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Infection Control by Antibody Disruption of Bacterial Quorum Sensing Signaling

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SUMMARY

Quorum sensing (QS) is the process through which bacteria communicate utilizing small diffusible molecules termed autoinducers. It has been demonstrated that QS controls a plethora of microbial processes including the expression of virulence factors. Here we report an immunopharmacotherapeutic approach for the attenuation of QS in the Gram-positive human pathogen *Staphylococcus aureus*. An anti-autoinducer monoclonal antibody, AP4-24H11, was elicited against a rationally designed hapten, and efficiently inhibited QS in vitro through the sequestration of the autoinducing peptide (AIP)-4 produced by *S. aureus* RN4850. Importantly, AP4-24H11 suppressed *S. aureus* pathogenicity in an abscess formation mouse model in vivo and provided complete protection against a lethal *S. aureus* challenge. These findings provide a strong foundation for further investigations of immunopharmacotherapy for the treatment of bacterial infections in which QS controls the expression of virulence factors.

INTRODUCTION

The ability of microorganisms to coordinate their gene expression in a population density-dependent manner has been coined "quorum sensing" (QS) [1]. This chemical exchange of information among single-cell organisms is mediated by secreted signaling molecules termed autoinducers (AI) [2]. Bacterial autoinducers can be classified into three major chemical groups: (1) N-acyl homoserine lactones (AHLs) that have been shown to be produced by over 70 species of Gram-negative bacteria [3, 4], (2) oligopeptides, which are generally employed by Gram-positive bacteria [5, 6], and (3) the ribose-like S-4,5-dihydroxy-2,3-pentanedione (DPD)/autoinducer (AI)-2, which is utilized by both Gram-negative and -positive bacteria

and, thus, can be regarded as an interspecies QS signaling molecule [7, 8].

Important biological and clinical aspects of QS include the regulation of bacterial virulence factors [9, 10], and hence inhibition of QS signaling could provide a promising new strategy for the attenuation of bacterial infections [11–13]. Indeed, small-molecule antagonists using AI analogs have been examined in a number of QS circuits as a means of signaling interference [14–18]. Alternatively, our laboratory pioneered an antibody-based strategy to inhibit AHL-mediated QS in Gram-negative bacteria [19]. Notably, following our initial report, an active immunization model in mice using an AHL-based vaccine was disclosed [20]. Ramifications arising from these studies have provided us further impetus to evaluate immunopharmacotherapeutic approaches targeting other bacterial QS systems.

Staphylococcus aureus is the most common cause of hospital-acquired infections [21] including various diseases ranging from skin infections and food poisoning to life-threatening nosocomial infections. Increasing resistance of *S. aureus* isolates to glycopeptide antibiotics, most prominently vancomycin, is a major concern in today's intensive care units, and therefore an alternative strategy to combat this pathogen is urgently required. *Accessory gene regulator* (*agr*) is the best-characterized QS circuit in *S. aureus*. The *agr* system utilizes cyclic oligopeptides, termed autoinducing peptides (AIPs), and these contribute to bacterial pathogenesis by orchestrating the temporal cell density-dependent expression of virulence genes [22]. Genes regulated by *agr* encode cell-surface proteins such as protein A, coagulase, and fibronectin-binding proteins, and secreted proteins including proteases, hemolysins, toxic shock syndrome toxin 1 (TSST-1), and enterotoxin B.

In addition, the *agr* QS system has also been linked to resistance with glycopeptide antibiotics in *S. aureus* [23]. Notably, Novick and coworkers have demonstrated that transient inactivation of the *agr* QS circuit might indeed be sufficient to prevent the deleterious effects of certain *S. aureus* infections [24]. Thus far, four different AIPs, with varying degrees of sequence similarities, have been identified as *agr* QS molecules (Figure 1) [25]. As a starting
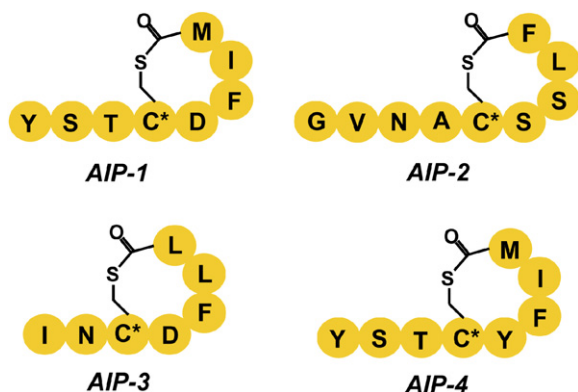
Figure 1. Structures of the AIPs Used by *S. aureus*
The oligopeptides are cyclized posttranslationally via a thioester linkage between the thiol moiety of the conserved (*) Cys and the carboxyl group of the C-terminal residue.

point for antibody-based interference with AIP-mediated QS, we focused on the AIP-4 QS system and its cognate strains RN4850 and NRS168 [16].

RESULTS AND DISCUSSION

Design and Synthesis of the AIP-4 Hapten
Based on the reported structural information of AIP-4 [26], we designed and synthesized the hapten AP4, **5** to elicit an anti-AIP-4 antibody immune response in mice (Figure 2). Our reasoning for the chemical switch from the native thiolactone to a lactone-containing hapten was based on a lactone's greater aminolytic stability [27]. This strategy ensured that the hapten conjugates remained structurally intact during the immunization process and subsequent immune response, thus avoiding the generation of degradation products with unknown chemical and biological properties as previously uncovered for other QS molecules by our laboratory [28]. Furthermore, this substitution was also intended to prevent a possible intramolecular thiol exchange between the conserved thiolactone and the pendant cysteine thiol. Therefore, Fmoc-serine(Trt)-OH was incorporated at position 4 in place of the native cysteine residue.

