Annexin A2 Regulates Autophagy in Pseudomonas aeruginosa Infection through the Akt1–mTOR–ULK1/2 Signaling Pathway

Rongpeng Li, Shirui Tan, Min Yu, Michael C. Jundt, Shuang Zhang and Min Wu

*J Immunol* 2015; 195:3901-3911; Prepublished online 14 September 2015;
doi: 10.4049/jimmunol.1500967
http://www.jimmunol.org/content/195/8/3901

Supplementary Material
http://www.jimmunol.org/content/suppl/2015/09/13/jimmunol.1500967.DCSupplemental

Why *The JI*?
- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

References
This article cites 67 articles, 20 of which you can access for free at:
http://www.jimmunol.org/content/195/8/3901.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Annexin A2 Regulates Autophagy in *Pseudomonas aeruginosa* Infection through the Akt1–mTOR–ULK1/2 Signaling Pathway

Rongpeng Li,*† Shirui Tan,*‡ Min Yu,*§ Michael C. Jundt,* Shuang Zhang,*‖ and Min Wu*  

Earlier studies reported that a cell membrane protein, Annexin A2 (AnxA2), plays multiple roles in the development, invasion, and metastasis of cancer. Recent studies demonstrated that AnxA2 also functions in immunity against infection, but the underlying mechanism remains largely elusive. Using a mouse infection model, we reveal a crucial role for AnxA2 in host defense against *Pseudomonas aeruginosa*, as *anxa2*−/− mice manifested severe lung injury, systemic dissemination, and increased mortality compared with wild-type littermates. In addition, *anxa2*−/− mice exhibited elevated inflammatory cytokines (TNF-α, IL-6, IL-1β, and IFN-γ), decreased bacterial clearance by macrophages, and increased superoxide release in the lung. We further identified an unexpected molecular interaction between AnxA2 and Fam13A, which activated Rho GTPase. *P. aeruginosa* infection induced autophagosome formation by inhibiting Akt1 and mTOR. Our results indicate that AnxA2 regulates autophagy, thereby contributing to host immunity against bacteria through the Akt1–mTOR–ULK1/2 signaling pathway. *The Journal of Immunology*, 2015, 195: 3901–3911.

Annexin A2 (AnxA2), a member of the annexin family, is expressed in various human cells, such as endothelial cells, mononuclear cells, macrophages, marrow cells, and some tumor cells (1). Accumulating evidence indicates that AnxA2 plays multiple roles in signal transduction, cell proliferation, differentiation, apoptosis, endocytosis, exocytosis, and inflammation (1, 2). AnxA2 downregulation is associated with the occurrence, invasion, and metastasis of cancer, whereas its upregulation is related to the development, invasion, metastasis, and drug resistance of hepatocellular carcinoma, colorectal cancer, breast cancer, acute promyelocytic leukemia, and renal cell carcinoma (3). In addition, AnxA2 was shown to play diverse roles in pulmonary diseases, such as non-small cell lung cancer, adenocarcinoma, chronic obstructive pulmonary disease (COPD), and chronic inflammatory diseases (4); thus, AnxA2 may be a novel diagnostic serum biomarker for lung cancer and other diseases (5). For instance, AnxA2 is involved in p53-induced apoptosis in lung cancer (6). Increasing secretion of collagen VI and pulmonary elasticity of bronchial epithelial cells (7), along with increased AnxA2 on lung epithelial cell surface, were recognized by severe acute respiratory syndrome–associated coronavirus spike domain 2 Abs (8). An epithelial cell surface AnxA2/p11 complex is also required for efficient invasion of *Salmonella typhimurium* (9). Evidence also suggests that AnxA2 directly interacts with Rab14 in alveolar type II epithelial cells (10). In addition, AnxA2 inducer or inhibitor dramatically modulates AnxA2-related cell functions. Investigators demonstrated that cationic lipid-guided AnxA2 short hairpin RNA attenuates tumor growth and metastasis in a mouse lung cancer stem cell model (11), whereas IFN-γ stimulates AnxA2 expression in lung epithelial cells to enhance apoptosis (8).

*Pseudomonas aeruginosa* is an opportunistic bacterial pathogen causing acute and chronic pulmonary infection in immunocompromised people (12), such as patients with cystic fibrosis and COPD, and most of the morbidity and pathophysiology associated with these diseases is due to innate hypersusceptibility to bacterial infection (13). Innate immunity, primarily through inflammatory cytokine production, cellular recruitment, and phagocytic clearance by neutrophils and macrophages, is key to host control of *P. aeruginosa* infection (14). Recently, macroautophagy (hereafter referred to as “autophagy”) in macrophages was found to be involved in host defense, and it directly impacts immunity and the inflammatory response (15). Autophagy participates in the elimination of invasive bacteria through autolysosome by functioning as downstream factors of pattern recognition receptors (such as TLRs) (16) and pathogen-associated molecular patterns (17).

Our previous work indicated that autophagy plays a role in immune defense against *P. aeruginosa* infection (15). However, the roles of AnxA2 in host defense against *P. aeruginosa* infection have not been defined. Investigations indicated that cell surface AnxA2 serves as a receptor for *P. aeruginosa* in mammalian
epithelial cells for bacterial internalization into host cells (18). Evidence also suggests that AnxA2 participates in endocytosis and EGFR-mediated signal transduction in macrophages (19), because suppression of AnxA2 impairs the phagocytic ability of peritoneal macrophages (20). In this study, we hypothesized that AnxA2 plays roles in host defense against P. aeruginosa infection. To this end, we used wild-type (WT) and anxa2−/− mice to investigate the role and underlying mechanism of AnxA2 in P. aeruginosa infection, particularly the participation of various autophagy-related factors. We demonstrate that AnxA2 regulates autophagosome formation after PAO1 infection through the Akt1–mTOR–ULK1/2 signaling pathway.

