***Staphylococcus aureus* nt5 gene contributes to bacterial infection ability to form kidney abscess**

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Abstract

Staphylococcus aureus (*S. aureus*) is a commonly conditional infection pathogen, in which several key virulence genes are responsible for bacterial infection ability. The *S. aureus nt5* gene, encoding 5'-nucleotidase, mediates bacterial nucleic acid pathway, yet it is nearly unknown of *nt5* function for staphylococcal infection ability. Herein we have constructed *S. aureus* mutant with the gene *nt5*^{C166T} silence (*S. aureus Δnt5*) by a CRISPR RNA-guided base editing system to investigate bacterial infection ability *in vitro* and *in vivo*. As expected, several *nt5*-related genes are disturbed in *S. aureus Δnt5*, in which gene transcription level of *py* is decreased compared with the wild-type *S. aureus*. Bacterial gene *nt5* is downregulated and *py/adk* are upregulated when *S. aureus* is exposed to antibiotics daptomycin, which indicates *nt5*-mediated nucleic acid pathway is interfered upon with daptomycin treatment. Furthermore, the mutant *Δnt5* displays about a 40-fold reduction of bacterial loading in mouse kidney on a mouse sepsis model, and the infection ability of *Δnt5* is reduced than the wild-type bacteria. The gene *nt5* contributes to *S. aureus*-infected abscess formation in mouse kidney, and the silence of *nt5* gene promotes phagocytosis of *S. aureus* by mouse and human immunocytes. In general, our findings reveal *nt5* silence impedes bacterial loading in kidney to form abscess but enhances *S. aureus* to be phagocytosed by host cell immune system *in vitro* and *in vivo*, which indicates that *nt5* gene plays an important role in bacterial infection and immune evasion.

Keywords: *Staphylococcus aureus*, *nt5* gene, 5'-nucleotidase, gene silence, abscess, phagocytosis, daptomycin

Introduction

Staphylococcus aureus (*S. aureus*) is a commonly conditional pathogen causing severe infections in almost every organ of human body, including sepsis, endocarditis, skin and wound infections, or osteomyelitis (1). Treatment of *S. aureus*-induced infections has become increasingly challenging due to emergence of drug-resistant bacteria (2). Drug resistance to many clinically used antibiotics becomes more and more serious, even several powerful antibacterial antibiotics, such as (DAP), vancomycin and linezolid, have suffered from antibiotics resistance (3,4). Emergence of drug resistance always overspeeds the development of new antibiotics. Hence, it is necessary to explore key pathogenicity-driven genes and their infection mechanism of *S. aureus*, which contributes to develop novel therapeutic means against *S. aureus* infections.

DAP is a novel cyclic lipopeptide antibiotic, which interacts with bacterial cell membrane to efficiently kill most clinically relevant Gram-positive bacteria (5). Our previous research has identified several key DAP-targeting bacterial proteins through quantitative drug proteomic analysis on the conditional pathogenic bacteria *S. aureus* (6). The downregulation of *nt5* (gi 446956624) upon DAP exposure leads to decrease of bacterial membrane potential and disruption of cell membrane, which contributes to bacterial DNA rapid release and finally causes bacteria death (6). Both

genes *py* (gi 487015361) and *adk* (gi 488431505) are essential in *nt5*-mediated adenine metabolism of *S. aureus* (7). DAP disrupts kinase activity of *py*-encoding pyruvate kinase (PY, protein ID: ODV52833.1) and ADK (protein ID: WP_002500890.1) to mediate adenine metabolism pathway (6). These evidences indicate bacterial *nt5* gene and bacterial adenine synthesis are tightly related with DAP antibacterial effect.

The *nt5*-encoding 5'-nucleotidase (NT5, protein ID: ABD29455.1) protein is ubiquitous among bacteria species, including *E. coli*, *S. aureus* and *Streptococcus suis* (8), which hydrolyzes the phosphate group of 5'-nucleotide. Most of the soluble intracellular NT5 from bacteria belongs to the vast haloacid dehalogenase superfamily, such as *E. coli* NagD (9) and *S. aureus* NT5. In *Bacillus subtilis*, YcsE and YktC as major NT5s both exhibit 5'-nucleotide phosphatase activity (10). *Streptococcus suis* NT5 is an important virulence factor enabling to inhibit neutrophil functions *in vitro* by converting adenosine monophosphate to adenosine (Ado) (8). More noticeably, bacterial NT5 activity is associated with immune evasion properties (11). But it is nearly unclear how does *S. aureus* NT5 function in *S. aureus*-induced infection before.

During infection, Staphylococcal nuclease and adenosine synthase A (*adsA*, protein ID: ALY19673.1) converts neutrophil extracellular traps to deoxyadenosine (dAdo), which is toxic to human immune cells and kills phagocytes and macrophages (12). The Ado and dAdo suppress immune responses and have immunoregulatory attributions (13,14). In *S. aureus*, *adsA* encoded by gene *adsA* (gi

59698588) is a cell wall-anchored enzyme to convert AMP/dAMP to Ado/dAdo, contributing to *S. aureus* escaping from phagocytic clearance and formation of organ abscesses *in vivo* (15). The *adsA* harbors NT5 signature sequence which is critical for catalytic activity of mammalian enzymes (16). NT5 activity of *S. aureus* is a mediator of virulence attributes of *adsA* (15). Although NT5s are widespread among all domains of life, it is unclear whether *S. aureus* NT5 with a 33.3kD-molecular weight has similar properties as *adsA*.