The hapten **5** was conjugated to the carrier proteins keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) via a bifunctional linker (see Figure S1 in the Supplemental Data available with this article online). Balb/c mice were immunized with the KLH conjugate using standard protocols [19]. Overall, the immunizations resulted in moderate titers (1600–3200) and, based on ELISA analysis, 20 monoclonal antibodies (mAbs) were prepared. The affinities of the AP4 mAbs were determined against all four natural AIPs using competition ELISA methodology (see Table S1). One of the mAbs, namely AP4-24H11, possessed strong binding affinity ($K_{d\ AIP-4} \approx$ 90 nM) and high specificity to AIP-4 while displaying little crossreactivity for the other AIPs ($K_{d\ AIP-1} \approx 5\ \mu M$, $K_{d\ AIP-2} \geq 25\ \mu M$, $K_{d\ AIP-3} \geq 25\ \mu M$). The ability of AP4-
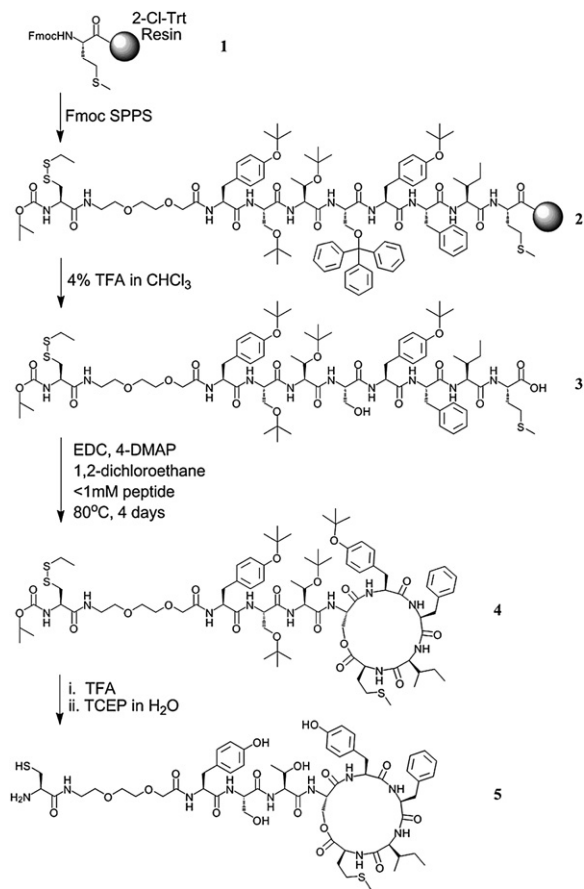

Figure 2. Synthesis of the AP4 Hapten 5
The linear peptide was synthesized on 2-chlorotrityl resin preloaded with Fmoc-methionine **1** using standard Fmoc chemistry employing DIC/HOBt as coupling reagents. The N-terminal pendant cysteine was incorporated for conjugation to a carrier protein and the short flexible linker was added between the hapten and the carrier protein as a spacer. The protected linear peptide was released from the resin using 4% trifluoroacetic acid in chloroform, which also selectively removed the trityl protection group from the serine. Intramolecular lactonization under dilute conditions was performed using EDC/4-DMAP, and subsequent side-chain deprotections afforded the AP4 hapten **5**. (For full details, see Experimental Procedures.)

24H11 to discriminate between AIP-1 and AIP-4 is noteworthy, as these two oligopeptides differ only at position 5 with an aspartic acid residue in AIP-1 and a tyrosine moiety in AIP-4. Thus, AP4-24H11 was selected for further biological evaluation.

AP4-24H11 Alters Expression of Virulence Factors in *S. aureus*
α-Hemolysin and protein A are two major virulence factors in *S. aureus*, and expression of these proteins is tightly regulated by *S. aureus* signaling networks including the AIP-based *agr* QS system. The *agr* QS system positively regulates expression of α-hemolysin, whereas protein A production is downregulated by QS signaling. In order to test our rationale that anti-AIP antibodies are able to interfere with QS signaling in *S. aureus*, we examined whether

the anti-AIP-4 mAb AP4-24H11 could modulate the expression of α-hemolysin and protein A in *agr* group IV strains RN4850 and NRS168. First, we observed that AP4-24H11 affects the expression and/or secretion of *S. aureus* exoproteins, some of which might also be regulated by the *agr* QS circuits (see Figure S2A). As seen in Figure 3A, mAb AP4-24H11 can successfully reduce the α-hemolysin expression in *S. aureus*; furthermore, no hemolytic activity was observed on blood agar plates with the AP4-24H11-treated supernatant (Figure S2B). In contrast, protein A expression was significantly increased by mAb AP4-24H11 in RN4850, which is also consistent with *agr* QS inhibition.

The only structural difference between AIP-1 and AIP-4 is position 5, and our data suggest that AP4-24H11 is able to bind to AIP-1 with moderate affinity ($\approx$5 μM). Therefore, we investigated whether AP4-24H11 could affect QS signaling in an *agr* group I strain, namely Wood 46. Clearly, AP4-24H11 was not able to block α-hemolysin expression in Wood 46 as effectively as in RN4850; however, a notable decrease in α-hemolysin production in Wood 46 grown in the presence of AP4-24H11 was evident (Figure 3A). These data suggest that it might be possible to generate crossreactive mAbs that suppress *S. aureus* QS signaling of two or more different *agr* groups.

