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Targeting Macrophage Activation for the Prevention and Treatment of *Staphylococcus aureus* Biofilm Infections

Mark L. Hanke,*1 Cortney E. Heim,*1 Amanda Angle,* Sam D. Sanderson,† and Tammy Kielian*

Biofilm infections often lead to significant morbidity due to their chronicity and recalcitrance to antibiotics. We have demonstrated that methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms can evade macrophage (MΦ) antibacterial effector mechanisms by skewing MΦs toward an alternatively activated M2 phenotype. To overcome this immune evasion, we have used two complementary approaches. In the first, a proinflammatory milieu was elicited by local administration of classically activated M1 MΦs and in the second by treatment with the C5a receptor (CD88) agonist EP67, which invokes MΦ proinflammatory activity. Early administration of M1-activated MΦs or EP67 significantly attenuated biofilm formation in a mouse model of MRSA catheter-associated infection. Several proinflammatory mediators were significantly elevated in biofilm-infected tissues from MΦ- and EP67-treated animals, revealing effective reprogramming of the biofilm environment to a proinflammatory milieu. A requirement for MΦ proinflammatory activity was demonstrated by the fact that transfer of MyD88-deficient MΦs had minimal impact on biofilm growth. Likewise, neutrophil administration had no effect on biofilm formation. Treatment of established biofilm infections with M1-activated MΦs also significantly reduced catheter-associated biofilm burdens compared with antibiotic treatment. Collectively, these results demonstrate that targeting MΦ proinflammatory activity can overcome the local immune inhibitory environment created during biofilm infections and represents a novel therapeutic strategy. *The Journal of Immunology*, 2013, 190: 2159–2168.

Biofilms are heterogeneous bacterial communities that can form on both natural body surfaces as well as foreign devices, such as indwelling catheters and orthopedic implants (1, 2). The presence of a foreign body increases the likelihood of infection and drastically lowers the threshold for device colonization (3). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a common etiologic agent of biofilms and often causes chronic and recurrent infections when associated with indwelling medical devices (4–6). The current therapeutic option for managing device-associated biofilm infections is a staged replacement of the hardware, either as a single-step exchange, whereby the entire implant is replaced in a single procedure, or, more commonly, as a two-stage exchange (7). In the latter case, patients receive extended antibiotic regimens in addition to surgical management, which generally consists of device removal and replacement with an antibiotic-impregnated temporary spacer, followed by insertion of a new prosthesis after a 2–8-wk period.

This long and debilitating process is associated with significant morbidity and economic impact for patients. Further complicating available treatment strategies is that antibiotics alone are generally ineffective for biofilm eradication (8, 9), which is thought to result from altered metabolism during biofilm growth (10, 11). The difficulties of biofilm treatment are further underscored in the context of more permanent implants such as artificial hips and knees, procedures that are particularly common in the elderly, who grow increasingly less immune responsive over time (12).

Based on these challenges, an urgent need exists for developing novel paradigms to prevent and/or facilitate biofilm eradication without the need for radical surgical interventions. One promising approach involves the exploitation of natural host immune mechanisms for therapeutic benefit. Targeting the host response rather than the pathogen itself offers certain advantages by largely avoiding selective pressures for the evolution of microbial resistance. Indeed, stimulating adaptive immunity through vaccination has remained resilient to microbial resistance over decades of clinical use, although not all pathogens have been amenable to vaccination strategies, most notably *S. aureus* (13, 14). Furthermore, the fact that innate immune defenses are geared to rapidly recognize an infinite pathogen repertoire, suggests that their modulation will afford broad-spectrum protection against a range of microbial pathogens, including *S. aureus*, and enable prophylactic use in high-risk groups or provide early treatment options prior to the identification of the causative infectious agent(s).

Earlier views regarding the host immune response to biofilm infections suggested that biofilms evaded immune recognition altogether (15, 16). Recent reports by our group and others (17, 18) have proposed an alternative possibility, namely that biofilms can skew the immune response to favor anti-inflammatory and profibrotic pathways, which contribute to biofilm persistence. Specifically, although macrophages (MΦs) are a prominent infiltrate in *S. aureus* biofilm infections, their penetration into the
biofilm itself is impeded by a robust fibrotic response surrounding the infection. In addition, biofilm-associated MΦs are polarized toward an alternatively activated M2 phenotype that possess anti-inflammatory and profibrotic properties that limit bacterial clearance (17). By extension, the programming of MΦs toward a microbical M1 response is diverted, which led us to examine whether the exogenous administration of M1-activated MΦs directly into sites of biofilm infection would overcome the local immune inhibitory environment and fibrotic barrier associated with biofilms. As a complementary approach to augment MΦ proinflammatory activity, we administered the MΦ-activating peptide EP67 to facilitate bacterial clearance by inducing a proinflammatory milieu. EP67 (Tyr-Ser-Phe-Lys-Asp-Met-Pro-Leu-Methyl-L-Leu)-d-Ala-Arg, or YSFKDMPE(MeL)aR), is a response-selective agonist of the human C5a receptor (C5aR/CD88) that preferentially elicits proinflammatory mediator production from MΦs without any effects on C5aR+ neutrophils (19, 20). In this study, we demonstrate that targeting MΦ activity with EP67 or the introduction of exogenous M1-activated MΦs inhibits S. aureus biofilm formation and provides a novel therapeutic treatment for these persistent infections. This therapeutic approach is not afforded by neutrophils, which agrees with our findings that neutrophils are not a prominent infiltrate in this model of S. aureus biofilm infection (21). Collectively, these findings suggest that immune-cell-based therapy using M1-activated MΦs may overcome the current confounds associated with biofilm treatment and control.

