The Zwitterionic Cell Wall Teichoic Acid of *Staphylococcus aureus* Provokes Skin Abscesses in Mice by a Novel CD4+ T-Cell-Dependent Mechanism

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Abstract

Zwitterionic polysaccharide (ZPS) components of the bacterial cell envelope have been shown to exert a major histocompatibility complex (MHC) II-dependent activation of CD4+ T cells, which in turn can modulate the outcome and progression of infections in animal models. We investigated the impact of zwitterionic cell wall teichoic acid (WTA) produced by *Staphylococcus aureus* on the development of skin abscesses in a mouse model. We also compared the relative biological activities of WTA and capsular polysaccharide (CP), important *S. aureus* pathogenicity factors, in abscess formation. Expression of both WTA and CP markedly affected the ability of *S. aureus* to induce skin abscess formation in mice. Purified wild-type zwitterionic WTA was more active in inducing abscess formation than negatively charged mutant WTA or purified CP8. To assess the ability of purified native WTA to stimulate T cell proliferation in vitro, we co-cultivated WTA with human T-cells and antigen presenting cells in the presence and absence of various inhibitors of MHC-II presentation. Wild-type WTA induced T cell proliferation to a significantly greater extent than negatively charged WTA. T cell activation was dependent on the presentation of WTA on MHC II, since inhibitors of MHC II-dependent presentation and antibodies to MHC II significantly reduced T cell proliferation. T cells activated in vitro with wild-type WTA, but not negatively charged WTA, induced abscess formation when injected subcutaneously into wild-type mice. CD4+/− mice similarly injected with WTA failed to develop abscesses. Our results demonstrate that the zwitterionic WTA of *S. aureus* induces CD4+ T-cell proliferation in an MHCII-dependent manner, which in turn modulates abscess formation in a mouse skin infection model. An understanding of this novel T cell-dependent host response to staphylococcal abscess formation may lead to the development of new strategies to combat *S. aureus* skin and soft tissue infections.

Introduction

Wall teichoic acid (WTA) of *Staphylococcus aureus* is a zwitterionic cell wall glycopolymer composed of ∼40 ribitol phosphate repeating units modified with N-acetylglycosamine and D-alanine [1,2]. The negatively charged phosphate groups in the ribitol repeating unit and the ester-linked D-alanine residues contribute to the zwitterionic charge of WTA (Fig. 1). The presence of WTA-like molecules in most Gram-positive bacteria prompted scientists to focus on the role of WTA in cell wall function [3,4] and turnover [5]. More recently, defined mutants of *S. aureus* that lack WTA or have altered WTA structures have facilitated experiments to elucidate in greater detail the role of WTA in staphylococcal pathogenesis [6–10]. WTA has been implicated in the adhesion of *S. aureus* to human epithelial and endothelial cells [9–11], and expression of WTA has been shown to be essential for *S. aureus* nasal colonization of cotton rats [9,11]. A *S. aureus* mutant lacking WTA showed attenuated virulence in a rabbit model of endocarditis [10]. Moreover, purified WTA was able to induce intraabdominal abscesses when rats were inoculated by the intraperitoneal route [12]. *S. aureus* mutants in the *dlt* operon, which mediates D-alanylation of WTA, exhibit a negatively charged cell surface and are more sensitive to cationic antimicrobial peptides than the parental strain [13,14].

WTA is expressed constitutively [1], whereas *S. aureus* capsular polysaccharides (CPs) are expressed in a tightly regulated manner [15,16]. Most clinical isolates of *S. aureus* express either capsule type 5 (CP5) or 8 (CP8). However, 20–25% of clinical isolates produce no capsule due to a variety of mutations within the conserved capsule biosynthesis operon [17,18].

WTA exhibits a zwitterionic charge, and advances in the field of glyco-immunology have led to the finding that zwitterionic polysaccharides (ZPS) exert a direct activity on the adaptive immune system, thereby modulating the development of bacterial infections [19]. ZPS, such as complex CPs produced by specific bacterial pathogens, directly elicit immune responses by activating various T cells subtypes in the absence of protein carriers [20,21]. ZPS activated T cells modulate the outcome of infection or colonization by different bacterial species [19,22]. CP5 and CP8 produced by *S. aureus* enhance staphylococcal virulence [23–25], and purified zwitterionic CP8 induces intraabdominal abscesses in rats in a CD4+ T cell dependent manner [12]. In a wound infection
model, McLoughlin et al. demonstrated that the presence of CD4 T cells modulated CXC chemokine production at the S. aureus infection site in an IFN-γ dependent manner, which led to a massive recruitment of neutrophils, and this effect was mediated by CP8 [26]. These findings suggest that staphylococcal ZPS are important factors for T cell dependent immune stimulation, which strongly influences the outcome of infections.