Although it could be argued that the decrease in toxin production and overall protein secretion is caused by an antibody-mediated growth defect, it is important to note that no significant growth changes of *S. aureus* were observed over a 24 hr growth period in the presence of AP4-24H11 (Figure 3B). In addition, no discernable growth effects were observed with mAb SP2-6E11, an unrelated isotype control ($\kappa\gamma_{2a}$) for AP4-24H11.

One of the important bacterial virulence factors regulated by QS is biofilm formation. In *S. aureus*, biofilm formation is known to be negatively regulated by *agr* QS signaling [29], which is indeed one of the problems in controlling *S. aureus* virulence through *agr* QS inhibition [30]. Consistent with previous studies, AP4-24H11-mediated QS inhibition led to increased biofilm formation in RN4850 (Figure 3C). Although the increase of biofilm formation poses a significant problem in chronic infection of *S. aureus*, it represents a lesser predicament in acute infections and, thus, mAb AP4-24H11 might still be an effective way to control such *S. aureus* infections.

Real-Time PCR Analysis
To further examine *agr* QS inhibition by AP4-24H11, we performed real-time polymerase chain reaction (real-time PCR) analysis to evaluate whether the observed changes in virulence factor expression were indeed caused by interference with the *agr* QS system, that is, whether the presence of AP4-24H11 affects the transcription of *rnaIII*, the immediate product of *agr* autoinduction and the main QS effector in *S. aureus* [31]. As expected, the *rnaIII* transcriptional level in RN4850 during stationary growth phase was reduced significantly (>50-fold) by AP4-24H11; this finding advocates that the alteration of α-hemolysin and protein A expression is a direct result

of the interference of AIP-4-mediated QS signaling by AP4-24H11 (Figure 3D). Yet, the subtle changes in overall exoprotein expression (see Figure S2A) might be misconstrued to mean that AP4-24H11 does not block the QS signaling efficiently; however, our real-time PCR analysis provides evidence that AP4-24H11 significantly inhibits AIP-4-based QS in *S. aureus* RN4850.

To gain greater appreciation of the specificity and potential limitations of antibody-based QS interference in *S. aureus*, we investigated the transcriptional level of two additional virulence regulators, namely *sarA* (*s*taphy*l*ococcal *a*ccessory *r*egulator) and *saeR* (*s*taphylococcal *a*ccessory protein *e*ffector) [6, 32, 33], which control the response to environmental stresses as well as virulence factor expression in *S. aureus*. Importantly, no significant changes ($\leq$2-fold) were observed in either *sarA* or *saeR* transcription, supporting that AP4-24H11 only affects the *agr* QS system (Figure 3D).

The transcription of α-hemolysin and protein A was analyzed by real-time PCR. As stated (vide supra), significant changes were seen in protein expression level. In terms of transcription, the *hla* and *spa* genes were suppressed and elevated, respectively, $\approx$3- to 5-fold, again confirming that *rnaIII* affects not only transcription but also translation of these proteins, as reported previously [31]. Finally, exofoliatin A (*eta*) transcription was investigated, which is another *agr* QS-regulated toxin exclusively produced by AIP-4 utilizing *S. aureus* strains [34]. Gratifyingly, our data indicated that AP4-24H11 also decreased *eta* transcription by $\approx$10-fold (Figure 3D).

