Genetic variation of DNA methyltransferase-3A contributes to protection against persistent MRSA bacteremia in patients

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The role of the host in development of persistent methicillin-resistant Staphylococcus aureus (MRSA) bacteremia is not well understood. A cohort of prospectively enrolled patients with persistent methicillin-resistant \( S. \) aureus bacteremia (PB) and resolving methicillin-resistant \( S. \) aureus bacteremia (RB) matched by sex, age, race, hemodialysis status, diabetes mellitus, and presence of implantable medical device was studied to gain insights into this question.

One heterozygous \( g.25498283A > C \) polymorphism located in the \( DNMT3A \) intronic region of chromosome 2p with no impact in messenger RNA (mRNA) expression was more common in RB (21 of 34, 61.8%) than PB (3 of 34, 8.8%) patients (\( P = 7.8 \times 10^{-4} \)). Patients with MRSA bacteremia and g.25498283A \( > C \) genotype exhibited significantly higher levels of methylation in gene-regulatory CpG island regions (\( \Delta \text{methylation} = 4.1\% \), \( P < 0.0001 \)) and significantly lower serum levels of interleukin-10 (IL-10) than patients with MRSA bacteremia without \( DNMT3A \) mutation (A/C: 9.7038 pg/mL vs. A/A: 52.9898 pg/mL; \( P = 0.0042 \)). Expression of \( DNMT3A \) was significantly suppressed in patients with \( S. \) aureus bacteremia and in \( S. \) aureus-challenged primary human macrophages. Small interfering RNA (siRNA) silencing of \( DNMT3A \) expression in human macrophages caused increased IL-10 response upon \( S. \) aureus stimulation. Treating macrophages with methylation inhibitor 5-Aza-2′-deoxycytidine resulted in increased levels of IL-10 when challenged with \( S. \) aureus. In the murine sepsis model, methylation inhibition increased susceptibility to \( S. \) aureus. These findings indicate that g.25498283A \( > C \) genotype within \( DNMT3A \) contributes to increased capacity to resolve MRSA bacteremia, potentially through a mechanism involving increased methylation of gene-regulatory regions and reduced levels of antiinflammatory cytokine IL-10.

Staphylococcus aureus | MRSA | persistence | host genetics | DNMT3A

Persistent methicillin-resistant \( S. \) aureus (MRSA) bacteremia in patients despite appropriate antibiotic therapy is common, incompletely understood, and associated with poor clinical outcome (1–3). A growing body of evidence indicates that genetic variation may influence patient risk for \( S. \) aureus infection (4–7). In this investigation, we used a comprehensive approach to identify potential host genetic determinants of persistent methicillin-resistant \( S. \) aureus bacteremia (PB) and resolving methicillin-resistant \( S. \) aureus bacteremia (RB) in a large cohort of patients with \( S. \) aureus bacteremia (SAB). First, patients with PB and RB were matched on important clinical variables to minimize potential confounding factors. Second, we used whole-exome sequencing (WES) to identify candidate genetic variants associated with PB and RB. Third, we interrogated the PB and RB patient groups for differences in epigenetic phenomena, whole-blood gene expression levels, and serum cytokine levels to establish a potential pathogenic mechanism for any associations found in the WES. Fourth, we performed in vitro and in vivo experiments to support the biological plausibility of our findings.

Significance

The severity and duration of methicillin-resistant \( S. \) aureus (MRSA) bacteremia varies widely between individuals. Host factors predisposing to persistent MRSA bacteremia are poorly understood, although genetic association studies are beginning to identify potentially influential variants. We found an association between the A/C heterozygous genotype in the \( DNMT3A \) correlating with shorter time to resolution of MRSA bacteremia. Using in vitro macrophage assays and murine sepsis models, we demonstrated that \( DNMT3A \) variants may alter host response to infection through increased methylation of key regulatory genes, resulting in reduced interleukin-10 production and in turn, allowing for a more protective immune response that clears infection. An improved understanding of the factors predisposing to persistent MRSA bacteremia may help to discover better treatment options.

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Results

Clinical Cohort. A total of 68 patients with PB or RB were matched 1:1 by sex, age (in deciles), race, hemodialysis status, diabetes mellitus, and presence of implantable medical device (Table 1).