Skin and soft tissue infections are the most common types of S. aureus infections and occur in the hospital as well as in the community, affecting hosts without predisposing risk factors [27,28]. Since bacterial ZPS are associated with abscess formation [12,29], we explored the contribution of zwitterionic WTA and CPs in a mouse skin infection model. We demonstrate here that WTA activates T cells in a MHC II dependent manner, and that T cells activated in vitro by WTA induce skin abscesses in mice. These novel findings link staphylococcal skin infections to recent discoveries of glyco-immunology and may lead to novel approaches to combat skin infections by S. aureus.

Results

WTA and Capsule Impact Abscess Formation When Live Bacteria Are Injected Subcutaneously into the Flanks of Mice

We used a mouse model to study the ability of various S. aureus strains to induce skin infections that closely resemble staphylococcal skin infections in humans. S. aureus cells mixed with cytodex beads were injected subcutaneously into the flanks of mice. The cytodec beads allow for formation of a localized abscess even at low inocula or with low concentrations of purified WTA.

To assess the relative roles played by S. aureus WTA and CPs in skin infections, we transduced the tagO mutation into the CP5+ strain Newman and its isogenic acapsular cap50 mutant. The resulting tagO deletion mutants lacked WTA in their cell wall as evidenced by the low phosphorus content of cell wall fractions, but their doubling times were similar to that of the parental strains under in vitro conditions (Fig. S1). The Newman tagO mutant was complemented by introducing a wild-type copy of tagO on plasmid pBl tagO. In addition, we used previously described mutants of the acapsular SA113 strain that either lack WTA [tagO] [9] or the D-alanine esters (dl4) [13] in the repeating WTA subunits. The dl4 mutation was transduced into Newman, and the phenotype of the resulting mutant was consistent with the absence of positive charges in the teichoic acids (Fig. S1).

S. aureus wild-type strains SA113 and Newman at inocula ranging from 10^8 to 10^9 cfu were potent inducers of subcutaneous abscesses in mice (Fig. 2). The tagO mutants of both bacterial strains were significantly attenuated in their ability to induce abscesses, whereas the dl4 mutants showed an intermediate phenotype at the lower inocula (Figs. 2A and 2B and Supplemental Fig. 2). Consistent with previous data with a CP8+ strain [22], the acapsular Newman cap50 mutant was also attenuated for subcutaneous abscess formation (Fig. 2C). When the bacteria were grown on Columbia salt agar to enhance capsule expression (Fig. 2C), strain Newman provoked a higher bacterial burden per abscess than when it was grown on tryptic soy agar (Fig. 2B). The Newman tagO and cap50 mutants were both significantly attenuated for abscess induction when compared to the parental strain. A double mutant of Newman that expresses neither capsule nor WTA was more attenuated for abscess formation than the single mutants (Fig. 2C), but the differences did not reach statistical significance. These data suggest that both WTA and CP promote S. aureus skin abscess formation when live bacteria are used as the inocula.

Purified WTA Induces Skin Abscesses More Efficiently than Purified CP8

To further assess its biological activity, we purified WTA from the acapsular strains SA113 wild-type (zwitterionic WTA) and SA113 dl4 (anionic WTA). WTA purity was assessed by 1H NMR, phosphate content, the absence of nucleic acids (<0.1%), protein (<1%), and endotoxin (<1 EU/mg). Sterile abscesses were induced by injection of sterile cytodec beads mixed with purified wt WTA at doses ranging from 200 to 0.02 μg. After 48 h, abscesses were excised and quantified by their weight. Purified wt WTA was a potent inducer of abscess formation at doses as low as 0.02 μg (Fig. 3A). WTA purified from the dl4 mutant or beads alone showed minimal activity under these conditions (Fig. 3A). Administration of dl4 WTA resulted in the recruitment of fewer neutrophils in the abscessed tissue than wt WTA (Fig. S2). We quantified the host response by measuring neutrophil accumulation at the infection site with the myeloperoxidase (MPO) assay (Fig. 3B) [30]. MPO is present in the azurophilic granules of neutrophils and is routinely used to assess the tissue inflammatory responses [31] and abscess formation. MPO activity in abscesses provoked by 20 μg wt WTA after 48 h was significantly (P<0.0001) higher than that induced by 20 μg dl4 WTA. Despite its potent activity in the induction of intraabdominal abscesses [12], purified CP8 exhibited only minimal potency in the s.c. abscess model, even at a 50 μg dose.

Purified WTA Fails to Induce Neutrophil Chemotaxis or Prolinflammatory Cytokines

To investigate whether purified WTA exhibited direct chemotactic activity, we assayed chemotaxis of human neutrophils in response to...
WTA in a transwell assay. WTA purified from *S. aureus* SA113 or the SA113 dltA mutant failed to induce significant chemotaxis compared to the authentic chemoattractant fMLP (Fig. 4A).

