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Cyclic di-AMP Released from *Staphylococcus aureus* Biofilm Induces a Macrophage Type I Interferon Response

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***Staphylococcus aureus* is a leading cause of community- and nosocomial-acquired infections, with a propensity for biofilm formation. *S. aureus* biofilms actively skew the host immune response toward an anti-inflammatory state; however, the biofilm effector molecules and the mechanism(s) of action responsible for this phenomenon remain to be fully defined. The essential bacterial second messenger cyclic diadenylate monophosphate (c-di-AMP) is an emerging pathogen-associated molecular pattern during intracellular bacterial infections, as c-di-AMP secretion into the infected host cytosol induces a robust type I interferon (IFN) response. Type I IFNs have the potential to exacerbate infectious outcomes by promoting anti-inflammatory effects; however, the type I IFN response to *S. aureus* biofilms is unknown. Additionally, while several intracellular proteins function as c-di-AMP receptors in *S. aureus*, it has yet to be determined if any extracellular role for c-di-AMP exists and its release during biofilm formation has not yet been demonstrated. This study examined the possibility that c-di-AMP released during *S. aureus* biofilm growth polarizes macrophages toward an anti-inflammatory phenotype via type I interferon signaling. DacA, the enzyme responsible for c-di-AMP synthesis in *S. aureus*, was highly expressed during biofilm growth, and 30 to 50% of total c-di-AMP produced from *S. aureus* biofilm was released extracellularly due to autolytic activity. *S. aureus* biofilm c-di-AMP release induced macrophage type I IFN expression via a STING-dependent pathway and promoted *S. aureus* intracellular survival in macrophages. These findings identify c-di-AMP as another mechanism for how *S. aureus* biofilms promote macrophage anti-inflammatory activity, which likely contributes to biofilm persistence.**

Staphylococcus aureus is a leading cause of prosthetic joint infections (1, 2), whereupon adherence to the implant surface facilitates biofilm formation. These infections are particularly challenging to treat and typically require a two-stage surgical process for insertion of a new device (1). Moreover, biofilms actively skew the host immune response toward an anti-inflammatory state, thereby contributing to the chronic nature of biofilm-mediated infections (3–5). This is evident by macrophage polarization toward an alternatively activated phenotype and the recruitment of myeloid-derived suppressor cells (MDSCs) (3, 6–8). While this immune deviation appears to be driven by *S. aureus* biofilm products, the effectors and their mechanism(s) of action remain to be fully identified.

To further understand mechanisms of immune modification by *S. aureus* biofilms, we investigated the role of the essential bacterial second messenger cyclic diadenylate monophosphate (c-di-AMP), since it has been identified as an emerging pathogen-associated molecular pattern (PAMP) that influences immune responsiveness (9, 10). While c-di-AMP has been found to regulate many important functions in bacteria, including cell wall stress and peptidoglycan homeostasis (11–15), antibiotic resistance (16–18), biofilm formation (19), growth and metabolism (20–23), and DNA damage response (24, 25), it can also modulate leukocyte activation. For example, c-di-AMP secretion into the macrophage cytosol induces a robust type I interferon (IFN [IFN-β]) response during intracellular bacterial infections, including those caused by *Listeria monocytogenes* (26, 27), *Chlamydia trachomatis* (28), and *Mycobacterium* spp. (29, 30). Cytosolic c-di-AMP is sensed by both the endoplasmic reticulum resident protein stimulator of interferon genes (STING) (31–33) and the helicase DDX41 (34); however, the individual contributions of these proteins in sensing c-di-AMP remain unclear.

While several intracellular proteins function as c-di-AMP receptors in *S. aureus* (11, 35–38), the spatial and temporal production as well as any extracellular role for c-di-AMP during biofilm development has yet to be determined. Furthermore, the ability of extracellular c-di-AMP to trigger type I IFN production in macrophages has not yet been demonstrated and may serve as a potential mechanism to polarize macrophages to an anti-inflammatory state characteristic of biofilm infections (3, 5–8). The data presented here demonstrate that the gene responsible for c-di-AMP synthesis (*dacA*) is expressed in a temporal manner during biofilm growth, and quantitative mass spectrometry revealed that c-di-AMP is released extracellularly from *S. aureus* biofilm during cell lysis. Additionally, macrophage exposure to extracellular c-di-AMP, coculture with *S. aureus* biofilms, and treatment with conditioned medium from mature *S. aureus* biofilm all induced a robust type I IFN response. In terms of functional implications, c-di-AMP promoted *S. aureus* intracellular survival in human

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TABLE 1 Bacterial strains and plasmids used in the study

Strain or plasmid	Characteristic(s)	Source(s)
Bacterial strains		
LAC-13C	Wild-type *S. aureus* CA-MRSA[a] (USA300) isolate cured of plasmid p03	40
Δ*gdpP*	Transduction of *gdpP*::ΦNΣ in LAC-13C	This study and ref. 40
CMG8	Transduction of SAUSA300_2126, SAUSA_2298, and SAUSA2360::ΦNΣ in LAC-13C	This study and ref. 40
RN4220	Highly transformable restriction-deficient strain	64
Plasmid		
pCMG2	Divergent P*dacA*::DsRed and P*gdpP*::GFP cloned into pDM4	This study and ref. 41

[a] CA-MRSA, community-acquired methicillin-resistant *S. aureus*.

monocyte-derived macrophages. Collectively, these data represent the first evidence for the recognition of exogenous c-di-AMP from an extracellular pathogen and provide support for bacterial cell lysis as a mechanism for polarizing macrophages toward a type I IFN response.

MATERIALS AND METHODS

Bacterial strains, plasmid construction, and growth conditions. *Escherichia coli* DH5α was used for cloning and grown at 37°C in lysogeny broth (LB) with ampicillin (100 μg ml^{-1}) for selection. DNA ligase and restriction enzymes were obtained from New England BioLabs (Beverly, MA). Plasmids were purified using the Wizard Plus SV Miniprep DNA purification system (Promega Corporation, Madison, WI) and analyzed using Vector NTI (Invitrogen, Carlsbad, CA). The *S. aureus* strain used (LAC) is a USA300 isolate from a skin and soft tissue infection, cured of plasmid p03, and designated LAC-13C (39). LAC-13C is referred to as LAC throughout the text. The strains and plasmid used in the study are listed in Table 1.

***S. aureus* growth, biofilm development, and analysis.** For static biofilm development, single isolated colonies grown on tryptic soy agar (TSA) were inoculated into 3 ml of RPMI 1640 plus 1% Casamino Acids (CAA; Becton, Dickinson, Franklin Lakes, NJ) and grown overnight at 37°C with orbital shaking at 250 rpm. Wells of a 12-, 24-, or 48-well plate were precoated with 20% human plasma in 0.1% carbonate-bicarbonate buffer (Sigma, St. Louis, MO) overnight at 4°C. Cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.05 in fresh RPMI 1640 plus 1% CAA and added to wells at a surface-to-volume ratio of 0.19. Chloramphenicol (10 μg ml^{-1}) was supplemented when plasmid maintenance was required. Biofilms were grown statically in a 37°C, 5% CO_2 incubator. After every 24 h of growth, 50% of the medium was removed and replaced with fresh medium.

