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TCA cycle inactivation in *Staphylococcus aureus* alters nitric oxide production in RAW 264.7 cells

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Abstract Inactivation of the *Staphylococcus aureus* tri-carboxylic acid (TCA) cycle delays the resolution of cutaneous ulcers in a mouse soft tissue infection model. In this study, it was observed that cutaneous lesions in mice infected with wild-type or isogenic aconitase mutant *S. aureus* strains contained comparable inflammatory infiltrates, suggesting the delayed resolution was independent of the recruitment of immune cells. These observations led us to hypothesize that staphylococcal metabolism can

modulate the host immune response. Using an in vitro model system involving RAW 264.7 cells, the authors observed that cells cultured with *S. aureus* aconitase mutant strains produced significantly lower amounts of nitric oxide (NO[•]) and an inducible nitric oxide synthase as compared to those cells exposed to wild-type bacteria. Despite the decrease in NO[•] synthesis, the expression of antigen-presentation and costimulatory molecules was similar in cells cultured with wild-type and those cultured with aconitase mutant bacteria. The data suggest that staphylococci can evade innate immune responses and potentially enhance their ability to survive in infected hosts by altering their metabolism. This may also explain the occurrence of TCA cycle mutants in clinical *S. aureus* isolates.

Chandrasegaran Massilamany and Arunakumar Gangaplara contributed equally to this study.

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Introduction

Staphylococcus aureus is a gram-positive bacterium capable of surviving extreme environmental conditions and causing severe infections in both immunocompetent and immunodeficient individuals. It is estimated that worldwide *S. aureus* colonize, both transiently and persistently, two billion people at any given time [1]. During an infection, phagocytes recognize bacteria through toll-like receptor (TLR)-2 and produce bactericidal oxidants such as, reactive oxygen species (ROS) and nitric oxide (NO[•]), via nicotinamide adenine dinucleotide phosphate-oxidase, myeloperoxidase, and inducible nitric oxide synthase (iNOS) [2–4]. Despite the presence of bactericidal oxidants, staphylococci can survive in macrophages through robust anti-oxidant defense mechanisms such as carotenoid

pigments, superoxide dismutases, manganese homeostasis, and catalases [5]. In addition, it was recently shown that *S. aureus* adapts to nitrosative stress by expressing the NO[•]-inducible L-lactate dehydrogenase (*ldh*) as one mechanism to evade the bactericidal effects of NO[•] [6].

The regulation of virulence in *S. aureus* is complex, involving the *agr* quorum-sensing system/riboregulator RNAIII, the SarA family of regulators, and an alternative sigma factor (σ^B) [7]. In addition to these regulatory elements, the authors have demonstrated a causal relationship between tricarboxylic acid (TCA) cycle activity and virulence factor synthesis [7, 8]. During those studies, the authors created a TCA cycle mutant of *S. aureus* by inactivating aconitase and demonstrated that mice infected with this TCA cycle mutant strain required a longer time to develop cutaneous ulcers and to resolve the infection as compared to mice infected with wild-type (wt) strain [7]. These observations led us to investigate function of the TCA cycle in the host-pathogen interaction.

Materials and methods

Bacterial strains and growth conditions

Staphylococcus aureus wt strains UAMS-1 and SA564 and the isogenic aconitase mutant strains UAMS-1-*acnA* and SA564-*acnA* have been described [7, 9]. Bacteria were grown in tryptic soy broth (TSB; BD Biosciences, San Jose, CA) or on TSB-containing agar (15 g per l) at 37°C with a flask volume-to-medium ratio of 10:1 aerated by shaking at 225 rpm.

Mouse soft-tissue infection model and histology

Immunocompetent, hairless outbred mice (CrI:SKH1-*hrBR*•SKH1) were procured from the Charles River Laboratories International (Wilmington, MA). The mice were maintained in accordance with the guidelines of the Animal Care and Use Committee at Rocky Mountain Laboratories, Hamilton, MT.

