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Atg7 Enhances Host Defense against Infection via Downregulation of Superoxide but Upregulation of Nitric Oxide

Xuefeng Li,*† Yan Ye,* Xikun Zhou,*† Canhua Huang,† and Min Wu*

Pseudomonas aeruginosa is an opportunistic bacterium that can cause serious infection in immunocompromised individuals. Although autophagy may augment immune responses against P. aeruginosa infection in macrophages, the critical components and their role of autophagy in host defense are largely unknown. In this study, we show that P. aeruginosa infection–induced autophagy activates JAK2/STAT1α and increases NO production. Knocking down Atg7 resulted in increased IFN-γ release, excessive reactive oxygen species, and increased Src homology-2 domain-containing phosphatase 2 activity, which led to lowered phosphorylation of JAK2/STAT1α and subdued expression of NO synthase 2 (NOS2). In addition, we demonstrated the physiological relevance of dysregulated NO under Atg7 deficiency as atg7−/− mice were more susceptible to P. aeruginosa infection with increased mortality and severe lung injury than wild-type mice. Furthermore, P. aeruginosa–infected atg7−/− mice exhibited increased oxidation but decreased bacterial clearance in the lung and other organs compared with wild-type mice. Mechanistically, atg7 deficiency suppressed NOS2 activity by downmodulating JAK2/STAT1α, leading to decreased NO both in vitro and in vivo. Taken together, these findings revealed that the JAK2/STAT1α/NOS2 dysfunction leads to dysregulated immune responses and worsened disease phenotypes. The Journal of Immunology, 2015, 194: 1112–1121.

Pseudomonas aeruginosa is a ubiquitous Gram-negative opportunistic bacterium and the most common cause of community-acquired pneumoniae (1–3). An improved understanding of the molecular pathogenesis of P. aeruginosa is urgently needed for developing novel strategies to combat its infection. Autophagy involves mammalian lysosomal degradation and was recently implicated in the degradation of intracellular bacteria, which protects host organisms against diverse pathogens (4). Atg7, an E1-like ubiquitin, is a critical factor for the formation of an Atg12-Atg5-Atg16L1 complex (5) and has been recently shown critical for immune response against bacterial infections (6–8).

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Abbreviations used in this article: AG, aminoguanidine; AM, alveolar macrophage; Atg7, autophagy-related protein 7; atg7−/−, atg7-deficient; BAL, bronchoalveolar lavage; DAPI, 4,6-diamidino-2-phenylindole; DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; IFN, IFN receptor; IRF1, IFN response factor-1; KO, knockout; 3-MA, 3-methyl adenosine; MPO, myeloperoxidase; NOS2, NO synthase 2; PARP, poly(ADP-ribose) polymerase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SHP2, Src homology-2 domain-containing phosphatase 2; siRNA, small interfering RNA; WT, wild-type.

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Alveolar macrophages (AMs), the resident mononuclear phagocyte in the respiratory tract, are part of the first line of host defenses against inhaled organisms by secreting chemokines and phagocytizing pathogens. Two antimicrobial mechanisms of tissue macrophages are production of reactive oxygen intermediates by the phagocyte oxidase (phox) and reactive nitrogen intermediates by NO synthase 2 (NOS2). Once phagocytized, macrophages will produce a spectrum of reactive oxygen species (ROS; mainly hydrogen peroxide, hydroxyl radical, and superoxide anion) and reactive nitrogen species (RNS; derived from NO or superoxide) to eradicate bacteria in lysosomes with the help of lysosome enzymes (9). The indigestible debris and excess ROS and RNS are subsequently evacuated from macrophages (9). However, the production of oxidative molecules needs to be tightly regulated as excessive ROS may impede the immune defense and hampering bacterial clearance, ultimately leading to tissue injury. Despite being involved in infection, the molecular mechanism of Atg7 during bacterial infection and clearance by AMs is largely unknown.

IFN-γ interacts with IFN receptor (IFNR) complex, which activates the JAK/STAT pathway, leading to synthesis of the transcription factor IFN response factor-1 (IRF1) and stimulation of NOS2 mRNA transcription. STAT1 is the most important IFN-γ-activated transcription factor for regulation of this response. JAK2 is activated to elicit phosphorylation of STAT1 (Tyr701). Phosphorylated STAT1 forms homodimers and translocates into the nucleus to bind the promoter of NOS2.

To investigate the molecular mechanism in oxidation regulation, we examined autophagy relevant proteins and found the involvement of Atg7 with NO levels. Using specific up- or downregulation approaches, we set out to elucidate the regulatory role of Atg7 in NO production and its relevance to bacterial killing in vitro and in vivo. Our investigation suggests that Atg7 may be critical for controlling P. aeruginosa infection progression through the JAK2/STAT1/NOS2 pathway to differentially impact NO production and H2O2 release.
Materials and Methods

