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BACTERIAL INFECTIONS

Bacterial Nucleotidyl Cyclase Inhibits the Host Innate Immune Response by Suppressing TAK1 Activation

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ABSTRACT Exoenzyme Y (ExoY) is a type III secretion system effector found in 90% of the *Pseudomonas aeruginosa* isolates. Although it is known that ExoY is a soluble nucleotidyl cyclase that increases the cytoplasmic levels of nucleoside 3′,5′-cyclic monophosphates (cNMPs) to mediate endothelial Tau phosphorylation and permeability, its functional role in the innate immune response is still poorly understood. Transforming growth factor β-activated kinase 1 (TAK1) is critical for mediating Toll-like receptor (TLR) signaling and subsequent activation of NF-κB and AP-1, which are transcriptional activators of innate immunity. Here, we report that ExoY inhibits proinflammatory cytokine production through suppressing the activation of TAK1 as well as downstream NF-κB and mitogen-activated protein (MAP) kinases. Mice infected with ExoY-deficient *P. aeruginosa* had higher levels of tumor necrosis factor (TNF) and interleukin-6 (IL-6), more neutrophil recruitment, and a lower bacterial load in lung tissue than mice infected with wild-type *P. aeruginosa*. Taken together, our findings identify a previously unknown mechanism by which *P. aeruginosa* ExoY inhibits the host innate immune response.

KEYWORDS nucleotidyl cyclase, ExoY, TAK1, MAPKs, NF-κB, proinflammatory cytokines

Received 4 April 2017 **Returned for modification** 12 May 2017 **Accepted** 13 June 2017

Accepted manuscript posted online 26 June 2017

Citation He C, Zhou Y, Liu F, Liu H, Tan H, Jin S, Wu W, Ge B. 2017. Bacterial nucleotidyl cyclase inhibits the host innate immune response by suppressing TAK1 activation. Infect Immun 85:e00239-17. https://doi.org/10.1128/IAI.00239-17.

Editor Beth McCormick, University of Massachusetts Medical School

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Pseudomonas aeruginosa infection usually causes pneumonia that can progress to sepsis and acute lung injury, especially in immunocompromised patients (1–3). During infection, *P. aeruginosa* utilizes a type III secretion system (T3SS) to inject effector proteins directly into host cells (4, 5). Four T3SS effector proteins have been well characterized in *P. aeruginosa*, including exoenzymes U, T, S, and Y (5), and recently new effectors secreted by T3SS were identified (6). Exoenzyme Y (ExoY) is a soluble nucleotidyl cyclase with similarities to *Bacillus anthracis* edema factor (EF) and *Bordetella pertussis* CyaA (7, 8). Most recently, filamentous actin (F-actin) was identified as a eukaryotic cofactor that is responsible for activation of ExoY in host cells (9). Once injected into a host cell, ExoY is recruited to actin filaments (9) where it massively increases the cytoplasmic levels of cyclic GMP (cGMP) and cUMP and modestly increases the levels of cAMP and cCMP (10, 11). These intracellular signals activate protein kinase A (PKA), which increases Tau phosphorylation, causing microtubule breakdown (12, 13). In the endothelium, microtubule disassembly initiates the formation of interendothelial cell gaps and increases macromolecular permeability (13, 14). ExoY also can disrupt the actin cytoskeleton (15) and mediate bleb-niche formation (16) in epithelial cells.

Bacterial infection activates pattern recognition receptors (PRRs), including Toll-like

receptor (TLR) signaling pathways (17). Upon recognition of a variety of molecular patterns specific for bacterial pathogens, TLRs recruit adaptor proteins such as MyD88, interleukin-1 (IL-1) receptor-associated kinase (IRAK), and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (17). Recruitment of these adaptors in turn triggers the activation of nuclear factor kappaB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling cascades, including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38. Activation of these signaling pathways leads to the biosynthesis of a group of immunoregulatory molecules such as TNF, IL-6, and arachidonic acid metabolites. Recent reports indicate that *P. aeruginosa* induces lung inflammation via a TLR4- or TLR5-dependent process, and recognition of *P. aeruginosa*-associated lipopolysaccharide (LPS) or flagellin by TLRs is necessary for induction of *Tnf* and *Il6* (18, 19).

Transforming growth factor β (TGF-β)-activated kinase 1 (TAK1, *Map3k7*) is a member of the MAPK kinase kinase family and was originally identified as a key regulator of MAPK activation in TGF-β-induced signaling pathways (20). Later, TAK1 was found to play a key role in the cellular response to a variety of stimuli (21–23). TAK1-deficient cells fail to activate transcription factor NF-κB and MAPK in response to IL-1β, TNF, and TLR ligands (24–26).

Much progress has been made to characterize ExoY in terms of the generation of nucleoside 3′,5′-cyclic monophosphates (cNMPs) and the regulation of integrity of the endothelial cell barrier. However, relatively little is known about the role of ExoY in the regulation of host innate immune responses. In this study, we demonstrate that ExoY inhibits TLR signaling pathways by downregulating the activation of TAK1.

RESULTS

ExoY inhibits cytokine production in epithelial cells. Airway epithelial cells play an important role in sensing and signal transduction during *P. aeruginosa* infection (27–29). We examined the nucleotidyl cyclase activity of ExoY by measuring the abundance of cAMP in *P. aeruginosa* PAO1- or PAO1 Δ*exoY*-infected A549 cells. We observed that a deficiency in ExoY yielded almost complete blockage of cAMP generation in response to *P. aeruginosa* infection (see Fig. S1A in the supplemental material), suggesting that ExoY from PAO1 possesses nucleotidyl cyclase activity.