Exponential growth phase bacteria (O.D.₆₀₀ ≈ 1.0; 25 ml) were harvested by centrifugation, washed two times with ice-cold phosphate buffered saline (PBS), centrifuged, suspended in 20 ml PBS, and frozen at -80°C until use. Prior to use and at the time of inoculation, the colony forming units (CFU) per ml were determined. Mice were inoculated subcutaneously in the dorsal neck region with 5 × 10⁷ CFU and monitored for the development and progression of cutaneous ulcers at the sites of infection [7]. A total of 40 animals were used with five mice per group, each inoculated with either wt or mutant bacteria. At 3, 6, 24, and 48 h postinfection, mice were sacrificed and skin

samples were taken from injection sites and fixed by immersion in 10% phosphate buffered formalin. Tissues were sectioned in 5 μm thickness and stained with hematoxylin and eosin [H and E; 10] and evaluated for inflammatory changes such as abscessation, epidermitis, and epidermal and dermal necrosis.

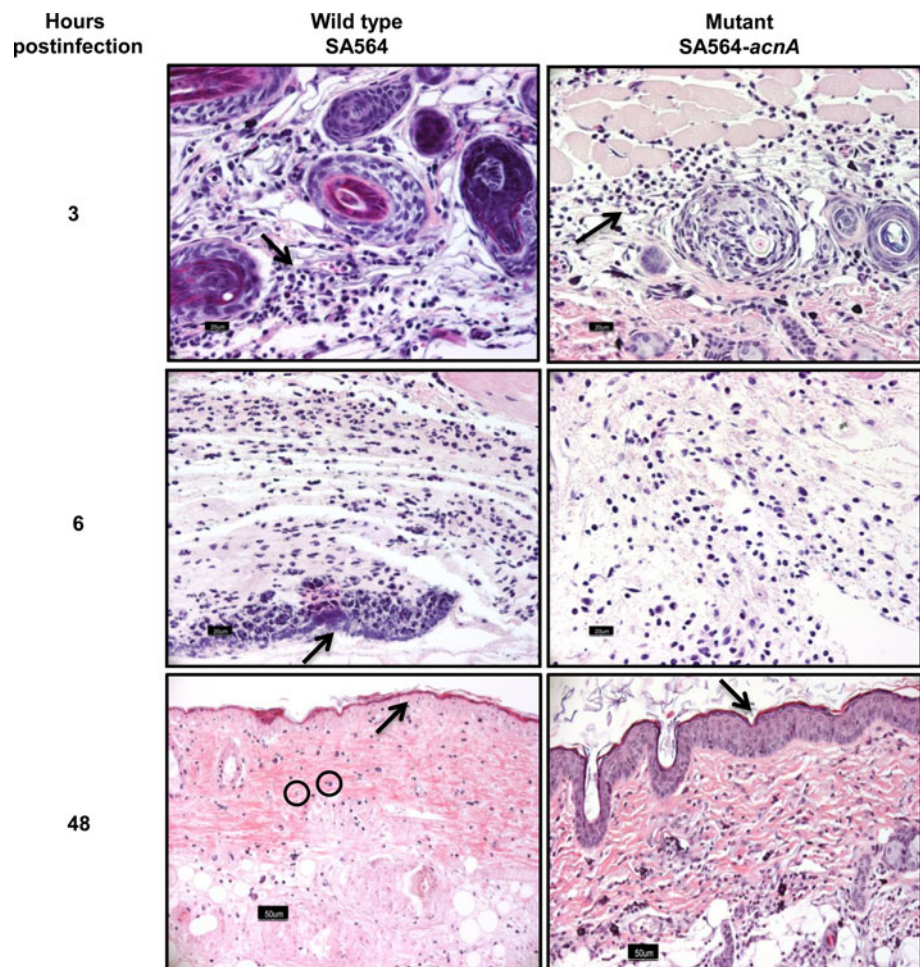
Intracellular detection of ROS and NO[•] by flow cytometry

