Type III Secretion Protein, PcrV, Impairs Pseudomonas aeruginosa Biofilm Formation by Increasing M1 Macrophage-Mediated Anti-bacterial Activities

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Pseudomonas aeruginosa biofilms employ a variety of strategies to hijack the host immune defense system to achieve chronic infection. However, the bacterial components that are involved in this process are not yet fully understood. PcrV, a needle tip protein of the P. aeruginosa type III secretion system (T3SS), was downregulated during P. aeruginosa biofilm infection. The impaired expression of the P. aeruginosa pcrV gene is associated with attenuated immune activation and an increased percentage of M2 macrophages following P. aeruginosa biofilm infection. Treatment with exogenous PcrV produced from Escherichia coli elevated tissue inflammation and the percentage of M1 macrophages, resulting in reduction in the biofilm burden. Further analyses demonstrated that the potential of PcrV to induce classically activated M1 macrophages as evidenced by the increased production of proinflammatory cytokines and anti-bacterial mediators, including inducible nitric oxide synthase (iNOS) and reactive oxygen species (ROS), as well as increased phagocytosis of bacteria. Mechanistically, PcrV-mediated promotion of macrophage M1 polarization and phagocytosis occurs through the activation of mitogen-activated protein kinases (MAPKs) and NF-κB signaling pathways. Collectively, these findings reveal a potential role of PcrV in skewing host immune defense to promote P. aeruginosa biofilm infection and provide new insights into the therapeutic strategies for P. aeruginosa biofilm infection.

Keywords: Pseudomonas aeruginosa, biofilm, PcrV, immune evasion, macrophage polarization, biofilm eradication

INTRODUCTION

Pseudomonas aeruginosa-mediated chronic infections are commonly associated with biofilm formation on host tissues and indwelling medical devices. Bacterial biofilm communities are encased in a self-produced extracellular matrix which precludes antibiotic penetration, which impedes eradication of biofilm bacteria by conventional antibiotic treatment. To combat biofilm infections, various strategies have been investigated, including utilization of antimicrobial peptides/lipids (Verderosa et al., 2019), quorum sensing inhibitors (Chang et al., 2019), and bacteriophages...
In this study, we demonstrated that pcrV gene expression is reduced during *P. aeruginosa* biofilm infection. Injection of PcrV into the milieu surrounding the biofilm-infected tissues induced a proinflammatory response with increased infiltration of M1-polarized macrophages, decreasing the biofilm burden. Further analyses demonstrated that PcrV promotes biofilm eradication, macrophage M1 polarization, and phagocytosis via the mitogen-activated protein kinases (MAPKs) and NF-kB signaling pathways. Taken together, these results suggest that decreased expression of PcrV during *P. aeruginosa* biofilm infection promotes biofilm persistence and provide novel clues into the therapeutic strategies against *P. aeruginosa* biofilm infection.

### MATERIALS AND METHODS

#### Mice and Ethics Statement
Male mice on a C57BL/6 background were purchased from biocytogen CO., Ltd. (Beijing, China). Animal experiments were conducted according to the experimental animal guidelines of the Army Medical University of China.

#### Expression and Purification of PcrV Protein

*Pseudomonas aeruginosa* PcrV gene was cloned into pQE31 (Qiagen, Germany), which introduces an N-terminal fusion of the protein to a His6 tag. Escherichia coli JM109 strain carrying expression plasmids was propagated in LB medium containing 100 μg/ml ampicillin until the OD600 nm reached 0.5 and was induced using 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 4 h. Cells were harvested and resuspended in phosphate buffered saline (PBS) and lysed by sonication. The fusion protein was purified by affinity chromatography using His-Trap HP (GE healthcare, Germany). Endotoxin was removed from PcrV by using Detoxi-Gel endotoxin removing gel (Thermo fisher, USA) following the manufacturer's instructions.