Mouse and cells

tag7f/-deficient (tag7−/−) mice (C57BL/6j) were provided by Dr. Y. He at Duke University, and these mice were originally generated by M. Komatsu at Tokyo Metropolitan Institute of Medical Science. Exon 14 encoded the active site cysteine residue, which was disrupted to generate tag7−/− mice. To conditionally delete the target gene, tag7−/− mice were bred with estrogen receptor cre mice and were injected with 0.1 mg tamoxifen (Sigma-Aldrich, St. Louis, MO) daily for 5 d before experiments (10). The knockout (KO) mice were based on C57BL/6j genetic background, so normal C57BL/6j mice were used as wild-type (WT) controls. Mice were kept and bred in the animal facility at the University of North Dakota, and the animal experiments were performed in accordance with the National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee (10). MLE-12 and MH-S cells were obtained from American Type Culture Collection and cultured in HITES medium (MUSE) and RPMI 1640 medium (MH-S) supplemented with 5% FBS (HyClone Laboratories, Logan, UT) and 100 U/ml penicillin/streptomycin from American Type Culture Collection and cultured in HITES medium (MLE-12) and RPMI 1640 medium (MH-S) supplemented with 5% FBS (HyClone Laboratories, Logan, UT) and 100 U/ml penicillin/streptomycin (Life Technologies, Carlsbad, CA) in serum-free HITES medium according to the manufacturer’s instructions for transient expression.

Bacterial infection

P. aeruginosa strain PA01 WT was provided by Dr. S. Lory (Harvard Medical School, Boston, MA). PA01-GFP was obtained from Dr. G. Pier (Channing Laboratory, Harvard Medical School). P. aeruginosa Xen-41 expressing luciferase bioluminescence was bought from Caliper Company (PerkinElmer, Waltham, MA). After culturing in Luria-Bertani broth at 37 °C with vigorous shaking overnight, the bacteria were centrifuged at 6000 × g for 5 min and then resuspended in 5 ml fresh Luria-Bertani broth to allow growing until mid-logarithmic phase. The concentration of the bacteria was counted by reading at OD600 (0.1 OD = 1 × 108 cells/ml).

After anesthesia with 40 mg/kg ketamine, mice were given 1 × 105 (six mice per group) CFUs (suspended in 50 μl PBS) of P. aeruginosa by intranasal instillation and sacrificed when they were moribund. If indicated, 1 h before infection, the mice were given i.p. injections of the NOS2 inhibitor aminoguanidine (AG; 100 mg/kg body weight) or the NO donor NOC-18 (10 mg/kg body weight). Survival was determined using Kaplan-Meier curve. After BAL procedures, lung and other tissues were fixed in 10% formalin using a routine histological procedure. The formalin-fixed tissues were used for H&E staining to examine tissue damage postinfection (11). The lung, spleen, liver, and kidney were homogenized with PBS. The homogenates were used for counting the CFUs.

Before infection, cells were washed once with PBS and replaced with serum and antibiotic-free medium immediately. Cells were infected by P. aeruginosa at multiplicity of infection (MOI) of 10:1 (bacteria/cell ratio) for 1 h and then washed three times with PBS to remove the floating bacteria. For required groups, 100 μM AG or NOC-18 was added 30 min before infection. Bacteria on the surface of the cells were killed by adding 100 μg/ml polymyxin B and left in incubation for another 1 h. Cells were lysed with 1% Triton X-100 dissolved in PBS. Cell homogenates were used for CFU counts.

In vivo imaging

Mice were infected with 1 × 107 CFU P. aeruginosa Xen-41 following anesthesia using ketamine. At various time points postinfection, the whole body of the infected mice was imaged under an IVIS XRII system following the user guides provided by the company (PerkinElmer-Caliper) (12).

Cell death and oxidation assays

AMs isolated from lavage fluid were cultured in 96-well plates overnight. TUNEL assay, MTI assay, dihydro-dichlorofluorescein diacetate (H2DCF-DA) to detect ROS, primarily hydrogen peroxide assay, EuTc (europium tetracycline hydrogen peroxide quantification) assay, and mitochondrial membrane potential (JC-1) assay were applied following the manufacturer’s instructions (13–15). NO production was determined using Griess reagent (Sigma-Aldrich) analysis. Lung and other organ tissues were homogenized, and equal protein amounts were used for myeloperoxidase (MPO) and lipid peroxidation assay. Cytokine concentrations in the first 0.6 ml BAL fluid collected at the indicated times postinfection were measured by standard ELISA kits following the manufacturer’s instructions (eBioscience, San Diego, CA) (16–19).

Immunoblotting

Samples taken from cells or lung tissues from same batch of mice infected as above were lysed with RIPA buffer (30 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, and complete mixture [Roche] and phosphatases [Sigma-Aldrich]). Lysates were centrifuged at 14,000 × g for 15 min, and the supernatants were collected, and the concentration was quantitated. The samples were boiled for 10 min, and an equal amount was applied to 12% SDS-polyacrylamide minigels and electrophoresed. The proteins in the gel were then transferred to nitrocellulose filter membranes (Thermo, Rockford, IL). HRP-linked secondary Ab (Rockland, Gilbertsville, PA) and x-ray film (Kodak) were used for exposure (20, 21). Mouse polyclonal Ab anti-LC3, Beclin1, Bcl-2, Bax, cytochrome c, rabbit polyclonal Ab anti-IFN-γ, p-JAK2, JAK2, STAT1α, IRF1, Src homology-2 domain-containing phosphate 2 (SH2P), β-actin, goat polyclonal Ab anti-Atg5-Atg12, and p-STAT1α were bought from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit mAb anti-IRF3 poly(ADP-ribose) polymerase (PARP) and cleaved caspase-3 were bought from Cell Signaling Technology (Danvers, MA).