One Polymorphism in DNMT3A Differentiates between Persistent and Resolving MRSA Bacteremia. To test whether host genetic variation is associated with development of the PB phenotype, WES was performed on the 68 study patients (8). The most significantly associated variant was a single-nucleotide polymorphism (SNP), g.25498283A > C, which is located in the DNMT3A intronic region of chromosome 2p. The SNP was more common in RB (21 of 34, 61.8%) than PB (3 of 34, 8.8%) with a P value of 7.8 × 10⁻⁶, which is just above a Bonferroni-corrected genome-wide level of significance (1.2 × 10⁻⁷) (Fig. 1 and SI Appendix, Table S1). The g.25498283A > C mutation overlaps with open chromatin regions and known transcription factors (SP1 and EGR1) binding sites (9), suggesting that the variant might be located within a regulatory region of DNMT3A. Given the role of DNMT3A in host immune response (10) and the fact that demographic characteristics of patients with and without the SNP were similar (SI Appendix, Table S2), we further pursued 2 lines of investigation: 1) the potential involvement of DNMT3A in host response to S. aureus infection and 2) the potential role of variation within DNMT3A in patients with PB vs. RB.

DNMT3A Is Involved in Host Response to S. aureus Infection. To evaluate the clinical relevance of DNMT3A in host response to S. aureus, we first compared the impact of S. aureus infection on DNMT3A expression using existing whole-blood microarray expression data from our previously published cohort of patients with SAB (n = 32) or Escherichia coli (n = 19) bacteremia (11). Relative to healthy controls, DNMT3A expression was significantly suppressed in patients with SAB but not E. coli bacteremia (Fig. 2A). Transcription of DNMT3A was also suppressed when we challenged primary human macrophages with S. aureus in vitro (Fig. 2B). Using publicly available Gene Expression Omnibus (GEO) datasets, DNMT3A transcription was suppressed at all tested time points in S. aureus-challenged human macrophages (12) (Fig. 2C) and at 2 and 3 h postinfection in S. aureus-challenged human neutrophils (13) (Fig. 2D). These data collectively suggest a potential role of DNMT3A in promoting protective immune responses to S. aureus infection and potential S. aureus-mediated dysregulation of such responses.

Patients with g.25498283A > C Exhibit Higher Methylation Levels in Gene Regulatory CpG Island Regions. We next tested whether DNA methylation patterns differed between patients with and without the g.25498283A > C mutation using reduced representation bisulfite sequencing (RRBS). The DNA methylation profile exhibited a bimodal distribution. To study the global methylation pattern at the population level, we investigated the average methylation level of each CpG region within patients with and without g.25498283A > C mutation. Study patients with the g.25498283A > C genotype exhibited significantly higher levels of methylation in gene-regulatory CpG island regions than patients with the homozygote genotype (Δmethylation = 4.1%, P < 0.0001) (Fig. 3). These results suggest that a global methylation increase at gene-regulatory regions is associated with the observed g.25498283A > C mutation in DNMT3A, and introduce a potential mechanism by which this mutation could influence host ability to resolve MRSA bacteremia.

DNMT3A Expression Levels Were Similar in Patients with and without g.25498283A > C. Next, we performed whole-blood gene expression analysis to consider whether the DNMT3A genotype influenced expression levels. No significant differences in DNMT3A expression were noted between patients with (n = 24) or without (n = 44) g.25498283A > C mutation (SI Appendix, Fig. S1A).

However, as the SNP of interest occurs within a noncoding region, it is possible that the mode of action occurs via a change in expression or activity of a regulatory RNA species. To test this possibility, we interrogated RNA sequencing (RNA-seq) data for the expression of regulatory RNA species. We found 2 RNA segments that were known to influence the DNMT3A promoter: NUP153, which has been shown to bind outside of the catalytic domain of DNMT3A with no effect in the DNMT3A activity, and CDH1 (E-cadherin), which binds to the catalytic domain and inhibits DNMT3A activity (14). When we compared the expression levels for NUP153 and CDH1 between the patients with and without the polymorphism of interest, we found no significant differences between the 2 populations. Collectively, these findings suggest that the mechanism by which the DNMT3A polymorphism contributes to resolving MRSA bacteremia is not due to differences in regulatory RNA species, although the number of samples available for this analysis was limited (A/A, n = 1; A/C, n = 2) (SI Appendix, Fig. S1 B and C).

