureus* by Langerin-expressing cells

Dynabeads-M280 Streptavidin (Thermo Fisher Scientific) and mid-exponential *S. aureus* (OD600= 0.6-0.7) were labeled with 0.5 mg/ml FITC (Sigma) in PBS for 30 minutes at 4°C. After extensive washing and coating of the beads with glycosylated RboP hexamers as described above, beads and bacteria were resuspended in RPMI 0.1% BSA at a concentration of $5x10^7$ beads/ml or $1x10^8$ CFU/ml (OD600= 0.4), respectively. Bacteria were stored at -20°C and beads at 4°C in the dark. For binding experiments, $1x10^5$ cells (THP-1 cells or muLCs) were incubated with FITC-labeled WTA beads or FITC-labeled S. aureus at different ratios in RPMI 0.1% BSA for 30 minutes at 4°C. Cells were washed (300 x g for 10 minutes at 4°C), fixed in PBS 1% formaldehyde and analyzed by flow cytometry as described above. To quantify internalization of β-GlcNAc WTA beads by THP-1 cells, we incubated WTA beads with $2x10^5$ cells in RPMI 0.1% BSA at a bead-to-cell ratio of 1

for 30 minutes at 4°C. Cells were washed twice to remove unbound beads, and the sample was divided over two separate tubes. Both samples were incubated for an additional 30 minutes, one at 4°C and the other at 37°C with 5% CO2 to allow phagocytosis. Cells were washed, and Fc-receptors were blocked with recombinant FLIPR-like (6 μg/ml) for 15 minutes at 4°C (54). Next, monoclonal antibodies specific for β-GlcNAc or α-GlcNAc WTA (4497/4461-IgG1, respectively) were added to all samples at 3 μg/ml for 20 minutes at 4°C, followed by goat anti-human kappa-Alexa Fluor 647 (5 μg/ml, Southern biotech) for another 20 minutes at 4°C to allow discrimination between cell adherent (FITC+/ Alexa fluor 647+) and internalized beads (FITC+/Alexa fluor 647-). Finally, cells were washed and fixed in 1% formaldehyde in PBS. The internalized fraction was calculated from the loss of Alexa Fluor 647 signal of FITC+ cells by flow cytometry, as previously described (36).

To confirm bead internalization by confocal microscopy, cells were stained with WGA-Alexa Fluor 647 (Thermo Fisher Scientific) and DAPI (Sigma) following incubation for 30 minutes at 37°C with FITC-labeled WTA beads and coated on 8 well chamber slides glass slides (Ibidi) before analysis by confocal laser scanning microscopy (SP5, Leica).

muLC stimulation

Gamma-irradiation of *S. aureus* and stimulation of muLCs was performed as previously described (21). Briefly, *S. aureus* strains were grown to exponential phase, washed with PBS, concentrated 10-fold in PBS with 17% glycerol, and stored at -80°C. Gamma-irradiation of bacteria was performed at Synergy Health Ede B.V., a STERIS company (Ede, The Netherlands). The loss of viability was confirmed by plating, and the bacterial concentrations were calculated using the MACSQuant Analyzer 10.

$1x10^5$ muLCs were stimulated with γ-irradiated RN4220 Δ*tarMS*+p*tarS*, RN4220 Δ*tarMS*+p*tarP* or RN4220 Δ*tarMS*+p*tarM* at bacteria to cell ratios of 0, 1, 10, and 50 for 24 hours at 37°C with 5% CO2 in IMDM containing 10% FBS. Supernatants for cytokine analysis were collected after centrifugation (300 x g, 10 min at 4°C), and stored at -80°C until further analysis. Cells were washed with PBS 0,1% BSA, stained with CD83 (clone HB15e) and CD86 (clone IT2.2, Sony Biotechnology), fixed and analyzed by flow cytometry. Cytokine production was analyzed by ELISA, for IL-8 (Sanquin) and TNFα (Thermo Fisher) following manufacturer's instructions.

Statistical analysis

Flow cytometry data was analyzed using FlowJo 10 (FlowJo, LLC). All data was analyzed using GraphPad Prism 8.3 (GraphPad Software), with a Two-way ANOVA followed by a Dunnett's multiple comparison test except for bacterial binding to langerin-FITC at one fixed concentration for which one-way ANOVA was performed with Dunnett's multiple comparison test. P values are depicted in the figures and $p < 0.05$ was considered significant.

Data availability

Upon request, the data supporting these findings are available from the corresponding author.

Acknowledgements

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Conflict of interest

A.H. 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 Utrecht University and participated in a post graduate studentship program at GSK.

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Supporting information

Table S1. Bacterial strains used in this study.

Strain (sequence type, clonal complex)	Source
N315 WT (ST5, CC5)	NARSA strain collection
N315 Δ*tarS*	(1)
N315 Δ*tarP*	(1)
N315 Δ*tarS*Δ*tarP*	(1)
N315 Δ*tarS*Δ*tarP*+pRB474-*tarS*	This study
N315 Δ*tarS*Δ*tarP*+pRB474-*tarP*	This study
RN4220 WT (ST8, CC8)	(2)
RN4220 Δ*dltA*	(3)
RN4220 Δ*tarM*Δ*tarS*	(4)
RN4220 Δ*tarM*Δ*tarS*+pRB474-*tarS*	(4)
RN4220 Δ*tarM*Δ*tarS*+pRB474-*tarP*	(1)
RN4220 Δ*tarM*Δ*tarS*+pRB474-*tarM*	(4)

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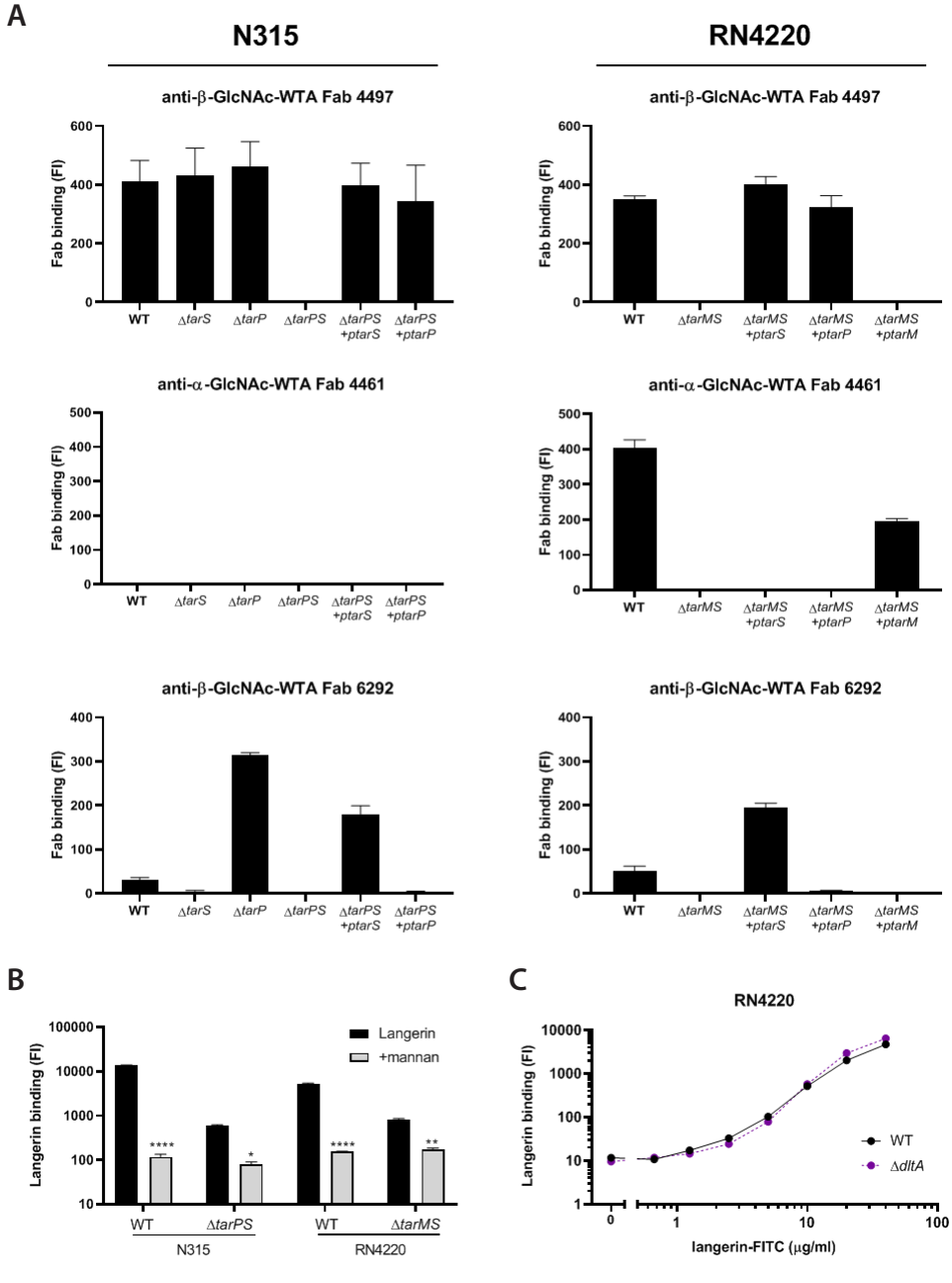
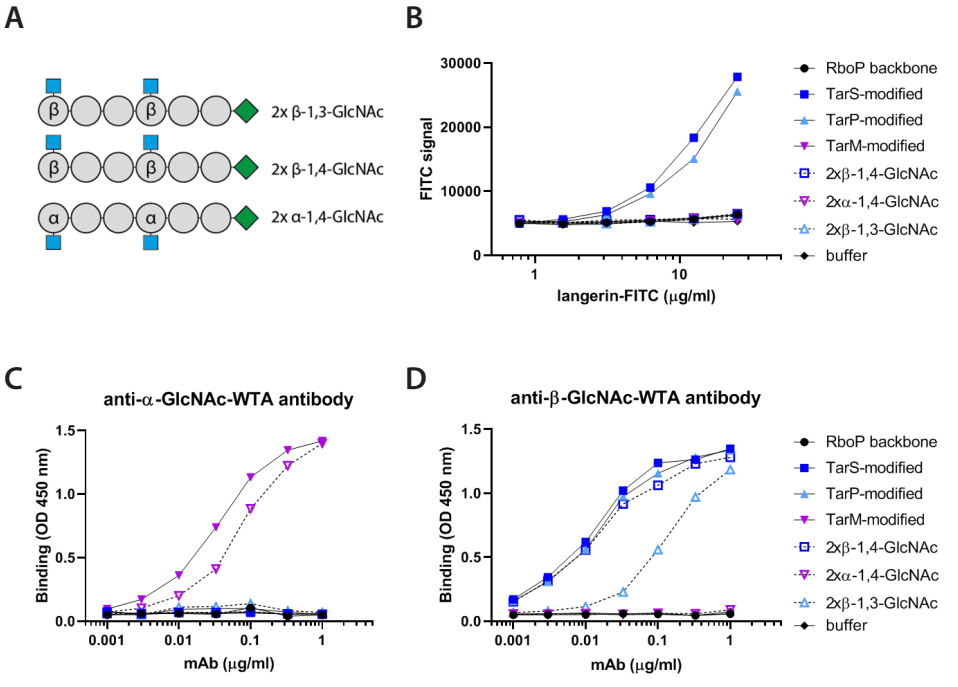
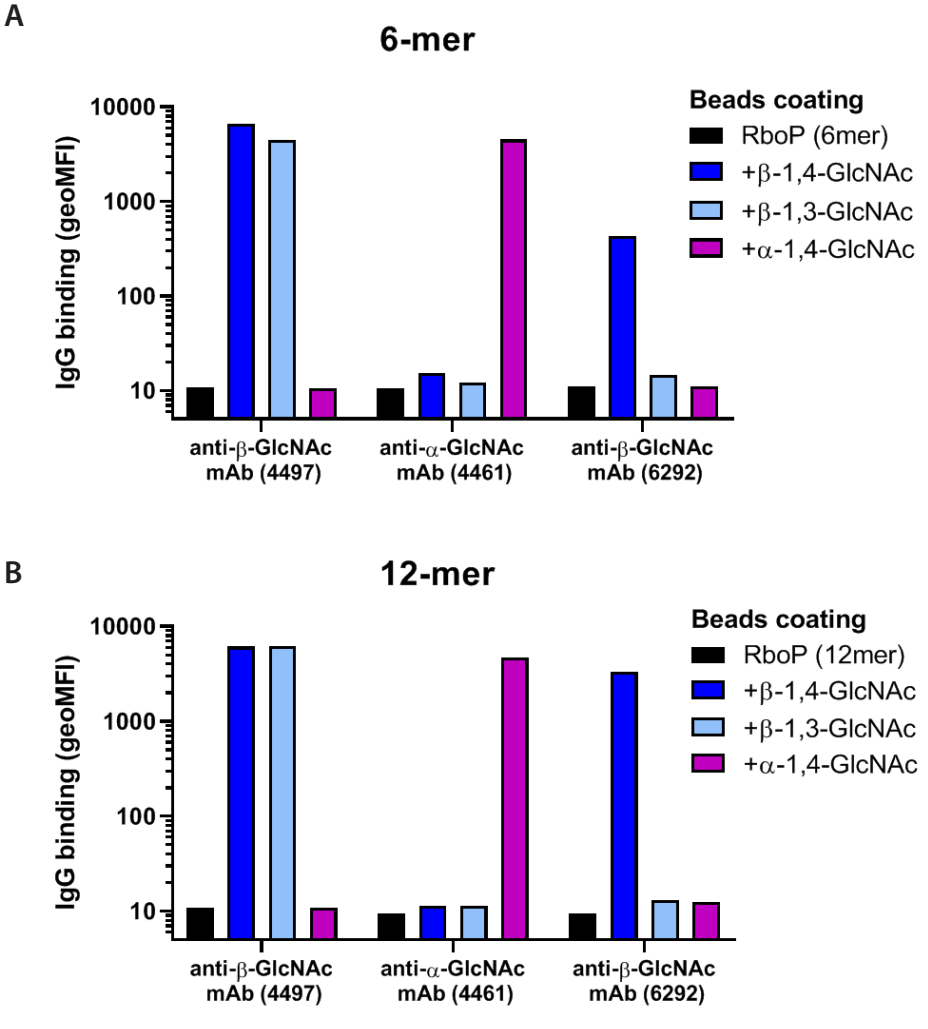


Supporting Figure 1. A) Binding of Fab specific for α-GlcNAc-WTA (4461), β-GlcNAc-WTA (4497) and β-1,4-GlcNAc-WTA (6292) to N315 (left) and RN4220 (right) mutant panels. B) Binding of recombinant langerin-FITC (40 μg/ml) to N315 WT, N315 Δ*tarS*Δ*tarP*, RN4220 WT and RN4220 Δ*tarM*Δ*tarS* in the presence or absence of mannan (20 μg/ml). C) Binding of recombinant langerin-FITC (0.6-40 μg/ml) to RN4220 wt and Δ*dltA*. Data is depicted as geometric mean fluorescence intensity (MFI) + standard error of mean (SEM) of biological triplicates. *p < 0.05, **p < 0.01, ****p < 0.0001



Supporting figure 2. A) Schematic overview of fully synthetic WTA oligomers. Grey circles indicate RboP subunit, blue square represents GlcNAc, green diamond indicates biotin tag, α and β indicate the type of linkage of GlcNAc to RboP subunit. B) Binding of recombinant langerin-FITC (0.8-25 μg/ml) to in vitro-glycosylated RboP hexamers, fully synthetic WTA oligomers and RboP backbone. C) Binding of monoclonal antibodies (0.001-1 μg/ml) specific for α-GlcNAc-WTA (4461) and β-GlcNAc-WTA (4497) to the same panel of WTA oligomers as in B, followed by anti-human IgG-HRP staining. Data for panel B is shown as fluorescence signal, for C the absorbance at 450nm.



Supporting figure 3. Binding of monoclonal antibodies (3 μg/ml) specific for α-GlcNAc-WTA (4461), β-GlcNAc-WTA (4497) and β-1,4-GlcNAc-WTA (6292) to beads coated with (A) RboP hexamers and (B) RboP dodecamers, in vitro-glycosylated by TarS, TarP or TarM. Unglycosylated RboP fragments are included as controls, binding is shown as geometric mean fluorescence intensity, after staining with goat anti-human kappa-Alexa Fluor 647.

3

"There is only one Michael Jordan"

MICHAEL JORDAN

Chapter 3

Fully synthetic *Staphylococcus aureus* wall teichoic acid fragments expose differences in fine specificities of monoclonal antibodies

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Abstract

Wall teichoic acid (WTA) is an important cell wall component of the opportunistic Gram-positive bacterium *Staphylococcus aureus*. Most commonly, WTA is composed of repeating ribitol phosphate (RboP) residues decorated with D-alanine and N-acetyl-D-glucosamine (GlcNAc) modifications, in a seemingly random manner. WTA GlcNAc modifications shape the host immune interaction with *S. aureus*, including recognition by antibodies. Indeed, WTA represents a promising target for antibody-based immune therapies and possibly vaccination. Currently, there is limited structural information on WTA-antibody interaction, which is mainly due to structural heterogeneity of endogenous WTA, precluding the isolation of pure and well-defined WTA molecules from bacterial sources. Here, we used a library of chemically-defined WTA fragments with specific incorporated GlcNAc modifications (α-GlcNAc at RboP C4; β-GlcNAc at RboP C4; β-GlcNAc at RboP C3) to study fine specificities of WTA-specific monoclonal antibodies (mAbs). In addition to the known impact of GlcNAc configuration and linkage to individual RboP subunits, we observe that GlcNAc positioning along the poly-RboP chain and/or co-presence of non-binding GlcNAc residues impacts mAb binding and subsequent complement deposition. In conclusion, the application of fully synthetic WTA oligomers represents a powerful glycobiology tool to perform structure-function studies with mAbs in the pursuit of WTA-directed therapies.

Introduction

Staphylococcus aureus is a Gram-positive bacterium that asymptomatically colonizes the nares and skin of healthy individuals, while also being the leading cause of hospital-acquired infections with associated high mortality and morbidity. Due to the rise in antibiotic-resistant *S. aureus* strains (e.g. methicillin-resistant *S. aureus*, MRSA), there is an increasing need and interest for alternative treatments, including antibody-based approaches (1–4). Wall teichoic acids (WTAs), abundant cell wall glycopolymers, form a promising target for immune-directed therapies (3,5–7). WTAs are covalently linked to the peptidoglycan layer of the bacterial cell wall, and are involved in numerous vital processes including host colonization, anti-microbial resistance and overall virulence (8–11). Antibody binding to WTA facilitates bacterial killing via antibody- and complement-mediated opsonophagocytosis by neutrophils, which are key effector cells for *S. aureus* eradication (12–15).

WTAs commonly consist of poly-ribitolphosphate (RboP) chains with additional modifications on individual RboP subunits, i.e. D-alanine residues on C2, α/β-linked N-acetyl-D-glucosamine (α/β-GlcNAc) on C4 or a β-GlcNAc on C3 (**Figure 1A**). Glycosylation of RboP with GlcNAc is mediated by three dedicated glycosyltransferases, TarS (β-1,4-GlcNAc), TarM (α-1,4-GlcNAc) and TarP (β-1,3-GlcNAc) (9,16,17). *TarS* is genetically encoded in nearly all *S. aureus* isolates and is present either alone (~50%) or in combination with *tarM* (~35%) or *tarP* (~5-10%) (18). The WTA glycosylation pattern of *S. aureus* is thus dependent on the strain-specific genetic presence and expression of these three glycosyltransferases, which likely varies during course of infection due to environment-driven regulation, resulting in the expression of different WTA glycoprofiles (19,20).

Antibodies raised against *S. aureus* WTA during natural exposure or infection appear to be specific for α- or β-linked GlcNAc moieties, but discrimination between β-1,3- and β-1,4-GlcNAc is not always apparent (13,14,21,22). The majority of WTA-specific IgG antibodies belong to the IgG2 subclass, but high affinity IgG1 antibodies also exist likely as a result of T cell-dependent B cell activation due to the zwitterionic properties of WTA (12,23). Although based on limited studies, different WTA-specific monoclonal antibodies (mAbs) are believed to bind β-1,4-GlcNAc WTA in more or less the same way through conserved binding principles that include the germline-encoded recognition of pyranose saccharides and involvement of the phosphate in the poly-RboP chain (3,22). There is currently limited structural information on WTA-antibody interaction due to the complexity and heterogeneity of endogenous WTAs. However, structure-activity studies would allow for rational design and optimization of WTAdirected mAbs for therapeutic applications. We have previously shown that semi-synthetic WTA oligomers represent a robust tool to study host antibody and lectin responses to *S. aureus* WTA (18,21,24). However, for true structure-activity studies, the use of fully-defined WTA fragments is required.

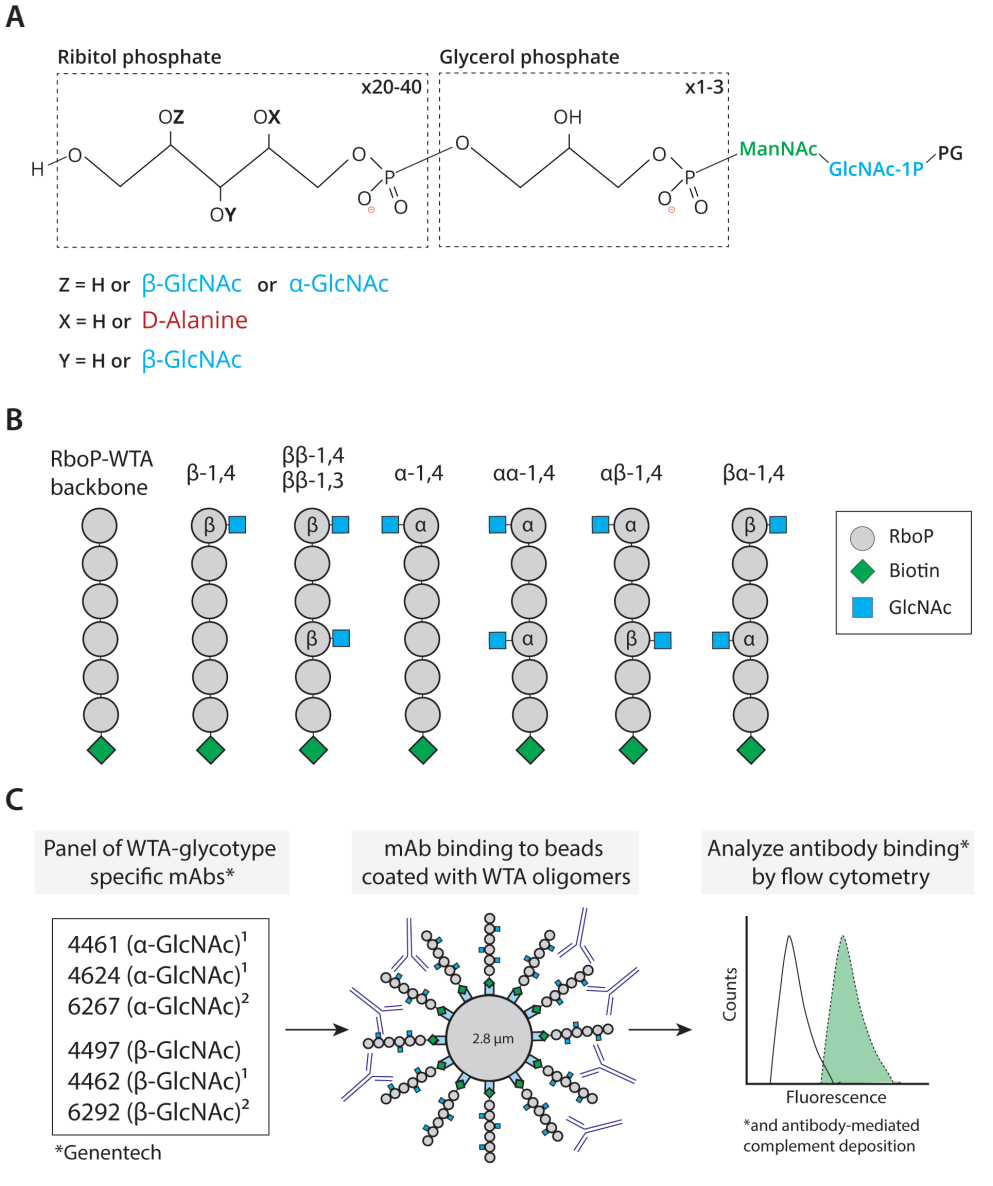

Figure 1. Structural composition of *S. aureus* WTA and fully synthetic WTA oligomers to analyze mAbs binding characteristics. A) Schematic structural representation of *S. aureus* WTA, linked to peptidoglycan (PG). B) Schematic representation of fully synthetic biotinylated RboP-WTA oligomers in the library. WTA oligomers consist of RboP backbone hexamer with anomeric and regioisomeric GlcNAc positioning on terminal RboP, or in combination with GlcNAc placement on internal RboP. C) Experimental set-up to measure mAb binding and complement deposition using synthetic WTA-coated beads by flow cytometry. Clones of recombinant mAbs used in this study are specific for α-GlcNAc- or β-GlcNAc-WTA and are derived from WO patent WO 2014/193722 A1 (6). Numbers indicate whether mAbs were derived from the same donor.

In this study, we used a small library of fully synthetic WTA oligomers (Figure 1B), with a defined number and configurations of GlcNAc moieties to perform in-depth structure-binding and structure-activity analyses. We applied this for a panel of recombinant WTA-specific mAbs to pinpoint differences in fine specificities, including cross-reactivity, and how these impact the ability to activate downstream functions (**Figure 1B**, C; 8, 12, 13).

Results

Impact of GlcNAc density and positioning on mAb binding

To investigate the minimal binding characteristics of WTA-specific mAbs, we recombinantly produced a panel of six mAbs, the sequences of which were derived from patients with invasive *S. aureus* infection. Three mAb clones are specific for α-GlcNAc WTA (clones 4461, 4624 and 6267) and three for β-GlcNAc WTA (clones 4497, 4462 and 6292)(3,6,21). Clone 6292 precipitated during the production process. Therefore, we altered the light chain to a kappa 3 variant (**Supplementary Figure 1A**), which strongly enhanced the overall yield (24), but did not affect the binding characteristics to β-GlcNAc WTA beads or β-GlcNAc WTA-producing *S. aureus* compared to the original clone (**Supplementary figure 1B, C**).