The bacterial suspensions were prepared to a final concentration of 1 × 10⁸ CFU per ml in RPMI supplemented with 50% human serum to opsonize bacteria at 37°C for 30 min. After pelleting, the bacteria were washed with 1 × endotoxin-free PBS and suspended in DMEM containing 10% fetal bovine serum (FBS). RAW 264.7 cells (clone, TIB-71) obtained from American Type Culture Collection (Manassas, VA) were maintained in antibiotic-free DMEM supplemented with 10% FBS, hereafter called growth medium. To determine intracellular production of ROS and NO[•] in RAW 264.7 cells exposed to bacteria, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H₂DCFDA) and 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM; Invitrogen, Eugene, CA) were used as ROS and NO[•] indicators, respectively [11–13]. In brief, 100 μl each of RAW 264.7 cells (4 × 10⁶ per ml) and bacteria (2 × 10⁷ per ml) were plated in 96-well plates at a 1:5 ratio, and the plates were incubated at 37°C for 6 h. The oxidation sensitive dyes CM-H₂DCFDA and DAF-FM were added at various concentrations (0–1 μM) during the last 20 min of incubation, and the cells were washed twice with 1 × PBS. After staining the cells with a cell death marker, 7-aminoactinomycin D (7-AAD; Invitrogen), cells were acquired by flow cytometry (FC; FACScan, BD Biosciences), and the fluorescence intensity of live cells (7-AAD⁻) positive for CM-H₂DCFDA and DAF-FM was analyzed by Flow Jo software (Tree Star, Ashland, OR).

Analysis of iNOS mRNA expression

RAW 264.7 cells (6 × 10⁶) and the bacteria (3 × 10⁷) were plated in 6-well plates at a 1:5 ratio in 3 ml of growth medium, and the plates were incubated for 6 h. In addition, lipopolysaccharide (LPS) (100 ng per ml) was used as a positive control [14]. To extract RNA, the medium was removed and the cells were lysed using RLT buffer containing guanidium thiocyanate (Qiagen, RNeasy kit, Valencia, CA), and the samples were treated with RNase-free DNase I according to the manufacturer's recommendations (Qiagen). To make certain that the RNA samples were free of residual DNA, a second round of DNase I digestion was performed using amplification grade DNase I (Invitrogen), and cDNAs were synthesized utilizing

Fig. 1 Histological evaluation of skin samples harvested from mice infected with wt and aconitase mutant *S. aureus*. Skin samples were harvested from mice infected with wt or mutant *S. aureus* at the indicated time intervals postinfection, and the cutaneous sections were stained with H and E to evaluate inflammatory changes (*arrows*). Note similar suppurative dermatitis at 3 h postinfection with wt or mutant bacteria. At 6 h, suppuration and bacterial colonization was more extensive in mice infected with wt than with mutant bacteria. Tissues sampled at 48 h from mice infected with wt but not mutant bacteria consistently showed epidermal and dermal necrosis, including pyknosis and karyorrhexis of dermal fibroblasts (shown in *circles*). Original magnification, $\times 400$ (*bar* 20 μm)



Superscript III reverse transcriptase kit as recommended (Invitrogen). First, it was qualitatively verified the expression of iNOS mRNA by PCR, the levels of which were compared with an endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, using a Gradient Thermal cycler (Eppendorf, Hauppauge, NY) as described [15]. The primer sets used were: iNOS-forward, 5' CCTCCTCCACCCTACCAAGT 3'; iNOS-reverse, 5' CACCCAAAGTGCTTCAGTCA 3'; GAPDH-forward, 5' CGGCAAATTCAACGGCACAGTCAA 3'; GAPDH-reverse, 5' CTTTCCAGAGGGGCCATCCACAG 3'. The PCR products were stained with ethidium bromide and resolved in 1% agarose gel electrophoresis. Second, it was quantitatively analyzed the relative fold induction of iNOS mRNA expression utilizing commercially obtained TaqMan PCR probes and primers (ABI Biosystems, Carlsbad, CA) using an iCycler (Bio-Rad Laboratories, Hercules, CA). The cDNAs derived from three replicates for each treatment group were used for analysis, and the fold induction of iNOS mRNA expression was calculated by normalizing to GAPDH mRNA [15, 16].

