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Enhanced Clearance of *Pseudomonas aeruginosa* by Peroxisome Proliferator-Activated Receptor Gamma

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The pathogenic profile of *Pseudomonas aeruginosa* is related to its ability to secrete a variety of virulence factors. Quorum sensing (QS) is a mechanism wherein small diffusible molecules, specifically acyl-homoserine lactones, are produced by *P. aeruginosa* to promote virulence. We show here that macrophage clearance of *P. aeruginosa* (PAO1) is enhanced by activation of the nuclear hormone receptor peroxisome proliferator-activated receptor gamma (PPARγ). Macrophages treated with a PPARγ agonist (pioglitazone) showed enhanced phagocytosis and bacterial killing of PAO1. It is known that PAO1 QS molecules are inactivated by PON-2. QS molecules are also known to inhibit activation of PPAR agonist (pioglitazone) showed enhanced phagocytosis and bacterial killing of PAO1. It is known that PAO1 QS molecules are inactivated by PON-2. QS molecules are also known to inhibit activation of PPARγ by competitively binding PPARγ receptors. In accord with this observation, we found that infection of macrophages with PAO1 inhibited expression of PPARγ and PON-2. Mechanistically, we show that PPARγ induces macrophage paraoxonase 2 (PON-2), an enzyme that degrades QS molecules produced by *P. aeruginosa*. Gene silencing studies confirmed that enhanced clearance of PAO1 in macrophages by PPARγ is PON-2 dependent. Further, we show that PPARγ agonists also enhance clearance of *P. aeruginosa* from lungs of mice infected with PAO1. Together, these data demonstrate that *P. aeruginosa* impairs the ability of host cells to mount an immune response by inhibiting PPARγ through secretion of QS molecules. These studies define a novel mechanism by which PPARγ contributes to the host immunoprotective effects during bacterial infection and suggest a role for PPARγ immunotherapy for *P. aeruginosa* infections.

*Pseudomonas aeruginosa* is an important opportunistic pathogen causing a variety of acute infections, including pneumonia, sepsis, keratitis, and urinary tract, wound, and skin infections. *P. aeruginosa* continues to be a leading cause of nosocomial and ventilator associated pneumonias, with a mortality as high as 50% even with antibiotic treatment (1, 2). Patients who are immunosuppressed, particularly transplant recipients, neutropenic patients, and patients with HIV, are also at increased risk for *P. aeruginosa* infections. *P. aeruginosa* also contributes to infections in lungs of patients with cystic fibrosis (CF), chronic obstructive airway diseases, and non-CF bronchiectasis. Emerging multidrug-resistant strains of *P. aeruginosa* contribute to the high mortality in these patients (2).

The pathogenic profile of *P. aeruginosa* is related to its ability to secrete a variety of virulence factors, including quorum-sensing (QS) molecules. QS molecules are small diffusible acyl-homoserine lactone (AHL) molecules that are produced as a means of communication to regulate virulence and biofilm formation (1, 3). *P. aeruginosa* predominantly makes two AHL autoinducers: N-3-oxododecanoyl homoserine lactone (3-oxo-C12-HSL) and N-butyryl-1-homoserine lactone. Activation of the QS cascade promotes formation of structured communities of bacteria that coat mucosal surfaces and invasive devices. Formation of biofilms makes conditions more favorable for bacterial persistence. In addition, bacteria in biofilms are inherently more difficult to eradicate than those in the planktonic form (4–10).

The host response to *P. aeruginosa* is complex and involves multiple cell types with induction of a variety of genes (3, 11, 12). Strategies that strengthen the ability of the host to inhibit virulence factors would promote enhanced bacterial clearance and could be used in the treatment of resistant infections. Paraoxonases (PONs) are a family of orphan enzymes with multiple activities and are comprised of three proteins: PON-1, PON-2, and PON-3 (13, 14). The enzymatic actions of PONs include arylesterase, organophosphatase, and lipo-lactonase activities, which bestow them with an ability to contribute to inflammation, toxicity, and infections. All three PON enzymes degrade oxidized lipids, protect against oxidative stress, and act to suppress inflammation. Paraoxonases have been shown to hydrolyze and thereby inactivate bacterial QS molecules, or N-acyl-homoserine lactones, such as 3-oxo-C12-HSL (13–17). In particular, PON-2 has the highest catalytic activity among the three PON proteins and is expressed in almost all human tissues (16). Inducers of PON-2 promote hydrolysis of QS molecules and have the potential to be used as quorum quenchers (18).

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors (19–23). Within the PPAR family, PPAR gamma (PPARγ) serves as a pharmacological target of agonists used in the treatment of type 2 diabetes to enhance insulin}

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sensitivity (21, 23, 24). Recent studies have shown a critical role for PPARγ in sepsis and in inflammatory lung diseases such as chronic obstructive pulmonary disease (COPD) and CF (22, 23). Growing evidence suggests that PPARγ plays an important role by regulating inflammation and host immune response to infections. By binding to PPAR response elements in target genes, PPARs control the expression of broad networks of genes that regulate diverse and fundamental cellular processes, including metabolism and inflammation (20). In recent years, many studies have described the enhancement of host defenses by PPARγ in response to infection by various pathogens (25–28). We sought to delineate one such mechanism by which PPARγ agonists can potentially induce downstream effects, such as the upregulation of PON-2, known for its quorum-quenching properties (15). PON proteins are regulated transcriptionally, and promoter analyses have revealed PPARγ binding sites within the proximal region of the PON-2 promoter. Moreover, the PON-1 promoter has been shown to have putative PPARγ binding sites.