Investigators have shown previously that RAW264.7 macrophages (30) or Chinese hamster ovary (CHO) cells (31) infected with *exoY* deletion strains of *P. aeruginosa* released lactose dehydrogenase (LDH) at levels similar to those of cells infected with wild-type *P. aeruginosa*. In accordance with these results, we found that A549 cells infected with ExoY-deficient PAO1 had death rates similar to those of cells infected with wild-type strains, as measured by LDH release assay (Fig. S1B). However, cells infected with PAO1 Δ*exoY* exhibited higher mRNA and protein levels of *Tnf* and *Il6* than cells infected with PAO1 (Fig. 1A to D). Similarly, infection of A549 cells with two other *P. aeruginosa* strains (PAK and PA388) in which ExoY had been deleted significantly enhanced mRNA expression of *Tnf* and *Il6* (Fig. S2). Complementation of PAO1 Δ*exoY* with pUCP*exoY* was sufficient to restore inhibition of *Tnf* and *Il6* expression (Fig. 1E to G).

The inhibitory effect of ExoY depends on nucleotidyl cyclase activity. ExoY is a soluble bacterial nucleotidyl cyclase. Complementation of PAO1 Δ*exoY* with pUCP*exoY*, but not with its catalytically inactive mutant, pUCP expressing the K-to-M change at position 81 encoded by *exoY* (pUCP*exoY*^{K81M}) (8), restored inhibition of *Tnf* and *Il6* mRNA and protein expression in epithelial cells (Fig. 2). Further, we used pUCP*exoY* or pUCP*exoY*^{K81M} to complement a PAK Δ*exoS exoT exoY* triple mutant strain and then infected A549 cells. Consistently, the PAK Δ*exoS exoT exoY* strain complemented with pUCP*exoY* but not with pUCP*exoY*^{K81M} inhibited *Tnf* and *Il6* mRNA expression in A549 cells (Fig. S3). Hence, the nucleotidyl cyclase activity of ExoY is required to suppress the host immune response.

ExoY inhibits cytokine production in macrophages. In response to *P. aeruginosa* infection, macrophages internalize and kill the pathogenic bacteria as well as induce

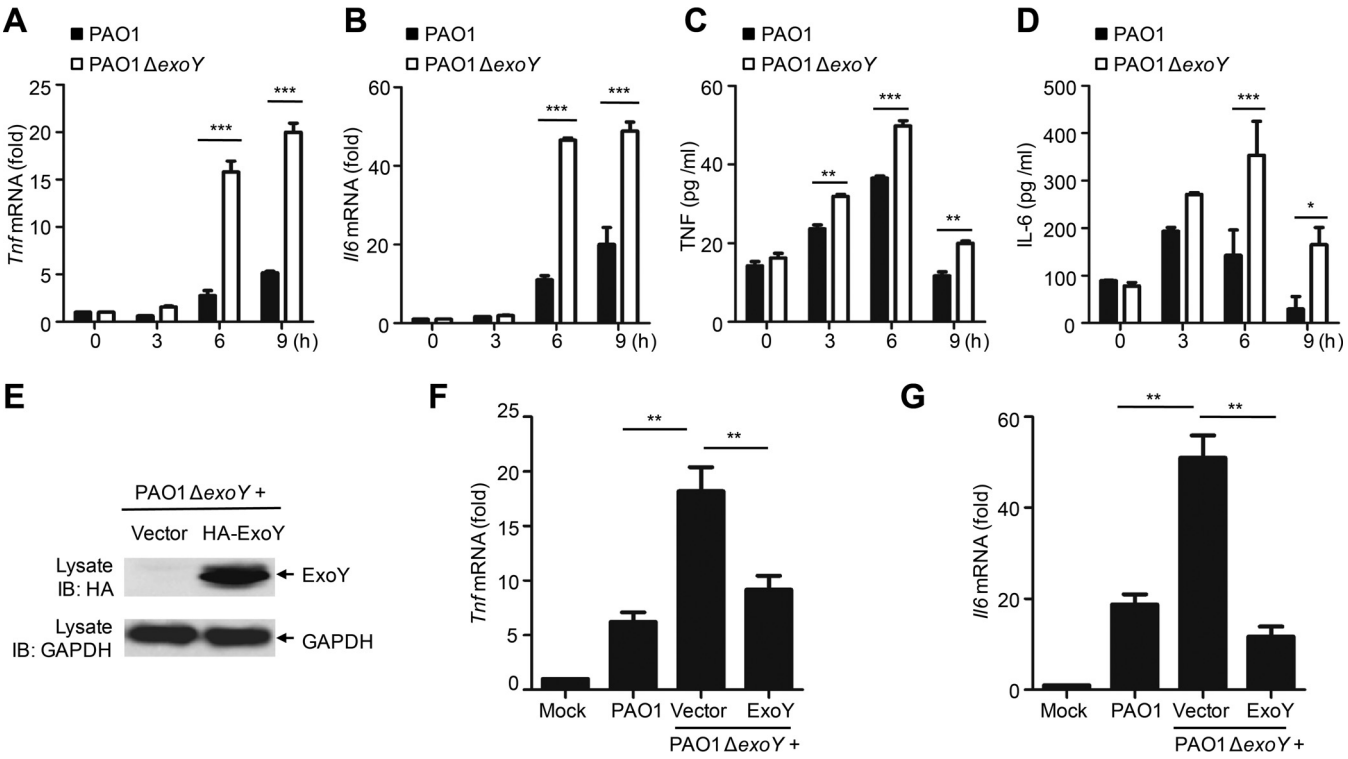

FIG 1 ExoY inhibits cytokine production in epithelial cells. (A and B) qPCR analysis of *Tnf* or *Il6* mRNA in A549 cells infected with PAO1 or PAO1 Δ*exoY* for 0 to 9 h. (C and D) ELISA of TNF or IL-6 release in supernatants of A549 cells infected as described for panels A and B. (E) Immunoblot (IB) of lysates from A549 cells infected with PAO1 Δ*exoY*(pUCP19) or PAO1 Δ*exoY*(pUCP*exoY*) for 6 h. (F and G) qPCR analysis of *Tnf* or *Il6* mRNA in A549 cells infected with the indicated bacteria for 6 h. Multiplicity of infection, 10 (A to G). Values are expressed as means ± standard deviations from three technical replicates (A to D, F, and G). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. All data shown are representative of at least three independent experiments.