Detection of surface molecules by flow cytometry

RAW 264.7 cells and the bacteria were plated as above at a 1:5 ratio, and after 6 h of incubation, the cells were harvested and stained with antibodies for TLR-2, CD80, CD86, major histocompatibility complex (MHC) class II, CD40 (eBioscience, San Diego, CA), and 7-AAD. The clones of the respective antibodies were 6C2, 16-10A1, GL1, M5/114.15.2, and 1C10. The reaction mixtures were incubated for 20 min on ice, and the cells were washed and acquired by FC (FACScan). Percentages of cells positive for each marker were then determined in the live (7-AAD⁻) subset.

Intracellular cytokine detection

RAW 264.7 cells and the bacteria were plated in 6-well plates at 1:5 ratio in growth medium. After incubating for 2.5 h, the cultures were supplemented with 2 mM monensin [Golgi stop, BD Biosciences; 10, 17] and incubated further for 4 h. It was also used cells in medium

Fig. 2 RAW 264.7 cells exposed to aconitase-mutant *S. aureus* produce less nitric oxide than wt strains. RAW 264.7 cells were cultured with wt or aconitase-inactivated *S. aureus* mutants at 1:5 ratio for 6 h at 37°C. The cultures were then exposed to DAF-FM during the last 20 min of incubation as an NO indicator. After washing, cells were stained with 7-AAD and acquired by FC. Percentages of DAF-FM⁺ cells were then determined in the live (7-AAD⁻) subset. Representative data from four individual experiments are shown

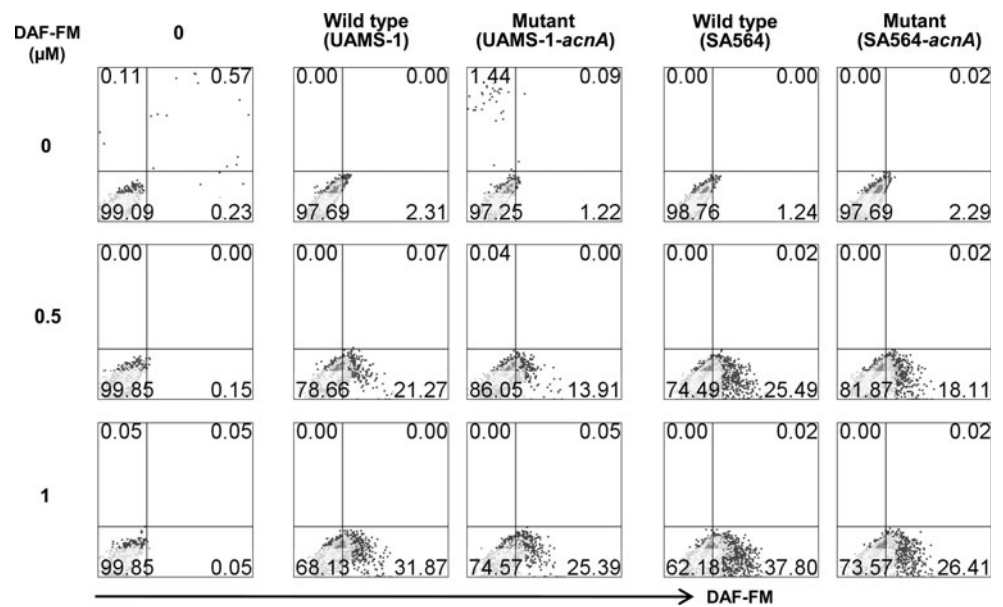


Table 1 Induction of reactive oxygen species and nitric oxide by wild type and aconitase-mutant *S. aureus* in RAW 264.7 cells

	Dye (μM)	Medium control	Wild type ^a (UAMS-1)	Mutant ^a (UAMS-1-acnA)	Wild type ^b (SA-564)	Mutant ^b (SA-564-acnA)
ROS	0	0.04 ± 0.04	1.41 ± 1.17	1.6 ± 1.29	0.75 ± 0.66	1.63 ± 1.22
	0.5	0.19 ± 0.085	34.36 ± 6.18	23.00 ± 5.43	36.18 ± 3.17	29.07 ± 7.64
	1	0.39 ± 0.15	62.78 ± 10.00	50.16 ± 8.44	71.48 ± 2.16	54.12 ± 5.92
NO	0	0.21 ± 0.11	3.08 ± 0.36	2.16 ± 0.51	1.20 ± 0.23	2.60 ± 0.39
	0.5	0.12 ± 0.02	23.01 ± 2.13 [†]	13.54 ± 1.88 [†]	29.37 ± 4.59 [‡]	18.03 ± 1.13 [‡]
	1	0.12 ± 0.05	39.41 ± 4.02	27.55 ± 5.33	50.20 ± 6.28	34.43 ± 2.99