Materials and Methods

Mice

Male C57BL/6 mice (8–10 wk old) were obtained from Charles River Laboratories (Frederick, MD), and MyD88 knockout (KO) animals (originally from Dr. S. Akira, Osaka University, Suita, Osaka, Japan) have been backcrossed with C57BL/6 mice for >10 generations (22–24). Mice were housed in restricted-access rooms equipped with ventilated microisolator cages and maintained at 21°C under a 12-h light/12-h dark cycle with ad libitum access to water (Hydropac; Lab Products, Seaford, DE) and Teklad rodent chow (Harlan, Indianapolis, IN). This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

S. aureus strains

For in vitro biofilms, USA300 LAC was transformed with the plasmid pCM1 to express GFP driven by the sarA P1 promoter (USA300 LAC-GFP), and plasmid expression was maintained with erm selection (10 μg/ml) (17, 25). For in vivo biofilm infections, a bioluminescent USA300 LAC::lux strain was used, as previously described (17, 26, 27).

In vitro S. aureus biofilms

Static biofilms were generated as previously described (17). Briefly, sterile two-well glass-chamber slides (Nunc, Rochester, NY) were treated with 20% human plasma in sterile carbonate-bicarbonate buffer (Sigma-Aldrich, St. Louis, MO) overnight at 4°C to facilitate bacterial attachment (26). Overnight cultures of USA300 LAC or USA300 LAC-GFP were prepared in MΦ culture medium (RPMI-1640, 10% FBS) supplemented with 10 μg/ml erm for the GFP strain. The following day, plasma-coating buffer was removed, and chambers were inoculated with bacteria (diluted to an OD600 of 0.050 in 2 ml) and incubated at 37°C under static aerobic conditions for 6 d. Medium was carefully replenished every 24 h to prevent disruption of the biofilm structure.

MΦ or neutrophil coculture with S. aureus biofilms in vitro

USA300 LAC-GFP static biofilms and bone marrow–derived MΦs (BMDMs) from either wild-type (WT) or MyD88 KO mice were prepared as previously described (28, 29). Neutrophils were recovered from the bone marrow using a three-layer Percoll gradient of 78, 69, and 52% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) as previously described (30). Neutrophils were further enriched using a Miltenyi Anti-Ly-6G MicroBead Kit (Miltenyi Biotec, Cambridge, MA) according to the manufacturer’s instructions. BMDMs and neutrophils were labeled with either 5 μM CellTracker Orange or CellTracker Blue (both from Molecular Probes, San Diego, CA) depending on the experimental setup. Cultures of S. aureus biofilms with 106 neutrophils, nonactivated MΦs, or M1-activated MΦs (pretreated with 10 ng/ml IFN-γ or 100 ng/ml TNF-α and 10 μg/ml S. aureus–derived peptidoglycan [PGN] for 6 h) were incubated at 37°C under static aerobic conditions and imaged using a Zeiss 510 META laser scanning microscope (Carl Zeiss, Oberkochen, Germany) at 2 and 24 h following the addition of immune cells. Neutrophil- and MΦ-biofilm cocultures were harvested 24 h after immune cell addition by mechanic disruption followed by sonication, whereupon bacterial enumeration was performed by serial dilution on tryptic soy agar plates supplemented with 5% sheep blood (Hemostat Laboratories, Dixon, CA).

Mouse model of S. aureus catheter-associated biofilm infection

S. aureus biofilm infections were performed as previously described (17, 26, 27). Briefly, a small s.c. incision was made in the flank under avertin anesthesia, and a blunt probe was used to create a pocket for insertion of a sterile 14-gauge teflon i.v. catheter 1 cm in length (Exel International, St. Petersburg, FL). The incision was sealed using Vetbond Tissue Adhesive (3M, St. Paul, MN), and 105 CFU USA300 LAC::lux in 20 μl sterile PBS was slowly injected through the skin into the catheter lumen. The health status of mice was regularly monitored throughout the course of infection, and any moribund animals were immediately euthanized.

Immune cell administration into biofilm infections in vivo

Mice received injections of 106 neutrophils, nonactivated MΦs, or M1-activated WT or MyD88 KO MΦs (10 ng/ml IFN-γ plus 10 μg/ml S. aureus–derived PGN) beginning at 12 h, with repeat administrations at 24 and 48 h postinfection (i.e., early treatment). Cells were introduced at four distinct sites surrounding infected catheters to ensure maximal immune cell access to the developing biofilm. A separate cohort of animals received M1-activated MΦs until 48 h postinfection, whereupon no additional cells were administered to determine the longevity of early MΦ treatment on inhibiting biofilm growth at day 14 postinfection. In some experiments, the numbers of injected neutrophils were increased by 1-log (i.e., 106) to confirm their inability to impact biofilm clearance. For established biofilms, mice were infected with 107 CFU USA300 LAC in the lumen of surgically implanted catheters, and on days 7 and 9 postinfection, animals received vehicle (PBS), 105 nonactivated MΦs, 106 M1-activated MΦs, or antibiotic (rifampicin and daptomycin; 0.125 and 0.25 mg/kg, respectively).

To determine the longevity of M1-activated MΦs following transfer into biofilms in vivo, mice received a single injection of 106 M1-activated MΦs loaded with near-infrared Quantum dots (Qtracker 800; Molecular Probes) at either 24 h or 7 d postinfection for the early and established biofilm paradigms, respectively. Mice were monitored daily using an in vivo imaging system (IVIS Spectrum; Caliper Life Sciences, Hopkinton, MA).

