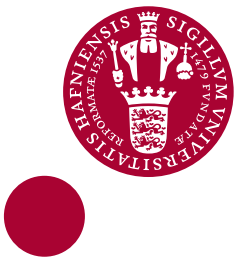

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BRIEF REPORT

Application of an *agr*-Specific Antivirulence Compound as Therapy for *Staphylococcus aureus*-Induced Inflammatory Skin Disease

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Atopic dermatitis (AD) is a chronic inflammatory skin disease where more than 90% of patients affected are colonized with *Staphylococcus aureus*. In AD, *S. aureus* δ-toxin is a major virulence factor causing cutaneous inflammation via mast cell degranulation. δ-toxin is controlled by the *S. aureus agr* quorum sensing system, and thus we addressed whether interference with *agr* signaling would limit skin inflammation. Indeed, treatment of *S. aureus* with the *agr*-inhibitor solonamide B (SolB) abolished δ-toxin production and reduced skin inflammation in a mouse model of inflammatory skin disease, demonstrating the potential of antivirulence therapy in treating *S. aureus*-induced skin disorders.

Keywords. *Staphylococcus aureus*; *agr* quorum sensing inhibition; atopic dermatitis; δ-toxin; antivirulence therapy.

Antibiotic resistance is a growing problem and alternative therapeutic strategies have been proposed such as antivirulence therapy, where virulence rather than viability is targeted [1]. Expression of bacterial virulence factors are often controlled by quorum sensing, as is the case in the human pathogen *Staphylococcus aureus*. *S. aureus* is part of the human commensal microbiota, but is also a serious opportunistic pathogen giving rise to acute and chronic infections [2]. Worryingly, methicillin-resistant *S. aureus* strains are spreading in the community and these strains, such as LAC USA300, are characterized by a hyperactive *agr*, the quorum sensing system of *S. aureus* [3, 4]. *Agr* responds to accumulation of self-produced peptides known as autoinducing peptides (AIPs) that bind to AgrC (a 2-component sensor histidine kinase) and stimulate the expression of regulatory RNAIII, the effector molecule of *agr* [3, 4]. Importantly, RNAIII also contains the *hld* gene, which encodes δ-toxin, a known *S. aureus* virulence factor and member of the cytolytic pore-forming secreted peptide family known as the phenol-soluble modulins (PSMs) [3, 4]. Recently, a link between *S. aureus* and acute dermatitis was reported, where δ-toxin was revealed as a hyperinducer of mast cell degranulation playing a key role in IgE-mediated allergic responses in atopic dermatitis (AD) [5]. AD is a chronically relapsing inflammatory skin disorder where over 90% of patients are colonized by *S. aureus*, 25% of which are USA300 [6]. Notably, *S. aureus* isolates from skin lesions of AD patients have been shown to produce high levels of δ-toxin [5]. Targeting δ-toxin production by *S. aureus* at sites of colonization in these patients might thus be of therapeutic value for the treatment of AD symptoms related to mast cell hyperinduction.

Several natural compounds have been found to target virulence gene expression in *S. aureus*, including solonamide B (SolB) and synthetic derivatives thereof [7]. Structurally, these compounds resemble the native *S. aureus* AIPs but competitively interfere with AIP binding to AgrC and inhibit *agr* [7]. Here we used a mouse model to investigate the potential use of solonamides to inhibit *S. aureus* δ-toxin–induced inflammatory responses in *S. aureus*-colonized skin, demonstrating the therapeutic applicability of an antivirulence approach against *S. aureus*-induced skin inflammation.

MATERIALS AND METHODS

Bacterial Strains

LAC, MW2, and their isogenic δ-toxin mutants (*Δhld* LAC and *Δhld* MW2 where *hld* gene start codon inactivation abolishes expression without interfering with RNAIII function [3]), were cultured in Tryptic Soy Broth (TSB, Oxoid) at 37°C overnight. For mast cell degranulation and western blotting, wild-type (WT) and *Δhld* strains were subjected to 10% native AIP-containing supernatant, with or without antivirulence compounds or nonnative AIP, at time of culture inoculation for induction and inhibition of *agr*, respectively. Bacterial supernatants were sterile filtered by passing through 0.2 μm filters.