Confocal analysis of fluorescence from transcription reporter plasmids during static biofilm growth was performed in sterile two-well glass chamber slides (Thermo Scientific Nunc) using a Zeiss 710 META laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) with ×40 magnification and analyzed using ZEN 2009 Black (Carl Zeiss) software. Green fluoresceint protein (GFP) and DsRed fluorescence were acquired with 488-nm and 560-nm excitation (Ex) wavelengths, respectively.

Analysis of *dacA* and *gdpP* transcription levels was performed by isolating RNA from static biofilms at 1 to 6 days of growth. Supernatants were removed and biofilms resuspended in 1 ml of TRIzol (Life Technologies). Cell suspensions were transferred to 2 ml lysing matrix B tubes containing 0.1-mm silica beads (MP Biomedicals, Santa Ana, CA), lysed in an OMNI Bead Ruptor 24 (Omni International, Kennesaw, GA), and bead beat twice for 23 s at speed 6. Subsequent RNA purification was performed according to the manufacturer's instructions. cDNA was generated using the high-capacity cDNA reverse transcription (RT) kit (Applied Biosystems, Foster City, CA), and quantitative RT-PCR (qRT-PCR) was performed using iTaq Universal SYBR green supermix (Bio-Rad, Hercules, CA) with primers specific to *sigA*, *dacA*, and *gdpP* (Table 2).

To assess fluorescence from the transcription reporter plasmids during planktonic growth, *S. aureus* was grown in trypic soy broth (TSB) overnight (16 to 18 h) at 37°C with shaking at 250 rpm. Cultures were diluted to an OD_{600} of 0.1 in TSB and added to a 96-well black-wall, clear-bottom plate (Corning, Inc., Corning, NY), where growth (OD_{600}), GFP fluorescence (Ex, 488 nm; emission [Em], 518 nm), and DsRed fluorescence (Ex, 560 nm; Em, 590 nm) were monitored in real-time by using

TABLE 2 Primers used in the study

Primer	Sequence[a]	Application	Source
dacA-F	TGCGGTTGGTATTTCAGAAG	*dacA* qRT-PCR	This study
dacA-R	TTTCTTTTGAAAGCGTGTGC	*dacA* qRT-PCR	This study
gdpP-F	TTAGTCGATGGGCAACTGAG	*gdpP* qRT-PCR	This study
gdpP-R	TTAATTGGGCACGATAACCA	*gdpP* qRT-PCR	This study
sigA-F	AACTGAATCCAAGTGATCTTAGTG	*sigA* qRT-PCR	65
sigA-R	TCATCACCTTGTTCAATACGTTTG	*sigA* qRT-PCR	65
CMG2	CCC*TGCAG*ATTGATAAATTGGGGATAAAGGAA	500 bp 5′ *dacA*	This study
CMG4	CC*GTCGAC*TTATTTCACACCTTTCTTTTGAAAG	3′ *dacA*	This study
CMG7	CCC*TGCAG*GCCGATGCGAATATGAC	500 bp 5′ *gdpP*	This study
CMG9	CC*GTCGAC*TCATGCATCTTCACTCCTAC	3′ *gdpP*	This study
CMG10	CC*GAATTC*GCGCGACTTTTCATTT	SAUSA300_2126	This study
CMG11	CC*GTCGAC*TAAAATTTCCTTCTATTACTTTCTATTTCT	SAUSA300_2126	This study
CMG12	CC*GAATTC*AAATTAAATCACTTAATGTTAAACAAGGTGA	SAUSA300_2298	This study
CMG13	CC*GTCGAC*TTATTCATGATTGATACTATTATCTGC	SAUSA300_2298	This study
CMG14	CC*GAATTC*ATGTACACAAGGAGTGAGT	SAUSA300_2360	This study
CMG15	CC*GTCGAC*TTATTGACGAGAATCAACTTC	SAUSA300_2360	This study

[a] Primer oligonucleotide sequences are provided in the 5′-to-3′ orientation. Italics indicate nonhomologous sequences added for cloning.

a TECAN 200 Infinite Pro system (Tecan Group Ltd., Männedorf, Switzerland). For fluorescence analysis during biofilm formation under flow conditions, methods previously described were employed for use with the Bio-Flux microfluidics system (40, 41).

Cyclic di-AMP measurements. Biofilms were propagated in 12-well plates containing 2 ml/well of medium as described above. At the desired time point, biofilm cells were resuspended in the existing 2 ml of biofilm-conditioned medium (no fresh medium change) and placed in a 2-ml tube on ice. A 10-μl aliquot was removed for bacterial numeration. Bacteria were centrifuged at 14,000 rpm for 5 min at 4°C. Bacterial cells were resuspended in ice-cold extraction buffer (methanol/acetonitrile/H_2O, 40:40:20) and stored at −80°C until assessed for c-di-AMP content. The insoluble fraction was pelleted in a benchtop centrifuge (15,000 rpm for 5 min), and 200 μl of the supernatant was filtered with a 0.45-μm-pore-size filter (Titan 4 mm; SUN-Sri, Rockwood, TN). Next, 100 μl of the filtered sample was transferred and the extraction buffer was evaporated using a vacuum manifold. The pellet was resuspended in 100 μl ultrapure water. Ten microliters of each sample was then analyzed on a Quattro Premier XE mass spectrometer (Waters, Milford, MA) coupled with an Acquity Ultra Performance liquid chromatography (LC) system (Waters) as previously described for c-di-GMP (42). Ion settings for the mass spectrometer were electrospray negative, and the specific ion pair being analyzed for quantification of c-di-AMP was 657 → 134.

For quantitation of c-di-AMP release from *S. aureus* biofilms, biofilm-conditioned medium was removed and lyophilized using vacuum centrifugation. Prior to high-performance LC/mass spectrometry (MS) measurements, lyophilized supernatant samples were suspended in extraction buffer and processed for c-di-AMP quantification as described above. For reporting both intracellular and extracellular c-di-AMP levels, the measured mass of c-di-AMP was normalized to viable bacteria collected from biofilms.

Preparation of bone marrow-derived macrophages, RNA isolation, qRT-PCR, and enzyme-linked immunosorbent assay (ELISA). To prepare bone marrow-derived macrophages (BMDMs), bone marrow was isolated from the long bones (femur and tibia) of C57BL/6 or STING knockout (KO) mice (Jackson Laboratories, Bar Harbor, ME) as previously described, with minor modifications (3). Briefly, bone marrow was cultured in a 12-well plate at 37°C in a 5% CO_2 incubator in RPMI 1640 medium supplemented with 1% penicillin/streptomycin/amphotericin B (Corning, Corning, NY), 50 μM beta-mercaptoethanol, 10 mM HEPES, 10% fetal bovine serum, and 20% supernatant from L-929 fibroblasts as a source of macrophage colony-stimulating factor (M-CSF). Medium was replaced at 2 to 3 and 4 to 5 days *in vitro*, and macrophages were used for experiments at days 7 to 10. These studies were conducted in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (43). The protocol was approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

BMDMs were either cocultured with mature (6-day) *S. aureus* biofilms for 3 h, or treated with purified c-di-AMP (InvivoGen, San Diego, CA) or biofilm-conditioned medium diluted to 22% for the indicated intervals, whereupon RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. In some experiments, diluted biofilm-conditioned medium was treated with 10 U/ml of snake venom phosphodiesterase (SVP; Affymetrix, Santa Clara, CA), an enzyme capable of degrading c-di-AMP (26), for 45 min at 37°C and handled according to the manufacturer's recommendations. Macrophage cDNA was generated using iScript RT supermix (Bio-Rad), and qRT-PCR was performed using TaqMan gene expression master mix (Applied Biosystems) in a Bio-Rad CFX Connect real-time system with TaqMan primers specific to *ifn*-β and *il-6* (interleukin-6 [IL-6]), and to *gadph* (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) as a housekeeping gene. Data were analyzed using CFX Manager software (Bio-Rad) with gene expression levels normalized to those of GAPDH and are presented as the fold induction ($2^{-\Delta\Delta CT}$) value relative to the level for untreated BMDMs. IFN-β and IL-6 ELISA kits were purchased from BioLegend (San Diego, CA, USA) and R&D BioScience (Minneapolis, MN, USA), respectively.