To assess the role of NT5 activity toward DAP treatment and Staphylococcal infection ability, herein we focus on gene function of *S. aureus nt5* and its effects on bacterial infection ability. Using lose-of-functional mutant strain with *nt5*^{C166T} gene silence performed by a CRISPR RNA-guided base editing system, we have revealed *S. aureus nt5* gene is required for *S. aureus*-induced abscess formation. Moreover, we have confirmed the antibiotics DAP exposure to *S. aureus* leads to downregulation of genes *nt5* and *adsA*, and upregulation of genes *py* and *adk*, therefore *nt5*-mediated bacterial nucleic acid metabolic pathway is disturbed by DAP. We also have revealed the silence of *nt5* gene promotes the killing of *S. aureus* by immune cells. Our findings indicate disruption of the *nt5*-involving bacterial activities provides a potentially novel therapeutic means against *S. aureus* even the emerging antibiotic-resistant pathogens.

Materials and methods

Reagents and chemicals

DAP with 99% purity was bought from Dalian Meilun Biotechnology Company, China. Kanamycin and chloramphenicol were ordered from Sigma-Aldrich for plasmid screening in *E. coli* and plasmid curing in *S. aureus* RN4220 and *S. aureus* ATCC 25923. *Bsa*I-HF and T4 DNA ligase were purchased from New England Biolabs for Golden gate assembly. The iScript cDNA Synthesis Kit and SsoAdvanced SYBR GreenSupermix were bought from Bio-Rad for qRT-PCR.

Human lymphocyte separation solution (DKW-LSH-0100) was ordered from Beijing Dakewei Company. MojoSort human CD14⁺ monocytes isolation kit was purchased from Biolegend. Cell culture media DMEM and RPMI-1640 were purchased from Hyclone.

Strains and plasmids

The bacterial strains including *S. aureus* ATCC 25923 and *S. aureus* RN4220 were stored with 20% glycerol (7). *S. aureus* was streaked from glycerol stocks to culture on tryptic soy broth (TSB), then was inoculated into 5 mL TSB to grow overnight at 37 °C with constant shaking for usage. *E. coli* was grown in Luria–Bertani broth.

The base editing plasmid pncasSA–BEC was endowed from Professor Ji of ShanghaiTech University. The pncasSA–BEC system mainly includes the APOBEC1–nCas9 that is a fusion protein composed of a deaminase APOBEC1 and a Cas9 nickase Cas9D10A, cap1A and rpsL promoters and a replication origin ColE1 (17). In addition, the plasmid contains an antibiotic kanamycin selection marker for plasmid amplification in *E. coli*, and another chloramphenicol-resistance marker for

plasmid selection in *S. aureus*. The repF is a temperature-sensitive origin for plasmid curing in *S. aureus*.

Plasmid construction of pnCasSA-BEC-NT5sp

We designed a 20 bp-spacer oligo to insert in *Bsa*I site of the pnCasSA-BEC plasmid by Golden Gate assembly. The plasmid from the Golden Gate assembly reaction was transformed into 100 μ L competent *E. coli* DH5 α cells to incubate at 37°C overnight, and the recombinant plasmid pnCasSA-BEC-NT5sp was sequenced for verifying correctness of plasmid construction.

***S. aureus* competent cells**

The electrocompetent cells of *S. aureus* strains were prepared as following procedures. A single colony of each *S. aureus* strain was inoculated into 2 mL TSB to incubate at 30°C for 12 h. Cells were diluted with a ratio of 1:100 into 100 mL fresh TSB medium, then were cultured up to OD₆₀₀ value with 0.3–0.4 and chilled on ice for 20 min. Cells were harvested by centrifugation with 5000 rpm at 4°C for 5 min, and washed twice with 0.5M sterile ice-cold sucrose. Cells were suspended into 1 mL 0.5M sucrose, from which 100 μ L aliquots were frozen in liquid nitrogen and stored at -80°C [18].

Modified plasmid transformation by electroporation

The pnCasSA-BEC-NT5sp plasmid was transformed into the competent cells of

laboratory strain *S. aureus* RN4220 by electroporation to get the modified plasmid. Before cell electroporation, 100uL competent cells and the plasmid pnCasSA-BEC-NT5sp were respectively thawed on ice for 5 min. The cells were mixed with 1 ug of the recombinant plasmid, and transferred into an electroporation cuvette with 1 mm-thickness at room temperature. After being pulsed at 2.1kV mm, 100U and 25mF, cells were immediately supplied with 900μL TSB and recovered at 30°C for 1.5h. Finally, 500μL cells were plated onto a TSB plate containing 5 mg/L chloramphenicol for incubation at 30°C for 24 h (18). The genomic DNAs of the target colonies were extracted to confirm base mutation in the target site by sequencing.

Base editing in *S. aureus* 25923

To acquire *nt5*^{C166T} gene silence ($\Delta nt5$) in the conditionally infectious pathogen *S. aureus* ATCC 25923 (*S. aureus* $\Delta nt5$), the assembled plasmid isolated from *S. aureus* RN4220 was transformed into *S. aureus* ATCC 25923 by electroporation performed as above. 1 mL cells were plated onto a TSB plate containing 5 mg/L chloramphenicol to incubate at 30 °C for 24 h. The target colonies were screened to extract genomic DNAs for sequencing to confirm *nt5*^{C166T} in *S. aureus* ATCC 25923. The method of plasmid curing was referenced Ji's article (18).