the generation of proinflammatory cytokines (27, 32). To investigate the effect of ExoY on proinflammatory cytokine production in macrophages, we infected THP-1 cells (a human monocytic cell line) and mouse peritoneal macrophages with PAO1 or PAO1 Δ*exoY*. THP-1 cells infected with PAO1 Δ*exoY* exhibited higher levels of *Tnf* and *Il1β* mRNAs (Fig. 3A and B), but a lower level of *Il6* mRNA (Fig. 3C), than cells infected with PAO1. Similarly, infection of mouse peritoneal macrophages by PAO1 Δ*exoY* resulted in higher levels of *Tnf* and *Il1β* (Fig. 3D and E) and a lower level of *Il6* (Fig. 3F).

ExoY suppresses the activation of TAK1. Expression of proinflammatory cytokines is induced by activation of NF-κB and MAPKs in TLR signaling pathways. Compared with A549 cells infected with PAO1, cells infected with PAO1 Δ*exoY* underwent high phosphorylation of p65, p38, and JNK (Fig. 4). The presence of ExoY efficiently blocked

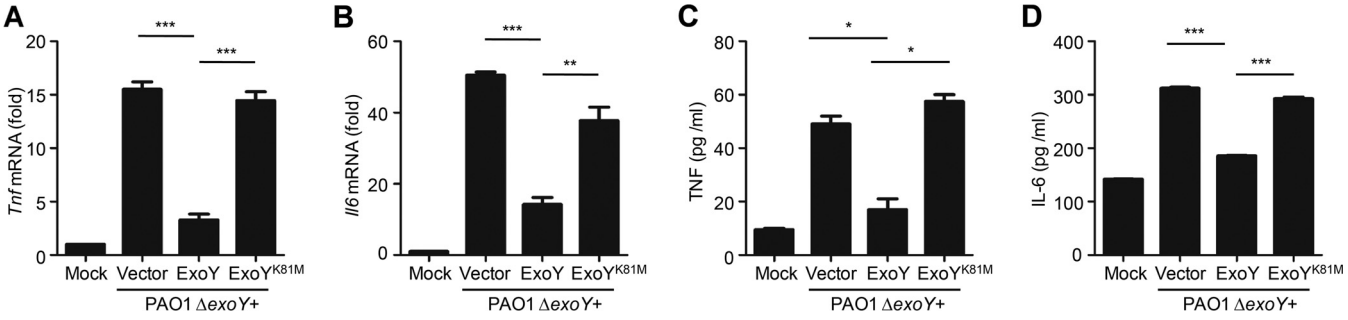

FIG 2 The inhibitory effect of ExoY on cytokine production is dependent on nucleotidyl cyclase activity. (A and B) qPCR analysis of *Tnf* or *Il6* mRNA in A549 cells infected with PAO1 Δ*exoY*(pUCP19), PAO1Δ*exoY*(pUCP*exoY*) or PAO1 Δ*exoY*(pUCP*exoY* ^{K81M}) for 6 h. (C and D) ELISA of TNF or IL-6 release in supernatants of A549 cells infected as described for panels A and B. Multiplicity of infection, 10. All data shown are representative of at least three independent experiments, and values are expressed as mean values ± standard deviations from three technical replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