Activation of pro-inflammatory responses via pattern recognition receptors like Toll-like receptors (TLRs) could also lead to pronounced inflammation and modulate abscess formation. Therefore, we tested whether purified WTA stimulates pro-inflammatory cytokines in macrophages or in HEK293 cells transfected with TLR-2. These experiments not only interrogated WTA proinflammatory activity but also enabled us to exclude functional contaminants in our purified WTA preparation. We measured production of TNF-α by human monocytes (THP-1 cells) or mouse macrophages (RAW 264.7 cells) after stimulation with WTA or with the TLR agonists LPS, MALP-2, or Pam3Cys as positive controls. We stimulated HEK293 cells transfected with TLR-2 with WTA, MALP-2, or Pam3Cys, and assayed for IL-8 production in the culture supernatants. Purified WTA at concentrations as high as 100 μg/ml showed no proinflammatory activity in THP-1 or RAW 264.7 cells (Fig. 4B) or in TLR-2 expressing HEK293 cells (Fig. 4C). This is consistent with previous reports that demonstrated the inability of WTA to directly activate host cells in a manner typical of classical pathogen associated molecular pattern molecules [32].

WTA activates proliferation of human T cells in a MHC II dependent manner

The induction of intraperitoneal abscesses by other zwitterionic polysaccharides is based on their ability to directly activate T-cells, which in turn modulate cellular responses at the infection site [14,28]. Therefore, we cultured nylon wool-purified human T-cells or column-purified CD4+ T cells with irradiated antigen-presenting cells (APCs) and wt or dltA WTA. *S. aureus* enterotoxin A (SEA) and polygalacturonic acid (GalU) were used as positive and negative controls, respectively. Wt WTA elicited a potent dose-dependent proliferative response in human T cells on day 6 (Fig. 5A). The T-cell response to wt WTA was significantly higher than that exhibited by CP8, dltA WTA, or beads alone (Fig. 5A). The negatively charged polygalacturonic acid failed to induce T-cell proliferation, and the *S. aureus* superantigen SEA induced a significantly higher T-cell response than proliferation induced by either of the WTA antigens (Fig. 5D).

To determine whether T-cell proliferation induced by purified WTA was mediated by MHC class I or II molecules, we performed assays in the presence of blocking antibodies (Abs) and isotype control Abs. Wt WTA induced T-cell proliferation was dependent on the MHC class II molecule HLA-DR since T cell proliferation was reduced significantly in the presence of blocking Abs to HLA-DR (Fig. 5B), but not with Abs to HLA-DP, HLA-DQ, or HLA-A, -B, or -C (data not shown). The residual activity of the dltA WTA was not inhibited by any of the Abs. In assays utilizing the human Burkitt lymphoma cell line (Raji) and its MHC class II transcriptional mutant cell line (RJ2.2.5), WTA stimulated T-cell proliferation only when the MHC-II–expressing Raji cells, but not the RJ2.2.5 cells, were incubated with T-cells and WTA (Fig. 5C). Inhibitors of the intracellular antigen-processing pathways that are required for MHC II-dependent antigen...
presentation were used at concentrations that had no effect on the stimulatory activity of the *S. aureus* superantigen SEA (Fig. 5D). The inhibitors Bafilomycin A (BafA), Brefeldin A (BrefA), Colchicine, and Cytochalasin D (CytoCalD) reduced the T-cell proliferation induced by wt WTA and dltA WTA, providing evidence that, unlike superantigens, WTA requires intracellular processing for efficient MHC II presentation to T cells.

**WTA Stimulated Mouse T Cells Modulate Abscess Formation in the S.C Abscess Model**

The role of T-cell activation in the modulation of WTA-induced abscess formation was assessed by T-cell transfer experiments. *S. aureus* wt WTA stimulated a potent proliferative response in mouse T cells after 6 days, whereas dltA WTA was less active (Fig. 6A). In T cell transfer assays, purified mouse CD4+ T cells were cultured in vitro for 6 days with WTA and irradiated APCs. The stimulated T cells were then purified, assayed for purity by FACS analysis, mixed with cytodelx beads, and injected s.c. into mouse flanks. Abscess formation was quantified by MPO activity in the abscessed tissue after 48 h. In dose-dependency experiments, we found that 3x10^5 activated T cells were sufficient to induce an abscess after 48 h (data not shown). T cells stimulated with wt WTA induced abscess formation in mice, whereas T cells stimulated with dltA WTA, naive T-cells, or APCs incubated with WTA in the absence of T cells were significantly less potent (Fig. 6B).

To lend further evidence to the premise that T cells are crucial for *S. aureus* abscess formation, we used CD4^+ T cell deficient mice. As predicted, WTA provoked abscesses in C57Bl/6 mice, but not in CD4-deficient C57Bl/6 tm1mac mice (Fig. 6C). These in vivo data clearly demonstrate the link between WTA dependent T cell activation and the modulation of abscess formation by activated CD4^+ T cells.