#### Isolation and Induction of BMDMs

Tibias and femurs from the euthanized C57BL/6 mice were excised, and marrow cells were washed out with a 25-gauge needle attached to a 5 ml-syringe. Cells were cultured in 10% FBS/dulbecco’s modified eagle medium (DMEM), penicillin, streptomycin, and 50 ng/ml recombinant murine macrophage colony stimulating factor (M-CSF) at 37°C and 5% CO2 for 3 days. At day 4, the medium was replaced, and cells were cultured at same condition for an additional 3 days.

#### Mouse Model of *P. aeruginosa* Catheter-Associated Biofilm Infection

A mouse model of catheter-associated biofilm infection was established as described previously with some modifications (Thurlow et al., 2011; Hanke et al., 2013). Briefly, a sterile 1 cm intravenous catheter was implanted subcutaneously into the flank of mice under pentobarbital sodium anesthesia. A suspension (20 μl) of log-phase PAO1 (1 × 10^5 colony forming unit, CFU) was injected through the skin into the catheter lumen. Biofilm formation was monitored throughout the course of infection, and mice were sacrificed on days 2, 5, and 8 post-infection. For scanning electron microscopy (SEM) analysis, biofilms on catheters were fixed and dehydrated according to a standard SEM protocol and were observed under a Crossbeam 340 SEM (Carl Zeiss, Germany). Tissues surrounding infected catheters were homogenized and weighed after freezing in liquid nitrogen. The bacterial burdens of catheters and surrounding tissues were enumerated using *P. aeruginosa* isolation agar (PIA) plates.
Macrophage Administration Into Biofilm Infections in vivo
To determine the efficacy of biofilm clearance by differentially polarized macrophages, 10° non-activated or PcrV-activated bone marrow-derived macrophages (BMDMs) were subcutaneously injected at sites surrounding infected catheters on days 5, 6, and 7 post-infection. The infected catheters and surrounding tissues were harvested on day 8 for subsequent analysis.

Generation of P. aeruginosa Static Biofilms in vitro
Static biofilms were generated as previously described (Thurlow et al., 2011) with minor modifications. Briefly, sterile 24-well cell culture plates were treated with 20% human plasma in sterile carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C to facilitate bacterial attachment. The PAO1 strain was cultured overnight at 37°C with shaking in DMEM supplemented with 10% FBS. The bacterial culture was adjusted to an initial OD600 of 0.05 and then incubated in the plasma pre-coated plate at 37°C under static aerobic condition for 3 days. Medium was carefully replenished every 24 h to prevent disruption of the biofilm structure.

Phagocytosis Assay
The phagocytic efficacy of macrophages against planktonic bacteria or biofilms was evaluated according to a previously described method with modifications (Thurlow et al., 2011). Briefly, planktonic bacteria or biofilms were co-cultured for 30 min with RAW264.7 cells at a ratio of 1:10 at 37°C under 5% CO₂. Cells were then treated with gentamicin (final concentration 400 μg/ml) for 2 h at 37°C under 5% CO₂ to remove extracellular bacteria. For immunofluorescence staining, the cytoskeleton and bacteria were visualized by phalloidin (red) and anti-PAO1 antibody (green), respectively. For evaluation of the intracellular bacteria, cells were lysed with 0.5% TritonX-100/PBS and enumerated on PIA plates.

Western Blot
Cell pellets were lysed using RIPA buffer (Beyotime, China) supplemented with protease inhibitor cocktail (Roche, USA). Equal amounts of proteins were separated on 10% SDS-PAGE and then transferred electrophoretically to PVDF membranes (Millipore, USA). The membrane was blocked using 5% skim milk in TBST at real time (RT) for 1 h. Then, the membrane was incubated with the appropriate first antibody at 4°C overnight and horseradish peroxidase-conjugated secondary antibody at RT for 1 h.