Biofilm autolysis assay. Triton X-100-induced autolysis assays were performed using a modification of previously published methods (44). Biofilms were grown in RPMI plus 1% Casamino Acids for 6 days as described above. In some experiments, polyanethole sulfonate (PAS) was added at day 4 of biofilm growth to a final concentration of 50 μg/ml to inhibit autolysis. Biofilms were disrupted, washed with ice-cold H_2O, and resuspended in autolysis buffer (50 mM Tris-HCl [pH 7.2] with 0.05% Triton X-100) to an OD_{600} of less than 1.0. Subsequently, 1 ml of cell suspension was placed in a 12-well plate and incubated at 30°C with constant orbital shaking in a Tecan 200 Infinite Pro apparatus. The OD_{580} was measured at 30-min intervals for a period of 13 h, and results are reported as the percentage of the initial OD_{580} for each sample.

Human monocyte-derived macrophages and gentamicin protection assays. Human monocytes were obtained from healthy human donors by the University of Nebraska Medical Center Elutriation Core Facility by countercurrent centrifugal elutriation, in full compliance and approval of the Institutional Review Board. Cells were cultured in suspension in Teflon flasks in Dulbecco's modified Eagle's medium supplemented with human M-CSF (100 ng ml^{-1}), 10% human serum, gentamicin, and ciprofloxacin for 7 days prior to experimentation.

To assess the effects of c-di-AMP on macrophage microbicidal activity, human MDMs were subjected to gentamicin protection assays conducted similarly to previously described methods (3). Briefly, macrophages were pretreated with various concentrations of c-di-AMP (0.01 to 1 μM) for 1 h, followed by a 2-h incubation with live *S. aureus* LAC at an multiplicity of infection (MOI) of 5:1. MDMs were washed three times with 1× phosphate-buffered saline and treated with gentamicin (100 μg ml^{-1}) for 1 h, whereupon fresh medium containing 1 μg ml^{-1} gentamicin was added and cells were incubated for 24 h. At this point, MDMs were washed and lysed using sterile double-distilled water and plated onto blood agar plates for enumeration of intracellular bacteria.

Statistics. Significant differences between experimental groups were determined with either an unpaired two-tailed Student's *t* test or a one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test, within Prism 6 (GraphPad, La Jolla, CA). For all analyses, a *P* value of less than 0.05 was considered statistically significant.

RESULTS

***S. aureus* expresses *dacA* but minimal *gdpP* during post-exponential-phase and biofilm growth.** To gain insight into the spatial and temporal production of c-di-AMP, we analyzed the expression patterns of the *S. aureus* diadenylyl cyclase (*dacA*) and phosphodiesterase (*gdpP*) genes, which are responsible for c-di-AMP synthesis and degradation, respectively. Utilizing divergent promoter-fusion plasmids (P*dacA*::DsRed and P*gdpP*::GFP), *dacA* expression was observed primarily during the post-exponential and early stationary phases of planktonic growth in TSB (Fig. 1A). Surprisingly, *gdpP* expression was nearly undetectable throughout the 18-h experiment. To assess *dacA* and *gdpP* expression in a developing biofilm under flow conditions, BioFlux microfluidic microscope technology was utilized. As shown in Fig. 1B, strong *dacA* expression was localized to developing biofilm tower structures, whereas *gdpP* expression was below the limit of detection. Regulation was also examined under static biofilm growth conditions in RPMI 1640 supplemented with 1% Casamino Acids. Widespread *dacA* expression was observed throughout the static biofilm, and *gdpP* expression was only detected in a small number of cells (Fig. 1C). Differential *dacA* and *gdpP* regulation during static biofilm growth was confirmed by qRT-PCR (Fig. 1D), showing constitutive *dacA* expression throughout 1 to 6 days of static