MC/9 Degranulation Assay

The MC/9 mast cell line was cultured in cRPMI (GIBCO) supplemented with interleukin-3 (IL-3) at 37°C with 5% CO_2. Degranulation was performed as previously described [5]. B-hexosaminidase release as a measure of mast cell degranulation was quantified by measuring absorbance at 405 and 570 nm using

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the Cytation3 imaging reader (Biotek) and Gen5 software. Results of the various stimuli are given as percentages, where freeze and thaw of total cell culture represents 100% degranulation.

δ-Toxin Immunoblotting

Bacterial culture supernatants in sample buffer (1:5 ratio in 20 µL) were loaded on an 18% polyacrylamide gel, run (60 minutes at 150 V) and transferred onto a nitrocellulose membrane (90 minutes at 25 V). After blocking, the membrane was incubated with shaking overnight at 4°C with primary rabbit-anti-δ-toxin antibody (1:1000), washed, and further incubated for 2 hours with secondary antirabbit peroxidase labeled antibody (1:5000) [5]. The washed membrane was incubated in ECL mix (Biorad), exposed and processed using Image Lab software.

Epicutaneous Colonization of Mice with *S. aureus,* Treatment with SolB, and Endpoint Sampling

The protocol with license number 28-168 27-3 was approved by the Chiba University Institutional Animal Care and Use Committee. All mice were kept under specific pathogen-free conditions and experiments were performed at Chiba University in strict accordance with the regulation of Animal Experimentation at Chiba University and with the national guidelines (Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan).

Six to 12-week-old female BALB/c mice (CLEA Japan) were used in sample sizes of 5–10 mice per group. The epicutaneous colonization was performed as previously described [5] with the exception that no tape stripping was performed. Briefly, overnight cultures of *S. aureus* cultured in fresh TSB for 4 hours were washed twice and resuspended in PBS at 10^8 CFU (colony forming units). Bacterial suspension (100 µL) was placed on a patch of sterile gauze (1 × 1 cm) and attached to the shaved skin with transparent bio-occlusive dressing. For SolB application, coadministration on the patch was performed at 10 ng/100 µL. Each mouse was exposed to *S. aureus* for 1 week and compound was readministered every 48 hours by injecting into the gauze. At day 7 and after visual scoring for disease [5], mice were sacrificed, the area under the gauze was excised, and divided into samples for CFU, tissue cytokines, histology, and RNA isolation [5].

For all samples, Prism software (GraphPad) was used for statistical analyses applying the 2-tailed Mann-Whitney test. *P* values of less than .05 were considered significant.

RESULTS

Solonamides Inhibit δ-Toxin Production and Mast Cell Degranulation In Vitro

The δ-toxin of *S. aureus* is encoded within RNAIII and given that we previously have seen dramatic reduction in RNAIII expression upon exposure to SolB and derivatives thereof (ESB and Am16-L) [8], we examined δ-toxin production by western blot in supernatants of treated bacterial cultures. We observed that δ-toxin was completely absent in the supernatants of SolB and ESB-treated LAC as well as in the supernatant of the control *Δhld* LAC strain (Figure 1A), indicating that SolB and ESB effectively inhibit δ-toxin production in *S. aureus*.

To analyze whether reduced δ-toxin production in treated cultures also affected mast cells, we measured degranulation of MC/9 cells after stimulation with 10% supernatant of