From previous studies, it is known that a single GlcNAc moiety on *S. aureus* WTA represents the minimal binding epitope for clone 4497 (22). However, it remains unclear whether this applies to other mAb clones as well. WTA fragments with one (to terminal RboP subunit) or two anomeric GlcNAc modifications (RboP subunits 3 and 6) were used to study the impact of GlcNAc abundance on mAb binding. Four out-of-six mAbs showed maximum binding to WTA hexamers containing a single GlcNAc moiety, except for clone 4461 and 4462, which exhibited significantly increased binding to WTA oligomers with two GlcNAc moieties (**Figure 2**). For clones 4461, 4497 and 6292-Vk3, full IgG and single Fab fragments behaved similarly in binding assays (**Supplementary figure 2A**). Overall, similar to previous data for clone 4497, these data suggest that a single GlcNAc moiety represents the minimal binding epitope for the majority of mAb clones analyzed.

In addition to GlcNAc density, we also assessed whether the position of the interacting GlcNAc affected antibody binding to its target. To this end, we analyzed the binding of mAbs to WTA oligomers that contained both anomeric GlcNAc modifications (α- and β-GlcNAc), with the interacting GlcNAc placed either internally (third RboP) or terminally (sixth RboP) on the poly-RboP chain (**Figure 1B**). Clone 4461 showed enhanced binding when α-GlcNAc was positioned internally as opposed to being positioned terminally (**Figure 3A**). In contrast, clone 6267 bound significantly better to fragments with a terminal GlcNAc (**Figure 3A**), whereas binding of clone 4624 was not affected by the position of the GlcNAc on the RboP hexamer (**Figure 3A**). For the β-GlcNAc-specific antibodies, clone 4497 did not discriminate based on GlcNAc

positioning, while clone 4462 showed slightly preferential binding to internal β-GlcNAc, and clone 6292-Vk3 only showed binding when β-GlcNAc was present at the terminal RboP subunit (**Figure 3B**). For clones that show reduced binding to internally-positioned GlcNAc, an alternative explanation may be related to binding interference by the presence of a terminal non-interacting GlcNAc modification. Again, Fab fragments of clones 4461, 4497 and 6292-Vk3 exhibited similar binding characteristics compared to full IgG, and the presence of non-binding GlcNAc did not appear to affect Fab binding to specific GlcNAc modifications (**Supplementary figure 2B**).

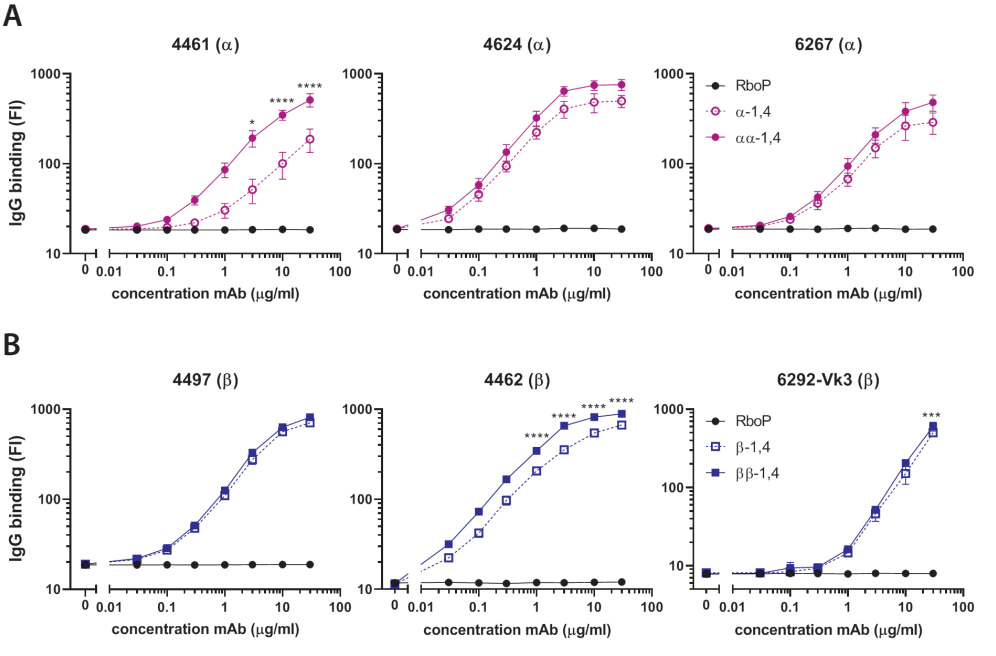

Figure 2. Impact of WTA GlcNAc abundance on mAb binding. Binding of (A) α-GlcNAc WTA-specific mAbs (clones 4461, 4624, 6267) and (B) β-GlcNAc WTA-specific mAbs (clones 4497, 4462, 6292-Vk3) to beads coated with RboP-WTA backbone, or RboP-WTA with one or two GlcNAc moieties in (A) alpha or (B) beta configuration, using a concentration range of mAbs (0.03-30 μg/ml). Data is shown as geometric mean fluorescence intensity (FI) + standard error of mean (SEM) of biological triplicates. *p < 0.05, ***p < 0.001, ****p < 0.0001.

Antibody-induced complement activation further enhances mAb binding differences based on GlcNAc positioning

WTA-specific antibodies potently activate the complement system, thereby inducing complement-mediated opsonophagocytosis and liberating C5a for further neutrophil recruitment (12–14). We wondered whether the discriminatory aspect in antibody binding to variable GlcNAc positioning on WTA would affect downstream immune functions, e.g. complement C3 deposition. For both α-GlcNAc-WTA and β-GlcNAc-WTA specific mAbs, antibody binding correlated with the capacity to activate complement (**Figure 4A, B**). For example, clones 4461 and 6267 showed lower C3 deposition for fragments that they showed reduced binding to, whereas the non-discriminatory clone 4624 activated complement equally when binding to either structure (**Figure 4A**). Only for clone 6292-Vk3, very limited C3 deposition on beads was observed for either structure despite detectable binding to the fragment with external β-GlcNAc (**Figure 4B versus 3B**). Overall, the observed differences in binding to terminal or internally placed GlcNAc are further amplified when analyzing antibody-mediated complement C3 deposition (**Figure 4B**). These data show that variable GlcNAc positioning on the WTA backbone can greatly affect antibody binding affinity and subsequent activation of downstream processes such as complement deposition.

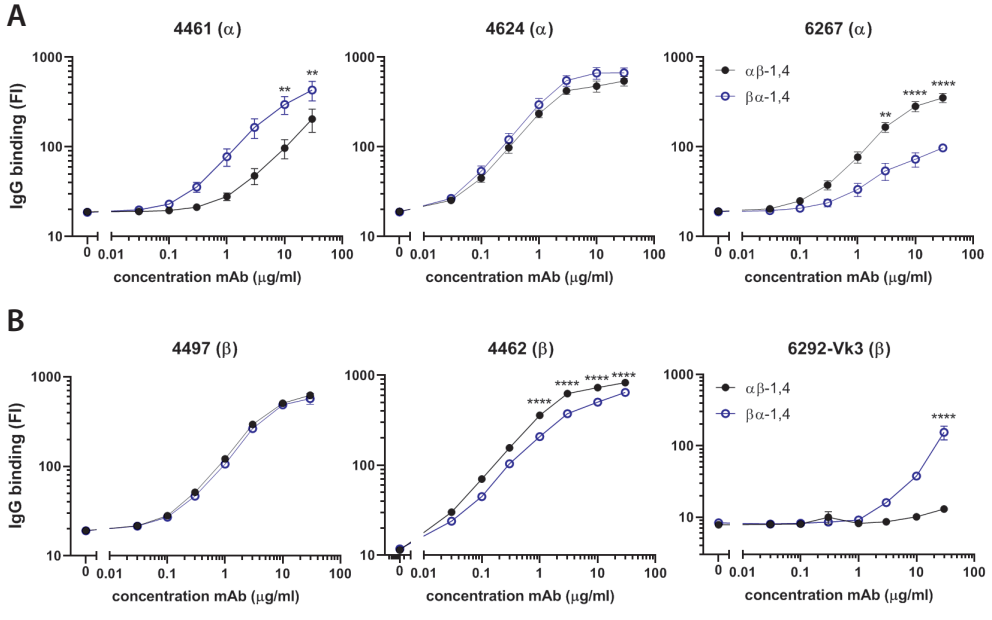

Figure 3 .The impact of WTA GlcNAc positioning and presence of non-binding GlcNAc moiety on mAb binding. Binding of A) α-GlcNAc WTA-specific mAbs (clones 4461, 4624, 6267) or B) β-GlcNAc WTA-specific mAbs (clones 4497, 4462, 6292-Vk3) to beads coated with RboP-WTA with a terminal α-GlcNAc and internal β-GlcNAc (αβ-1,4) or terminal β-GlcNAc and internal α-GlcNAc (βα-1,4) using a concentration range of mAbs (0.03-30 μg/ml). Data is depicted as geometric mean fluorescence intensity (FI) + standard error of mean (SEM) of biological triplicates. **p < 0.01, ***p < 0.001, ****p < 0.0001.

Cross-reactivity of mAbs for β-1,4-GlcNAc and β-1,3-GlcNAc-WTA

S. aureus shows strain-specific expression and regulation of WTA glycosyltransferases *tarS*, *tarP* and *tarM*, resulting in different WTA glycoprofiles. In plasma from most healthy donors, antibodies against TarS-modified WTA are dominant (13,21). However, a small proportion of antibodies that bind the TarS-mediated β-1,4-GlcNAc epitope are able to cross-react with the TarP-mediated β-1,3-GlcNAc epitope (21). Moreover, clones 4462 and 4497 bind both TarS and TarP-modified synthetic WTA fragments equally, while clone 6292 partially discriminates between these two modifications (21). To further investigate the cross-reactive potential of these three β-GlcNAc-specific mAbs, we analyzed their binding to RboP-WTA hexamers containing either two β-1,4-GlcNAc (ββ-1,4-GlcNAc) or two β-1,3-GlcNAc moieties (ββ-1,3-GlcNAc), coated to beads. Interestingly, clone 4462 appeared to have a higher cross-reactive potential compared to clone 4497, as 4497 showed significantly increased binding to ββ-1,4-GlcNAc compared to ββ-1,3-GlcNAc, where clone 4462 bound both structures equally well (**Figure 5A**). Similar to previous findings, clone 6292-Vk3 only bound specifically to ββ-1,4-GlcNAc at the tested antibody concentrations (**Fig. 5A**).

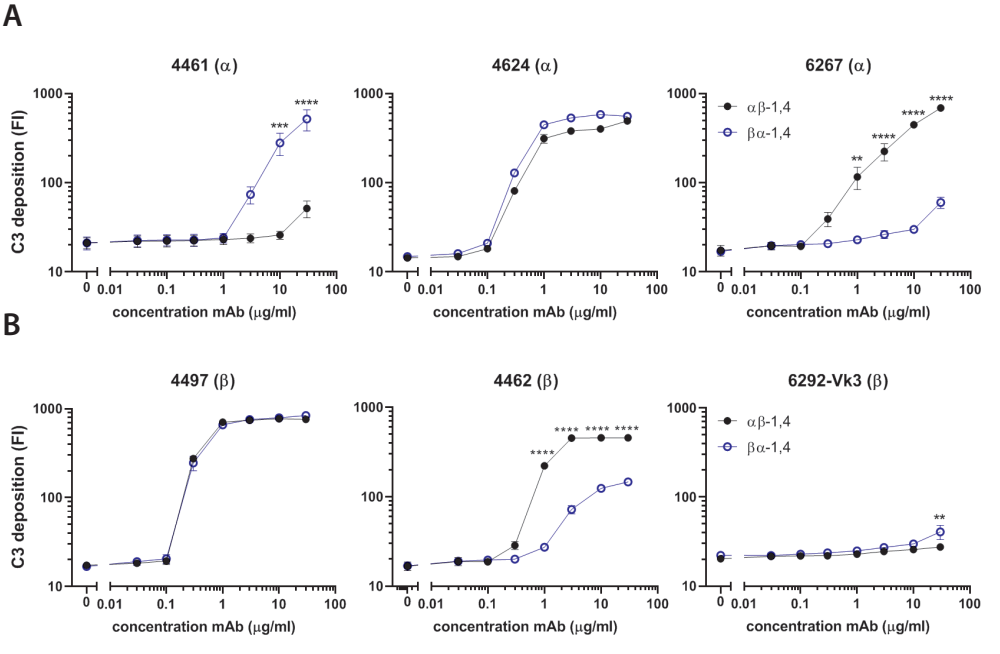

Figure 4. The impact of WTA GlcNAc positioning and presence of non-binding GlcNAc moiety on mAb-mediated complement activation. Complement C3 deposition on beads coated with RboP-WTA with a terminal α-GlcNAc and internal β-GlcNAc (αβ-1,4) or terminal β-GlcNAc and internal α-GlcNAc (βα-1,4), preopsonized with A) α-GlcNAc WTA-specific mAbs (clones 4461, 4624, 6267) or B) β-GlcNAc WTA-specific mAbs (clones 4497, 4462, 6292-Vk3) using a concentration range of mAbs (0.03-30 μg/ml). Data is depicted as geometric mean fluorescence intensity (FI) + standard error of mean (SEM) of biological triplicates. **p < 0.01,***p < 0.001, ****p < 0.0001.

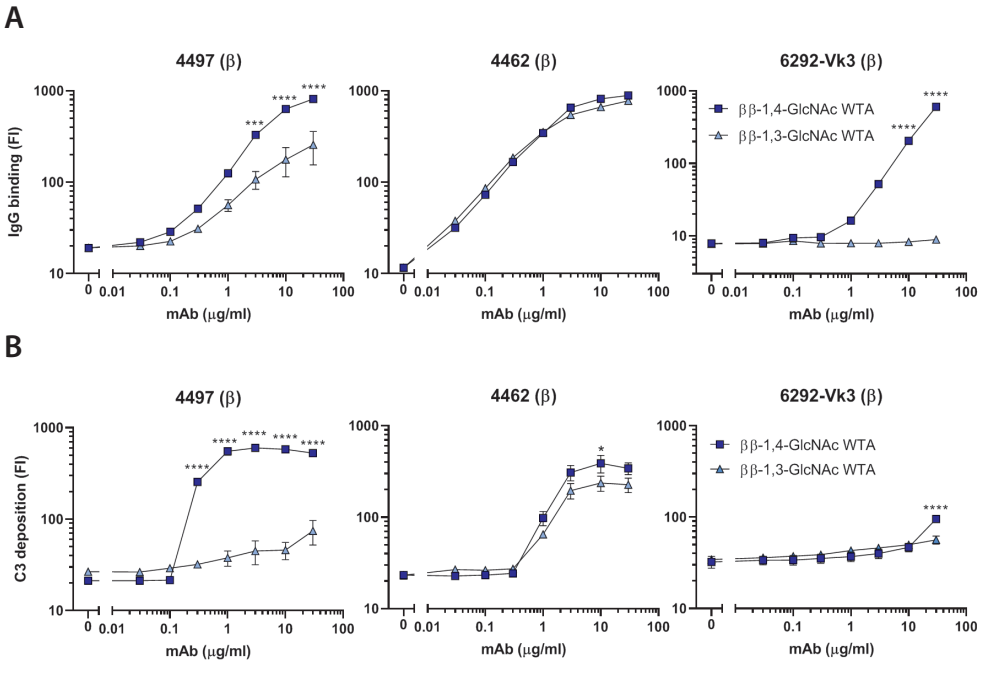

Figure 5 Cross-reactivity of β-GlcNAc WTA-specific mAbs for β-1,4-GlcNAc WTA and β-1,3-GlcNAc WTA. (A) Binding of and (B) C3 deposition on ββ-1,4- or ββ-1,3-GlcNAc WTA beads, preopsonized with β-GlcNAc WTA-specific mAbs (clones 4497, 4462, 6292-Vk3) across a concentration range (0.03-30 μg/ml). Data are depicted as geometric mean fluorescence intensity (FI) + standard error of mean (SEM) of biological triplicates. *p < 0.05, ***p < 0.001,****p < 0.0001.

We also tested complement C3 deposition on both ββ-1,4-GlcNAc and ββ-1,3-GlcNAc coated beads, pre-opsonized with IgG from clones 4497, 4462 or 6292-Vk3. Again, complement deposition fully reflected and further amplified the observed differences in antibody binding patterns; 4462 binding induced C3 deposition equally well on both β-GlcNAc beads, while 4497 only induced C3 deposition on ββ-1,4-GlcNAc beads (**Figure 5B**). For 6292-Vk3, limited C3 deposition was observed only at the highest concentration on ββ-1,4-GlcNAc beads (**Figure 5B**). The use of fully synthetic WTA fragments can further elucidate the cross-reactive potential of β-GlcNAc WTA specific antibodies.

Impact of WTA density on mAb binding

Thus far, we obtained information on antibody binding characteristics to *S. aureus* WTA using a robust bead-based flow cytometry assay. In this experimental set-up, we work with a fixed density of WTA oligomers coated to beads, which might influence binding efficiency. We set out to analyze and validate antibody binding characteristics using a different approach and to investigate the potential impact of WTA density on antibody binding. A glycan microarray is another well-established high-throughput

approach to study antibody binding to bacterial glycans. This technology allows the analysis of density-dependent antibody binding preferences to various glycan antigens simulateanously using minimal amount of sample (27,28). Synthetic *S. aureus* WTA oligomers were printed on microarray slides in different densities (3, 10 and 30 μM), and reacted with a fixed concentration of the mAbs. At lower WTA densities, binding characteristics for the different mAbs were comparable to observations with the bead-based assay, whereas binding was saturated at the highest WTA density (**Figure 6**). In line with previous results, binding of clone 4461, but not clone 4624 and 6267, to a single terminally placed α-GlcNAc was lower compared to WTA fragments containing both terminal and internal α-GlcNAc independent on WTA density. Similarly, clone 4462 displayed decreased binding to terminal β-GlcNAc at a lower WTA density while this was not the case for clone 4497. Furthermore, unlike 4462, 4497 bound significantly less to β-1,3-GlcNAc compared to β-1,4-GlcNAc at the lowest WTA density, which is in line with our previous findings. Interestingly, the presence of internal β-GlcNAc negatively affected the binding of 6267 to terminal α-GlcNAc only at the lowest WTA density which was not the case in the beads-based assay. While our findings could be reproduced in different assays, microarray results suggest that WTA density might further impact mAb binding characteristics.

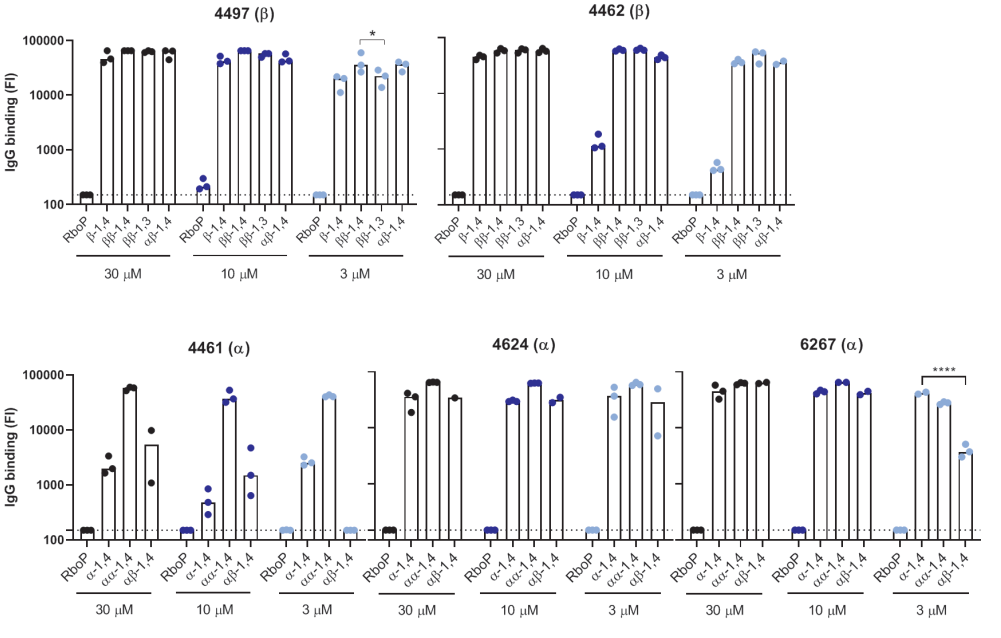

Figure 6. Impact of WTA density on mAb binding. Microarray analysis of mAb binding at a fixed concentration (0.5 μg/ml, clones 4497, 4462, 4461, 4624 and 6267) to printed synthetic WTA oligomers in different densities (30, 10 and 3 μM) Binding is shown as fluorescence intensity (FI), bar graphs show median of biological triplicates (shown as individual symbols), dotted line represents background signal.

Discussion

In this study, we performed an in-depth analysis on a panel of six anti-WTA mAbs using an unique library of synthetic WTA fragments to obtain more insights in WTA characteristics that affect antibody binding and induction of complement activation, an important downstream effector function. We observed differences in fine specificities between mAbs that discriminate between anomeric α- and β-GlcNAc modifications with regard to minimal binding requirements. In addition, the anomeric configuration of the GlcNAc moiety, the number of GlcNAcs as well as the GlcNAc position on the poly-RboP chain and/or presence of non-binding GlcNAc moieties represent discriminatory factors for binding of some, but not all, mAbs. Furthermore, we show that antibody binding to WTA, correlates with the capacity of mAbs to activate complement.

We have previously suggested that a proportion of antibodies directed against the dominant TarS-modified WTA-GlcNAc epitope (β-1,4-GlcNAc-WTA) is likely cross-reactive in their ability to bind β-1,3-GlcNAc-WTA (TarP-modified) (21). Such antibodies may thereby possibly counteract the suggested WTA-mediated antibody immune evasion strategies of *S. aureus* (16). We zoomed in on the ability of two mAbs (4497 and 4462), which were originally isolated based on reactivity with β-1,4-GlcNAc WTA (6), to bind β-1,3-GlcNAc-WTA. These antibody clones bind equally well to RboP hexamers that are in vitro glycosylated by TarS- and TarP, i.e. semi-synthetic structures (21). However, limiting substrate availability through the use of fully synthetic WTA fragments, carrying either two β-1,4- or β-1,3-GlcNAc moieties, revealed differences in binding capacity between the two clones; 4462 exhibited full cross-reactive potential, whereas 4497 bound significantly more to the structure carrying two β-1,4-GlcNAc. This suggest that for the 4497 clone, β-1,3-GlcNAc density is probably a major determinant for binding as a higher GlcNAc density, achieved through enzymatic glycosylation, did not reveal this difference (21). Furthermore, at the lowest WTA density coated in our microarray set-up, only 4497 and not 4462 showed significantly decreased binding to β-1,3-GlcNAc compared to β-1,4-GlcNAc. In addition, GlcNAc density also appears to be an important determinant for clone 6292, as binding to the fully synthetic WTA oligomers required 10-100 fold higher concentrations compared to the other mAbs. Continued in-depth structural analysis of these antibody clones will greatly aid in understanding the underlying molecular characteristics that enable cross-reactivity.

WTAs represent an important target for the immune system, specifically opsonic antibodies (18). Antibody binding to WTA mediates complement activation, recruitment of and opsonophagocytosis by neutrophils, which are key effector cells to clear *S. aureus* infection (12–15). Screening for the presence of WTA-specific antibodies has indicated that all tested donors produce antibodies against at least one of three GlcNAc-WTA modifications, which likely reflects the host's exposure to *S. aureus* expressing a particular WTA glycoprofile. Although the WTA glycoprofile primarily depends on the strain-

dependent expression and enzymatic activity of WTA glycosyltransferases TarS, TarP and TarM, we now show that also the position of the attached GlcNAc on the poly-RboP chain can greatly influence antibody binding and downstream effector immune processes. This raises the question whether *S. aureus* is able to control the position of GlcNAc attachment on the poly-RboP backbone, in addition to regulation of WTA glycosyltransferase expression (19,20). Currently, there is no insight on the arrangement of GlcNAc on *S. aureus*-expressed WTA nor the regulation of the Tar enzymes (10).

Antibody binding to specific GlcNAc moieties on WTA might be inhibited by the nearby presence of non-binding GlcNAc moieties or D-alanine modifications. Since our library did not contain oligomers with a single internally positioned α or β-GlcNAc, we cannot definitively state whether mAbs such as clone 6267 and 6292 have a true binding preference for terminally-positioned GlcNAc. Binding to the internally-positioned GlcNAc might be blocked by terminal presence of a non-binding GlcNAc through steric hindrance. Although for clone 4461 the presence of terminal β-GlcNAc did still allow binding to the internal α-GlcNAc, this might not be the case for other antibody clones. For this purpose, we aim to expand the existing WTA oligomer library to include longer oligomers, differently spaced GlcNAc moieties and the introduction of positively-charged D-alanine modifications to study the occurrence of steric hindrance and charge, respectively. In any case, the use of longer RboP-WTA oligomers has helped to gain more insights in the fine binding specificities, including the impact of GlcNAc position on the poly-RboP chain, while previous studies with monomeric GlcNAc-RboP fragments failed to show similar aspects (3,22). In line with this, STD-NMR analysis of clone 4461 showed that the internal GlcNAc contributed more to the binding than the terminal sugar which could be mainly attributed to higher STD signals for both the acetyl group and proton H1 of the internal GlcNAc (Prof. Alba Silipo, personal communication).

In order to effectively target *S. aureus* strains expressing different WTA glycoprofiles, the host's immune system will benefit from raising a heterogeneous antibody repertoire to *S. aureus* WTA. Although there may be an intrinsic difference in immunogenicity among the three WTA glycotypes (16,21), we show here that all GlcNAc modifications in our small library of WTA oligomers can be recognized by mAbs that were generated from patients recovering from invasive *S. aureus* infections and thus appear immunogenic (3,6). These high affinity IgG1 antibodies are the result of rigorous T cell-dependent B cell somatic hypermutation and affinity maturation (3,22). However, the majority of the WTA antibody repertoire in healthy donors belongs to the IgG2 subclass indicating T cell-independent B cell activation (12). This may impact antibody binding characteristics as a result of differences in affinity maturation and their ability to trigger downstream effector functions (18,21). Still, the antibody binding principles to WTA have been suggested to be conserved and might not dramatically change following affinity maturation (22). Interestingly, different antibody clones were isolated from the same donor (4462/4461/4624 vs 6267/6292), thereby underlining the importance of a heterogeneous antibody response during infection. How GlcNAc positioning on the

poly-RboP chain induces antibody clones with different specificities is not clear. However, antibodies recognizing terminal GlcNAc moieties will likely be less effective in activating the complement system and inducing opsonophagocytosis compared to antibodies recognizing internally positioned GlcNAc, as terminal GlcNAcs are less abundant on the bacterial surface. Overall, some WTA-specific IgG antibodies will be more effective than others, and future vaccination studies should take this into account when considering WTA as a vaccine antigen. Employing synthetic WTA fragments might support the design of the most optimal vaccine antigen to induce potent and broadly reactive antibody clones. In general, synthetic glycoconjugate vaccines are receiving increasing attention, and larger multivalent glycan structures might induce more potent immune responses than minimal epitopes alone (29–31). Importantly, synthetic WTA oligomers serve as an extremely valuable glycobiology tool to study molecular and structural characteristics of diverse antibody pools and might ultimately aid in identifying potent clones suitable for mAb therapies against *S. aureus*.