Numbers are Mean ± SEM ($n = 4$)

^a, [†] $P = 0.01$

^b, [‡] $P = 0.04$

alone or cells treated with LPS (1 μg per ml) as negative and positive controls, respectively. At the end of incubation, cells were harvested and washed once with 1 × PBS. After fixation and permeabilization, the cells were stained with antibodies for interleukin (IL)-1β (rabbit polyclonal), IL-6 (clone, MPS-20F3), and tumor necrosis factor (TNF)-α (clone, MP6-XT22) and their respective isotype controls (eBioscience) and 7-AAD. Cells were acquired by FC, and frequencies of cytokine-secreting cells were then enumerated in live (7-AAD⁻) cell populations [10].

Statistics

Differences in the levels of iNOS mRNA, ROS, NO[•], and cytokine induction in RAW 264.7 cells cultured with wt or mutant strains of *S. aureus* were analyzed by Student's *t* test. To determine differences in inflammatory changes in cutaneous tissues at each time point postinfection with wt or mutant bacteria, skin sections were examined for abscessation, epidermitis, and necrosis and the differences

between groups were compared using one-tailed Fisher's exact test. $P \leq 0.05$ values were considered significant.

Results and discussion

Aconitase inactivation in *S. aureus* altered the temporal synthesis of secreted virulence and cell-associated adhesion factors [7]. Using a mouse soft tissue infection model, mice infected with an aconitase mutant strain (SA564-acnA) took longer to develop lesions, re-epithelialize the sites of infection, and to regain their pre-inoculation body weight relative to mice infected with an isogenic wild-type strain (SA564) [7], suggesting that disruption of the TCA cycle in *S. aureus* alters the host's response to the bacteria. In this report, it was demonstrated that *S. aureus* metabolism does not affect the recruitment of inflammatory cells but does alter the host immune response, raising the possibility that one mechanism by which *S. aureus* evades the immune response is by altering its central metabolism. Support for