Materials and Methods

**Mice**

C57BL/6J female mice (6–8 wk) were obtained from the Jackson Laboratory (21), and anxa2−/− mice that are constructed based on C57BL/6J mice were kindly provided by Dr. K. Hajar (Cornell University) (22). Exons 3 and 4 of anxa2 were disrupted with a cassette containing neo-mycin phosphotransferase driven by the phosphogluconokinase promoter to generate anxa2−/− mice (22). Animals were kept in a specific pathogen-free facility at the University of North Dakota (23). All animal studies were approved by the University of North Dakota Institutional Animal Care and Use Committee and performed in accordance with the animal care and institutional guidelines (IACUC approval #1204–4). The animal experimental procedures, including treatment, care, and end point choice, followed Animal Research: Reporting In Vivo Experiment guidelines.

**Primary cells and cell lines**

Mice were sacrificed, and the thoracic cavity and trachea were dissected. A small incision was made in the trachea via a 1-ml syringe with an angiocath (BD Biosciences, Franklin Lakes, NJ). The lungs were lavaged three times with 1 ml PBS containing 1% FBS (Life Technologies, Grand Island, NY). The retained bronchoalveolar lavage fluid (BALF) was centrifuged at 300 × g for 5 min at 4°C. The cell pellets were resuspended in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS and incubated on a culture plate for 1 h at 37°C/5% CO2 in an incubator to allow attachment of macrophages. Nonadherent cells were removed by washing with normal saline. Murine MLE-12 lung type II epithelial cells and MH-S alveolar macrophages (AMs) were obtained from American Type Culture Collection (Manassas, VA) and cultured following the manufacturer’s instructions (24).

**Bacterial preparation and infection experiments**

The P. aeruginosa WT strain, PAO1, was kindly provided by Dr. S. Lory (Harvard University) (25). PAK and PAO1-EGFP were obtained from Dr. G. Pier (Harvard University) (26). PAO1 Xen-41 was obtained from PerkinElmer-Caliper (Waltham, MA); Klebsiella pneumoniae was provided by Dr. V. Miller (University of North Carolina) (27); and Escherichia coli DH5α was obtained from New England Biolabs (Ipswich, MA). Bacteria were grown for ~16 h in lysogeny broth (LB) at 37°C for 220 rpm with shaking and pelleted by centrifugation at 5000 × g. Various mammalian cells were changed to antibiotic-free medium and infected with bacteria at a multiplicity of infection (MOI) of 20. To control for LC3 degradation, 5 mM NH4Cl was added to the medium for 2 h of bacterial infection in in vitro LC3 immunoblotting experiments (28). This short duration of culture with a low NH4Cl concentration did not cause significant cellular alterations. Mice were anesthetized with 45 mg/kg ketamine and instilled intranasally with 0.5 × 107 CFU PAO1 in 50 μl PBS (six mice/group) (29). Mice were monitored for symptoms and euthanized when they were moribund.

**In vivo imaging**

At various time points postinfection, P. aeruginosa Xen-41-infected mice were imaged with an IVIS XRII system (PerkinElmer, Waltham, MA), following the user guides provided by PerkinElmer-Caliper (30).

**RNA isolation and quantitative real-time PCR**

RNA was isolated from primary AMs. A total of 50-ng DNA-free RNA was subjected to first-strand cDNA synthesis using the SuperScript III First-Strand Synthesis System (Life Technologies). Quantitative real-time PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA) and gene-specific primers (Supplemental Table I, synthesized by Integrated DNA Technologies, Coralville, IA) in a CFX Connect Real-Time PCR Detection System (Bio-Rad). Relative transcript levels were obtained using 2–ΔΔCt threshold methodology following normalization with GAPDH (31).

**Plasmid construction**

Fami13A gene was amplified from MH-S genome DNA with specific primers (Supplemental Table I, synthesized by Integrated DNA Technologies) by PCR and cloned into the BamHI and HindIII sites of the pcDNA3.1 vector (Addgene, Cambridge, MA). Constructed plasmids were electroporated into DH5α using an Electroporator 2510 system (settings: 25 μF, 200 V, 2.5 kV, Eppendorf, Hauppauge, NY). Transformants were selected and maintained in LB medium containing 100 μM ampicillin (Sigma-Aldrich, St. Louis, MO). All of the nuclease, polymerase, and ligase used in molecular cloning were purchased from New England Biolabs.

**Transfection of small interfering RNA, plasmids, activators, and inhibitors**

AnxA2, Fam13A, Atg5, Atg7, Beclin1, and scrambled small interfering RNAs (siRNAs) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). MH-S cells were transfected with small interfering RNA (siRNA; 5 pm), LC3-RFP G120A, pcDNA3.1, and pcDNA3.1-Fami13A (100 ng) plasmids using Lipofectamine 2000 (Life Technologies) for 24 h, following the manufacturer’s instructions. In some cases, anxa2−/− AMs were treated with 5 μM autophagy activator Trehalose (Trex; Sigma-Aldrich), and WT AMs were treated with 5 μM 3-methyladenine autophagy inhibitor (3-MA; Sigma-Aldrich) for 2 h before and during PAO1 infection, as indicated.

**Bacterial burden assay**

AMs from BALF and ground lung, spleen, liver, and kidney tissues were homogenized with PBS and spread on LB dishes to enumerate bacteria. The dishes were cultured in a 37°C incubator overnight, and colonies were counted. Duplicates were performed for each sample and control (32).