Materials & Methods

Recombinant expression of mAbs and Fab fragments.

For mAb expression, we cloned the human IgG1 heavy chain (hG) and kappa light chain (hK) constant regions (sequences as present in pFUSE-CHIg-hG1 and pFUSE2-CLIg-hk; Invivogen) in the XbaI-AgeI cloning sites of the pcDNA34 vector (ThermoFisher). VH and VL sequences from mAbs specific for α- GlcNAc-WTA (clones 4461, 4624, 6267), β-GlcNAc-WTA (clones 4497, 4462) and β-1,4-GlcNAc-WTA (clone 6292, (21,24)) were described previously and are derived from patent WO 2014/193722 A1.50 (3,6). As the VL of anti-WTA antibody 6292 resulted in protein precipitation, it was adapted toward a Vκ3 (6292-Vκ3), leaving the complementarity-determining regions (CDRs) intact (**Supplementary figure 1A**). The VH and VL sequences, preceded by a Kozak sequence (ACCACC) and the HAVT20 signal peptide (MACPGFLWALVISTCLEFSMA), were codon-optimized for human expression and ordered as gBlocks (IDT). We cloned VH and VL gBlocks into the pcDNA34 vector, upstream of the IgG1 heavy chain (hG) and kappa light chain (hK) constant regions, respectively, by Gibson assembly (New England Biolabs) according to the manufacturer's instructions. NheI and BsiWI were used as the 3' cloning sites for VH and VL, respectively, to preserve the immunoglobulin heavy and kappa light chain amino acid sequence. The constructs were transformed in *E. coli* TOP10F' by heat shock, and clones were verified by PCR and Sanger sequencing (Macrogen). Plasmids were isolated by NucleoBond Xtra Midi kit (Macherey-Nagel) and sterilized using 0.22 μm Spin-X centrifuge columns (Corning). EXPI293F cells (Thermo Fisher), grown in EXPI293 Expression medium (Thermo Fisher) at 37 °C, 8% CO2 in culture filter cap conical flasks (Sigma) on a rotation platform (125 rotations/min) were used for protein production. One day before transfection, cells were diluted to 2 × 106 cells/mL and 100 mL cell culture was used for transfection the next day. In 10 mL of Opti-MEM (Thermo

Fisher), 500 µL PEI-max (1 µg/µL; Polysciences) was mixed with DNA (1 µg/mL cells) in a 3:2 ratio of hK and hG vectors. After 20 min incubation at room temperature, the DNA/PEI mixture was added dropwise to 100 mL of EXPI293F cells. After 5 days, we verified IgG expression by SDS-PAGE and harvested cell supernatant by centrifugation and subsequent filtration through a 0.45 µM filter. IgG was purified using a HiTrap Protein A column (GE Healthcare) and Äkta Pure (GE Healthcare). Protein was eluted in 0.1 M citric acid, pH 3.0, and neutralized with 1 M Tris, pH 9.0. The IgG fraction was dialyzed overnight against PBS at 4°C. Purified mAbs were stored at −20°C.

Fab fragments were cloned and expressed similar as the full-length mAbs, except that the Fab heavy chain ends with 211 VEPKSC 216. A flexible linker (GGGGS), an LPETG sequence, and a 6xHIS tag were added at the C-terminus of each Fab. EXPI293F expression supernatant was dialyzed against 50 mM Tris, 500 mM NaCl; pH 8.0, before Fab purification on a HISTrap FF column (GE Healthcare). Fab fragments were dialyzed against 50 mM Tris, 300 mM NaCl; pH 8.0 and stored at −20 °C.

Coating fully synthetic *S. aureus* WTA oligomers on beads

A library of fully synthetic RboP-WTA oligomers was synthesized as described elsewhere (25)(**Figure 1B**). Synthetic RboP-WTA hexamers (0.17 mM) were coupled to beads by adding 5×10^7 magnetic streptavidin beads (Dynabeads M280 Streptavidin, Thermo Fisher Scientific) in PBS and incubated for 15 min at room temperature. Next, the coated beads were washed three times with PBS 0.1% BSA 0.05% Tween-20 using a magnetic sample rack (Thermo Fisher Scientific) and stored at 4 °C.

Analysis of antibody deposition and complement activation on WTA beads

To study binding of mAbs to beads coated with synthetic WTA oligomers (WTA beads), 1 x 10^5 WTA beads were incubated with a concentration range of the different mAbs (0.3-30 µg/ml) in a 96-wells round bottom plate (Greiner) for 20 minutes at 4°C in PBS containing 0.1% BSA and 0.05% Tween-20 (PBS-BT). The beads were washed once with PBS-BT using a plate magnet and incubated with Protein G-Alexa Fluor 488 (1 µg/ml, Sigma) for an additional 20 minutes at 4°C. For Fab fragments and 6292-Vk3 IgG1, goat F(ab')2 anti-human kappa-Alexa Fluor 647 (5 µg/mL, Southern Biotech) was used as secondary detection instead of Protein G-Alexa Fluor 488. Beads were washed and analyzed by flow cytometry (BD FACSVerse). A general of the workflow for measuring antibody binding to WTA beads is shown in **figure 1C**.

To measure C3 complement deposition, WTA beads were opsonized with mAbs (0.3-30 µg/ml) for 20 minutes at 4°C, washed and incubated with 1% ΔIgG ΔIgM human serum (26) for 30 minutes at 37°C in Veronal Buffered Saline pH 7.4, 0.25 mM MgCl2, 0.5 mM CaCl2, 0.05% Tween-20, 0.5% human serum albumin (HSA). Next, the beads were

washed, stained with goat anti-C3 IgG-FITC (1:300, De Beer Medicals) for 20 minutes at 4°C and analyzed by flow cytometry (BD FACSVerse).

Microarray analysis

The amino-spacer-equipped synthetic fragments were dissolved in spotting buffer (Nexterion Spot, Schott Nexterion) with 10% DMSO in 384-wells V-bottom plates (Genetix, New Milton, UK). The fragments were printed in three final concentrations (30 μM, 10 μM and 3 μM) in triplicate on epoxysilane-coated glass slides (Slide E, Schott, Nexterion) by contact printing using the Omnigrid 100 microarrayer (Genomic Solutions, Ann Arbor, MI) equipped with SMP3 pins with uptake channels that deposit 0.7 nl at each contact. The slides were rested in a high humidity chamber for 18 hours and were stored in the dark until used. The slides were washed with PBS (3x) and all unreacted sites on the arrays were blocked by shaking the slides for 1 hour with ethanolamine (0.10 ml, 0.05 M in PBS containing 20 mg/ml of BSA). The slides were flushed with PBS containing 5% Tween-20, followed by PBS and finally rinsed with PBS containing 1% Tween-20. After removal of the PBS 1% Tween-20, the arrays were incubated with designated mAbs at 0.5 μg/ml (100 μl, diluted in PBS 1% Tween-20 and 10 mg/ml BSA) for 60 minutes. The slides were washed subsequently with PBS 5% Tween-20, PBS and PBS 1% Tween-20. After removal of the PBS 1% Tween-20, the arrays were incubated with Alexa Fluor-488 conjugate goat anti-human IgG (Invitrogen, A-11013; 100 μl, 0.5 μg/ml final dilution in PBS 1% Tween-20 10 mg/ml BSA) for 30 minutes in the dark. The slides were washed with subsequently PBS 5% Tween-20, PBS and MilliQ, dried by centrifugation and analyzed for fluorescence on 532 nm and 635 nm using a G2565BA scanner. Data and image analyses were performed with GenePix Pro 7.0 software (Molecular Devices, Sunnyvale, CA, USA) as described previously (27). Fluorescence intensities were quantified and corrected for background/nonspecific antibody adhesion by subtracting the fluorescence at blank spots, where spotting buffer was printed without GTA fragment. The average of the triplicate spots was normalized to the highest intensity on the array.

Statistical analysis

Flow cytometry data was analyzed using FlowJo 10 (FlowJo LLC). A two-way ANOVA with Sidak's multiple comparison test was performed for statistical analysis with Prism software (version 8.3; GraphPad). Two-sided p values <0.05 were considered significant, and are depicted in the figures.

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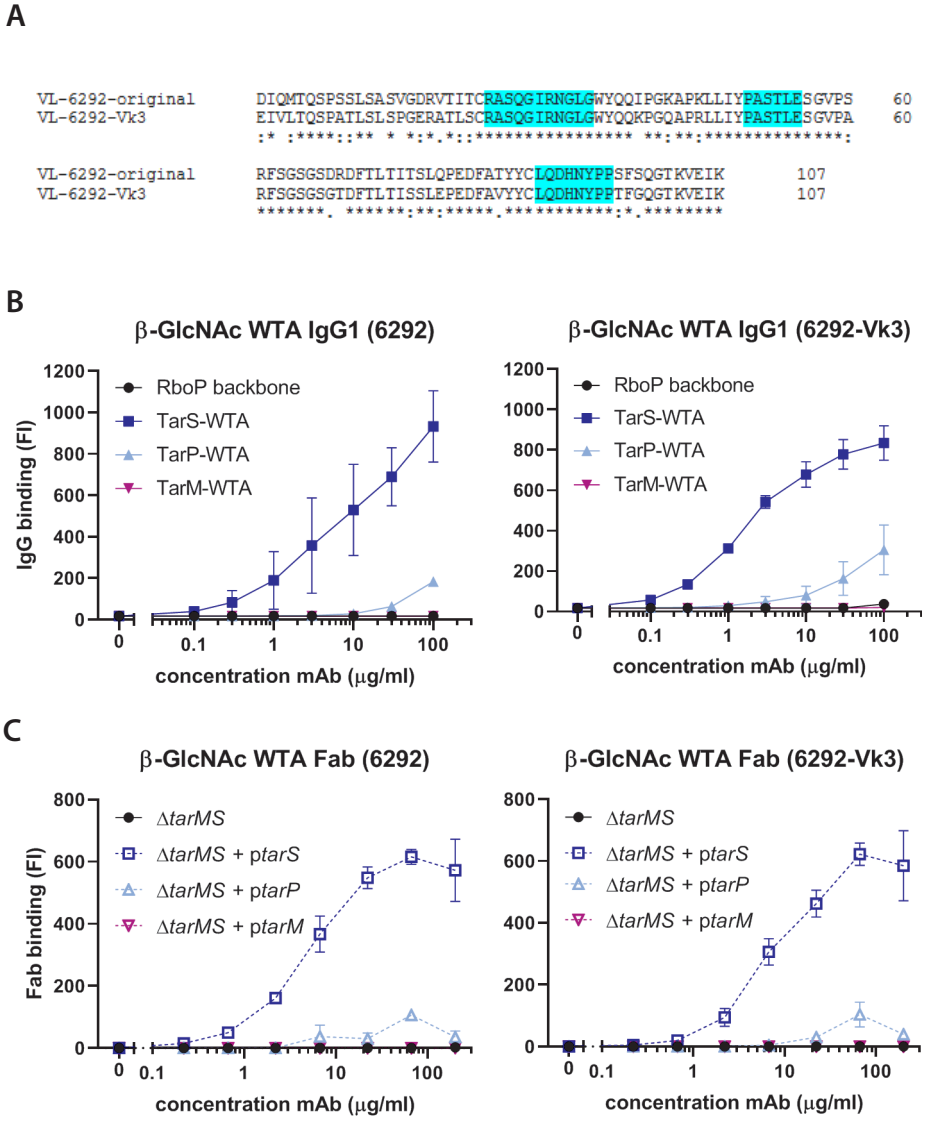
Supplementary information

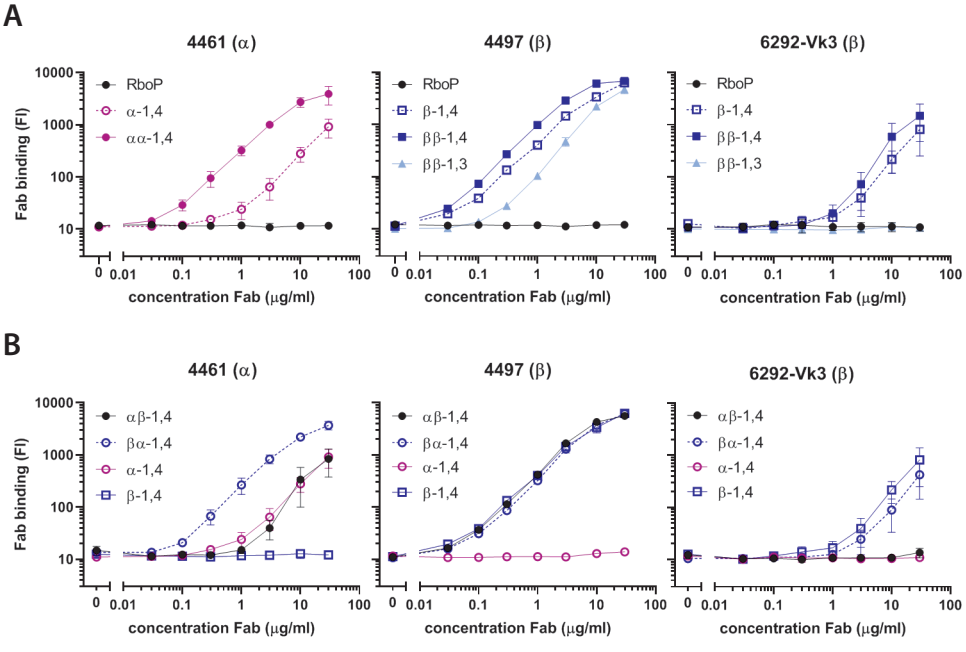
Supplementary materials and methods

WTA-specific Fab binding of *S. aureus*

S. aureus RN4220 Δ*tarMS*, and *tarS, tarP* and *tarM*-plasmid-complemented strains were grown overnight in 3 mL of Todd-Hewitt broth (THB; Oxoid) at 37°C with agitation. Plasmidcomplemented strains were cultured in the presence of 10 μg/ml chloramphenicol (Sigma). Overnight cultures were subcultured the next day in fresh THB with chloramphenicol and grown to a mid-exponential growth phase, corresponding to an optical density of 0.6–0.7 at 600 nm (OD600). Bacteria were collected by centrifugation (10 min @ 3,000g, 4°C) and resuspended in PBS 0.1% BSA to an OD600 of 0.4 (~ 1x108 CFU/ml). Bacteria were stained with a concentration range of Fab fragments (0.2-200 μg/ml) from clone 6292 and 6292-Vk3 for 20 min at 4°C, washed and next stained with goat F(ab')2 anti-human kappa-AF647 (5 μg/mL, Southern Biotech). Bacteria were washed, and fixed in PBS 1% formaldehyde prior to analysis by flow cytometry (FACSVerse).



Supplementary figure 1. (A) Protein sequence alignment of original 6292 and modified 6292-Vk3 to improve protein solubility, CDRs shown in blue. (B) Binding characteristics of 6292-IgG1 versus adapted clone 6292-Vk3-IgG1 (0.1-100 μg/ml) to beads coated with RboP backbone or enzymatically glycosylated RboP by TarS, TarP or TarM (C) Binding characteristics of 6292-Fab versus adapted 6292-Vk3 Fab (0.2-200 μg/ml) to glycosylated WTA deficient *S. aureus* strain RN4220 Δ*tarM*Δ*tarS* (Δ*tarMS*) and *tarS, tarP* or *tarM*-complemented Δ*tarMS* strains. Data is depicted as geometric mean fluorescence intensity (FI) + standard error of mean (SEM) of biological triplicates.



Supplementary figure 2. Binding of Fab fragments to fully synthetic *S. aureus* WTA oligomers. Fab binding (0.03-30 μg/ml) of clones 4461, 4497 and 6292-Vk3 to A) beads coated with RboP backbone only, one or two α-GlcNAc or β-GlcNAc moieties and B) beads coated with RboP-WTA with a terminal α-GlcNAc and internal β-GlcNAc (αβ-1,4), terminal β-GlcNAc and internal α-GlcNAc (βα-1,4) or terminal GlcNAc only (α-1,4 or β-1,4). Data is depicted as geometric mean fluorescence intensity (FI) + standard error of mean (SEM) of biological triplicates

4

"A lack of passion can do more harm than simple inexperience"

MARCEL MINNAERT

Chapter 4

Mapping the antibody repertoire to *Staphylococcus aureus* wall teichoic acid: a protective role for IgM in invasive infection?

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Abstract

Staphylococcus aureus is one of the leading causes of hospital-acquired infections with high overall mortality. Pre-existing immunity to *S. aureus* is common among healthy individuals due to natural exposure, although this is not always sufficient to protect from (re-) infection. Antibodies are believed to play a key role in bacterial killing through antibody opsonization and complement activation, which induces subsequent bacterial uptake as well as neutrophil recruitment. However, the exact correlates of protection remain to be fully elucidated. A large proportion of the anti-*S. aureus* antibody pool is directed against Wall Teichoic Acid (WTA), an abundant cell wall glycopolymer, which shows structural variation through glycosylation with N-acetylglucosamine (GlcNAc). Overall, three WTA glycotypes are currently distinguished and IgG antibody responses reactive with these different WTA glycotypes can be detected in plasma from healthy individuals. How these antibody profiles are affected during invasive *S. aureus* infection is not known but may help to identify protective responses. Here, we analyzed and compared the antibody repertoire to *S. aureus* WTA in plasma from healthy individuals (n=31) and ICU patients with culture-confirmed *S. aureus* bacteremia (n=38) using in vitro glycosylated synthetic WTA fragments that resemble the three *S. aureus* WTA glycotypes. Robust IgM responses to all three WTA modifications were detected in 30 out-of-31 healthy individuals. In contrast, WTA-specific IgM responses were significantly decreased (three-fold lower) in 31 out-of-38 (82%) *S. aureus* sepsis patients, whereas no differences in IgG2 responses were observed. For four patients, we correlated the specific WTA glycoprofile of the infecting *S. aureus* isolate to the detected WTA-specific antibody responses and observed that the absence of glycotype-specific IgM, but also decreased glycotype-specific IgG2 responses, was associated with patient mortality. This study supports the existence of a broad antibody repertoire to *S. aureus* WTA glycotypes, and hints towards a protective role of WTA-specific IgM against invasive *S. aureus* infections.

Introduction

Staphylococcus aureus is an important opportunistic pathogen and among the leading causes of hospital-acquired infections with continuously limiting treatment options due to antibiotic resistance (1,2). Infections caused by *S. aureus* vary from mild superficial skin infections to more invasive and even systemic infections with high mortality rates when the bacterium enters the bloodstream (3). Natural exposure or infection does not guarantee the induction of protective immunity since re-infection occurs frequently within months of primary infection (4). Consequently, the correlates of protection currently remain unknown, which has complicated the development of an effective vaccine for *S. aureus* despite rigorous efforts (5).

Neutrophils are key immune effector cells for *S. aureus* eradication (6,7). Indeed, defective neutrophil responses increase the susceptibility to *S. aureus* infection (8). Both the cellular and humoral arms of the adaptive immune system, including T cells and antibodies, promote *S. aureus* killing by neutrophils by enhancing their effector functions and recruitment (9,10). Antibodies and complement bound to surface antigens act in concert to increase phagocytosis and killing of *S. aureus* by neutrophils (11). However, it remains unclear which *S. aureus* surface antigen represents the best target for opsonic antibodies and thereby contributes to protective immunity. Among a large body of literature reporting on specific antibody profiles for *S. aureus* protein antigens, only a limited number of studies observed a small but significant correlation between specific antibody titers and decreased risk of developing *S. aureus*-induced septic shock (12–14). Thus far, *S. aureus* glycan antigens, including the highly immunogenic Wall Teichoic Acid (WTA), have largely been excluded from antibody profiling studies due to technical challenges associated with these non-proteinaceous antigens.

S. aureus WTAs are cell wall-anchored glycopolymers that are involved in essential processes including metal homeostasis, bacterial colonization, antibiotic resistance, and numerous host-pathogen interactions (15–21). WTAs represent an important target for the immune system, with an estimated 70% of the human *S. aureus* surface-specific antibody repertoire directed to WTAs (22–24). WTA-specific IgG induces complement activation via the classical pathway, resulting in neutrophil recruitment and *S. aureus* opsonophagocytosis and killing (24–27). These antibodies are predominantly, but not exclusively, of the IgG2 subclass and are believed to arise in a T cell-independent manner through direct crosslinking of the B cell receptor (28). In addition to IgG2, IgM antibodies play an important role in the binding of various microbial glycans. Furthermore, IgM can display poly-reactivity due to its high avidity, which compensates for often low affinity properties of this antibody isotype. IgM does not induce Fc-mediated phagocytosis and killing of Gram-positive bacteria by neutrophils, but can strongly activate the complement system to support complement-mediated phagocytosis and neutrophil recruitment via C5a generation (29–32). Despite the potential relevance of IgM in *S. aureus* defense, to our knowledge no studies have analyzed the presence of WTA-specific IgM.

In addition to T cell-independent responses, experimental studies have demonstrated that the zwitterionic properties of WTA enable presentation on MHC-II, thereby inducing T cell-dependent responses (33,34). This route would support affinity maturation and class switching of WTA-specific B cells to for example IgG1 or IgG3 (35,36).

The majority of *S. aureus* strains express WTA that consists of a linear polymerized ribitol phosphate (RboP) backbone. This RboP backbone is further modified with N-acetylglucosamine (GlcNAc) and positively charged D-alanine groups, the latter of which confers bacterial resistance to killing by cationic antimicrobial peptides (21,37). Three distinct WTA glycotypes have been described that vary in their specific glycosylation with GlcNAc and is conferred by three dedicated glycosyltransferases; TarS, TarP and TarM. TarS and TarM both modify WTA at C4 of the RboP subunit with a β-linked GlcNAc (β-GlcNAc) and α-GlcNAc, respectively (38), whereas TarP modifies the WTA RboP backbone at C3 with β-GlcNAc (39). Importantly, these three WTA glycotypes differently impact immune interaction and antibiotic resistance. For WTA-specific antibodies, IgG antibodies directed against β-1,4-GlcNAc modified WTA are dominant in serum from healthy individuals and can display cross-reactivity towards β-1,3-GlcNAc-modified WTA but not α-1,4-GlcNAc-modified WTA (40). Generally, antibodies specific for α-1,4-GlcNAc-WTA are least abundant in serum from healthy individuals (40), which may result from different intrinsic immunogenicity (39) or limited exposure to tarM-expressing strains (16). To add an additional level of variation, over half of *S. aureus* isolates express a combination of two tar genes, i.e. *tarS*/*tarM* (approximately 30% of human isolates) or *tarS*/*tarP* (5-10% of *S. aureus* isolates)(16,39). Consequently, *S. aureus* strains can simultaneously express different WTA modifications, depending on tar expression, environmental conditions and enzymatic activity, which gives rise to a strain-specific WTA glycoprofile. Consequently, antibody-immune escape could arise in case induced antibody responses do not align with the WTA glycoprofile of the infecting strain.

Here, we aimed to gain insight in the systemic antibody responses to the three main *S. aureus* WTA glycotypes using our recently developed bead-based flow cytometric assay that employs synthetic WTA hexamers (41) modified with specific TarS-, TarP- or TarM-modifications (17,19,40). We analyzed WTA-specific antibody responses, not only focusing on IgG but also IgM, IgA and IgG subclasses 1, 2 and 3 in plasma samples from 31 healthy individuals and 38 patients with culture-confirmed *S. aureus* bacteremia to elucidate the potential contribution of WTA-specific antibodies to protective immunity to *S. aureus*. Finally, we analyzed the correlation between the WTA glycoprofile of the infecting *S. aureus* isolate and the induced WTA antibody profile for a select number of patients with different disease outcomes.

Results

The antibody repertoire to *S. aureus* WTA in healthy individuals

To study the antibody repertoire to specific *S. aureus* WTA glycotypes, we adapted a previously described assay to measure antibody binding to synthetic WTA oligomers coated to beads (40). Specifically, we optimized the assay using a isotype- and subclass-switched mAb (clone 4497) to allow simultaneous analysis of multiple antibody isotypes (IgM, IgG, IgA) or IgG subclasses (IgG1, IgG2, IgG3) to a specific WTA glycotype in a single sample (**Supplementary figure 1A-C**). We observed specific IgM binding to all three WTA glycotypes in at least 30 out of 31 of healthy donors (97%) (Figure 1A). No statistically significant differences were observed for IgM responses against TarS-, TarP- and TarM-modified WTA (median (range); TarS 1.3 (0.1-6.8); TarP 1.0 (0.1-5.8); TarM 1.5 (0.3-7.3)) (**Figure 1A**). Unfortunately, data obtained on WTA-specific IgG responses was considered inconclusive due to the absence of dilution linearity across the tested plasma concentrations and these data were therefore excluded from further analysis. WTA-specific IgA could be detected in approximately half of the donors (S**upplementary figure 2**).

We were able to reliably measure the presence of WTA-specific IgG1, IgG2 and IgG3. In line with previous findings, all healthy donors had WTA-specific IgG2 antibodies against at least one of the WTA glycotypes (**Figure 1B, Supplementary Figure 2**), whereas only low levels of WTA-specific IgG1 and IgG3 were detected in approximately half of the donors (**Supplementary figure 2**). Overall, IgG2 responses to TarS-modified WTA were the highest and most uniform among the healthy donors (median 18.8; range 2.3-82.6), and not significantly different from IgG2 responses against TarP-modified WTA (median 14.4; range 0.4-123.3), which may be explained by the existence of β-GlcNAc-WTA cross-reactive antibody clones (40). IgG2 responses against the two β-GlcNAc-modified WTAs were significantly higher than IgG2 responses towards TarM-modified WTA (median 5.5; range 0.1-122.3) (**Figure 1B**). Only two donors lacked detectable IgG2 antibodies against TarM-modified WTA (**Figure 1B**).

Correlations between antibody responses may indicate the existence of cross-reactivity for different WTA glycotypes. For IgM, antibody binding to all three WTA glycotypes correlated, suggesting the existence of poly-reactive IgM clones binding to GlcNAcylated WTA independent of GlcNAc configuration or linkage position (**Figure 1C**). For IgG2 however, we only observed a correlation between TarS-WTA and TarP-WTA, which was expected based on our previous study (40) (**Figure 1D**). This data supports the existence of a broadly-reactive antibody repertoire to *S. aureus* WTA in healthy individuals, with potential differences in binding specificities and characteristics within specific antibody isotypes.