 Regulation of PON-2 in macrophages by PPARγ agonists has been previously established by Shiner et al. However, their study was conducted in an atherosclerosis model, whereby PON-2 was induced by polyphenolic antioxidants in pomegranate juice, in a PPARγ- and AP-1-dependent manner (29).

Our study shows that clearance of *P. aeruginosa* (PAO1) is enhanced by PPARγ activation in the macrophages and lungs of mice infected with PAO1. By using a gene-silencing approach, we show that the immunostimulatory effects of PPARγ are mediated through the induction of PON-2 in macrophages. PON-2 expression was significantly increased in cells overexpressing PPARγ (Ad-PPARγ), whereas PON-2 expression was attenuated by silencing PPARγ (siPPARγ). Infection with PAO1 or treatment of cells with recombinant QS molecules attenuated the expression of PPARγ and PON-2 in macrophages. However, the infection of cells with mutant PAO1 (lacking expression of QS molecules) did not attenuate expression of PPARγ or PON-2. These data suggest that PAO1 impairs the capacity of host immune cells by suppressing the expression of PPARγ through expression of QS molecules. Therefore, activating PPARγ can enhance the immune capacity of cells to clear *P. aeruginosa*. These data demonstrate, for the first time, an immunostimulatory role of PPARγ through the induction of PON-2 in *P. aeruginosa* infection in an acute lung infection model.

**MATERIALS AND METHODS**

**Cell culture.** A macrophage cell line (RAW264.7 [RAW]) and a macrophage-like monocytic cell line (THP-1) were purchased from the American Type Culture Collection (Manassas, VA). RAW cells were maintained in Dulbecco modified Eagle medium (DMEM), whereas THP-1 cells were cultured in RPMI 1640 (HyClone, Logan UT). Media were supplemented with 10% fetal bovine serum (FBS), 50 IU of penicillin/mL, and 50 mg of streptomycin/mL (HyClone). Cells were grown in a 5% CO₂ incubator with humidity at 37°C. THP-1 cells were differentiated into macrophages using 200 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO) for 3 days (30).

**BMDMs.** Bone marrow-derived macrophages (BMDMs) from C57BL/6J mice were prepared as described previously. Briefly, mice were euthanized by asphyxiation with CO₂. Cellular material was aspirated from femurs and spine at 400 × g at 4°C for 5 min. The cells were then resuspended in DMEM with 10% FBS and 10% 1929 cell-conditioned medium containing macrophage colony-stimulating factor. The cells were allowed to mature into phenotypic macrophages by incubation in the presence of L929 cell-conditioned medium for 5 days before the experiments were done. The purity of the resulting macrophages was confirmed by flow cytometry (>90% CD11b+ F4/80+).

**Reagents.** Pioglitazone (PIO), rosiglitazone, prostaglandin D₂ (PGD₂), prostaglandin J₂ (PGJ₂), GW9662, and 3-oxo-C₁₂-HSL were obtained from Cayman Chemicals (Ann Arbor, MI). All these compounds were dissolved in dimethyl sulfoxide (DMSO) according to the manufacturer’s instructions. All cell types were treated with 10 μM concentrations of the respective compounds, except for 3-oxo-C₁₂-HSL, which was used at a concentration of 50 μM for 24 h.

**Bacterial stocks.** The wild-type (WT) *P. aeruginosa* strain (PAO1), mutant strains lacking QS genes, and the PAO1 strain expressing green fluorescent protein (PAO1-GFP) were kindly provided by Joanna Goldberg (31). The parental and the mutant strains were used at a multiplicity of infection (MOI) of 10 for all in vitro experiments. *P. aeruginosa* stock was prepared as described previously (2, 3). Briefly, bacteria from frozen stocks were streaked onto Luria-Bertani (LB) plates with agar and grown in LB broth (Sigma) in a shaking incubator. Overnight cultures were centrifuged at 8,500 × g for 5 min, and the bacterial pellet was washed twice in phosphate-buffered saline (PBS). Cultures were adjusted to an optical density at 600 nm (OD₆₀₀) of 0.2 (~1.86 × 10⁶ CFU/ml).