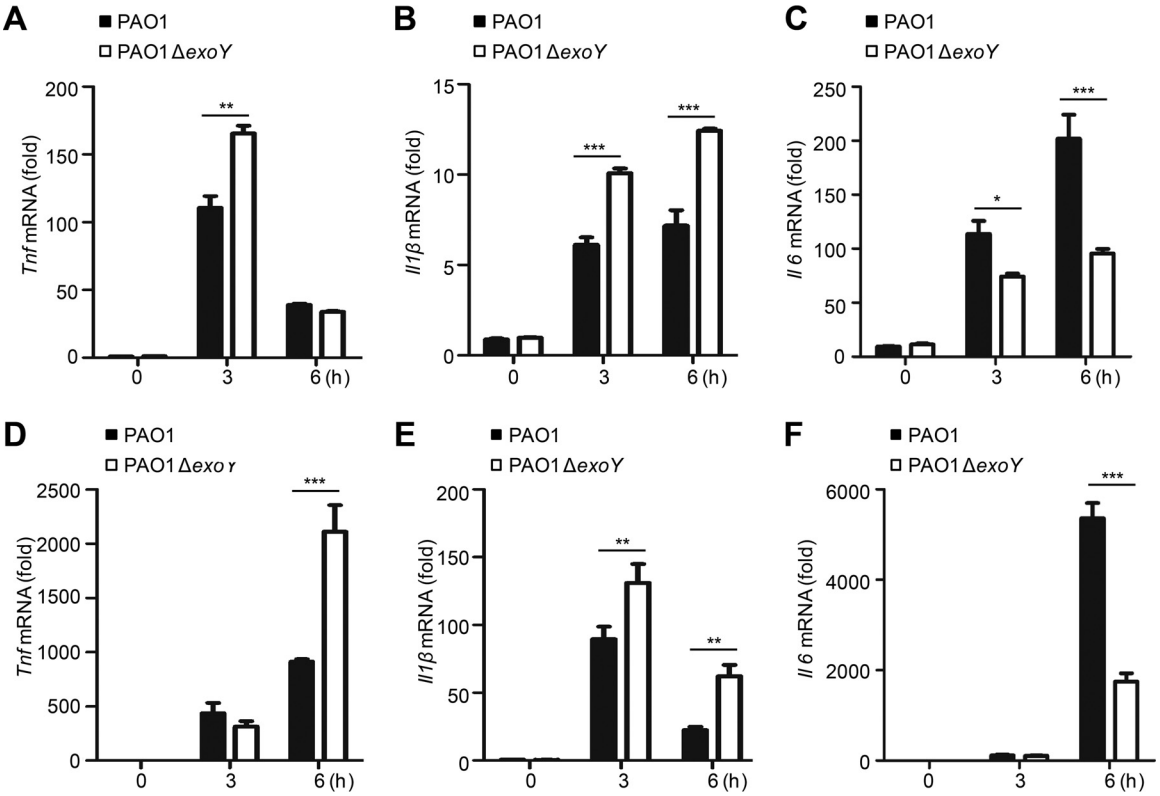

FIG 3 ExoY inhibits cytokine production in macrophages. (A to C) qPCR analysis of *Tnf*, *Il1β*, or *Il6* mRNA in THP-1 cells infected with PAO1 or PAO1 Δ*exoY* for 0 to 6 h. (D to F) qPCR analysis of *Tnf*, *Il1β*, or *Il6* mRNA in mouse peritoneal macrophages infected as described for panels A to C. Multiplicity of infection, 10. All data shown are representative of at least three independent experiments, and values are expressed as mean values ± standard deviations from three technical replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

activation of NF-κB and AP-1 in HEK293T cells stimulated by coexpression of TAK1 and TAB1 in a luciferase reporter assay (Fig. 5A and B). In contrast, ExoY had no effect on TRAF6-stimulated activation of NF-κB (Fig. S4A). Infection of A549 cells with PAO1 Δ*exoY* also resulted in higher phosphorylation of TAK1 at Thr187 (33, 34) (Fig. 5C), indicating that ExoY disrupted activation of TAK1. In line with these results, A549 cells

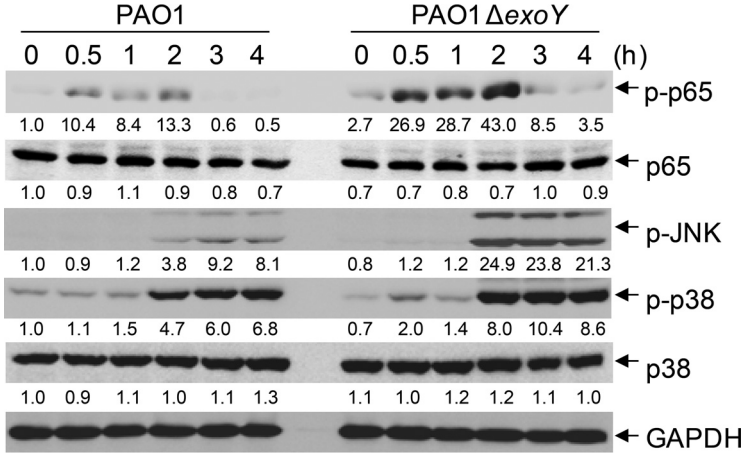

FIG 4 ExoY inhibits the phosphorylation of p65, p38, and JNK. (A) Immunoblotting of lysates from A549 cells infected with PAO1 or PAO1 Δ*exoY* at the indicated times (multiplicity of infection, 10). Densitometry quantification (under the band) of results (ratio of the expression of the indicated protein to that of GAPDH) is presented relative to those of uninfected (0 h, PAO1) cells, set as 1. Data shown are representative of at least three independent experiments.

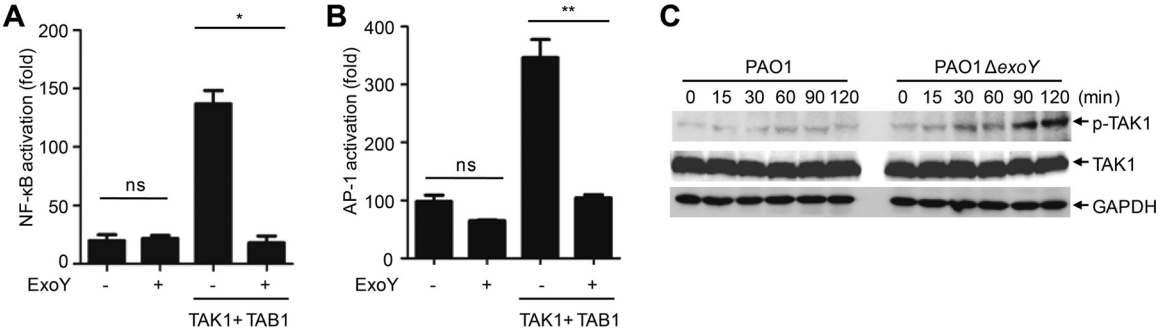

FIG 5 ExoY suppresses the activation of TAK1. (A and B) Luciferase assay of NF-κB or AP-1 reporter activation in HEK293T cells transfected with various plasmids. (C) Immunoblotting of lysates from A549 cells infected with PAO1 or PAO1 Δ*exoY* at the indicated times (multiplicity of infection, 10). All data shown are representative of at least three independent experiments, and values are expressed as means ± standard deviations from three technical replicates. *, $P < 0.05$; **, $P < 0.01$; ns, nonsignificant.

infected with the PAK Δ*exoS exoT exoY* strain complemented with pUCP*exoY* had lower activation of TAK1 and subsequent activation of NF-κB and MAPKs than cells infected with the PAK Δ*exoS exoT exoY* strain complemented with an empty vector or pUCP*exoY*^{K81M} (Fig. S4B).