EP67 synthesis and treatment

EP67 (YSFKDMP(MeL)aR) and its inactive scrambled sequence [sEP67; (MeL)RMYKPRa FDS] were generated by a solid-phase Fmoc method of orthogonal purification, purified by analytical and preparative reverse-phase HPLC, and characterized by electrospray mass spectrometry according to previously published methods (31). Although EP67 was derived from the C-terminal portion of human C5a, it is a well-characterized agonist for the mouse C5aR (CD88), and the peptide is devoid of activity in CDB88 KO mice (20). Animals were treated with either 200 μg EP67 or the inactive scrambled derivative (sEP67) directly into the catheter at the time of S. aureus infection followed by 300 μg peptide distributed equally at four different sites surrounding the catheter at 24 and 48 h postinfection. To limit animal numbers, sEP67 was not used in all experiments, because our initial studies established that this control peptide did not exert any biological activity.

Recovery of catheters and surrounding tissues for S. aureus enumeration

Mice were euthanized at the indicated time points following infection, whereupon catheters were removed and sonicated in 1 ml PBS to dissociate bacteria from the catheter surface. Tissues surrounding infected catheters were also collected, weighed, and disrupted in 500 μl homogenization buffer (PBS supplemented with 100 μl RNasin and a protease inhibitor tablet [Roche Diagnostics, Indianapolis, IN]) using a Bullet Blender (Next Advance, Averill Park, NY). Bacterial titers associated with catheters and
surrounding tissues were enumerated using tryptic soy agar plates supplemented with 5% sheep blood and are expressed as Log_{10} CFU per milliliter for catheters or Log_{10} CFU per gram wet tissue weight.

**MILLIPLEX multianalyte bead array**
To evaluate the ability of M1-activated MoFs or EP67 to alter the inflammatory milieu associated with MRSA biofilm infections, a custom-designed mouse microbead array was used according to the manufacturer’s instructions (MILLIPLEX; Millipore, Billerica, MA), which detects the following inflammatory mediators: IL-1α, IL-1β, TNF-α, IFN-γ, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-15, IL-17, CXCL1, CXCL2, CXCL9, CXCL10, CCL2, CCL3, CCL4, and CCL5. Results were analyzed using a Bio-Plex workstaton (Bio-Rad, Hercules, CA) and normalized to the amount of total protein recovered to correct for differences in tissue sampling size.

**Flow cytometry**
BMDMs from C57BL/6 mice were activated with 10 ng/ml IFN-γ plus 10 μg/ml PGN for 6 h to generate M1-activated MoFs or incubated with medium alone. At the end of the 6-h treatment period, cells were evaluated for surface marker expression (MHC class II-PE and CD86-AF700; both from BD Biosciences) and production of mitochondrial and total cellular CXCL9, CXCL10, CCL2, CCL3, CCL4, and CCL5. Results were analyzed using a Bio-Plex workstation (Bio-Rad, Hercules, CA) and normalized to the amount of total protein recovered to correct for differences in tissue sampling size.

**Immunofluorescence staining and confocal microscopy**
Tissues surrounding infected catheters were fixed in 10% formalin and embedded in paraffin, whereupon 10-μm thick sections were deparaffinized in xylene and a graded series of alcohols followed by Ag retrieval as previously described (17). Sections were processed for immunofluorescence staining using primary Abs specific for the MΦ markers Iba-1 (Biocare Medical, Concord, CA) and arginase-1 (Santa Cruz Biotechnol., San Diego, CA), followed by donkey anti-rabbit FITC or biotinylated secondary Abs (Jackson ImmunoResearch Laboratories, West Grove, PA) and a streptavidin-594 conjugate for the latter (Invitrogen, Carlsbad, CA). Confocal imaging was performed using a Zeiss 510 META laser scanning microscope (Carl Zeiss) with staining specificity confirmed by incubating tissues with a primary isotype-matched control Ab and appropriate secondary Ab. Importantly, control-stained tissues consistently revealed no background signals, indicating that S. aureus protein A was not influencing Ab staining. Quantification of arginase-1 or Iba-1 fluorescence was calculated from at least 10 random fields of view using Axiovision software 4.8 (Carl Zeiss).

**Statistical analysis**
Significant differences between experimental groups were determined using an unpaired two-tailed Student t test, a one-way ANOVA with Bonferroni’s multiple comparison post hoc analysis, or a Kruskal-Wallis one-way ANOVA with Dunn’s multiple comparison post hoc analysis in GraphPad Prism 4 (La Jolla, CA). For all analyses, a p value <0.05 was considered statistically significant.

**Results**

**Activated MoFs exhibit S. aureus biofilm bactericidal activity in vitro**
We have previously demonstrated that MRSA biofilms are capable of attenuating traditional proinflammatory responses explaining, in part, why these infections persist in an immunocompetent host (17). To determine whether MoFs that were preprogrammed toward a proinflammatory M1 phenotype were capable of overcoming the immune inhibitory aspects of biofilms, MoFs were stimulated with IFN-γ or TNF-α and S. aureus–derived PGN for 6 h prior to their addition to S. aureus–GFP biofilms or planktonic cultures. Several attributes characteristic of M1-activated MoFs were detected using this treatment paradigm, including significant increases in CD86 and reactive oxygen species production (Supplemental Fig. 1). All MoF populations were capable of phagocytosing planktonic bacteria regardless of their activation state (Fig. 1A, left panel, Planktonic), whereas only M1-activated MoFs stimulated with either IFN-γ or TNF-α plus PGN were capable of phagocytosing biofilm-associated organisms (Fig. 1A, right panel, Biofilm), which resulted in significant reductions in bacterial burdens following a 24-h coculture period (Fig. 1B). In contrast, nonactivated MoFs displayed no indication of intracellular bacteria when incubated with biofilms, confirming our earlier report (17), but were still able to decrease biofilm bacterial burdens (Fig. 1B). The ability of nonactivated MoFs to reduce biofilm burdens without any evidence of phagocytic activity suggests that antimicrobial mediator(s) are secreted upon contact with either organisms dispersed from the biofilm and/or bacterial components shed during biofilm growth. The ability of M1-activated MoFs to reduce biofilm burdens required MyD88-dependent signals, because MyD88 KO MoFs treated with IFN-γ and PGN had no impact on biofilm growth (Fig. 1B).