**Phagocytosis and bactericidal assay.** RAW and THP-1 cells were infected with PAO1-GFP at an MOI of 50. The cells were plated at a concentration of 10⁶/ml in 24-well plates and infected for 30 min at 37°C with 5% CO₂ with simple agitation every 5 min in the same 24-well plates. To remove extracellular bacteria, the cells were washed with DMEM (4.5 g/ml glucose) containing 10% heat-inactivated FBS and 100 μg of gentamicin (Sigma)/ml. The cells were then incubated at 37°C with 5% CO₂ in the same media and harvested at 1, 2, 3, 5, and 7 h by trypsination with 0.05% trypsin-EDTA (HyClone) diluted in PBS. The cells were then washed and fixed in 2% paraformaldehyde in PBS. Cell populations were analyzed using BD FACSCalibur using BD CellQuest software to determine the mean fluorescence intensity (MFI) (32). Phagocytosis was also quantitated using relative fluorescence units (RFU) (33). Cells were cultured in 96-well plates and then infected with PAO1-GFP at an MOI of 100:1 in culture medium containing 10% FBS only for 1 h at 37°C. After incubation, the wells were washed twice with 1× PBS and treated with 100 μg of gentamicin/ml for 1 h to kill any remaining extracellular bacteria. The numbers of phagocytosed bacteria were counted using a Victor Multi-Label plate reader (Perkin-Elmer, Boston, MA) with excitation (485 ± 20 nm) and emission (528 ± 20 nm) filters. Background correction was performed for autofluorescence. Replicates (eight wells) were performed for each sample and control. Phagocytosis was quantitated as RFU. Alternatively, classical CFU were counted to quantitate phagocytosis as described below.

**Bacterial killing assay (also referred to as gentamicin protection assay) was performed as described previously (34). Briefly, overnight plates with PAO1 were adjusted to an OD₆₀₀ of 0.2 (~2 × 10⁶ CFU/ml) in antibiotic-free RPMI 1640 medium containing 10% heat-inactivated FBS. Unstimulated and stimulated THP-1-derived macrophages were washed and adjusted to 10⁵ cells/ml in antibiotic-free RPMI 1640 medium containing 10% heat-inactivated FBS. The cells were then infected with PAO1 at an MOI of 50. The cell mixture was then centrifuged at 600 × g for 3 min and washed three times with medium containing 10% heat-inactivated FBS and 100 μg of gentamicin/ml to kill extracellular bacteria. Infected monocytes were resuspended using fresh antibiotic-free medium containing 10% heat-inactivated FBS and gentamicin. Cell suspensions were then transferred into 24-well tissue culture plates at a concentration of 10⁵/ml and further incubated at 37°C for 1, 2, 3, 5, and 7 h. The cell suspensions were harvested at the respective time points by centrifugation at 300 × g for 3 min and then washed three times with PBS. The cell pellet was suspended in 0.2% (vol/vol) Triton X-100 in sterile PBS, incubated for 5 min at 37°C, and then vortexed thoroughly to permeate the monocyte membranes (for the retrieval of intracellular bacteria). The lysed cell mixture was serially diluted in sterile PBS and cultured on LB agar plates.
followed by overnight incubation at 37°C with 5% CO₂, after which viable colonies were counted.

**PPARγ adenoviral transfection.** Undifferentiated and differentiated THP-1 cells (1 × 10⁶ and 5 × 10⁵ cells, respectively) were infected with human PPARγ in adenovirus (Ad-hPPARγ) or Ad-GFP (Vector Biolabs, Philadelphia, PA) at an MOI of 25 in growth media containing 2% serum for 5 h, as we recently reported (35). After incubation with adenoviral vectors, the cells were washed twice with sterile PBS and then transferred into regular growth media without antibiotics. Ad-hPPARγ or Ad-GFP-transfected cells were then treated with 10 μM rosiglitazone at 6 h post-infection to activate the overexpressed PPARγ receptor. To keep the PPARγ receptor in an active state, fresh media with rosiglitazone were replaced every 24 h. Control cells transfected with Ad-GFP were similarly treated with rosiglitazone corresponding to the Ad-hPPARγ-treated cells. mRNA and protein were extracted from the cells for analysis at 24, 48, 72, and 96 h and processed as described previously.

**RNAi.** Gene silencing in THP-1 cells was performed with silencer select RNA interference (RNAi) oligonucleotides (Life Technologies, Grand Island, NY) targeting PPARγ and PON-2 mRNA and a nonrelated small interfering RNA (siRNA) control (scrambled control [sc); Santa Cruz and Life Technologies). Transfection was performed using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. Transfections in RAW and THP-1 cells were also conducted, using a cell line Nucleofector kit (Lonza, Walkersville, MD). The cells were incubated with siRNA complexes for 24, 48, and 72 h; mRNA and protein were then extracted for reverse transcription-PCR (RT-PCR) and Western blot analysis.

**Quantitative real-time PCR.** Total cellular RNA was extracted using an RNeasy RNA extraction kit (Qiagen) and a TRizol mRNA extraction kit (Life Technologies) according to the manufacturers’ protocols. The cDNA was synthesized from 1 μg or 500 ng of total RNA using the SuperScript first-strand synthesis system (Fermentas, Pittsburgh, PA). Real-time PCR was performed with TaqMan Universal PCR master mix (Life Technologies) to analyze PON-2 and PPARγ expression in cells. PPARγ and PON-2 mouse and human primers were purchased from Life Technologies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping control for normalization. PCR was conducted using Applied Biosystems (ABI Prism, 7900HT). Data were analyzed using the 2⁻ⁿδⁿδm method (36).