Patients with g.25498283A > C Exhibit Lower Levels of Interleukin-10 than Patients without the SNP. The Th2 antiinflammatory cytokine interleukin-10 (IL-10) has been previously shown to be significantly elevated in patients with poor clinical outcome among SAB patients, including mortality (15, 16), endocardial infection (17), and PB (15), and was also significantly higher in PB patients in our study (PB: 114.7472 pg/mL vs. RB: 13.1849 pg/mL; P = 0.0009). Thus, we hypothesized that patients heterozygous for DNMT3A g.25498283A > C, who were significantly less likely to exhibit PB, would also have significantly lower levels of IL-10. To test this hypothesis, we measured IL-10 levels in acute-phase serum from the study patients using a Luminex-based 38-plex cytokine assay (SI Appendix, Fig. S2). Consistent with our hypothesis, patients who were A/C heterozygotes had significantly lower serum levels of IL-10 (A/C: 9.70 pg/mL vs. A/A: 52.99 pg/mL; P = 0.0042) than A/A patients without the DNMT3A mutation (Fig. 4). This pattern of lower IL-10 among heterozygotes occurred among both patients with PB (A/C: 16.88 pg/mL vs. A/A: 68.94 pg/mL) and patients with RB (A/C: 8.68 pg/mL vs. A/A: 14.95 pg/mL), although the small number of A/C patients who developed PB (n = 3) limited statistical comparisons. These findings suggest our working model in which patients with the A/C heterozygote genotype of DNMT3A were more likely to resolve MRSA bacteremia in part due to lower levels of IL-10 regulated through higher levels of methylation in gene-regulatory regions (Fig. 5).

Small Interfering RNA Knockdown of DNMT3A Increases IL-10 Production in Human Macrophages. We next sought to assemble biological evidence to support the plausibility of this working model. To further define the role of DNMT3A in regulation of the macrophage response to S. aureus infection, cells were treated with small interfering RNA (siRNA) specific to DNMT3A. Successful knockdown of DNMT3A was confirmed by qRT-PCR (SI Appendix, Fig. S3). Macrophages were then stimulated with S. aureus, and a Luminex-based 10-plex cytokine assay was used to evaluate the impact of DNMT3A knockdown on the macrophage inflammatory response to S. aureus. Although the expression of several cytokines was affected, only IL-10 levels were significantly altered among S. aureus-challenged human macrophages with siRNA-mediated knockdown of DNMT3A (siRNA knockdown: 232.63 pg/mL vs. scramble: 98.40 pg/mL; P = 0.0209) (Fig. 6A and SI Appendix, Fig. S4A).

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Table 1. Characteristics of patients with persistent and resolving MRSA bacteremia