TAK1 is essential for activation of NF-κB and MAPKs in TNF receptor (TNFR) and TLR/IL-1R signaling pathways (24, 25). To further confirm the inhibitory effect of ExoY on TAK1 activation, we transiently transfected the *exoY* or *exoY*^{K81M} strain into HEK293T cells or A549 cells and then stimulated these cells with TNF or IL-1β, respectively. Expression of ExoY but not ExoY^{K81M} efficiently blocked TNF- and IL-1β-stimulated TAK1 activation as well as downstream activation of NF-κB and MAPKs (Fig. S4C and D). These results indicated that ExoY had a generally inhibitory effect on TAK1 activation downstream of the TNFR and TLR/IL-1R signaling pathways.

To investigate whether ExoY inhibited cytokine production through TAK1, we constructed a stable TAK1 knockdown in A549 cells via a lentiviral delivery system. Knockdown efficiency was determined by Western blotting using a TAK1-specific antibody (Fig. 6A). The production of *Tnf* and *Il6* mRNA in TAK1 knockdown cells was significantly reduced upon PAO1 infection compared to the levels in A549 cells without the knockdown construct. Moreover, the effects of ExoY on cytokine production were absent in TAK1 knockdown cells (Fig. 6B and C). Therefore, ExoY may inhibit cytokine production through TAK1.

ExoY suppresses cytokine production and enhances *P. aeruginosa* pathogenicity in mice. To explore the functional role of ExoY in an animal model, mice underwent intratracheal instillation of 1×10^6 CFU of PAO1 or PAO1 Δ*exoY*. Infection with PAO1 Δ*exoY* resulted in elevated levels of *Tnf* and *Il6* in mouse lung homogenates compared to levels with PAO1 infection (Fig. 7A to D). Complementation of PAO1 Δ*exoY* with

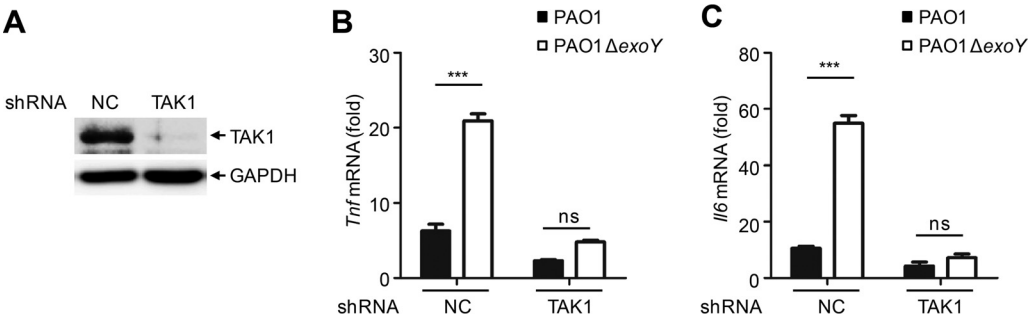

FIG 6 ExoY inhibits cytokine production through TAK1. (A) Immunoblotting of lysates from TAK1 knockdown or control A549 cells. (B and C) qPCR analysis of *Tnf* or *Il6* mRNA in control or TAK1 knockdown A549 cells infected with PAO1 or PAO1 Δ*exoY* for 6 h. Multiplicity of infection, 10. All data shown are representative of at least three independent experiments, and values in panels B and C are expressed as means ± standard deviations from three technical replicates. ***, $P < 0.001$; ns, nonsignificant. shRNA, short hairpin RNA; NC, negative control.