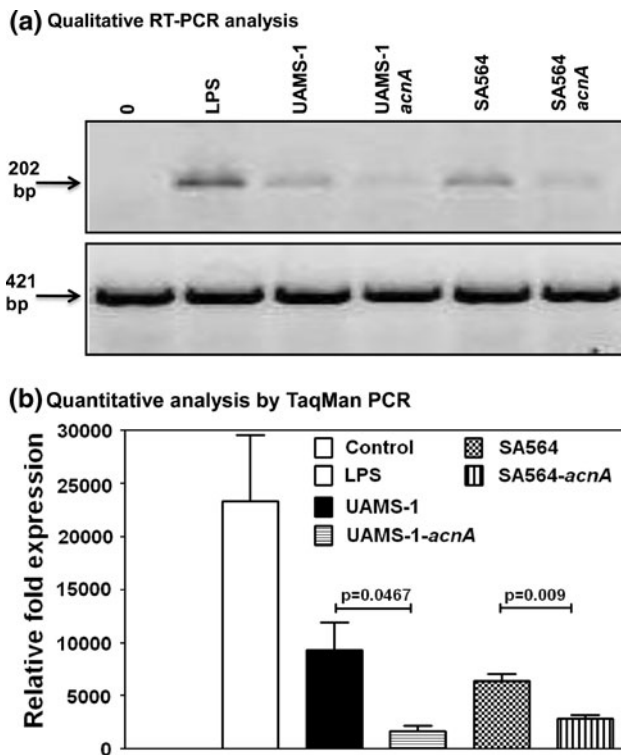


Fig. 3 RAW 264.7 cells exposed to aconitase-mutant *S. aureus* express lower amounts of iNOS mRNA than wt strains. **a** Qualitative RT-PCR analysis. RAW 264.7 cells were cultured with wt or aconitase-inactivated *S. aureus* mutants at 1:5 ratio for 6 h at 37°C. After washing, total RNA was extracted, and synthesized cDNAs and iNOS mRNA expression was examined by PCR using sequence specific primers. The ethidium bromide-stained PCR products were resolved in 1% agarose gel electrophoretic analysis, and shown are the expected sizes of iNOS (*top panel*) and GAPDH (*bottom panel*) PCR products. **b** Quantitative analysis by TaqMan PCR. cDNAs were generated from RAW 264.7 cells cultured with wt or aconitase-mutant bacteria as above and the relative fold induction of iNOS mRNA expression was determined by TaqMan PCR analysis by normalizing the expression levels of iNOS mRNA to GAPDH mRNA. Mean \pm SEM values are shown ($n = 3$)

this possibility was recently reported by Richardson et al. [6]. To test this possibility, skin samples were harvested from mice infected with *S. aureus* wt (SA564) or aconitase mutant (SA654-*acnA*) strains and assessed by histological evaluation for inflammatory changes. At 3 h postinfection, histological evaluation of skin sections from mice infected with either wt or aconitase mutant strains had identical inflammatory infiltrates, predominantly comprised of neutrophils and a few macrophages (Fig. 1). As the infection progressed, cellular infiltrates and bacteria were less pronounced in tissues sampled at 6, 24, and 48 h from mice infected with strain SA654-*acnA* as compared to strain SA564. Notably, skin samples obtained from mice infected with wt (10/10) but not mutant bacteria (4/10) consistently showed necrosis of the epidermis and dermis beyond 24 h ($P = 0.0054$). Similarly, at 48 h there was remarkable

epidermitis in mice infected with wt bacteria (5/5) but not in the aconitase mutant (1/5)-infected mice ($P = 0.024$). These observations indicate the host recognizes TCA cycle inactivated bacteria and recruits phagocytes to the sites of infection; however, it does not address whether differences in the activation state of infiltrates exist. To address this question, an *in vitro* culture system using the mouse macrophage cell line, RAW 264.7, was established to examine the production of bactericidal oxidants in response to wt and mutant strains of *S. aureus* at a single cell level by FC. Specifically, CM-H₂DCFDA was used as a broad range indicator of ROS, such as hydrogen peroxide, hydroxyl radicals, peroxy radicals, and peroxy nitrite anions, while NO[•] production was assessed using DAF-FM [11–13]. Figure 2 and Table 1 show that cells cultured with *S. aureus* aconitase mutants UAMS-1-*acnA* and SA564-*acnA* produced significantly lower amounts of NO[•] as indicated by the lower frequency of DAF-FM-positive cells when compared with the cells exposed to wt bacteria ($P < 0.05$). Similarly, cells cultured with wild-type *S. aureus* strains produced greater amounts of ROS relative to the aconitase mutant strains when analyzed using CM-H₂DCFDA-positive cells (Table 1). Taken together, these data suggest that TCA cycle inactivation does not alter the recruitment of phagocytes; however, it does alter the activation of those infected phagocytes.

The decreased synthesis of NO[•] by RAW 264.7 cells exposed to aconitase mutant strains UAMS-1-*acnA* and SA564-*acnA* relative to the isogenic wild-type strains (Fig. 2) could occur at the level of transcription or by bacterial interference with the iNOS protein-mediated NO[•] synthesis [18, 19]. To determine if the decreased production of NO[•] in cells exposed to aconitase mutant strains was due to a decrease in iNOS transcription, iNOS mRNA expression was analyzed by RT-PCR in cells exposed to wt or mutant bacteria using LPS as a positive control. As shown in Fig. 3, both wt strains (UAMS-1 and SA564) induced the expression of iNOS mRNA in RAW 264.7 cells; however, iNOS mRNA expression was significantly decreased in cells exposed to the bacterial mutants ($P \leq 0.05$). Together these data indicate that aconitase inactivation in *S. aureus* suppresses iNOS synthesis, resulting in decreased NO[•] production. This has important implications for the host-pathogen interaction because NO[•] is a critical mediator of bacterial killing [5, 20] and when produced in excess, NO[•] can contribute to tissue damage during inflammation [21, 22]. These observations and the fact that naturally occurring *S. aureus* TCA cycle mutants have been reported [23], suggest there is immune system selective pressure on bacterial TCA cycle activity. Any selective pressure that results in decreased TCA cycle activity will create metabolic signals within the bacteria [24] that result in enhanced bacterial survival, decreased