Cytokine profiling

Cytokine concentrations in cell culture supernatant or BAL fluid were measured by ELISA kits following the manufacturer’s instructions (eBioscience, San Diego, CA).

Measurement of mRNA expression

Total RNA was extracted using TRIzol (Invitrogen, Grand Island, NY) according to the manufacturer’s instructions. RNA was eluted in RNase-free water and stored at −70 °C. The expression of NOS2 mRNA was detected by Quantitect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA) forward 5′-TCTGGGATGACTGCTGTGC-3′ and reverse 5′-GGCTTCTGACCGTTTCTGG-3′. The mRNA expression was analyzed using the 2−ΔΔ Ct method (22).

Confocal laser scanning microscopy

AM, MH-S, and MLE-12 cells were cultured in glass-bottom dishes (MatTek, Ashland, MA). The fluorescence images were obtained by LSM 510 Meta confocal microscope (Carl Zeiss Micro Imaging, Thornwood, NY) (22).

Statistical analyses

Each experiment was conducted in triplicate or repeated independently at least three times. The differences in outcomes of tag7−/− mice are presented as percent or amount changes compared with WT controls after P. aeruginosa infection. Data were analyzed by Mann–Whitney U test using Prism 5.0 statistical software (GraphPad Software, La Jolla, CA) (23). The survival percentage was generated using Kaplan–Meier curve, with p < 0.05 from a log-rank test.

Results

Atg7 knockdown aggravated apoptosis in lung cells after P. aeruginosa infection

To determine whether Atg7 is involved in P. aeruginosa pathogenesis, we employed siRNA silencing to repress gene expression in murine alveolar macrophage cell line (MH-S). We first measured cell viability by an MTT assay and found that P. aeruginosa infection resulted in more cell death that is associated with Atg7 loss and infection extent (Fig. 1A). Similar results were also observed in primary AMs. In both cell types, Atg7 deficiency aggravated infection-induced cell death (Fig. 1A). Next, we analyzed cell death patterns and noticed that P. aeruginosa infection induced apoptosis in both MH-S and primary AMs by measuring mitochondrial potential (Fig. 1B). We also evaluated apoptosis-associated signals and found that P. aeruginosa infection increased...
P. aeruginosa survival of and NOC-18, a diazeniumdiolate small-molecule NO donor, the release was suppressed by Atg7 siRNA interference (Fig. 2B). To demonstrate the induction of autophagy by infection, we examined the punctate foci following infection to indicate LC3-phospholipid conjugation using fluorescence microscopy. As shown in Supplemental Fig. 1B, Atg7 knockdown resulted in less LC3 puncta than control siRNA in MH-S cells upon PAO1-GFP infection (MOI 10:1), suggesting that an impaired autophagy due to Atg7 loss may be associated with increased host cell death (19).

Atg7 deficiency promoted ROS release, whereas it limited NO release

ROS and RNS serve as important intracellular signaling molecules that influence cell metabolism and survival (24). We sought to determine levels of ROS and RNS in MH-S cells by H$_2$DCF (to detect primarily hydrogen peroxide) and Griess test, respectively, and observed that ROS generation was increased in Atg7 knockdown groups upon P. aeruginosa infection (Fig. 2A), whereas NO release was suppressed by Atg7 siRNA interference (Fig. 2B). After treatment with ROS inhibitor diphenyleneiodonium (DPI) and NOC-18, a diazeniumdiolate small-molecule NO donor, the survival of P. aeruginosa–infected Atg7$^{-/-}$ cells was restored (Fig. 2C). Although the addition of NOC-18 did not affect total ROS production in Atg7$^{-/-}$ cells upon P. aeruginosa infection (Fig. 2D), addition of DPI increased NO release in these cells (Fig. 2E). Although the overall levels of ROS were not affected by NOC-18, insufficient NO release may be related to the infection-induced cell death. Next, we used a bactericidal killing assay to measure the ex vivo clearance of intracellular P. aeruginosa by macrophages. We found that Atg7 siRNA silencing led to decreased P. aeruginosa clearance in MH-S cells after 2 h infection by CFUs (Fig. 2F). Importantly, DPI or NOC-18 was found to be able to augment bactericidal activities (Fig. 2F). There is no difference in uptake (phagocytosis) of P. aeruginosa between WT and Atg7$^{-/-}$ cells (Supplemental Fig. 1C). Note the uptake assay was performed at 30 min upon P. aeruginosa infection, whereas the clearance assay was performed at 2 h after P. aeruginosa infection, in which the cells were treated with polymyxin B to remove the surface bacteria and then detect the survived intracellular bacteria. These findings indicate that Atg7 deficiency dampens the innate immunity of macrophages by affecting ROS and NO production and bacterial eradication.

Induction of excessive inducible NO synthase (also known as NOS2) often occurs in an oxidative environment, and thus, high levels of NO have the opportunity to react with superoxide, leading to peroxynitrite formation and cell toxicity (25). NOS2 was found to be inhibited by Atg7 knockout with P. aeruginosa infection (Fig. 3A, Supplemental Fig. 2A). Besides, knockdown of NOS2 significantly diminished the production of NO (Fig. 3B, Supplemental Fig. 2B). When treated with DPI or NOC-18, NOS2 mRNA expression showed a strong correlation with NO production (Fig. 3C).