To compare the efficacy of MoFs versus neutrophils in regulating MRSA biofilm growth, neutrophils were isolated from murine bone marrow and cocultured with biofilms. Unlike MoFs, neutrophils were not preactivated prior to biofilm addition, because this would lead to rapid degranulation and reduced cell viability. Interestingly, neutrophils were able to phagocytose MRSA biofilms, yet this did not translate into reduced bacterial numbers (Supplemental Fig. 2), revealing disconnect between the two processes. This may result from additional virulence determinants released by S. aureus during biofilm growth, because the organism is known to produce numerous factors that interfere with neutrophil function (32–34). Alternatively, S. aureus can survive inside neutrophils, which could explain why phagocytosis was observed without concomitant reductions in bacterial burdens (35). On a comparative basis, biofilm formation did afford some protection against phagocytic uptake compared with planktonic growth conditions, because both MoFs and neutrophils actively phagocytosed planktonic S. aureus but were less capable of internalizing biofilm-associated bacteria (Fig. 1A, Supplemental Fig. 2).

**M1-activated MoFs limit MRSA biofilm formation in vivo**
Based on our in vitro studies demonstrating the ability of M1-polarized MoFs to phagocytose biofilm-associated S. aureus and reduce bacterial burdens, we next examined whether this would translate in vivo. These experiments used a mouse model of MRSA catheter-associated biofilm infection that we have previously shown limits MoΦ invasion into biofilms and skews these cells toward an alternatively activated M2 phenotype (17). We first employed an approach in which M1-activated MoFs were administered beginning at 12 h following MRSA infection, with repeat injections occurring at 24 and 48 h after bacterial exposure. The introduction of M1-activated MoFs directly into the biofilm infection site significantly reduced bacterial burdens on both infected catheters and in surrounding tissues at day 3 postinfection (Fig. 2A, 2B). More importantly, this early intervention with M1-activated MoFs led to long-term effectiveness against biofilm formation, because catheters showed minimal evidence of biofilm growth at day 14 without any additional MoΦ treatment, and although some bacteria were observed in the surrounding tissues, this was significantly reduced compared with vehicle treatment (Fig. 3A, 3B). Interestingly, the introduction of nonactivated MoFs also reduced biofilm burdens, although significant differences were only observed at day 14 postinfection (Figs. 2, 3). To better illustrate the superior efficacy of M1-activated compared with nonactivated MoFs, a dose–response experiment was performed in which animals were treated with increasing numbers (10^5, 10^6, or 10^7) of either nonactivated or M1-activated MoFs. Results from this experiment indicated that 10^5 or 10^6 M1-activated MoFs were...
capable of significantly reducing biofilm burdens compared with vehicle controls, whereas nonactivated MΦs were not statistically effective at any dose (Fig. 4). Similar to the in vitro studies, MyD88-dependent mechanisms were critical, because MΦs from MyD88 KO mice did not demonstrate any efficacy in controlling biofilm burdens on either infected catheters or surrounding tissues.

**FIGURE 1.** M1 MΦ polarization enhances phagocytosis and killing of S. aureus biofilms. (A) Nonactivated MΦs and M1-activated MΦs (10 ng/ml IFN-γ or 100 ng/ml TNF-α + 10 μg/ml PGN) from C57BL/6 mice, as well as MyD88 KO MΦs were labeled with CellTracker Blue (blue) and cocultured with S. aureus–GFP (green) during biofilm or planktonic growth for 2 h and imaged to observe their phagocytic ability (original magnification ×63). (B) After 24 h, biofilms were sonicated to quantitate bacterial burdens to evaluate the ability of the various MΦ populations to attenuate biofilm growth. Biofilms without MΦs were used as untreated controls. Results are representative of at least three independent experiments. White arrows indicate phagocytic cells, and significant differences are denoted by asterisks. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 2.** Activated MΦs, but not neutrophils, impair MRSA biofilm formation in vivo. C57BL/6 mice were infected with 10⁷ CFU of USA300 LAC in the lumen of surgically implanted catheters to establish biofilm infection. Animals were treated with vehicle, 10⁶ neutrophils (PMN), 10⁶ nonactivated MΦs, or 10⁶ M1-activated MΦs at 12, 24, and 48 h postinfection, whereupon catheters (A) and surrounding tissues (B) were collected at 72 h to quantitate bacterial burdens. Results are expressed as the number of CFU per milliliter for catheters or CFU per milligram tissue to correct for differences in tissue sampling size. Results are presented from individual animals combined from at least two independent experiments. Significant differences are denoted by asterisks (*p < 0.05).

**FIGURE 3.** Activated MΦs provide long-lasting defense from MRSA biofilm infections in vivo. C57BL/6 mice were infected with 10⁷ CFU of USA300 LAC in the lumen of surgically implanted catheters to establish biofilm infection. Animals were treated with vehicle, 10⁶ nonactivated, or 10⁶ M1-activated MΦs at 12, 24, and 48 h postinfection, whereupon catheters (A) and surrounding tissues (B) were recovered at day 14 to quantitate bacterial burdens. Results are expressed as the number of CFU per milliliter for catheters or CFU per milligram tissue to correct for differences in tissue sampling size. Significant differences in bacterial burdens between vehicle and MΦ-treated mice are denoted by asterisks (*p < 0.05).
in vivo (Fig. 5). In addition, neutrophils had no impact on biofilm formation, even when the number of cells was increased to $10^7$ per injection (Fig. 2 and data not shown), which confirmed our in vitro findings and the fact that neutrophils are not a significant infiltrate in the MRSA catheter-associated biofilm model used in this study (Supplemental Fig. 3).