**Discussion**

Ribitol-phosphate WTA is a conserved cell wall-associated ZPS produced by *S. aureus*. CP produced by the majority of staphylococcal strains may mask WTA on the bacterial surface. However, neither CP5 nor CP8 is expressed by 20–25% of clinical isolates [17,18]. Even in encapsulated strains, WTA is most likely surface exposed during the logarithmic growth phase when little CP is produced [16,17,33]. WTA is present both in the logarithmic and stationary phases of bacterial growth, and it appears to be expressed constitutively by the bacterial cell. Structural genes encoding enzymes of WTA biosynthesis machinery can be upregulated under certain in vivo conditions, such as the early phases of nasal colonization, as demonstrated in the cotton rat model [34].

We were interested in the role of WTA in the development of skin infections caused by *S. aureus*, since these are among the most frequent staphylococcal infections. Moreover, WTA was shown to induce abscesses in a rat model of intraabdominal abscess formation [12]. Our data demonstrate that *S. aureus* WTA plays a critical role in the development of staphylococcal skin lesions. Mutants that lack WTA (tagO) or have an altered WTA structure (dltA) are significantly impaired in the mouse s.c. abscess model. The attenuated phenotype of the *dltA* mutant may be partially attributed to its susceptibility to killing by neutrophils and cationic microbial peptides [9,13], which are highly abundant in skin infections [35]. The absence of *S. aureus* WTA or the presence of an altered WTA structure influences the molecular events that modulate abscess formation since purified wt WTA induced abscesses more efficiently than dltA WTA. T cells have been shown to play a role in animal models of *S. aureus* intraperitoneal and subcutaneous abscess formation [12,22] and in a staphylococcal surgical wound infection model [22]. In this study we identify a novel role for zwitterionic WTA, together with CP, in the induction of subcutaneous abscess formation through a mechanism dependant upon T-cell activation. We demonstrated that purified WTA stimulated CD4+ T-cell proliferation in vitro, and that this was dependent upon the zwitterionic charge of WTA. WTA was unable to induce T cell proliferation when APCs lacking MHC II were used in co-culture, and the stimulatory activity of wt WTA, but not the residual activity of the *dltA* WTA, was diminished by HLA-DR blocking Abs. In contrast, inhibitors of intracellular processing pathways blocked the activity of wt WTA and the residual activity of *dltA* WTA. This argues for a charge-dependency of WTA presentation but not intracellular processing, which is consistent with reports characterizing other bacterial ZPS polymers [36]. Thus, WTA qualifies as a bacterial ZPS that is processed and presented by APCs via the MHCII pathway [19]. Having defined a mechanism by which WTA can activate T-cells, we then established that the T cell stimulatory activity of WTA could modulate skin abscess formation. CD4^+ T cells activated by purified wt WTA were sufficient to provoke abscesses when injected s.c. into the flanks of healthy mice. Moreover, CD4^-/- mice injected with WTA failed to develop abscesses. Taken together, we present a novel function of WTA as a ZPS that can modulate CD4^+ T cell-dependent development of skin abscesses in mice.

*S. aureus* mutants defective in CP production were attenuated in the s.c. abscess model of infection, but purified CP8 was less active than WTA in T-cell proliferation assays and in the skin abscess model. Both CP8 and WTA showed similar potency in the rat intraperitoneal abscess model [12], suggesting that the underlying molecular mechanisms leading to abscess formation differ between the skin and peritoneal cavity. USA300 strains that are responsible for most SSTIs in the U.S. are negative for CP production [37], and thus WTA may be a critical factor that promotes USA300 abscess formation in humans.