Autophagy is required to activate JAK2/STAT1/NOS2 signaling upon P. aeruginosa infection

Microorganisms use an array of mechanisms to survive in host environments, including ROS, NO, and cytokines (IFN-$\gamma$) (9, 26). NOS2 produces large quantities of NO upon stimulation by proinflammatory cytokines or other mediators. To define how Atg7 influences host defense, we assessed the production of IFN-$\gamma$, a critical cytokine for resistance against acute bacterial infection (27). As shown in Fig. 4A, P. aeruginosa infection induced IFN-$\gamma$ release in a dose-dependent manner, and after Atg7 knockdown, the production of IFN-$\gamma$ was further increased (Fig. 4B). However, knocking down NOS2, and adding DPI or NOC-18 did not affect the IFN-$\gamma$ production (Supplemental Fig. 2C–E).

Binding of IFN-$\gamma$ to its receptor IFNR results in rapid autophosphorylation and activation of IFN$\gamma$R-mediated JAK2, which in
turn regulates the phosphorylation and activation of STAT1. STAT1 modulates inflammatory responses through the regulation of NOS2 production (28). In this study, we found that JAK2/STAT1 signaling was significantly activated in MH-S cells upon *P. aeruginosa* infection. Despite a higher IFN-γ production, the JAK2/STAT1/NOS2 pathway was inhibited by Atg7 knockdown (Fig. 4C, Supplemental Fig. 2F), indicating a link of Atg7 to STAT1 activation. To determine whether JAK2/STAT1 activation was dependent on integrity of autophagy, we used rapamycin and 3-methyl adenine (3-MA) and found that 3-MA could effectively inhibit *P. aeruginosa*–induced JAK2/STAT1 activation (Fig. 4D).

Additionally, similar results were found in Atg5- and Beclin1-siRNA–transfected cells (Fig. 4E, Supplemental Fig. 3A, 3B). To further study the specific role of JAK2/STAT1/NOS2 activation by IFN-γ, we treated the cells with IFN-γ as a positive control and IFN-γ neutralizing Ab as a blocker. As shown in Fig. 4F, *P. aeruginosa*–induced JAK2/STAT1/NOS2 activation was inhibited by an anti–IFN-γ Ab. Finally, NO production detected by Griess test recapitulated the above results (Fig. 4G). These data indicate that autophagy blockade may hamper the JAK2/STAT1/NOS2 pathway, thereby impairing IFN-γ activation.

**FIGURE 2.** ROS and NO production are reversely correlated with *atg7* deficiency in MH-S cells upon *P. aeruginosa* infection. MH-S cells were transfected with control (Ctrl) siRNA or Atg7 siRNA for 24 h and then infected with PAO1 at different MOIs for 2 h. ROS generation (A) and NO release (B) in supernatant were determined by H$_2$DCF assay or Griess test, respectively. (C–E) Cells were pretreated with DPI (5 μM) or NOC-18 (100 μM) for 30 min, then infected with PAO1 for 2 h. (C) Cell viability was measured by MTT assay. ROS generation (D) and NO release (E) were determined by an H$_2$DCF assay or Griess test, respectively. (F) Bacterial killing of *P. aeruginosa* (Pa) by MH-S cells treated with DPI or NOC-18. Data are representative as means ± SD of three independent experiments. *p < 0.05, **p < 0.01. RFU, relative fluorescence units.

**FIGURE 3.** NOS2 contributes to NO release upon *P. aeruginosa* (Pa) infection. Twenty-four hours after the transfection of the MH-S cells with control (Ctrl) siRNA or Atg7 siRNA, the cells were infected with *P. aeruginosa* as above. (A) NOS2 mRNA abundance was detected by quantitative PCR. (B) NO release was determined by Griess test. (C) Cells were pretreated with DPI or NOC-18 as above and then infected with *P. aeruginosa*. NOS2 mRNA abundance was detected by quantitative PCR. Data are representative as means ± SD of three independent experiments. *p < 0.05, **p < 0.01.

**Autophagy facilitated IFN-γ signaling by negatively regulating ROS**

Because autophagy deficiency was shown to cause the accumulation of ROS, we surmised that ROS may play a role in *P. aeruginosa*–activated STAT1 signaling. To determine whether infection specifically induces high levels of hydrogen peroxide (H$_2$O$_2$, the most important ROS in regards to pathogen stimulation), we used EuTc assay to quantify the level of H$_2$O$_2$ and found that *P. aeruginosa* infection did cause H$_2$O$_2$ production 2 h postinfection (Fig. 5A), and Atg7 deficiency enhanced this process (Fig. 5B). In addition, 4-amino benzoyl hydrazide (mechanism-based inhibitor of peroxidase), apocynin (a potent inhibitor of NADPH-dependent ROS production), and N-acetylcysteine (a scavengers of hydrogen peroxide [H$_2$O$_2$]) could all abolish *P. aeruginosa*–induced H$_2$O$_2$, whereas DPI showed the best inhibitory efficiency in all ROS including H$_2$O$_2$ (Supplemental Fig. 3C). Further, IFN-γ induced H$_2$O$_2$ similarly as *P. aeruginosa* did, and these processes were inhibited by DPI (Fig. 5C). Next, we used exogenous H$_2$O$_2$ to mechanistically define the role of ROS against *P. aeruginosa* infection. Fig. 5D showed that NO production induced by IFN-γ or *P. aeruginosa* was inhibited by H$_2$O$_2$. We first showed that DPI further boosted activation of STAT1 and...
NOS2 (Fig. 5E), whereas exogenous H₂O₂ inhibited it. These results indicate that Atg7 deficiency put a brake on NO production (while increasing H₂O₂), thereby suppressing results indicate that Atg7 deficiency put a brake on NO production (while increasing H₂O₂), thereby suppressing.