| Characteristic                                      | PB, n = 34 | RB, n = 34 | Fisher P value |
|-----------------------------------------------------|------------|------------|---------------|
| Age, median (P25, P75)                              | 63 (55, 69)| 61.5 (53, 70)| 0.9462        |
| Sex, no. (%)                                        |            |            | 1.00000       |
| Male                                                | 23 (67.6)  | 23 (67.6)  |               |
| Female                                              | 11 (32.4)  | 11 (32.4)  |               |
| Race, no. (%)                                       |            |            | 1.00000       |
| White                                               | 18 (52.9)  | 17 (50)    |               |
| African American                                    | 16 (47.1)  | 16 (47.1)  |               |
| Unknown                                             | 0 (0.0)    | 1 (2.9)    |               |
| Underlying comorbidity, no. (%)                    |            |            | 0.9462        |
| Neoplasm                                            | 0 (0.0)    | 11 (32.4)  | 0.00037       |
| Diabetic                                             | 17 (50)    | 20 (58.8)  | 0.62666       |
| Hemodialysis dependent                              | 13 (38.2)  | 9 (26.5)   | 0.43722       |
| HIV positive                                        | 2 (5.9)    | 0 (0.0)    | 0.49254       |
| Transplant recipient                                | 1 (2.9)    | 6 (17.6)   | 0.10543       |
| Injection drug use                                   | 1 (2.9)    | 0 (0.0)    | 1.00000       |
| Corticosteroid use (30 d)                           | 8 (23.5)   | 9 (26.5)   | 1.00000       |
| Surgery past 30 d                                   | 7 (20.6)   | 9 (26.5)   | 0.77568       |
| Endocarditis, previous episode                      | 1 (2.9)    | 1 (2.9)    | 1.00000       |
| Site of acquisition, no. (%)                        |            |            | 0.52113       |
| Hospital acquired                                   | 2 (5.9)    | 5 (14.7)   |               |
| HCA community acquired                              | 30 (88.2)  | 26 (76.5)  |               |
| Non-HCA community acquired                          | 2 (5.9)    | 3 (8.8)    |               |
| Source of bacteremia, no. (%)                       |            |            | 0.20791       |
| Endovascular infection                              | 12 (35.3)  | 6 (17.6)   |               |
| GI/GU infection                                     | 4 (11.8)   | 8 (23.5)   |               |
| Respiratory/lung                                    | 2 (5.9)    | 1 (2.9)    |               |
| Skin, soft tissue, joint/bone infection             | 12 (35.3)  | 10 (29.4)  |               |
| None/unknown                                        | 4 (11.8)   | 9 (26.5)   |               |
| Other                                               |            |            |               |
| Metastatic infection, no. (%)                       | 25 (73.5)  | 14 (41.2)  | 0.01356       |
| Metastatic abscess                                  | 6 (18.2)   | 3 (8.8)    | 0.30467       |
| Metastatic arthritis                                | 5 (15.2)   | 1 (2.9)    | 0.10543       |
| Metastatic epidural abscess                         | 3 (9.1)    | 1 (2.9)    | 0.35591       |
| Metastatic vertebral osteomyelitis                  | 5 (15.2)   | 1 (2.9)    | 0.10543       |
| Metastatic nonvertebral osteomyelitis               | 5 (15.2)   | 3 (8.8)    | 0.47628       |
| Metastatic psoas abscess                            | 3 (9.1)    | 2 (5.9)    | 0.67284       |
| Metastatic septic emboli                            | 5 (15.2)   | 1 (2.9)    | 0.10543       |
| Metastatic septic thrombophlebitis                  | 1 (3)      | 1 (2.9)    | 1.00000       |
| Metastatic kidney abscess                           | 0 (0.0)    | 2 (5.9)    | 0.49254       |
| Metastatic endocarditis                             | 10 (30.3)  | 3 (8.8)    | 0.03331       |
| APACHE II, mean (SD)                                | 18.3 (7.7) | 15.8 (4.5) |               |
| LOS categories, d, no. (%)                          |            |            | 0.00010       |
| <9                                                  | 0 (0.0)    | 13 (38.2)  |               |
| 9–14                                                | 9 (26.5)   | 10 (29.4)  |               |
| 15–20                                               | 13 (38.2)  | 5 (14.7)   |               |
| >20                                                 | 12 (35.3)  | 6 (17.6)   |               |
| Type of procedures used to treat the infection, no. (%) |            |            |               |
| Surgical removal of foreign device                  | 21 (67.7)  | 9 (40.9)   | 0.09057       |
| Surgical debridement                                | 11 (35.5)  | 2 (9.1)    | 0.04965       |
| Surgical insertion of foreign device                | 4 (12.9)   | 3 (13.6)   | 1.00000       |
| Abscess drainage                                    | 6 (19.4)   | 5 (22.7)   | 1.00000       |
| Other                                               | 15 (48.4)  | 17 (77.3)  | 0.04752       |
| Outcome (90 d), no. (%)                             |            |            | 0.08432       |
| Cure                                                | 27 (79.4)  | 33 (97.1)  |               |
| Recurrent SAB infection                             | 4 (11.8)   | 1 (2.9)    |               |
| Death due to SAB infection                          | 3 (8.8)    | 0 (0.0)    |               |
| Death due to other causes                           | 0 (0.0)    | 0 (0.0)    |               |

For metastatic infection, some patients have more than 1 type of metastatic infection. Some patients have more than 1 type of procedure used to treat the infection. APACHE II, Acute Physiology and Chronic Health Evaluation II; GI/GU, gastrointestinal/genitourinary; HCA, health care associated; LOS, length of stay; P, percentile.
Inhibition of DNA Methylation Also Increases IL-10 Production in Human Macrophages. Because of the documented role of \( \text{DNMT3A} \) in DNA methylation (18) and our finding that A/C heterozygotes had higher methylation levels, we next evaluated the role of DNA methylation on human macrophage cytokine response to \( S. \text{aureus} \). To do this, human macrophage methylation was inhibited by pretreatment with 5-Aza-2'-deoxycytidine (DAC), a drug known to inhibit DNA methyltransferase (19). Consistent with the increases in IL-10 production elicited by \( S. \text{aureus} \) challenge of human macrophages with siRNA knockdown of \( \text{DNMT3A} \), inhibiting the methylation capacity of human macrophages also caused significant increases in IL-10 production after \( S. \text{aureus} \) challenge (DAC treatment: 353.19 pg/mL vs. phosphate-buffered saline (PBS) treatment: 235.62 pg/mL; \( P = 0.0209 \)) (Fig. 6B and SI Appendix, Fig. S4B). These data agree with our working model (Fig. 5) and further support a role of \( \text{DNMT3A} \) in regulating cytokine production, possibly mediated by changes in DNA methylation.