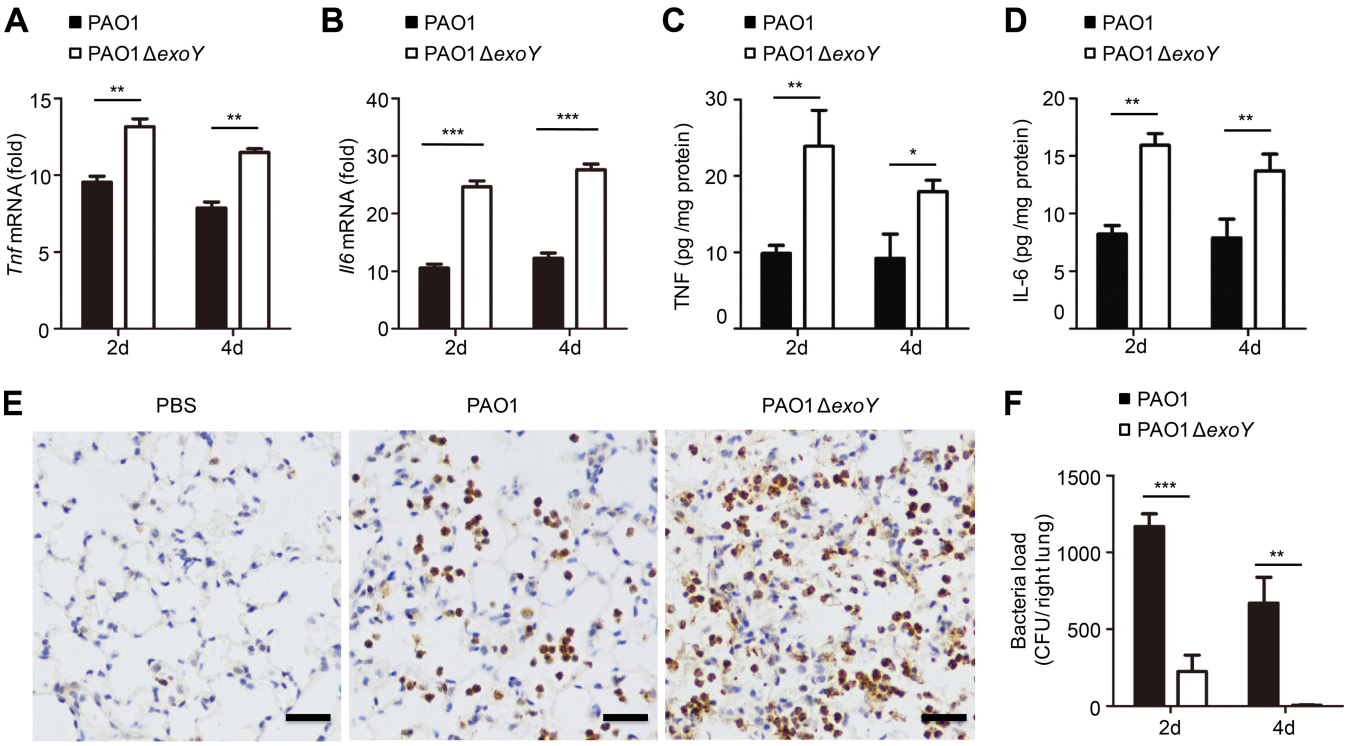

FIG 7 ExoY suppresses cytokine production and enhances *P. aeruginosa* pathogenicity in mice. (A and B) qPCR analysis of *Tnf* or *Il6* mRNA in lung homogenates from mice infected with PAO1 or PAO1 Δ*exoY* (1×10^6 CFU) for 2 or 4 days (d) ($n = 5$ per group). (C and D) ELISA of TNF or IL-6 release in supernatants of mouse lung homogenates infected as described in panels A and B. (E) MPO staining of mouse lung infected with PAO1 or PAO1 Δ*exoY* (1×10^6 CFU) for 24 h. Scale bar, 50 μm. $n = 3$ per group. (F) Bacterial load in lung tissue of mice infected as described in panels A and B. All data shown are representative of three independent experiments. Values in panels A to D and F are expressed as mean values ± standard deviations from five mice per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

pUCP*exoY*^{K81M} eliminated the inhibitory effect of ExoY on cytokine production in the mouse lung (Fig. S5).

Neutrophils are recruited into infected airways via inflammatory mediators during lung infection (35, 36), and they play an essential role in the clearance of *P. aeruginosa* from the lung (37, 38). We evaluated infiltration of neutrophils into the lung after *P. aeruginosa* infection by means of immunohistochemical staining of myeloperoxidase (MPO). Lung tissue infected with PAO1 Δ*exoY* had substantially higher neutrophil infiltration (Fig. 7E) and lower bacterial load (Fig. 7F) than lung tissue infected with PAO1. Therefore, *P. aeruginosa* ExoY inhibits the host immune response to allow the bacteria to establish a successful infection.

DISCUSSION

P. aeruginosa strains that harbor *exoY* can induce severe lung damage in the host (1). Despite considerable evidence that ExoY is an essential effector for the disassembly of microtubules (12, 13), relatively little is known about how ExoY regulates the innate inflammatory response. Here, the results obtained from our study demonstrate that ExoY disrupts activation of TAK1, thereby suppressing the production of proinflammatory cytokines during *P. aeruginosa* infection.

Appropriate inflammatory responses are crucial for controlling acute *P. aeruginosa* infection (39–41). Proinflammatory cytokines, induced following pathogen stimulation, recruit and activate factors of the acute-phase response to stimulate T and B cells for pathogen clearance (42). Inhibition of cytokine production by ExoY could block the innate and adaptive immune responses and improve the likelihood of a bacterial infection. Indeed, mice infected with PAO1 Δ*exoY* had a smaller bacterial load in their lungs than mice infected with PAO1. Consistently, results from a study involving a mouse model of burn injury showed that mice infected with a *P. aeruginosa* strain that

carried a knockout of ExoY had a higher survival rate than mice infected with wild-type *P. aeruginosa* (strain 388; 10^3 CFU, injected subcutaneously) (43). In a BALB/c mouse model of acute pneumonia, Lee et al. deleted ExoY in the PAK strain and found only minor effects on the bacterial load in the lungs of infected female mice (31). Vance et al. reported that infection with an ExoY-deficient PAO1 strain had no significant effect on bacterial colonization in the lungs of neutropenic C57BL/6 mice (30). The differences between the results of our study and those of others may be due to the use of a different mouse model. Sufficient recruitment of neutrophils to the site of infection was essential for clearance of *P. aeruginosa*. BALB/c mice have a lower inflammatory response than mice with a C57BL background and decreased accumulation of neutrophils upon bacterial stimulation (44, 45). In Δ*exoY* strain-infected BALB/c mice, recruitment of neutrophils to the lungs may be blunted compared with that of C57BL/6 mice infected with the Δ*exoY* strain. In the neutropenic C57BL/6 mouse model, the effects of ExoY on neutrophil recruitment and subsequent bacterial clearance may be masked.

P. aeruginosa ExoY produces four types of nucleoside 3′,5′-cyclic monophosphates (cNMPs) with a preference for cGMP and cUMP formation (10). cAMP and cGMP are well-characterized second messengers that regulate numerous cellular functions via specific effectors (46, 47). However, only in recent years have cCMP and cUMP been unequivocally identified as second messengers in mammalian cells by means of highly specific and sensitive mass spectrometry analyses (48, 49). The functional roles of cCMP and cUMP are poorly understood, and their effect on the immune response remains to be clarified (46, 50, 51). Research regarding cGMP focuses primarily on the cardiovascular system, neurophysiology, and metabolic diseases (52–54). Baldissera et al. reported that BAY 60-2770, an activator of soluble guanylate cyclase, could inhibit airway inflammation to relieve ovalbumin-induced allergic asthma (55). Chang et al. reported that plecanatide-mediated activation of guanylate cyclase could reduce the production of multiple proinflammatory cytokines in dextran sodium sulfate (DSS)-induced colonic inflammation (56). The results of these two studies support the idea that cGMP has an inhibitory effect on inflammation, but the exact mechanism remains unknown. Increases in intracellular cAMP have been shown to suppress the expression of inflammatory mediators, such as TNF and IL-12 (57–59). The cross talk between the cAMP pathway and NF-κB/MAPKs varies by cell type and stimulus, with many potential mechanisms and discrepancies described in the literature (60–62). Here, we found that second messengers produced by *P. aeruginosa* ExoY inhibited cytokine production via suppression of TAK1, which may shed light on the relationship between second messengers and the production of inflammatory mediators.