Primer design for gene mutation and qRT-PCR

We selected a 20 bp-spacer sequence before a PAM site (NGG) in the target gene

locus for designing sgRNAs. Two oligos were designed as qRT-PCR primers (Table 1). The total RNA was isolated using bacterial RNA extraction kit following the manufacturer's protocol (RE-03111, FOREGENE Company). The expression levels of genes *nt5* and *py*, *adk* were normalized to that of the reference *Tpi* gene. Each gene expression was quantified by qPCR for three times (n=3).

Minimal inhibitory concentration (MIC) of DAP

The MIC of DAP on *S. aureus* 25923/ $\Delta nt5$ was determined by broth microdilution following the Clinical Laboratory Standards Institute guidelines. DAP was serially diluted to final concentrations ranged from 0.03125 to 16 $\mu\text{g}/\text{mL}$. The MIC was measured by three experiment repeats.

Mouse sepsis model

The following experiments on BALB/c mice, including a mouse sepsis model, immune evasion and phagocytosis, were approved by the Animal Care Committee of Sichuan University (Chengdu, China). The infection ability of *S. aureus* ATCC 25923 (WT) and $\Delta nt5$ was respectively assayed on a mouse sepsis model. Overnight cultures of each *S. aureus*, including the WT and $\Delta nt5$, were diluted 1:100 into fresh TSB and grown for 3 h at 37°C. Then each *staphylococci* culture was centrifuged, washed twice, and diluted in PBS to yield an OD₆₀₀ up to 0.5 with 10⁸ colony-forming units (CFU)/mL. 10⁶ CFU bacterial suspension was administered via tail intravenous into a 6-week-old male BALB/c mouse. Totally ten mice were

inoculated with each strain culture, however, two mice died of *S. aureus* WT infection during experiment progression.

On the 5th day of bacteria inoculation, mice were killed to observe the abscess formation in kidneys and staphylococcal survival in kidneys and livers. Mouse's left kidney and left lobe liver were respectively collected to homogenize with PBS containing 1% Triton X-100. Aliquots of homogenates were diluted and plated on TSB agar plates for triplicate measurements of CFUs. Histopathology analysis of mouse right kidney tissue was performed as following. Tissues were incubated at room temperature in 10% formalin for 24 h, and embedded in paraffin to be sectioned, stained with hematoxylin-eosin to examine by microscopy. Data were obtained from each representative group samples with cohorts of six mice.

Cell culture

The murine macrophage RAW 264.7 cells and human myeloid leukemia mononuclear THP-1 cells were respectively cultured in Dulbecco's Modified EagleMedium (DMEM, Hyclone) and Roswell Park Memorial Institute (RPMI 1640, Hyclone) media supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO₂. Cells were cultured for 48h, and collected to adjust cell density to 10⁷ cells/mL for assay.

Phagocytosis to *S. aureus* 25923/ Δ nt5 *in vitro*

S. aureus 25923 WT and mutant Δ nt5 were cultured overnight, from which 1 mL

bacteria was cultured with 100 mL TSB medium for additional 3 h at 37°C to detect their phagocytosis by monocytes. As mentioned above, each staphylococci culture was centrifuged, washed twice, and diluted in PBS to yield an OD600 up to 0.5 with 10^8 colony-forming units (CFU) /mL. 10^6 CFU bacterial suspension was mixed with 5×10^5 RAW264.7 or THP-1, and cultured at 37°C from 15 to 90 min respectively to plate on TSA and count on the next day.

The following experiments were approved by the West China Hospital Medical Center Institutional Review Board of Sichuan University, following the guidelines of the West China Hospital Medical Center Institutional Review Board of Sichuan University. The enrolled volunteers provided written consent to participate in this study. To further measure *S. aureus* 25923/ Δ nt5 survival ability under the existence of *ex vivo* neutrophils, 1mL peripheral blood was respectively collected from 8 BALB/c mice and 5mL peripheral blood was collected from 3 human volunteers. 2.5×10^4 CFU bacteria was mixed with 225 μ L of mouse blood, and 10^6 CFU bacteria was mixed with 900 μ L of human blood to incubate at 37°C with slow rotation [15]. The mixture of bacteria with mouse or human blood were sampled after incubation for 30 and 90 minutes, from which aliquots were incubated with 0.5% saponin/PBS on ice for 30 min. Dilutions of staphylococci were plated on TSA for enumeration of surviving CFUs.

Isolation of human serum CD14⁺ monocyte to test phagocytosis

The remaining 4ml peripheral blood samples were collected from each donor and diluted with isotonic PBS to isolate lymphocyte using separation solution (DKW-LSH-0100, Beijing Dakewei Company). After centrifugation with 250g for 10 min, cells in the plasma layer were collected to isolate CD14⁺ monocytes using a commercial kit (MojoSort human CD14⁺ monocytes isolation kit, 480047, Biolegend) according to the manufacturer's protocol. Generally, the cells were resuspended with MojoSort™ buffer to adjust to 10⁸ cells, from which 100 uL suspension was taken into a new centrifuge tube to mix with 10 uL Biotin-antibody cocktail and 5 uL Human TruStain FcX™ to incubate at room temperature for 10 min. Then 10 uL Streptavidin nanobeads were mixed to incubate on ice for 15 min, and cells were cleaned and collected by centrifugation at 300g for 5 min, then 2.5 mL of Mojosort™ was added to collect the decanted liquid on a centrifuge tube via a magnet device. Finally, 5x10⁵ cells were mixed with 10⁶ CFU bacteria, cultured at 37°C, sampled at 30, 60 and 90 min respectively, plated and counted the survival rate of bacteria.