Inactivation of AP4-24H11 by the Synthetic AIP-4
Balaban and coworkers have proposed that the RNAIII-activating protein (RAP) regulates *agr* QS signaling in *S. aureus* in an AIP-independent manner [35]. In addition, the same group has reported that a linear peptide, termed RNAIII-inhibiting peptide (RIP), could blunt RAP-mediated QS signaling, resulting in a decrease in RNAIII transcription, although it is still debatable [36, 37]. Although we have presented evidence that AP4-24H11 inhibited *agr* QS through binding to AIP-4 and sequestering it from the cell growing medium, there was still the possibility that AP4-24H11 might affect other signaling systems in *S. aureus* including RAP, which in turn could affect the *agr* QS network. As such, we investigated whether external addition of AIP-4 could restore the *agr* QS signaling network in *S. aureus* RN4850 in the presence of AP4-24H11. Thus, we treated AP4-24H11 with an equimolar amount of synthetic AIP-4 before addition to the *S. aureus* growth medium; this then would assure saturation of the antibody binding sites with the AIP-4 peptide. As seen in Figure 3E, the addition of synthetic AIP-4 efficiently reduced the quorum quenching effect of AP4-24H11, and as a result fully restored expression of α-hemolysin in *S. aureus* RN4850. As expected, this finding provides additional confirmation that AP4-24H11 indeed sequesters AIP-4 in *S. aureus* growth medium and inhibits AIP-dependent QS signaling in *S. aureus* in a strictly AIP-4-dependent manner.
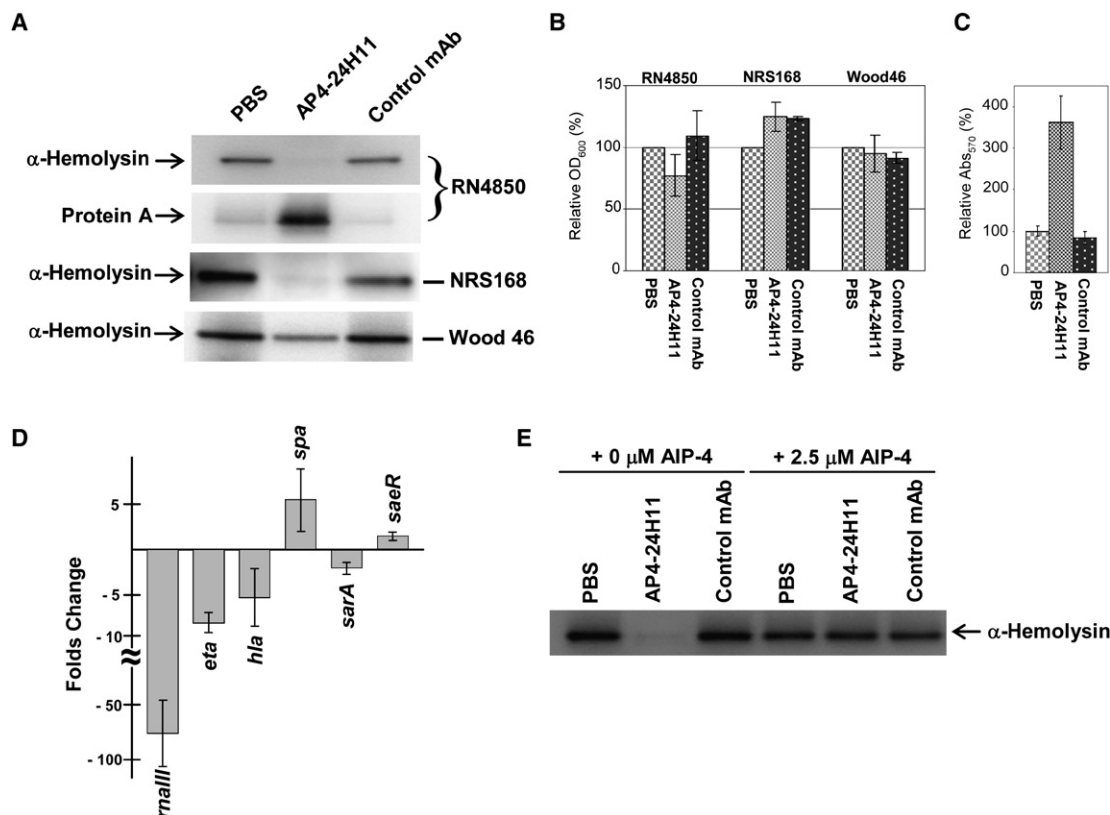
Figure 3. Inhibition of Quorum Sensing Signaling in *S. aureus* by AP4-24H11

(A) Western blot analyses of α-hemolysin and protein A expression in *S. aureus* (RN4850 and Wood 46). *S. aureus* culture supernatants were prepared as described in Experimental Procedures.

(B) Relative OD_{600} (%) of RN4850, NRS168, and Wood 46 after a 20–24 hr incubation in the presence/absence of AP4-24H11.

(C) Analysis of static biofilm formation in RN4850.

(D) Real-time PCR analysis. The amounts of the selected mRNAs were measured in RN4850 grown in the presence or absence of AP4-24H11. Relative quantification was performed using *gyrA* as a calibrator. At least two independent experiments were carried out for each experiment in duplicate. Actual numbers of fold change: *rnaIII* (-77 ± 48), *eta* (-8.1 ± 1), *hla* (-5.2 ± 3.1), *spa* ($+5.7 \pm 3.6$), *sarA* (-2.1 ± 0.6), and *saeR* ($+1.4 \pm 0.4$).

(E) Suppression of AP4-24H11-mediated QS inhibition in *S. aureus* by AIP-4. AP4-24H11 (≈ 1.3 μM) was incubated with the native AIP-4 (2.5 μM) in CYPG medium for 20 min at room temperature. Overnight cultured *S. aureus* cells were diluted into the above medium ($OD_{600} \approx 0.03$) and grown for 20–24 hr at 37°C under the static condition. The supernatants were prepared and analyzed. Error bars indicate the standard deviation (σ)) of the experiment. (For full details, see Experimental Procedures.)

AP4-24H11 Inhibits *S. aureus*-Induced Apoptosis in Mammalian Cells

Recent studies have shown that incubation of Jurkat T cells with supernatant of *S. aureus* culture results in induction of apoptosis [38]. We treated Jurkat cells with the supernatants of *S. aureus* (RN4850 and Wood 46) cultures grown in the presence or absence of AP4-24H11. After incubation for 4 hr with the supernatant, the cleavage of poly(ADP-ribose) polymerase (PARP), a biochemical marker indicative of apoptosis induction, was evaluated in Jurkat cell protein extracts. As shown in Figure 4, AP4-24H11 prevented RN4850 supernatant (1%) to induce PARP cleavage in Jurkat cells, and also partially inhibited the effect of Wood 46 supernatant. In fact, previous mechanistic studies proposed that one of the major factors in *S. aureus* supernatant responsible for inducing apoptosis might be α-hemolysin; our findings (Figures 3A and 4), support this positive correlation between expression of α-hemolysin and *S. aureus*-induced apoptosis.