We found that ExoY inhibited *Il6* production in infected A549 cells but promoted *Il6* production in infected macrophages. Consistent with our results, Aronoff et al. reported that treatment with the cAMP analog 6-Bnz-cAMP promoted LPS-induced IL-6 production but suppressed TNF production in alveolar macrophages and peritoneal macrophages (58). These authors also showed that cAMP analogs decreased LPS-stimulated generation of TNF and IL-6 in dendritic cells (58). In addition, TAK1-deficient peritoneal macrophages were found to express similar levels of IL-6 but lower levels of TNF and IL-1β than wild-type peritoneal macrophages upon LPS stimulation (63). Therefore, modulation of IL-6 production by cAMP in different cell types may depend on different mechanisms.

It has been reported that ExoU can activate NF-κB and JNK signaling pathways to promote IL-8 production (64, 65). And recently ExoS and ExoT were reported to inhibit generation of reactive oxygen species in neutrophils by downregulating phosphatidylinositol 3-kinase (PI3K) signaling and thus decreasing bacterial clearance (66). Here, we demonstrated that the *P. aeruginosa* virulence factor ExoY, which is known to generate cNMPs in host target cells, downregulates the activation of TAK1, as well as downstream NF-κB and MAPKs, to inhibit production of proinflammatory cytokines during *P. aeruginosa* infection. These findings implicate ExoY as a negative regulator of the TLR signaling pathways to dampen the host's ability to clear bacteria in a mouse model. Our results provide a molecular basis for the pathological actions of ExoY in relation to the

immune system and may inform the development of novel therapies for *P. aeruginosa* infection.

MATERIALS AND METHODS

Animals. C57BL/6 mice were bred under specific-pathogen-free conditions at the Laboratory Animal Center of Tongji University. Female mice, aged 6 to 8 weeks, were used in the preparation of peritoneal macrophages; male mice, aged 12 to 14 weeks, were used for bacterial infection. All animal studies were conducted in accordance with institutional guidelines and complied with protocols that had been approved by the Animal Experiment Administration Committee of Tongji University.

Bacterium preparation and mouse infection. PAO1 and its Δ*exoY* strains were kindly provided by K. M. Duan (Northwest University, Xi'an, China) (67). PAK, PA388, PAK Δ*exoY*, PA388 Δ*exoY*, and PAK Δ*exoS exoT exoY* strains were from S. G. Jin (University of Florida, USA). Electrocompetent PAO1 Δ*exoY* and PAK Δ*exoS exoT exoY* cells were prepared as described previously (68). A 3′ hemagglutinin (HA)-tagged *exoY* wild-type or K81M mutant gene was cloned into the pUCP19 vector and introduced into Δ*exoY* or Δ*exoS exoT exoY* competent cells by electroporation (capacitance [C], 25 μF; resistance [R] = 200, voltage [V], 2.5 kV). Bacteria were streaked from frozen cultures onto Luria-Bertani (LB) agar, and then a single colony was cultured for 16 to 18 h at 37°C with shaking in LB broth supplemented with 100 μg/ml of ampicillin. For mouse infection, the bacteria were washed at least three times with phosphate-buffered saline (PBS), and 50 μl of a suspension of *P. aeruginosa* in PBS was delivered into the lung of an anesthetized mouse via intratracheal instillation.

Antibodies. Anti-TAK1 antibody (sc-7162) was obtained from Santa Cruz Biotech. Anti-p-TAK1 Thr187 (4536S), anti-p-p65 (3033S), anti-p-p38 (9215S), anti-p-JNK (9251S), anti-IκBα (4812S), and anti-p38 (9212S) antibodies were obtained from Cell Signaling Technology. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (G9545) and anti-HA (SAB1306169) antibodies were obtained from Sigma-Aldrich.

Cell culture. All cell lines were tested to be mycoplasma negative by a commonly used PCR method. HEK293T and A549 cells obtained from the ATCC were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone). Human monocytic THP-1 cells (ATCC) and mouse peritoneal macrophages were cultured in RPMI 1640 medium (HyClone). All media were supplemented with 10% (vol/vol) fetal bovine serum (FBS; Gibco), 2 mM L-glutamine (HyClone), and 100 U/ml penicillin and streptomycin. Cells were grown at 37°C in a 5% CO_2 incubator. To induce differentiation of THP-1 cells, 50 ng/ml phorbol 12-myristate 13-acetate (PMA) was added for 48 h. Thioglycolate-elicited peritoneal macrophages were generated by injecting 2 ml of 4% thioglycolate solution into the peritoneal cavity of mice. Two to three days later, macrophages were collected by peritoneal lavage with RPMI 1640 medium. Infection assays were performed with 1×10^6 cells unless otherwise noted. All infection assays were performed in medium without antibiotics.

Generation of knockdown cell lines. To construct stable knockdown cell lines, pLKO.1 plasmids harboring the desired genes, together with the packing plasmids pSPAX2 and pMD2.G, were transfected into HEK293T cells at a ratio of 4:3:1 in a six-well plate for 6 h. Subsequently, the medium was replaced with 2 ml of fresh medium per well, and cells were maintained for another 48 h. Supernatants were collected, mixed with an equal volume of complete DMEM, and added to A549 cells. Cells were maintained for 24 h; the medium was removed, and the mixture was added again for another 24 h. Puromycin (2 μg/ml; Santa Cruz Biotech) was used to screen for positive cells.