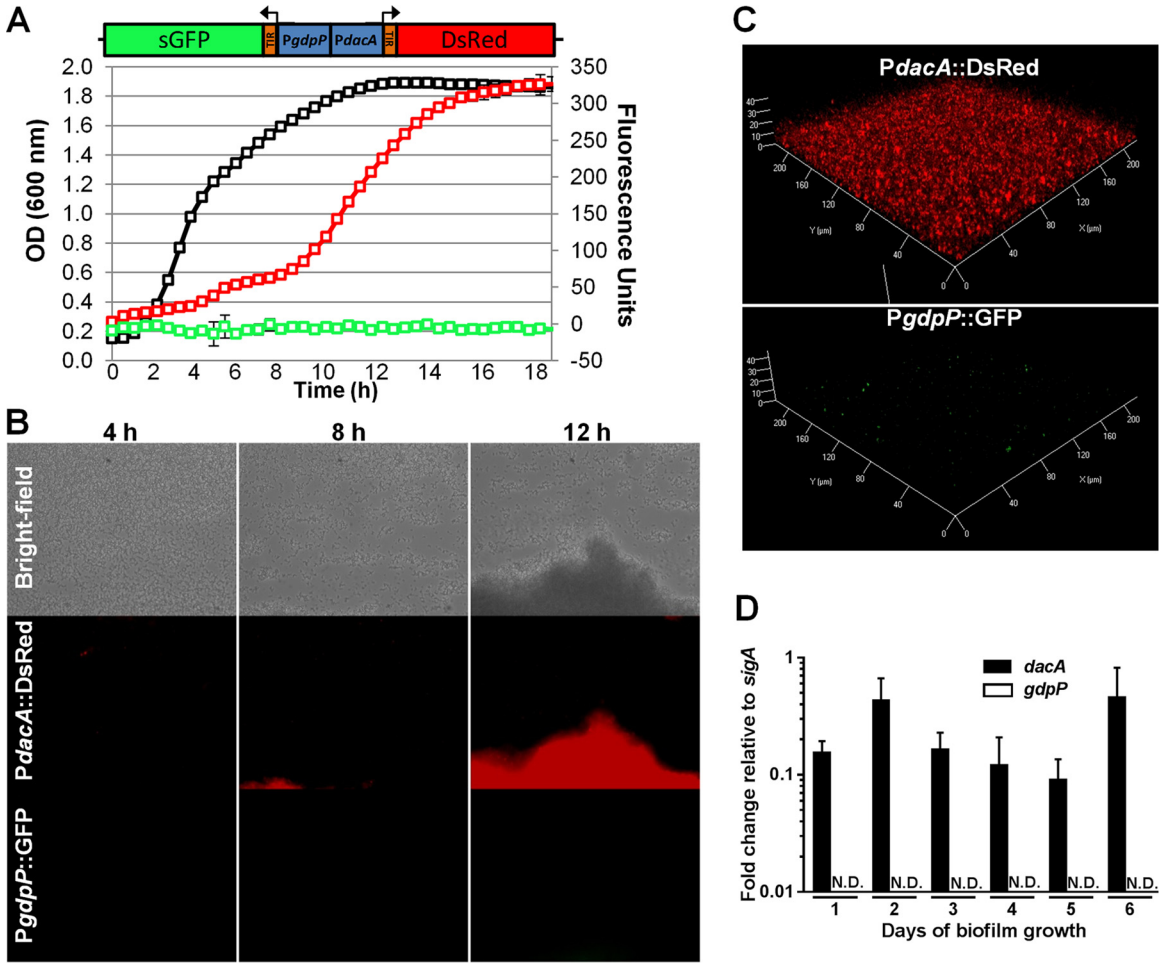

FIG 1 *S. aureus* expresses *dacA* but minimal *gdpP* during post-exponential and biofilm growth. (A to C) To assess *dacA* and *gdpP* promoter activity, fluorescence from a transcriptional reporter plasmid (divergent P*dacA*::DsRed and P*gdpP*::GFP) was monitored in *S. aureus* LAC during planktonic growth in TSB by using a TECAN fluorescent plate reader (black line indicates the OD_{600}; green reflects GFP fluorescence; red line indicates DsRed fluorescence) (A); at 4, 8, and 12 h in a flow cell biofilm grown in 50% TSB in a Bio-Flux system (B); or in a 5-day static biofilm grown in RPMI 1640 plus 1% Casamino Acids and examined using confocal microscopy (C). (D) Expression levels of *dacA* and *gdpP* in static biofilms grown for 1 to 6 days were analyzed using qRT-PCR. No significant changes in *dacA* expression were observed across the time points based on a one-way ANOVA with Tukey *post hoc* analysis. Results are representative of at least two independent experiments. ND, not detected.

biofilm development, whereas *gdpP* was below the limit of detection.

Cyclic di-AMP is released extracellularly from *S. aureus* biofilm. To compare *dacA/gdpP* expression patterns with c-di-AMP production by *S. aureus* biofilms, quantitative analysis of intracellular and extracellular c-di-AMP levels from wild-type (WT) and isogenic *gdpP* mutant (Δ*gdpP*) static biofilms was performed using ultrahigh-performance LC–tandem MS (UPLC-MS/MS) and results were normalized for viable bacteria (see Fig. S1A in the supplemental material). A *dacA* mutant could not be examined, since this is an essential gene in *S. aureus* (45). As shown in Fig. 2A, 59.1% ± 8.9% (mean ± standard deviation) of total c-di-AMP detected was extracellular following 2 days of static biofilm growth. Despite the inability to detect significant *gdpP* expression during static biofilm growth using the reporter-fusion plasmid or qRT-PCR (Fig. 1C and D), the Δ*gdpP* strain resulted in significantly more intracellular and extracellular c-di-AMP during static biofilm growth (~5-fold increase). These results are consistent with previous findings assessing planktonic *S. aureus* (12). Interestingly, a similar percentage of total c-di-AMP (48.8% ± 12.9%) was extracellular in Δ*gdpP* biofilm-conditioned medium after 2 days of growth. These phenotypes continued through days 4 and 6, with ~30 to 40% of total c-di-AMP detected extracellularly in biofilm-conditioned medium (Fig. 2B and C).

In silico analysis identified three proteins in the *S. aureus* USA300 reference strain FPR3757 that share homology with the *L. monocytogenes* MdrM and MdrT c-di-AMP secretion proteins (Table 1 and data not shown). A triple mutant of all three homologues (CMG8) revealed minor overall changes in extracellular c-di-AMP levels (see Fig. S2 in the supplemental material), suggesting that other mechanisms exist for c-di-AMP release from *S. aureus* biofilms.

Extracellular c-di-AMP induces a STING-dependent type I IFN response in macrophages. Cyclic di-AMP released from intracellular pathogens is sensed by STING within the host cytosol and induces a type I IFN response (26–34). A previous report described IFN-β production following c-di-AMP treatment *in vivo* and in human and murine dendritic cells *in vitro* (46); how-

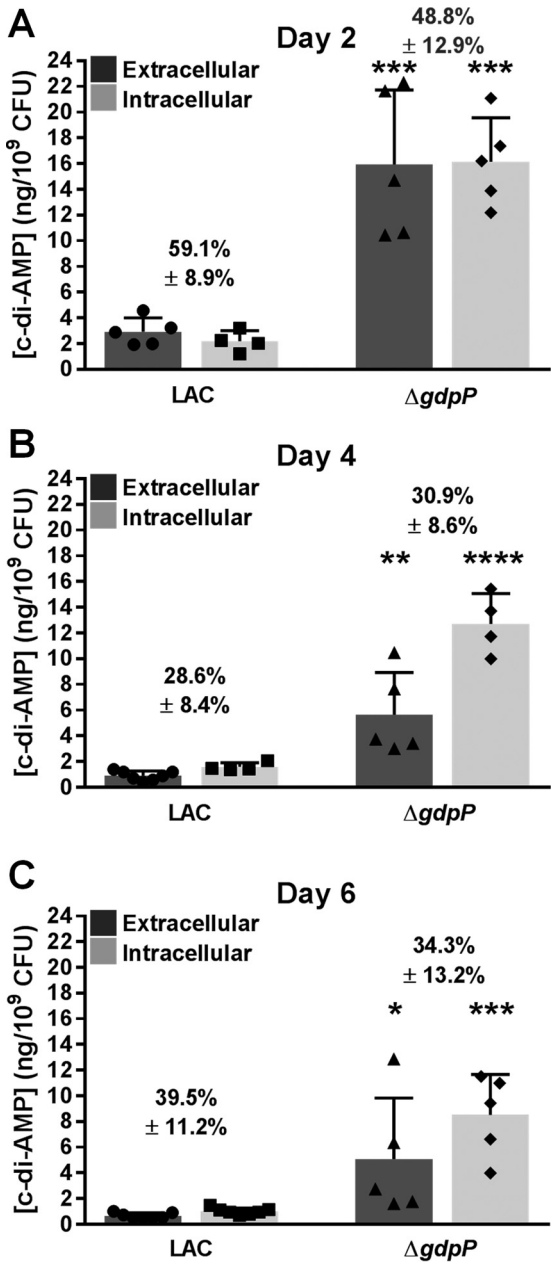

FIG 2 c-di-AMP is released from *S. aureus* biofilms. c-di-AMP levels were quantified in extracellular fractions (biofilm-conditioned medium) and intracellular fractions from wild-type *S. aureus* (LAC) and isogenic Δ*gdpP* static biofilms by using UPLC-MS/MS. Viable bacteria were quantified, and c-di-AMP values were plotted per 10^9 CFU for normalization. The percentage of total c-di-AMP detected in biofilm-conditioned medium after 2 (A), 4 (B), and 6 (C) days of growth are indicated. Error bars represent standard deviations of the average results from at least two independent experiments, with individual data points plotted. Results were analyzed using Student's *t* test to compare wild-type LAC and Δ*gdpP* fractions. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