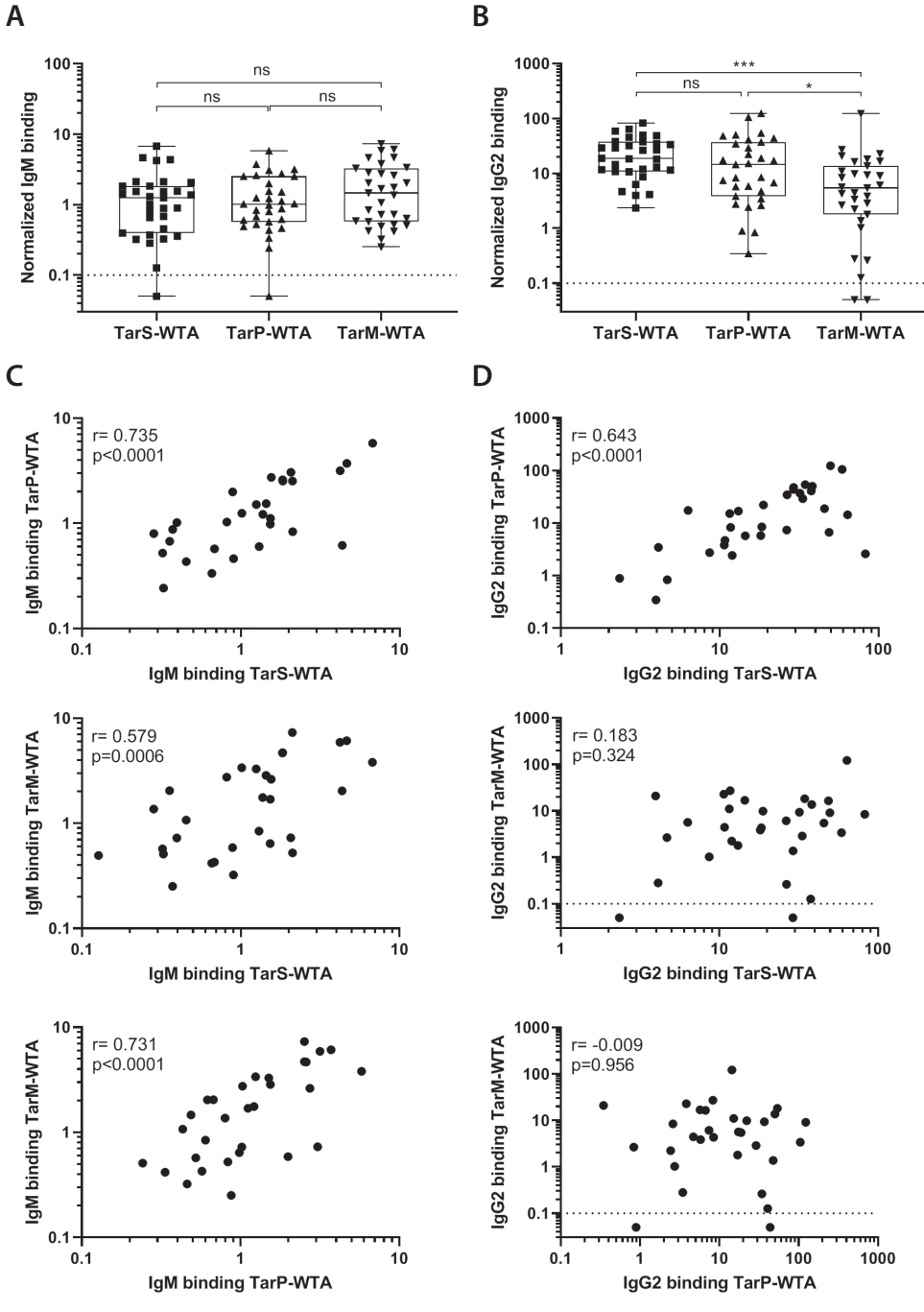

A
Normalized IgM binding
ns
ns
ns
TarS-WTA
TarP-WTA
TarM-WTA
B
Normalized IgG2 binding
***
ns
*
TarS-WTA
TarP-WTA
TarM-WTA
C
IgM binding TarP-WTA
r= 0.735
p<0.0001
IgM binding TarS-WTA
IgM binding TarM-WTA
r= 0.579
p=0.0006
IgM binding TarS-WTA
IgM binding TarM-WTA
r= 0.731
p<0.0001
IgM binding TarP-WTA
D
IgG2 binding TarP-WTA
r= 0.643
p<0.0001
IgG2 binding TarS-WTA
IgG2 binding TarM-WTA
r= 0.183
p=0.324
IgG2 binding TarS-WTA
IgG2 binding TarM-WTA
r= -0.009
p=0.956
IgG2 binding TarP-WTA

Figure 1. Antibody repertoire against specific WTA glycotypes in healthy subjects. A, B) Normalized binding of (A) IgM and (B) IgG2 to beads coated with TarS-WTA, TarP-WTA and TarM-WTA. Boxplots extend from the 25th to 75th percentiles and the line inside the box represents the median. C, D) Correlation between binding of (C) IgM and (D) IgG2 reactivity toward distinct WTA glycotypes within individual donors. Each dot represents an individual donor (n=31). Dotted line represents the lower limit of detection, symbols shown below line present extrapolated values. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns=not significant.

WTA-specific IgM responses are reduced in patients with invasive *S. aureus* infection

Having established a 'base-line' level of WTA-specific antibody reactivity in healthy individuals, we sought to determine how these antibody profiles were different or affected in patients (n=38) suffering from culture-confirmed *S. aureus* bacteremia (**Table 1**). Plasma samples derived from patients with culture-confirmed invasive *Streptococcus pyogenes* bacteremia (n=13) were included as an infection control. There were no significant differences in IgG2 responses to any of the three WTA glycotypes between healthy donors and both patient groups (**Figure 2A**). One patient did not have detectable WTA-specific IgG2 responses (**Figure 2A**), and also lacked WTA-specific IgG1 and IgG3 (data not shown). This particular patient underwent allogenic hematopoietic stem cell transplantation one year prior to infection, which likely resulted in the loss of all *S. aureus*-specific immunological memory. In contrast to IgG2, IgM responses to all WTA glycotypes were significantly decreased in *S. aureus* patients compared to healthy donors (median IgM binding healthy donors vs patients; 1.3 vs 0.4; average from TarS, TarP and TarM-WTA) and were undetectable in 7 out-of-38 patients (18%) (**Figure 2B**). Importantly, patients with a *S. pyogenes* infection did not show a similar decrease in WTA-specific IgM responses, indicating that this is an infection-specific phenomenon (**Figure 2B**). For individual patients, there was no clear correlation between IgG2 and IgM responses to *S. aureus* WTA (**Figure 2C**).

Association between WTA-specific IgM levels and patient mortality on the ICU

Next, we investigated whether decreased WTA-specific IgM responses were associated with disease outcome following *S. aureus* bacteremia. Of the patients included in this study, 7 out-of-38 patients (18%) died on the ICU (**Table 1**). We stratified the antibody responses of the *S. aureus* patient cohort based on mortality (n=7 deceased vs n=31 survived). IgM, but not IgG2, responses to TarS- and TarM-modified WTA were significantly lower in deceased patients (**Figure 3**). For TarP-modified WTA, only IgG2 levels were significantly decreased in deceased patients (**Figure 3A**), whereas IgM responses were not significantly different (p=0.053, **Figure 3B**). Importantly, low WTA-specific IgM levels were not a result of low total IgM levels in these patients (**Supplementary Figures 3C, D**), nor were total IgM or IgG levels different between surviving or deceased patients (**Supplementary Figures 3B, D**). In addition, there is no correlation between total IgM antibody titers and WTA-specific IgM responses (**Supplementary Figure 3E**). These findings suggest a potential link between the absence of WTA-specific IgM and negative disease outcome following *S. aureus* bacteremia.

Table 1. Patient characteristics of ICU patients with *S. aureus* bacteremia.

Characteristic	ICU patients (n=38)
Male, n (%)	27 (71)
Age, years mean (SD)	59 (16)
Admission type, n (%)	
Medical	23 (60.5)
Planned surgical	6 (15.8)
Emergency surgical	9 (23.7)
Site of infection, n (%)	
Pulmonary	5 (13.2)
Cardiovascular	10 (26.3)
Musculoskeletal	7 (18.4)
Surgical site	4 (10.5)
Abdominal	2 (5.3)
Soft tissue	6 (15.8)
Unknown	4 (10.5)
Comorbidities prior to ICU admission, n (%)	
Diabetes mellitus	8 (21.1)
Non-metastatic solid tumor	5 (13.2)
Immunodeficiency	2 (5.3)
APACHE IV score, median (range)	84 (44-162)
Septic shock/sepsis, n (%)	15 (39.5)
Length of stay ICU in days, mean (SD)	17 (18)
ICU mortality, n (%)	7 (18.4)

Mismatch between WTA-specific antibody responses and WTA glycoprofile of infecting *S. aureus* isolate is associated with negative disease outcome

So far, we have studied antibody responses in healthy individuals and patients to the three known GlcNAc modifications of *S. aureus* WTA, and compared responses within and between these groups. However, it is known that *S. aureus* can express a mixture of different WTA glycotypes, depending on strain-specific expression of WTA glycosyltransferases TarS, TarP and TarM. Therefore, infection with *S. aureus* does not result in immune exposure to all three GlcNAc-WTA epitopes. To assess whether the induced WTA-specific antibody responses matched with the WTA glycoprofile of the infecting strain, we analyzed clinical isolates from six patients within the *S. aureus* cohort; three patients that had survived the infection (patient 4, 18 and 29) and three patients that passed away on the ICU (patient 16, 20 and 35). We determined the presence of specific tar genes by PCR analysis and verified the expressed WTA glycoprofile by bacterial staining with Fab fragments that specifically recognize α-GlcNAc moieties (4461) and β-GlcNAc moieties (4497, 6292; β-1,4-GlcNAc) on *S. aureus* RboP-WTA (19,22,23). All six isolates contained

tarS and expressed the corresponding β-1,4-GlcNAc moiety on their surface (**Figure 4A**). Four isolates co-expressed *tarM* in addition to *tarS* with corresponding surface expression of α-GlcNAc-WTA, and one isolate contained *tarP* in addition to *tarS* (**Figure 4A**). Of the four patients that had been infected by *S. aureus* strains that coexpressed TarM and TarS, two survived the infection (patient 4 and 18) and two passed away (patient 16 and 35) (**Figure 4A**). No clear differences in antibody reactivity, including IgG1, IgG2 and

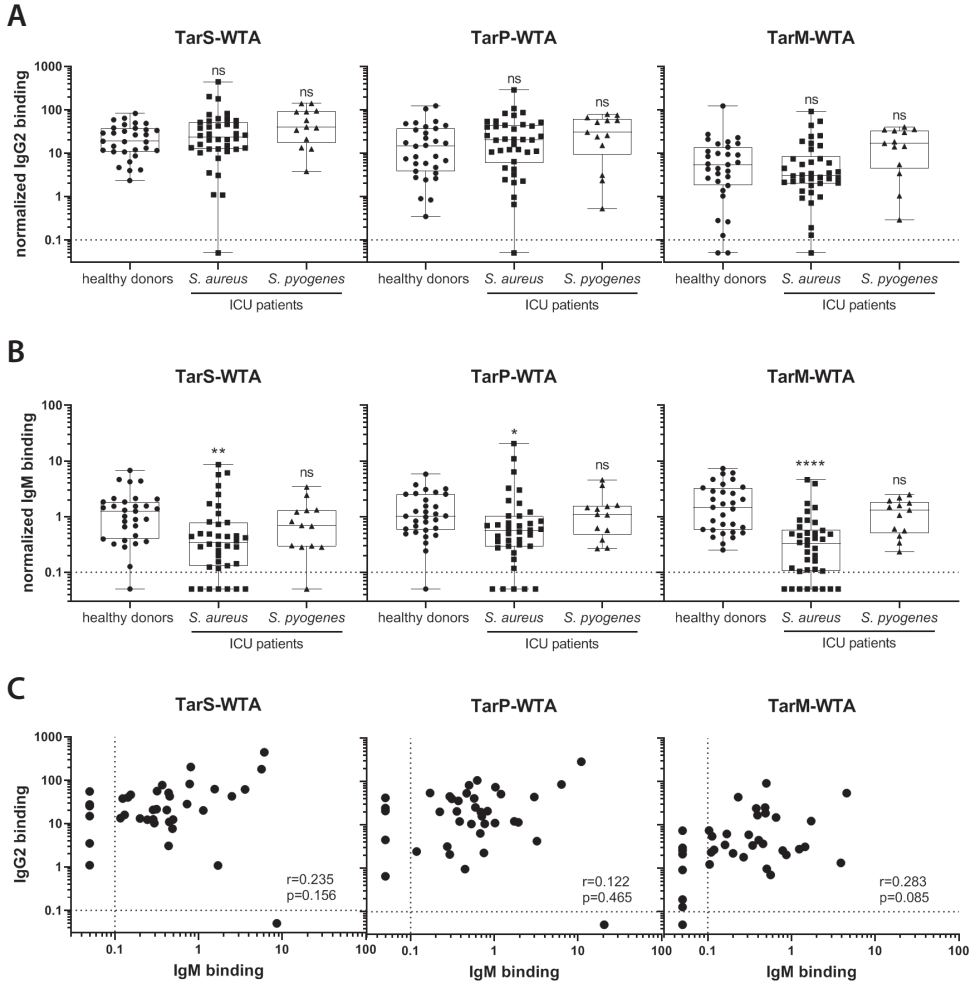


Figure 2. Antibody profile against specific WTA glycotypes in patients with invasive bacterial infection. A, B) Normalized binding of (A) IgG2 and (B) IgM to beads coated with TarS-WTA, TarP-WTA and TarM-WTA. Boxplots represent data for healthy donors (n=31), ICU patients with *S. aureus* (n=38) or *S. pyogenes* (n=13) bacteremia, and extend from the 25th to 75th percentiles. The line inside the box represents the median, with symbols representing individual donors. C) Spearman correlation between IgM and IgG2 binding to all three WTA modifications, symbols indicate individual patients with S. aureus infection. Dotted line represents the lower limit of detection, symbols shown below line present extrapolated values. *p < 0.05, **p < 0.01, ****p < 0.0001, ns=not significant.

IgM, to TarS-modified WTA could be observed based on disease outcome in these four patients (**Figure 4B**). Intriguingly, IgG2 and IgM reactivity towards TarM-modified WTA was higher in the two surviving patients (4 and 18) compared to the two deceased patients (16 and 35), and patient 18 also displayed high IgG1 reactivity to TarM-modified WTA (**Figure 4B**). Overall, the mounted antibody responses in the deceased patients was not aligned with the WTA glycoprofile of the infecting strains.

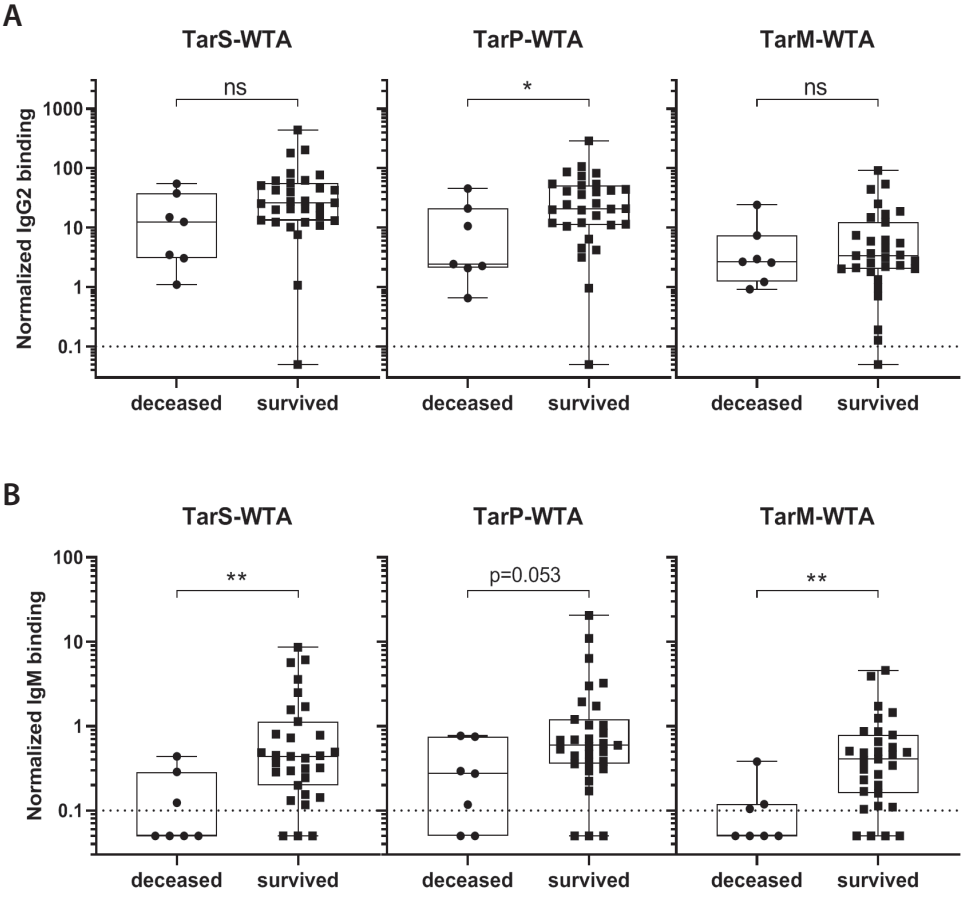


Figure 3. Association between WTA-specific antibody response and mortality on ICU in *S. aureus* bacteremia patients. A, B) Normalized binding of plasma-derived WTA-specific A) IgG2 and B) IgM according to the three WTA glycotypes from ICU patients with *S. aureus* bacteremia, stratified by mortality. Symbols indicate individual patients with *S. aureus* infection (n=38). Dotted line represents the lower limit of quantification, symbols shown below line present extrapolated values. *p < 0.05, **p < 0.01, ns=not significant.

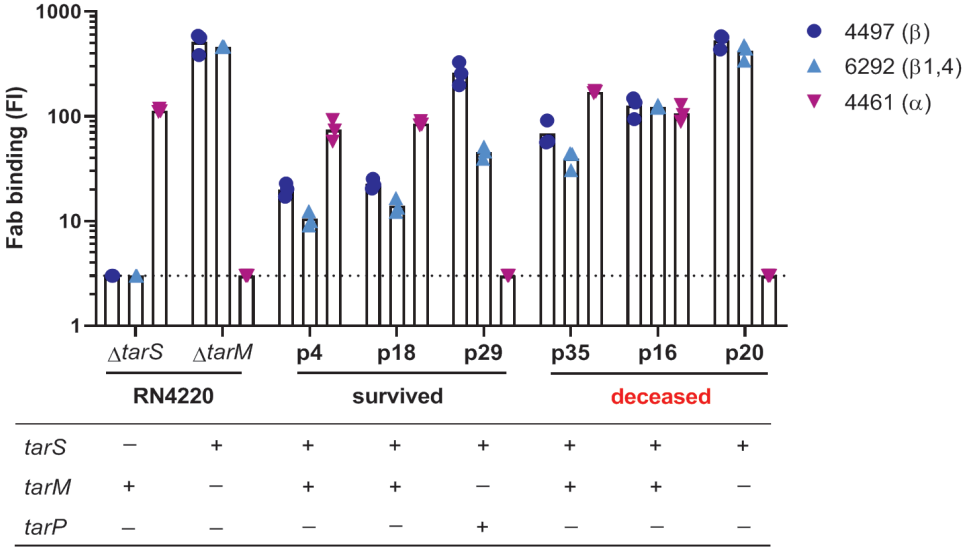
To test whether low WTA-antibody binding impacted downstream functions, we analyzed complement C3 deposition on glycotype-specific WTA beads pre-opsonized with plasma from individual patients. The differences in C3 deposition to WTA beads paralleled the observed differences in antibody binding, as there was less C3 deposition on TarM-WTA beads opsonized with plasma from deceased patients 16 and 35 compared to surviving patients 4 and 18 (**Figure 4C**). Such a difference in C3 complement deposition was not observed for TarS-WTA beads (**Figure 4C**). The results may indicate that decreased antibody binding to the specific WTA glycoprofile of the infecting *S. aureus* strain results in impaired complement activation, which likely impacts bacterial clearance through opsonophagocytosis but also for neutrophil recruitment.

Discussion

In this study, we analyzed the human antibody repertoire to glycosylated *S. aureus* RboP-WTA, an abundant glycopolymer expressed by nearly all *S. aureus* isolates. To measure antibody binding in a robust and reproducible way in human plasma, we used in vitro glycosylated synthetic WTA fragments that resemble the three distinct *S. aureus* WTA glycotypes, i.e. modified with α-1,4-GlcNAc (TarM), β-1,4-GlcNAc (TarS) and β-1,3 GlcNAc (TarP). Robust IgM responses to all three WTA modifications were detected in plasma from at least 95% of healthy individuals (median IgM binding 1.3; average from TarS, TarP and TarM-WTA), whereas WTA-specific IgM responses were approximately three-fold lower in *S. aureus* bacteremia patients (median 0.4) and absent altogether in 20% of patients. Moreover, patients with a negative disease outcome (i.e. died on the ICU) had significantly lower WTA-specific IgM responses within the *S. aureus* bacteremia cohort. As a possible explanation, we observed that there was a mismatch between the glycotype-specific IgM responses and the specific WTA glycoprofile of the patient infecting strain in deceased patients, which resulted in aberrant complement activation, a critical process for bacterial opsonization and neutrophil recruitment.

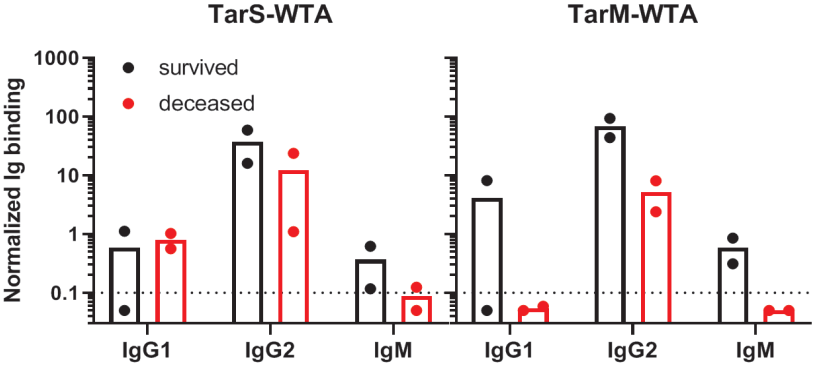
Until now, the complete antibody repertoire to WTA has not yet been fully elucidated for a number of mainly technical reasons. It has proven difficult to dissect specific antibody responses to WTA using intact bacteria, since many additional immunogenic antigens are present on the bacterial surface (26,44), generating high background levels and lowering assay sensitivity. Furthermore, the isolation of endogenous bacterial WTA yields instable preparations with high heterogeneity which are not suitable for robust and reproducible testing. Here, we have used synthetic RboP-WTA oligomers of defined length, which were glycosylated in vitro with recombinant WTA glycosyltransferases, and consequently mimic the three possible *S. aureus* WTA-GlcNAc modifications (40). Resemblance to bacteria-expressed WTA was previously demonstrated using WTA-specific monoclonal antibodies and confirmation of recognition by the C-type lectin receptor langerin (19,40). In a bead-based flow cytometry assay, we could limit the amount of patient material required through multiplex analysis of three antibodies isotypes and subclasses. In addition, similar *S. aureus*

A



tarS	–	+	+	+	+	+	+	+
tarM	+	–	+	+	–	+	+	–
tarP	–	–	–	–	+	–	–	–

B



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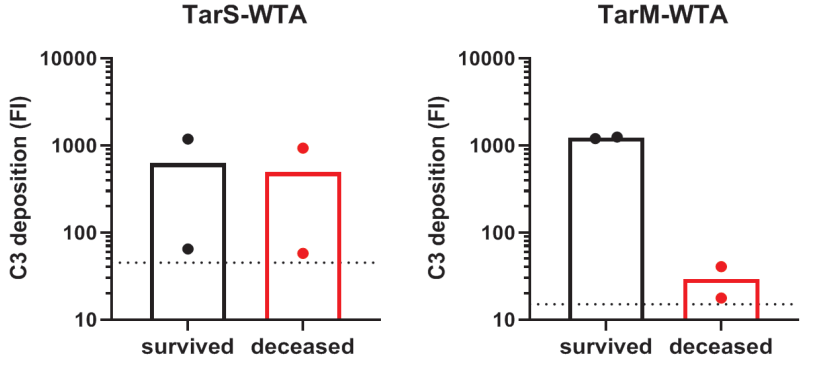

Figure 4. Correlation between WTA-specific antibody presence and functionality with the WTA glycoprofile of the infecting *S. aureus* isolate. A) Fab staining (10 μg/ml, clones 4461, 4497 and 6292) of α-GlcNAc-WTA and β-GlcNAc-WTA in matching clinical isolates from six patients (4, 18, 29, 35, 16, 20) and reference strains RN4220 Δ*tarS* and Δ*tarM*, with corresponding WTA glycosyltransferase genotype (tarS, tarP and tarM) as determined by PCR analysis. B) Normalized antibody binding (IgG1, IgG2 and IgM) to TarS-WTA and TarM-WTA (n=4) in patients stratified by ICU mortality. Dotted line represents the lower limit of quantification, symbols shown below line present extrapolated values. C) C3 deposition on TarS-WTA and TarM-WTA coated beads, pre-opsonized with 3% plasma from same patients as in panel B. Dotted line represents C3 deposition to non-opsonized WTA beads. Fab binding and C3 deposition is shown as fluorescence intensity (FI) from biological triplicates.

antibody profiling studies have only included a fixed concentration of plasma (45–47), while in our experience the inclusion of a plasma concentration range ensures a more reliable determination of specific antibody levels among healthy individuals and patients by checking dilution linearity (48,49). Indeed, for WTA-specific IgG, the included plasma dilutions were still too high to reach dilution linearity and hence the data was considered inconclusive.