Determination of cAMP. A549 cells were infected with PAO1 or PAO1 Δ*exoY* at the times indicated in the figures, and the level of cAMP was measured with a cAMP-Glo assay (V1501; Promega) according to the manufacturer's instructions. Briefly, 1×10^4 cells per well were infected in a 96-well plate. Cells were lysed with 20 μl of cAMP-Glo lysis buffer for 20 min at room temperature and then incubated with 40 μl of cAMP-Glo detection solution for another 20 min. Subsequently, 80 μl of cAMP-Glo reagent was added, and cells were incubated at room temperature for 10 min. Luminescence was measured with a plate-reading luminometer.

Dual-luciferase reporter assay. HEK293T cells were transiently transfected with pRL-NF-κB-Luc (or pRL-AP-1-Luc), pRL-TK, and the plasmids indicated in the figures for 48 h in a 24-well plate (a total of 500 ng of DNA). Cells were lysed and measured with a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Preparation of mouse lung homogenates. For RNA extraction, comparable sizes and sites of mouse lungs were collected in 500 μl of TRIzol reagent (Invitrogen). For cytokine measurement, whole lungs were harvested in 5 ml of cold PBS plus protease inhibitor cocktail. For determination of bacterial numbers, the right lungs of mice were aseptically removed and harvested in 1 ml of cold PBS. Lungs were homogenized on ice with a tissue homogenizer. The homogenizer was carefully cleaned and disinfected with 75% alcohol after each homogenization. The bacterial load was determined by plating serial 10-fold dilutions (in sterile PBS) of lung homogenates onto LB agar with ampicillin and incubation at 37°C for 24 h. For cytokine measurement, the homogenates were spun at 8,000 $\times$ *g* for 20 min at 4°C. Supernatants were collected for subsequent analysis.

Quantitative RT-PCR analysis. Total RNA was extracted with 500 μl of TRIzol reagent (Invitrogen) in a 12-well plate. A total of 1 μg of RNA was reverse transcribed with a ReverTra Ace quantitative PCR (qPCR) reverse transcription (RT) kit (Toyobo). A SYBR RT-PCR kit (Toyobo) was used with diluted cDNA (10:1) and primers according to the manufacturer's instructions. An Applied Biosystems 7500 real-time PCR system was used for subsequent analyses. The mRNA levels were normalized to those of *Gapdh*. The primer sequences are as follows for human genes: Tnf-F, CTGGCCCAGGCAGTCAGAT; Tnf-R, AGCTGCCC CTCAGCTTGAG; Il-6-F, ACTCACCTCTTCAGAACGAATTG; Il-6-R, CCATCTTTGGAAGGTTCAGGTTG; Gapdh-F,

CTGGGCTACACTGAGCACC; Gapdh-R, AAGTGGTCGTTGAGGGCAATG. The primer sequences are as follows for mouse genes: Tnf-F, GTCCCCAAAGGGATGAGAAGTT; Tnf-R, GTTTGCTACGACGTGGGCTACA; Il-6-F, AGATAAGCTGGAGTCACAGAAGGAG; Il-6-R, CGCACTAGGTTTGCCGAGTAG; Il-1β-F, CAACCAACAAGTGATATTCTCCATG; Il-1β-R, GATCCACACTCTCCAGCTGCA; Gapdh-F, TGGAGAAACCTGCCAAGTATGA; Gapdh-R, CTGTTGAAGTCGCAGGAGACAA.

Cell death assay. Cell death was determined with an LDH assay using a CytoTox 96 nonradioactive cytotoxicity assay kit (Promega) according to the manufacturer's instructions.

Cytokine release assay. Concentrations of cytokines in cell supernatants or mouse lung homogenates were measured with enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Westang Bio-Tech Co., Ltd.).

Transfection, immunoblot analysis, and densitometry quantification. HEK293T cells and A549 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) or ExFect transfection reagent (Vazyme) according to the manufacturers' instructions. The infected or transfected cells were harvested by 1× sodium dodecyl sulfate (SDS) loading buffer and were boiled at 95°C for 10 min. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose blotting membrane (GE Healthcare Life Science). The blots were blocked with 5% nonfat dry milk for 1 h at room temperature and subsequently incubated overnight at 4°C with the primary antibodies indicated in the figures. Following three washes (10 min each) with Tris-buffered saline plus Tween (TBST), the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000) for 1 h at room temperature. After three washes with TBST, the blots were developed with SuperSignal West Pico Plus chemiluminescent substrate (34578; Thermo Fisher Scientific) according to the manufacturer's instructions. Densitometries were measured with ImageJ software.

Immunohistochemistry. The lung tissues of mice infected with PAO1 or PAO1 Δ*exoY* were fixed in 4% paraformaldehyde (PFA). The tissues were dehydrated, embedded in paraffin, and cut into 5-μm sections. Paraffin sections were dewaxed with xylene and a graded alcohol series. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide, and nonspecific binding sites were blocked with 3% bovine serum albumin (BSA). Slices were incubated with polyclonal rabbit antibody against MPO overnight at 4°C. Sections were then incubated with secondary antibody at room temperature for 1 h.

Statistical analysis. Statistical significance between groups was determined by a two-tailed Student's *t* test and two-way analysis of variance (ANOVA). GraphPad Prism, version 5.0, software was used for all analyses. A *P* value of <0.05 was considered to indicate a significant difference.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00239-17.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

ACKNOWLEDGMENTS

We thank K. Duan (Northwest University) for PAO1 and its Δ*exoY* strains and members of Ge's laboratory for helpful discussions and technical assistance.

This work was supported by the National Natural Science Foundation of China (projects 91542111, 81330069, and 31030028).

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