Immune evasion ability of *S. aureus* 25923 / Δ nt5 detected on mouse

In order to detect the immune evasion ability of *S. aureus* 25923/ Δ nt5 *in vivo*, we injected 10⁶ CFU bacteria of *S. aureus* 25923/ Δ nt5 from the tail vein for 5 BALB/c mice, and collected 100 μ L blood from mouse orbit after infection for 30, 90 min. Aliquots were incubated with 0.5% saponin/PBS on ice for 30 min to lyse host

eukaryotic cells. Dilutions were plated on TSA plates for enumeration of surviving CFUs.

Hemolysis assay *in vitro*

S. aureus WT and $\Delta nt5$ were grown in TSB at 37 °C overnight and diluted in TSB to yield an OD600 up to 0.5 with 10^8 . Then 1 μ L of culture of each strain including *S. aureus* 25923 and $\Delta nt5$ was loaded onto a sheep blood plate [17] to incubate at 37 °C for 24 h for observing their hemolytic circle size.

Results

Construction of $nt5^{C166T}$ mutant by a CRISPR RNA-guided editing system

To investigate the loss-of-function of *nt5* gene, we constructed gene silence mutant strain $\Delta nt5$ by a base editing CRISPR RNA-guided cytidine deaminase system pnCasSA-BEC which includes APOBEC1-nCas9, cap1A and rpsL promoters [17]. In pnCasSA-BEC system, the deaminase is directed to any genomic locus by the Cas9/sgRNA complex and directly catalyzes the conversion of cytidine (C) to uridine (U) through a deamination reaction. This base editor has an editable window from positions 4 to 8 in the spacer sequence and it can achieve C/T conversion without homologous recombination (Fig. 1A), which is efficient for gene inactivation in *S. aureus*.

We constructed the pnCasSA-BEC-NT5sp plasmid by Golden Gate assembly, then each of 200 ng/ μ L plasmids was transformed into the laboratory strain *S. aureus*

283 RN4220 with 100% efficiency by electroporation. The base C166 of *nt5* gene in *S.*
 284 *aureus* RN4220 was mutated to T (*nt5*^{C166T}), which led to produce a premature stop
 285 codon with TAA (Fig. 1B). Correspondingly, the mutant *nt5*-encoding protein
 286 5'-nucleotidase (mNT5) was a truncated mutant in which the natural amino acid Q56
 287 of the wild-type NT5 was mutated and terminated due to emergence of the premature
 288 stop codon TAA.

289 *S. aureus* RN4220 retains the methylation function of the *Sau* I system, which thus
 290 appropriately methylates DNA for subsequent uptake by other target *S. aureus*
 291 strains containing restriction–modification systems. Firstly, we transferred the
 292 recombinant plasmids pnCasSA-BEC-NT5sp modified by *S. aureus* RN4220 to our
 293 target object *S. aureus* ATCC 25923, in which the pnCasSA-BEC-NT5sp system
 294 enabled highly efficient C/T conversion for target base editing. The C at position 7
 295 of spacer in *nt5* gene was mutated to T with 100% efficiency (Fig. 1C), which
 296 indicated that C166 of *nt5* gene of the *S. aureus* ATCC 25923 strain was mutated to
 297 T so that Q56 amino acid of the wild-type NT5 protein was interrupted and
 298 terminated at translational level due to emergence of the mutant premature stop
 299 codon TAA.

300 Although the gene *nt5* was not deleted, the endogenous encoding wild-type
 301 protein NT5 was interrupted to lose their normal functions. After plasmid curing,
 302 four randomly picked colonies grew normally in the absence of chloramphenicol
 303 whereas none of them could grow in the presence of the antibiotic after two passages
 304 (Fig. 1D). Meanwhile, no assembled plasmid was detectable in *S. aureus* $\Delta nt5$ (Fig.

1E), which confirmed a stable removal of the pnCasSA-BEC-NT5sp plasmid from the host strain *S. aureus* ATCC 25923. All base mutations were verified by sequencing.

Fig. 1. Construction of *nt5*^{C166T} gene silence *S. aureus* Δ*nt5*. (A) Composition of the base editor pnCasSA-BEC system. It mainly includes APOBEC1-nCas9, cap1A and rpsL promoters, and it can be directed to any genomic locus by the Cas9/sgRNA complex to achieve C/T conversion in positions 4 to 8 of the spacer sequence without homologous recombination. PAM: protospacer adjacent motif. (B-C) The C166 of *nt5* gene was mutated to T in the edited nucleotide strand in the *S. aureus* RN4220 (B) and *S. aureus* 25923 (C) respectively. (D-E) The pnCasSA-BEC-NT5sp plasmid was easily cured from *S. aureus* Δ*nt5* after base editing. The individual colonies obtained after the curing steps were streaked onto the TSB agar plates in the

presence (D, right) or absence (D, left) of Cm to test the effectiveness of curing (D), and (E) the PCR result showed that the assembled plasmids disappeared in $\Delta nt5$. Cm: chloramphenicol, WT: wild type.

The *nt5* mutation leads to expression decrease of gene *py* and *adk*

The gene *adk*, and *py* are essential in *nt5*-mediated nucleic acid metabolism of *S. aureus* based on the reported literature and bioinformatics analysis by STRING software (<http://string-db.org/cgi/input.pl>) (19). We further verified if *nt5* gene mutation had influences on the two gene transcription levels. Therefore, the gene transcription levels of *nt5*, *adk* and *py* were detected on mutant $\Delta nt5$ to learn about their gene level changes along with the single base mutation of gene *nt5*.