**Western blotting.** Protein extraction, electrophoresis, and gel transfer were performed as previously described (37, 38). Anti-mouse and anti-human PON-2 was purchased from GenScript (Piscataway, NJ) and Abcam (Cambridge, MA). Anti-PPARγ and β-actin antibodies were purchased from Santa Cruz Biotechnologies (Dallas, TX). Cell lysates were prepared using cell lysis buffer (Cell Signaling Technology, Danvers, MA). The protein content was determined with a Bio-Rad protein assay. Equal amounts of protein (20 to 25 μg) were analyzed by SDS–10% PAGE and transferred onto nitrocellulose membranes (Millipore). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline–0.1% Tween 20 (TBST) and incubated overnight at 4°C with primary antibodies. Membranes were washed with TBST and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnologies) for 1 h at room temperature in TBST. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Pierce) on a molecular imager ChemiDoc XRS system (Bio-Rad). Band densities were quantitated using ImageJ and normalized to their respective controls, as well as housekeeping controls such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnologies) or β-actin.

**In vivo treatment with PPARγ ligands.** C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed under specific-pathogen-free conditions at the Veteran’s Affairs Medical Center (VAMC; Decatur, GA). All experiments were approved by the VAMC Institutional Animal Care and Use Committee. As previously reported (39), male C57BL/6J mice, 8 to 10 weeks of age, were treated with PIO dissolved in methylcellulose (United States Biological, Salem, MA) at 10 mg/kg body weight or with an equivalent volume of methylcellulose alone used as a vehicle control (Veh) by oral gavage. After 24 h, the mice were infected with PAO1 intranasally (40–42) with 10⁶ CFU. Briefly, PAO1 stocks were grown as described above and adjusted to 10⁶ CFU based on an OD₆₀₀ of 0.2 (which yields ~10⁶ CFU/ml). The inoculum was adjusted to a concentration of 10⁵/0.1 ml. The inoculum was placed just above the nostril with a sterile pipette tip. Ketamine-xylazine combination anesthesia, i.e., ketamine (KetaVed [Vedco, St. Joseph, MO]); 9 mg/10 g [body weight]) and xylazine (Anased [Lloyd Laboratories, Shenandoah, IA]; 1 mg/g [body weight]), was administered intraperitoneally. At 4 h postinfection, the mice were again treated with vehicle or pioglitazone (10 mg/kg). Then, 24 h later, all the mice were sacrificed and lungs harvested. Quantitative PCR and Western blotting were performed on the lung samples harvested from these mice.

Each entire right lung was gently homogenized in 1 ml of PBS, the lysates were titrated in PBS, and 50-μl aliquots were plated on blood agar plates with 5% sheep blood (BD Biosciences). The plates were incubated overnight at 37°C, and the colonies were counted. The colony counts were normalized to the wet weight of the lung tissue.

**BAL.** Bronchoalveolar lavage (BAL) was performed after sacrificing mice postinfection as described previously (37, 43). Briefly, mouse tracheas were exposed through a small skin incision on the anterior neck. Once exposed, a 21-gauge lavage needle was inserted into the trachea. Each mouse was lavaged three times with 1 ml of PBS. The BAL fluid was then spun at 300 × g for 5 min at 4°C. The supernatant was collected and stored at −80°C. The cellular fraction was lysed using 0.5% Triton X-100. Lysates were tritiated and plated in a volume of 50 μl on blood agar plates with 5% sheep blood. CFU counting was performed after 24 h. The cells harvested from the BAL fluid were also subjected to ex vivo analysis using quantitative RT-PCR (qRT-PCR) for studying the expression levels of PON-2 and PPARγ.

**Statistical analysis.** All experiments were repeated at least three times, and data were expressed as means ± the standard errors. Statistical analysis was performed using a paired Student t test and one-way and two-way analyses of variance using GraphPad Instat; a P value of <0.05 was considered statistically significant.

**RESULTS**

**PPARγ agonists enhanced phagocytosis and bacterial clearance.** We have previously demonstrated that lipid mediators, such as 15d-PGJ₂, are potent immunostimulators and play a pivotal role in host response to *P. aeruginosa* (44). However, the immunostimulatory mechanisms of these lipid mediators are not well defined (45). Since 15d-PGJ₂ is a PPARγ ligand, we investigated whether these effects were mediated by PPARγ and whether PPARγ agonists enhance bacterial phagocytosis and killing. BMDMs from wild-type mice were treated with 10 μM PGD₂, PGJ₂, or PIO for 24 h prior to infecting cells with *P. aeruginosa* (PAO1) at an MOI of 10. As shown in Fig. 1A, treatment with PGD₂, PGJ₂, or PIO reduced bacterial numbers, and this effect was attenuated by cotreatment with the PPARγ antagonist GW9662, indicating that bacterial clearance was mediated through activation of the PPARγ.

In order to examine whether the clearance of bacteria has an effect on phagocytosis, RAW cells were infected with PAO1-GFP at an MOI of 50. The cells were then cultured with gentamicin-containing media for 5 h to remove any extracellular bacteria. RAW cells were harvested at each indicated time point using 0.05% trypsin-EDTA, with subsequent washing with 1× PBS, followed by fixation in 0.5% paraformaldehyde. Cells were visualized and quantitated by flow cytometry. Increased phagocytosis was observed in RAW cells infected with PAO1 at 5 h postinfection.
The fluorescent cells were quantitated (Fig. 1C).