AP4-24H11 Blocks *S. aureus*-Induced Dermal Injury in Mice

Next, we investigated the potential of mAb AP4-24H11 to mitigate *S. aureus*-induced injury in vivo by employing a murine subcutaneous infection model. Freshly grown log phase *S. aureus* RN4850 was suspended in PBS containing Cytodex beads and, where indicated, AP4-24H11 or control IgG. Subcutaneous injections of bacterial suspension or vehicle control were made in the flank of SKH1 hairless mice followed by close monitoring over 7 days. Doses administered were 10^7 or 10^8 bacteria (colony forming units; cfu) and 0.6 or 0.06 mg AP4-24H11 or control IgG. Mice receiving 10^7 cfu developed minimal hyperemia/edema followed by limited induration over 7 days
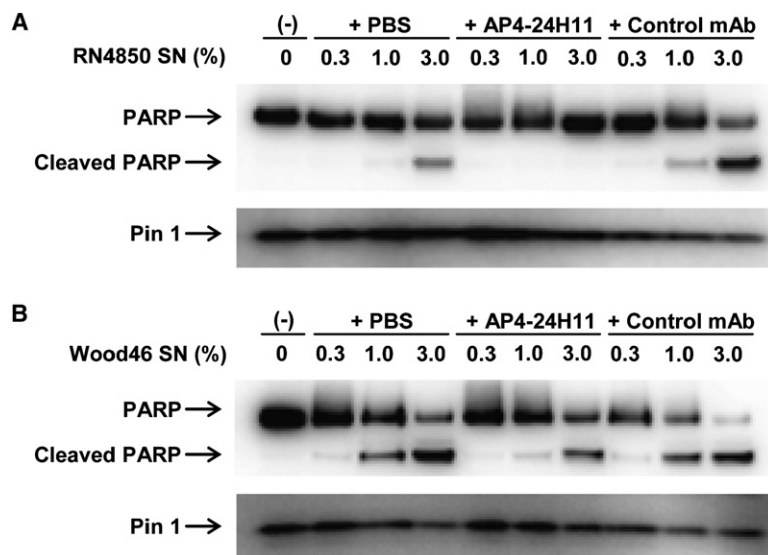
Figure 4. Inhibition of *S. aureus*-Induced PARP Cleavage by AP4-24H11

PARP cleavage in Jurkat cells after treating with *S. aureus* (A) RN4850 and (B) Wood 46 supernatants (SN). Human Jurkat leukemic T cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 10 mM L-glutamine, and 50 mg/ml streptomycin and penicillin (GIBCO, Invitrogen). *S. aureus* supernatants were prepared as described in Experimental Procedures, and the supernatants of RN4850 were further concentrated to one third of the original volume using Amicon Ultra-4 (5000 NMWL) centrifugal filter devices (Millipore). Confluent cells were distributed to 24-well plates in fresh medium (0.5 ml) and incubated for 6 hr before addition of the *S. aureus* supernatants. After a 4 hr incubation with the indicated amount of *S. aureus* supernatants, cell extracts were prepared and analyzed by western blotting using anti-PARP antibody.

(see Figure S3A). However, as early as 6 hr after injection, mice receiving 10^8 cfu suspended in saline or control IgG showed early-stage hyperemia/redness at the injection site and extending 3–5 mm horizontally and 5–10 mm vertically in a diagonal pattern along the flank (Figure 5A). Upon reexamination at 18 hr, the same areas surrounding the injection site were devitalized, and the skin was transformed to a brittle, reddish-brown scab. Over the 7 day observation period, the hardened scab began to detach from the surrounding relatively normal appearing skin, and small amounts of purulent exudate were observed at the normal/necrotic junction. In contrast, skin injury

was abrogated in mice that received 10^8 bacteria with 0.6 mg AP4-24H11 (Figure 5C). As anticipated, the lower dose of AP4-24H11 (0.06 mg) was not protective (Figure 5B), and control mice receiving 10^8 cfu with 0.6 mg control IgG were not protected (see Figure S3B). Mice that received an injection of PBS/Cytodex alone or containing 0.6 mg AP4-24H11 remained normal over the observation period with the exception of occasional local induration (Figure 5D). Excitingly, animals that had received the protective dose of 0.6 mg AP4-24H11 in combination with *S. aureus* RN4850 did not develop any significant lesions over the 7 day observation period.
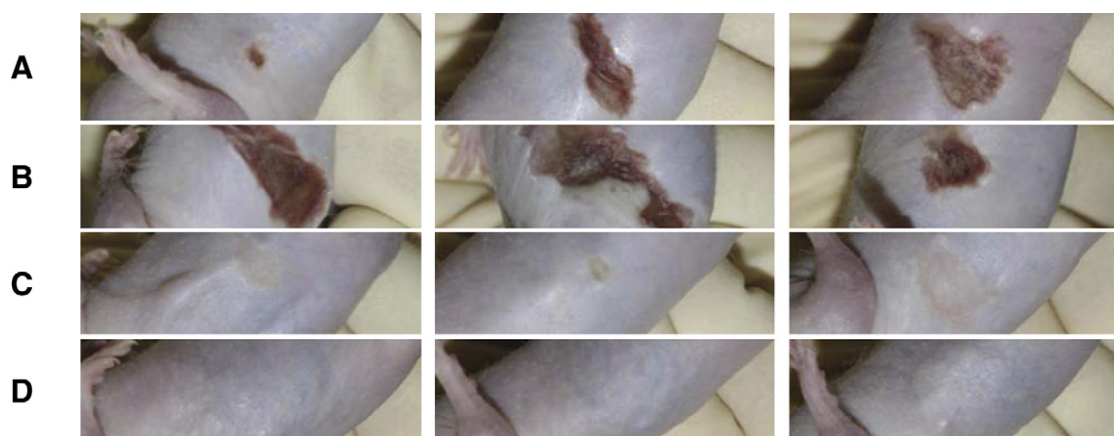