As results, compared with WT, the RNA levels of *nt5* and *py* in *S. aureus* $\Delta nt5$ were significantly decreased by 2.82 and 1.90 times respectively ($p < 0.05$, $p < 0.01$) (Fig. 2A and 2B). Gene transcriptional level of *adk* was 1.55-times of downregulation, which indicated the silencing of *nt5* gene led to the destruction of this nucleic acid pathway ($p < 0.01$) (Fig. 2C). However, the growth rate of mutant *S. aureus* $\Delta nt5$ was similar with the wild-type *S. aureus* (*S. aureus* WT) (Fig. 2D), which indicated *nt5* gene had little direct influence on the growth rate of *S. aureus* ATCC 25923.

Fig. 2. The mRNA expression level of target gene in gene silence mutant $\Delta nt5$.

(A) The *nt5* gene expression in $\Delta nt5$ mutant was decreased in comparison with WT. (B) The mRNA level of *py* gene was downregulated in $\Delta nt5$ compared with WT. (C) Compared with WT, lower *adk* mRNA was detectable in $\Delta nt5$. (D) The mutant strain $\Delta nt5$ exhibited a similar growth rate to the WT. WT: wild type, *py*: pyruvate kinase, *adk*: adenylate kinase, * $P < 0.05$, ** $p < 0.01$.

***nt5*^{C166T} mutant phenotype and relative gene expression changes in response to DAP exposure**

We further studied the phenotype changes of the *nt5*^{C166T} mutant $\Delta nt5$ upon DAP treatment. The MIC values of DAP on $\Delta nt5$ mutant was proximately 0.5 mg/L, which had little significant differences from the WT upon DAP treatment. Upon 0.5 MIC (0.25mg/L) DAP treatment, the mRNA level of *nt5* was downregulated by 2.90

351 times in *S. aureus* WT, while there was no significant change of *nt5*^{C166T} in *S. aureus*
 352 $\Delta nt5$ when compared to without DAP-exposed bacteria ($p < 0.05$) (Fig. 3A). The
 353 results indicate *S. aureus nt5* gene is key and sensitive to antibiotics DAP exposure,
 354 which has been confirmed in our previous studies [6]. The mRNA level of *py* was
 355 1.96-times upregulation in WT upon 0.25mg/L DAP exposure, but *py* had no
 356 significant change in $\Delta nt5$ with DAP treatment ($p < 0.05$) (Fig. 3B). In addition, the
 357 mRNA level of *adk* gene was upregulated respectively by 2.06 and 2.35 times in WT
 358 and $\Delta nt5$ upon DAP exposure ($p < 0.001$, $p < 0.0001$) (Fig. 3C).

359 Above all, it showed that mutation of the key pathogenic gene *nt5* would induce
 360 gene transcription changes of *nt5*-related genes *py* and *adk* upon DAP exposure.
 361 Although that the silence of *nt5* gene leads to the destruction of nucleic acid pathway,
 362 but the silence of *nt5* gene has no direct influence on growth of bacteria. So far, we
 363 speculate that the silence of *nt5* gene may affect the virulence of bacteria, and we
 364 further investigate *nt5* roles for *S. aureus* pathogenesis, specially focusing on abscess
 365 formation.

366 **Fig. 3. The *nt5*-mediated *py* and *adk* levels in response to DAP exposure in *S.***

367 ***aureus* $\Delta nt5$.** (A) The *nt5* was decreased in WT upon 0.25 mg/L DAP treatment. (B)

368 The mRNA level of *py* was upregulated by 0.25 mg/L DAP treatment in *S. aureus*

WT and $\Delta nt5$. (C) The mRNA level of *adk* was upregulated in WT and $\Delta nt5$ upon 0.25 mg/L DAP treatment. DAP: daptomycin, * $P < 0.5$, *** $p < 0.001$, **** $P < 0.0001$.

***nt5* deficiency impedes *S. aureus*-infected abscess formation**

10^6 colony-forming units (CFU) of *S. aureus* WT, $\Delta nt5$ was respectively inoculated into BALB/c mice by intravenous injection to investigate the role of gene *nt5* for abscess formation. Ten mice were included for each group of *S. aureus* inoculation, but two mice died of *S. aureus* WT infection before infection experiment ending. Mice were killed after bacterial infection for 5d, and right kidneys were collected for observation of abscess profiles by H&E staining and colony counting respectively.

It was noticed that WT formed more abscesses in mouse kidneys compared with the $\Delta nt5$ (Fig. 4A). The abscess lesions were further observed in kidney tissues by H&E staining analysis. There were several obvious lesions surrounded by infiltrating neutrophils in the kidney of mice inoculated with *S. aureus* WT.

Moreover, 6×10^6 (CFU/g tissue) *S. aureus* WT bacteria were detectable in mouse apostematic kidney. Under same conditions, but there was 1.5×10^5 (CFU/g tissue) *S. aureus* $\Delta nt5$ surviving in mouse apostematic kidney tissue (Fig. 4B). So, the living *S. aureus* $\Delta nt5$ in mouse kidney was about a 40-times amount reduction compared with that of *S. aureus* WT infection ($P < 0.05$), which indicates *nt5* gene silence greatly impedes infection ability of *S. aureus*.