**NBT assay**

This assay is based on the color change of NBT dye upon reduction by released superoxide. AMs from BALF were grown in a 96-well plate in serum-containing medium at 37°C for 4 h, and NBT (1 μg/ml) dye (Sigma-Aldrich) was added to each well. Cells were incubated for an additional hour or until color developed. The dye is yellow and gives a blue-colored formazan product upon reduction by superoxide. The reduction was terminated by adding 100 μl stop solution (10% DMSO; 10% SDS in 50 mM HEPES buffer). The plate was kept at room temperature overnight, and complete dissolution of formazan, and absorbance at 560 nm was recorded using a multispec plate reader to quantify the concentration of superoxide anion (24). Triplicates were performed for each sample and control.

**Dihydrorhodochlorofluorescein diacetate assay**

Dihydrorhodochlorofluorescein diacetate (H2DCF) dye (Life Technologies) does not normally fluoresce, but it emits green fluorescence upon reaction with superoxide inside of cells. AMs were treated as above, and an equal amount of dye was added. After 10 min of incubation, fluorescence was measured using a fluorometer at 485 nm excitation with a 528-nm emission filter (33).

**MITT assay**

This assay measures the color change in MTT (Sigma-Aldrich) upon reduction by enzymes to assess the viability of cells. Cells were treated as above, and an equal amount of dye was added. After 1 h of incubation, the reaction was stopped by stop solution and left at room temperature overnight for complete dissolution of formazan. Absorbance at 550 nm was recorded using a multispec plate reader to quantify the concentration of superoxide anion (34).

**Mitochondrial potential assay**

A JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical, Ann Arbor, MI) was used for this assay, following the manufacturer’s instructions. The cytofluorometric lipophilic cationic dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1), can selectively enter mitochondria and reversibly change the color from green to red as the membrane potential increases. AMs were treated as above, and an equal amount of dye was added. After 30 min of incubation, fluorescence was measured using a fluorometer at 560-nm excitation and
with a 595-nm emission filter to detect healthy cells and at 485-nm excitation and with a 535-nm emission filter to detect dead cells (35).

**Histological analysis**

Lung tissues of three independent mice were fixed in 10% formalin (Sigma-Aldrich) for 24 h and then embedded in paraffin using a routine histologic procedure. Four-micrometer sections were cut, stained with standard H&E, and examined for differences in morphology postinfection (36).

**Inflammatory cytokine profiling**

Cytokine concentrations of TNF-α, IL-6, IL-1β, and IFN-γ were measured, using ELISA kits (eBioscience, San Diego, CA), in BALF samples collected at the indicated times postinfection. Bacterial CFU was collected, and 100-μl aliquots were added to the coated microtiter wells. The cytokine concentrations were determined with corresponding detection HRP-conjugated Abs. The values were read at 450 nm (36).

**CFU count assay**

Phagocytosis and intracellular killing were assessed with a CFU assay, as previously described (37). AMs were challenged with the indicated bacteria at a 20:1 ratio. The numbers of internalized and killed bacteria were assessed after 1 h of incubation. After 1 h, gentamicin (300 μg/ml) was added to the medium for 30 min to kill extracellular bacteria. Cells were counted using a hemocytometer (Sigma-Aldrich); subsequently, cells were lysed, and bacterial CFU were determined. Phagocytosed bacteria were calculated as CFU (1 h)/cell number. A second series of internalization assays was run in parallel to determine the number of viable bacteria following 2 h of incubation. After the same treatment to remove extracellular bacteria, cells were incubated for an additional hour and lysed for analysis of intracellular bacterial CFU. The killing efficiency was calculated as CFU (1 h) − CFU (2 h)/CFU (1 h) and normalized to the control group (37).

**Phagocytosis assay**

AMs were challenged with PAO1 EGFP at an MOI of 20 for 2 h and then stained with cholera toxin B chain (CTB; Sigma-Aldrich) to track membrane sphingolipid-rich lipid rafts for 30 min. Cells were observed under an LSM 510 Meta Confocal Microscope (Carl Zeiss Micro Imaging, Thornwood, NY). After observation, cells were washed three times with PBS, centrifuged to remove extracellular bacteria, and lysed. Intracellular bacteria were incubated on LB dishes and analyzed using an IVIS XRII system (30).

**Autothagy-based PCR primer assay**

RNA was isolated from AnxA2 silenced or negative MH-S cells 4 h post-PAO1 infection. DNA-free RNA (100-ng) was used for first-strand cDNA synthesis using the SuperScript III First-Strand Synthesis System (Life Technologies). A PCR primer assay was performed using Taq Universal SYBR Green Supermix and gene-specific primers that attached to the bottom of the Autophagy M384 predesigned 384 well plate in a CFX Connect Real-Time PCR Detection System (both from Bio-Rad). PCR primer assay data were analyzed using PrimerPCR analysis software (Bio-Rad).

**Immunoblotting**

Mouse mAbs against LC3, β-actin, and Akt1 and rabbit polyclonal Abs against AnxA2, mTOR, ULK1, p-Akt1, and p-mTOR were obtained from Santa Cruz Biotechnology, Inc. Rabbit polyclonal Abs against Fam13A was obtained from Proteintech Group (Chicago, IL), whereas Phospho-ULK1 (Ser317) was obtained from Cell Signaling Technology (Danvers, MA). The samples derived from cells and lung homogenates were lysed in RIPA buffer, separated by electrophoresis on 12% SDS-PAGE gels, and transferred to nitrocellulose transfer membranes (GE Amersham Biosciences, Pittsburgh, PA). Proteins were detected by Western blotting using primary Abs at a concentration of 1/200 (Santa Cruz Biotechnology) or 1/1000 (Cell Signaling Technology) and were incubated overnight. Binding of the first Abs was determined using corresponding secondary Abs conjugated to HRP (Santa Cruz Biotechnology) and signals were developed using the ECL reagent (Santa Cruz Biotechnology) (38). Gel bands were quantified by Quantity One software (Bio-Rad), and data are presented as means ± SEM from three independent immunoblotting assays (38). Phosphorylated and total protein levels were determined and quantified by three independent successive immunoblotting membranes.