Figure 5. Inhibition of *S. aureus*-Induced Abscess Formation by AP4-24H11 in Mice Models

SKH1 euthymic hairless mice (6–8 weeks old) received 200 µl intradermal flank injections containing *S. aureus* (1 × 10^8 bacteria), 4 µl packed volume Cytodex beads, DPBS, and mAb AP4-24H11 or control IgG (0.06 or 0.6 mg). Additional control animals received 200 µl intradermal injections containing Cytodex beads or beads plus antibody. After injections were made, the mice were monitored at least three times each day over a period of 4–7 days. At the conclusion of the monitoring period, the mice were euthanized and tissues were harvested for bacteriologic and histologic analysis.
(A) *S. aureus* + PBS.
(B) *S. aureus* + AP4-24H11 (0.06 mg).
(C) *S. aureus* + AP4-24H11 (0.6 mg).
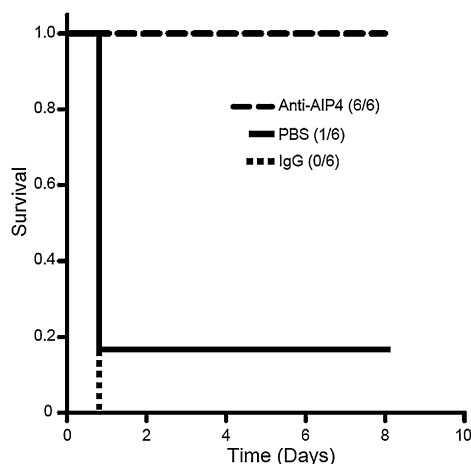(D) Cytodex + AP4-24H11 (0.6 mg).

Figure 6. Passive Immunization of Mice with AP4-24H11 against *S. aureus* Infection

Survival in mice that were pretreated with mAb AP4-24H11 or control IgG followed 2 hr later by *S. aureus* injection (3×10^8 i.p.). The numbers in parentheses show the number of survivors/number per group. Log rank statistic, $p = 0.001$; $n = 6$ for each group.

Passive Immunization with AP4-24H11 Protects Mice from *S. aureus*-Induced Fatality

To evaluate the effectiveness of a passive immunization approach using AP4-24H11 against a lethal challenge with *S. aureus*, SKH1 hairless mice received a 1 ml intraperitoneal (i.p.) injection of AP4-24H11, control IgG, or vehicle (Dulbecco's phosphate-buffered saline; DPBS) followed 2 hr later by 0.5 ml DPBS$^-$ containing 3×10^8 *S. aureus* RN4850. As shown in Figure 6, all of the mice receiving AP4-24H11 (6/6) survived through the 8 day observation period. In contrast, only one of the DPBS-treated control mice (1/6) and none of the control IgG-treated mice (0/6) survived longer than 24 hr. These data further validated our immunopharmacotherapeutic approach for combating acute *S. aureus* infections.

SIGNIFICANCE

We have sought to elucidate the consequences of quorum sensing signaling using highly specific anti-autoinducer antibodies elicited against a rationally designed synthetic hapten. Our data indicate that the scavenging of an AIP by a monoclonal antibody is sufficient to suppress QS-controlled expression of virulence factors in *S. aureus*, which to our knowledge establishes this as the first report of AIP removal from bacterial culture without genetic manipulations of the organism. Antibodies now comprise over one third of the molecules undergoing clinical evaluation, mostly for cancer immunotherapy [39]. However, antibody-based therapies for the treatment of bacterial infections using QS as a molecular target are still in their infancy [39–41]. Conventional QS interference strategies utilizing small-molecule antagonists are based on a competition between the bacterial autoinducer

and the inhibitor for the bacterial AI receptor protein. In contrast, quorum quenching antibodies engage in competition with the AI receptor for the autoinducer. In this context, an immunopharmacotherapeutic strategy is highly appealing, as the antibody acts as a "decoy-receptor" while possessing well-documented pharmacokinetic behavior and pharmacodynamic response.

In total, antibodies generated against bacterial autoinducers represent a new and valuable set of immunological tools for both the study of QS-controlled processes and potentially an alternative strategy engaging immunopharmacotherapy for the prevention or treatment of infections in which QS signaling contributes to bacterial pathogenesis.

EXPERIMENTAL PROCEDURES

Synthesis of the Linear Protected Peptide 3

All N-α-Fmoc-protected amino acids, coupling reagents, and resins for peptide synthesis were purchased from EMD Biosciences (San Diego, CA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). ESI-MS analyses were performed with API150EX (PE SCIEX, Foster City, CA, USA), and Hitachi L-7300 (Hitachi, San Jose, CA, USA) and Shimadzu SCL-10A (Shimadzu, Columbia, MD, USA) were used for analytical and preparative HPLC experiments, respectively.

The peptide was synthesized by Fmoc solid-phase peptide synthesis on 2-chlorotrityl resin preloaded with the Fmoc-Met **1**. An Fmoc-Ser(Trt)-OH was incorporated at the position of lactonization. All other residues were chosen with side-chain protecting groups stable to dilute TFA and labile in 95% TFA. A short flexible linker was incorporated penultimate to the N terminus by coupling Fmoc-8-amino-3,6-dioxaoctanoic acid. The N-terminal residue was Boc-Cys(SEt)-OH for eventual use in conjugation to carrier proteins.

