**Staphylococcus aureus** targets the purine salvage pathway to kill phagocytes

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**Staphylococcus aureus** colonizes large segments of the human population and causes invasive infections due to its ability to escape phagocytic clearance. During infection, staphylococcal nucleases and adenosine synthase A convert neutrophil extracellular traps to deoxyadenosine (dAdo), which kills phagocytes. The mechanism whereby staphylococcal dAdo intoxicates phagocytes is not known. Here we used CRISPR-Cas9 mutagenesis to show that phagocyte intoxication involves uptake of dAdo via the human equilibrative nucleoside transporter 1, dAdo conversion to dAMP by adenoscytidine kinase and adenosine kinase, and signaling via subsequent dATP formation to activate caspase-3-induced cell death. Disruption of this signaling cascade confers resistance to dAdo-induced intoxication of phagocytes and may provide therapeutic opportunities for the treatment of infections caused by antibiotic-resistant *S. aureus* strains.

The pathogen *Staphylococcus aureus* persistently colonizes the nasopharynx of large segments of the human population and is also a frequent cause of soft tissue infections, pneumonia, osteomyelitis, septic arthritis, bacteremia, endocarditis, and sepsis (1–3). *S. aureus* colonization represents a key risk factor for invasive disease, which in hospital environments frequently manifests as surgical wound and medical device infections or ventilator-associated pneumonia (4, 5). Owing to the high incidence of hospital-acquired infection, antibiotics are used both for *S. aureus* decolonization and prophylaxis of nosocomial disease (6, 7). However, large-scale use of antibiotics selects for antibiotic-resistant strains, designated methicillin-resistant *S. aureus* (MRSA) (8). Due to limited efficacy of antibiotics to eradicate drug-resistant strains, MRSA infections, i.e., 22% of hospital-acquired *S. aureus* infections in the United States, are associated with increased morbidity and mortality, compared with infections caused by antibiotic-sensitive strains (1).

The hallmark of *S. aureus* disease is the formation of abscess lesions, where staphylococci replicate within fibrin-encapsulated communities and attract neutrophils and other phagocytes to implement the purulent destruction of host tissues (9). Activated neutrophils release extracellular traps (neutrophil extracellular traps, NETs), an extracellular matrix composed of nuclear and mitochondrial DNA armed with granular proteins, cell-specific proteases, and antimicrobial peptides, to degrade antimicrobial peptides and DNA (12, 13). Interestingly, nucleosome-mediated degradation of neutrophil NETs triggers the formation of deoxyadenosine monophosphate (dAMP), which is converted by *S. aureus* adenosine synthase A (AdsA) into deoxyadenosine (dAdo) (14). Macrophages and other immune cells are highly sensitive to dAdo intoxication, enabling staphylococci to block phagocyte infiltration of mouse abscess lesions and preventing bacterial clearance in vivo (14, 15). Recent work revealed further that other bacterial pathogens, including pathogenic bacilli and streptococci, utilize secreted nucleases and 5′-nucleotidases (homologs of *S. aureus* AdsA) to escape phagocyte clearance, suggesting that dAdo production represents a general immune evasion mechanism for microbial pathogens (16–18). However, the mechanisms whereby *S. aureus* or other pathogen-derived dAdo is able to kill phagocytes is not known. Here, we used a genome-wide CRISPR-Cas9 knockout screen to identify genes required for dAdo intoxication. The results suggest that *S. aureus* targets the purine salvage pathway to eliminate host phagocytes. The identification of genes affecting phagocyte intoxication may aid in the development of therapeutic strategies that can improve the outcome of MRSA infections.

**Results**

A CRISPR-Cas9 Screen Identifies Genes Contributing to Deoxyadenosine Intoxication of Phagocytes. To conduct CRISPR-Cas9 dAdo knockout screens in the human U937 macrophage cell line, we determined the concentration of dAdo required to kill U937 cells within 24 and 48 h of incubation, respectively (Fig. 1 A and B). Human genome-scale CRISPR-Cas9 dAdo knockout (GeCKO) (19, 20) library-transduced U937 cells were treated with 5 µM dAdo or left untreated (nontreatment control) (Fig. 1 C and D). U937 cells with defective genes whose products otherwise contribute to intoxication are expected to proliferate in the presence of dAdo, leading to the enrichment of the corresponding small-guide RNAs (sgRNA) in treatment samples compared with control samples. This experimental scheme led to the isolation of dAdo-resistant U937 cell populations after 28 d of dAdo selection (Fig. 1D). Next, cells were subjected to a combined strategy of rapid Sanger sequencing of cloned sgRNAs and of next generation sequencing (NGS) of all sgRNAs in the samples (Fig. 1C). Sanger sequencing of 80 independent *Escherichia coli*-derived plasmids containing the