Methylation Inhibition in Mice Confers Susceptibility to \( S. \text{aureus} \) Infection. To further elucidate the importance of \( \text{DNMT3A} \)-mediated methylation in host response to \( S. \text{aureus} \), we inhibited methylation capacity in mice by pretreatment with DAC followed by infection with \( S. \text{aureus} \). DAC-treated mice exhibited significantly greater mortality than PBS-treated controls following challenge with either methicillin-susceptible \( S. \text{aureus} \) (Fig. 7A) or MRSA (Fig. 7B). Bacterial load was significantly higher in the kidney, spleen, liver, and lung of DAC-treated mice than infected PBS-treated controls (Fig. 7C). DAC-treated mice also exhibited higher serum concentration of cytokines/chemokines than PBS-treated controls overall, although none of these differences achieved the level of statistical significance (SI Appendix, Fig. S5). These results suggest that methylation capacity is vital in mice to control \( S. \text{aureus} \) infection.

MRSA Isolates from Patients with PB and RB Elicit Similar Cytokine Response from Primary Human Macrophages but Different Levels of Macrophage Uptake. To test whether the associations between \( \text{DNMT3A} \) polymorphisms and the PB clinical phenotype might be specific to the infecting bacterial isolates rather than host response, we challenged primary human macrophages with representative PB or RB isolates (n = 5 of each) and assessed uptake and the elicited cytokine response using a 25-plex Luminex array. PB isolates exhibited significantly reduced median levels of phagocytosis by primary human macrophages compared with MRSA isolates (45,111.11 vs. 66,666.67 colony-forming unit [cfu]/mL; \( P = 0.0292 \)) (Fig. 8). When we conducted the experiment with extended incubation to 24 h, RB isolates were again isolated in higher numbers from macrophages than PB isolates, although the difference did not achieve statistical significance (SI Appendix, Fig. S6). In the 25-plex Luminex array, only IL-8 production differed significantly in primary human macrophages challenged by PB isolates vs. RB isolates (12,610.6 vs. 14,764.4 pg/mL; \( P = 0.0008 \)) (SI Appendix, Fig. S7).

Discussion

This study demonstrates that a specific polymorphism in \( \text{DNMT3A} \) is associated with a reduced risk for persistent MRSA bacteremia in patients, potentially through reducing IL-10 response by way of enhanced methylation. These conclusions are based on the following experimental evidence. First, g.25498283A > C in \( \text{DNMT3A} \) was associated with the RB phenotype at a level of significance of \( P = 7.8 \times 10^{-6} \). Second, \( \text{DNMT3A} \) expression was suppressed by \( S. \text{aureus} \) in both primary human macrophages challenged with...
S. aureus and patients with SAB. Third, IL-10 levels were significantly higher in patients with PB and among patients without the g.25498283A > C SNP in DNMT3A. Fourth, inhibition of both DNMT3A transcription and methylation activity led to increased IL-10 production by human macrophages upon S. aureus stimulation. Fifth, overall DNA methylation in patients with the g.25498283A > C mutation in DNMT3A was increased within gene-regulatory regions and was consistent with expectations based on our in vitro studies of DNMT3A. Sixth, methylation inhibition in mice rendered them significantly more susceptible to S. aureus. Collectively, these findings support the observation that DNA methylation is important for protective host immune defenses against S. aureus by way of a mechanism outlined in our working model (Fig. 5).

Although the mechanism remains incompletely understood, the observed association between g.25498283A > C mutation in DNMT3A and PB is consistent with previous studies. Cao et al. (20) found that a polymorphism in DNMT3A was associated with increased risk of Helicobacter pylori infection, a gram-negative bacterium responsible for gastric cancer. Other investigators have shown that mice deficient in Dnmt3a were susceptible to vesicular stomatitis infection (21) via impaired production of type I interferons (IFNs; IFN-α and IFN-β) (22). In T cells, Dnmt3a is known to regulate cytokine production with IL-13, in particular being increased in the absence of Dnmt3a but not Dnmt3b (23). These data extend insights in this regard to S. aureus, as IL-13 is a hallmark of Th2 immune responses that recent vaccine studies suggest do not adequately protect against S. aureus (24). Likewise, in mast cells, loss of Dnmt3a is accompanied by increased IL-13, IL-6, and tumor necrosis factor-α (TNF-α) production after stimulation with Immunoglobulin E antigen (10). However, as transforming growth factor-β (TGF-β) is required along with IL-6 and TNF-α to induce protective Th17 polarization, change-in-function polymorphisms of DNMT3A could alter protective immunity against S. aureus. It has also been shown that mice with B cells deficient in Dnmt3a/Dnmt3b have increased cell activation when immunized with phycoerythrin emulsified in complete Freud's adjuvant (25). Consistent with the pattern of generally increased cytokine production in DNMT3A-deficient cells, we found that siRNA knockdown of DNMT3A or inhibition with DAC in human macrophages resulted in increased levels of cytokine IL-10 production upon S. aureus stimulation.