The presence of near universal antibody responses to *S. aureus* antigens, including WTA, in healthy individuals suggests widespread exposure to this bacterium in the absence of clinical disease (40,50,51). Although past studies have mainly focused on IgG responses to WTA (22,24,26,52), we now report the presence of lower but robust IgM responses to WTA as well. It is well established that IgM potently activates the complement system, and is better suited to bind structures with repetitive epitopes, e.g. microbial carbohydrates due to higher avidity than other Ig isotypes (29,31,53). In addition, IgM can be poly-reactive since it generally displays lower affinity interactions compared to for example IgG allowing the binding to a broader range of glycan structures. This may explain the observed differences in WTA glycotype binding between IgM and IgG in healthy individuals, where IgM binding to GlcNAcylated RboP WTA is not affected by GlcNAc configuration or linkage position (**Figure 1**). IgM GlcNAc-WTA poly-reactivity may be lost in the process of class-switching and affinity maturation, which consequently limits the ability of IgG to recognize different WTA glycotypes. While IgM production has historically been linked to acute infection due to the short serum half-life, the presence of IgM in the absence of antigen exposure can be maintained by IgM memory B cells (29,54). Overall, poly-reactive IgM binding to structurally divergent WTA may contribute to protective immunity already at early onset of *S. aureus* infection.

Lower antibody responses to different *S. aureus* protein antigens are associated with increased risk of invasive infection and worsened disease progression including septic shock (12,13,47,55). These studies have mainly reported on the impact of decreased IgG and IgA responses, with less focus on IgM-specific responses. Here, we observed lower WTA-specific IgM responses in ICU patients with *S. aureus* bacteremia compared to healthy individuals, whereas IgG2 levels were not affected (**Figure 2**). Moreover, within this patient cohort, patients that died on the ICU had on average even lower WTA-specific IgM responses compared to *S. aureus* bacteremia patients that survived (**Figure 3**), thereby suggesting a protective role for WTA-specific IgM during invasive *S. aureus* infection.

In general, IgM has been proposed to contribute to protection against bacteremia at the onset of infection (56). Impaired IgM responses may therefore be a risk factor for bacterial dissemination which increases risk of mortality.

Glycosylation of *S. aureus* RboP-WTA is mediated by three WTA glycosyltransferases, TarS, TarP and TarM. Thus, the strain-specific ability to express these enzymes will determine the WTA glycosylation profile on the bacterial surface. We sought to determine whether the WTA glycoprofile in a selection of clinical isolates from patients in our cohort matched the detected antibody profile in that particular patient. We confirmed that the genetic presence of WTA glycosyltransferases resembled the actual WTA glycoprofile of clinical isolates by staining in vitro grown *S. aureus* isolates with GlcNAc-WTA specific Fab fragments. This specific staining excludes the possibility that naturally-acquired mutations had affected enzymatic activity of the encoded Tar enzymes. This analysis revealed that two patients that died on the ICU and were infected with a tarM-carrying strain showed lower IgG2 and IgM levels to the TarM-modified WTA glycotype, resulting in impaired complement deposition (**Figure 4**). Therefore, in these two patients, there appears to be a mismatch between the specificity of the WTA-directed immunological response and the WTA glycoprofile of the infecting strain, which may have contributed to impaired bacterial clearance and negative disease outcome. More donors and matched isolates need to be analyzed to confirm these findings and gain a better understanding of protective host immune responses to specific WTA glycotypes. A possible caveat to our strategy is the fact that the regulation of WTA glycosyltransferases expression remains unknown. Consequently, the WTA glycosylation pattern of a specific strain analyzed in vitro could be different from its in vivo-expressed glycoprofile (57).

Patients with bacteremia often contain lower serum immunoglobulin levels compared to healthy individuals as a result of extravasation due to leaky vessels, excessive fluid administration or underlying pre-existing immunodeficiencies. Interestingly however, patients with S. pyogenes bacteremia did not show decreased WTA-specific IgM responses while both patient groups showed similar lower IgM titers (**Supplementary Figure 3C**). Therefore, our findings appear to be pathogen specific, which raises the question whether the absence of WTA-specific IgM in these patients is a cause or a consequence of infection. Additional studies, including the analysis of WTA-specific IgM responses over time, preferably even from before onset of infection, are required to address this question. Previous studies have shown that *S. aureus*-specific IgM responses were absent or decreased over the course of infection in some patients, although total IgM levels were not tested (14,45,58). In our study, we cannot exclude a lack of free serum IgM due to the presence of antibodies elsewhere in the body, e.g. bound to bacteria. However, we do not expect that consumption of *S. aureus*-specific antibodies accounts for the low or absent levels of WTA-specific IgM in these patients for two reasons. Firstly, WTA-specific IgG2 responses are not decreased in patients compared to healthy donors (**Figure 2A**), which would be expected if invading *S. aureus* would consume surface-specific antibodies. Secondly, we did not observe a uniform loss of α-GlcNAc-WTA specific antibodies in patients that

have been infected with a *tarM*-expressing *S. aureus* strain. In contrast, the presence of α-GlcNAc-WTA specific IgM, and also IgG2, seems to be linked to a positive disease outcome in patients that are infected with a *tarM*-carrying strain.

In conclusion, this study highlights the immunogenic properties of *S. aureus* WTA for systemic antibody responses, and shows the existence of a broad antibody repertoire even in healthy donors. Furthermore, we provide evidence that suggests a key role for IgM in targeting *S. aureus* WTA and its different glycotypes presumably through poly-reactivity. IgM binding will activate complement through the classical pathway, thereby facilitating bacterial killing by neutrophils via enhanced opsonophagocytosis and neutrophil recruitment. Therefore, *S. aureus* WTA-specific IgM may be important for a protective immune response during invasive disease. In addition to IgG, the classic opsonic and sought-after vaccine-induced antibody isotype, studies should explore the potential therapeutic application of IgM in preventing mortality during *S. aureus* bacteremia (28,59,60).

Materials and methods

Sample collection – Ethics statements

Blood from healthy individuals (n=31) was collected in EDTA tubes with full informed consent in accordance with the Declaration of Helsinki and after approval from the Institutional Review Board of the University Medical Center Utrecht (METC protocol 07-125/C, approved March 1, 2010). EDTA-plasma was obtained by centrifugation (10 min, 2,000 g at 4°C), and stored at -80°C. Patients, admitted to the intensive care unit (ICU), were included on the basis of specific inclusion criteria within the framework of the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) study (ClinicalTrials.gov, NCT01905033). The Institutional Review Board approved an opt-out consent method (protocol number 10-056C). Plasma samples were collected from leftover blood drawn for routine care. Samples used in this study were collected in EDTA-treated tubes on day one after a positive S. aureus (n=38) or S. pyogenes (n=13) blood culture. EDTA-plasma samples were stored at -80°C within 4 hours after collection from the patient.

Glycosylation of synthetic RboP-WTA hexamers and coating to beads

The synthesis of biotinylated RboP-WTA hexamers and their enzymatic glycosylation by recombinant TarS, TarP and TarM has been described previously (19,39–41). In brief, biotinylated RboP oligomers (0.17 mM) were incubated with recombinant glycosyltransferases TarS, TarP or TarM (6.3 μg/ mL) for 2 h at room temperature with UDP-GlcNAc (2 mM, Merck) in glycosylation buffer (15 mM HEPES, 20 mM NaCl, 1 mM EGTA, 0.02% Tween 20, 10 mM MgCl2, 0.1% bovine serum albumin (BSA), pH 7.4) (Supplementary figure 1A). Next, glycosylated RboP hexamers were coupled to beads

by adding 5×10^7 magnetic streptavidin beads (Dynabeads M280 Streptavidin, Thermo Fisher Scientific) to the individual glycosylation reaction mixtures. After incubation for 15 min at room temperature, the coated beads were washed three times with PBS 0.1% BSA 0.05% Tween-20 using a magnetic sample rack and stored at 4°C. The coating of WTA beads was validated as previously described (19), by binding of monoclonal IgG1 antibodies (3 μg/ml) specific for α-GlcNAc-WTA (TarM-modified WTA, clone 4461), β-GlcNAc WTA (TarS- and TarP-modified WTA, clone 4497) and β-1,4-GlcNAc WTA (TarS-modified WTA, clone 6292), followed by staining with detection antibody goat-anti-human kappa-Alexa Fluor 647 (5 μg/ml, Southern Biotech) and analysis by flow cytometry (BD FACSVerse).

Production of recombinant monoclonal antibodies

Monoclonal antibodies (mAbs) specific for β-GlcNAc-WTA (clone 4497), in different Ig isotypes and IgG subclasses, were expressed in EXPI293F cells (Thermo Fisher) as previously described (19,42), with minor changes for IgM and IgA. We cloned the heavy chain (hG) and kappa light chain (hK) constant regions for human IgG1, IgG2, IgG3, IgM and IgA1 (**Supplementary Table 1**) in the XbaI-AgeI cloning sites of the pcDNA34 vector (ThermoFisher). Sequences for variable regions (VH and VL) were previously described and are derived from patent WO 2014/ 193722A1.50 (22,23). The VH and VL sequences, preceded by a Kozak sequence (ACCACC) and the HAVT20 signal peptide (MACPGFLWALVIST- CLEFSMA), were codon-optimized for human expression and ordered as gBlocks (IDT). We cloned VH and VL gBlocks into the pcDNA34 vector, upstream of the Ig heavy chain (hG) and kappa light chain (hK) constant regions, respectively, by Gibson assembly (New England Biolabs) according to the manufacturer's instructions. NheI and BsiWI were used as the 3' cloning sites for VH and VL, respectively, to preserve the immunoglobulin heavy and kappa light chain amino acid sequence. For IgM, BamHI was used as 3' cloning site for VH. The constructs were transformed in E. coli TOP10F' by heat shock and clones were verified by PCR and Sanger sequencing (Macrogen). Plasmids were isolated by NucleoBond Xtra Midi kit (Macherey-Nagel) and sterilized using 0.22 μm Spin-X centrifuge columns (Corning). We used EXPI293F cells (Thermo Fisher), grown in EXPI293 Expression medium (Thermo Fisher) at 37 °C, 8% CO2 in culture filter cap conical flasks (Sigma) on a rotation platform (125 rotations/ min) for protein production. One day before transfection, cells were diluted to 2×10^6 cells/mL, and 100 mL cell culture was used for transfection the next day. In 10 mL of Opti-MEM (Thermo Fisher), 500 μL PEI-max (1 μg/μL; Polysciences) was mixed with DNA (1 μg/mL cells) in a 3:2 ratio of hK and hG vectors. After 20 min of incubation at room temperature, this DNA/PEI mixture was added dropwise to 100 mL of EXPI293F cells (2×10^6 cells/mL). After 5 days, Ig expression was verified by SDS-PAGE and cell supernatant was harvested by centrifugation and subsequent filtration through a 0.45 μM filter. IgG1 and IgG2 were purified using a HiTrap Protein A column (GE Healthcare) and Äkta Pure (GE Healthcare), eluted in 0.1M citric acid, pH 3.0, and neutralized with 1M Tris, pH 9.0. IgG3 was purified using a HiTrap Protein G column (GE Healthcare),

eluted in 0.1M Glycine-HCl, pH 2.7, and neutralized with 1M Tris, pH 8.0. IgM was purified after dialysis against PBS. Extra NaCl was added to the IgM preparation to a final of 500 mM before application to a POROS™ CaptureSelect™ IgM Affinity matrix (Thermo Scientific) column. IgM was eluted with 0.1M Glycine-HCL pH 3.0. on the ÄKTA Pure. 0.5M NaCl was added to the pooled fraction, which was neutralized with 1M Tris pH 7.5. For IgA1, a Jacalin agarose (Thermo scientific) column was used for purification, followed by elution with 0.1M Melibiose (Sigma). All Ig fractions were dialyzed overnight in PBS at 4°C, and purified mAbs were stored at −20°C.

Deposition of antibodies and complement activation on WTA beads

To analyze the presence of WTA-specific antibodies in human plasma, 1 x 10^5 beads coated with glycosylated RboP-WTA oligomers (WTA beads) and non-coated beads were incubated with a 3-fold serial dilution range of human plasma (concentrations ranging between 0.01% - 3%) in a 96 wells round bottom plate (Greiner) for 20 minutes at 4°C in PBS containing 0.1% BSA and 0.05% Tween-20 (PBS-BT). The beads were washed once with PBS-BT using a plate magnet, incubated with a mixture of (1) goat anti-IgG-PE (clone , goat anti-IgM-FITC and goat F(ab)$_2$ anti-IgA-Alexa Fluor 647 or (2) mouse anti-IgG1 Fc-PE, anti-IgG2 Fc-Alexa Fluor 488 and mouse anti-IgG3 hinge-Alexa Fluor 647 (1 μg/ml, all from Southern biotech) for an additional 20 minutes at 4°C, washed and immediately analyzed by flow cytometry (Supplementary figure 1B).

To measure the deposition of complement C3, WTA beads were opsonized with 3% plasma for 20 minutes at 4°C, washed and incubated with 1% IgG- and IgM-depleted pooled human serum (43) for 30 minutes at 37°C in Veronal Buffered Saline pH 7.4, 0.25 mM MgCl2, 0.5 mM CaCl2, 0.05% Tween-20, 0.5% human serum albumin (HSA). Next, the beads were washed, stained with goat anti-C3 IgG-FITC (1:300, De Beer Medicals) for 20 minutes at 4°C and analyzed by flow cytometry (BD FACSVerse).

Data analysis of antibody binding to WTA beads

Antibody binding to WTA beads was measured by flow cytometry in triplicate or duplicate and median geometric mean fluorescence intensity (geoMFI) values were corrected for median background binding (geoMFI values) to non-coated beads. Background-corrected geoMFI values were interpolated using a standard curve of the β-GlcNAc WTA specific mAb (clone 4497, in IgG1/IgG2/IgG3/IgM/IgA isotype) binding to TarS-WTA beads (**Supplementary figure 1C**). Interpolated values were corrected for the dilution factor and values from at least two dilutions were used to calculate the mean normalized binding for each donor. Values lower or equal to background signals (binding to non-coated beads) were given the set value 0.05. Pooled human EDTA-plasma (from n=9 healthy donors) was included in every measurement as control sample to determine the interassay variation, resulting in a coefficient of variation (CV) <25% (**Supplementary figure 1D**).

ELISA to determine total IgG and IgM levels in human plasma

To determine total IgG and IgM levels in human plasma samples, 96 wells Maxisorp plates (Nunc) were coated overnight at 4°C with sheep anti-IgG or sheep anti-IgM (2 μg/ml in PBS, ICN Biomedicals). The next day, the plates were washed three times with PBS 0.05% Tween-20 (PBS-T), blocked for 1 h at 37°C with PBS-T containing 4% BSA (Serva). After three washing cycles with PBS-T, plates were incubated for 1 h at 37°C with a concentration range of plasma samples in duplicate (5-fold serial dilution starting at 1:10,000 for IgG, 1:1,000 for IgM) as well as a standard for either IgG (0.56-200 ng/ml, ChromPure human IgG, Jackson Immunoresearch) or IgM (3.12-400 ng/ml, ChromPure human IgM, Jackson Immunoresearch). Following three washing steps, horseradish peroxidase (HRP)-conjugated goat anti-human IgG or goat anti-human IgM (1:6,000, Southern Biotech) was added for 1 h at 37°C, and after washing the plates were developed using tetramethylbenzidine (TMB). After 5-10 minutes, the reaction was stopped by adding 1N H2SO4, absorbance was measured at 450 nm in an iMark Microplate Absorbance Reader (Bio-Rad), and values were corrected for background signals at 595 nm. Pooled human plasma (n= 9 healthy donors) was included in every measurement as control sample to determine the interassay variation, resulting in a coefficient of variation (CV) <25% (data not shown).

WTA glycoprofiling of *S. aureus* isolates

DNA was isolated from *S. aureus* isolates, including RN4220 Δ*tarS* and RN4220 Δ*tarM* by incubation with lysostaphin and achromopeptidase (both at 100 μg/ml, Sigma) in 0.5M NaCl, 10 mM Tris-HCl pH 8.0 at 37°C for 30 minutes. Samples were boiled for 5 minutes at 100°C, diluted five-fold in 1 mM EDTA, 10 mM Tris-HCl pH 8.0 and stored at -20°C until further analysis. The presence of tarS, tarP and tarM was determined by PCR analysis using the following primers: tarP (up) 5'-CTTCACGAAAGAGCACTAGAAG-3' and tarP (dn) 5'-TTCCCGGCAAGTTGGTG-3', tarS (up) 5'- GTGAACATATGAG-TAGTGCGTA-3' and tarS (dn) 5'-CATAATGTCCTTCGCCAATCAT-3' and tarM (up) 5'-GGGATACCCATATATTTCAAGG-3' and tarM
(dn) 5'- CAATTCGCTTCGTTGGTACCATTC-3'.

To analyze the correlation between the tar genotype and expressed WTA glycoprofile on the *S. aureus* surface, *S. aureus* isolates were grown overnight in 3 mL of Todd-Hewitt broth (THB; Oxoid) at 37°C with agitation. Overnight cultures were subcultured the next day in fresh THB and grown to a mid-exponential growth phase, corresponding to an optical density of 0.6–0.7 at 600 nm (OD600). Bacteria were collected by centrifugation (10 min @ 3,000g, 4°C) and resuspended in PBS 0.1% BSA to an OD600 of 0.4 (~$1x10^8$ CFU/ml). Bacteria were stained with Fab fragments specific for α-GlcNAc WTA (clone 4461), β-GlcNAc WTA (clone 4497) and β-1,4-GlcNAc WTA (clone 6292-Vk3, all at

10 μg/ml) as described previously (19), followed by staining with goat F(ab')2 anti-human kappa-Alexa Fluor 647 (5 μg/mL, Southern Biotech), fixation in PBS 1% formaldehyde and analysis by flow cytometry (BD FACSVerse).

Statistical analysis

Data obtained by flow cytometry was analyzed using FlowJo 10 (FlowJo LLC). Statistical analysis was performed with Prism software (version 8.3; GraphPad). Data between 3 groups were analyzed using the Kruskal-Wallis test, between 2 groups the Mann-Whitney U test was used. Correlations between antibody binding to WTA beads were assessed using the Spearman correlation. Two-sided p values < 0.05 were considered significant, and are depicted in the figures.

Acknowledgements

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Conflict of interest

A.H. 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 Utrecht University and participated in a post graduate studentship program at GSK.

Funding and additional information

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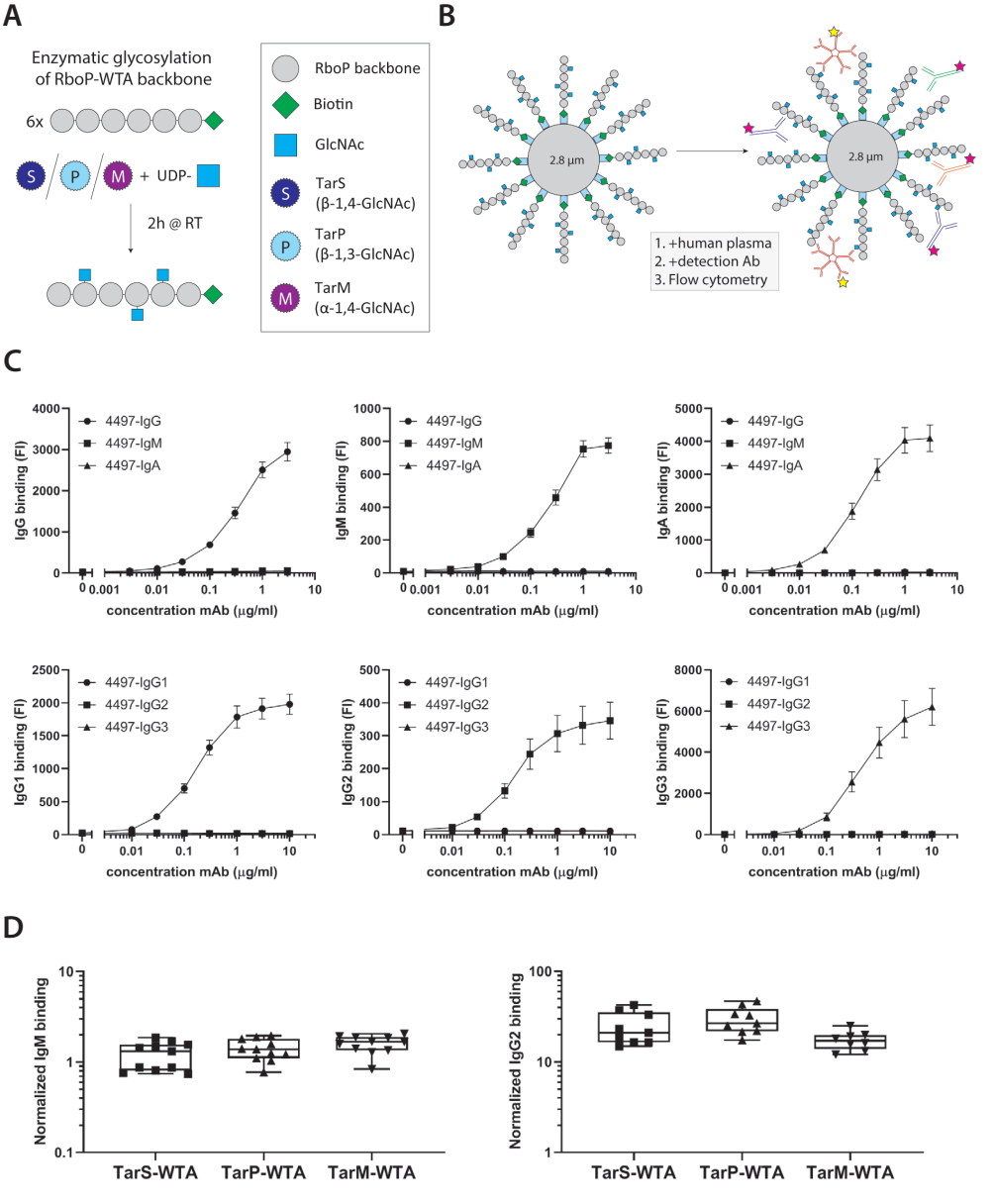
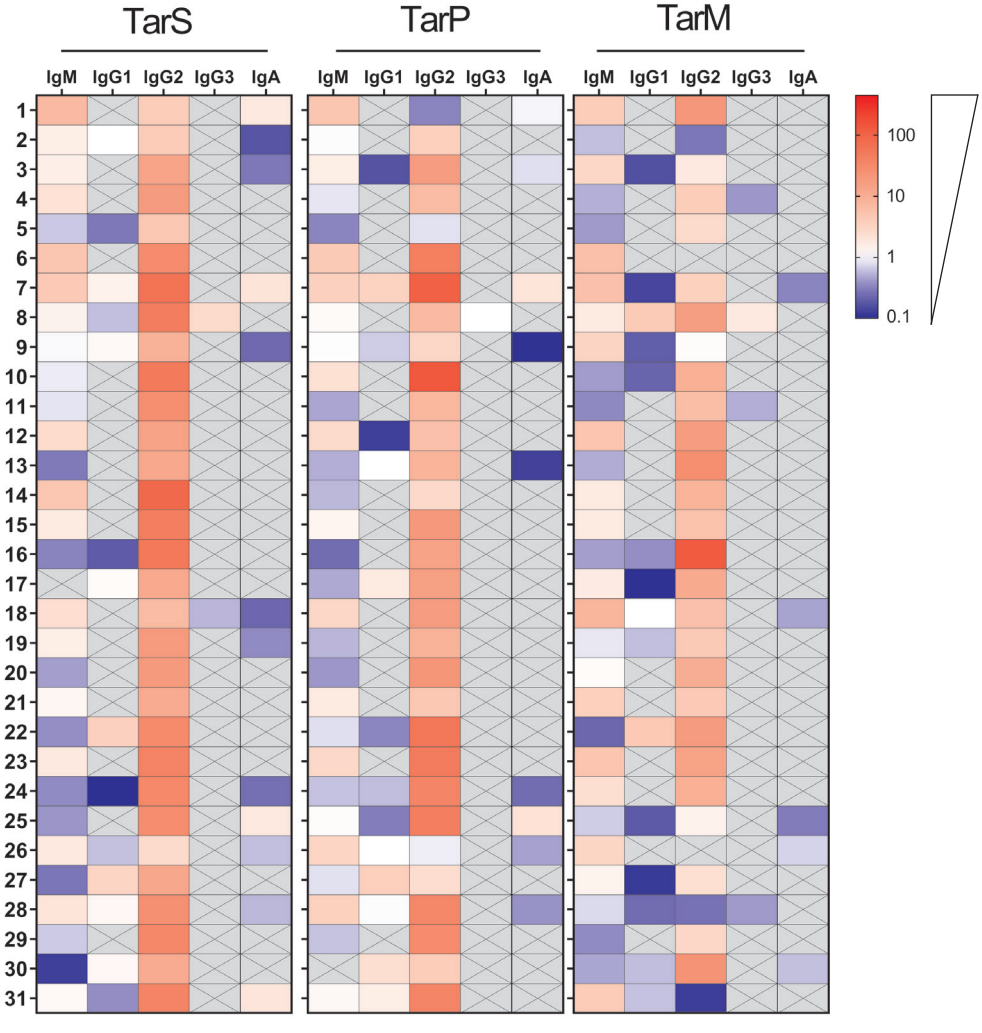
Supplementary figures

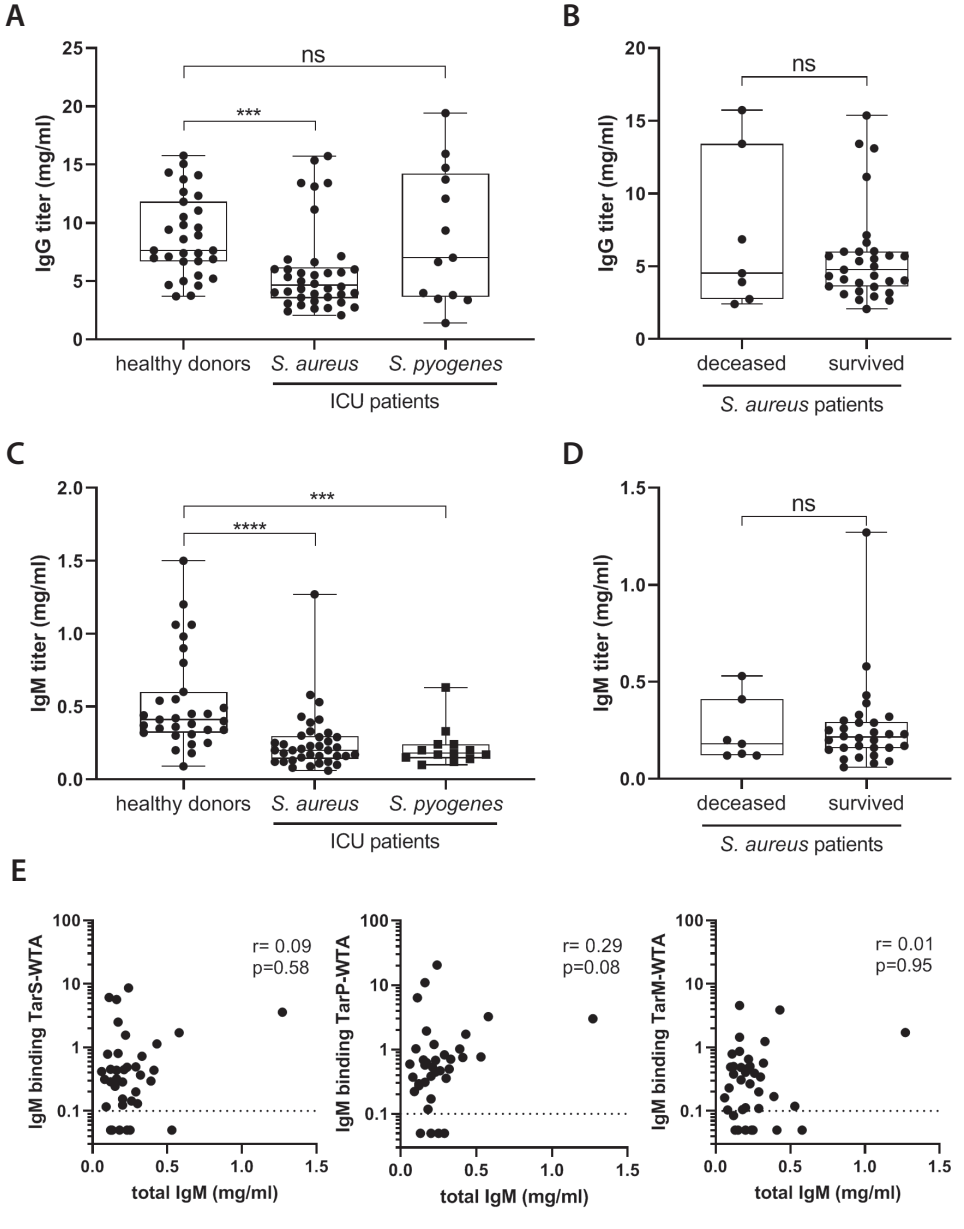


Supplementary Figure 1. Schematic overview of semi-synthetic WTA fragments, bead-based assay to study WTA-specific antibodies in human plasma and interassay variation. A) Schematic representation of in vitro enzymatic glycosylation of biotinylated RboP-WTA hexamers by recombinant enzymes (TarS, TarP and TarM), which are then coated to beads (WTA beads). B) General work-flow to measure antibody binding to WTA beads by flow cytometry. WTA beads are incubated with human plasma (step 1), subsequently stained with a combination of three detection antibodies to analyze multiple antibody isotypes/subclasses in a single sample (Step 2) to measure antibody binding by flow cytometry (step 3).