Previous work from our laboratory demonstrated that MRSA biofilms attenuated the expression of numerous proinflammatory mediators compared with a sterile foreign body (17). The introduction of M1-activated, but not nonactivated, MΦs significantly increased CXCL9, CCL5, and IFN-γ expression within biofilm-infected tissues (Fig. 6) revealing the successful redirection toward a proinflammatory milieu. CXCL9 is an IFN-γ–induced T cell chemoattractant, whereas CCL5 recruits a broader array of leukocytes, including T cells, eosinophils, and basophils, although the influx of these target populations was not further examined in these studies following M1 MΦ treatment. The proinflammatory activity of M1-activated MΦs is likely a key mechanism responsible for limiting biofilm growth. Interestingly, no significant changes in IL-10 were detected following M1 MΦ transfer (Fig. 6D), which suggests that the broader balance of pro- versus anti-inflammatory factors may be a better predictor of inflammatory outcome compared with individual mediators.

**Introduction of M1-activated MΦs for the treatment of established MRSA biofilm infections**

Based on the efficacy of our M1 MΦ early treatment paradigm, we next examined whether this would extend to attenuate bacterial growth in established MRSA biofilm infections. We employed a similar strategy to the early treatment regimen for MΦ administration except that M1 MΦs were initially given at day 7 following *S. aureus* infection, a point at which robust biofilm has formed (17), with a repeat injection occurring at day 9. Similar to the early treatment paradigm, the introduction of M1-activated MΦs directly into the biofilm infection site led to significant reductions in bacterial burdens on infected catheters at day 10 postinfection, although no effect was seen in surrounding tissues (Fig. 7A, 7B, respectively). In contrast to M1 MΦ delivery, antibiotic treatment had no effect on biofilm formation (Fig. 7). As expected, Iba-1 immunofluorescence was significantly increased following the administration of both nonactivated and M1-activated MΦs compared with the endogenous MΦ population after introduction into established biofilms augmented CXCL9, CXCL2, IL-17, and IL-6 expression (Fig. 6D), which suggests that the broader balance of pro- versus anti-inflammatory factors may be a better predictor of inflammatory outcome compared with individual mediators.

FIGURE 4. M1-polarized MΦs display superior efficacy at impairing MRSA biofilm formation. C57BL/6 mice were infected with $10^7$ CFU of USA300 LAC in the lumen of surgically implanted catheters to establish biofilm infection. Animals were treated with either vehicle or increasing numbers of nonactivated or M1-activated MΦs at 12, 24, and 48 h postinfection, whereupon catheters (A) and surrounding tissues (B) were recovered at day 3 to quantitate bacterial burdens. Results are expressed as the number of CFU per milliliter for catheters or CFU per milligram tissue to correct for differences in tissue sampling size. Significant differences in bacterial burdens between vehicle and MΦ-treated mice are denoted by asterisks ($^*p < 0.05$).

**FIGURE 5.** The ability of M1-polarized MΦs to impair MRSA biofilm development is mediated by MyD88-dependent signals. C57BL/6 mice were infected with $10^3$ CFU of USA300 LAC in the lumen of surgically implanted catheters to establish biofilm infection. Animals were treated with vehicle or $10^6$ M1-activated MΦs derived from WT or MyD88 KO mice at 12, 24, and 48 h postinfection, whereupon catheters (A) and surrounding tissues (B) were recovered at day 3 to quantitate bacterial burdens. Results are expressed as the number of CFU per milliliter for catheters or CFU per milligram tissue to correct for differences in tissue sampling size. Results are presented from individual animals combined from at least two independent experiments. Significant differences are denoted by asterisks ($^*p < 0.05$).
still visible at 4 d postinjection (Fig. 9), which likely accounts for their ability to significantly limit MRSA biofilm formation. Although it is well-established that Qdots are retained in intact cells, it remains possible that they could be internalized by neighboring phagocytic cells if donor MΦs are dying in situ.

**EP67 attenuates MRSA biofilm formation in vivo and stimulates local proinflammatory responses**

As a complementary approach to the introduction of exogenous M1-activated MΦs, we next examined whether the CD88 agonist EP67 would reprogram the endogenous MΦ infiltrates associated with MRSA biofilms in vivo from an anti-inflammatory M2 to a proinflammatory M1 phenotype to facilitate bacterial clearance. Animals were initially treated with EP67 at the time of infection with additional injections occurring at 24 and 48 h. Bacterial burdens associated with biofilm-infected catheters as well as surrounding tissues were significantly decreased following EP67 treatment compared with animals receiving an inactive scrambled sequence of EP67 (sEP67) or vehicle control (Fig. 10). Early EP67 treatment was key to restricting MRSA biofilm establishment, because minimal bacterial growth was detected at day 14 following infection, even though the last dosing interval of EP67 occurred at 48 h (Fig. 10C, 10D).

To determine whether EP67 could skew the biofilm environment to a proinflammatory state, we evaluated cytokine and chemokine expression in biofilm-infected tissues. Several inflammatory mediators predominantly expressed by activated MΦs, such as IL-12p40 and RANTES, were significantly increased in EP67-compared with vehicle-treated animals (Fig. 11A, 11B). To further investigate mechanisms of EP67 action during biofilm infections, we compared the degree of MΦ influx into tissues surrounding MRSA biofilms using two complementary approaches. Immunofluorescence staining revealed that MΦ accumulation into EP67-treated biofilms was significantly increased at day 3 postinfection compared with vehicle (Fig. 12A). Importantly, although only a few MΦs were recruited to the biofilm surface in vehicle-treated mice, EP67 administration dramatically increased the numbers of MΦs that migrated into the biofilm (Fig. 12B). The ability of EP67 to augment MΦ infiltrates in MRSA biofilms was confirmed by FACS (Fig. 12C). Collectively, these findings demonstrate that EP67 induces a proinflammatory milieu by augmenting MΦ recruitment and cytokine/chemokine production, which effectively counteracts the anti-inflammatory environment elicited by MRSA biofilms. We also investigated whether EP67 treatment could impact established biofilms; however, the peptide did not exert any beneficial effects in this setting, suggesting its optimal use as a prophylactic modality under the conditions used in this study.