SHP2 is an important regulator in the IFN-γ feedback loop, which inactivates the JAK2–STAT1 pathway (29). We hypothesized that ROS-mediated SHP2 activation is critical for inhibiting P. aeruginosa–induced STAT1 in autophagy-deficient cells is dependent upon ROS-mediated SHP2 activation. Thus, Atg7/SHP2 knockdown reactivated STAT1 under P. aeruginosa infection (Fig. 5G).

To determine the role of SHP2 in Atg7–associated immune response and bacterial clearance, we used bacterial killing assay to measure intracellular P. aeruginosa in MH-S cells. Consistent with the aforementioned data, bacterial burdens decreased in Atg7/SHP2 siRNA–cotransfected cells compared with Atg7 siRNA–transfected groups, whereas bacterial burdens further decreased with only SHP2 knockdown in a phagocytosis assay (Fig. 5H). Besides, SHP2 siRNA interference restored viability under Atg7 knockdown upon P. aeruginosa infection (Fig. 5I). These findings indicate that autophagy negatively regulates ROS-activated SHP2, which, in turn, facilitates P. aeruginosa–induced STAT1/ NOS2 activation.

atg7 deficiency led to increased lung injury and bacterial dissemination

AMs are the first line of innate host defense to eradicate bacteria in early infection in the lung (30). To confirm the essential role of AMs in bacterial clearance, we infected atg7−/− mice (31). atg7−/− mice and WT mice were infected with 1 × 10⁷ CFU of PAO1. Primary AMs were isolated by BAL to evaluate viability using an MTT assay. As shown in Fig. 6A, survival of AMs decreased by ~60% in atg7−/− mice compared with that of WT mice 24 h postinfection, suggesting that AM phagocytic function may be impaired in atg7−/− mice. Mitochondrial membrane potential was also found to be decreased in atg7−/− AM using a JC-1 fluorescence assay (Fig. 6B). To investigate whether atg7 deficiency is associated with ROS production in vivo during infection, we examined levels of superoxide in AMs. AMs of atg7−/− mice showed an ~2.3-fold increase in oxidative stress at 24 h postinfection compared with those of WT, as determined by an H₂DCF-DA assay (Fig. 6C). Besides, H₂O₂ production in KO mice was higher than that in WT mice (Fig. 6D). NO release in BAL was similarly increased as determined using the Griess reagent (Fig. 6E). By immunoblotting of lung homogenates, Atg7 deficiency disrupted the formation of Atg5–Atg12 complex both with and without infection and reduced conversion of LC3-I to LC3-II upon P. aeruginosa infection (Fig. 6F).
expression of NOS2 and phosphorylation of STAT1 for 30 min. Griess reagent was used to detect the generation of nitrite after IFN-γ infection. 

P. aeruginosa for double knocked down using Atg7 siRNA and SHP2 siRNA, MH-S cells were infected with P. aeruginosa or Atg7 siRNA transfection. (I) Bacterial killing of P. aeruginosa by MH-S cells treated with H2O2 with Ctrl siRNA, SHP2 siRNA, or Atg7 siRNA transfection. (J) Cell viability was measured by MTT assay. Data are representative as means ± SD of three independent experiments. *p < 0.05, **p < 0.01. RFU, relative fluorescence units.

FIGURE 5. ROS-regulated SHP2 inhibited P. aeruginosa–activated STAT1 in the absence of Atg7. (A) MH-S cells were infected with PAO1 (MOI 10) for different times. H2O2 production was determined using EuTc assay (absorbance at 617 nm). (B) MH-S cells were transfected with control (Ctrl) siRNA or Atg7 siRNA for 24 h and then infected with PAO1 (MOI 10, 2 h). H2O2 was measured as above. (C) MH-S cells were pretreated with DPI (5 μM) for 30 min. EuTc assay was used to measure H2O2 after IFN-γ or P. aeruginosa (Pa) infection. (D) MH-S cells were pretreated with H2O2 (10 mM) or DPI (5 μM) for 30 min. Griess reagent was used to detect the generation of nitrite after IFN-γ or P. aeruginosa infection. (E) Immunoblotting was used to determine NOS2 and phosphorylation of STAT1α after P. aeruginosa infection. (F and G) Twenty-four hours after being transfected with Ctrl siRNA or SHP2 siRNA or double knocked down using Atg7 siRNA and SHP2 siRNA, MH-S cells were infected with P. aeruginosa as above. Immunoblotting shows the expression of NOS2 and phosphorylation of STAT1α. (H) Bacterial killing of P. aeruginosa by MH-S cells treated with H2O2 with Ctrl siRNA, SHP2 siRNA, or Atg7 siRNA transfection. (I) Cell viability was measured by MTT assay. Data are representative as means ± SD of three independent experiments. *p < 0.05, **p < 0.01. RFU, relative fluorescence units.