Real-Time Quantitative PCR
Total RNA extraction and reverse transcription to cDNA were performed according to the manufacturer's instructions. Quantitative PCR was performed using an ABI 7500 RT PCR system (Applied Biosystems, Germany). For macrophages, the relative gene expression levels of cd11c, inos, ptg2, cd206, and pparγ were normalized to GAPDH. For PAO1 strain, the relative gene expression level of ndk was normalized to gyrB, rpoD, or rplU gene. The primers used in this study were provided in Supplementary Table S1.

Enzyme-Linked Immunosorbent Assay
Supernatants of stimulated macrophages or biofilm infected tissues were assessed for their levels of TNFα, IL 12 p40/p70, and IL6 by using the sandwich enzyme-linked immunosorbent assay (ELISA) kits (BD biosciences, USA) according to the manufacturer's instructions.

Measurement of Intracellular ROS
Macrophages were harvested and incubated with 2′-dichlorodihydrofluorescein diacetate (H2DCFDA; Santa Cruz, USA) dye in DMEM medium at a final concentration of 5 μM for 30 min at RT. Intracellular ROS were measured by flow cytometry.

Statistical Analysis
Data were expressed as means ± standard errors of the means (SEM). The statistical analysis was carried out with GraphPad (GraphPad Software Inc., San Diego, CA). Data were analyzed by unpaired t test when comparing two groups and one-way analysis of variance (ANOVA) with Tukey’s multiple-comparison test for multiple groups. A value of p < 0.05 was considered statistically significant.

RESULTS
P. aeruginosa Biofilm Persistence Is Associated With Attenuated Activation of Host Proinflammatory Responses and Decreased Bacterial Eradication Ability
To evaluate the condition of the host immunity and bacterial killing efficacy in response to a P. aeruginosa biofilm, we used a mouse model of catheter-associated biofilms to mimic bacterial biofilm formation on medical devices in humans (Hanke et al., 2013). Bacterial biofilm formation involves a transformation from an immature to a mature structure involving bacterial surface attachment and bacterial colony formation in which bacteria are surrounded by matrix materials. In this study, we primarily observed the different stages of P. aeruginosa biofilm formation on catheters by SEM. Examination of the catheter lumen by SEM revealed that an immature biofilm was formed with few extracellular matrix-encompassed bacteria within 4 days of the initial infection (Figure 1A). With the extension of time post-infection (7 days), a mature biofilm was formed with a contiguous bacterial layer in which extracellular matrix and interior holes were found (Figure 1A), suggesting the successful establishment of a mature P. aeruginosa biofilm.

To compare the host immune responses and bacterial eliminating efficacy between P. aeruginosa biofilms and planktonic bacteria-associated infections, we analyzed the production of inflammation-related cytokines, arginase, and inducible nitric oxide synthase (iNOS), as well as the bacterial burdens in the tissues surrounding...
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At 1 day post-infection, bacterial loads in planktonic bacteria-infected tissues were lower than biofilm bacteria-infected group ($p < 0.05$). At 7 days post-infection, the planktonic bacteria were almost eradicated, while the bacterial loads in the biofilm remained high ($p < 0.001$). In contrast to the decreased bacterial loads, the levels of proinflammatory cytokines, TNFα ($p < 0.001$) and IL12 p40/70 ($p < 0.05$), and the percentages of iNOS$^+$ cells were higher in planktonic bacteria-infected tissues (1 day post-infection) than those in biofilm-infected tissues (Supplementary Figure S1). Arg1, which is involved in activating anti-inflammatory responses, was also higher in biofilm-infected tissues than in planktonic bacteria-infected tissues (Supplementary Figure S1), suggesting a lower ability of P. aeruginosa biofilms to activate proinflammatory responses to infection.