The molecular effector mechanisms that are downstream of WTA-induced T cell activation and lead to abscess formation have yet to be resolved. CD4^+ T cells have been shown to influence neutrophil recruitment into *S. aureus*-infected wound tissues via production of IFN-γ [26]. High local IFN-γ production leads to upregulation of CXC chemokines, thereby resulting in a pronounced neutrophil influx into the infected host tissues [26]. Here we provide evidence that *S. aureus* WTA and CP are both crucial for SSTIs provoked by *S. aureus*, and that WTA induces
Figure 5. WTA stimulates in vitro T-cell activation by a MHC-II dependent mechanism. T cells and APCs were incubated with different concentrations of WTA purified from SA113 wt or the dltA mutant. The stimulation index was calculated by normalizing on wells with APCs and T-cells but no stimulating antigen. A) wt WTA stimulated T-cell proliferation in a dose-dependent manner, whereas dltA WTA and CP8 were considerably less active (p < 0.0001 for wt WTA vs. dltA WTA at 20 and 2 μg, p = 0.0003 for wt WTA vs. CP8). The negatively charged GalU showed no activity. SEA was considerably more active than WTA (panel D). Values represent means ± SEM, n = 6. B) T-cell proliferation assays with WTA from wt and dltA were performed in the presence of blocking Abs to the MHC class II molecules and the appropriate isotype control. An antibody to HLA-DR (5 μg/ml) inhibited T-cell proliferation induced by zwitterionic wt WTA (p = 0.0001) but not negatively charged dltA WTA. Shown are means ± SEM, n = 5. C) WTA induced T-cell proliferation only with Raji cells as APCs but not when APCs were from the MHC II negative cell line RJ2.5.5. Shown are means and SEM, n = 4. D) T cells were incubated with different inhibitors of the MHC II antigen processing pathway. The inhibitors bafilomycin A (BafA), brefeldin A (BrefA), colchicine, and cytochalasin D (CytoCalD) decreased T-cell proliferation induced by wt WTA (wt WTA with BafA: p = 0.0399; wt WTA with BrefA: p = 0.0220; wt WTA with Colchicine: p = 0.0042; wt WTA with CytoCalD: p = 0.0036) and dltA WTA (dltA WTA with BafA: p = 0.0220; dlt WTA with...
skin abscesses through MHC II-dependent activation of CD4+ T cells. A recent study by Cho et al. demonstrated the importance of γδ T cells in controlling neutrophil recruitment and influencing the outcome of intradermal S. aureus infections in mice [30]. The discrepancies between the results of Cho et al. and our own results may reflect subtle differences between the two infection models, as well as characteristics of the S. aureus strains utilized by the different labs. The immune mechanisms modulating the onset of infection likely differ depending on the site of infection, the specific virulence factors produced by the microbe, and the repertoire of effector cells responding to the bacterial insult.

Multiple virulence determinants likely affect S. aureus induction of SSTIs [39], and the host response to infection is currently under investigation by several laboratories. WTA is present in all S. aureus strains, and synergistic interactions among leukocidins, CPs, α-toxin [40], phenol-soluble modulins [41] and WTA may increase the frequency or severity of staphylococcal SSTIs. The ribitol phosphate WTA produced by S. aureus differs from the glycerol phosphate WTA polymer synthesized by Staphylococcus epidermidis, the most common staphylococcal species found on human skin [42,43]. Whether the biological properties of the two polymers differ is a question that has not yet been addressed.

Our results demonstrate a novel function for the zwitterionic WTA polymer of S. aureus. We link the novel finding that WTA can stimulate CD4+ T cells to the development of staphylococcal SSTIs in an in vivo infection model. We offer new insights into the pathology of SSTIs caused by S. aureus and propose a novel host mechanism involved in staphylococcal skin infections.

Methods

Ethics Statement

Human blood was collected from healthy volunteers giving written informed consent for venipuncture, as approved by the Institutional Review Board of The Brigham and Women’s Hospital (Human Subject Assurance Number 00000484). Animal experiments were performed in accordance with the guidelines of the Harvard Medical School Standing Committee on Animals (Animal Welfare Assurance Number A3431-01) under approved protocol 86-02131. The Harvard Medical School animal management program is accredited by the American Association for Accreditation of Laboratory Animal Care and meets National Institutes of Health standards as set forth in “Guide for the Care and Use of Laboratory Animals” (DHSS Publication No. (NIH) 85-23 Revised 1983). The institution also accepts as mandatory the Public Heath Service “Policy on Humane Care and Use of Laboratory Animals by Awardee Institutions” and NIH “Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training.”

Bacterial Strains and Growth Conditions

S. aureus SA113 (ATCC 35556) is a previously described laboratory strain [44]. The WTA-deficient Sa113 tagO mutant was generated by replacing the tagO gene with an erythromycin resistance cassette [9]. The dltA mutant of S. aureus SA113 was generated by replacing the dltA gene with a spectinomycin resistance cassette [13], and this mutation was transduced into strain Newman with phage 80α. The phenotype of the dltA mutant was assessed by its ability to bind more positively charged cytochrome C (Sigma) than the wildtype strain, as described previously [45]. S. aureus Newman and the isogenic Newman cag5O mutant were described previously [46]. The tagO::tet mutation from RN4220 tagO::tet gdh::tagO (kindly provided by Drs. Timothy Meredith and Suzanne Walker, Harvard Medical School) was introduced into strain Newman by transduction with phage 80α. The strain Newman tagO mutation was verified by PCR, and the absence of WTA was confirmed by measuring the phosphorus content of cell wall fractions prepared from the isogenic wild-type and mutant strains [9]. For genetic complementation studies, the mutants were complemented with a plasmid containing a wild-type copy of the tagO gene (pRBtagO). Bacterial strains were grown in tryptic soy broth (BBL) or B-Medium BM [9] unless otherwise noted.