IL-10, an immunosuppressive cytokine produced primarily in the Th2 paradigm, has been identified as a potential marker of immune dysregulation associated with persistent SAB and/or mortality (15). IL-10 is known to block the activation of Th1 cells, thereby promoting susceptibility to certain intracellular pathogens (26, 27). One role for IL-10 is in regulation of the production of proinflammatory cytokines and has been shown to play an essential role during S. aureus infection: too much IL-10 expression biases toward death of the infected host (28). IL-10 has also been shown to inhibit microbial killing by limiting the production of reactive oxygen and nitrogen intermediates (29, 30). In Mycobacterium tuberculosis infection, IL-10 blocks phagosome maturation and facilitates M. tuberculosis survival (31). Transgenic mice overexpressing IL-10 in T cells are more susceptible to M. tuberculosis infection (32).
pulmonary tuberculosis (33), thereby establishing a direct correlation between IL-10 level and disease severity.

Our study had limitations. First, the polymorphism is located in the noncoding region of *DNMT3A*. Thus, it is not possible to predict any direct effect of the polymorphism on *DNMT3A* function. Second, the mutation did not impact the transcription of *DNMT3A* messenger RNA (mRNA); therefore, the siRNA model system used in this manuscript may not accurately represent the effect of the polymorphism observed with WES. Our proof of concept study using DAC-treated mice has the potential for broader, off-target effects of DAC. Thus, additional experiments are underway in our laboratory to determine the specific association between *DNMT3A* polymorphisms and persistent *S. aureus* infection using knockout mice. Third, we recognize that certain subsets of IL-10-producing CD4+ T cells are proinflammatory (34). Thus, IL-10 should not be exclusively defined as antiinflammatory; rather, IL-10 likely functions in context of other responses that collectively shape protective vs. nonprotective immunity to a given pathogen in a specific host. Fourth, the causes of persistent MRSA bacteremia are complex. For example, we found that bacteria from patients with PB and RB interacted differently with human macrophages, eliciting different levels of phagocytosis and triggering different levels of IL-8 from these cells. Thus, it is likely that a number of clinical and bacterial characteristics in addition to the patient’s *DNMT3A* genotype may also contribute to this clinical syndrome. Fifth, there are undoubtedly additional cytokines or other molecular and cellular immune responses not presently studied that contribute to the PB vs. RB outcome. Susceptibility to host defense peptides and binding to endothelial cells are 2 examples of MRSA phenotypes that also seem to influence PB vs. RB outcomes (35).

Despite these limitations, this study provides evidence that the g.25498283A > C polymorphism in *DNMT3A* protects against persistent MRSA bacteremia by changing patterns in DNA methylation. The findings establish epigenetic modification of DNA as influencing host response to *S. aureus* infection. Future studies are underway to elucidate the molecular mechanisms of how this polymorphism influences the risk for PB. The ability of advanced bioinformatics tools to assess global genome methylation patterns across all regions of the human genome will greatly facilitate this goal.
Fig. 5. Working model of DNMT3A polymorphism and persistent MRSA bacteremia.

SAB episode, had no signs or symptoms of infection, had an additional clinically significant bacterial pathogen that was isolated from their blood culture, or declined informed consent. Only the initial presentation for eligible patients was included in the study. Clinical data were collected on a standardized case report form and entered into an electronic database (Microsoft Access). The outcomes of SAB per study protocol recorded deaths (attributable to S. aureus or other causes), cure, or recurrent bacteremia within the 90-d follow-up period. The study was approved by the Duke Institutional Review Board. Written informed consent was obtained from patients or their legal representatives. If a patient died prior to the notification of their blood culture results, the subjects were included using an Institutional Review Board-approved Notification of Decedent Research.

Patients were defined as having either PB or RB. PB patients (n = 34) had ongoing MRSA bacteremia for at least 5 d after initiation of effective antibiotics. Patients with PB had initial blood cultures that were positive for MRSA, but all subsequent blood cultures were sterile. PB and RB patients were eligible for inclusion if all required biological samples were available (sera/plasma, RNA, DNA, and bloodstream isolate), and they were successfully matched 1:1 by sex, age (by deciles), race, hemodialysis status, diabetes mellitus, and presence of any implantable medical device using nearest neighbor propensity scores (36) generated from logit regression models fit separately across 4 strata.

WES and Analysis. DNA-Seq data were processed using the TrimGalore toolkit (37) (v0.4.0), which uses Cutadapt (v1.8.3) to trim low-quality bases and Illumina sequencing adapters from the 3′ end of the reads. Reads were aligned to the b37 version of the human genome with the BWA algorithm (v0.7.12). Alignment processing and variant calling were performed using the GATK toolkit (v3.7) following the Broad Institute’s Best Practices Workflow (38). Association testing was carried out using the Fisher’s exact test from the RVTESTS algorithm (39) (v20150104).