ever, effects of extracellular c-di-AMP on macrophages have not yet been reported. To assess the impact of extracellular c-di-AMP on macrophage activation, murine primary BMDMs were treated with purified c-di-AMP (0.01 to 10 μM) for 3 or 6 h, whereupon RNA was isolated and analyzed for cytokine expression. Although all but one concentration of c-di-AMP examined was higher than those detected in biofilm-conditioned medium (see Fig. S1B in the supplemental material), the known heterogeneity in biofilms and the fact that *dacA* expression was localized to biofilm towers (Fig. 1B) suggest that macrophages may be exposed to higher local concentrations of c-di-AMP that are reminiscent of the levels examined here. The expression of 14 proinflammatory (i.e., IL-1β, IFN-β, and inducible nitric oxide synthase [iNOS]) and anti-inflammatory (i.e., IL-10, arginase) mediators was examined (data not shown), and the two most strongly induced genes were *ifn*-β (Fig. 3A) followed by *il-6* (Fig. 3C), which were selected for further analysis. Increases in gene expression correlated with elevated IFN-β and IL-6 protein secretion (Fig. 3B and D). The increase in both cytokines by c-di-AMP was dose dependent and showed early, yet sustained induction. Interestingly, no concentration of c-di-AMP tested induced *il-1*β, *ifn*-γ, or *inos* expression (data not shown).

To examine the mechanism of *ifn*-β and *il-6* induction by exogenous c-di-AMP, BMDMs from STING KO mice were examined. As shown in Fig. 3, the response to c-di-AMP was predominantly STING dependent at all concentrations tested, indicating that extracellular c-di-AMP is detected in the macrophage cytosol and induces a type I IFN response via a STING-mediated signaling process.

Cyclic di-AMP is released from *S. aureus* biofilm during cell lysis to induce STING-dependent *ifn*-β expression. To directly assess whether c-di-AMP released from *S. aureus* biofilm induces a type I IFN response, WT and STING KO BMDMs were cocultured for 3 h with mature (day 6) WT LAC and isogenic Δ*gdpP* static biofilms. As shown in Fig. 4A, WT BMDMs expressed significantly higher levels of *ifn*-β following coculture with the Δ*gdpP* strain compared to WT LAC biofilms. This increase was absent in STING KO BMDMs. To further assess the impact of c-di-AMP released from the biofilm, WT and STING KO BMDMs were treated for 3 h with biofilm-conditioned medium from mature (day 6) WT LAC and isogenic Δ*gdpP* static biofilms (Fig. 4B). Due to the rapid killing of BMDMs with undiluted biofilm-conditioned medium (data not shown), supernatants were diluted to examine effects on macrophage *ifn*-β induction. Conditioned medium from Δ*gdpP* biofilms induced significantly greater *ifn*-β expression than WT biofilms, reflecting similar trends to the concentrations of extracellular c-di-AMP released from the respective biofilms (see Fig. S1B in the supplemental material). This increase in *ifn*-β expression was absent following treatment of STING KO BMDMs, indicating that the excess c-di-AMP released from Δ*gdpP* biofilms triggered a STING-dependent type I IFN response. Importantly, there were no observable increases in *ifn*-β expression following treatment of BMDMs with planktonic Δ*gdpP* supernatant compared to WT LAC, indicating a mechanism of c-di-AMP release specific for biofilm growth (see Fig. S3 in the supplemental material).

As previously shown, an isogenic *S. aureus* mutant in which all three homologues of the *L. monocytogenes* c-di-AMP secretion proteins were interrupted had a minimal effect on extracellular c-di-AMP release (see Fig. S2 in the supplemental material), suggesting that alternative mechanisms were involved. To assess the possibility that c-di-AMP is passively released from *S. aureus* biofilms via cell lysis, mature biofilms were treated for 2 days with 50 μg/ml PAS. PAS has been shown to inhibit *S. aureus* autolysis without affecting cell viability (47, 48). PAS had no observable

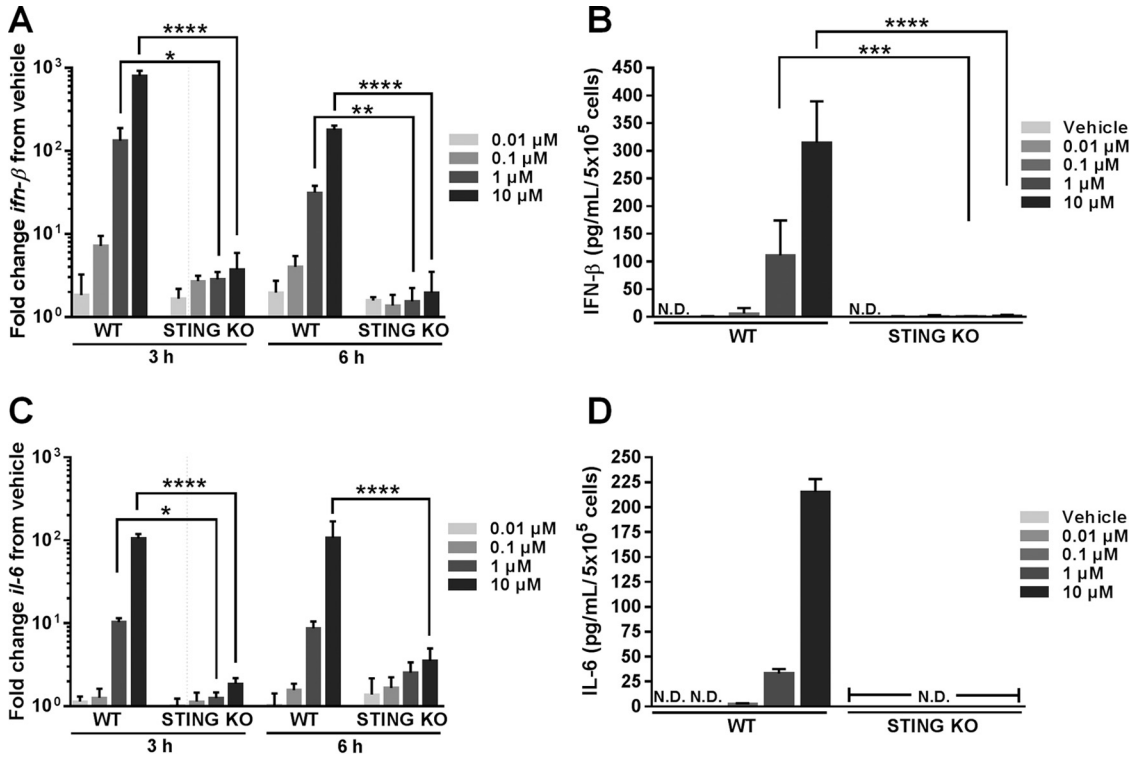

FIG 3 Extracellular c-di-AMP induces macrophage IFN-β and IL-6 production in a STING-dependent manner. BMDMs were treated with c-di-AMP (0.01 to 10 μM) for 3 or 6 h, whereupon RNA and supernatants were collected to quantify IFN-β and IL-6 mRNA (A and C) and protein (B and D) expression by qRT-PCR and ELISA, respectively. (A and C) Gene expression levels were normalized to the housekeeping gene GAPDH and are presented as the fold induction ($2^{-\Delta\Delta CT}$) value relative to that for untreated BMDMs (vehicle). (B and D) Cytokine levels were normalized to cell number and are expressed per milliliter of supernatant per 5×10^5 cells. Results depict averages from three independent experiments with standard deviations shown. Statistical analysis was performed using a one-way ANOVA with Tukey's *post hoc* analysis. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. ND, not detected.