Cells pretreated with PIO overnight and exposed to PAO1-GFP at an MOI of 10 or with 25 and 50 μM 3-oxo-C12-HSL (QS molecules) for 18 h. The mRNA and protein expression levels of lasI, lasR, and lasO genes were significantly reduced in macrophages treated with PAO1; however, cells that were treated with PAO1 mutants that lack the expression of QS genes.

Concurrent with increased phagocytosis, significantly lower numbers of bacterial colonies were seen in PIO-treated THP-1 cells infected with PAO1 (Fig. 1D). Starting bacterial CFU was noted at 1.86 × 10⁵ at 1 h postinfection; bacterial colonies were 2-fold lower in PIO-treated cells than in controls. Colonies obtained from control cells decreased minimally at 5 h but by 7 h started to increase (data not shown). Bacterial colony counts from PIO-treated cells remained low throughout the course of the experiment. Collectively, these data suggest that PPARγ activation in macrophages inhibits bacterial growth and stimulates phagocytosis and bacterial killing by macrophages.

**P. aeruginosa** QS molecules downregulate PPARγ and PON-2 in monocytes and macrophages. A recent study by Griffin et al. showed that patients with CF infected with *P. aeruginosa* show decreased expression of PPARγ and PON-2 in cells from their BAL fluid. Furthermore, the same samples were also positive for the expression of lasI, an autoinducer responsible for the downstream synthesis of 3-oxo-C₁₂-HSL. However, the investigators did not establish a link between PON-2 and PPARγ.

Based on our initial observations suggesting that PPARγ agonists attenuate bacterial growth, we questioned whether PAO1 can directly modulate expression of PPARγ and PON-2 in host cells and whether the same effect is elicited by the QS molecules. To determine whether PAO1 and QS molecules induce alterations in PPARγ levels in host cells, we infected THP-1 and RAW cells with PAO1 (which expresses QS genes) or with las system QS mutants at an MOI of 10 or with 25 and 50 μM 3-oxo-C₁₂-HSL (QS molecules) for 18 h. The mRNA and protein expression levels of PPARγ was determined using qRT-PCR and Western blotting, respectively. PPARγ mRNA and protein expression was significantly reduced in macrophages treated with PAO1; however, cells that were treated with PAO1 mutants that lack the las system were able to induce PPARγ similar to control cells (Fig. 2A, C, and D, respectively). Similar to PPARγ, we found that PON-2 mRNA (Fig. 2B) and protein (Fig. 2C and D) were significantly reduced in cells that were treated with PAO1, whereas the expression of PON-2 was relatively preserved in cells that were treated with mutant strains (lasI and lasR) that lack the expression of QS genes. Quantitation of protein expression by densitometry indicated a significant reduction in PPARγ and PON-2 protein expression.
Pseudomonas aeruginosa Clearance and PPARγ

FIG 2 PAO1 QS molecules inhibit expression of PPARγ and PON-2. THP-1 cells were infected with wild-type PAO1 or las QS mutants at an MOI of 10 for 18 h. (A and B) The mRNA expression levels of PPARγ and PON-2 were significantly reduced by the parental PAO1 strain but not by its lasI and lasR QS mutants, as determined by qRT-PCR. (C and D) The protein levels of PPARγ and PON-2 were also reduced in cells infected with PAO1, as shown by a representative image and densitometry, as determined by Western blotting. THP-1 monocytes were treated with 3-oxo-C12-HSL at 25 and 50 μM for 18 h. (E and F) PPARγ and PON-2 mRNA was significantly reduced by 50 μM, as detected by qRT-PCR. (G and H) PPARγ and PON-2 protein levels were significantly reduced by 50 μM 3-oxo-C12-HSL but not by 25 μM, as shown in panel H. Representative Western blots for PPARγ and PON-2 protein expression, as shown in a representative Western blot (Fig. 2G). The reduction in protein expression was quantitated by densitometry and determined to be significant (~50% reduction) in cells treated with 50 μM 3-oxo-C12-HSL (Fig. 2H). Similar results were observed in RAW cells (data not shown). These data suggest that PAO1 downregulates the expression of PPARγ and PON-2 molecules that contribute to the clearance of PAO1. Furthermore, QS molecules of PAO1 are responsible for the downregulation of PPARγ and PON-2 in host cells, and this may be a mechanism by which bacteria evade host defenses by seizing host protective molecules.

PPARγ modulates PON-2 expression in monocytes and macrophages. Since our data show that PAO1 inhibits the expression of PPARγ and PON-2, we sought to determine whether PON-2 is regulated by PPARγ. By using the JASPAR computational software prediction program, we found three PPARγ transcriptional binding sites in the promoter of the PON-2 gene. In order to demonstrate a direct modulatory effect of PPARγ on PON-2 expression in monocytes and macrophages in vitro, we performed a series of PPARγ gain and loss of expression studies using a PPARγ adenovirus and siPPARγ. Both undifferentiated and PMA-differentiated THP-1 and RAW cells were infected with PPARγ adenovirus or control adenovirus (MOI of 25) for 24, 48, 72, and 96 h. Overexpression of PPARγ by an adenoviral construct significantly increased the mRNA and protein expression of PPARγ and, concomitantly, that of PON-2 (Fig. 3A, B, and C) in undifferentiated THP-1 monocytes. Similar results were also obtained in RAW cells (data not shown).