**FIGURE 6.** M1-activated MΦ therapy augments the local proinflammatory milieu during MRSA biofilm infection. Tissues surrounding *S. aureus* biofilms of vehicle, nonactivated MΦ (NA MΦ), and M1-activated MΦ-treated (A MΦ) mice were collected at days 3 (early treatment) or 10 (established biofilm treatment) postinfection and homogenized to quantitate CXCL9 (A, B), CCL5 (B), IFN-γ (C), IL-10 (D), IL-17 (F), CXCL2 (G), and IL-6 (H) expression by MILLIPLEX analysis (Millipore). Results were normalized to the amount of total protein recovered to correct for differences in tissue sampling size. Significant differences are denoted by asterisks (*p < 0.05) and are representative of five to eight mice per group. N.M., Not measured.

**FIGURE 7.** M1-activated MΦs attenuate established MRSA biofilm infection. C57BL/6 mice were infected with 10³ CFU of USA300 LAC in the lumen of surgically implanted catheters to establish biofilm infection. On days 7 and 9 postinfection, animals received injections of vehicle, antibiotic (rifampicin + daptomycin), 10⁶ nonactivated MΦs, or 10⁶ M1-activated MΦs, whereupon catheters (A) and surrounding tissues (B) were recovered at day 10 to quantitate bacterial burdens. Significant differences between groups are denoted by asterisks (*p < 0.05) and represent animals from two independent experiments.
Collectively, our results have identified a previously unappreciated role for signals provided by M1-activated MΦs in biofilm containment and bacterial clearance. By extension, it is not unexpected that MRSA biofilms have the capacity to thwart this response by skewing MΦs away from a proinflammatory M1 to an anti-inflammatory M2 phenotype, which ensures biofilm persistence in an immunocompetent host.

**Discussion**

*S. aureus* is a frequent etiological agent of biofilm infections on indwelling devices and orthopedic implants (9, 36), and recent reports by our group and others have demonstrated that biofilms can skew the immune response to favor anti-inflammatory and profibrotic pathways, which likely contribute to biofilm persistence (17, 18). To overcome this immune deviation and provide a novel treatment strategy for biofilm infections, we augmented antimicrobial activity through the local administration of classically activated M1 MΦs or treatment with the CD88 agonist EP67, which invokes MΦ proinflammatory responses. Early administration of M1-activated MΦs or EP67 limited biofilm formation, and treatment of established biofilm infections with M1-activated MΦs also significantly reduced catheter-associated biofilm burdens. Based on this evidence, we have identified a novel therapeutic strategy to limit *S. aureus* catheter-associated biofilm infections by targeting MΦ activation, which may extend to other artificial implants.

The greatest therapeutic benefit of both MΦ-targeting strategies in this study was achieved with early interventions to boost pro-inflammatory activity against biofilm infections. By extension, targeting MΦ proinflammatory activity may prove useful when administered to patients undergoing orthopedic surgery or other device-related implants to prevent nosocomial infections, particularly for individuals who are at high risk for developing infectious complications. Although our M1-activated MΦ therapy did not completely eliminate established biofilms on infected devices, this strategy may prove beneficial in combination with antibiotics for patients who are unable or unwilling to undergo additional surgeries to manage the infection and maintain the implanted device. The significance of this approach is even more pronounced against the backdrop of the rapidly increasing elderly population, which becomes progressively less immune responsive and represents the primary recipients of hip and knee replacements. To achieve enhanced efficacy against established biofilms, we are currently refining our M1-activated MΦ delivery; nonetheless, these results clearly demonstrate proof-of-principle that induction of a proinflammatory milieu during MRSA biofilm infection is beneficial for bacterial clearance.

The therapeutic potential of activated MΦs is supported by our results demonstrating that early treatment with proinflammatory M1-activated MΦs significantly limited *S. aureus* biofilm growth in vivo and provided long-term protection from biofilm colonization. Likewise, activated MΦs were also effective at reducing *S. aureus* burdens in established biofilms. The cytokine/chemokine milieu elicited following M1-activated MΦ transfer reflects products derived from both T cells (i.e., IFN-γ and IL-17) and...
and the functional impact of these mediators on biofilm burdens remains to be determined. When querying the inflammatory milieu associated with established biofilms following M1 MΦ injection, only IL-17 was significantly increased. Although the other mediators examined did not show statistically significant increases, the trends toward elevated production may translate into increased efficacy when considering their combined action. This may explain why bacterial burdens were decreased on the catheter itself but not in the surrounding tissue because heightened inflammatory mediator levels may be required to impact the latter. It was not feasible to measure all of the microbicidal effectors associated with biofilm infections; therefore, it is likely that alternative factors not examined in this study could be significantly elevated after M1 MΦ treatment to account for the decreased bacterial burdens observed on infected catheters. It was unexpected that M1-activated MΦ transfer had no effect on tissue burdens in established biofilms. One explanation to account for this finding is that the number of MΦs injected was not sufficient to effectively manage bacterial burdens within the infected tissue. It is clear that M1-activated MΦs limit biofilm growth on the catheter itself, however, it remains to be determined whether this results from direct killing of the biofilm and/or enhanced dispersal of organisms from the biofilm into the surrounding tissue. In the latter case, this would lead to increased tissue-associated bacteria, which would likely overwhelm the microbicidal capacity of M1-activated MΦs injected at the injection site. In future studies, it would be interesting to determine whether M1-activated MΦs display synergy with antibiotics to facilitate biofilm clearance, because the former facilitates the dispersal of organisms from the biofilm, which, in turn, would restore their metabolic activity and potential susceptibility to antibiotics. However, this is beyond the scope of the current report.