Continued figure S1 legend
C) Binding of β-GlcNAc WTA specific mAb (clone 4497 in different Ig isotypes/IgG subclasses) to TarS-WTA coated beads. Concentration curves were used for data interpolation, data is depicted as geometric mean fluorescence intensity (FI) + standard error for the mean (SEM) of 6 (for IgG subclasses) or 11 (for IgM, IgA and IgG) independent experiments. D) Interassay variation within control sample (pooled plasma, n=9 healthy donors) in normalized IgM and IgG2 binding to TarS-WTA, TarP-WTA and TarM-WTA beads.



Supplementary figure 2. Heatmap of WTA-specific antibody responses in healthy individuals. Colors match log-transformed normalized binding data for IgM, IgG1, IgG2, IgG3 and IgA to the three WTA glycotypes (mediated by TarS, TarP and TarM). Grey boxes with crosses indicate values equal to or below detection limit (0.1).



Supplementary figure 3. Total IgG and IgM titers in tested donors and correlation between IgM titer and WTA-specific IgM. A) IgG titers of healthy donors (n=31) and patients with *S. aureus* (n=38) or *S. pyogenes* (n=13) bacteremia. B) IgG titers in patients with *S. aureus* bacteremia, grouped on ICU mortality. C) IgM titers of healthy donors and patients with *S. aureus* or *S. pyogenes* bacteremia. D) IgM titers in patients with *S. aureus* bacteremia, grouped on ICU mortality. E) Spearman correlation between WTA-specific IgM responses and total IgM titers in patients with *S. aureus* bacteremia, dotted line represents detection limit. Symbols indicate individual donors, boxplots extend from the 25th to 75th percentiles and the line inside the box represents the median. ***p < 0.001, ****p < 0.0001, ns=not significant.

Supplementary table 1. protein sequence for 4497 antibody isotypes.

	Sequence
Variable heavy chain	
Anti-WTA-IgG (4497)	EVQLVESGGGLVQPGGSLRLSCSASGFSFNSFWMHWVRQVPGKGLVWISFTNNEGTTTAY-ADSVRGRFIISRDNAKNTLYLEMNNLRGEDTAVYYCARGDGGLDDWGQGTLVTVSS
Variable light chain	
Anti-WTA-IgG (4497)	DIQLTQSPDSLAVSLGERATINCKSSQSIFRTSRNKNLLNWYQQRPGQPPRLLIHWASTRKSG-VPDRFSGSGFGTDFTLTITSLQAEDVAIYYCQQYFSPPYTFGQGTKLEIK
Constant heavy chain	
IgG1	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS-GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPS-VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY-RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKN-QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVF-SCSVMHEALHNHYTQKSLSLSPGK
IgG2	ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS-GLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCVECPPCPAPPVAGPSV-FLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNST-FRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTLPPSREEMT-KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSR-WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
IgG3	ASTKGPSVFPLAPCSRSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS-GLYSLSSVVTVPSSSLGTQTYTCNVNHKPSNTKVDKRVELKTPLGDTTHTCPRCPEPK-SCDTPPPCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRCPAPELLGGPSVFLFPPKPK-DTLMISRTPEVTCVVVDVSHEDPEVQFKWYVDGVEVHNAKTKPREEQYNSTFRVVSVLTV-LHQDWLNGKEYKCKVSNKALPAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVK-GFYPSDIAVEWESSGQPENNYNTTPPMLDSDGSFFLYSKLTVDKSRWQQGNIFSCSVMHEAL-HNRFTQKSLSLSPGK
IgM	GSASAPTLFPLVSCENSPSDTSSVAVGCLAQDFLPDSITFSWKYKNNSDISSTRGFPSVLRGG-KYAATSQVLLPSKDVMQGTDEHVVCKVQHPNGNKEKNVPLPVIAELPPKVSVFVPPRDGFF-GNPRKSKLICQATGFSPRQIQVSWLREGKQVGSGVTTDQVQAEAKESGPTTYKVTSTL-TIKESDWLSQSMFTCRVDHRGLTFQQNASSMCVPDQDTAIRVFAIPPSFASIFLTKSTKLT-CLVTDLTTYDSVTISWTRQNGEAVKTHTNISESHPNATFSAVGEASICEDDWNSGERFTCT-VTHTDLPSPLKQTISRPKGVALHRPDVYLLPPAREQLNLRESATITCLVTGFSPADVFVQW-MQRGQPLSPEKYVTSAPMPEPQAPGRYFAHSILTVSEEEWNTGETYTCVVAHEALPNRVTER-TVDKSTGKPTLYNVSLVMSDTAGTCY
IgA1	ASPTSPKVFPLSLCSTQPDGNVVIACLVQGFFPQEPLSVTWSESGQGVTARNFPPSQDASGD-LYTTSSQLTLPATQCLAGKSVTCHVKHYTNPSQDVTVPCPVPSTPPTPSPSTPPTPSPSCCH-PRLSLHRPALEDLLLGSEANLTCTLTGLRDASGVTFTWTPSSGKSAVQGPPERDLCGCYSVS-SVLPGCAEPWNHGKTFTCTAAYPESKTPLTATLSKSGNTFRPEVHLLPPPSEELALNELVTLT-CLARGFSPKDVLVRWLQGSQELPREKYLTWASRQEPSQGTTTFAVTSILRVAAEDWKKG-DTFSCMVGHEALPLAFTQKTIDRLAGKPTHVNVSVVMAEVDGTCY
Constant light-chain (kappa)	
IgG1,2,3,IgM, IgA1	RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSK-DSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

5

"A pancia piena si ragiona meglio"
(with a full stomach, we reason better)
COMMON ITALIAN SAYING

Chapter 5

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.

Key words: *Staphylococcus aureus*, infection, immunity, human skin, tissue-resident memory T cells, $CD4^+$ T cells

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 represent 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.

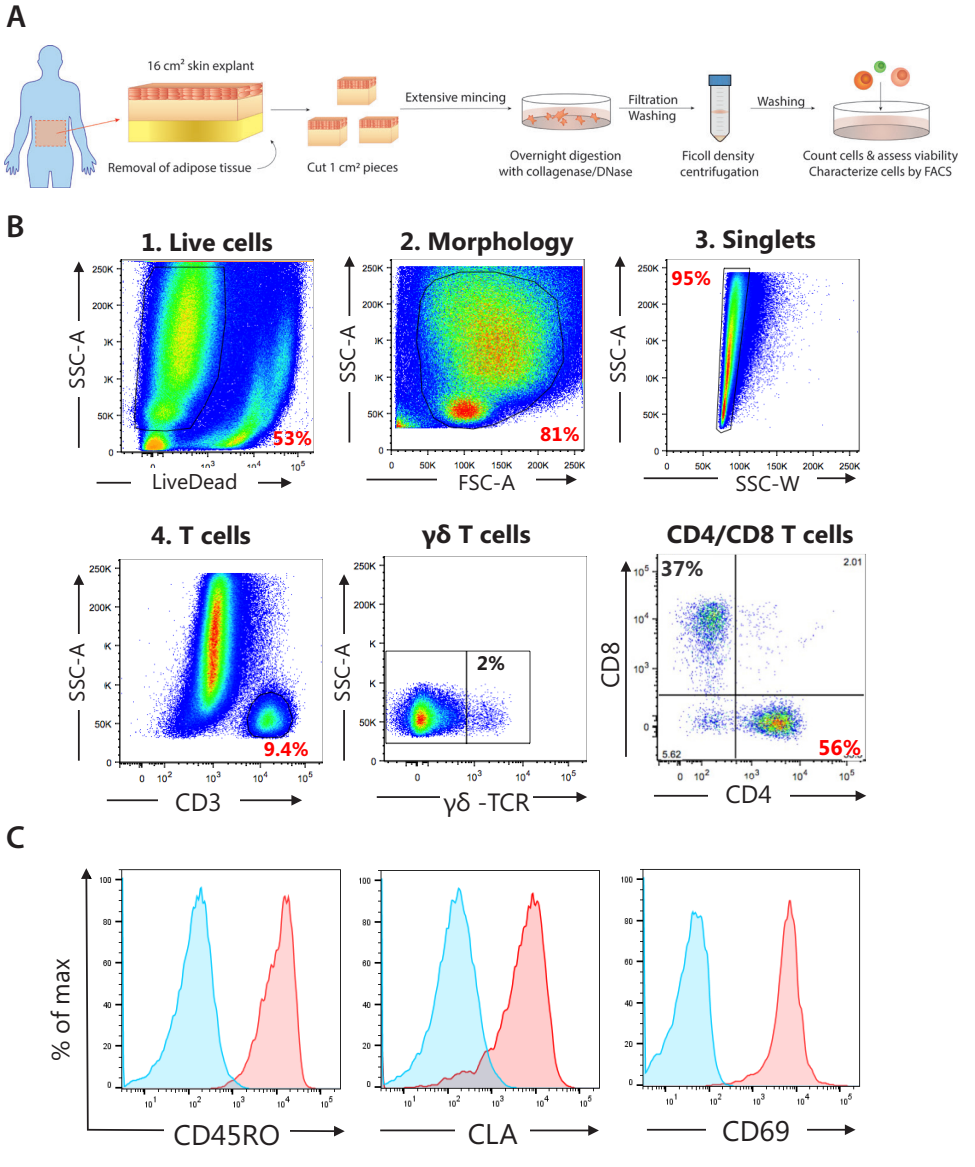

Figure 1. Characterization of T cell subsets in cell suspensions obtained from human skin explants. (A) Schematic overview of the protocol used to obtain single cell suspensions from human skin explants. (B) Gating strategy to analyze T cell subsets, γδ-, $CD4^+$ and $CD8^+$ T cells, in single cell suspensions. (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.

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 the isotype control to the skin cell cultures. Indeed, in the presence of MHC-II blocking antibodies, CD4$^+$ 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.

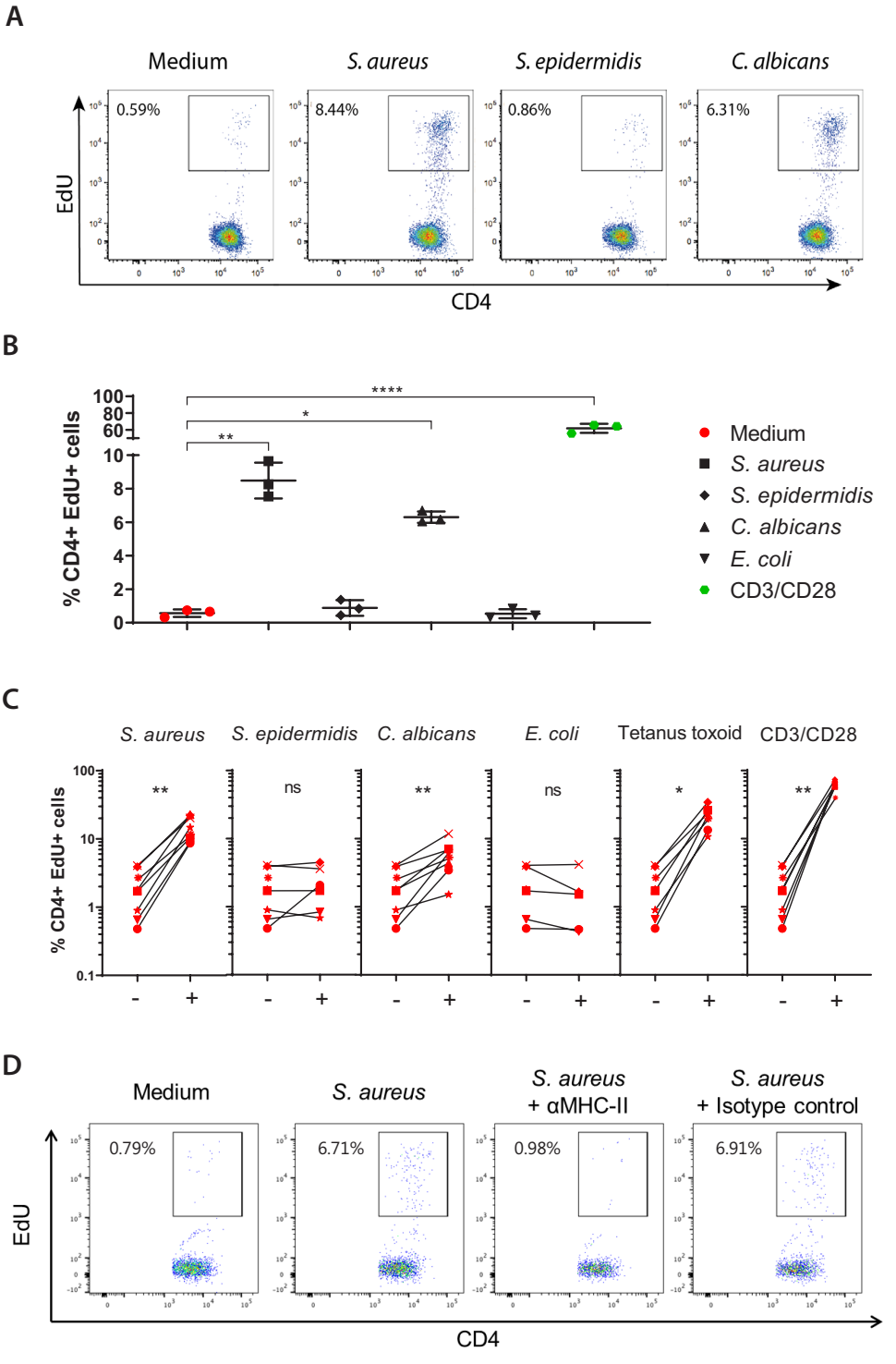
A
Medium
S. aureus
S. epidermidis
C. albicans
0.59%
8.44%
0.86%
6.31%
EdU
CD4
B
% CD4+ EdU+ cells
Medium
S. aureus
S. epidermidis
C. albicans
E. coli
CD3/CD28
C
S. aureus
S. epidermidis
C. albicans
E. coli
Tetanus toxoid
CD3/CD28
% CD4+ EdU+ cells
ns
D
Medium
S. aureus
S. aureus + αMHC-II
S. aureus +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

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. 2C**), 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-α 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).

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 5 x 10^6 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 has been 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*

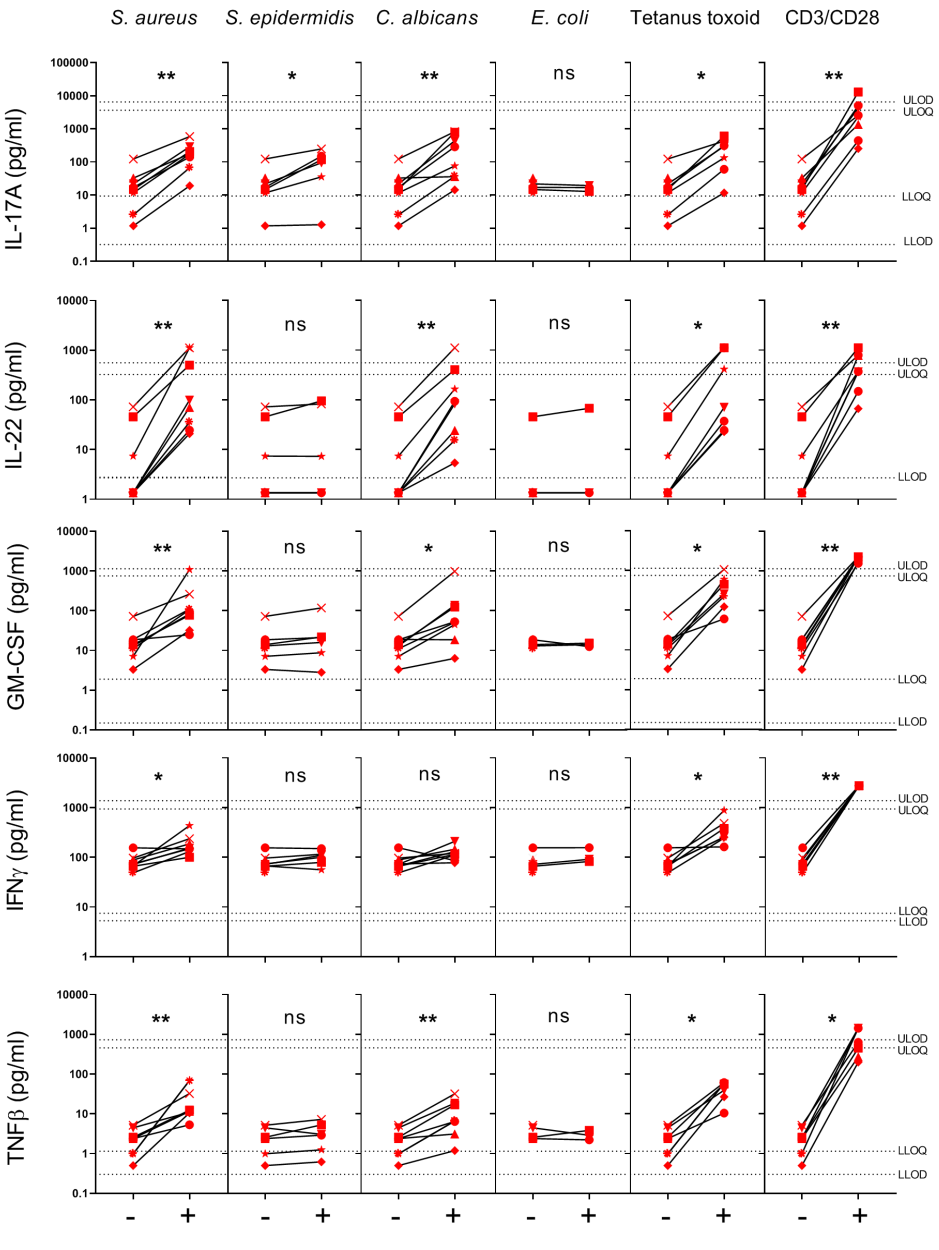

Figure 3. Cytokines secreted in the supernatants of skin cell cultures stimulated with different HK microbes. Supernatants were collected from samples used to determine CD4+ Tsrm cell proliferation by the Click-iT EdU assay. Cytokines were quantified by V-PLEX assays. Cytokine production in response 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.

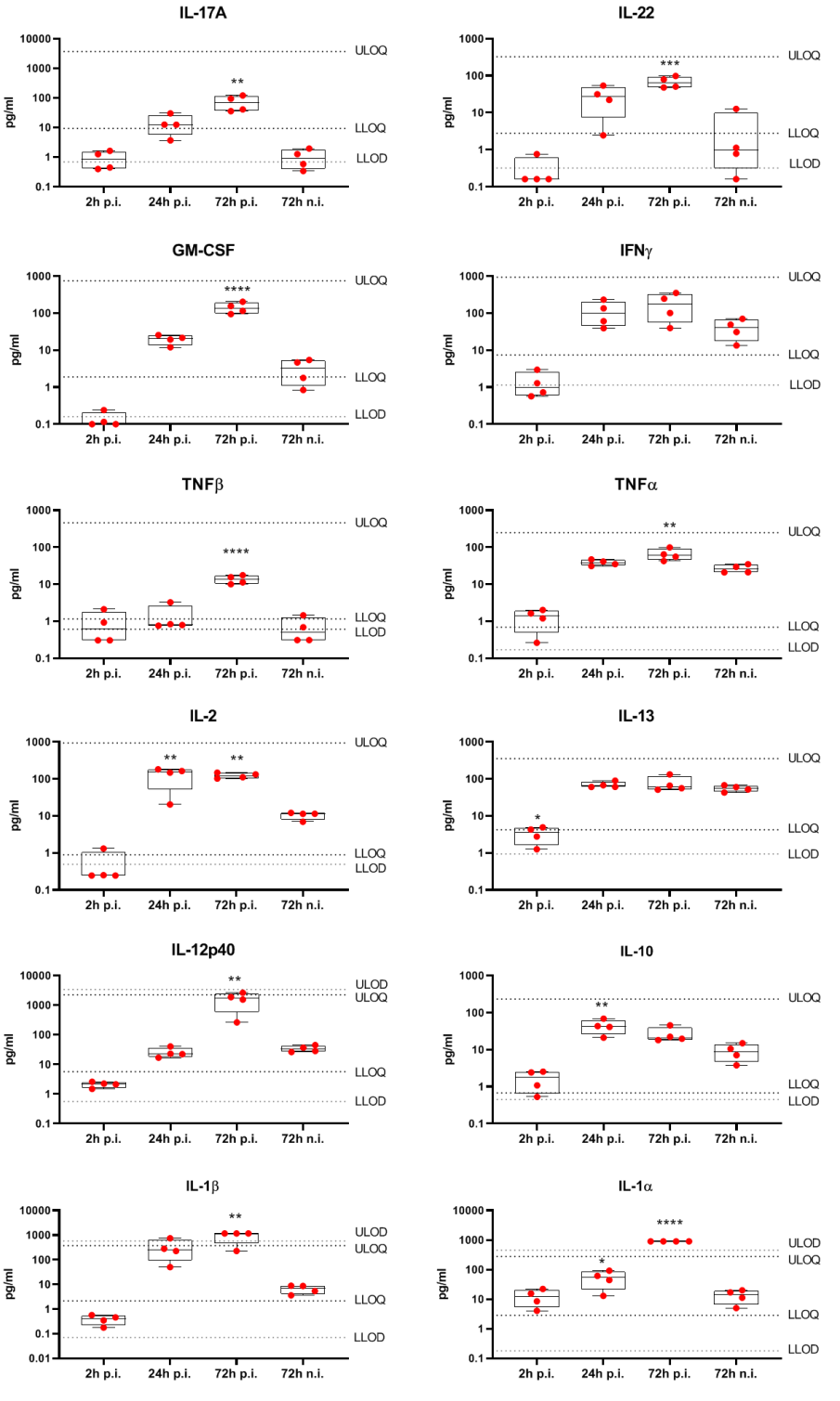
IL-17A
IL-22
GM-CSF
IFNγ
TNFβ
TNFα
IL-2
IL-13
IL-12p40
IL-10
IL-1β
IL-1α
pg/ml
2h p.i.
24h p.i.
72h p.i.
72h n.i.
ULOQ
LLOQ
LLOD
ULOD

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 5 x 10^6 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

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 murine 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.

Materials and Methods

Preparation of single cell suspensions from human skin explants

Fresh human skin explants (16 cm2) 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 cm2 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% CO2 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 x106 cells/cm2 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 (OD600 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.

$CD4^+$ 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% CO2 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) 106 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 2x ULOD (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). Gating strategy is shown in **figure 1B**. 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 ex vivo 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% CO2. 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.