effect on biofilm structure or viability (see Fig. S4 in the supplemental material), likely because treatment was initiated with established biofilms. PAS treatment of both WT and Δ*gdpP* biofilms significantly reduced *ifn*-β expression in both WT and STING KO BMDMs (Fig. 4B). Importantly, supplementation of PAS-treated biofilm-conditioned medium with 1 μM c-di-AMP restored *ifn*-β expression only in WT but not STING KO BMDMs (see Fig. S5 in the supplemental material). An *S. aureus gdpP* mutant was previously reported to have an altered rate of autolysis under planktonic conditions, which resulted from increased peptidoglycan cross-linking (12). To examine the impact of the *gdpP* mutation on autolysis in a mature (6-day) biofilm, WT LAC and Δ*gdpP* biofilms were subjected to a Triton X-100-induced autolysis assay. As shown in Fig. 4C, the *gdpP* mutation significantly increased the rate of cellular autolysis from a biofilm, suggesting that elevated extracellular c-di-AMP likely results from both the lack of GdpP hydrolysis and release due to cell lysis.

To further assess the contribution of extracellular c-di-AMP released from *S. aureus* biofilm on macrophage type I IFN expression, biofilm-conditioned medium from WT LAC and Δ*gdpP* was treated with SVP, an enzyme known to degrade c-di-AMP (26). SVP treatment of WT LAC biofilm-conditioned medium significantly reduced *ifn*-β induction in both WT and STING KO BMDMs compared to results with untreated supernatant (Fig. 4D). However, SVP treatment of Δ*gdpP* biofilm-conditioned medium did not affect *ifn*-β expression in STING KO BMDMs, further supporting a STING-dependent type I IFN response elicited by extracellular c-di-AMP. Together, these data provide direct evidence that biofilm-derived c-di-AMP is responsible for macrophage *ifn*-β induction and signals primarily via a STING-dependent pathway.

c-di-AMP promotes *S. aureus* intracellular survival in macrophages. To evaluate the functional and translational implications of c-di-AMP on macrophage-*S. aureus* interactions, the type I IFN response and intracellular survival of *S. aureus* were examined in human monocyte-derived macrophages (MDMs). Human MDMs were treated with exogenous c-di-AMP (0.01 to 10 μM), after which *ifn*-β expression was assessed. As shown in Fig. 5A, *ifn*-β was induced in a dose-dependent manner following c-di-AMP treatment. To determine whether extracellular c-di-AMP modulated macrophage functional activity, human MDMs were treated with purified c-di-AMP (0.01 to 1 μM) prior to live *S. aureus* exposure, whereupon intracellular survival was assessed in gentamicin protection assays. As shown in Fig. 5B, pretreatment with 0.1 μM c-di-AMP significantly promoted *S. aureus* intracellular survival, as revealed by elevated bacterial counts, which was less evident at a 10-fold higher concentration of c-di-AMP (1 μM). Currently, we cannot determine whether this results from increased *S. aureus* fitness in response to potential changes in the macrophage intracellular milieu following c-di-AMP exposure or from reduced microbicidal activity of c-di-AMP-treated macrophages. Collectively, these data indicate that c-di-AMP-induced signaling pathways bias macrophages toward a nonproductive phenotype, reflected by impaired *S. aureus* clearance.

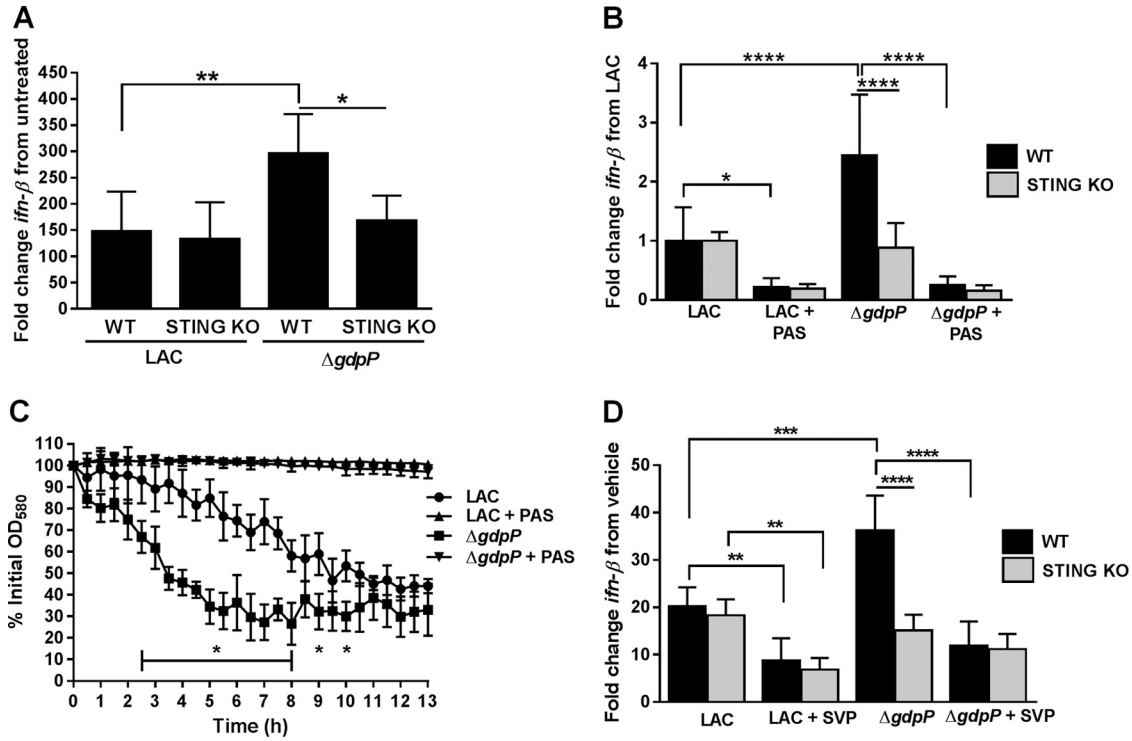

FIG 4 *S. aureus* biofilms release c-di-AMP via cell lysis to induce STING-dependent macrophage type I IFN production. (A) WT and STING KO BMDMs were cocultured for 3 h with mature (6-day) WT LAC or Δ*gdpP* static biofilms, whereupon RNA was isolated to assess changes in *ifn*-β expression by qRT-PCR. (B) Expression of *ifn*-β in WT and STING KO BMDMs exposed for 3 h to biofilm-conditioned medium collected from WT LAC or Δ*gdpP* biofilms, where lysis was inhibited with PAS. (C) Autolytic rates of cells harvested from WT LAC and isogenic Δ*gdpP* static biofilms with and without PAS treatment. (D) WT and STING KO BMDMs were exposed for 3 h to LAC or Δ*gdpP* biofilm-conditioned medium treated with SVP to degrade c-di-AMP. For qRT-PCR (A, B, and D), *ifn*-β expression was normalized to expression of the housekeeping gene *gapdh* and is presented as the fold induction ($2^{-\Delta\Delta CT}$) value relative to untreated BMDMs (A and D) or wild-type LAC biofilm-conditioned medium (B). Results depict averages from three independent experiments with standard deviations shown. Statistical analysis was performed using a one-way ANOVA with Tukey's *post hoc* analysis. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