Similarly, the colony formation quantity of *S. aureus* $\Delta nt5$ surviving in mouse

liver showed a 300-times reduction as compared with WT ($P < 0.05$) (Fig. 4C), which demonstrated infection ability of $\Delta nt5$ to mouse liver was also obviously attenuated.

***nt5* silence recovers *S. aureus*-infected mouse body weight**

The *S. aureus* $\Delta nt5$ and WT strains were assessed to have influence on the body weight of the infected mice. The mouse weight with $\Delta nt5$ injection was about 20g, which showed no significant change in body weight compared with the normal BALB/c mice injected with PBS. But the weight of mouse infected with *S. aureus* WT was greatly reduced to a mean value of 13g, which indicated *S. aureus* WT-induced infection led to an obvious decrease of mouse body weight. Compared with *S. aureus* WT, the mouse weight with $\Delta nt5$ injection was recovered and increased up to the normal mouse weight ($p < 0.001$) (Fig. 4D). The *nt5* gene expression attenuates *S. aureus*-infected mouse body weight.

In addition, the weight of each kidney from *S. aureus* $\Delta nt5$ -infected mouse was about 0.18g, while a kidney *ex vivo* was reduced to 0.14g in *S. aureus* WT-infected mouse ($P < 0.0001$) (Fig. 4E). It was consistent with changes of *S. aureus*-infected mouse body weight. In general, *nt5* gene contributes to the infection capacity of *S. aureus*, and *nt5* silence recovers *S. aureus*-infected mouse body weight as similar to the normal mouse without infection.

Fig. 4. Numbers of kidney abscess and liver bacterial loading were less in *S. aureus* $\Delta nt5$ -infected mouse than WT infection. (A) *S. aureus* $\Delta nt5$ -infected kidney abscess. Yellow arrows indicated abscess lesions. (B-C) Staphylococcal loading in mouse kidneys (B) and livers (C) after intravenous injection of 10^6 CFU of *S. aureus* WT and $\Delta nt5$ for 5 d. Ten mice were included in each group, but only 8 mice were finally enumerated in the *S. aureus* WT-infected group (n = 8) due to 2 mice died before experiment ending. (D) Body weight of the mice inoculated with

$\Delta nt5$ was increased compared with WT. Normal group: normal BALB/c mice injected with PBS. (E) Mass weight of mouse kidneys infected with *S. aureus* $\Delta nt5$ was nearly recovered to that of normal mouse. * $P < 0.5$, *** $p < 0.01$, **** $P < 0.0001$.

nt5* gene silence promotes phagocytosis to *S. aureus* by macrophages *in vitro

In order to investigate the mechanism of *nt5*^{C166T}-weakened abscess formation, we respectively co-cultured *S. aureus* WT or *S. aureus* $\Delta nt5$ with macrophage cell line RAW264.7 and THP-1 *in vitro* to study *nt5* role in immune response (20). As a result, *S. aureus* $\Delta nt5$ was more easily phagocytosed by RAW 264.7 and THP-1 cells when co-culture from 15 to 60 min by counting bacterial colonies on TSA at timed intervals (Fig. 5A and 5B). After co-culture for 75-90 min, *S. aureus* WT and $\Delta nt5$ were almost clear away.

In addition, we incubated bacteria with host peripheral blood *in vitro* respectively collected from 8 mice and 3 human volunteers at 37°C for 30, 90 min to examine the survival ability of *S. aureus* WT and $\Delta nt5$. There was little difference between WT and $\Delta nt5$ on bacterial load at incubation with mouse blood for 30 min (Fig. 5C). We speculated the anticoagulant EDTA on the blood anticoagulation tube probably influenced biological activity of immune cells, and EDTA affected release of complement C5a, thus affecting the phagocytosis effect of macrophages (21,22). Under same conditions at incubation with each donor's blood for 30 min, the remaining bacterial load of $\Delta nt5$ was decreased than the WT ($p < 0.01$) (Fig. 5D), which indicated *nt5* gene silence improves phagocytosis of macrophages to *S. aureus*.

But at incubation for 90 min, the surviving bacterial quantity of the mutant *Δnt5* was less than the *S. aureus* WT in both of two groups ($p < 0.05$, $p < 0.01$) (Fig. 5C and 5D), which indicated *S. aureus Δnt5* displayed a defect in staphylococcal escape from phagocytic killing compared with the *S. aureus* WT.

Human innate immune system firstly responds to *S. aureus* infection, so we further isolated CD14⁺ monocytes from human peripheral blood mononuclear cells (PBMC) to detect the immune response to *S. aureus Δnt5* exposure. We sorted out CD14⁺ cells from 3 volunteers' blood samples and co-cultured CD14⁺ cells with *S. aureus* WT and *Δnt5* respectively. The survival of bacteria was detected by recording bacterial load at timed intervals via the formation of colonies on TSA (Fig. 5E). *S. aureus Δnt5* was more easily cleared out by human CD14⁺ cells, which indicates that *nt5* gene plays an important role in immune escape for *S. aureus* infection. Because CD14⁺ monocyte cells bind to cell wall peptidoglycan of *S. aureus* and then activate phagocytosis to protect human body suffering from *S. aureus* infection (23).

***nt5* gene is required for bacterial survival in mouse blood**

In order to further verify the role of *nt5* gene in host immune response, we directly injected 10⁶ CFUS. *aureus* WT and *Δnt5* into BALB/c mice to detect the killing effect of host immune system on bacteria. In short time of bacterial infection for 30 and 90 min *in vivo*, the quantity of surviving *S. aureus* WT was higher than the mutant *Δnt5*, which shows the mutant *Δnt5* is more easily cleared by mouse immune system ($p < 0.01$) (Fig. 5F).