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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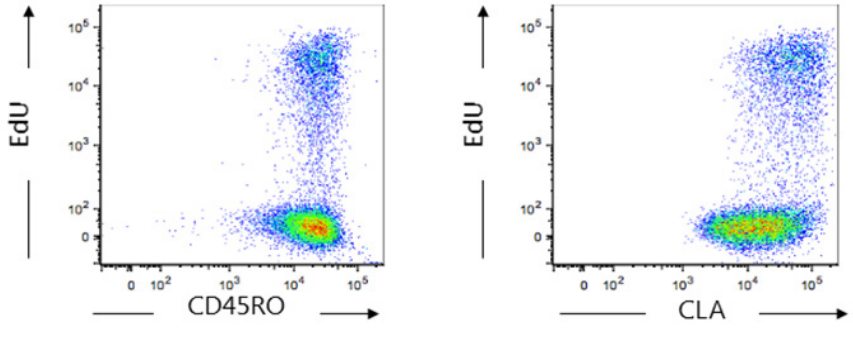
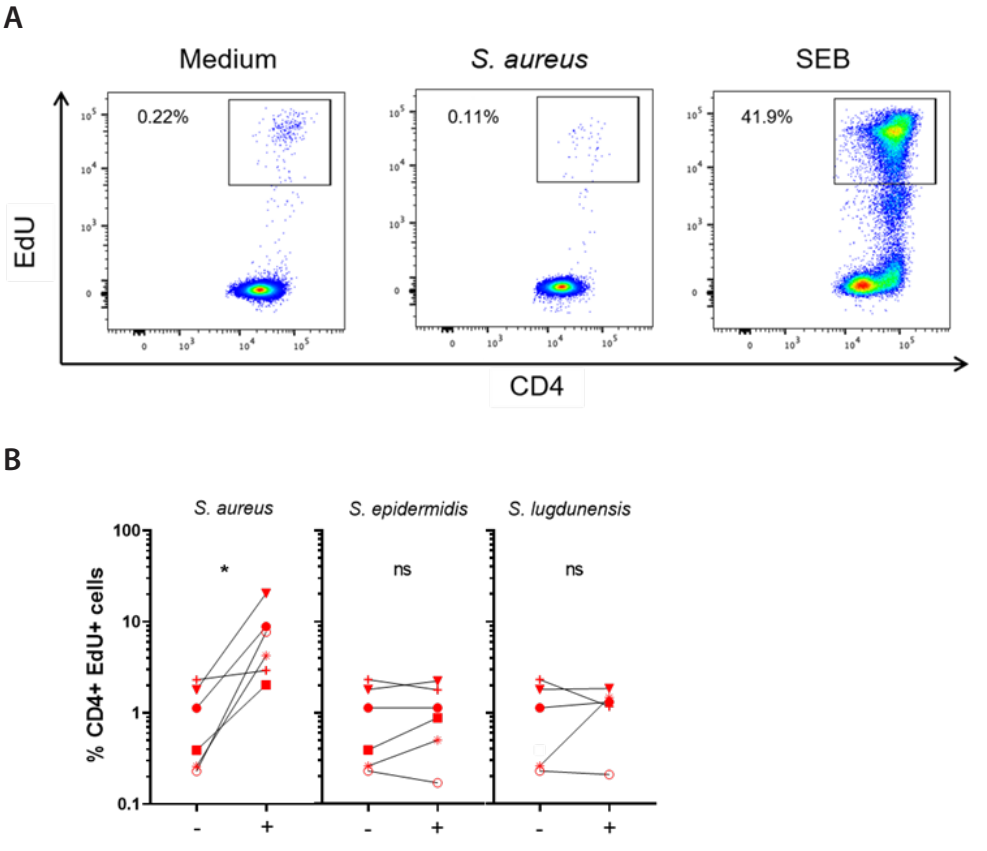
Supplementary information

Supplementary Table 1. List of antibodies used in this study

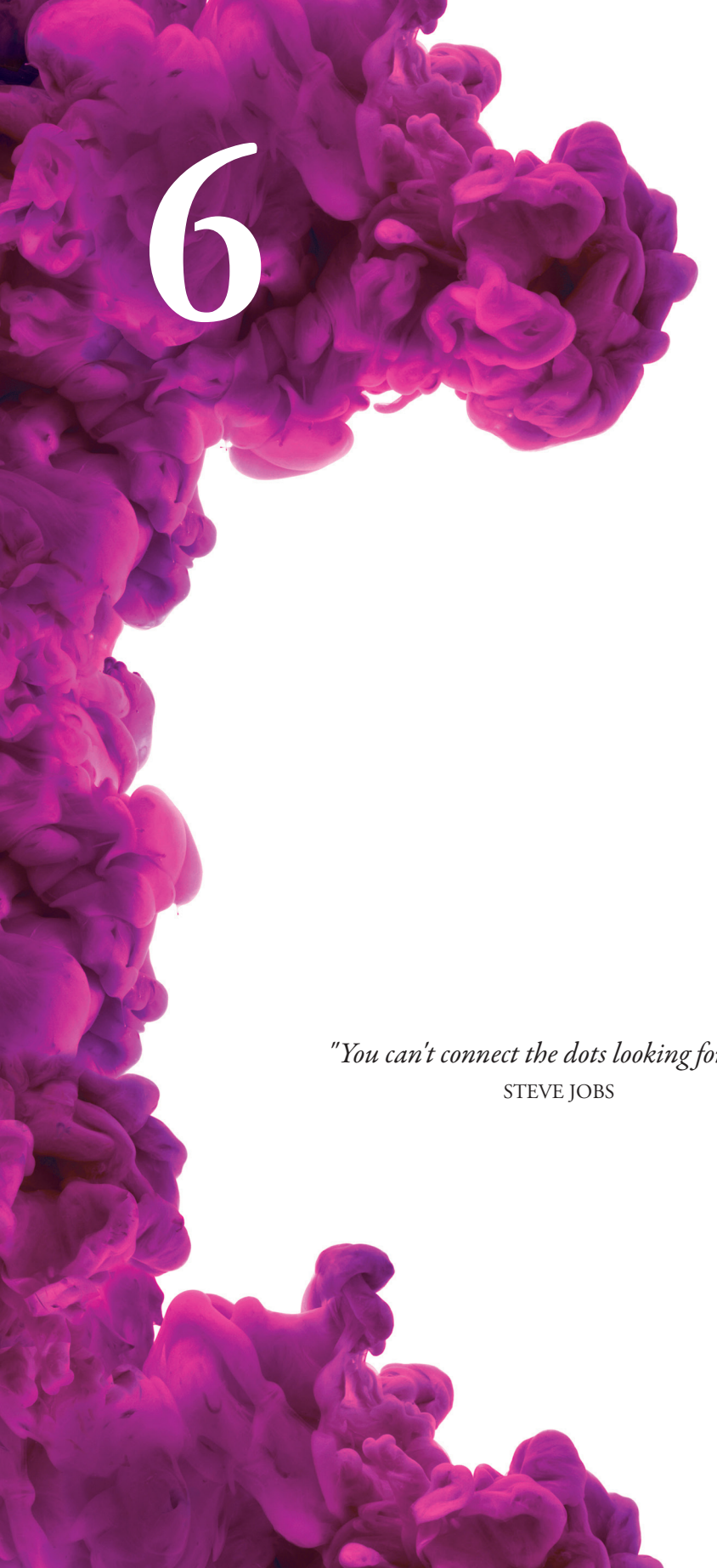
Antibody	Supplier	Clone	Catalogue number
anti-human CD3-PE-CF594	BD biosciences	UCHT1	562280
anti-human CD4-BV421	BD biosciences	RPA-T4	562424
anti-human CD8-APC	Biolegend	RPA-T8	301014
anti-human-γδ-TCR-PECy7	Biolegend	B1	331222
anti-human CLA-BV605	BD biosciences	HECA-452	563960
anti-human CD45RO-BV650	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. ludgunensis* 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.



6

"You can't connect the dots looking forward"

STEVE JOBS

Chapter 6

Summarizing discussion:

A spoon full of sugar – the role of wall teichoic acid in protective immunity against *Staphylococcus aureus*

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Introduction: Lessons learned from past experiences

Staphylococcus aureus represents a significant threat to global health due to the high incidence of invasive infections in combination with increasing acquisition of antibiotic resistance. Consequently, there is need for the development of non-antibiotic alternative treatments such as vaccines. Thus far, all vaccines and antibody-based therapies have failed to show considerable protection in clinical trials, thereby underlining our incomplete understanding on how to boost immunity to this intricate pathogen. Indeed, there is no clear consensus on the immune correlates of protection to *S. aureus*. More insights into the human immune responses to *S. aureus* are needed to rationally design and develop effective immune-based therapies including vaccines.

The selection of conserved and immunogenic antigens is crucial in the development of an efficacious vaccine. Glycoconjugate vaccines targeting capsular polysaccharides have proven very successful for various encapsulated bacterial pathogens including *Streptococcus pneumoniae*, *Haemophilus influenza* and *Neisseria meningitides* (1). These vaccines induce high affinity opsonic antibodies that mediate opsonophagocytosis and killing by neutrophils, key effector cells in the elimination of extracellular bacteria. Since neutrophil-mediated killing is also critical in elimination of *S. aureus*, mimicking this successful vaccine strategy appeared to be a good starting point for *S. aureus* vaccine development. However, glycoconjugate vaccine of *S. aureus* capsular polysaccharides CP5 and CP8, being either the sole component or part of a multicomponent vaccine (2–4), failed to provide protection against infection in various clinical trials. Most likely, this is because capsule expression is variable for different *S. aureus* isolates, and even absent in 20% of clinical strains including in the highly virulent USA300 strains (5,6). Still, other cell wall polysaccharides might be sufficiently conserved and immunogenic as alternative targets for vaccines (7).

Over the past ten years, *S. aureus* wall teichoic acid (WTA) has emerged as an important target for the immune system, with WTA-specific antibodies possessing strong opsonic properties (8–11). WTAs are abundant cell wall incorporated glycopolymers that constitute about 60% of the total cell wall mass, and are involved in various key processes including bacterial adhesion, antimicrobial resistance and cation homeostasis (12–15). Therefore, WTA presents a universally-expressed antigen with promising potential as a vaccine target based on its abundant expression on the *S. aureus* surface, requirement for bacterial survival and overall immunogenicity. However, WTA shows a certain level of diversity with subsequent impact on immune activation. A hallmark feature of *S. aureus* the commonly produced ribitol phosphate (RboP)-WTA is the abundant decoration with D-alanine and N-acetylglucosamine (GlcNAc) residues, with variation in GlcNAc configuration, linkage position and overall abundance on the WTA backbone (16). The strain-specific WTA glycosylation profile or glycoprofile has a massive impact on host immune recognition, and depends at least partially on the genetic presence and expression of dedicated WTA glycosyltransferases TarS (β-1,4-GlcNAc), TarP (β-1,3-GlcNAc) and TarM (α-1,4-GlcNAc)(12,17–19).

This thesis describes novel insights into the human immune recognition of *S. aureus* WTA, that builds on previously published findings (shown in **figure 1**). We also report findings on skin immune responses to *S. aureus* by skin resident Langerhans cells and memory T cells. Here, I will summarize and connect these novel findings to the discussion whether WTA-induced immune responses contribute to protective immunity and the implications for the use of WTA as vaccine antigen. In addition, I will discuss general considerations for future immune-based therapies to tackle *S. aureus* infection.

Importance of local tissue immunity

The development of an efficacious vaccine for *S. aureus* has been hindered by a lack of relevant preclinical models to study human immune responses to *S. aureus* (20). Often, preclinical animal models for vaccine-related studies failed to show translational relevance for a number of reasons. Firstly, *S. aureus* produces a variety of human-specific virulence factors that are not at play in animal models, including numerous immune evasion proteins (21). Furthermore, many animal models exhibit naïve immunity to *S. aureus*, whereas pre-existing immunity to *S. aureus* in humans greatly influences the ensuing vaccine-induced immune response and different immune subsets. For example, in murine skin infection, γδ-T cells have been identified as key IL-17-producing cells that mediate protection to S. aureus. However in the skin, γδ-T cells are replaced by CD4+ memory T cells upon immune system maturation as a result of general aging and consequent antigen exposure (22–24). To obtain a comprehensive understanding of the human immune response to *S. aureus* at relevant infection sites, both in vitro and ex vivo human tissue models should be explored.

S. aureus can asymptomatically colonize human skin. However, *S. aureus* is also the leading cause of skin and soft tissue infections as it often invades the host through the skin as primary entry site. Local immunity at barrier sites, such as the skin but also mucosal surfaces, is believed to be vital for rapid initiation of pro-inflammatory responses that contribute to pathogen clearance. Skin is an immunocompetent organ, with many different immune cells present in homeostatic conditions (25). In **chapter 2**, we expand on how Langerhans cells (LCs), key antigen-presenting cells (APCs) in the epidermis, act as first responders to *S. aureus* skin invasion. Previous work demonstrated that LCs are able to sense *S. aureus* through an interaction with the human C-type lectin receptor langerin (CD207), allowing discrimination from other skin commensals such as coagulase negative staphylococci (26). Langerin specifically recognized TarS-modified WTA. **Chapter 2** shows that langerin is also able to bind TarP-modified WTA, and we show that human langerin specifically binds β-GlcNAc-WTA without the need for additional bacterial co-factors. Langerin-mediated binding to β-GlcNAc-WTA-producing *S. aureus* activates LCs as indicated by maturation and cytokine production. Thus, we suggest that langerin functions as one of the main pattern-recognition receptors (PRRs) that detects *S. aureus*

in the early stages of skin invasion. Besides β-GlcNAc-WTA, there are indications that a possible secondary langerin ligand is expressed on the *S. aureus* surface, as strains lacking glycosylated WTA showed residual and saturable binding to recombinant human langerin. Various glycosylated proteins are anchored to the cell wall (see also **chapter 1**), and may represent targets for langerin, even in strains that show dominant WTA glycosylation with α-GlcNAc.

Activated LCs will enhance early neutrophil recruitment to the site of infection via IL-8 production (27). In addition, activated LCs will migrate to peripheral lymph nodes to either activate naïve T cells, which will differentiate into effector memory T cells, or reactivate memory T cells that migrate to the site of infection or reside in the lymph nodes to mediate B cell responses. Memory T cell responses to *S. aureus* skin infection were initially believed to be solely composed of circulating memory T cells that home to the skin after reactivation (28,29). However, in **chapter 5** we show that *S. aureus*-specific memory T cells reside in healthy human skin. These T cells express classical markers of tissue-resident memory cells, proliferate rapidly in response to heat-killed *S. aureus* and produce IL-17, IL-22 and IFNγ to promote an antibacterial environment, enhance recruitment of neutrophils to the infection site, and induce wound healing. Although antigen presentation by skin APCs is involved in activation of these T cells, it remains unknown which antigens induce this T cell reactivity, or whether these responses contribute to protective immunity.

For many viral, fungal and parasitic pathogens, including herpes simplex virus, *Candida albicans* and *Leishmania major*, skin resident memory T cells have been shown to provide protection against reinfection by rapid activation upon pathogen encounter (23,30,31). For *S. aureus*, local T cell memory could contribute immensely to protective immunity especially in the context of skin infection. This protective effect may have been overlooked in murine infection models in the past since these models often represent primary infections when skin resident T cell memory has not yet been formed. In case memory T cells responses were induced and indeed analyzed, this only concerned the circulating but not resident memory T cells. This potentially novel correlate of protection offers new perspective for future vaccination studies (32). Conventional routes of immunization, such as intramuscular vaccination, may not be efficient for the induction of tissue resident memory T (Trm) cells (33,34). In contrast, tissue-specific signals are likely required to properly prime and induce tissue homing signals in activated T cells, raising the question if targeted antigen delivery to tissue resident DC subsets is crucial in the formation of Trm cells (35,36). Targeting skin resident APCs including LCs via intradermal antigen delivery could provide an attractive alternative vaccination strategy for *S. aureus* as well as a multitude of other skin pathogens e.g. *Candida albicans* and *Streptococcus pyogenes*.

Besides host defense responses, skin resident immune cells such as LCs and T cells also help maintain tissue homeostasis by promoting tolerogenic responses to skin commensals to prevent excessive inflammation. Under homeostatic conditions, LCs preferentially activate skin resident regulatory T cells (Tregs), which in turn control inflammatory T cell

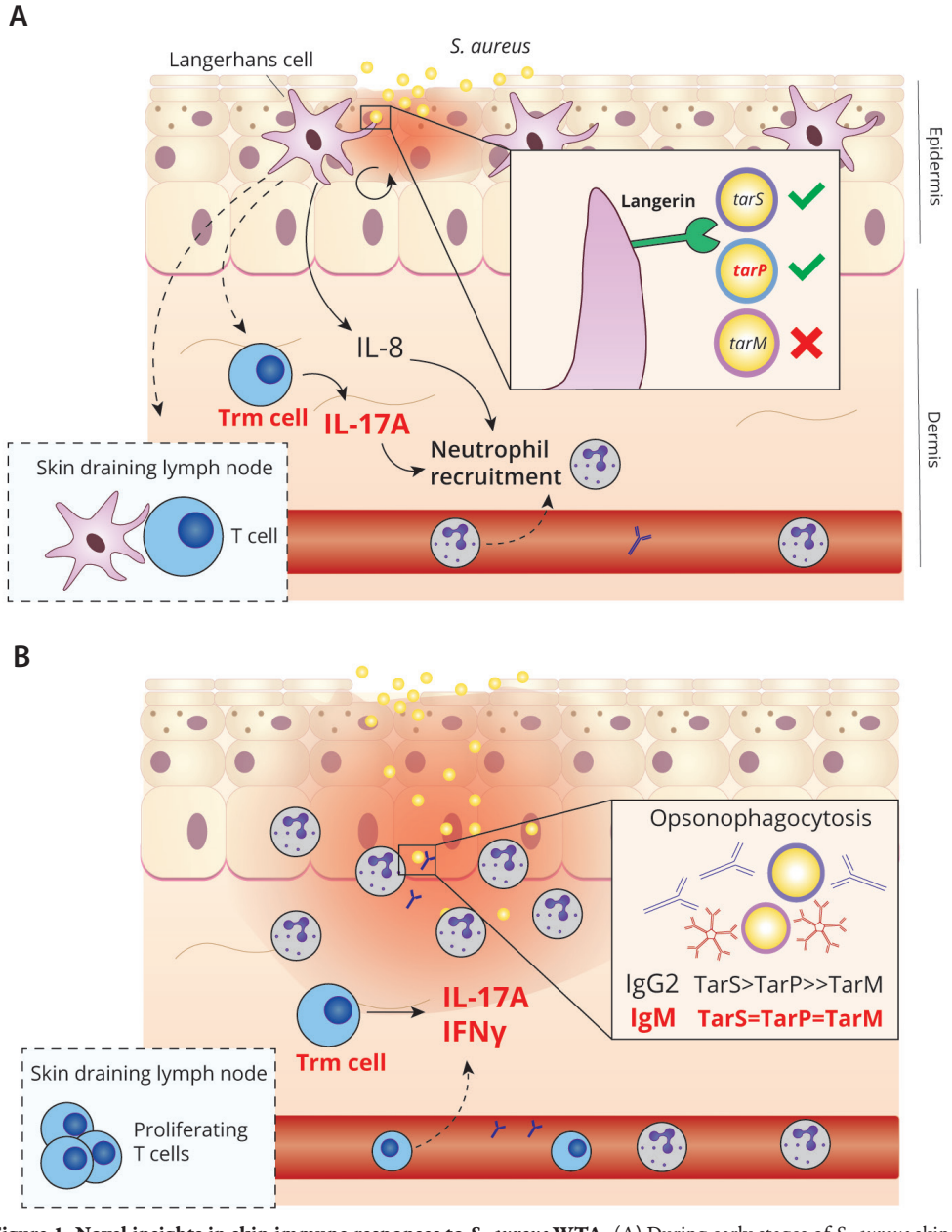

Figure 1. Novel insights in skin immune responses to *S. aureus* WTA. (A) During early stages of *S. aureus* skin invasion, LCs sense β-GlcNAc WTA producing *S. aureus* (modified by TarS or TarP) via the CLR Langerin. Activated LCs produce pro-inflammatory cytokines including IL-8 and migrate out of the epidermis to activate *S. aureus*-specific skin resident memory T (Trm) cells in the dermis, as well as T cells in the skin draining lymph nodes. Upon activation, *S. aureus* specific Trm cells will proliferate locally and produce IL-17A that, in addition to IL-8, promotes neutrophil recruitment to the site of infection. (B) During later stages of skin infection, *S. aureus* is opsonized by WTA-specific antibodies, both IgG2 and IgM, that leads to increased neutrophil-mediated opsonophagocytosis and killing of *S. aureus*. Novel findings presented in this thesis are indicated in red.

responses (37). A loss of tolerance or changes in the skin microbiome composition may contribute to the development of skin inflammatory disorders e.g. atopic dermatitis (AD) (38). AD is a common skin inflammatory disorder caused by a genetic epidermal barrier defect and overall disturbed skin homeostasis (39). Interestingly, dominant *S. aureus* skin colonization is very common, especially in lesional skin, of AD patients. It is hypothesized that *S. aureus* aggravates AD disease progression via the production of numerous toxins and pro-inflammatory factors (40). In a genome-wide association study, a single nucleotide polymorphism in the promoter region of langerin correlated with decreased incidence of AD, suggesting that aberrant langerin expression or functioning contributes to AD pathogenesis (41). Given the established interaction between *S. aureus* and langerin, LCs may be central players in the AD inflammatory cycle by connecting the *S. aureus*- T cell axis. Indeed, LCs may activate local T cells to produce IL-17 and IL-13, which are prime targets of immune therapies against AD (42).

Immune recognition of different WTA glycotypes

Tools that closely mimic *S. aureus* antigens are extremely powerful to study host immune responses in a robust and reliable manner. Specifically for *S. aureus* WTA, the isolation of endogenous WTA yields heterogeneous and instable preparations. In **chapters 2 and 4**, we use synthetic WTA oligomers, that resemble the three *S. aureus* WTA glycotypes through enzymatic glycosylation with recombinant WTA glycosyltransferases, to study human lectin (**chapter 2**) and polyclonal antibody responses (**chapter 4)** to the different WTA glycotypes. In **chapter 3**, we describe the use of fully synthetic WTA oligomers, with defined GlcNAc modifications, to perform an in-depth structure-binding and structure-function analysis of WTA-specific monoclonal antibodies (mAbs). These WTA oligomers could be further optimized to better resemble *S. aureus* WTA by including D-alanine modifications, a technically challenging residue due to its lability.

In **chapter 2,** we show that innate immune recognition of *S. aureus* WTA is affected by anomeric GlcNAc modifications since the C-type lectin receptor langerin only binds β-GlcNAc WTA, and not α-GlcNAc WTA. Adaptive immune recognition of the three WTA glycotypes also greatly differs (8,18). In line with previous studies, **chapter 4** shows that healthy donors exhibit the highest IgG2 titers to TarS-modified WTA, followed by TarP-modified WTA and lastly significantly lower IgG2 levels are present for TarM-modified WTA. Interestingly, in **chapter 4** we also show the existence of robust WTA-specific IgM levels in healthy donors, which show less discriminatory recognition of the different WTA glycotypes. This could be due to increased poly-reactive properties of IgM through low avidity interactions compared to IgG with various microbial glycans (43,44). Besides the antibody binding difference to the three GlcNAc-WTA modifications, in **chapter 3** we also provide compelling new evidence that the spatial distribution of GlcNAc as well as GlcNAc abundance on the poly-RboP chain can impact antibody binding.

We observe a variation in the ability of the adaptive immunity to recognize specific WTA glycotypes. This raises the question whether this antibody variation simply reflects the host's immune exposure to *S. aureus* strains with a specific WTA glycotype or whether the different WTA glycotypes intrinsically differ in terms of immunogenicity. To address the latter, the high IgG titers to TarP-modified β-1,3-GlcNAc WTA likely results from cross-reactive β-1,4-GlcNAc specific antibodies (45). The existence of these cross-reactive antibodies is supported by our data with mAbs (**chapter 3**) and correlation between polyclonal IgG2 responses to TarS- and TarP-WTA (**chapter 4**). Therefore, the presence of high IgG titers that recognize β-1,3-GlcNAc WTA does not necessarily reflect the immunogenicity of this structure (18). To address the question of host immune exposure to different WTA glycotypes, more insight is required in both the tar genotype of colonizing and infecting isolates as well as our understanding of the regulation of WTA glycosylation, since the presence of genes does not warrant its expression. This is clearly illustrated by a recent paper identifying the interaction between *S. aureus* strains of the ST395 lineage and the CLR Macrophage Galactose-type lectin (MGL) (46). This interaction was conferred by the α-N-acetylgalactosamine (α-GalNAc) modification expressed on WTA, which requires the enzyme TagN. Although tagN homologues are expressed by several coagulase-negative staphylococci, no binding to MGL was observed, unless overexpressed on a plasmid using a constitutive promoter (46). A similar situation may occur for *S. aureus* strains encoding two tar enzymes, i.e. *tarM/tarS* or *tarS/tarP*. Although during in vitro growth, WTA glycosylation by TarM and TarP is dominant over TarS due to higher enzymatic activity and/or expression, TarS-mediated WTA glycosylation becomes dominant during in vivo infection and under stress conditions (47). In contrast, *tarM* expression likely increases in response to oxidative stress signals via the GraRS two component system (48,49). In addition to the glycosylation profile, also WTA density in general is linked to bacterial virulence in vivo, which is increased through activation of the important quorum sensing system Agr (50). Caution should therefore be applied when interpreting the genetic profile of WTA biosynthesis genes including glycosyltransferases in clinical *S. aureus* isolates as the genotype may not be directly translate to the specific WTA glycoprofile in vivo (51). Given the possibility for antigenic variation through changes in WTA glycosylation, it seems beneficial to raise a heterogeneous antibody response to *S. aureus* WTA to cover all WTA glycotypes.

Protective antibody responses to *S. aureus* WTA

Antibodies play an important role in battling *S. aureus* infections, by neutralizing secreted toxins and by promoting neutrophil mediated-opsonophagocytosis through binding of surface antigens including WTA. In healthy individuals, WTA-specific IgG antibodies are abundantly present as we have also shown in **chapter 4**. Decreased antibody levels to bacterial cell lysates or individual *S. aureus* protein antigens have been associated with increased susceptibility to invasive *S. aureus* disease (52–55), however for WTA such studies were not previously performed until now. In **chapter 4**, we show that ICU patients

with invasive *S. aureus* infection have decreased WTA-specific IgM titers, and that low or absent WTA-specific IgM might signal increased risk for negative disease progression including increased mortality.

The role of polysaccharide-specific IgM in protective immunity to invasive bacterial infection has not received much attention. However, the importance of anti-glycan IgM is supported by an observation in patients with common variable immune deficiencies (CVID); CVID patients without a history of bacterial pneumonia had a normal frequency of memory IgM B cells and produced anti-pneumococcal polysaccharide IgM antibodies whereas CVID patient with recurrent bacterial pneumonia lacked such cells (56). For *S. aureus*, several reviews have discussed the discrepancy in observations as to whether primary antibody deficiencies (PADs) increase susceptibility to *S. aureus* infection, which led to the conclusion that preexisting *S. aureus* specific antibodies do not confer protection (57,58). However, many of these studies focused on the absence of IgG responses. In light of our findings, it may be important to know whether some patients with PAD still produced IgM, whereby protection is at least partially maintained. Indeed, selective IgM deficiencies increase susceptibility to infections with encapsulated bacteria (59). Interestingly, pre-clinical immunization studies in mice, such as a study with using WTA as vaccine antigen, focus on induction of IgG responses while IgM responses are not been taken into account (10). Overall, IgM may play a more important role in protective immunity to *S. aureus* and other encapsulated bacteria than previously envisaged, by harboring broad reactivity towards bacterial surface polysaccharides resulting in potent complement activation. Future studies should explore the ability of WTA-reactive IgM to bind other glycosylated structures in the cell wall of *S. aureus*, and other bacteria, that contain terminal GlcNAc modifications, such as the family of serine-aspartate dipeptide-repeats (SDR) proteins (60). While S. aureus has evolved different mechanisms to circumvent functional IgG-mediated responses (e.g. through expression of staphylococcal protein A (SpA) (61,62)), IgM-mediated effector functions may not suffer from a similar level of interference, increasing their therapeutic potential.