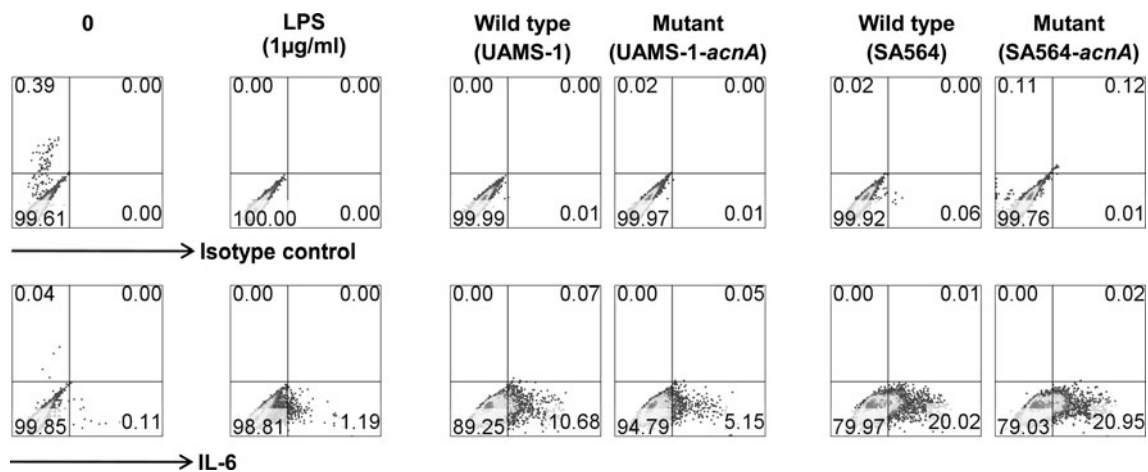


Fig. 4 RAW 264.7 cells exposed to aconitase-mutant *S. aureus* produce lower amounts of IL-6 than wt strains. RAW 264.7 cells were incubated with wt or aconitase-inactivated *S. aureus* mutants at 1:5 ratio for 2.5 h in growth medium, and after adding Golgipost, cells were further incubated for 4 h. Cells were then stained with 7-AAD,

fixed and permeabilized, followed by staining with anti-IL-6 or isotype control. Cells were acquired by FC, and the percentage of IL-6-secreting cells was enumerated in the live (7-AAD⁻) subset. Representative data from three individual experiments are shown

Table 2 Comparative analysis of antigen-presentation and costimulatory molecules induced by wild type and aconitase-mutant *S. aureus* in RAW 264.7 cells

	Medium control	LPS (100 ng/ml)	Wild type (UAMS-1)	Mutant (UAMS-1- <i>acnA</i>)	Wild type (SA-564)	Mutant (SA-564- <i>acnA</i>)
TLR-2	99.96 ± 0.01	99.92 ± 0.024	97.98 ± 0.77	98.11 ± 0.47	98.51 ± 0.70	98.33 ± 0.32
CD80	59.16 ± 8.77	70.65 ± 6.13	90.52 ± 3.08	83.90 ± 8.48	88.8 ± 3.62	78.22 ± 12.44
CD86	73.24 ± 13.38	76.58 ± 12.06	72.96 ± 12.74	64.24 ± 16.23	69.84 ± 13.71	71.56 ± 13.67
MHC-II	0.57 ± 0.46	2.42 ± 1.94	1.49 ± 0.46	1.57 ± 0.36	1.28 ± 0.22	1.047 ± 0.62
CD40	6.05 ± 2.18	81.35 ± 2.89	8.97 ± 1.71	7.77 ± 1.00	8.51 ± 0.83	8.34 ± 0.82

Numbers are Mean ± SEM ($n = 3$)

virulence factor synthesis, and an alteration of bacterial metabolism [7, 25]; thus, staphylococci may use to evade the host's defense mechanisms. Finally, these observations may explain the long duration needed to establish and resolve infections in mice inoculated with aconitase mutant bacteria [7].