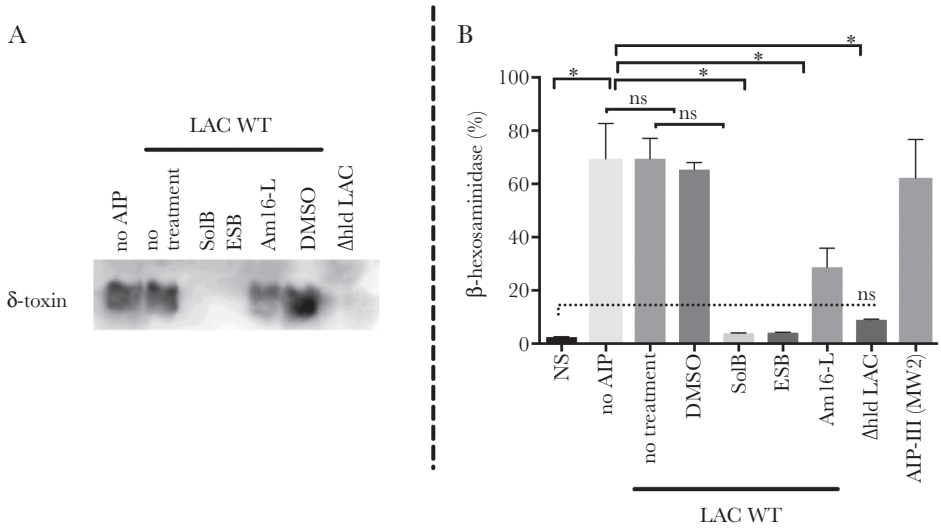

Figure 1. Solonamides inhibit δ-toxin production and mast cell degranulation. *A*, Immunoblot analysis (20 µL/well) of δ-toxin in overnight culture supernatant of *Staphylococcus aureus* wild-type (LAC WT) alone or treated with 20 µg/mL of the solonamides: solonamide B (SolB), epi-solonamide B (ESB), and the solonamide analogue Am16-L. Externally added native autoinducing peptides (AIP1) was included for induction of *agr*. Culture supernatants of LAC WT treated with compound vehicle (dimethyl sulfoxide, DMSO), and supernatant of the δ-toxin mutant strain (*Δhld* LAC) were included as controls. *B*, β-hexosaminidase released from MC/9 cells stimulated by medium alone (NS), or the same overnight culture supernatants assessed in *A*. Data represent means ± standard deviation of triplicate cultures. **P* < .001 to .05; ns, not significant. Both *A* and *B* are representative of 3 reproducible independent experiments.

solonamide-treated LAC. The absence of δ-toxin in treated culture supernatants correlated with lack of MC/9 degranulation activity (Figure 1B). To demonstrate that this effect is down to lack of δ-toxin and not to other *agr*-regulated toxins, we performed additional experiments (Supplementary Figure 1) where we added nonnative AIP3 (a competitive inhibitor of the LAC native AIP1) to *agr*-active WT and *Δhld* LAC strains, respectively. There was no significant difference between with and without AIP3 samples, while the difference between WT vs *Δhld* LAC remained intact, indicating that whether there is an active (−AIP3) *agr* or an inhibited *agr* (+AIP3) it is the absence of the δ-toxin production specifically that inhibits mast cell degranulation. Thus, inhibition of RNAIII by the solonamides can indeed abolish δ-toxin–induced mast cell degranulation.

Solonamide B Attenuates *S. aureus* δ-Toxin–Induced Allergic Immune Responses in a Modified Epicutaneous Colonization Mouse Model of AD-like Inflammatory Skin Disease

To examine whether solonamides suppress δ-toxin–induced disease, we applied a modified epicutaneous disease model for AD, in which the skin of BALB/c mice was colonized with LAC *S. aureus*. Here, SolB was added at the time of *S. aureus* inoculation, and readministered topically every 48 hours until the time of sacrifice (Figure 2A). One week after colonization, the mice developed severe inflammation at the site of inoculation with pronounced edema, erythema, thickening of the skin, and exudate formation (Figure 2B and 2C). Histology of excised tissue from the LAC-colonized skin revealed severe disruption of skin morphology, with prominent spongiosis, parakeratosis, and infiltration of inflammatory cells (Figure 2C bottom). In contrast, mice colonized with LAC but treated with SolB showed a significantly reduced skin disease score, a marked decrease in skin inflammatory cell infiltrate (Supplementary Figure 2), and a much less disrupted skin structure (Figure 2B and 2C) that was not due to differences in bacterial load (Figure 2D). Importantly, the disease score and histological results of the SolB-treated LAC group was almost identical to that presented by the *Δhld* LAC colonized group, suggesting that SolB inhibition of *agr* and consequently RNAIII expression reduces *S. aureus*-induced inflammatory responses in vivo, including that towards δ-toxin. Quantitative polymerase chain reaction assay on lesional skin samples showed that SolB treatment resulted in