Phagocytosis by phagocytes, such as macrophages, accelerates the clearance of bacteria following infection; therefore, we compared the phagocytic ability of macrophages against P. aeruginosa in the planktonic and biofilm forms in vitro. The results showed that the ability of macrophages to phagocytose biofilm bacteria was inferior to that observed for planktonic bacteria ($p < 0.001$). Collectively, these results indicated that P. aeruginosa biofilms enhance bacterial chronic infection by circumventing the host immune response and anti-bacterial activities.
**P. aeruginosa Biofilm-Mediated Programming of Macrophages Toward an M2 Phenotype Attenuates Immune Activation, and Promotes Biofilm Persistence**

Based on the above results, we further investigated the association of the impaired activation of host immune responses to *P. aeruginosa* biofilm infection with alternatively activated M2 macrophages. Result revealed that a higher percentage of F4/80+/Arg1+ macrophages and a lower percentage of F4/80+/iNOS+ macrophages in biofilm-infected tissues (7 days post-infection) than those in planktonic bacteria-infected tissues (1 day post-infection; **Figures 1D,E**), reflecting a less M1-like phenotype of macrophages under a biofilm-associated infection. To further verify this phenomenon, we analyzed the gene expression profiles of *P. aeruginosa* planktonic bacteria or biofilms *in vitro*. In accordance with our *in vivo* findings, macrophages associated with the biofilm infection exhibited decreased expression of proinflammation-related genes, including *inos*, *il1β*, and *ptgs2*, compared with those associated with planktonic bacterial infection (**Figure 1F**), whereas the expression of *arg1* was increased (**Figure 1F**; *p < 0.05).

The decreased phagocytic ability of M2 macrophages skewed by bacterial biofilms is also responsible for the delayed bacterial clearance and biofilm persistence. To this end, we analyzed the phagocytic ability of differentially polarized macrophages against *P. aeruginosa* biofilms. Although, lipopolysaccharide (LPS)- or IL4-primed M1 or M2 macrophages both displayed higher phagocytic ability than non-activated macrophages (**Figures 1G,H**), the M1 macrophages exhibited a superior ability for phagocytosis of biofilm bacteria than M2 macrophages (**Figures 1G,H; *p < 0.001**). Collectively, these results suggested that *P. aeruginosa* biofilm-mediated skewing of macrophages toward the M2 phenotype enhances biofilm persistence.

**PcrV Production Is Attenuated in Chronic *P. aeruginosa* Biofilm Infection, and the Addition of Exogenous PcrV Accelerates Macrophage-Mediated *P. aeruginosa* Clearance in Biofilms**

The reduced activation of host proinflammatory responses that allows *P. aeruginosa* biofilms to persist may be due to the inactivation of the T3SS, whereby components of the T3SS could be immunostimulatory. Previous research has shown that components of the *P. aeruginosa* needle tip complex, including PcrV, are involved in enhancing proinflammatory responses in mouse lung tissues following infection (Wangdi et al., 2010). Therefore, we speculated that *pcrV* gene expression is downregulated during *P. aeruginosa* biofilm infection, and the decrease in PcrV production in the milieu surrounding the biofilms accounts for the impaired activation of immune responses mediated by M2 macrophages following biofilm infection, which ultimately results in biofilm persistence. To verify this hypothesis, we first analyzed *pcrV* gene expression in *P. aeruginosa* biofilm-infected catheters *in vivo* by real-time quantitative PCR (RT-qPCR) analysis of three different *P. aeruginosa* reference genes. Compared to the immature biofilm bacteria (1 day post-infection), *pcrV* gene transcription was decreased both in the incompletely matured (4 days post-infection) and mature biofilm bacteria (7 days post-infection; **Figure 2A**). A similar trend was also observed in the *in vitro* static biofilm system (**Figure 2B; *p < 0.001***). To further investigate the correlation between PcrV production and biofilm eradication, PcrV protein was subcutaneously injected into the tissues surrounding the biofilm-infected catheters. Bacterial burdens in PcrV-treated catheters (**Figure 2C; *p < 0.001*** and surrounding tissues (**Figure 2C; *p < 0.05**)) were lower than those in the PBS-treated control group, whereas the levels of TNFα (**Figure 2D; *p < 0.01** and IL12 p40/70 (**Figure 2D; *p < 0.001**)) were elevated.