Specific Conditions

Batch synthesis was carried out on 1 mmol resin swollen in DMF for at least 1 hr. A solution of the protected amino acid, N,N'-diisopropylcarbodiimide (DIC), and HOBt (4 equivalents each) in 5 ml DMF was prepared and allowed to sit for 5 min for preactivation, followed by the addition of 0.5 ml sym-collidine. The cocktail was added to the resin for coupling, which was generally complete in 1 hr. The resin was then washed with DMF and subjected to Fmoc deprotection with 20% (v/v) piperidine in DMF (2 × 7 min). The resin was then washed with DMF and the next coupling reaction was carried out. When synthesis was complete, the resin was washed with DMF, then methylene chloride, and finally with ether before it was placed in a desiccator.

Cleavage (and Trityl Deprotection)

The resin was added to a cocktail of 4% TFA, 4% triisopropylsilane (TIS), and 0.5% H_2O in chloroform, and shaken for 6 hr. The mixture was filtered, allowing the filtrate to drip into cold ether to precipitate the peptide. The ether mixture was centrifuged and the supernatant was decanted. The peptide was then washed (twice) with ether by resuspending the solid in ether, centrifuging, and decanting the supernatant. The resulting solid was placed in a desiccator.

Purification

The fully protected peptide **3** was dissolved in methylene chloride and purified by normal-phase silica gel chromatography eluted with 5% methanol in methylene chloride.

Lactonization of 3

The protected linear peptide **3** was dissolved in 1,2-dichloroethane (previously dried over anhydrous $MgSO_4$) to give a final concentration of no greater than 1.0 mM. The solution was stirred and heated to 80°C and 3 equivalents each of 1-ethyl-3-(3-dimethylaminopropyl)-

carbodiimide hydrochloride (EDC) and 4-dimethylaminopyridine (4-DMAP) were added; another equivalent each of EDC and 4-DMAP were added at both 24 and 48 hr into the reaction. The reaction was monitored by HPLC. After 4 days, the reaction mixture was cooled to room temperature, washed with 2 × 200 ml of 0.2 M KHSO$_4$ (aqueous), dried over anhydrous Na$_2$SO$_4$, and evaporated to dryness. The cyclized peptide **4** was purified by prep-HPLC. Yields ranged from 30%–60% as determined by analytical HPLC integration.

Global Deprotection and Disulfide Deprotection of 4

The solid, purified peptide was dissolved in TFA containing 2% TIS and stirred for 1 hr. The mixture was then evaporated to dryness. Water was added and the mixture was frozen and lyophilized. The lyophilized solid was then dissolved in H$_2$O with tris(2-carboxyethyl)phosphine hydrochloride (TCEP). The mixture was stirred for 1 hr and injected directly into the prep-HPLC for purification, yielding AP4 hapten **5**. The collected pure fractions were pooled, frozen, and lyophilized. ESI-MS: m/z calculated for C$_{57}$H$_{80}$N$_{10}$O$_{17}$S$_2$ (M + H), 1241.5; found, 1242.2.

Conjugation of 5 to KLH/BSA
Attachment of Sulfo-SMCC

Five milligrams of the carrier protein was resuspended in 0.9 ml PBS (pH 7.4). To this solution was added 1 mg of the linker sulfo-SMCC (sulfosuccinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate). The solution was stirred for 6–8 hr and the protein-linker conjugate was purified by dialysis in PBS at 4°C.

Conjugation of the Hapten 5

To the protein-linker conjugate in PBS was added 100 μl DMF containing 2 mg of the hapten **5**. The solution was shaken overnight and the protein-hapten conjugate was purified by dialysis. MALDI-TOF analysis confirmed the attachment on average of ≈6 haptens per BSA molecule.

Analysis of Exoprotein Secretion in *S. aureus*

After overnight growth on an agar plate at 37°C, a single colony of *S. aureus* (RN4850 or Wood 46) was inoculated into 3 ml CYGP medium and grown overnight (18 hr) [42]. The overnight cultured cells were diluted to OD$_{600}$ ≈ 0.03 in fresh CYGP medium, and distributed to 5 ml polystyrene cell-culturing tubes, where each tube contained 0.5 ml of the diluted cells and the appropriate antibody (0.2 mg/ml). After growth for 20–24 hr at 37°C in a humid incubator without agitation, the samples were transferred to microcentrifuge tubes (1.5 ml) and centrifuged at 13,000 rpm for 5 min. The supernatants were sterilized by filtration through a Millex-GV filter unit (0.22 μm; Millipore, Billerica, MA, USA) and analyzed by SDS-PAGE (10% bis-Tris gel; Invitrogen, Carlsbad, CA, USA). To confirm α-hemolysin and protein A expression, western blot analyses were performed using an HRP-conjugated sheep polyclonal α-hemolysin antibody (Abcam, Cambridge, MA, USA) and anti-protein A mouse monoclonal antibody (Sigma-Aldrich), and murine mAb SP2-6E11 (J.P. and K.D.J., unpublished data) was used as a control antibody. To test hemolytic activity, the *S. aureus* supernatants (75 μl × 3) were applied onto the sheep blood agar plate, and the plates were incubated at 37°C for 18 hr and at room temperature for another 24 hr.

Static Biofilm Analysis

The biofilm assay was conducted by following a literature procedure with a few modifications [43]. After *S. aureus* cells (200 μl) were grown in tryptic soy broth medium containing 0.2% glucose with or without the antibody (0.2 mg/ml) in the polystyrene 96-well plate for 20–24 hr without agitation, the plate was washed by submersion in water and dried. A crystal violet solution (200 μl, aqueous 0.1%) was added to stain the biofilm, and then the plate was washed vigorously with water followed by the addition of acetic acid (250 μl, aqueous 30%) to solubilize the remaining crystal violet. Absorbance was measured at 570 nm with a Spectramax 250 (Molecular Devices, Sunnyvale, CA, USA).