**Immunoprecipitation**

To obtain whole-cell lysates, AMs and MH-S cells were homogenized in lysis buffer containing phosphatase inhibitor (1:10000) and protease inhibitors (1:50; Thermo Fisher Scientific, Waltham, MA). Then total-cell lysates were mixed with immunoprecipitation (IP) Abs, which were coupled to agarose beads (50:50; Thermo Fisher Scientific). Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose transfer membranes. Membranes were incubated with the primary Abs overnight. Labeling of the first Abs was detected using secondary Abs conjugated to HRP and detected using ECL reagents (23).

**LC3 puncta observation**

AMs and MH-S cells were re transfected with LC3-RFP G120A plasmids for 24 h. Cells were infected with PAO1-EGFP at an MOI of 20 for 2 h. Cells were observed under an LSM 510 Meta Confocal Microscope. LC3 puncta values were derived from 100 cells/sample (33).

**Immunostaining**

AMs were isolated from WT mice 24 h post-PAO1 infection, as well as from control mice. Cells were incubated individually with primary anti-AnxA2 Ab and anti-Fam13A Ab, and then with the secondary FITC-conjugated Abs, as described (31). Colocalization was observed under a Zeiss LSM 510 Meta Confocal Microscope.

**Rho GTase activity assay**

Rho GTase activity in AMs and MH-S cells was detected with a Pierce active Rho GTase Pull-down and Detection Kit (Life Technologies), following the manufacturer’s instructions. Briefly, whole-cell lysates were prepared from AMs using the lysis buffer provided in the kit. Lysate (containing active and inactive Rho GTase) was incubated with the GST-protein binding domain fusion protein from the respective downstream effector protein and glutathione resin. Unbound lysate proteins, including inactive or GDP-bound GTase, were removed using spin columns. The active GTase population was recovered from the glutathione resin using SDS-PAGE loading buffer and analyzed by immunoblotting (39).

**Statistical analysis**

Most experiments were conducted in triplicate. Differences between two groups were compared by one-way ANOVA (Tukey post hoc test) using GraphPad Prism 5 software, whereas survival rates were calculated using a Kaplan–Meier curve (40).

**Results**

**anxa2−/− mice are highly susceptible to PAO1 infection**

To investigate the role of AnxA2 in the *P. aeruginosa* infection process, we instilled PAO1 (0.5 × 10⁷ CFU/mouse) intranasally in *anxa2−/−* and WT mice (with otherwise similar genetic backgrounds) to establish an acute pneumonia model and compared the survival rates of these two groups of mice (six mice/group). As shown in Fig. 1A, *anxa2−/−* mice exhibited increased lethality (50% died within 38 h postinfection). By 68 h, all *anxa2−/−* mice had died, whereas 80% of WT mice remained alive. This result is shown in Kaplan–Meier survival curves (p = 0.021, log-rank test).

To directly examine the in vivo infection process, we also instilled mice with PAO1 Xen-41 (an engineered bacterium emitting bioluminescence for imaging) at the same dose/mouse and observed the pattern of bacterial dissemination. We found that *anxa2−/−* mice displayed a much broader distribution of bioluminescence in the thoracic cavity area at 2 h postinfection, and it continuously expanded, compared with WT mice (Fig. 1B, 1C). These results suggest that AnxA2 contributes to the host defense against *P. aeruginosa* in pneumonia models by slowing bacterial dissemination.

**AnxA2 loss results in increased oxidation, lung injury, and inflammatory response**

To further analyze the cause of infection lethality in *anxa2−/−* mice, we examined bacterial burdens in the lung, liver, spleen, kidney, BALF, and blood from PAO1-infected mice. Bacterial CFU increased significantly in the organs of *anxa2−/−* mice compared with the same organs in WT mice (Fig. 2A, 2B, Supplemental Fig. 1A–D), demonstrating severe lung injury and...
AnxA2 REGULATES PSEUDOMONAS-INDUCED AUTOPHAGY IN MACROPHAGES

AnxA2 loss results in impaired PAO1-induced autophagy in AMs

Bacteria burdens in BALF from anxa2−/− mice were much higher than in WT mice (Fig. 2B), indicating that the bacterial clearance capacity in anxa2−/− mice was substantially dampened. To confirm this, we determined the survival of AMs, which is essential for maintaining bacterial clearance, 24 h post-PAO1 infection. The results showed a significant decrease in the viability of AMs in anxa2−/− mice (Fig. 3A); consistent with this reduced survival rate, the capacity for bacterial clearance, as quantified by CFU assays, also was impaired (Fig. 3B). We then transfected the model murine lung macrophages (MH-S cells) and lung epithelial cells (MLE-12 cells) with an siRNA against AnxA2 to inhibit AnxA2 expression, as well as with siNC as a negative control. Two hours post-PAO1 infection, similar decreases in survival and bacterial clearance capacity were detected in AnxA2-silenced MH-S cells and negative controls (Fig. 3C, 3D). Note that the survival of AnxA2-silenced MLE-12 cells showed no significant difference from controls (Fig. 3C), indicating that AnxA2 may play more dominant roles in macrophages than in epithelial cells. To determine whether the role of AnxA2 is limited to P. aeruginosa or it plays a general role in bacterial defense, other bacterial strains and species were investigated. When infected with P. aeruginosa strains PAK and PA14, as well as K. pneumoniae and E. coli, both the survival and bacterial clearance capacity were significantly decreased in AnxA2-silenced MH-S cells (Supplemental Fig. 1E, 1F), suggesting that the role of AnxA2 may extend to a broad range of Gram-negative bacteria.