WTA Isolation and Purification

Staphylococcal WTA was isolated and analyzed as described [9]. The phosphorus content in WTA samples was determined by colorimetric assays [9,13]. WTA was then dialyzed against 20 mM Bis/Tris buffer and run on a Q-Sepharose column with a NaCl gradient (0-1 M) followed by a Sephacryl S-300 size exclusion column. All buffers and water were pyrogen-free. The instruments and devices used in the WTA purification process were deproteinized by treatment with sulfaric acid and depyrogenated by heat inactivation for 4 h at 240°C or by treatment with 1–2 M sodium hydroxide buffer. WTA was analyzed for protein by the Biorad method and by UV absorbance at 280 nm; for nucleic acid by UV absorbance at 260 nm and by agarose gel electrophoresis; and for endotoxin by the Limulus amebocyte lyse test (Charles River Laboratories, Charleston, SC). 1H-nuclear magnetic resonance spectroscopy (NMR) was performed for structural evaluation.

Mouse Model of Subcutaneous Abscess Formation

Male Swiss Webster ND4 mice (4–6 weeks old) were purchased from Charles River or Harlan, and C57Bl/6 and C57Bl/6 tmlmac CD4−/− mice were purchased from The Jackson Laboratory. 10^5 to 10^6 CFU S. aureus or purified WTA was mixed with sterile dextran beads (Cytodex 1, Sigma), and the mixture (0.2 ml) was injected s.c. into the shaved flanks of mice as described previously [47]. The abscesses were either weighed or homogenized in TSB for quantitative culture. Alternatively, the myeloperoxidase (MPO) activity in abscess homogenates was measured with a colorimetric assay [48]. For histological analysis, excised tissue was fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin for microscopic examination.

T-Cell Activation, Neutrophil Chemotaxis, and Cytokine Production Assays

Mononuclear cells were purified by density gradient centrifugation in Polymorphoprep (Axis-Shield) from blood drawn from various healthy human donors. Total T cells were purified from the mononuclear cell fraction with nylon wool columns (Polysciences, Inc.), and CD4 T cells were purified with CD4 enrichment columns (R&D Systems). Purity was assessed by FACCS with CD3 and CD4 antibody staining. T cells (1×10^5) were cultured with 1×10^5 irradiated antigen-presenting cells and S. aureus WTA. Dose-dependency experiments were performed using WTA at concentrations of 20 and 2 μg/ml using SEA (5 ng/ml) and polygalacturonic acid (20 μg/ml) as positive and negative controls.
respectively. Cellular proliferation was measured after 6 days by a 3H-thymidine incorporation method [21]. In some experiments splenic mouse T cells were purified over nylon wool columns and stimulated with WTA as described above. In addition, T-cell proliferation assays were performed in the presence of blocking Abs to the MHC class I molecules HLA-A, HLA-B, and HLA-C, the MHC class II molecules HLA-DR, HLA-DP, and HLA-DQ, and their respective isotype controls. The human Burkitt lymphoma cell line (Raji) expressing MHC class II and MHC class I molecules and its MHC class II transcriptional mutant cell line (RJ2.2.5) were used as APCs in certain experiments [21]. WTA was incubated with T cells purified from human blood and either MHCII+/- or Raji B-cells or MHCII-/+- cells for 6 days, and cellular proliferation was measured as described above. WTA was tested for chemotactic activity on human neutrophils as previously described [49].

For cytokine detection assays HEK293 cells were cultured in Iscove’s Modified Dulbecco’s Medium (Invitrogen) with 10% fetal calf serum. THP-1 human monocytes were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% fetal calf serum. RAW 264.7 mouse macrophages were cultured in Dulbecco’s modified Eagle’s medium (GIBCO) with 10% fetal calf serum.

After stimulation with purified WTA, LPS, MALP-2 or Pam3Cys for 8 h (TNF alpha) or 10 h (IL-6), suppressant mutants were harvested and stored at −20°C until assayed by ELISA (R&D Biosystems) according to the manufacturer’s instructions. All assays were performed with triplicate or quadruplicate replicates.

Adoptive Transfer Assays

The role of T-cell activation by WTA in the modulation of abscess formation was determined by transferring T cells stimulated with WTA into mice. Mouse T cells purified with nylon wool columns were cultured in vitro for 6 d with irradiated APCs and either 20 µg wt or dltA WTA. CD4+ T cells were isolated from the co-culture with mouse CD4+ enrichment columns (R&D Systems), mixed with cytodex beads, and 3 x 10^5 T cells were injected s.c. into the flanks of mice. Control mice were injected with native T cells isolated from co-cultures with APCs and no stimulating antigen, APCs incubated with WTA (no T cells), or cytodextrin beads alone. Abscess formation was quantified by MPO activity.