Real-Time PCR Analysis

Overnight cultured *S. aureus* RN4850 cells were diluted to OD$_{600}$ ≈ 0.03 in fresh CYGP medium (1 ml) containing the antibody and grown for 20–24 hr (OD$_{600}$ ≈ 2) at 37°C without shaking. RNA from the cells was isolated using an RNeasy mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Isolated RNA was further purified by treating with RNase-free DNase (QIAGEN) for 30 min at room temperature. The first-strand DNA was synthesized using the SuperScript First-Strand synthesis system for real-time PCR (Invitrogen) using ≈300 ng of purified RNA. Real-time PCR experiments were performed with at least two independent samples, and each experiment was set up in duplicate using LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche Applied Science, Indianapolis, IN, USA). Generic SYBR Green Protocol (Roche) was used for the PCR conditions, and relative quantification analyses were performed with a LightCycler 2.0 system (Roche Applied Science) using the housekeeping *gyrA* gene as a reference. The sequence information of the primers used in our experiments is listed in Supplemental Data [44].

Dermal Infection Model in Mice

All experiments on mice were performed in accordance with TSRI guidelines and regulations. SKH1 euthymic hairless mice, 6–8 weeks old, were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed in the biocontainment vivarium for 1 week before use in experiments. Brain heart infusion (BHI) agar was from BBL (Sparks, MD, USA; 211065), and CYGP broth contained 1% casamino acids (Fisher, Pittsburgh, PA, USA; BP1424), 1% yeast extract (EMD Biosciences, San Diego, CA, USA; 1.03753), 0.59% sodium chloride, 0.5% dextrose, and 60 mM β-glycerol phosphate disodium salt (Fluka, Milwaukee, WI, USA; 50020) as described by Novick [42]. Cytodex 1 beads (GE Healthcare, Piscataway, NJ, USA; 17-0448-01) were suspended (1 g in 50 ml) in Dulbecco's phosphate-buffered saline without calcium/magnesium (GIBCO Invitrogen) overnight at 20°C. The supernatant was decanted and the beads were washed three times by suspension in DPBS and 1G sedimentation followed by autoclaving (121°C, 15 psi, 15 min). *Staphylococcus aureus* RN4850 (AIP-4) was grown from frozen stock (BHI + 20% glycerol) on BHI agar plates at 35°C overnight. Three representative colonies were combined to inoculate 2 ml CYGP broth, and after overnight incubation without shaking, 0.25 ml of the culture was used to inoculate 5 ml of CYGP followed by incubation at 35°C, 200 rpm for 3 hr. The culture was centrifuged at 1300 × g at 4°C for 20 min, the supernatant was poured off, and the bacterial pellet was suspended in 1 ml DPBS without calcium/magnesium. The SKH1 mice received 200 μl intradermal flank injections containing *S. aureus* (1 × 10^7 or 1 × 10^8 bacteria), 4 μl packed volume Cytodex beads, DPBS, and anti-AIP-4 antibody or control IgG (0.6 or 0.06 mg). Additional control animals received 200 μl intradermal injections containing Cytodex beads or beads plus antibody. After the injections were made, the mice were monitored at least three times each day over a period of 4–7 days. At the conclusion of the monitoring period, the mice were euthanized and the tissues were harvested for bacteriologic and histologic analysis.

Passive Immunization of Mice with AP4-24H11

S. aureus RN4850 were stored at −80°C in 20% glycerol/BHI medium, thawed, and grown on BHI agar plates overnight, and three separate colonies were sampled to inoculate 2 ml CYGP medium. The inoculum culture was maintained 1 hr at 35°C without shaking, followed by shaking at 200 rpm for 3 hr. Aliquots of the freshly grown inoculum culture were transferred to 5 ml CYGP medium in 50 ml conical polypropylene tubes (1/20 dilution) followed by shaking at 200 rpm, 35°C for 3 hr. The bacteria were pelleted by centrifugation at 3000 rpm (1300 × g) for 10 min, 4°C. The bacterial pellets were resuspended in Dulbecco's phosphate-buffered saline without calcium/magnesium (DPBS$^-$) and enumerated using a Petroff-Hausser counting chamber. Final dilutions were made in DPBS$^-$ so that 3 × 10^8 bacteria were administered i.p. in 0.5 ml. To maintain viability, bacteria were administered within 2 hr of harvest.

mAb AP4-24H11, isotype-matched control IgG (1 mg each), or DPBS was administered i.p. in DPBS to SKH1 mice (6–9 weeks old; six animals per treatment group) followed 2 hr later by 0.5 ml DPBS$^-$ i.p. containing 3×10^8 S. aureus. The mice were monitored several times on the day of injection and twice each day on subsequent days, observing ambulation, alertness, response to handling, and skin temperature measured by infrared thermometry (Raytek Mini-Temp MT4; Santa Cruz, CA, USA) using a 1 cm diameter infrasternal skin site. Animals showing surface temperature consistently below 30°C and also diminished response to handling and weakened righting reflex were considered moribund and were euthanized.

Other Experimental Procedures

See the Supplemental Data for details of syntheses of AIPs (also see Figure S4), competition ELISA, and sequence information of the primers for real-time PCR.

Supplemental Data

Supplemental Data include four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://www.chembiol.com/cgi/content/full/14/10/1119/DC1/.

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