**Significance**

Immune evasion by the human pathogen *Staphylococcus aureus* includes the synthesis of cytotoxic deoxyadenosine (dAdo) from neutrophil extracellular traps to eliminate macrophages. The signaling pathway that triggers dAdo-mediated apoptosis in macrophages is not known. By using a genome-wide CRISPR-Cas9 knockout screen, we demonstrate that *S. aureus*-derived dAdo targets the purine salvage pathway to provoke caspase-3-dependent cell death in human macrophages, thereby antagonizing host immune responses and promoting the establishment of invasive disease. Interference with the staphylococcal dAdo signaling pathway may boost macrophage survival and phagocytic clearance of bacteria, providing new strategies for the treatment of *S. aureus* infections.

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HENT1 Mediates Uptake of Cytotoxic Deoxyadenosine into Macrophages. Secreted NXPH1 inhibits the proliferation of hematopoietic progenitor cells via neurexin 1α (NRXN1α) signaling (21). To test whether NXPH1 contributes to dAdo-mediated killing of phagocytes, U937 cells or U937-derived macrophages were incubated with human NXPH1, and cell viability was recorded. NXPH1 did not induce macrophage cell death (SI Appendix, Fig. S1). Conversely, inhibition of HENT1 (encoded by SLC29A1), a polytopic membrane protein, with dipyridamole (22) rendered U937 cells and U937-derived macrophages resistant to dAdo-induced cell death, suggesting that dipyridamole-mediated inhibition of HENT1 transport activity protects macrophages from dAdo intoxication (Fig. 2A). Targeted CRISPR-Cas9 mutagenesis with a SLC29A1-specific sgRNA was used to generate biallelic disruptions of SLC29A1, which were confirmed by sequencing exon 4 on human chromosome 3 (Fig. 2B). SLC29A1−/− cells were further analyzed by immunoblotting with HENT1-specific antibodies, which confirmed that HENT1 production had been abolished (Fig. 2C). Of note, U937 SLC29A1−/− cells as well as U937-derived SLC29A1−/− macrophages were resistant to dAdo intoxication (Fig. 2D). Together these data indicate that macrophage expression of HENT1 is essential for dAdo-mediated intoxication of these cells.

As a member of the human equilibrative transporter family, HENT1 mediates uptake of nucleosides and of nucleoside derivatives used for the treatment of cancer and viral infections (23). Recent work demonstrated that HENT1 binds to dAdo in HENT1-overexpressing HEK293F cells (24). To test whether HENT1 transports dAdo into macrophages, we incubated U937 cells with [3H]dAdo and measured uptake via scintillation analysis of cell lysates. U937 cells imported extracellular [3H]dAdo into their cytoplasm (Fig. 2E). Mutations in genes encoding purine salvage pathway kinases ADK, DCK, and NXPH1 were confirmed by Sanger sequencing of 80 plasmids containing cloned sgRNA cassettes. Sanger sequencing of the SG-6 and DCK-6 U937 cells have mutations in SLC29A1 and NXPH1, respectively.

Purine Salva Dresden Pathway Kinases Are Required for Deoxyadenosine Intoxication of Macrophages. Earlier work reported that TAOK1 activates the r38 MAP kinase pathway (25). To test whether TAOK1 and the p38 MAP kinase pathway are involved in dAdo-mediated killing of macrophages, we incubated U937 cells and their derived macrophages with SB 203580, a p38-specific inhibitor. SB 203580 caused modest increases in the survival of dAdo-treated macrophages only at high concentrations, suggesting that the p38 MAP kinase pathway and TAOK1 do not play a major role in the killing of macrophages (SI Appendix, Fig. S3). To explore the role of ADK and DCK in dAdo-mediated cell death of macrophages, we first sought to inhibit the corresponding protein products. The substance 5′-amino-5′-deoxyadenosine is an inhibitor of ADK (26), whereas deoxycytidine is an inhibitor of ADK (27). To explore whether ADK and DCK contribute to dAdo-induced cell death, we tested inhibitors of ADK and DCK (Fig. 2F). Both inhibitors reduced the survival of U937 cells and differentiated macrophages carrying mutations in the corresponding structural genes, which were confirmed by sequencing of the mutant alleles (Fig. 3A). Immunoblotting experiments with specific antibodies further confirmed the absence of ADK and DCK in ADK−/− and DCK−/− mutant cells, respectively (Fig. 3B). Both ADK−/− and DCK−/− cells and macrophages exhibited increased resistance to dAdo intoxication (Fig. 3C and D). U937 cells and differentiated macrophages carrying mutations in the structural genes for both ADK and DCK exhibited increased resistance to dAdo-induced cell death (SI Appendix, Fig. S4). dAdo resistance in ADK−/−, DCK−/−, or ADK−/−, DCK−/− cells was not associated with a defect in the uptake of [3H]dAdo, indicating that HENT1 is fully functional in cells with defects in the purine salvage pathway (Fig. 3E). Together these experiments suggest that purine