DISCUSSION

Since its discovery in 2008 (24), c-di-AMP has been reported to function in a variety of essential processes in various bacterial species (49, 50). However, the role of c-di-AMP in the major human pathogen *S. aureus* has been limited to mediating ion homeostasis by regulating channel activity (11, 36, 37). In this study, we investigated an extracellular role for c-di-AMP during *S. aureus* biofilm growth and regulation of macrophage activation and functional activity. Biofilms represent an interesting paradigm in the bacterial lifestyle, since as a community of cells organized within a self-produced matrix, their gene expression patterns, metabolism, and fate are dramatically altered from those for planktonic cells (51). In contrast to planktonic cells, the immune response to *S. aureus* biofilm is suppressive, as reflected by macrophage polarization toward an anti-inflammatory state and MDSC recruitment (3, 7). To better understand how *S. aureus* biofilms are able to skew host immune responses and promote their own survival, we sought to identify molecules that alter macrophage signaling.

c-di-AMP secretion during intracellular bacterial infections is known to induce a robust type I IFN response (26, 27). These reports have established the importance of c-di-AMP in bacterial signaling and innate immune sensing of intracellular pathogens; however, a role for exogenous c-di-AMP produced by extracellular bacteria has not yet been examined. It has been proposed that c-di-AMP production is triggered during metabolic switches involved in biofilm formation (52). Indeed, we found rapid induction of *dacA* but minimal *gdpP* expression during the post-exponential and early stationary phases of planktonic growth that extended to biofilm formation, where *dacA* was concentrated within biofilm tower structures formed within a flow cell and ubiquitously throughout a static biofilm. These data defining the spatial and temporal expression of *dacA* and *gdpP* suggest that c-di-AMP production is induced as *S. aureus* cultures reach high cell densities, such as those found within biofilms, and that c-di-AMP synthesis is lower in rapidly growing, planktonic cells and maximal in post-exponential-phase cells. While it is tempting to speculate that c-di-AMP may be acting as a quorum-sensing molecule, the addition of exogenous c-di-AMP to *S. aureus* planktonic or biofilm cultures had no effect on *dacA* and *gdpP* expression (data not shown).

To understand the relationship between *dacA* and *gdpP* expression on c-di-AMP production and release, intracellular and extracellular c-di-AMP levels from *S. aureus* static biofilms were quantified using UPLC-MS/MS. These data demonstrated that 30 to 50% of the total c-di-AMP produced by *S. aureus* biofilms was extracellular. Surprisingly, despite the small amount of *gdpP* promoter activity detected, Δ*gdpP* biofilms displayed a 5-fold increase in total c-di-AMP; however, no difference in the percentage of extracellular c-di-AMP was observed. This appears to be due to a

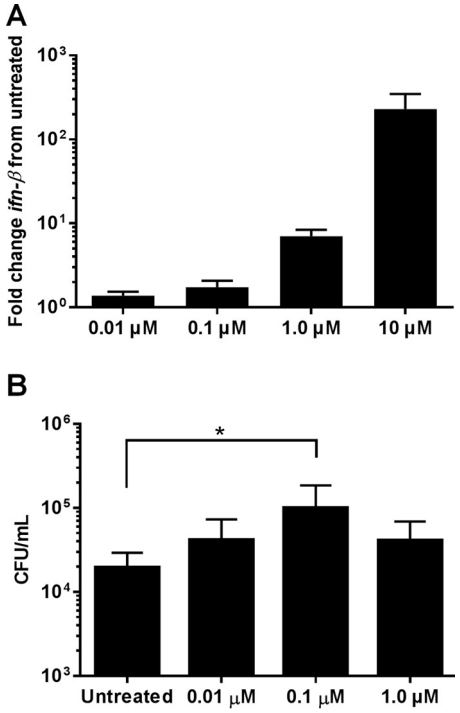

FIG 5 c-di-AMP induces *ifn*-β expression and promotes *S. aureus* intracellular survival in human MDMs. (A) Human MDMs were treated with c-di-AMP (0.01 to 10 μM) for 3 h, whereupon RNA was isolated and analyzed for changes in *ifn*-β expression by qRT-PCR. Gene expression levels were normalized to the housekeeping gene *gapdh* and are presented as the fold induction ($2^{-\Delta\Delta CT}$) value relative to untreated MDMs. (B) MDMs were pretreated with c-di-AMP (0.01 to 1.0 μM) for 1 h, whereupon cells were incubated with live *S. aureus* at an MOI of 5 for 2 h and subjected to a gentamicin protection assay, in which MDMs were lysed 24 h later to quantify intracellular bacteria. Statistical analysis was performed using a one-way ANOVA method with Tukey *post hoc* analysis. *, $P < 0.05$.

combination of highly efficient c-di-AMP hydrolysis by GdpP and an increased rate of autolysis by the Δ*gdpP* strain. Mutations in all three *S. aureus* MdrM orthologues revealed minor overall changes in extracellular c-di-AMP, and while these data do not exclude the possibility for c-di-AMP secretion, they do suggest other mechanisms exist for c-di-AMP release. To this end, we identified a novel mode of c-di-AMP release via cell autolysis, revealed by the finding that macrophage IFN-β induction was attenuated when biofilm autolysis was inhibited by PAS and the phenotype could be complemented with the addition of exogenous c-di-AMP.

c-di-AMP secreted by intracellular bacterial pathogens is sensed by the endoplasmic reticulum-associated protein STING (28), a potent inducer of type I IFN signaling (26), via phosphorylation of the transcription factor interferon response factor 3 (IRF3) (53). Prior to this study, it was unknown whether extracellular c-di-AMP is sensed and what effect(s) it may have on macrophage function. Many of the transcription factor activation pathways that induce anti-inflammatory cytokine production are regulated by type I IFN signaling (54). Secreted type I IFNs (IFN-α and -β) bind to the same receptor (IFNAR) in an autocrine and paracrine manner, triggering Janus kinase (JAK)/tyrosine kinase (TYK)-mediated phosphorylation of signal transducer and activator of transcription (STAT) proteins. STATs then translocate into the nucleus and promote the expression of many cytokines, including IFN-α and IFN-β, forming a positive feedback loop. Anti-inflammatory pathways of type I IFN signaling result from STAT3 phosphorylation (55) and can drive an alternatively activated macrophage phenotype and MDSC development, both of which are immune hallmarks of *S. aureus* biofilm-mediated infections (8, 56). It is currently unknown what STAT pathways are elicited in response to *S. aureus* biofilms, although the anti-inflammatory profile of infiltrating macrophages and MDSCs suggests that STAT3 may play a role (3, 6–8). IFN-β protein expression was significantly elevated in our *S. aureus* orthopedic implant biofilm infection model at days 7 to 14 compared to animals receiving sterile implants (data not shown). Our preliminary studies suggest that IFN receptor (IFNAR)-deficient mice do not display drastic changes in biofilm burdens or leukocyte infiltrates during early infection; however, the impact of IFNAR signaling may be more evident during later disease, which remains to be determined. Alternatively, due to the complexity of host immune responses during biofilm formation, it is possible that multiple pathways involved in type I IFN production exert redundant effects that would necessitate the generation of double/triple mutant mice to discern overt phenotypes (i.e., IFNAR/STING or IFNAR/SOCS KO).