RNA-seq in PB and RB Patients. Total RNA was isolated with the Qiagen RNA Blood kit, and quality control was performed with Nanodrop 8000 and Agilent Bioanalyzer 2100. Globin RNA was removed with the Life Technologies GLOBINCLEAR (human) kit. Libraries for RNA-seq were prepared with the KAPA Stranded mRNA-Seq Kit. The workflow consists of mRNA enrichment, complementary DNA (cDNA) generation, and end repair to generate blunt ends; A tailing, adaptor ligation, and PCR amplification. Different adaptors were used for multiplexing samples in 1 lane. Sequencing was performed on an Illumina Hiseq3000 for a single-read 50 run. Each sample gets an average of 15 million reads. Data quality check was done on Illumina SAV. Demultiplexing was performed with the Illumina Bcl2fastq v 2.17 program.

DNA Methylation Levels in A/A and A/C Patients. The DNA methylation levels in patients with and without the g.25498283A > C mutation were measured by RRBS as previously described (40). The detectable CpGs were determined based on the filter of 15× coverage. The average methylation level for each CpG in patients with or without g.25498283A > C mutation was calculated using python package scpy. The violin plot demonstrated the distributions of the CpG counts at each averaged methylation level generated by python library seaborn. The median test was computed to compare the differences between patients with and without g.25498283A > C mutation in STATA.

Serum/Plasma 38-Plex Cytokine/Chemokine Array. Luminex assay and analysis were performed by the University of California, Los Angeles Immune Assays Core. Human 38-plex magnetic cytokine/chemokine kits (EMD Millipore; HCYTMAG-60K-PX38) were used per the manufacturer’s instructions. Fluorescence was quantified using a Luminex 200 instrument. Cytokine/chemokine concentrations were calculated using Milliplex Analyst software version 4.2 (EMD Millipore).

Measurement of Cytokines in In Vitro Assays. Cytokine production was assayed from the supernatant of either S. aureus-challenged siRNA-transfected macrophages or S. aureus-challenged DAC-treated macrophages using a multiplex cytokine kit and Luminex technology available at the Duke Human Vaccine Institute. Ten cytokines were tested: TNF-α, IL-1β, IFN-γ, IL-5, IL-6, IL-10, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Of the 10 tested cytokines, 5 for siRNA (IL-1β, IL-5, IFN-γ, IL-2, and IL-4) and 6 for DAC (IL-1β, IL-5, IFN-γ, IL-2, IL-4, and GM-CSF) were excluded from our analysis due to lack of response to S. aureus; 1 cytokine GM-CSF for siRNA and 1 cytokine TNF-α for DAC were excluded for weak signal or unproven running, and 1 cytokine (IL-8 for both siRNA and DAC) was excluded for cross-contamination (SI Appendix, Fig. S5). For cytokines/chemokines production in human macrophages challenged with persistent and resolving isolates, 25 cytokines were tested: IL-1β, IL-10, IL-6, RANTES, Eotaxin, IL-17A, MIP-1α, GM-CSF, MIP-1β, MCP-1, IL-15, IL-5, IFN-γ, IL-1RA, TNF-α, IL-2, IL-7, IP-10, IL-2R, MIG, IL-4, IL-8, IL-12, and IL-13. Of those, 9 (IL-1β, RANTES, Eotaxin, IL-17A, GM-CSF, IL-5, IL-2, IL-7, and IL-13) were excluded from our analysis due to lack of response to S. aureus; 2 (IP-10 and IFN-γ) were excluded for weak signal, and 3 (IL-1RA, IFN-α, and MCP-1) were excluded cross-contamination.

Fig. 6. (A) siRNA knockdown or (B) DAC treatment of human macrophages increases IL-10 production. Primary human macrophages were treated with either DNMT3A siRNA or DAC, then incubated with S. aureus at a multiplicity of infection of 10 for 5 h, after which macrophage culture supernatants were collected for cytokine analysis. The IL-10 profile was determined by Luminex-based multiplex cytokine assay. Data are presented as the average and SD of points from 4 biological replicates. Significance was determined using the Mann–Whitney U test. *P < 0.05.
Bacterial Strains and Growth Conditions. Sanger 476, a methicillin-susceptible *S. aureus* (41), and LAC, an MRSA isolate (42), were used in this study. *S. aureus* was prepared as previously described (43) with minor modifications. After overnight culture, an appropriate number of cells were diluted to 500 mL fresh tryptic soy broth to achieve an optical density at wavelength 600 nm (OD 600) of 0.1. The flasks were incubated at 37 °C and 220 rpm to log-phase growth (OD 600 ∼ 1.0). Cells were harvested by centrifugation, washed twice with PBS, resuspended in PBS containing 20% glycerol to a concentration of ∼ 10^6 cfu/μL, aliquoted into individual cryovials, and immediately stored at −80 °C until further use. An aliquot of PBS-washed culture was serially diluted and plated on Trypticase soy agar plates to enumerate cfu. Frozen stocks were titered for cfu in triplicate on separate occasions before use in any experiment.