To explore the involvement of macrophages in PcrV-mediated elimination of biofilm bacteria, M1 and M2 polarization was analyzed in the macrophages associated with *P. aeruginosa* biofilm catheters-infected tissues following treatment with or without PcrV. The percentages of M1 macrophages (F4/80+/iNOS+) in the PcrV-treated groups were higher than those in the PBS-treated groups (**Figure 2E**), whereas the opposite trend was observed in M2 macrophages (F4/80+/Arg1+; **Figure 2F**). These results suggested that PcrV promotes the elimination of biofilm bacteria through polarization of macrophages toward an M1 phenotype.

**PcrV Is Involved in Skewing Macrophages Toward an M1 Phenotype**

Given that subcutaneous injection of PcrV promoted biofilm clearance and increased the percentages of M1-polarized macrophages around the infected catheter, we investigated the ability of PcrV to directly drive macrophage differentiation toward an M1 phenotype. To this end, we first evaluated the extensive inflammatory modulation effects of PcrV on BMDMs by gene and protein chip analysis. Gene chip analysis showed that PcrV treatment extensively upregulated macrophage M1 activation-related genes including proinflammatory cytokines (e.g., *tnf*, *il1β*, *il12*, and *il6*), chemokines (e.g., *cxcl3*, *cxcl9*, and *cxcl11*), bacterial killing molecules (*inos*), antigen presentation (e.g., *MHCI*, *MHCIi*, and *CD86*), and others (e.g., *cd11c*, *ptgs2*, *egf3*, and *inhbα*), whereas M2 activation-related genes, such as *cd206*, *ppary*, *cd83*, and *egr2*, were downregulated (**Figure 3A**). Protein chip assays revealed that PcrV-primed BMDMs displayed increased production of macrophage M1 polarization-related cytokines (e.g., GM-CSF, TNFα, IFNγ, IL12 p40/70, and IL11α/β), chemokines (e.g., CXCL1 and CCL4/6), and IL2, which is responsible for T cell activation (**Figure 3B**). Despite the extensive upregulation of M1 markers following PcrV treatment, and decreased expression of M2 markers in BMDMs, some of the M2 molecules, such as *cd22*, *ccl2*, *il6*, and *il10* were also elevated in PcrV-primed BMDMs (**Figures 3A,B**), suggesting a balance of immune responses following PcrV treatment.

To observe the dose-dependent effects of PcrV on macrophages, BMDMs and the RAW264.7 cells were treated with different concentrations of PcrV. Similar to LPS-primed M1 macrophages, PcrV-pulsed BMDMs and RAW264.7 cells showed upregulated M1-related gene expression (*inos*, *ptgs2*, and *cd11c*; **Figure 3C; Supplementary Figure S2A**), increased production of iNOS (**Figure 3E**), and NO (**Figure 3F**, TNFα, IL12 p40/70, IL6 (**Figure 3D; Supplementary Figure S2A**), and ROS (**Figures 3G,H**),
whereas expression of M2-related genes, including cd206, pparγ (Figure 3C; Supplementary Figure S2B), and arg1 (Supplementary Figure S2B) was downregulated in a dose-dependent manner compared with the untreated- or hydrolyzed PcrV (PK)-treated groups. Taken together, these results suggested that PcrV plays a role in skewing macrophage differentiation toward an M1 phenotype.

**PcrV Repolarizes Macrophages From M2 to M1 Phenotype**

To evaluate the ability of PcrV to promote a macrophage switch from the M2 to M1 phenotype, BMDMs were pretreated with the M2 inducer IL4, before PcrV treatment. IL4 significantly increased the expression of M2-specific genes, including cd206, pparγ, and arg1 in BMDMs (Figure 4); however, the IL4-mediated upregulation of these genes was abolished by PcrV treatment (Figure 4). Despite the anti-inflammatory conditions of macrophages pretreated with IL4, PcrV treatment significantly elevated the expression of proinflammatory M1-related genes in BMDMs, such as inos, ptgs2, and cd11c (Figure 4), demonstrating that PcrV plays a role in inducing transformation from the M2 to M1 phenotype.