Fig. 1. Genome-wide CRISPR-Cas9 screen for mutations conferring resistance to cytotoxic deoxyadenosine in human macrophages. (A) Chemical structure of dAdo. (B) Survival of U937 cells treated with variable concentrations of dAdo (0–10 μM) in the presence of 50 μM dCF (2′-deoxycoformycin or pentostatin), an inhibitor of adenine deaminase. Cell viability was analyzed after 24 h and 48 h of incubation; 5 μM dAdo (red) was used for selection of resistant variants. Data points represent the mean ± SD of three independent determinations. (C) Experimental scheme for CRISPR-Cas9 knockout screen to identify genes conferring resistance to deoxyadenosine (dAdo) intoxication in U937 cells. (D) Analysis of cell viability for GeCKO library-transduced U937 cells intoxicated with 5 μM dAdo and 50 μM dCF for 28 d (red squares) or treated with 50 μM dCF alone (black circles). (E) Distribution of sgRNAs in dAdo-resistant U937 cells identified by Sanger sequencing of 80 plasmids containing cloned sgRNA cassettes. (F) Identification of the top candidate genes following dAdo treatment of U937 cells by next generation sequencing. Data were analyzed using the MaGeCK-based robust rank aggregation (RRA) score analysis.
Disruption of the Purine Salvage Pathway Causes Deoxyadenosine-Intoxicated Macrophages from Caspase-3-Induced Cell Death. Cytoplasmic dAdo can be detoxified by adenosine deaminase (ADA), which catalyzes the irreversible conversion of dAdo to deoxyinosine (27). Alternatively, ADK and DCK can convert dAdo into dAMP for subsequent synthesis of dADP and dATP via adenylate kinase and nucleoside-diphosphate kinase, respectively. If so, S. aureus-mediated synthesis of dAdo may trigger not only import of dAdo into macrophages but also intracellular synthesis of dAMP and the accumulation of dADP and dATP. To test this, we extracted nucleotides from [14C]dAdo-treated U937 cells and analyzed the abundance of dAMP, dADP, and dATP via TLC. The experiments revealed the accumulation of [14C]dATP, [14C]dADP, [14C]dAMP, and [14C]AMP following incubation of U937 cells with [14C]dAdo (Fig. 4B). Compared with their U937 parent, ADK−/−, DCK−/−, DCK−/−, and SLC29A1−/− cells exhibited reduced abundance of intracellular [14C]dATP, [14C]dADP, and [14C]AMP (Fig. 4B). Quantification of [14C]dAdo-derived nucleotides using liquid scintillation counting (LSC) confirmed that [14C]dAdo exposure caused accumulation of dATP and its precursors (dADP/dAMP), which was diminished in ADK−/−, DCK−/−, DCK−/−, and SLC29A1−/− cells (Fig. 4C). Thus, mutations that disrupt the purine salvage pathway prevent the accumulation of dATP following dAdo exposure, suggesting that dATP may represent a key signaling molecule triggering cell death in macrophages. Earlier work suggested that intracellular dATP activates the apoptotic protease-activating
Mutations disrupting the purine salvage pathway of deoxyadenosine-intoxicated macrophages provide resistance to caspase-3-induced cell death. (A) Uptake of [3H]dAdo into wild-type U937 cells (WT, black) and their DCK<sup>−/−</sup> (white), ADK<sup>−/−</sup> (red), ADK<sup>−/−</sup> DCK<sup>−/−</sup> (gray), or SLC29A1<sup>−/−</sup> (blue) variants was quantified by LSC. As a control, dipyridamole (Dipy) (10 μM) inhibition of hENT1 blocked uptake of [3H]dAdo into WT U937 cells. (B) Analysis of [<sup>14</sup>C]dAdo-derived nucleotides from WT U937 cells and their ADK<sup>−/−</sup>, DCK<sup>−/−</sup>, ADK<sup>−/−</sup> DCK<sup>−/−</sup>, or SLC29A1<sup>−/−</sup> variants. An autoradiograph of the TLC plate is shown with migrational positions of dAMP, AMP, dADP, and dATP. (C) Nucleotides extracted from [<sup>14</sup>C]dAdo-treated cells were analyzed by TLC and LSC for the abundance of dATP, dADP, and dAMP. (D) WT U937 cells and U937-derived macrophages (Mφ) and their ADK<sup>−/−</sup>, ADK<sup>−/−</sup> DCK<sup>−/−</sup>, or SLC29A1<sup>−/−</sup> variants were treated with dAdo and cell lysates were analyzed for caspase-3 activity using a colorimetric assay. As controls, lysates were treated with the caspase-3 inhibitor Ac-DEVD-CHO (+Inh). All data are the mean ± SD of three independent determinations. Statistically significant differences were analyzed with one-way ANOVA and Bonferroni’s multiple comparison test. ns, not significant (P > 0.05); *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