One interesting finding from this study was that nonactivated MΦs demonstrated a trend toward reduced biofilm burdens in vivo, although most of these differences did not reach statistical significance. This suggests that MΦ activation signals are present at sites of biofilm infection; however, additional stimuli are required to achieve maximal MΦ microbicidal action. One such signal could be direct MΦ contact with the biofilm, which is impeded by the host-derived fibrotic matrix deposited around the infected device. Another possibility is that the degree of endogenous MΦ recruitment is insufficient to prevent biofilm establishment, and therefore, the injection of a large number of MΦs at the site of infection is sufficient to limit biofilm growth, regardless of their activation state. Nonetheless, the superior action of M1-activated compared with nonactivated MΦs at thwarting S. aureus biofilm formation was demonstrated by the ability of the former to significantly reduce bacterial burdens in vivo.

The use of EP67 may overcome the principal issues of biofilm immune dysfunction, as EP67 appears to provide the correct activation signals to CD88+ MΦs (and perhaps other APCs) (37) to engage a robust microbicidal response in the developing biofilm and surrounding tissues. Indeed, EP67 has been shown to enhance the immune status of aged mice by re-establishing an immunologically productive Th1/Th2 balance (38), indicating that this peptide may be a valuable therapeutic option in the aged population in whom multiple surgeries to manage infected devices is not desirable. However, unlike M1-activated MΦ transfer, EP67 treatment did not impact established biofilms, suggesting that additional therapeutic obstacles are present. One such hurdle is the fibrotic capsule that typically surrounds biofilm infections (39–41). Although it is presumed that biofilm encapsulation by the host represents a protective response to contain the infection, this process may inadvertently provide survival advantages to the

**FIGURE 9.** M1-activated MΦs remain localized at the site of biofilm infection. Mice received one dose of $10^7$ Quantum Dot–labeled M1-activated MΦs (red) either at the time of S. aureus challenge (A) or at day 7 following infection (B), representing early and established therapies, respectively. The same cohort of animals was subjected to daily IVIS imaging (Caliper Life Sciences) to visualize MΦ persistence. Results are representative of 10 individual animals per group.

MΦs (i.e., CXCL9, CCL5, and IL-1), suggesting the coordinate activity of both cell types. This complex profile was only significant in the early intervention paradigm with M1-activated MΦs, and the functional impact of these mediators on biofilm burdens remains to be determined. When querying the inflammatory milieu associated with established biofilms following M1 MΦ injection, only IL-17 was significantly increased. Although the other mediators examined did not show statistically significant increases, the trends toward elevated production may translate into increased efficacy when considering their combined action. This may explain why bacterial burdens were decreased on the catheter itself but not in the surrounding tissue because heightened inflammatory mediator levels may be required to impact the latter. It was not feasible to measure all of the microbicidal effectors associated with biofilm infections; therefore, it is likely that alternative factors not examined in this study could be significantly elevated after M1 MΦ treatment to account for the decreased bacterial burdens observed on infected catheters. It was unexpected that M1-activated MΦ transfer had no effect on tissue burdens in established biofilms. One explanation to account for this finding is that the number of MΦs injected was not sufficient to effectively manage bacterial burdens within the infected tissue. It is clear that M1-activated MΦs limit biofilm growth on the catheter itself, however, it remains to be determined whether this results from direct killing of the biofilm and/or enhanced dispersal of organisms from the biofilm into the surrounding tissue. In the latter case, this would lead to increased tissue-associated bacteria, which would likely overwhelm the microbicidal capacity of M1-activated MΦs injected at the injection site. In future studies, it would be interesting to determine whether M1-activated MΦs display synergy with antibiotics to facilitate biofilm clearance, because the former facilitates the dispersal of organisms from the biofilm, which, in turn, would restore their metabolic activity and potential susceptibility to antibiotics. However, this is beyond the scope of the current report.

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bacteria (42, 43). The signals responsible for eliciting this fibrotic response are currently under investigation; nonetheless, in the current study, the injection of MΦs immediately adjacent to the biofilm bypasses this fibrotic barrier and enables MΦ activation to occur.

Neutrophils represent a first line of defense against bacterial infections and possess a potent arsenal of bactericidal compounds, including defensins, cathelicidins, and lysozyme (44, 45). In terms of their bactericidal activity, neutrophils are most notable for their ability to produce large amounts of reactive oxygen intermediates catalyzed by NADPH oxidase. In addition, neutrophils also degranulate and generate neutrophil extracellular traps, a meshwork of DNA and enzymes that lead to the extracellular killing of *S. aureus* and other bacteria (46). Despite these microbialic mechanisms, neutrophil transfer did not attenuate *S. aureus* biofilm growth even when higher numbers of cells were injected. One possibility to explain this finding is that neutrophils rapidly degranulated following in vivo transfer and did not survive long enough to provide a measurable effect on biofilm growth. Nevertheless, it is important to acknowledge that neutrophils may contribute to biofilm clearance at other sites of infection, which remains to be determined. The reasons responsible for differential neutrophil recruitment in various biofilm models may be influenced by the degree of tissue vascularization and/or extent of biofilm development. Another factor to consider is the type of device. For example, bacteria colonizing the lumen of a hollow catheter are initially shielded from immune recognition by the catheter wall, which may afford additional protection. In the case of a solid device, bacteria are immediately exposed to host tissues, in theory enabling an immediate proinflammatory response. We are currently investigating these possibilities using other in vivo models of staphylococcal biofilm infection.