Transcription of iNOS and the synthesis of NO[•] are regulated by inflammatory cytokines [26–28]. To determine if aconitase mutant strains altered the production of inflammatory cytokines, the authors enumerated by FC the frequencies of cells producing IL-1 β , IL-6, and TNF- α at the single cell level. The expression of IL-1 β and TNF- α in RAW 264.7 cells infected with wt and those infected with mutant strains was similar; however, the number of IL-6-secreting cells was approximately twofold lower in cells cultured with UAMS-1-*acnA* but not strain SA564-*acnA* (Fig. 4). Since both mutant strains carry the same mutations, it was anticipated that cytokine responses would be similar; however, *S. aureus* strain-dependent variations in host immune responses are common [29, 30]. Traditionally, IL-6 is regarded as one of the prototypical inflammatory cytokines produced in response to *S. aureus*

infection [31], but recent reports indicate that IL-6 can also have an anti-inflammatory function [32, 33]. Although the data provide an association between aconitase inactivation and decreased secretion of IL-6, additional studies are required to investigate whether suppressed IL-6 production favors bacterial survival or their destruction.

Upon entry into a host, *S. aureus* produce numerous virulence determinants [34, 35] that can induce phagocyte recognition through TLR-2 via pathogen-associated molecular patterns. In addition, during the formation of an abscess, T cells are activated through the CD28-CD80/CD86 costimulation pathway [36], while CD4 helper cells interact with macrophages via the CD40-CD40 ligand pathway to potentiate their bactericidal effects [37]. *S. aureus* aconitase mutants have been shown to decrease synthesis of virulence determinants such as lipase (*geh*), enterotoxin C (*sec*), α -toxin (*hla*), β -toxin (*hlyB*), δ -toxin (*hlyD*), σ^B (*sigB*), capsule (*cap*), and clumping factor (*clfA*) [7]; thus, it is possible that antigen presentation could be affected by aconitase inactivation. To determine if the decreased synthesis of virulence determinants by the aconitase mutant strains affect adaptive immunity, the

authors assayed for the presence of antigen-presentation (MHC class-II) and costimulatory molecules (CD80, CD86, and CD40; Table 2). Both the wt and mutant strains of *S. aureus* induced CD80, MHC class-II, and CD40 molecules in RAW 264.7 cells to a similar level. Although the expression of CD80 tended to be lower in cells cultured with aconitase mutant strains as compared to those cultured with the wt bacteria, the differences were not significant. These data strongly suggest that phagocyte antigen presentation is independent of *S. aureus* TCA cycle activity.

Staphylococcus aureus have evolved several mechanisms that favor their survival in infected hosts. These include interference with chemotaxis, opsonin-mediated phagocytosis, and detoxification of bactericidal oxidants [5]. Recently it was shown that staphylococci can adapt to nitrosative stress and maintain virulence by promoting the production of lactate through NO[•]-inducible *ldh* [6]. Normally, bacteria induce lactate dehydrogenases when the oxidation of NADH through the respiratory chain is inhibited. In the case of the *S. aureus* NO[•]-inducible *ldh*, NO[•] prevents the respiratory chain from oxidizing NADH and forces carbon through an NO[•]-inducible *ldh* to oxidize NADH [6, 38, 39]. Similarly, inactivation of the TCA cycle redirects carbon away from the TCA cycle and into overflow metabolism pathways, including *ldh*. In other words, when phagocyte-produced NO[•] decreases bacterial respiratory activity, NADH accumulates, synthesis of the NO[•]-inducible *ldh* is activated, and carbon flow-through the TCA cycle is redirected into *ldh*, allowing for the oxidation of NADH. Concomitantly, the decrease in TCA cycle activity results in a decrease in phagocyte-produced NO[•] (Fig. 2 and Table 1) and an increase in *S. aureus* survival [7]. In total, it is tempting to speculate that mammalian immune responses evolved to recognize bacterial metabolic signatures, and this has resulted in the repurposing of bacterial metabolic pathways to suppress the host's ability to mount an effective innate immune response.

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