A previous study reported that human epithelial cell surface AnxA2 could recognize PAO1, indicating that AnxA2 may be required to allow internalization of P. aeruginosa (18). However, whether AnxA2 facilitates phagocytosis in AMs remains unclear. In this study, CFU assay showed that, 2 h post-PAO1 infection, the number of PAO1 phagocytized by anxa2−/− AMs is similar to that of WT AMs (Fig. 3E). Confocal laser scanning microscopy (CLSM) also showed a close colocalization of PAO1 EGFP with CTB stained from WT and anxa2−/− AMs (Fig. 3F). In addition, we lysed these infected AMs to measure phagocytized PAO1 on LB dishes using an IVIS imaging system. Phagocytosis of PAO1 EGFP by anxa2−/− AMs was similar to that of WT AMs (Fig. 3G). Collectively, these data suggest that AnxA2 is not required for bacterial phagocytosis in AMs.

In contrast, autophagy plays an essential role in the clearance of PAO1 by macrophages (15). We speculated that AnxA2 is involved in autophagy in AMs to facilitate bacterial clearance. To test this hypothesis, we used immunoblotting to determine the expression
were infected with 0.5 × 10^7 CFU PAO1/mouse for 24 h. (A) Bacterial burdens in the lungs after homogenization in PBS. (B) Bacterial loads in BALF derived from infected mice. (C) PMN cell percentages evaluated in BALF versus total nuclear cells using HEMA-3 staining. Superoxide production in AMs was detected by an NBT assay at a wavelength of 560 nm (D) and an H2DCF assay at a wavelength of 488 nm (E). (F) Mitochondrial potential of AMs derived from infected mice. (G) PMN cell percentages evaluated in BALF versus total nuclear cells using HEMA-3 staining. (H) Lung injury and inflammation assessed by histology. Lungs from indicated mice were embedded in formalin. Sections were analyzed by H&E staining (arrows indicate the region of insets showing typical tissue injury and inflammatory influx). Original magnification ×200, insets, ×1000. (I) Inflammatory cytokines in BALF were assessed by ELISA. Data (mean ± SEM) are representative of three independent experiments. *p ≤ 0.05, **p ≤ 0.005.

patterns of LC-3, which is a marker of microtubule-associated protein in the autophagosomal membrane, in mouse AMs. We found that endogenous conversion of LC3-I to LC3-II was dramatically increased in AMs of WT mice 24 h post-PAO1 infection (Fig. 3H). However, this conversion was not detected in AMs of anxa2^−/−^ mice (Fig. 3H), indicating that loss of AnxA2 inhibited PAO1-induced autophagy in AMs. Similar results were observed in vitro using AnxA2-silenced MH-S cells 2 h post-PAO1 infection (Fig. 3I). A significant increase in LC3-II was detected in MH-S control cells, whereas the level of LC3-II remained unchanged in AnxA2-silenced MH-S cells (Fig. 3I). We also transfected an RFP-LC3 plasmid into anxa2^−/−^ and WT AMs and infected them with PAO1 EGFP. PAO1 infection induced significant LC3 puncta in AMs from WT mice, but AnxA2 knockdown resulted in an ~80% decrease in RFP-LC3 puncta (Fig. 3I). In addition, CLSM showed colocalization of PAO1 GFP and LC3 (Fig. 3I), indicating that PAO1 clearance is actually due to autophagy.

AnxA2 loss results in impaired PAO1-induced autophagy through the Akt1–mTOR–ULK1/2 pathway

To further investigate the role of AnxA2 in autophagy, we assessed expression patterns of autophagy-related factors in AnxA2-silenced MH-S cells and negative controls, using an autophagy array-based real-time PCR primer assay. Postinfection with PAO1, the quantitative real-time PCR primer array assay revealed that three genes were significantly (>2-fold) upregulated, whereas 73 genes were downregulated (Fig. 4A, Supplemental Table II), indicating that AnxA2 repression may profoundly impair expression of autophagy-related factors. Among the upregulated factors, NFKB is a key transcription regulator that controls diverse gene expression related to host defense against PAO1 infection (45), whereas Akt1 and mTOR are interconnected junctions within the autophagy regulation pathway (46). Among the downregulated factors, ULK1 and ULK2 were the most significantly decreased factors that directly participate in autophagosome formation (47). Previous studies suggest that mTOR phosphorylates ULK1/2 and Atg13, thereby inhibiting the translocation of ULK1/2–Atg13–FIP200 complex to preautophagosomal membrane to induce autophagy (46). According to data from the autophagy array-based quantitative real-time PCR assay, we hypothesized that AnxA2 functions in PAO1-induced autophagy through an Akt1–mTOR–ULK1/2 signaling pathway.