To assess the effects of exogenous c-di-AMP on macrophage activation, cytokine expression was first evaluated in BMDMs treated with purified c-di-AMP. The gene most strongly induced was *ifn*-β, followed by *il-6*. Importantly, this response was STING dependent, which represents the first evidence linking extracellular c-di-AMP to STING in macrophages. IL-6 can also regulate anti-inflammatory pathways (57) and MDSC expansion (58), as cells expressing the IL-6 receptor (macrophages, MDSCs) respond to IL-6 by STAT3 activation (57, 59). The ability of c-di-AMP to promote *S. aureus* intracellular survival further indicates that extracellular c-di-AMP biases macrophages toward an anti-inflammatory state. These data are the first to demonstrate that macrophages are able to internalize c-di-AMP, where it can be sensed by STING; however, the mechanism of c-di-AMP uptake remains unknown. Pretreatment of BMDMs with cytochalasin D, a drug which depolymerizes actin filaments and inhibits endocytosis (60), had no effect on c-di-AMP-mediated *ifn*-β expression (data not shown). Additionally, analysis of MyD88 KO BMDMs revealed that this major Toll-like/IL-1 receptor adaptor protein also plays no role in the type I IFN response to c-di-AMP (data not shown). Other possibilities are that c-di-AMP enters the cell either through fluid-phase pinocytosis or protein-mediated membrane transport, which remain to be investigated. Another interesting concept is whether other PRRs are being activated concurrently with STING to modulate macrophage type I IFN production in response to *S. aureus* biofilms. One possibility is NOD2, which recognizes muramyl dipeptides following PGN degradation (61, 62) and could account for the minor STING-independent induction of type I IFN that was observed in our study, although this remains speculative. The failure of STING KO macrophages to augment type I IFN production in response to *gdpP* mutant biofilms coupled with the finding that SVP treatment of biofilm-conditioned medium is capable of inhibiting macrophage *ifn*-β expression implies a dominant action of c-di-AMP; however, a contribution from the cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS) in detecting extracellular DNA present in biofilm-conditioned medium cannot be excluded (63).

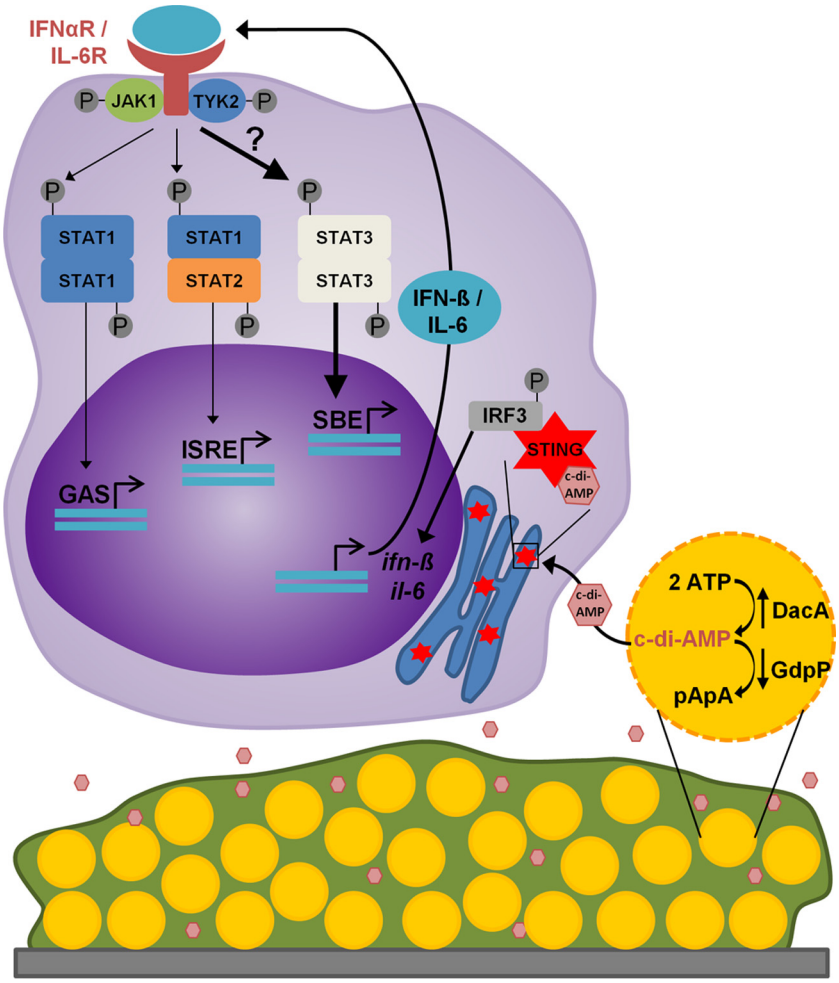

FIG 6 Model for the *S. aureus* biofilm c-di-AMP-induced macrophage type I IFN response. Increased expression of *dacA*, the enzyme responsible for c-di-AMP synthesis, relative to minimal expression of the phosphodiesterase *gdpP*, suggests that c-di-AMP production is favored during *S. aureus* biofilm growth. Furthermore, autolytic events occurring during biofilm growth allow for c-di-AMP release from *S. aureus*. STING-mediated sensing of c-di-AMP by surrounding macrophages induces a type I IFN response, including the expression of IL-6. Upon secretion, type I IFNs and IL-6 bind their cognate receptors in an autocrine and paracrine manner. It is proposed that type I IFN and IL-6 signaling in macrophages induces a STAT3-mediated anti-inflammatory response, ultimately contributing to biofilm persistence. GAS, gamma interferon activation site; ISRE, IFN-stimulated response element; SBE, STAT-binding element.

While the anti-inflammatory response to *S. aureus* biofilms appears to be driven by biofilm products, the effectors and their mechanism(s) of action remain ill-defined. These data provide evidence for the ability of *S. aureus* biofilms to induce macrophage type I IFN production via extracellular c-di-AMP (Fig. 6). Lending support to this hypothesis, increased c-di-AMP levels observed in Δ*gdpP* biofilms corresponded with increased *ifn*-β expression in BMDMs and was STING dependent. Furthermore, inhibiting biofilm autolysis dampened the *ifn*-β response to levels similar to those in untreated macrophages, revealing that c-di-AMP is released passively during cell lysis. Remarkably, despite the multitude of toxins and other bacterial antigens released from *S. aureus* biofilms that can cause macrophage dysfunction (5), the macrophage type I IFN response has high specificity to extracellular c-di-AMP. Taken together, these data identify a novel extracellular role for *S. aureus* c-di-AMP in polarizing macrophages toward an anti-inflammatory state.

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