In conclusion, we have revealed that *nt5* is required for staphylococcal survival in host as an extracellular pathogen, and *S. aureus nt5* gene plays a critical role for staphylococcal escape from the phagocytic killing of host immune defense system to promote their survival in host blood and tissues.

Fig. 5. Phagocytosis of *S. aureus* by host immune cells. (A-B) Phagocytosis of *S. aureus* by rat macrophage cell line RAW264.7 (A) and human monocyte cell line THP-1 (B). The survival rate of bacteria was calculated by recording the surviving CFU of *S. aureus* on TSA. (C-D) The surviving ability of *S. aureus* $\Delta nt5$ in peripheral blood *in vitro*. *S. aureus* WT, $\Delta nt5$ was co-cultured *in vitro* with blood sample collected from 8 BALB/c mice (C) and 3 human volunteers (D) by recording

474 bacterial load at timed intervals. (E-F) Phagocytosis of *S. aureus* $\Delta nt5$ by human (E)
 475 and mouse (F) CD14⁺ immune cells *in vitro*. The survival rate of bacteria was
 476 calculated by recording the surviving CFU of *S. aureus* on TSA.

477

478 ***nt5* silence has no influence on hemolysis**

479 We detected whether the silence of *nt5* gene affected the hemolytic activity of
 480 bacteria. We inoculated 10⁵ CFU bacteria on blood agar plate and cultured at 37°C
 481 for 16 h. The silence of *nt5* gene had no influence on the hemolytic activity of
 482 bacteria (Fig. 6A and 6B). Therefore, the attenuation of bacterial virulence caused by
 483 the silence of *nt5* gene is mainly reflected in the formation of abscess.

484 In addition, we verified gene stability of *nt5* silence mutant grown on the TSA
 485 plate that was derived from the liver and kidney tissue homogenate extracted from *S.*
 486 *aureus* $\Delta nt5$ -infected mouse. The DNA sequencing showed the growing bacteria
 487 were consistent with *S. aureus* $\Delta nt5$ (Fig 6C), which indicates the *nt5*^{C166T} mutation
 488 via pnCasSA–BEC single base editing system is genetically stable.

Fig. 6. Hemolytic activity of *S. aureus* $\Delta nt5$. (A-B) Size of hemolytic ring formed by WT and $\Delta nt5$ on blood agar plate. (C) The *nt5* gene sequencing of *S. aureus* $\Delta nt5$ colony from mouse liver homogenate and kidney homogenate.

Discussion

Almost all archaea and many bacteria achieve adaptive immunity through a diverse set of CRISPR-Cas systems, which has proven to be extraordinarily simple and efficient in DNA and RNA editing, nucleic acid detection, and disease diagnosis (24,25). The CRISPR/Cas9 system has been engineered for gene editing in various organisms which is originally confirmed as a bacterial immune system (18). The

500 pCasSA system is one kind of specific CRISPR/Cas9 designed for *S. aureus* gene
 501 knockdown (18). But most of *S. aureus*, especially clinical isolates, cannot survive
 502 due to the double-stranded DNA breaks induced by the pCasSA system and the lack
 503 of the homologous recombination repair process. A novel base editing system based
 504 on CRISPR RNA-guided cytidine deaminase (17) is available to construct *S. aureus*
 505 $\Delta nt5$ since this genome editing approach does not require formation of
 506 double-stranded DNA breaks or provision of a donor DNA template, which allows
 507 for rapid and efficient genetic manipulation and drug-target exploration in *S. aureus*
 508 (24).

509 Base editing method is an efficient gene silence strategy, which induces
 510 single-nucleotide changes in the DNA of living cells. There are two kinds of base
 511 editors including the cytidine editor and the adenosine editor. Efficient editing by the
 512 C/T conversion “base editor” requires the presence of a PAM motif with NGG
 513 nucleotide sequence, which has an editable window from positions 4 to 8 in the
 514 spacer sequence (18). This PAM requirement limits the number of sites in the
 515 bacterial genome. An editable site of the pCasSA–BEC system is essential for
 516 highly efficient base editing. In addition, it has a disadvantage that mutation site
 517 could not be verified by mRNA levels compared with CRISPR/Cas9 system. For
 518 example, the mRNA expression level of *nt5* gene in our study is still observed in
 519 $\Delta nt5$, so DNA sequencing is required to confirm gene mutations into terminate
 520 codons (26).

521 Herein, we focus on physiological changes in *S. aureus* with *nt5* gene silence. The

silence of *nt5* gene influences the expression of *py* and *adk* genes, but it does not affect the growth of bacteria, which indicates metabolic compensation of nucleic acid pathway in bacteria (27). And we have revealed that *nt5*-mediated nucleic acid pathway in *S. aureus* is obviously disturbed due to increase of its pathogenic genes *py* /*adk* and *nt5* decrease in response to antibiotics treatment. These observations are in line with the bacterial proteome profiling of *S. aureus* ATCC 25923 upon DAP treatment (7). Meanwhile, it also shows that DAP not only disrupts bacterial cell membrane, but also affects the *nt5*-mediated nucleic acid pathway in *S. aureus*, which contributes to the effective antibacterial activity of DAP (28). Therefore, disruption of *nt5*-mediated signaling paves possible therapeutic opportunities for treatment of infections and provides a direction for finding new target of DAP.