To determine the effects of PPARγ loss of expression, similar experiments as described above were performed in macrophages transfected with siPPARγ. Upon transfection with siPPARγ, maximal reduction in PPARγ mRNA level was observed at 48 h. PPARγ knockdown with siPPARγ in undifferentiated THP-1 cells reduced PPARγ as well as PON-2 mRNA levels (Fig. 3D). Similar results were obtained in RAW cells (data not shown). The mRNA and protein expression of PON-2 protein was also significantly reduced in PPARγ-silenced cells that were transfected with siPPARγ for 24 and 48 h, suggesting that expression of PON-2 is dependent on PPARγ (Fig. 3E and F).

Enhanced clearance of PAO1 in macrophages by PPARγ is partially dependent on PON-2. Thus far, our results suggest that PPARγ enhances bacterial killing and induces the expression of PON-2 in vitro. We next examined whether the enhanced bacterial killing by PPARγ is mediated through induction of PON-2. We used siRNA to silence the PON-2 gene in macrophages and treated them with PPARγ agonists. Knockdown of PON-2 was optimized in cells, and we noted maximal inhibition after 48 h of transfection with siPON-2 (Fig. 4A).

Based on these observations, THP-1 cells were transfected with

(>50%) in cells stimulated by PAO1 parental strain (Fig. 2D). This effect was attenuated in cells stimulated by the QS mutants. To further confirm that QS molecules are responsible for reduced expression of PPARγ, we treated cells with the recombinant QS molecule 3-oxo-C12-HSL (25 and 50 μM). We found that 50 μM 3-oxo-C12-HSL significantly reduced the mRNA expression of PPARγ and PON-2 mRNA (Fig. 2E and F) in macrophages. Furthermore, THP-1 cells treated with 50 μM but not 25 μM 3-oxo-C12-HSL showed a significant reduction in PPARγ and PON-2 protein expression, as shown in a representative Western blot (Fig. 2G). The reduction in protein expression was quantitated by densitometry and determined to be significant (~50% reduction) in cells treated with 50 μM 3-oxo-C12-HSL (Fig. 2H). Similar results were observed in RAW cells (data not shown). These data suggest that PAO1 downregulates the expression of PPARγ and PON-2 molecules that contribute to the clearance of PAO1. Furthermore, QS molecules of PAO1 are responsible for the downregulation of PPARγ and PON-2 in host cells, and this may be a mechanism by which bacteria evade host defenses by seizing host protective molecules.

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Based on these observations, THP-1 cells were transfected with
siPON-2 for 48 h, followed by the infection of cells with PAO1 at an MOI of 50. Bacterial colony counts were determined from the cell lysates at 1, 3, and 6 h. Silencing PON-2 in THP-1 cells increased bacterial CFU 3 h postinfection (Fig. 4B) compared to scrambled (Sc) siRNA controls. Adding PIO to PON-2-silenced cells partially rescued the effect on bacterial clearance to control levels. These data show that PON-2 plays a major role in PIO-induced PAO1 clearance.

To further confirm our findings, we used a natural activator of PPARγ, magnolol (29,30). Macrophages were treated with vehicle or 5 μM magnolol. A time course study was established to determine the induction of PPARγ by magnolol (data not shown). In agreement with the data presented above, we found that magnolol induced expression of PPARγ and PON-2 by 6 h (Fig. 4C). Induction of PON-2 was attenuated in cells that were treated with magnolol and GW9662 (data not shown), suggesting that magnolol induced expression of PON-2 in a PPARγ-dependent manner. Next, we wanted to determine whether magnolol exhibited bacterial killing effects comparable to those observed with PPARγ agonists. THP-1 cells treated with magnolol also demonstrated significantly higher (~5-fold) bacterial killing capacity than vehicle-treated cells upon infection with PAO1 at an MOI of 50 (Fig. 4D). Furthermore, we wanted to confirm that increased bacterial killing by magnolol involved PON-2. Macrophages (THP-1 and RAW cells) were transfected with siPON-2 or control siRNA in the presence of magnolol (5 μM). Cells treated with magnolol in the presence of siPON-2 were unable to clear bacteria compared to cells treated with control scrambled siRNA, suggesting that the bacterial killing by magnolol which induces PPARγ is also dependent on PON-2 (Fig. 4E). Collectively, these data show that PPARγ agonists enhance clearance of PAO1 in a PON-2-dependent manner.

Since our initial studies were conducted in a transient PON-2 knockout (KO) model, we wanted to confirm the role of PON-2 in PPARγ-dependent enhanced bacterial clearance in BMDMs derived from PON-2 KO mice. BMDMs derived from wild-type (WT) and PON-2 KO mice were pretreated with PIO and stimulated with PAO1. Bacterial clearance assay was performed on these cells as described above. WT BMDMs stimulated with PIO showed significantly enhanced bacterial clearance effects compared to the Veh-treated cells (Fig. 4F). In comparison, PON-2 BMDMs treated with PIO had a significantly attenuated effect on bacterial clearance (Fig. 4F). Interestingly, Veh-treated BMDMs derived from both WT and PON-2 KO mice were equivalent in their ability to clear bacteria.