Infection (Fig. 6F). Due to inflammatory responses, we assessed signaling proteins in lung tissues and found that IFN-γ markedly increased in the lungs of atg7−/− mice as compared with those of WT mice upon P. aeruginosa infection; on the contrary, the phosphorylation of JAK2 and STAT1α increased in WT mice compared with atg7−/− mice upon P. aeruginosa infection (Fig. 6F). Besides, the levels of IRF1 and NOS2 were found to be increased in WT mice compared with those of atg7−/− mice (Supplemental Fig. 3E) occurred in the lungs of atg7−/− mice. Besides, atg7−/− mice showed elevated bacterial dissemination after P. aeruginosa infection, and CFU numbers in the lung increased significantly as compared with WT mice in a time-dependent manner (Fig. 6I). Similar results were found in the liver, kidney, and spleen (Supplemental Fig. 4A). Taken together, these findings suggest that Atg7 is crucial for resistance to P. aeruginosa in the respiratory tract.

Impaired NO generation contributed to immune impotency in atg7−/− mice against P. aeruginosa infection

To assess the physiological significance of Atg7 in P. aeruginosa infection, we intranasally infected atg7−/− and WT mice with 1 × 107 CFUs of P. aeruginosa Xen-41 strain (derived from parental strain PAO1) through the intratracheal route (22). Xen-41 emits bioluminescence for in vitro and in vivo imaging using Caliper Xenogen IVIS XRII (PerkinElmer-Caliper). The dissemination of infection illuminated by bioluminescence was monitored for 7 d postinfection (Fig. 7A, 7B). atg7−/− mice were highly susceptible to P. aeruginosa infection and died as early as 15 h postinfection, with 66.7% mortality within 27 h, and finally only 16.7% of mice survived to day 7. However, 83.3% of WT mice survived up to 7 d after P. aeruginosa infection (Fig. 7C). The increased mortality was associated with uncontrolled bacterial growth, as atg7−/− mice exhibited wider dissemination of bioluminescence in the area of thoracic cavity 12 h postinfection. In contrast, WT mice showed significant initial clearance of the instilled bacteria, whereas the dissemination areas were more constrained than those in atg7−/− mice, as reflected by decreased bioluminescence starting 24 h postinfection (Fig. 7B). These data indicate that resistance to P. aeruginosa profoundly decreased in atg7−/− mice.
We next detected MPO activity of the lung and other organs. As expected, increased MPO in the lung, liver, spleen, and kidney suggests that oxidative stress has resulted from systemic spread of the invading bacteria (Supplemental Fig. 4B) or higher leukocyte recruitment and cytokine production. Lipid peroxidation indicates oxidative degradation of lipids. We detected lipid peroxidation in the lungs, liver, kidneys, and spleen and noticed that lipid peroxidation increased significantly in all *P. aeruginosa*–infected organs of *atg7*−/− mice compared with those of WT mice (Supplemental Fig. 4C), suggesting that superoxide release may be related to the systemic spread of the invading *P. aeruginosa* bacteria.

In macrophages, NOS2 is produced following exposure to endotoxins or cytokines to increase NO levels to control invading microorganisms or inhibit neoplasm growth (25, 32). To confirm that the impairment of NO release during infection contributes to the impaired host defense against *P. aeruginosa*, we elucidated whether exogenous supplementation of NO enhances the bactericidal capability to increase host defense. Indeed, we found that addition of NOC-18 significantly reduced the mortality of *atg7*−/− mice infected with *P. aeruginosa* (Fig. 7D, Supplemental Fig. 4D, 4E). Treatment with AG (an irreversible and selective inhibitor of NOS2) impeded bacterial clearance in WT mice, whereas combined treatment with AG and NOC-18 prior to infection substantially increased mouse survival rates postinfection (Fig. 7E, Supplemental Fig. 4D, 4E). These findings indicate that NOS2 expression and NO release induced by NOC-18 at least partially restored the bacterial killing capability of AMs in *atg7*−/− mice and protect mice from *P. aeruginosa*–inflicted death. To summarize the discoveries of this study, Fig. 7F illustrates a model delineating the role of Atg7 in *P. aeruginosa* infection and the underlying cell signaling process.

**Discussion**

In this study, we demonstrate that *P. aeruginosa* infection of *atg7*−/− mice exhibits a severe disease phenotype, implying a crucial role of this gene in host defense against infection. We find that *atg7* deficiency significantly increases ROS release but decreases NO production and bacterial clearance. The majority of previous work has focused on the role of Atg7 in cellular process induced by starvation or various other stress factors (31, 33); however, Atg7-dependent autophagy may also serve as an innate immunologic effector in antimicrobial responses. Different bacteria may have divergent pathogenesis because bacterial pathogens have developed a variety of virulence factors to subvert host defense to establish persistent infection (34). Previous studies have revealed that Atg7 contributes to plant basal immunity toward fungal infection (35), accompanied by production of reactive oxygen intermediates. The literature demonstrated that *atg7* deletion in the hematopoietic system resulted in loss of hematopoietic stem cell functions, severe myeloproliferation, and mortality within weeks (36). Another report, however, showed that knockdown of autophagy enhances the innate immunity in hepatitis C virus–infected hepatocytes (37). Our recent report revealed a role of Atg7 in inflammatory response against *Klebsiella pneumoniae* infection (38). Autophagy has also been reported to enhance
bacterial clearance against *P. aeruginosa* infection in in vitro models (39). These observations laid out the foundation for us to dissect the molecular mechanism for Atg7, which may be also pathogen specific.