**The Proinflammatory M1 Macrophages Polarized by PcrV Are Involved in Biofilm Elimination**

Considering that PcrV is involved in skewing macrophage toward the M1 phenotype, we further investigated the efficacy of PcrV-pulsed M1 macrophages in biofilm elimination both in vitro and in vivo. In vitro studies revealed that PcrV treatment significantly increased the phagocytosis ability of macrophages against biofilm
bacteria in a time-dependent manner (Figures 5A,B; p < 0.001). Following injection of BMDMs pretreated with PcrV for 24 h into the tissues surrounding biofilm catheters in vivo, the percentage of F4/80\(^{+}\)iNOS\(^{+}\) macrophages was higher in PcrV/BMDMs treated mice than that in mice treated with non-activated BMDMs (Figure 5C), suggesting that functional M1 macrophages were present at the site of infection. Analyses of bacterial burdens and tissue inflammation status revealed that PcrV-primed M1 macrophages reduced bacterial loads in catheters and infected tissues (Figure 5D; p < 0.01), whereas the production of TNF\(\alpha\) and IL12 p40/70 was augmented (Figure 5E; p < 0.001). Collectively, these results suggested that PcrV-primed M1 macrophages are effective in accelerating biofilm clearance.

**MAPKs and NF-\(\kappa\)B Signaling Pathways Play Dominant Roles in Promoting PcrV-Mediated Macrophage M1 Activation and Phagocytosis**

MAPK and NF-\(\kappa\)B signaling pathways are among the most extensively reported pathways that are involved in activating anti-bacterial immune responses, involving macrophage M1 polarization (Akhtar et al., 2019; Justino et al., 2020). Our results demonstrated that PcrV promoted the phosphorylation of extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPKs and IKB\(\alpha\) (an indicator of NF-\(\kappa\)B pathway activation) in RAW264.7 treated cells by western blot and flow cytometry, respectively. The concentration of nitric oxide (NO) in the culture supernatant of RAW264.7 treated with the indicated compound for 24 h was detected by NO detection kit. One-way ANOVA (Tukey’s post hoc, C,D,F,G) was used for statistical analysis. *p < 0.05; **p < 0.01; ***p < 0.001; n.s. indicates no significance.
SP600125, SB203580 for ERK, JNK, and p38 MAPKs, respectively, revealed that PcrV-mediated activation of the three pathways was successfully inhibited (Figure 6A). Since the nuclear translocation of NF-κB p65 results in the activation of NF-κB signaling pathway, we used JSH23, an inhibitor of NF-κB p65 nuclear translocation and transcriptional activity, to treat the cells. Given NF-κB p65 might regulate the transcriptional level of itself, the JSH23 treatment also lead to a massive reduction of the expression of NF-κB p65 in the JSH23 treated groups (Figure 6B). In spite of that, LPS or PcrV-mediated promotion of nuclear translocation of NF-kB p65 was impaired by JSH23 (Figure 6B). Analyses of the downstream cytokines, gene expression, and phagocytosis showed that the levels of TNFα, IL6 (Supplementary Figure S3C; p < 0.001), and ROS (Figures 6C,D), expression of the M1-related genes, cd11c and ptgs2 (Supplementary Figure S3D), and phagocytosis (Figures 6G,H) were reduced in PcrV-treated RAW264.7 in the presence of both MAPK and NF-kB inhibitors; however, ERK inhibition elevated iNOS (Figure 6E; Supplementary Figure S3D) and NO (Figure 6F; p < 0.001), while JNK and p38 inhibition did not alter NO production (Figure 6F). Meanwhile, MAPK inhibition did not elevate ppara expression in RAW264.7 cells primed with PcrV (Supplementary Figure S3D). In contrast, JSH23 treatment reduced the levels of iNOS (Figure 6E; Supplementary Figure S3D; p < 0.001) and NO (Figure 6F; p < 0.001) and reversed the decrease in ppara expression in PcrV-stimulated RAW264.7 cells (Supplementary Figure S3D; p < 0.05). Collectively, these results indicated that the MAPK and NF-kB signaling pathways are involved in PcrV-mediated regulation of macrophage M1 polarization and phagocytosis.