**PPARγ induces PON-2 in mouse lungs in vivo and enhances clearance of PAO1.** Since our data show that PPARγ induces PON-2 in vitro and enhances clearance of PAO1, we wanted to investigate whether the PPARγ agonist can induce similar effects in vivo in mouse lungs. Wild-type mice (n = 10 to 12) were treated with PIO at 10 mg/kg in methylcellulose 24 h prior to infection. Mice were infected with PAO1 (10⁸ CFU/ml) via the intranasal route. Control mice were treated with vehicle and infected with PAO1. At 4 h postinfection, the mice were treated again with the same dose of PIO or vehicle. The mice were sacrificed at 18 h postinfection. BAL was performed, and the mouse lungs were har-

![FIG 3](https://example.com/fig3.png) **PPARγ modulates PON-2 expression in macrophages.** THP-1 monocytes were transfected with AdPPARγ or AdGFP (MOI of 25) for 24, 48, and 72 h. (A) mRNA for PPARγ and PON-2 at 24, 48, and 72 h posttransfection showed significantly increased levels of PPARγ. (B and C) Representative Western blotting and densitometry for PPARγ and PON-2 protein expression at the indicated times showed a concomitant increase in PON-2 protein expression. THP-1 monocytes were transfected with siPPARγ for 24 and 48 h. (D, E, and F) mRNA and protein levels for PPARγ and PON-2 were concomitantly reduced, as determined by qRT-PCR, Western blotting, and band densitometry. One-way ANOVA with Tukey’s test for multiple comparisons was performed on all the data sets. Significance: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
vested. The expression of PON-2 mRNA and protein was determined from the lungs. In agreement with our in vitro data, control mice that were infected with PAO1 showed a reduced expression of PON-2 mRNA (Fig. 5A) and protein (Fig. 5B). However, mice infected with PAO1 that were treated with PIO showed an increased expression of PON-2 mRNA and protein (Fig. 5A, B, and C). In addition to analyzing lung samples, we also studied PON-2 expression ex vivo in cells harvested from the BAL fluid of uninfected or PAO1-infected WT mice. qRT-PCR analysis showed a significant reduction in PON-2 expression levels in cells harvested from the BAL fluid of PAO1-infected mice.

Bacterial colony counts were determined from the lungs and BAL fluid. We found a significant decrease in the number of colony counts from lung homogenates of mice treated with PIO that were infected with PAO1 (Fig. 5C). A significant decrease in the CFU was also noted from the cells in the BAL fluid (Fig. 5D). These data demonstrate that PIO has an immunostimulatory effect and enhances the clearance of PAO1 in the lungs primarily via the induction of PON-2. Figure 6 shows a proposed schematic mechanism for our findings. Infection with PAO1/QS molecules (3-oxo-C12-HSL) downregulates expression of PPARγ, thus leading to an attenuated expression of PON-2, which is required for lysing the QS molecules. Reduction in PON-2 compromises the ability of host cells to lyse 3-oxo-C12-HSL and allows the bacteria to establish infection by expressing other virulence factors. Upon stimulation with PPARγ agonists, PPARγ nuclear receptors are activated, thereby increasing PPARγ transcriptional activity. Since PON-2 is a potential downstream target of PPARγ, PPARγ agonists induce PON-2 mRNA and protein expression level and subsequent lactonase activity, thereby attenuating PAO1 virulence and infection.

**DISCUSSION**

In this study, we show that PPARγ agonists modulate host responses to enhance the clearance of *P. aeruginosa*. Our data show that phagocytosis and bacterial clearance were significantly augmented by PPARγ agonists in macrophages in vitro and in the lungs of mice infected with PAO1 in vivo. Our data establish that the immunostimulatory effects of PPARγ are partially mediated through the induction of PON-2, a downstream target of PPARγ (29). Furthermore, our data show that PPARγ and PON-2 expression is attenuated when cells are infected with PAO1 or 3-oxo-C12-HSL but not with the lasI QS mutant strains of PAO1 (47).
These data show for the first time that PPARγ agonists can immunomodulate host response through induction of PON-2. PPARγ is a nuclear hormone receptor involved in metabolic regulation. Synthetic thiazolidinedione PPARγ agonists are currently used in patients with type 2 diabetes to increase insulin sensitivity. PPARγ agonists have also been shown to have anti-inflammatory properties. However, the role of PPARγ in immune responses is less well defined. Recent studies have shown that PPARγ is essential for the differentiation of alveolar macrophages whereas PPARγ is dispensable for development of macrophages in other organs, such as the heart and kidneys (48–50). These studies highlight a key role for PPARγ in alveolar macrophages and suggest that PPARγ may play a pivotal role in pulmonary host response to infections. Aronoff et al. showed that the activation of PPARγ in alveolar macrophages upregulates the phagocytosis of apoptotic neutrophils through increased expression of the CD36 surface receptor (25).