Mammalian Cell Culture. Primary human macrophages were derived from peripheral blood monocytes isolated from whole blood taken from healthy volunteers (Nebraska Medical School). Monocytes were cultured in Dulbecco’s Modified Eagle’s Medium containing 10% (vol/vol) true pooled human serum (serocase) supplemented with 0.1% gentamycin (Sigma) and recombinant human macrophage colony-stimulating factor (M-CSF) at 6 ng/mL (RD systems). After 3 d of culture, fresh medium was added to dilute out M-CSF and antibiotic, and every other day, half of the medium was replaced with fresh medium for up to 7 d, at which point cells were ready for functional study.

siRNA Transfection. Differentiated primary human macrophages were transfected with either siRNA targeting DNMT3A or control scramble siRNA (Dharmacon). Stocks of siRNA along with the scramble siRNA were diluted to 10 μM with RNase free water. Prior to transfection, siRNAs were added to Opti-MEM (Thermo Fisher Scientific); then, the solution was combined with lipofectamine RNAiMAX (Thermo Fisher Scientific) and incubated at room temperature for 10 min for complex formation. After the siRNA complex was added to the cells to a final concentration of 50 nM and cells were incubated in a 37 °C and 5% CO2 incubator for 24 h, the cells were washed with PBS and then, challenged with *S. aureus*. The efficiency of DNMT3A knockdown was measured by qRT-PCR.

Fig. 7. Mice treated with DAC are hypersusceptible to *S. aureus* with high bacterial tissue burden. (A) Survival curve of 8-wk-old C57BL/6NJ-DAC and C57BL/6NJ-PBS mice after intraperitoneal injection of 10^8 cfu of methicillin-susceptible *S. aureus* (MSSA) strain Sanger 476 per mouse (n = 15 per group). (B) Survival curve of 8-wk-old C57BL/6NJ-DAC and C57BL/6NJ-PBS mice after intraperitoneal injection of 4 × 10^7 cfu of methicillin-resistant *S. aureus* (MRSA) LAC strain per mouse (C57BL/6NJ-DAC, n = 10; C57BL/6NJ-PBS, n = 9). Survival analysis was done using the log rank test (P < 0.001). (C) Increased bacterial load in DAC-treated and PBS-treated mice after *S. aureus* infection. DAC-treated and PBS-treated controls were injected intraperitoneally with *S. aureus* strain Sanger 476, and the bacterial loads in kidneys, spleens, livers, and lungs were determined 18 h postinfection. Significant difference in bacterial load between DAC-treated and PBS-treated control mice was done using the Mann–Whitney U test (P < 0.05). At least 4 mice per group were analyzed.
DAC Treatment of Human Macrophages. Cells were seeded at a density of 5 x 10^5 cells per well in a 24-well plate (VWR). Dissolved DAC (Sigma) was added to cells to achieve a final concentration of 5 μM, and cells were incubated in a 37 °C and 5% CO2 incubator for 72 h. After the treatment, the medium was replaced with fresh medium without DAC, and the cells were challenged with S. aureus.

RNA Extraction, cDNA Conversion, and qRT-PCR. Total RNA was extracted using the Direct-zol RNA MiniPrep Kit (Zymo Research) and was subsequently digested with DNase I (Zymo Research). The concentration and purity were evaluated using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA was reverse transcribed using the SuperScript III Reverse Transcriptase Kit (Thermo Fisher). qRT-PCR was performed using a Power SYBR Select Mater Mix (Life Technologies) and an ABI Prism 7500 Fast real-time PCR system (Life Technologies). All kits were used according to the manufacturer’s instructions. The mRNA of DNMT3A was normalized to Actin ribosomal RNA. The DNMT3A and Actin primers used here are as follows: DNMT3A (5′-ATT-GATCGGCCACACAGAGAC-3′/5′-GGGGTTTCCAGGGTAACATTGAG-3′) and Actin (5′-CACCACACTGGGAGCAT-3′/5′-ACAGCCTGGATAGCAACG-3′).

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