**DISCUSSION**

Bacterial biofilm formation on human tissues and implanted/indwelling devices provides a basis for persistent infections. The remodeling of host immune responses by biofilms following infection is one of the most important factors that benefit bacterial survival and chronicity of infection (Gonzalez et al., 2018; Campoccia and Mirzaei, 2019). Previous studies have revealed
that S. aureus biofilms attenuated the production of proinflammatory cytokines/chemokines, including IL1β, TNFα, and CXCL2, as well as iNOS, and exacerbated bacterial biofilm infection (Thurlow et al., 2011). Chronic P. aeruginosa biofilm infections in cystic fibrosis patients are dominated by a Th2 response with increased and decreased levels of IL4 and IFNγ, respectively (Moser et al., 2000; Hartl et al., 2006), suggesting that P. aeruginosa biofilms persist by reducing host proinflammatory responses to infection. In vitro co-culture of P. aeruginosa biofilm matrix exopolysaccharides (EPS) and extracellular DNA (eDNA) with RAW264.7 cells induced a lower-grade inflammatory response than that induced by planktonic bacteria- or LPS-treated cells (Ramirez et al., 2019). In accordance with these reports, we have demonstrated that compared to planktonic bacteria, P. aeruginosa biofilms impaired the production of proinflammatory cytokines, as well as iNOS, while promoting the expression of the anti-inflammatory enzyme, Arg1, both in vitro and in vivo. Thus, our findings further confirm that P. aeruginosa biofilm-associated infection reduces proinflammatory responses to benefit biofilm bacterial survival.

Accumulating evidence demonstrates that the biofilm-mediated hijacking of host immune defense relies on several processes, such as interference in the release of antimicrobial peptides (AMPs), enzymes, ROS, RNS, and NO from leukocytes; impaired phagocytosis; and the recruitment of immunosuppressive cells,
such as MDSCs (Campoccia and Mirzaei, 2019). In addition, studies have demonstrated that the re-education of M1 macrophages to an anti-inflammatory M2 phenotype is also involved in biofilm-mediated immune suppression (Thurlow et al., 2011; Hanke et al., 2013). In accordance with this phenomenon observed in *S. aureus*, we demonstrated that *P. aeruginosa* biofilms obstructed the host immune response by activation of M2 macrophages. Mechanistically, it has been shown that the cyclic di-AMPs, alpha-toxin (Hla), and leukocidin AB (LukAB; Scherr et al., 2015), released from *S. aureus* biofilms promote biofilm persistence by enhancing macrophage anti-inflammatory polarization or inhibiting phagocytosis (Gries et al., 2016). In *P. aeruginosa*, the biofilm formation-related exopolysaccharide alginates (Leid et al., 2005) and rhamnolipids (Alhede et al., 2014) have been implicated in the protection of biofilm bacteria against macrophage-mediated phagocytosis or have been shown to exert direct cytotoxic effect against macrophages. However, the mechanisms by which *P. aeruginosa* biofilms skew macrophage phenotypes to favor their survival following infection still remain obscure. Although excessive activation of *P. aeruginosa* T3SS during acute infection might aggravate inflammation-mediated tissue damage and immune dysfunction, activation of the host immune response by T3SS might also accelerate bacterial recognition and eradication by host immune cells (Galle et al., 2012; Klockgether and Tummler, 2017). However, during a chronic infection, bacterial T3SS is inactivated (Jain et al., 2008), which enhances the ability of bacteria to evade host immune recognition and clearance, ultimately resulting in biofilm persistence. Due to the reverse correlation between T3SS and biofilms (Kuchma et al., 2005), it is highly possible that regulatory elements that control T3SS inhibition/activation might also regulate biofilm persistence. In this study, we found that the T3SS protein PcrV, which is involved in enhancing proinflammatory polarization and phagocytosis of macrophages, is downregulated during *P. aeruginosa* biofilm formation in vitro and in vivo. The addition of exogenous PcrV or PcrV-pulsed M1 macrophages reversed M2 macrophage-mediated immune inhibition and increased biofilm bacterial elimination, indicating that the decreased expression of PcrV during biofilm formation might impair the M1 macrophage-mediated proinflammatory response and bacterial clearance, ultimately promoting biofilm persistence. Studies have demonstrated that *P. aeruginosa* T3SS genes, including *pcrV*, are negatively regulated in bacteria by the intracellular second messenger cyclic di-GMPs (Romling and Galperin, 2017) and the three-component SadARS regulatory system (Kuchma et al., 2005). Given that cyclic di-GMPs and the SadARS system are also involved in positively regulating *P. aeruginosa* biofilm formation (Kuchma et al., 2005; Ha and O’Toole, 2015; Sharp and Retsch, 2019), it is likely that these factors are involved in modulation of PcrV-mediated regulation of biofilm persistence during infection in vivo. Considering that PcrV, which is involved in inducing macrophage M1 polarization and enhancing phagocytosis, is downregulated during biofilm infection, the addition of exogenous PcrV shows promise as a therapeutic strategy in patients with biofilm infections or chronic immune suppression.