Discussion

Histopathological features of <i>S. aureus</i> invasive disease include infiltration of large numbers of neutrophils, a process that is dependent on staphylococcal lipoproteins as inducers of TLR2 signaling by host immune cells (30). Neutrophils discharge their degradative enzymes and NETs at the site of infection, triggering liquefaction necrosis and purulent drainage of abscess lesions (9, 31). Unlike treated with antibiotics (and surgical drainage), staphylococci replicating in deep-seated abscess lesions cannot be cleared by the immune system, subsequently causing disseminated abscess lesions and lethal bacteremia (9, 31). In the United States, <i>S. aureus</i> causes each year 3.4 million community-acquired diseases and 460,000 hospital-acquired infections. Antibiotic-resistant MRSA infections (7% of community- and 22% of hospital-acquired infections) are associated with poor clinical outcomes and represent a frequent cause of mortality (1).

Development of new therapeutic strategies to combat MRSA infections requires deeper understanding of the immune evasive attributes that enable staphylococci to survive in host tissues. Unlike neutrophils, macrophages are not found in close proximity to staphylococcal abscess communities (14, 31). Exclusion of these immune cells is at odds with physiological clearance mechanisms, which rely on macrophages for the removal of neutrophil remnants and the killing of bacteria. Earlier work showed that <i>S. aureus</i> secretes nucrese, an enzyme that degrades neutrophil NETs into its monophosphate deoxynucleosides (12). Staphylococcal AdsA, a cell wall-anchored surface protein, catalyzes the conversion of adenosine nucleotides (released by damaged host tissues) to adenosine and deoxyadenosine nucleotides (derived by nuclease digestion of neutrophils NETs) to deoxyadenosine (14, 18, 32). Increases in adenosine concentration dampen inflammatory and pathogen-specific immune responses, whereas increases in deoxyadenosine trigger macrophage apoptosis in the vicinity of staphylococcal abscess communities formed during the infection of mice (14, 18). Unlike neutrophils, macrophages and other myeloid cells retain the ability to synthesize DNA and divide. Due to reduced levels of de novo nucleotide...
synthesis, these cells rely on the import of nucleosides for their DNA synthesis and cell division.