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Notably, previous studies have shown that deletion of PPARγ in lung macrophages impaired host defense when challenged with *Streptococcus pneumoniae* by delaying bacterial clearance and accelerating infection-induced mortality (28). Enhancement in host defense by PPARγ agonists has also been noted in other infectious agents such as *Candida albicans*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* (25–28, 51–54). Overall, these findings point toward a key role of PPARγ in the macrophage immune response, where the function of PPARγ goes beyond an anti-inflammatory role and extends into modulation of host defense. In chronic pathological states such as CF, infection by *P. aeruginosa*
further exacerbates inflammation and mortality by reducing already low endogenous PPARγ levels. It does so by its QS sensing molecule 3-oxo-C₁₂-HSL, which has been shown to bind and modulate the function of PPARγ (55, 56). Other mechanisms by which PPARγ may enhance host defense include the regulation of defensins, the increased expression of CD36, and the production of adiponectin (57, 58). Our data provide evidence for a novel mechanism by which PPARγ modulates macrophage function by inducing PON-2.

PON-2 is an intracellular protein which because of its lactonase activity hydrolyzes and thereby inactivates bacterial QS molecules (N-acyl-homoserine lactones), such as 3-oxo-C₁₂-HSL (59, 60). The concentration of 3-oxo-C₁₂-HSL that induces a proinflammatory response in resting macrophages is typically observed in vitro at 50 to 100 μM (61–65). 3-Oxo-C₁₂-HSL has been detected in vivo at a concentration of 1 to 20 nM in cystic fibrosis patient sputum and 1 to 2 μM in a murine P. aeruginosa model of acute lung infection (66, 67). P. aeruginosa culture secretes 3-oxo-C₁₂-HSL at low micromolar concentrations; however, 3-oxo-C₁₂-HSL can reach concentrations as high as 600 μM in biofilms (68), which is clinically relevant since biofilms are formed in vivo as in the lungs of CF patients (9, 69).

Devarajan et al. showed that PON-2-deficient macrophages have a marked impairment in their ability to hydrolyze 3-oxo-C₁₂-HSL. PON-2 KO macrophages showed an increase in endoplasmic reticulum (ER) and oxidative stress and reduced phagocytosis function. PON-2 KO mice also displayed reduced clearance of P. aeruginosa from the lungs (15). We show that activation of PON-2 is PPARγ dependent, and thus PPARγ agonists such as thiazolidinediones or natural agents such as magnolol can increase expression of PON-2. PON-2 has also been shown to have both anti-inflammatory and antioxidant properties and can protect cells against the effects of the P. aeruginosa virulence factor pyocyanin (16, 70). Griffin et al. (46) noted that PPARγ and PON-2 were lower in BAL fluid cells of patients with CF who had P. aeruginosa infection; however, these researchers did not establish a link between PPARγ and PON-2. Our results indicate that activation of PPARγ induces the expression of PON-2 and may therefore contribute to the immunostimulatory effects of PPARγ agonists. Studies to further define the molecular mechanisms by which PPARγ regulates PON-2 are ongoing in our laboratory.

Our data show that P. aeruginosa inhibits the expression of PPARγ in host macrophages, thus suggesting that this may be a mechanism by which bacteria evade host defenses. The mechanism by which QS molecules and bacteria downregulate PPARγ needs to be defined. We speculate that multiple transcriptional mechanisms may contribute to this effect, including the modulation of NF-κB, Nrf2, or API (42, 43). Cho et al. (71) have shown that Nrf2 may regulate PPARγ, which is an important antioxidant transcriptional factor that contributes to host defense. Other potential mechanisms may involve modulation of calcium signaling through the QS molecules. Recent studies have demonstrated that increased cellular calcium mediates degradation of PON-2 protein and mRNA. Since 3-oxo-C₁₂-HSL can increase cytosolic calcium, it can be hypothesized that 3-oxo-C₁₂-HSL may mediate changes in PPARγ and PON-2 levels by modulating the activity of key transcriptional factors (16, 17, 72, 73). The induction of NF-κB and interleukin-10 may also play a role in the inhibition of PPARγ, as these are upregulated by 3-oxo-C₁₂-HSL (74, 75).

In conclusion, our studies for the first time show that PPARγ agonists enhance host immune response against P. aeruginosa. PAO1 and QS molecules downregulate PPARγ and PON-2 in host cells. Central to the success of P. aeruginosa as an opportunistic pathogen is the genetic flexibility provided by its large genome and ability to form biofilms (49, 50). Because of its ability to develop resistance to multiple antibiotics (3), many P. aeruginosa strains have become multidrug resistant and totally drug resistant and therefore continue to be problematic from a treatment perspective. The development of novel therapeutics that block virulence mechanisms rather than simply kill or inhibit the growth of bacteria is urgently needed in order to complement the existing antibacterial arsenal. Therefore, immunomodulatory strategies to enhance host defenses are an attractive strategy, and such approaches are sorely needed for P. aeruginosa infections. Our data suggest that PPARγ-activating therapies may prove to be effective in treating resistant P. aeruginosa and perhaps other infections.

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