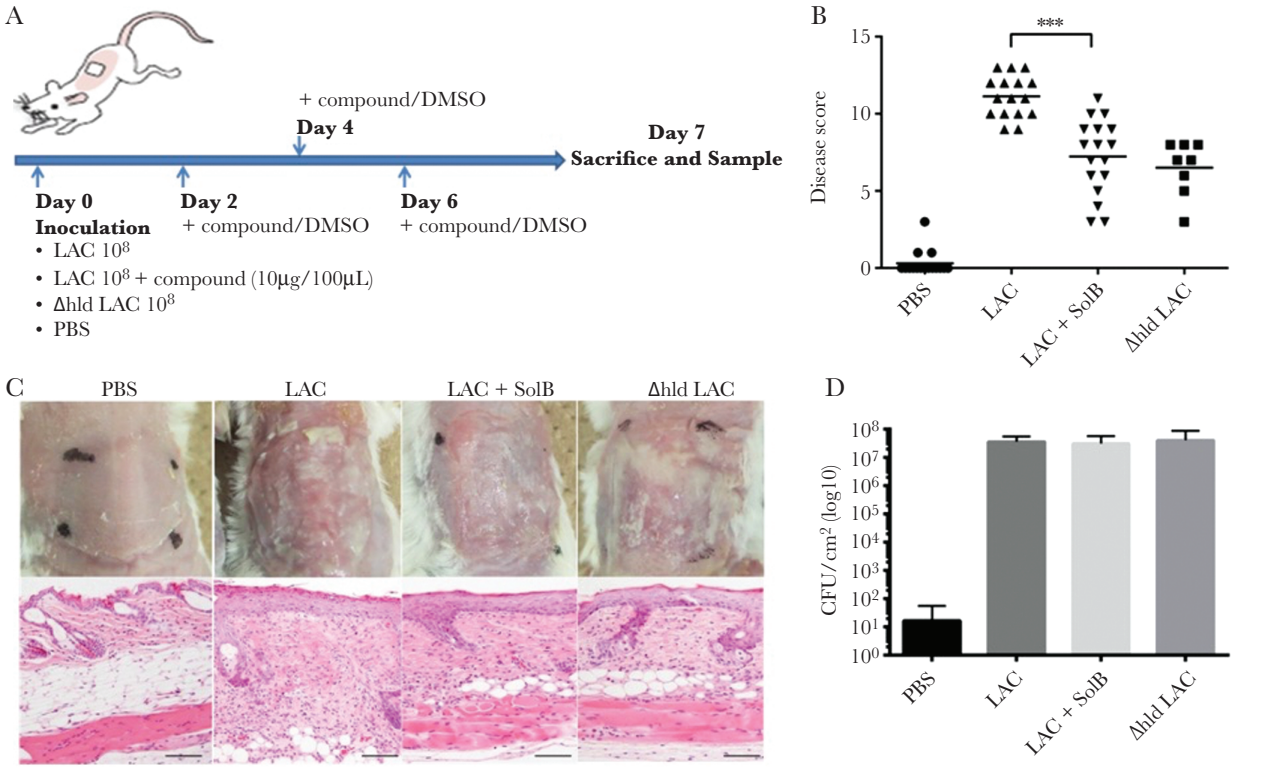

Figure 2. Solonamide B (SolB) treatment significantly reduces inflammatory skin disease. *A, Staphylococcus aureus* (LAC*; Δhld* LAC) colonization and compound treatment protocol. BALB/c mice were colonized epicutaneously with 10^8 colony-forming units of *S. aureus* using a gauze patch and treated every 48 hours with SolB for 1 week. *B*, Skin disease score 1 week after colonization with δ-toxin mutant (*Δhld* LAC), wild-type LAC, LAC treated with SolB, and in a control group treated with phosphate-buffered saline (PBS). The total clinical score of the skin lesions was designated as the sum of individual scores for 0, 1, 2, and 3 (none, mild, moderate, and severe, respectively) for thickness, erythema, edema, erosion, and scaling [5]. ***$P < .001$, Mann-Whitney test. *C*, Skin phenotype and histopathology of BALB/c mice 1 week after treatment with PBS, or colonization with *Δhld* LAC, LAC, or LAC treated with SolB. Skin sections were stained with H & E. Scale bar indicates 100 μm. *D*, Colony forming units (CFU) of *S. aureus* in excised skin tissue. Representative of 5 to 10 mice per group.

a 10-fold reduction in RNAIII expression in LAC *S. aureus* (Supplementary Figure 3), further supporting the evidence for in vivo SolB-suppression of RNAIII and subsequent δ-toxin production.