Supporting Information

Figure S1 Characterization of tagO and dltA mutants. A) WTA content of the wt and tagO mutant as determined by phosphorous content of cell wall fractions in Newman and C) the isogenic CP5 mutant cap50. The tagO mutants show only residual P3 which indicates the lack of WTA. Growth was monitored in LB broth after inoculation from a pre culture grown to log-phase (B and D). E) WTA content of the Newman wt and dltA mutant as determined by the phosphorous content of cell wall fractions. WT and mutant had similar amounts of WTA. F) Cytochrome C binding to whole cells. Bacterial cells in PBS were incubated with 0.5 µg/ml cytochrome C. The positive charge conferred by the ester linked D-alanine in the wt WTA diminishes binding of the positively charged cytochrome C. This observed phenotype of the dltA mutant depends on the lack of D-alanine esters.

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Figure S2 Abscess formation with purified WTA. 20 µg of wt WTA or dltA WTA was mixed with cytodex beads and injected s.c into the flanks of mice. After 48 h, the mice were euthanized and representative abscesses photographed (A). The abscesses were excised, fixed in formalin, embedded in paraffin, and stained with hematoxylin and cosin for histological analysis (B).

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Author Contributions

Conceived and designed the experiments: CW RMM JCL. Performed the experiments: CW. Analyzed the data: CW RMM JCL. Contributed reagents/materials/analysis tools: RMM. Wrote the paper: CW JCL.
11. Weidenmaier C, Kokai-Kun JF, Kulauzovic E, Kohler T, Thumm G, et al. (2008) Differential roles of sortase-anchored surface proteins and wall teichoic acid in Staphylococcus aureus nasal colonization. Int J Med Microbiol 298: 503–513.

12. Tzianabos AO, Wang JY, Lee JC. (2001) Structural rationale for the modulation of abscess formation by Staphylococcus aureus capsular polysaccharides. Proc Natl Acad Sci U S A 98: 9365–9370.

13. Peschel A, Otto M, Jack RW, Kalbacher H, Jung G, et al. (1999) Inactivation of the dlt operon in Staphylococcus aureus confers sensitivity to defensins, proteoglycans, and other antimicrobial peptides. J Biol Chem 274: 8805–8810.

14. Peschel A, Sahi HG. (2006) The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat Rev Microbiol 4: 529–536.

15. Herbert S, Neswell SW, Lee C, Wieland KP, Dassy B, et al. (2001) Regulation of Staphylococcus aureus type 5 and type 8 capsular polysaccharides by CO(2). J Bacteriol 183: 4609–4613.

16. Luong T, Sau S, Gomes M, Lee JC, Lee CY. (2002) Regulation of Staphylococcus aureus capsular polysaccharide expression by αg and αc. Infect Immun 70: 444–450.

17. O’Riordan K, Lee JC. (2004) Staphylococcus aureus capsular polysaccharides. Clin Microbiol Rev 17: 219–234.

18. Cocchiara JL, Gomez MI, Ridey A, Solinga R, Sordelli DO, et al. (2006) Molecular characterization of the capsule locus from non-typeable Staphylococcus aureus. Mol Microbiol 59: 948–960.

19. Mazmanian SK, Kasper DL. (2006) The love-hate relationship between bacterial polysaccharides and the host immune system. Nat Rev Immunol 6: 849–858.

20. Cobb BA, Kasper DL. (2005) Zwitterionic capsular polysaccharides: the new MHCII-dependent antigens. Cell Microbiol 7: 1398–1403.

21. Kalka-Moll WM, Tzianabos AO, Bryant PW, Niemeyer M, Ploegh HL, et al. (2002) Zwitterionic polysaccharides stimulate T cells by MHC class II-dependent interactions. J Immunol 169: 6149–6153.

22. McLoughlin RM, Solinga RM, Rich J, Zaleski KJ, Cocchiaro JL, et al. (2006) CD4+ T cells and CXCL chemokines modulate the pathogenesis of Staphylococcus aureus wound infections. Proc Natl Acad Sci U S A 103: 10408–10413.

23. Nilsson IM, Lee JC, Bremell T, Ryden C, Turkowski A. (1997) The role of staphylococcal polysaccharide macrocapsule expression in septicemia and septic arthritis. Infect Immun 65: 4216–4221.

24. Thakker M, Park JS, Ulrich M, Kiser KB, Doring G, Lee JC, et al. (2000) IFN-gamma regulated chemokine production determines the outcome of Staphylococcus aureus infection. J Immunol 161: 1233–1322.

25. Stevens DL. (2009) Treatments for skin and soft-tissue and surgical site infections due to MDRO Gram-positive bacteria. J Infect 59 Suppl 1: S32–39.

26. Iordanescu S, Surdeanu M. (1976) Two restriction and modification systems in Staphylococcus epidermidis. J Gen Microbiol 96: 277–281.