Macrophage polarization is influenced by a variety of factors, such as different types of inflammatory cytokines/chemokines and infiltrated immune cells, as well as cell membrane and intracellular molecule-related mechanisms (Zhou et al., 2014). In this study, we demonstrated that PcrV-mediated polarization of M1 macrophages is through the activation of MAPK and NF-κB signal pathways. Similar to our findings, the bacterial pathogenicity associated molecular patterns (PAMPs) derived from Gram-negative bacteria, such as *Yersinia enterocolitica* LcrV (Sing et al., 2002), *Brucella abortus* cell-surface protein 31 (BCSP31) protein (Li et al., 2014), and *Vibrio cholerae* porin OmpU (Khan et al., 2015), have also been demonstrated to induce macrophage M1 polarization via MAPK and NF-κB signaling pathways. Peroxisome proliferator activated receptor (PPARy), which is mainly expressed in adipose tissue and immune cells, is a member of the nuclear receptor superfamily of ligand-inducible transcription factors that regulate a variety of biological activities, including adipogenesis, lipid metabolism, and insulin sensitization, as well as inflammation (Janani and Ranjitha Kumari, 2015). PPARy-mediated regulation of inflammation often leads to an inhibitory effect on the activation of immune cells, as well as the production of inflammatory factors through the suppression of signaling pathways, such as NF-κB and JNK/p38 signal pathways (Wang et al., 2018). In this study, we found that PcrV treatment significantly decreased the expression of *ppary* gene in macrophages, suggesting that the impaired *ppary* expression might exacerbate NF-κB and MAPK pathway-mediated inflammation in PcrV-treated macrophages.

In summary, this study reveals a role for PcrV in altering biofilm persistence of *P. aeruginosa*, and subsequently the potential therapeutic effects of treating *P. aeruginosa* biofilm infections with PcrV to reverse biofilm-mediated immune suppression.

**DATA AVAILABILITY STATEMENT**

The datasets generated for this study are available on request to the corresponding authors.

**ETHICS STATEMENT**

The animal study was reviewed and approved by ethics committee of Army Medical University.

**AUTHOR CONTRIBUTIONS**

HY and KZ conceived and designed the experiments. HY, JX, JQ, XH, HS, QD, DL, RX, LJ, QL, QC, JP, and MW performed the experiments. HY, XR, and KZ analyzed the data. HY, XR, and KZ wrote the paper. All authors contributed to the article and approved the submitted version.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01971/full#supplementary-material.
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.