To validate the results of the autophagy array-based quantitative real-time PCR assay and confirm our proposed roles for AnxA2 in PAO1-induced autophagy, we examined the expression levels of predicted factors in AMs of PAO1-infected anxa2^−/−^ and WT mice by immunoblotting using related Abs. In anxa2^−/−^ AMs, protein expression of Akt1 and mTOR was highly increased; in contrast, ULK1 expression was significantly decreased in anxa2^−/−^ AMs compared with WT AMs (Fig. 4B). We also observed that phosphorylation of Akt1 and mTOR was increased in anxa2^−/−^ AMs (Fig. 4B), indicating that, in addition to increased protein levels, AnxA2 deficiency led to enhanced Akt1 and mTOR activation. In WT AMs, phosphorylation of Akt1 remained at the normal level compared with AMs without PAO1 infection, whereas phosphorylation of mTOR seemed to be inhibited (Fig. 4B). Phosphorylation of Ser^317^ of ULK1 is an independent marker for ULK1/2–Atg13–FIP200 and mTOR (48). We found that, after PAO1 infection, ULK1 Ser^317^ was dramatically phosphorylated in WT AMs but was not phosphorylated in anxa2^−/−^ AMs (Fig. 4B), indicating that AnxA2 deficiency inhibited the translocation of ULK1/2–Atg13–FIP200 complex to preautophagosomal membrane.
AnxA2 deficiency blunted PAO1-induced autophagy in AMs. anxa2−/− and WT mice were infected with 0.5 × 107 CFU PAO1/mouse for 24 h. (A) Viability of the indicated AMs was determined by MTT assay at a wavelength of 570 nm. (B) Bacterial killing by AMs was assessed with a CFU assay. (C) Anxa2-silenced MH-S and MLE-12 cells were infected with PAO1 at an MOI of 20 for 2 h. Viability of the indicated MH-S and MLE-12 cells was determined by an MTT assay at a wavelength of 570 nm. (D) Bacterial killing by MH-S cells was detected using a CFU assay. (E) Phagocytosis of anxa2−/− and WT AMs infected with PAO1 was assessed by CFU count. (F) CTB-rhodamine-stained anxa2−/− and WT AMs (red) were challenged with PAO1 EGFP at an MOI of 20 for 1 h. Uptake of bacteria was observed using an immunofluorescence confocal microscope. (G) Phagocytized PAO1 EGFP was cultured on LB dishes and detected using an IVIS XRII imaging system. (H) PAO1-infected anxa2−/− and WT AMs were lysed to measure LC3 levels by immunoblotting. (I) LC3 levels in Anxa2-silenced and MH-S control cells were determined by immunoblotting. During PAO1 infection, 5 mM NH4Cl was added to exclude LC3 degradation. (J) Purified anxa2−/− and WT AMs were transfected with RFP-LC3 G120A plasmids for 24 h and then infected with PAO1-EGFP for 1 h (MOI = 20). Cells with apparent puncta (>10/cell) were considered LC3-RFP+ cells (arrows indicating colocalization of LC3 puncta and PAO1 EGFP). Values are derived from 100 cells/sample. Data (mean ± SEM) are representative of three independent experiments. *p ≤ 0.05, **p ≤ 0.005, one-way ANOVA with Tukey post hoc test.

To further confirm this result, we prepared total-cell lysates of anxa2−/− and WT AMs to perform co-IP analysis. At 24 post-PAO1 infection, we used anti-mTOR or anti-ULK1 Ab to pull-down the target protein and determined ULK1 and mTOR interaction by immunoblotting using the appropriate Ab. Our results showed that, in the absence of PAO1 infection, mTOR and ULK1 showed a stable association in anxa2−/− and WT AMs (Fig. 4C). However, after PAO1 infection, this association was disrupted in WT AMs but not in anxa2−/− AMs (Fig. 4C). This result strongly suggests that AnxA2 plays a role in mTOR–ULK1 association, which impacts autophagy initiation.

To demonstrate that AnxA2 specifically acts by regulating the Akt1–mTOR–ULK1/2 signaling pathway, we treated WT AMs with 3-MA to inhibit the Akt1-mTOR signaling pathway and simultaneously treated anxa2−/− AMs with Tre to activate the Akt1-mTOR signaling pathway, to mediate autophagy (Fig. 4D). Two hours post-PAO1 infection, the viability of 3-MA–treated WT AMs decreased to a level similar to that seen in anxa2−/− AMs, whereas the survival of Tre-treated anxa2−/− AMs increased to equal the level in WT AMs (Fig. 4E). Similar results were observed in bacterial clearance ability detection in these indicated AMs (Fig. 4F).

Fam13A is required in PAO1-induced autophagy

Previous studies revealed several new factors that are involved in diverse pulmonary diseases, including COPD and lung cancer, but it remains unknown whether they are required for host defense against bacterial infection in macrophages. We randomly selected 15 of these factors and measured their transcripts levels in AMs from WT and anxa2−/− mice 24 h post-PAO1 infection, by quantitative real-time PCR using specific primers (Supplemental Table I). After PAO1 infection, transcripts of APOB, Brd4, Cd1d, Fam13A, Malt1, NSF, Scgb, Sirt1, and Tob1 were significantly increased, whereas transcript levels of Sirga were reduced (Fig. 5A). Among these 10 proteins, only transcripts of Fam13A and Malt1 showed significant differences between anxa2−/− and control AMs (Fig. 5A), indicating that they played roles associated with AnxA2 in AMs during PAO1 infection. We focused further on Fam13A, because the change in the levels of its expression in anxa2−/− AMs was more significant compared with Malt1 (Fig. 5A).

As reported, Fam13A is involved in COPD and lung cancer, but little is known about its role in cystic fibrosis and bacterial defense (49). We next detected Fam13A protein levels in AMs by immunoblotting. We observed a significant induction of Fam13A in PAO1-infected WT AMs, but not in anxa2−/− AMs (Fig. 5B), again indicating that Fam13A plays a role in AMs during PAO1 infection. To further confirm our hypothesis, we introduced Fam13A-interfering RNA into MH-S cells to block Fam13A expression, while using siNC as a negative control (Fig. 5C). Our results showed that the survival and bacterial clearance capacity of Fam13A-silenced MH-S cells were decreased after PAO1 infection.
and ULK1 in AMs from postinfection with PAO1. Gel data were quantified from three independent experiments using densitometry with Quantity One. During PAO1 infection, we lysed primary AMs isolated from WT MICE and performed a co-IP assay with anti-AnxA2 and anti-Fam13A Abs. A stable interaction between AnxA2 and Fam13A was detected (Fig. 6A). CLSM also showed a significant colocalization of AnxA2 and Fam13A in AMs isolated from WT mice 24 h post-PAO1 infection (Fig. 6B), strongly indicating that AnxA2 and Fam13A are associated in AMs and likely act together in host defense as a single complex. This protein–protein interaction was also detected in siNC-transfected MH-S cells after PAO1 infection (Fig. 6C), but it was abolished when either AnxA2 or Fam13A was repressed by RNA interference (Fig. 6C), and it could not be rescued with Tre treatment in AnxA2-inhibited MH-S cells (Fig. 6C). In addition, we saw reduced macrophage viability and bacterial killing in autophagy-inhibited MH-S cells by directly silencing some key autophagy factors, including Atg5, Atg7, and Beclin1 (Fig. 6D, Supplemental Table II). To validate these data, we also examined expression levels of predicted factors in Fam13A-silenced MH-S cells after PAO1 infection, using the autophagy-based quantitative real-time PCR primer assay. The results showed that, in Fam13A-silenced MH-S cells, Akt1, mTOR, and NF-κB were significantly induced, and many factors, including ULK1 and ULK2, were downregulated (Fig. 5G, Supplemental Table II). To validate these data, we also examined expression levels of predicted factors in Fam13A-silenced MH-S control cells and found that phosphorylation of Akt1 and mTOR was significantly increased in Fam13A-silenced MH-S cells, whereas phosphorylation of Ser^{317} in ULK1 was dramatically inhibited, compared with MH-S control cells (Fig. 5H). This pattern was extremely similar to that of AnxA2-silenced MH-S cells (Fig. 4A, 4B, Supplemental Table II), revealing an extremely critical function for Fam13A in macrophage autophagy during PAO1 infection.