Autophagy protein Rubicon has been reported to mediate phagocytic NADPH oxidase activation upon microbial infection (40), but the role of autophagy in regulating oxidation in *P. aeruginosa* remains undemonstrated. Our previous data have demonstrated that autophagy plays an essential role in *P. aeruginosa* clearance by alveolar macrophages (19). However, detailed pathogenic molecular mechanisms of *P. aeruginosa* remain to be learned. In NOS2 KO mice, it has been demonstrated that reactive nitrogen intermediate contributes to host defense against a restricted set of pathogens (41, 42). IFN-γ could induce MD-2 protein expression in corneal epithelial cells, which is mediated by JAK/STAT1 signaling during *P. aeruginosa* infection. However, whether this process is similar in macrophages is still unknown. In this report, we linked these processes and reveal that Atg7, perhaps to a bigger perspective, autophagy is indeed essential in immune response against bacterial infection. Atg7 loss leads to a spontaneous inhibition of critical innate immunity and severe lung injury, which may be due to the impairment of both epithelial and macrophage functions (43). However, tissues of uninfected *atg7*−/− mice display no significant pathological signs. We speculate that *P. aeruginosa* infection may trigger complex pathogenesis using multiple virulence factors (44), which is even more complex when intertwined with host multilayer signaling pathways, thereby leading to the inflammatory cascade. Our recent studies indicate that critical cysteines in *P. aeruginosa* are responsible for the oxidative sensing (44, 45). Elevated levels of IFN-γ and MPO in *atg7*−/− mice may be due to higher leukocyte recruitment and stronger proinflammatory cytokines (46). One of the striking findings of this study is that *atg7* deficiency could contribute to uncontrolled inflammatory responses, such as TNF-α, IL-1β, IL-6, and IFN-γ secretion. A sudden rise of IFN-γ may activate JAKs/STATs and their related host response (NO release) to help clear bacteria. NO may be an immunity player in host defense by blocking the synthesis of microorganism DNA or other mechanisms (47). Cell wall components of bacteria and fungi, pathogen-associated molecular patterns, trigger innate immunity, leading to increased expression of NOS2. LPS-mediated TLR4 activation functions on releasing transcription factor NF-κB, which interacts with IkB elements in the NOS2 5′, triggering NOS2 transcription. Cytokines, including TNF-α and IL-1β, also activate NO production (32, 48, 49). In this study, we demonstrated that under *P. aeruginosa* infection, IFN-γ could interact with the IFNR1 and IFNR2 complex that activates

![FIGURE 7.](http://www.jimmunol.org/)

**FIGURE 7.** NO generation contributes to resistance to *P. aeruginosa* infection in *atg7*−/− mice. WT mice and *atg7*−/− mice were infected with 1 × 10⁷ CFU of *P. aeruginosa*-Xen41 (six mice per group). Arrows indicate illuminated areas of different bacterial loads in the lung. (A) Images of the lung in different time points were obtained using digital camera in an IVIS XRII system. (B) Statistic analysis of lung infection level by measure luminescence signal using IVIS XRII software. Data are presented as means ± SD from six mice. (C) Survival test was represented by Kaplan-Meier survival curves (*p < 0.05, 95% confidence interval, log-rank test). (D) Survival of *atg7*−/− or WT mice treated with NOC-18, AG, or PBS after pulmonary *P. aeruginosa* infection. (F) A schematic diagram showing how Atg7 activates NOS2 and regulates the JAK2/STAT1 pathway to modulate the inflammatory response to *P. aeruginosa* (Pa) infection. *p < 0.05, **p < 0.01, ***p < 0.001. RLU, relative luminescence units.
JAK/STAT pathways, resulting in the synthesis of transcription factor IRF1 and stimulation of NOS2 transcription. Hydrogen peroxide is a factor that going too far is as bad as not going far enough. Our current study analyzed the involvement of H$_2$O$_2$ in detail using several critical specific inhibitors together with exogenous ROS species, suggesting that Atg7 loss augmented H$_2$O$_2$ release, whereas it reduced NO. A previous report demonstrated that atg7$^{-/-}$ mouse embryonic fibroblasts had higher levels of ROS under basal and starved conditions (50), but the authors did not determine the role and changes in each subtype of ROS. Despite its importance in killing bacteria, uncontrolled accumulation of ROS may cause lung injury, and bacteria also have oxidation-sensing mechanisms modulate the oxidation-mediated host response (51). For example, excessive ROS inhibits IFN-γ-induced JAK2/STAT1 activation in neurons (52). In astrocytes and BCR/ABL chronic myelogenous leukemia, ROS-mediated signaling positively regulates SHP2. ROS-generating mitochondria accumulates in mouse embryonic fibroblasts with an autophagy deficiency, and ROS-regulated SHP2 inhibits JAK2/STAT1 signaling (53).