27. Watts A, Ke D, Wang Q, Pillay A, Nicholson-Weller A, et al. (2005) Staphylococcus aureus strains that express serotype 5 or serotype 8 capsular polysaccharides differ in virulence. Infect Immun 73: 3502–3511.

28. McLoughlin RM, Lee JC, Kasper DL, Tzianabos AO. (2006) IFN-gamma reguluated chemokine production determines the outcome of Staphylococcus aureus infection. J Immunol 181: 1233–1322.

29. Stevens DL. (2000) Treatments for skin and soft-tissue and surgical site infections due to MDRO Gram-positive bacteria. J Infect 39 Suppl 1: S32–39.

30. Saxena S, Thompson P, Birger R, Bottle A, Spyriths N, et al. Increasing skin infections and Staphylococcus aureus complications in children, England, 1997-2006. Emerg Infect Dis 16: 530–533.

31. Tzianabos AO, Chandraker A, Kalka-Moll W, Stingle F, Dong VM, et al. (2000) Bacterial pathogens induce abscess formation by CD4+ T-cell activation via the CD28-B7-2 costimulatory pathway. Infect Immun 68: 6650–6655.

32. Magnotti LJ, Upperman JS, Xu DZ, Lu Q, Deitch EA. (1998) Gut-derived peptiodes are crucial but not the sole neutrophil attractants produced by Staphylococcus aureus. Cell Microbiol 8: 207–217.

33. van der Veen BS, de Winther MP, Heeringa P. (2009) Myeloperoxidase: molecular mechanisms of action and their relevance to human health and disease. Antioxid Redox Signal 11: 2899–2937.

34. Moreillon P, Majcherczyk PA. (2000) Inflammatory activity of cell-wall constituents from gram-positive bacteria. Scand J Infect Dis 33: 632–641.

35. Lee JC, Takeda S, Livolsi PJ, Padgett LC. (1995) Effects of IFN γ and in vivo growth conditions on expression of type 8 capsular polysaccharide by Staphylococcus aureus. Infect Immun 61: 1653–1656.

36. Burton M, Rastenberg M, Kohler T, Fritz M, Kramer B, et al. (2010) Temporal expression of adhesion factors and activity of global regulators during establishment of Staphylococcus aureus nasal colonization. J Infect Dis 201: 1414–1421.

37. Cho JS, Pietras EM, Garcia NC, Ramos R, Farzam DM, et al. (2010) IL-17 is essential for host defense against cutaneous Staphylococcus aureus infection in mice. J Clin Invest 120: 1762–1773.

38. Cheng AG, Kim HK, Burtis ML, Krausa T, Schnerwind O, et al. (2009) Genetic requirements for Staphylococcus aureus abscess formation and persistence in host tissues. FASEB J 23: 3393–3404.

39. Bubeck Wardenburg J, Bae T, Otto M, Deleo FR, Schnerwind O. (2007) Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in Staphylococcus aureus pneumonia. Nat Med 13: 1405–1406.

40. Wang R, Braughton KR, Kretschmer D, Bach TH, Quey SY, et al. (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat Med 13: 1510–1514.

41. Kloos WE, Musselwhite MS. (1975) Distribution and persistence of Staphylococcus coccus and Micrococcus species and other aerobic bacteria on human skin. Appl Microbiol 30: 381–385.

42. Kloos WE. (2009) Staphylococcus epidermidis—the accidental’’ pathogen. Nat Rev Microbiol 7: 555–567.

43. Jordanescu S, Surdeanu M. (1976) Two restriction and modification systems in Staphylococcus aureus NCTC12325. J Gen Microbiol 96: 277–281.

44. Kraus D, Herbert S, Kristian SA, Khosravi A, Nizet V, et al. (2008) The GmRS regulatory system controls Staphylococcus aureus susceptibility to antinociceptive host defenses. BMC Microbiol 8: 85.

45. Pohlmann-Dietze P, Ulrich M, Kiser KB, Doring G, Lee JC, et al. (2000) Adherence of Staphylococcus aureus to endothelial cells influences capsular polysaccharide, global regulator agr, and bacterial growth phase. Infect Immun 68: 4865–4871.

46. Portoles M, Kiser KB, Bhatnag N, Chan KH, Lee JC. (2001) Staphylococcus aureus CapSO has UDP-ManNAc dehydrogenase activity and is essential for capsule expression. Infect Immun 69: 917–923.

47. Mullane KM, Kraemer R, Smith B. (1983) Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. J Pharmacol Methods 14: 157–167.

48. Durr MC, Kristian SA, Otto M, Manteoli G, Margolis PS, et al. (2006) Neutrophil chemotaxis by pathogen-associated molecular patterns—formylated peptides are crucial but not the sole neutrophil attractants produced by Staphylococcus aureus. Cell Microbiol 8: 207–217.