Concomitantly, we measured production of cytokines often found associated with acute AD, namely members of the IL-1 and IL-17 family, IL-6, tumor necrosis factor-alpha (TNF-α), IL-12, interferon-gamma (IFN-γ), and IL-10. In samples obtained from lesional skin we found that SolB treatment significantly reduced production of only the proinflammatory IL-1 family (α and β) and IL-6 cytokines, all 3 of which play a central role in innate immune responses, with the IL-1 family orchestrating immune responses to both infections and tissue injury [9] (Supplementary Figure 4).

DISCUSSION

Here we provide evidence that an antivirulence approach targeting *S. aureus agr* may attenuate *S. aureus*-induced inflammation in complex disorders of the skin such as AD, by showing that SolB, an *agr* quorum sensing inhibitor, impedes δ-toxin production both in vitro and in vivo, resulting in reduced inflammation and better disease outcome without interfering with bacterial growth and viability. Many factors contribute to AD, such as skin barrier defects, impaired immunity, imbalances in skin microbiota, allergens, and bacterial superantigens [10], while recent work has also documented a central role of *S. aureus* colonization and δ-toxin production in severity of the disease [5]. There exist a number of murine models of atopic skin disease available to study each of these factors, for example the OVA sensitization model, several transgenic models (IL-3, IL-4 overexpression), the spontaneous AD Nc/Nga and DS-Ng models, and the filaggrin-deficient flaky tail mouse model [5, 11]; however, the recent link between *S. aureus* δ-toxin production and disease severity was made using the modified epicutaneous colonization model [5]. This model has the advantage that, unlike others that rely on subepidermal inoculation or epicutaneous colonization after physical disruption of the epidermis (eg, by tape stripping), any inflammation observed is solely in response to *S. aureus* colonization [11].

SolB treatment not only reduced inflammatory cell infiltrates at the site of *S. aureus* colonization, but also resulted in a significant drop in the production of proinflammatory cytokines IL-1α, IL-1β, and IL-6. In AD, IL-1α, IL-1β, and IL-6 cytokine levels are statistically higher than in healthy individuals and contribute to the robust T helper 2 (T_H2) response seen in the acute phase of AD [9, 12]. Interestingly, this significant drop was not observed in the *Δhld* LAC group, implying that SolB has an impact on the inflammatory response in addition to that due to interference with δ-toxin expression. We have previously shown that SolB reduces the transcription of the *psma* operon (encoding the PSMα virulence peptides under strict regulation of *agr*), due to reduced expression of *agrA* [7]. These observations, as well as those of others, that δ-toxin and PSMα are potent inducers of IL-18 (another key mediator of skin inflammation) production from keratinocytes [13], suggest that other *agr*-regulated virulence factors also modulate skin inflammation. Further supporting this is the recent finding that PSMα induces release of IL-1α from keratinocytes in the same murine epicutaneous model used in this study. This IL-1α release, together with IL-36α, results in corresponding receptor signaling and induction of IL-17–producing cells that are key mediators of skin inflammation [14]. Interestingly, in another study looking at epicutaneous versus intradermal challenge with *S. aureus* in an AD murine model [15], an IL-36–mediated response by keratinocytes took precedence over that of IL-1α and IL-18, which were instead observed following intradermal challenge. In the same study, PSMα and not δ-toxin appeared to be the main inflammation driver [15]. These results imply that *S. aureus* skin inflammation in the context of atopic-like disease is complicated and warrants further investigations.

In summary, we hypothesize that SolB effectively targets multiple *S. aureus*-induced inflammatory responses and in turn dampens T_H2-type skin inflammation, so common in an atopic setting, by blocking mast cell degranulation and interfering with IL-1α and IL-36 skin inflammatory responses. As the solonamides have been reported to be nontoxic towards murine and human immune cells, this group of compounds may potentially be safe as antivirulence therapeutics [8] and beneficial for the treatment of inflammatory symptoms related to epicutaneous *S. aureus* colonization in AD.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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