The in vivo imaging technology allowed us to monitor the pathophysiology and dynamics of P. aeruginosa infection in real time. This animal imaging revealed infection dynamic and showed a wider and quicker infection spread with P. aeruginosa infections. The animal imaging may help with confocal imaging. We thank S. Rolling of the University of North Dakota imaging core for assistance with confocal imaging.

Acknowledgments
We thank S. Rolling of the University of North Dakota imaging core for help with confocal imaging.

Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Fig. 1

A

![Graph showing relative density of various proteins](image)

B

![Immunofluorescence images](image)

C

![Bar graph showing internalized bacteria](image)
FIGURE S1. (A) Densitometric quantification of the immunoblotting gel data presented in Fig. 1C (in text) using Quantity one software. (B) MH-S cells were transfected with Ctrl siRNA or Atg7 siRNA respectively, combined with LC3-RFP plasmid for 24 h and then infected with PAO1-GFP (MOI=10:1) for 2 h. LC3 congregations were found in Ctrl siRNA transfected cells upon Pa infection. Data are representative from three independent experiments. Scale bar=5 μm. (C) Bacteria internalization assay in MH-S cells 30 min after infection with pretreatment of DPI or NOC-18 either with Ctrl siRNA or Atg7 siRNA transfection, respectively. Average values and SDEVs were calculated from triplicate samples.
Supplementary Fig. 2
FIGURE S2. (A) MH-S cells were transfected with Ctrl siRNA or Atg7 siRNA at 50 nM for 24 h, respectively. The cells were infected with PAO1 for 2 h (MOI=10). Atg7 and NOS2 were detected by immunostaining. (B-D) MH-S cells were transfected with Ctrl siRNA or NOS2 siRNA, respectively. The cells were infected as above. (B) The expression of NOS2 was detected by real-time qPCR. (C) NOS2 knock-down was determined by immunoblotting. (D) ELISA was used to measure IFN-γ in MH-S cell supernatant. (E) MH-S cells were transfected as above, and pretreated with DPI (5 μM) or NOC-18 (100 μM) for 30 min, then infected with PAO1. ELISA was used to measure IFN-γ in cell supernatant. (F) Cells were transfected as above, and pretreated with STAT1 inhibitor Fludara (50 μM, 2 h). pSTAT1α was immunostained for nuclear translocation detection. The data are representative of three independent experiments. Scale bar=10 μm. (Average values and SDEVs were calculated from triplicate samples; *, p<0.05 by one-way ANOVA with Turkey’s post-hoc).
Supplementary Fig. 3

A) Western blots showing Atg5, Beclin1, and β-actin expression levels.

B) Western blots showing Beclin1 and β-actin expression levels.

C) Bar graph showing RFU with Ctrl, ABH, DPI, APO, and NAC treatments.

D) Bar graph showing NOS2 (fold change) with Ctrl siRNA, SHP2 siRNA, H2O2, and Pa treatments.

E) Bar graphs showing TNF-α, IL-1β, and IL-6 production with Normal and Infected conditions.
**FIGURE S3.** MH-S cells were transfected with Ctrl, Atg5 or Beclin1 siRNA at 50 nM, respectively. Protein lysates prepared 24 hours post-transfection were analyzed for Atg5 (A) and Beclin1(B) expression by immunoblotting. The data are representative of three independent experiments. (C) MH-S cells were pretreated with ABH (150 µM), DPI (5 µM), APO (100 µM) and NAC (100 µM) for 30 min, respectively. EuTc assay was used to measure H$_2$O$_2$ after Pa infection (MOI=10, 2 h). (D) MH-S cells were transfected with Ctrl siRNA or SHP2 siRNA, respectively. Cells were pretreated with H$_2$O$_2$ (10 mM, 30 min). The cell were then infected with Pa as above. The expression of NOS2 was detected by real-time qPCR. (E) WT mice and atg7$^{-/-}$ mice were infected with 1×10$^7$ CFU of PAO1. After 24 h, BAL fluids were collected. The pro-inflammatory cytokines were determined by ELISA, respectively. Data are showed as means ± SD of three independent experiments (*, $p$<0.05; **, $p$<0.01). One-Way ANOVA with Turkey's post-hoc. RFU, relative fluorescence units.
FIGURE S4. (A) WT mice and atg7−/− mice were infected with $1 \times 10^7$ CFU of PAO1. After 24 h, organs were homogenized in PBS and used for assessing bacterial colonies. (B) MPO activity in lung, liver, spleen, and kidney of the mice. (C) Levels of lipid peroxidation were observed in the lung, liver, kidney and spleen tissues as assessed by the thiobarbituric acid-reactive substance assay. (D) WT mice and atg7−/− mice were treated with AG (100 mg/kg body weight), NOC-18 (10 mg/kg body weight), or PBS 1 h before pulmonary Pa infection. Lungs were homogenized for RNA isolation 24 h post infection. NOS2 mRNA was detected by qPCR. (E) NOS2 mRNA expression in different organs were determined after Pa infection. The data are shown as means ± SD from three mice (one-way ANOVA (Tukey’s post hoc); *, p<0.05; **, p<0.01). RLU, relative luciferase units.