Fam13A binds directly to AnxA2 and activates Rho GTPase to facilitate autophagosome formation during PAO1 infection

To test the potential association between Fam13A and AnxA2 during PAO1 infection, we lysed primary AMs isolated from WT mice at 24 h post-PAO1 infection and performed a co-IP assay with anti-AnxA2 and anti-Fam13A Abs. A stable interaction between AnxA2 and Fam13A was detected (Fig. 6A). CLSM also showed a significant colocalization of AnxA2 and Fam13A in AMs isolated from WT mice 24 h post-PAO1 infection (Fig. 6B), strongly indicating that AnxA2 and Fam13A are associated in AMs and likely act together in host defense as a single complex. This protein–protein interaction was also detected in siNC-transfected MH-S cells after PAO1 infection (Fig. 6C), but it was abolished when either AnxA2 or Fam13A was repressed by RNA interference (Fig. 6C), and it could not be rescued with Tre treatment in AnxA2-inhibited MH-S cells (Fig. 6C). In addition, we saw reduced macrophage viability and bacterial killing in autophagy-inhibited MH-S cells by directly silencing some key autophagy factors, including Atg5, Atg7, and Beclin1 (Fig. 6D, Supplemental Fig. 2A, 2B), again indicating that autophagy plays key roles in bacterial defense in macrophages. The association of AnxA2 with Fam13A was stable in all of these autophagy-inhibited MH-S cells after PAO1 infection (Fig. 6E). Collectively, our results suggested that the AnxA2–Fam13A complex, rather than canonical autophagy pathways, may be a critical regulator of autophagy.

Protein function prediction analysis suggests that Fam13A contains two conserved Rho GTPase-activating domains (www.ebi.ac.uk/interpro/protein/O94988). We suspected that Fam13A regulates Rho GTPase activity in the defense against P. aeruginosa. To confirm this, we analyzed Rho GTPase activities in WT and Fam13A-silenced MH-S cells, with or without PAO1 infection. We found that Fam13A deficiency directly reduced Rho GTPase activation in MH-S cells, even without PAO1 infection (Fig. 6F, Supplemental Fig. 2E). After PAO1 infection, MH-S control samples exhibited dramatically increased levels of activated protein 3-MA for 4 h before and during PAO1 infection. During PAO1 infection, 5 mM NH_{4}Cl was added to exclude LC3 degradation. LC3 levels of indicated AMs were measured by immunoblotting. AnxA2 deficiency impaired PAO1-induced autophagy through the Akt1–mTOR–ULK1/2 pathway. AnxA2 binds to Fam13A to facilitate autophagosome formation during PAO1 infection. We again analyzed the expression of autophagy-related factors, including Atg5, Atg7, and Beclin1 (Fig. 6D, Supplemental Table II). To validate these array data, we also examined expression levels of predicted factors in Fam13A-silenced and MH-S control cells after PAO1 infection for 4 h. Genes with >2-fold changes among subsets are shown (n = 2 biological replicates). Upper left dots of double lines indicate increased proteins, and lower right dots indicate decreased proteins. (B) Autophagy-related factors and cell signaling protein levels of anxa2^{−/−} and WT AMs were determined by immunoblotting 24 h postinfection with PAO1. Gel data were quantified from three independent experiments using densitometry with Quantity One. (C) Association of mTOR and ULK1 in AMs from anxa2^{−/−} and WT mice was determined by immunoblotting with the indicated Ab for IP and detection. (D) Purified anxa2^{−/−} AMs were treated with 5 μM Tre and WT AMs were treated with 5 μM 3-MA for 4 h before and during PAO1 infection. During PAO1 infection, 5 mM NH_{4}Cl was added to exclude LC3 degradation. LC3 levels of indicated AMs were measured by immunoblotting. (E) Viability of the indicated AMs was determined by MTT assay at a wavelength of 570 nm. (F) Bacterial killing by indicated AMs was tested with a CFU assay. Data (mean ± SEM) are representative of three independent experiments. *p ≤ 0.05, **p ≤ 0.005, one-way ANOVA with Tukey post hoc test.
GTpase compared with control cells (Fig. 6F, Supplemental Fig. 2E), but the level of activated Rho GTpase in Fam13A-silenced cells was still low (Fig. 6F, Supplemental Fig. 2E). We further cloned Fam13A gene into the pcDNA3.1 vector and transfected this plasmid into MH-S cells to overexpress Fam13A; MH-S cells transfected with empty pcDNA3.1 vector were used as negative control (Fig. 6D). High expression of Fam13A did not change macrophage viability or bacterial killing in MH-S cells (Supplemental Fig. 2C, 2D) after PAO1 infection, but it directly induced Rho GTpase activation, even in the absence of PAO1 infection (Fig. 6G, Supplemental Fig. 2F). Together, these data indicate that Fam13A regulates PAO1-induced Rho GTpase activation and contributes to host immunity in bacterial defense. The impaired GTpase activation also was detected in anxa2−/− AMs after PAO1 infection compared with WT AMs (Fig. 6H, Supplemental Fig. 2G), suggesting that AnxA2 plays a role in the activation of GTpase via Fam13A.