To identify genetic determinants associated with *S. aureus* deoxyadenosine intoxication, we used CRISPR-Cas9 mutagenesis of human U937 macrophages and identified *SLC29A1*, *ADK*, and *DCK* as top hits. CRISPR-Cas9–specific sgRNAs were used to generate U937 macrophage variants unable to produce hENT1, ADK, or DCK, which resulted in resistance against dAdo-mediated killing. *S. aureus* intoxication involves hENT1 import of dAdo into macrophages, ADK/DCK catalyzed conversion of dAdo to dAMP, subsequent adenylyl kinase- and nucleoside-diphosphate kinase-dependent accumulation of dADP and dATP, as well as caspase-3–induced apoptosis. Thus, *S. aureus* intoxication targets the purine salvage pathway of macrophages with rapid increases in intracellular dATP, likely activating caspase-3–induced apoptosis. Other signaling pathways may also contribute to macrophage cell death, including p53 activation via DNA damage. Nevertheless, adenylyl kinase (AK), nucleoside-diphosphate kinase (NDPK), and caspase-3 (CASP3) were not identified as top hits in the CRISPR-Cas9 screen. Various isoforms have been described for AK and NDPK (AK1–3, NDPK A–D), and accordingly, only deletion of all variants in a single cell may provide for dAdo resistance (33, 34). U937 *CASP3*−/− cells presumably remain sensitive to dAdo intoxication, as earlier work reported that *CASP3*−/− colon cancer cells are more sensitive to DNA damaging agents than their parent (35). If so, dAdo may suppress proliferation of *CASP3*−/− cells while selecting *SLC29A1*−/−, *DCK*−/−, and *ADK*−/− cells during the CRISPR-Cas9 screen. While *ADK* is essential for viability and dCK is required for the development of B and T lymphocytes (36–38), the immune system of mice lacking *SLC29A1* is not impaired (39). *SLC29A1*−/− mice exhibit accelerated bone synthesis with pericardial calcifications due to increases in blood adenosine and adenosine receptor signaling (40). In humans, *SLC29A1* is the genetic determinant of the Augustine (At) blood type (41). The single nucleotide polymorphism rs45458701 (c.1171G > A in *SLC29A1*) gives rise to a missense mutation and identifies individuals with At− blood type, which occurs predominantly in individuals with African ancestry (41). At− individuals expressing variant hENT1 (p.Glu391Lys) may produce allotype antibodies recognizing wild-type hENT1 on erythrocytes, which trigger severe hemolytic reactions following transfusion with At+ blood. *SLC29A1* null mutations have been identified in the genomes of individuals with European ancestry where they are associated with pericardial calcifications (41). Some of the identified SNPs in human *SLC29A1*, DCK, or ADK may provide for resistance against bacterial pathogens, which could explain their abundance in human populations. In this regard, we note that homologs of staphylococcal nuclease and *adsA* are found in other Firmicutes causing invasive disease in mammals, including members of the genera *Bacillus*, *Streptococcus*, and *Staphylococeus* (18). As analyzed in *Streptococcus pyogenes*, similar to *S. aureus* *AdmA* (16, 17, 42). The compelling findings of our CRISPR-Cas9 screen suggest that inhibitors of AdmA, adenosine signaling, hENT1, as well as ADK or DCK, may disrupt the immune evasive signaling cascades associated with AdmA 5′-nucleotidase and Nuc nuclease activities. If so, knockout mutation in the mouse gene for ENT1 (*SLC29A1*) as well as pharmacological inhibitors of AdmA, ENT1, ADK, or DCK may exhibit improved outcomes for MRSA infections in animal models for staphylococcal diseases.

**Materials and Methods**

**Tissue Culture.** U937 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) supplemented with 10% heat-inactivated FBS. HEK293FT cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% FBS, 0.1 mM MEM nonessential amino acids, 6 mM L-glutamine, 1 mM sodium pyruvate, and 500 μg/mL geneticin. Cells were grown at 37 °C under 5% CO₂.

**CRISPR-Cas9 Screen and Mutagenesis.** The human CRISPR-Cas9 GeCKO v2 library was a gift from Feng Zhang (Massachusetts Institute of Technology, Cambridge, MA) and mutagenesis was performed as described (19, 20). sgRNA library–transduced U937 were intoxicated with dAdo and dCF, or dCF alone for 28 d. The genomic DNA was isolated to prepare a sgRNA library by a two-step PCR (19, 20), which was sequenced with HiSeq2500 (Illumina). Analysis of essential sgRNAs and genes was performed using MAGeCK (43). CRISPR–Cas9–mediated mutagenesis was performed using lentCRISPR v2 plasmids expressing specific sgRNAs as described before (19). Details are described in SI Appendix.
Cytotoxicity Assays. dAdo-mediated cytotoxicity was analyzed as described earlier (14). Cytotoxicity mediated by S. aureus-derived dAdo was analyzed by incubating S. aureus strains in the presence of thymus DNA to generate dAdo-containing culture supernatants. Cell viability was analyzed after a 24-h incubation by Trypan Blue staining and microscopy (SI Appendix).

Biochemical Assays. Caspase-3 activity was determined with the colorimetric caspase-3 detection kit (Sigma). dAdo uptake was analyzed by incubating cells with 1 μCi [3H]dAdo (specific activity: 38.1 Ci/mmol; Moravek Biochemicals, Inc.) and 50 μM dCF. Cellular nucleotides were extracted using ice-cold 60% methanol (44) and separated by TLC (SIL G plates, Macherey-Nagel) using a water/isopropanol/ammonium bicarbonate mixture (25%:75%:0.2 M). Chromatograms were developed by autoradiography. Spots were excised and radioactivity was quantified by LSC to calculate nucleotide as picomoles per milligrams of protein (SI Appendix).

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