Collectively, these studies have identified a previously unappreciated role for M1-activated MΦs in biofilm containment and bacterial clearance. By extension, it is not unexpected that *S. aureus* biofilms have the capacity to thwart this response by skewing MΦs away from a proinflammatory M1 to an anti-inflammatory M2 phenotype, which ensures biofilm persistence in an immunocompetent host. The implementation of our M1-activated MΦ transfer therapy would allow MΦs to be on board to neutralize potential device contamination from normal skin flora during surgical insertion. Although conventional antibiotics are ineffective for treating biofilms, they are commonly used to control bacteria that escape the biofilm matrix to prevent their colonization of other tissue sites. Such use of antibiotics imposes mutational pressures on the bacteria and portends the possibilities of developing antibiotic resistance. MΦ-based immune cell therapy is not only efficacious at controlling biofilm infections, but also has the added advantage of doing so by using the host’s endogenous innate immune cells, thus eliminating mutational pressures imposed directly on the bacteria and decreasing the likelihood of the emergence of antibiotic resistant strains.

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Disclosures

The authors have no financial conflicts of interest.

References

Corrections


In Fig. 2 of the original publication, the bacterial titer data points displayed for the “Non-activated MΦ” group in panel B were an accidental duplication of the data points for the same group in panel A. Reassessment of the raw data confirmed that the “Non-activated MΦ Tissue” data in panel B was inadvertently populated with the “Non-activated MΦ Catheter” data in panel A from the same figure. The replacement figure presented here displays the correct “Non-activated MΦ Tissue” data set obtained from the same series of experiments collected at the same time as the rest of the data in Fig. 2.

The final corrected figure is shown below. The main conclusions and statistical analysis remain unchanged. The figure legend was correct as originally published and is shown below for reference.

In Fig. 5 of the original publication, the bacterial titer data points displayed for the “MyD88 KO MΦ group in panel B were an accidental duplication of the data points for the same group in panel A. Reassessment of the raw data confirmed that the “MyD88 KO MΦ Tissue” data in panel B was inadvertently populated with the “MyD88 KO MΦ Catheter” data in panel A from the same figure. The replacement figure presented here displays the correct “MyD88 KO MΦ Tissue” data obtained from the same series of experiments collected at the same time as the rest of the data in Fig. 5.

The final corrected figure is shown on the next page. The main conclusions and statistical analysis remain unchanged. The figure legend was correct as originally published and is shown on the next page for reference.

FIGURE 2. Activated MΦs, but not neutrophils, impair MRSA biofilm formation in vivo. C57BL/6 mice were infected with $10^5$ CFU of USA300 LAC in the lumen of surgically implanted catheters to establish biofilm infection. Animals were treated with vehicle, $10^6$ neutrophils (PMN), $10^6$ nonactivated MΦs, or $10^6$ M1-activated MΦs at 12, 24, and 48 h postinfection, whereupon catheters (A) and surrounding tissues (B) were collected at 72 h to quantify bacterial burdens. Results are expressed as the number of CFU per milliliter for catheters or CFU per milligram tissue to correct for differences in tissue sampling size. Results are presented from individual animals combined from at least two independent experiments. Significant differences are denoted by asterisks ($*p < 0.05$).
FIGURE 5. The ability of M1-polarized MΦs to impair MRSA biofilm development is mediated by MyD88-dependent signals. C57BL/6 mice were infected with $10^3$ CFU of USA300 LAC in the lumen of surgically implanted catheters to establish biofilm infection. Animals were treated with vehicle or $10^6$ M1-activated MΦs derived from WT or MyD88 KO mice at 12, 24, and 48 h postinfection, whereupon catheters (A) and surrounding tissues (B) were recovered at day 3 to quantitate bacterial burdens. Results are expressed as the number of CFU per milliliter for catheters or CFU per milligram tissue to correct for differences in tissue sampling size. Results are presented from individual animals combined from at least two independent experiments. Significant differences are denoted by asterisks (*$p < 0.05$).

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Supplemental Figure 1. M1 macrophage polarization enhances co-stimulatory molecule and reactive oxygen species (ROS) production. Bone marrow-derived macrophages (MΦs) from C57BL/6 mice were stimulated with 10 ng/ml IFN-γ + 10 μg/ml PGN for 6 h to induce M1-activation or medium alone (non-activated MΦs). Expression of the cell surface markers MHC Class II and CD86 was assessed by FACS analysis (A). MΦs were also incubated with MitoSOX (B) or CM-H₂DCFDA (C) to measure mitochondrial superoxide (mROS) and determine total cellular H₂O₂, respectively. Significant differences are denoted by asterisks (*, p < 0.05; **, p < 0.01; *** , p < 0.001).
Supplemental Figure 2. Neutrophils are capable of phagocytosing *S. aureus* biofilms but do not reduce bacterial burdens. A) Neutrophils (PMN) were isolated from the bone marrow of C57BL/6 mice, labeled with CellTracker Orange (orange-yellow), and co-cultured with *S. aureus*-GFP (green) during biofilm or planktonic growth for 2 h and imaged to observe their phagocytic ability. B) After 24 h, biofilms were sonicated to quantitate bacterial burdens to evaluate the ability of PMNs to attenuate biofilm growth. Biofilms without PMNs were used as untreated controls. White arrows indicate phagocytic cells.
Supplemental Figure 3. Neutrophil infiltrates into catheter-associated biofilms are minimal compared to abscesses. C57BL/6 mice were infected with $5 \times 10^5$ CFU USA300 LAC either in the lumen of surgically implanted catheters or s.c. in the absence of any indwelling device to establish biofilm and abscess infections, respectively. Animals were sacrificed at days 3, 7, or 14 following *S. aureus* exposure, whereupon tissues surrounding infected catheters or s.c. injection sites were collected to quantitate neutrophil infiltrates by FACS. Results are expressed as the percent of Ly6G$^+$ neutrophils after correction for isotype control staining and represent the mean ± SEM of three independent experiments.