In addition to IgM, IgG responses to WTA also seem to limit the risk of invasive *S. aureus* infection and mortality. In **chapter 4**, we have analyzed WTA glycotype-specific Ig responses and their possible match to the strain-specific WTA glycoprofile of clinical isolates within individual patients, which revealed a potential correlation between low specific IgG2 responses and increased mortality. This correlation was not evident when analyzing overall differences in WTA glycotype-specific IgG2 responses within ICU patients, since these patients were obviously not all infected with strains exhibiting similar WTA glycoprofiles. This finding supports an approach where the analysis of antibody responses within large cohorts of donors to specific *S. aureus* antigens occurs in conjunction with molecular analysis of the infecting strain to interpret the potential protective capacity of observed antibody responses. This may be particularly important in vaccination studies, where inter-strain variability of vaccine antigen expression in vivo could result in misinterpretation of obtained results on vaccine efficacy.

MHC-II presentation of WTA

Zwitterionic polysaccharides (ZPS), including WTA and capsular polysaccharides from *B. fragilis*, *S. pneumoniae* and *S. aureus*, can be presented on MHC-II thereby activating T cells (63). T cell help is needed for the formation of germinal centers and subsequent affinity maturation of specific B cells. Indeed, DCs can effectively present WTA on MHC-II to activate T cells, since intradermal immunization with WTA induces $CD4^+$ T cells in mice (10). Moreover, WTA-specific memory T cells are commonly found in healthy donors, indicating the ability of human APCs to present WTA to naïve T cells (28). However, we observed that for WTA IgM and IgG2 are the predominant isotypes and these are classically associated with T cell-independent B cell responses. In contrast, we observed only low levels in some donors of T cell-dependent WTA-specific IgG1, IgG3 and IgA. This finding suggests a suboptimal T cell participation in WTA-specific B cell activation, which could be the result of suboptimal MHC-II presentation of WTA compared to other ZPS such as B. fragilis polysaccharide A (PSA). Possible explanations may include the loss of zwitterionic properties after antigen uptake or ineffective processing of WTA altogether. Since labile D-alanine ester bonds are generally more stable at a lower pH, it seems unlikely that zwitterionic properties of WTA are lost in the acidic environment of the endolysosome (64). Unlike large polysaccharides such as PSA, WTA could possibly even be directly loaded on MHC-II as ZPS fragments of ~15 kDa do not require processing prior to MHC-II presentation (65). Furthermore, it may be possible that the difference in WTA uptake or co-stimulation through PRRs between professional APCs and B cells results in differential MHC-II presentation. Indeed, DC-SIGN and TLR2 expression by human DCs is required for PSA-specific T cell activation (66,67). Both DC-SIGN and TLR2 are also expressed on B cells, and could therefore play a role in enhancing MHC-II presentation following BCR-mediated uptake of PSA (68). Subsequently, MHC-II presentation of PSA by B cells may not be representative of the ability of B cells to present other ZPS including WTA. Contradicting results on T cell-dependent B cell activation by ZPS Sp1 from *S. pneumoniae* have been published, where in mice Sp1 immunization induces both IgG1 and IgG2b responses while in humans unconjugated Sp1 immunization did not induce memory B cells although IgG1 titers have not been measured here (69,70).

Finally, we cannot exclude the occurrence of aberrant T cell activation via *S. aureus* secreted superantigens, which could preclude effective and specific T cell help to activated B cells (71). Future studies should explore the capacity of human B cells to process and present WTA to T cells in the assumption that this may be different compared to other ZPS. Zwitterionic synthetic WTA oligomers, with incorporated D-alanine modifications, will be excellent tools to study MHC-II presentation and WTA-specific T cell responses in more detail. Alternatively, WTA presentation may be improved through protein conjugation, as has been shown for non-zwitterionic polysaccharides in different childhood vaccines (63,72).

WTA as vaccine antigen

WTA has all the characteristics of a good target antigen for vaccination against *S. aureus*, since it is highly immunogenic and universally expressed by all strains. However, given the limited but existing variation in WTA glycosylation, incorporation of all three WTA glycotypes could ensure coverage of nearly all *S. aureus* infecting strains. Promising data on the use of WTA as vaccine antigen were obtained in mice, where endogenous WTA, isolated from a *tarM/tarS*-containing strain, provided protection from MRSA infection (10). However, humans are repeatedly exposed to *S. aureus*. Consequently, nearly every individual has acquired a certain level of immunity specific for *S. aureus*, and WTA in particular. The impact of pre-existing immunity on vaccine efficacy should not be discarded. Since WTA antibody responses are predominantly T cell-independent, the administration of conjugated WTA may not just boost existing IgG2 memory B cells, but possibly also recruit T cell help to naïve or existing memory B cells to isotype-switch and produce higher affinity IgG1 and IgG3 antibodies similar to pneumococcal vaccination (73,74). Moreover, synthetic WTA could be explored as potential vaccine antigen as is already the case for synthetic glycans for other bacterial pathogens (75). In general, synthetic glycoconjugate vaccines can be improved to enhance T cell activation and T cell-dependent B cell responses. These vaccines consist of short glycan fragments covalently linked to carrier peptides thereby facilitating MHC-II presentation without additional antigen processing (76). To prevent immune interference by carrier peptide mediated immunity, synthetic zwitterionic WTA molecules will theoretically also enable T cell-dependent B cell responses although more research is needed to test this hypothesis.

Vaccination is not always the best option to promote protective immunity, especially when the host immune system is underdeveloped or defective for instance in neonates or patients with immunodeficiencies. Furthermore, vaccination is not feasible in time-sensitive situations with high risk of infection such as emergency surgeries. In these cases, passive immunization strategies present a promising alternative. Monoclonal antibody therapies are receiving increasing attention in the context of bacterial infections, with cell surface polysaccharides being prime targets for opsonic antibodies. WTA falls in this category and current clinical trials are exploring the efficacy of WTA-specific antibody-antibiotic conjugates as novel therapy (77). Moreover, while the main focus in antibody-based therapies has been on IgG, studies in this thesis highlight the possible importance of IgM, which has not been explored yet as a therapeutic isotype to target bacterial polysaccharides.

In conclusion, our findings and those made by others warrant the inclusion of WTA in a future multicomponent vaccine for *S. aureus*, in which both neutralizing antibodies for secreted toxins as well as highly opsonic antibodies are induced with appropriate T cell help. In general, besides the intensively studied capsular polysaccharides as target antigen in successful glycoconjugate vaccines, other surface glycans might be equally excellent targets for immune-based therapies or vaccines against bacterial pathogens for which such strategies have proven unsuccessful.

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"Nog eentje voordat het gedonder begint"

JOOP HENDRIKS

Closing pages

Nederlandse samenvatting

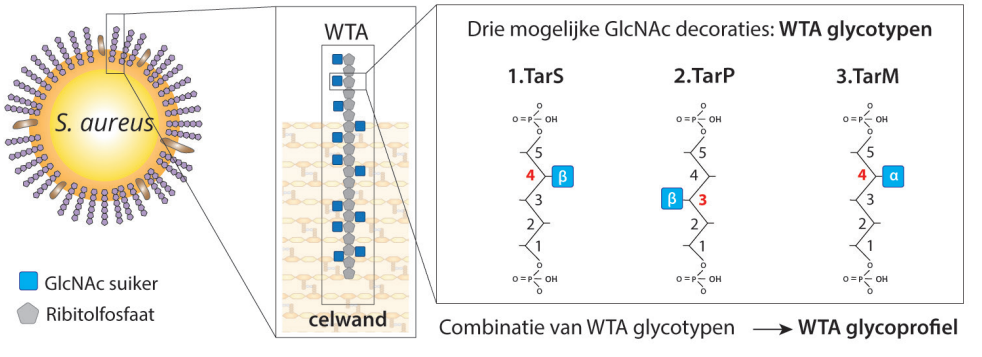
Ons immuunsysteem komt dagelijks in contact met gevaarlijke bacteriën en virussen. Een van die bacteriën is *Staphylococcus aureus* (de stafylokok). Ongeveer een derde van de mensen draagt deze bacterie bij zich, in de neus, keel of op de huid, zonder enige klachten. Echter is deze bacterie ook in staat om verschillende soorten infecties te veroorzaken. Veel van de *S. aureus*-gerelateerde infecties vinden plaats in het ziekenhuis, na bijvoorbeeld operatieve ingrepen, maar ook buiten het ziekenhuis komen *S. aureus* infecties veel voor. Dit kunnen oppervlakkige huidinfecties zijn zoals krentenbaard, of onderhuidse abcessen en longontstekingen. Door meervoudige blootstelling heeft bijna iedereen natuurlijke afweer opgebouwd tegen deze bacterie. Helaas biedt deze natuurlijke afweer onvoldoende bescherming, omdat terugkerende infecties vaak voorkomen. De bacterie kan namelijk op verschillende manieren het immuunsysteem actief onderdrukken of omzeilen. Daarnaast worden steeds meer stammen van *S. aureus* resistent tegen antibiotica, waardoor de noodzaak voor een alternatieve behandelingsmethode toeneemt. Een voorbeeld van een alternatieve methode is het boosten van de immuunrespons tegen *S. aureus* door middel van vaccinatie of passieve immunisatie door antistof-therapieën.

Ondanks meerdere pogingen is er tot op het heden nog geen vaccin ontwikkeld dat voldoende bescherming biedt tegen *S. aureus* infecties. Hier zijn verschillende redenen voor. Ten eerste is het onduidelijk hoe een goede immuunrespons tegen *S. aureus* eruit ziet, en welke componenten van het immuunsysteem betrokken moeten zijn om volledige bescherming te bieden. Het is dus belangrijk om een zo volledig mogelijk beeld te krijgen van de immuunreacties tegen *S. aureus* in de mens. Deze inzichten kunnen bijdragen aan de ontwikkeling van een goed vaccin. Ten tweede is het onduidelijk welke componenten van *S. aureus* het beste gebruikt kunnen worden als target voor vaccinatie.

Het rijksvaccinatieprogramma in Nederland bevat meerdere succesvolle vaccins tegen schadelijke bacteriën zoals pneumokokken en meningokokken. Deze vaccins boosten het immuunsysteem met behulp van specifieke suikerkapsels die op het oppervlak van veel bacteriën te vinden zijn, waarna het immuunsysteem beter in staat is de bacterie te herkennen en op te ruimen. Deze strategie is ook gebruikt voor *S. aureus*, maar helaas zonder succes. Echter, zijn er naast het suikerkapsel nog veel meer andere suikerstructuren aanwezig op het oppervlak van de bacterie die een goede target zouden kunnen zijn voor vaccinatie.

In dit proefschrift hebben we gefocust op specifieke celwand suikers van *S. aureus*, namelijk de wall teichoic acids (WTAs), en hebben we onderzocht hoe het immuunsysteem hierop reageert. Daarnaast hebben we ingezoomd op specifieke immuunreacties tegen deze bacterie in de huid, omdat dit een van de voornaamste plekken is waar *S. aureus* het lichaam binnendringt.

Voordat ik verder in ga op de inhoud van dit proefschrift, is het eerst belangrijk om te begrijpen hoe *S. aureus* WTA er op moleculair niveau uit ziet, en waarom het belangrijk is voor de bacterie. Over het algemeen bestaat *S. aureus* WTA uit één rechte keten, gemaakt van ribitolfosfaat, die verder gedecoreerd is met GlcNAc suikers (zoals te zien in **figuur 1**). Gedecoreerd (of geglycosyleerd) WTA speelt een rol in de celdeling, is belangrijk voor de hechting aan epitheelcellen in de neus, en draagt bij aan antibiotica resistentie. De decoratie van GlcNAc suikers kan plaatsvinden op drie unieke manieren (ook wel WTA glycotypen genoemd). Er zijn dan ook drie enzymen beschreven die de WTA keten met GlcNAc suikers kunnen decoreren, namelijk TarS, TarP en TarM (Figuur 1). TarS en TarM decoreren de WTA keten met GlcNAc suikers op dezelfde positie (C4), maar in andere orientatie (β-GlcNAc voor TarS, α-GlcNac voor TarM). Net als TarS, decoreert TarP WTA ook met β-GlcNAc maar dan op een andere positie (C3 in plaats van C4). Niet alle *S. aureus* stammen zijn in staat om alle drie de enzymen te maken, de helft van alle *S. aureus* stammen maakt alleen TarS, ongeveer een derde (35%) maakt zowel TarS als TarM, en een klein deel (5-10%) maakt TarS en TarP. De combinatie van de aanwezige enzymen, en dus ook de aanwezige WTA glycotypen, bepaalt het WTA suikerprofiel van een desbetreffende stam. Uit eerder onderzoek is gebleken dat het immuunsysteem de verschillende WTA glycotypen niet altijd even goed kan herkennen, dus de mate van activatie van het immuunsysteem zal verschillen op basis van het WTA glycoprofiel van een binnendringende *S. aureus* stam. In dit proefschrift hebben we in meer detail bestudeerd hoe verschillende componenten van het immuunsysteem reageren op de drie WTA glycotypen.



Figuur 1. Overzicht van de structuur van *S. aureus* WTA celwandsuikers en de drie verschillende GlcNAc decoraties door TarS, TarP en TarM. TarS decoreert de lineaire WTA keten, bestaande uit ribitolfosfaat (5 koolstofatomen en een fosfaatgroep), op positie 4 met β-GlcNAc suikers, TarP plaatst β-GlcNAc suikers op positie 3 en TarM modificeert de ribitolfosfaat keten met α-GlcNAc suikers op positie 4. De combinatie van aanwezige enzymen TarS, TarP en TarM en desbetreffende GlcNAc decoraties bepaalt het WTA glycoprofiel van een *S. aureus* stam

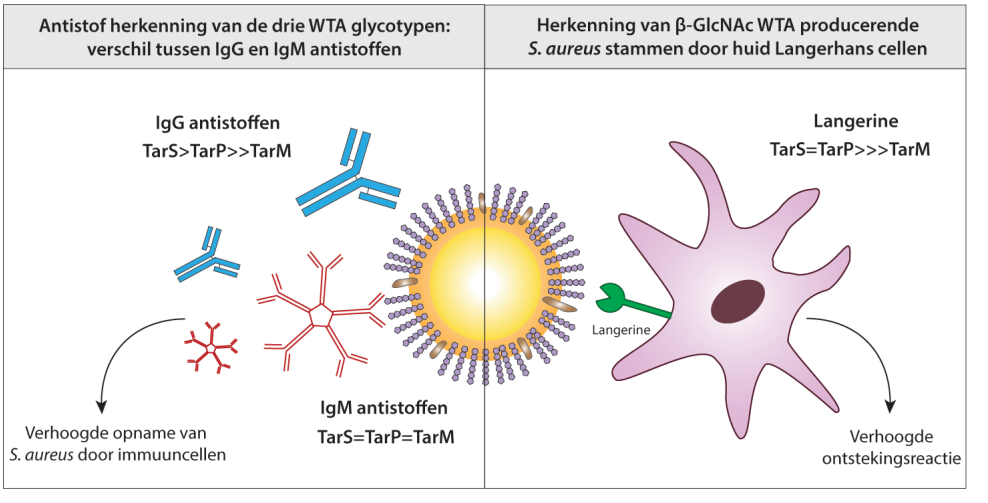
Bevindingen beschreven in dit proefschrift

Hoofdstuk 2 beschrijft hoe belangrijke immuuncellen in de opperhuid, Langerhans cellen, *S. aureus* herkennen en dus vroeg tijdens een huid infectie het immuunsysteem activeren en zo bijdragen aan een effectieve afweerreactie tegen de bacterie. Deze studie bouwt voort op een vorige studie waar de interactie tussen Langerhans cellen en *S. aureus*voor het eerst is beschreven. Deze interactie is afhankelijk van de aanwezigheid van een specifieke immuunreceptor op het oppervlak van Langerhans cellen, langerine. Langerine behoort tot een familie van immuunreceptoren die lichaamsvreemde suikerstructuren, van zowel bacteriën en virussen, kan herkennen. Tijdens de vorige studie is duidelijk geworden dat langerine *S. aureus* WTA kan herkennen dat gedecoreerd is door TarS, maar niet TarM. Toentertijd was het derde enzyme, TarP, echter nog niet geïdentificeerd. **Hoofdstuk 2** laat zien dat langerine ook in staat is WTA te herkennen dat gedecoreerd is door TarP. Verder hebben in dit hoofdstuk een nieuwe tool gebruikt, namelijk synthetische WTA fragmenten. Het is heel lastig om WTA uit bacteriën te isoleren, de WTA ketens zijn erg instabiel en er zit veel variatie in qua lengte en GlcNAc suikerdecoratie. De synthetische fragmenten die hier gebruikt zijn, lijken op de WTA ketens zoals die op de bacterie voorkomen, maar zijn in het lab geproduceerd. Deze fragmenten zijn erg stabiel, hebben allemaal dezelfde lengte, en kunnen we vervolgens individueel decoreren met GlcNAc suikers door één van de drie enzymen (TarS, TarP of TarM) toe te voegen. Deze fragmenten kunnen ook gekoppeld worden aan plastic bolletjes (of beads) om bacteriën na te bootsen. Met behulp van deze WTA fragmenten hebben we laten zien dat langerine specifiek bindt aan WTA gedecoreerd met β-GlcNAc (TarS en TarP), maar niet α-GlcNAc (TarM). De oriëntatie van de GlcNAc suikers op WTA ketens bepaalt dus of langerine kan binden, en Langerhans cellen geactiveerd kunnen worden (**figuur 2**).

In **hoofdstuk 3** hebben we net als in hoofdstuk 2 gebruik gemaakt van de synthetische WTA fragmenten, maar dan in de context van antistof binding (IgG). IgG antistoffen zijn belangrijke componenten van het immuunsysteem die bacteriën of virussen kunnen herkennen, en blijven in hoge concentraties aanwezig in het bloed na bijvoorbeeld vaccinatie of natuurlijke blootstelling aan pathogenen. *S. aureus* WTA suikers vormen een belangrijke target voor IgG antistoffen om te binden, waarna de bacteriën worden opgenomen en gedood door immuuncellen. Gezonde mensen hebben ook IgG antistoffen tegen de *S. aureus*WTA suikers, maar er is verschil tussen de WTA glycotypen. De meeste IgG antistoffen binden aan WTA gedecoreerd door TarS (β-GlcNAc), waarvan een klein deel ook TarP gelinkte GlcNAc suikers kan binden en dus kruis-reactief is. Hier hebben we geprobeerd in kaart te brengen hoe IgG antistoffen, afkomstig van patiënten met een ernstige *S. aureus* infectie, binden aan een collectie van verschillende synthetische WTA fragmenten. Deze collectie bevat meerdere combinaties van GlcNAc suiker decoraties op verschillende posities op de WTA keten (te zien in **figuur 1B**, **pagina 60**). We laten hier zien dat niet alleen de oriëntatie van GlcNAc suikers aan de WTA keten (α- of β-GlcNAc) bepalend kan zijn voor IgG antistof binding, maar ook de positie op de lineaire WTA streng. Sommige IgG antistoffen maken echter geen onderscheid tussen GlcNAc suikers in

het midden of uiteinden van de WTA keten. Er is meer onderzoek nodig om deze antistof binding criteria verder uit te pluizen/uit te diepen, maar het biedt perspectief voor de ontwikkeling van toekomstige antistof therapieën tegen *S. aureus* WTA.

In **hoofdstuk 4** hebben we wederom gebruik gemaakt van de synthetische WTA fragmenten, gekoppeld aan plastic beads, om de aanwezigheid van antistoffen tegen de drie WTA glycotypen te onderzoeken in het bloed van zowel gezonde mensen en IC patiënten met een *S. aureus* bloedvergiftiging. Uit eerder onderzoek is gebleken dat gezonde mensen veel antistoffen tegen *S. aureus* WTA hebben, alleen is hier vooral gekeken naar IgG antistoffen. Hier laten we zien dat gezonde mensen ook een ander type antistof hebben tegen WTA: namelijk IgM. De aanwezigheid van IgM antistoffen werd vroeger vooral geassocieerd met een actieve infectie, maar ook in de afwezigheid van infectie blijven lage maar stabiele IgM concentraties in het bloed. Onze resultaten laten zien dat IgG antistoffen over het algemeen binden aan 1 specifiek WTA glycotype, maar dat de IgM antistoffen meer kruis-reactief lijken te zijn (**figuur 2**). IC patiënten hebben minder IgM antistoffen tegen WTA dan gezonde mensen, en de afwezigheid van deze antistoffen lijkt samen te gaan met een negatief ziekteverloop. Daarnaast hebben we infecterende stammen, geïsoleerd van een aantal patiënten, geanalyseerd op het specifieke WTA glycoprofiel en vervolgens vergeleken met het antistofpatroon van de patiënt. In gevallen waar de patiënt de infectie niet had overleefd, waren de aanwezige antistoffen niet in staat het desbetreffende WTA glycoprofiel te herkennen. Dit suggereert dat de aanwezigheid van antistoffen tegen de WTA suikers lijkt bij te dragen aan een goede afweerreactie tegen *S. aureus* infectie, en een gunstig ziekteverloop.



Figuur 2. Herkenning van de drie *S. aureus* WTA glycotypen (TarS, TarP en TarM) door het immuunsysteem.

In **hoofdstuk 5** hebben we gekeken naar de lokale afweer tegen *S. aureus* in de huid. *S. aureus* is de nummer 1 oorzaak van bacteriële huidinfecties, en de huid vormt een prominente plek voor de bacterie om het lichaam binnen te dringen. Het is dus belangrijk dat immuuncellen in de huid snel en adequaat kunnen reageren op deze bacterie bij overbrugging van de natuurlijke huidbarrière, om zo verdere verspreiding te voorkomen. In dit hoofdstuk hebben we in de huid van gezonde mensen een gespecialiseerde populatie immuuncellen gevonden, namelijk de T cellen, die specifiek geactiveerd werden na blootstelling aan *S. aureus*. Deze T cellen blijven voor lange tijd in de huid aanwezig, en kunnen lokaal bijdragen aan bescherming tegen *S. aureus* huidinfecties.

Conclusies

Zoals in dit proefschrift staat beschreven, kan het immuunsysteem op verschillende manieren *S. aureus* WTA suikers herkennen, onder andere via antistoffen in bloed en Langerhans cellen in de huid. Aan de andere kant is de bacterie in staat herkenning door het immuunsysteem tegen te gaan door de variatie in de compositie van de WTA suikers, namelijk de drie verschillende WTA glycotypen. Een robuuste immuunrespons tegen alledrie de WTA glycotypen zou kunnen bijdragen aan bescherming tegen *S. aureus* infecties. Er is veel bewijs dat de WTA suikers een goede target zouden kunnen zijn voor vaccinatie en antistoftherapieën, als alternatief voor de veelgebruikte suikerkapsels. Daarnaast is het belangrijk om de bijdrage van de lokale afweer in de huid niet te vergeten, aangezien de meeste infecties door *S. aureus* hier plaatsvinden. De bevindingen beschreven in dit proefschrift dragen hopelijk bij aan de toekomstige ontwikkeling van een effectief vaccin tegen *S. aureus*.

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About the author

Astrid Hendriks was born May 2nd, 1992 in Utrecht, The Netherlands. In 2010, she graduated from secondary school (VWO) at the st. Bonifatiuscollege in Utrecht. That same year, she started the bachelor's programme Biomedical sciences at Utrecht University which she completed in 2014, including an additional 1-year minor in education. In 2014, Astrid was admitted to the master's programme Infection and Immunity within the Graduate School of Life Sciences at Utrecht University. Within this programme, she followed a 9-month research internship at the department of Pathology at University Medical Center Utrecht (UMCU) under the supervision of dr. Niels Bovenschen. During this time, she studied the effects of granzymes, produced by cytotoxic T cells, on human cytomegalovirus infection. For her second research internship, Astrid joined the group of prof. Simon Foster at the University of Sheffield in the UK for 6 months. Here, she was first introduced to the elusive pathogen *Staphylococcus aureus*, as she studied the mechanisms of S. aureus infection augmentation in a zebrafish model. In 2016, Astrid obtained her Master's degree.

In the summer of 2017, Astrid started her PhD as part of the EU-funded Marie Curie Industrial Training Network (ITN) programme called DISSection under the supervision of prof. Jos van Strijp, prof. Nina van Sorge (Department of Medical Microbiology, UMCU, the Netherlands), dr. Elisabetta Soldaini and dr. Fabio Bagnoli (GlaxoSmithKline Vaccines, Siena, Italy). She spent 18 months working in Siena on the characterization of *S. aureus* specific tissue resident memory T cells in human skin before moving back to Utrecht to work on human immune responses to *S. aureus* wall teichoic acid. The main findings of this work are described in this thesis, have been presented at several international scientific conferences and have been published in peer-reviewed scientific journals.

List of publications

Related to this thesis

Hendriks A, Codée JDC, van Strijp JAG, Bagnoli F, van Sorge NM. Planned patent application on innovative antigen design for vaccination and development of monoclonal antibodies (2022).

Ali S, **Hendriks A**, van Dalen R, Bruyning T, Meeuwenoord N, Overkleeft H, et al. (Automated) synthesis of well-defined *Staphylococcus aureus* wall teichoic acid fragments. Chem – A Eur J. 2021;27(40):10461–9.

Hendriks A[*], Mnich ME[*], Clemente B, Cruz AR, Tavarini S, Bagnoli F, et al. *Staphylococcus aureus*-specific tissue-resident memory CD4+ T cells are abundant in healthy human skin. Front Immunol. 2021;12:642711.

Hendriks A, Van Dalen R[*], Ali S[*], Gerlach D, Van Der Marel GA, Fuchsberger FF, et al. Impact of glycan linkage to *Staphylococcus aureus* wall teichoic acid on langerin recognition and Langerhans cell activation. ACS Infect Dis. 2021;7(3):624–35.

Hendriks A[*], Cruz AR[*], Soldaini E, Manetti AGO, Bagnoli F. Human organotypic models for anti-infective research. Current Topics in Microbiology and Immunology. 2021;430:77-99.

Other publications

Mnich ME, van Dalen R, Gerlach D, **Hendriks A**, Xia G, Peschel A, et al. The C-type lectin receptor MGL senses N-acetylgalactosamine on the unique *Staphylococcus aureus* ST395 wall teichoic acid. Cell Microbiol. 2019;21(10):e13072.

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