S. aureus usually causes serious infections, with an annual incidence of 20 to 50 cases per 100,000 people. Approximately 10% to 30% of these patients die from *S. aureus* infection, which accounts for a greater number of deaths than for AIDS, tuberculosis, and viral hepatitis combine (29). Although antibiotics, such as vancomycin and DAP, have powerful antibacterial activity on *S. aureus*, the increase of bacterial drug resistance leads to the gradual failure of antibiotics. It is necessary to explore the pathogenic mechanism of *S. aureus*.

By now, this is the first study to reveal role of *nt5* gene in abscess formation and immune evasion. The *in vivo* analysis in mouse sepsis model indicates that *S. aureus* *nt5* gene is required for kidney abscess formation and is helpful for bacterial evasion from the host immune response during infection. Immune evasion is an important

process in which microorganisms directly modulate host-immune system in response to pathogens (30). Of note, in some bacteria, NT5 contributes to immune evasion by dephosphorylating adenosine mono-, di-, or tri-phosphates, thereby increasing the concentration of adenosine.

In *S. aureus*, NT5 activity is the mediator of the virulence attributes of *adsA* (16). And NT5 has classic lipoprotein signal sequence which belongs to the haloacid dehalogenase superfamily and is similar to haemophilus influenzae lipoprotein E. A critical role of NT5 in haemophilus influenzae is the degradation of external riboside (30). *S. aureus adsA* is an adenosine synthase to own NT5 activity. AdsA converts neutrophil extracellular traps to dAdo during infection, and dAdo production represents a general immune evasion mechanism for microbial pathogens (31). Pathogenic bacteria use synthesized dAdo to evade host defenses (14). The production of dAdo by *S. aureus adsA* induces caspase-3-mediated death of human macrophages (14). *Streptococcus suis* produces dAdo by NT5 causing monocytopenia in mouse blood *in vivo*, which suggests the immunosuppressive activity of Ado on immune responses *in vivo* (8). Perturbation of adenosine levels is likely to affect host immune responses during infection (31,32).

Moreover, the global proteome and phosphor-proteome of sepsis kidney injury also reveal that caspase-1 is significantly up-regulated in the cecal ligation and puncture-induced sepsis, which mediates gasdermin D cleavage and activation (33). Gasdermin D dependent pyroptosis cell death has been revealed for the first time in nonseptic acute kidney injury (34). In the kidney abscess caused by *S. aureus*, it's

valuable to further investigate whether does exist a similar mechanism with cecal ligation and puncture-induced sepsis.

Ex vivo infection of whole blood is a valuable tool to study *S. aureus* virulence factors and the host innate immune responses. In order to consider effects of cellular mediators, the coagulation cascade must be inhibited to avoid clotting. However, EDTA, a strong chelator of Ca^{2+} as well as Mg^{2+} , blocks coagulation as well as complement entirely (21). Blocking of C5a or C5aR1 attenuates phagocytosis and increases the extracellular growth of *S. aureus* in blood (22). Besides the host response, the anticoagulants might directly affect the bacteria growth. EDTA as a chelator of Ca^{2+} is present on the blood tube, which has a strong inhibitory effect on *S. aureus* growth when *S. aureus* is phagocytosed by macrophages *in vitro*. Hence, the citrate or heparin as anticoagulant should be selected to collect host blood sample, which has little effect on host immune response and bacterial growth (21).

S. aureus secretes a number of host-injurious toxins, among the most prominent of which is the small β -barrel pore-forming toxin α -hemolysin. Initially named based on its properties as a red blood cell lytic toxin, early studies suggested a far greater complexity of α -hemolysin action as nucleated cells also exhibited distinct responses to intoxication (35). However, our experimental results show that gene *nt5* does not affect the hemolytic activity of *S. aureus*. We speculate that the virulence of gene *nt5* is mainly manifested in sepsis and immune escape.

In conclusion, we have provided direct evidences of the role of *nt5* gene toward staphylococcal infection ability. We have revealed *nt5* gene of *S. aureus* is required

for host infection and abscess formation. These results are helpful for the development of NT5 inhibitors as a new strategy for rendering *S. aureus* susceptible to human host defence.

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S1 Table. Primers used in this study

Primer	Sequence (5'-3')
<i>nt5</i> spacer For (Forward primer)	GAAATAATCAAAATATTATGGCAG
<i>nt5</i> spacer Rev (Reverse primer)	AAACCTGCCATAATATTTTGATTA
<i>nt5</i> check spacer For	CATCATTATCGGTAGCGGT
<i>nt5</i> check spacer Rev	GTAGACGTCCAATTCATATA
PnCAsSA-BEC check for	AGCCTATGGAAAAACGCCAG
pnCAsSA-BEC check rev	GTAAACACTTACTAATTCTC
<i>nt5</i> For	ACAGTGCAAAGACTCAGTTAGA
<i>nt5</i> Rev	TGCATAGCCTTGATATGGAGAA
<i>py</i> For	GCTGTATGTGCAACCGAAATAC
<i>py</i> Rev	TGAAGCAGCCCAAGATTACA
<i>adk</i> For	TGTTCTTGGAAGCCATCTAAT
<i>adk</i> Rev	GTCCTGATGAAGTGACTGTAGG
<i>tpi</i> For	AGCTAACGATGTTGTAGGTGAG
<i>tpi</i> Rev	GATTGCCCAGATTGGTTCATAAG