Discussion

In this study, we identified a critical role for AnxA2 in host defense against P. aeruginosa infection. We observed a severe disease phenotype in anxa2−/− mice in our experiments, which included decreased survival, increased inflammatory response, and more severe lung injury compared with WT mice (Figs. 1, 2, Supplemental Fig. 1A–D). The increased bacterial burdens in the lung and other organs of anxa2−/− mice indicate that AnxA2 is a critical contributor to bacterial clearance. Importantly, additional data demonstrate that AnxA2 plays an essential role in PAO1-induced autophagy in AMs (Fig. 6I). Furthermore, we identified the underlying mechanism by which AnxA2 impacted autophagy during infection: by directly binding to Fam13A and activating Rho GTpase to regulate the Akt1–mTOR–ULK1/2 signaling axis. Similar phenotypes (increased cell death and decreased bacterial clearance) were observed in AnxA2-silenced MH-S cells infected with different bacterial strains or species, such as P. aeruginosa strains PA14 and PAK, K. pneumoniae, and E. coli (Supplemental Fig. 1E, 1F). Thus, AnxA2 may render a critical immune defense against a wide range of microorganisms.

Our study established that AnxA2 is involved in host inflammatory response regulation during P. aeruginosa infection. As reported previously, AnxA2 plays multiple roles in host defense against bacteria. For instance, AnxA2 is predominantly an anti-inflammatory agent, largely because of its similarities to Annexin A1, which was universally demonstrated to play such a role (50). Existing data support a role for AnxA2 in the proinflammatory process; AnxA2 elicits activation of MAPK and nuclear translocation of NF-κB, resulting in inflammatory cytokine production, as well as chemokine production. This function was reflected in enhanced macrophage effector function, because AnxA2 dramatically facilitated phagocytosis of E. coli bacteria (51). Other studies also illustrated the role for AnxA2 in regulating the inflammatory response (52). Our results are in line with this...
observation, because significantly enhanced inflammatory response, lung injury, ROS accumulation, and cytokine production were found in Anxa2 knockout mice following PAO1 infection (Fig. 2), and the autophagy array-based quantitative real-time PCR assay showed that AnxA2 negatively regulates the host inflammatory response by NF-κB (Fig. 4A).

AnxA2 was reported to serve as a receptor for P. aeruginosa in mammalian epithelial cells and is involved in its internalization (18). Whether AnxA2 is required for phagocytosis of PAO1 by AMs is unknown. Our data showed that phagocytosed PAO1 by Anxa2 knockout AMs is similar to that of WT AMs, indicating that AnxA2 does not play a critical role in bacterial uptake by AMs (Fig. 3E–G). Instead, we observed that inhibition of AnxA2 resulted in suppression of autophagosome formation in AMs after PAO1 infection (Fig. 3H–J). No study has reported a mechanistic role for AnxA2 in regulating autophagy and bacterial clearance. The only previous study demonstrated that AnxA2 expression increased along with autophagy-regulated genes, such as GABARAP, LC3B, and ATG3, in HUVECs after repeated treatment with resveratrol (53). We found that the reduction in survival occurred in AnxA2-silenced MH-S cells, and not in MLE-12 cells (Fig. 3C), indicating that AnxA2 might play important roles specifically in macrophages rather than epithelial cells. Autophagy array-based quantitative real-time PCR assay and immunoblotting showed increased expression and activation of Akt1 and mTOR in AnxA2-silenced MH-S cells (Fig. 4A, 4B), suggesting that AnxA2 regulates PAO1-induced autophagy via an Akt1-mTOR pathway. Autophagy is normally inhibited in metabolically repressed cells by the metabolic checkpoint kinase mTOR (54); how the Akt1-mTOR signaling pathway induces autophagy upon bacterial infection remains unclear. A previous study determined that infection of epithelial cells with Shigella and Salmonella triggers acute intracellular amino acid starvation as the result of host membrane damage, and pathogen-induced amino acid starvation was shown to cause downregulation of mTOR activity, resulting in the induction of autophagy (55). Our data provide evidence that AnxA2 regulates pathogen-induced host autophagy through the Akt1-mTOR signaling pathway. Generally, PAO1 infection stimulates AnxA2 to interact with free Fam13A in the cytoplasm and activate Rho GTPase. Activated Rho GTPase inactivates Akt1 and mTOR; thus, ULK1/2 is released to form the autophagosome (46). In contrast, AnxA2 deficiency impaired Rho GTPase activation; hence, activated mTOR strongly binds ULK1/2 and inhibits the formation of the autophagosome (Fig. 6I). However, AnxA2

FIGURE 6. Fam13A binds AnxA2 and activates Rho GTPase to facilitate autophagy during PAO1 infection. (A) Association of AnxA2 and Fam13A in AMs from WT mice was determined by immunoblotting with the indicated Ab for IP and detection 24 h postinfection with PAO1. (B) AMs were purified from control WT mice and PAO1-infected mice 24 h postinfection. CLSM showed the colocalization of AnxA2 and Fam13A (arrows) after PAO1 infection by immune staining. (C) Association of AnxA2 and Fam13A in MH-S cells was determined by immunoblotting with AnxA2 Ab for IP and detection 2 h postinfection with PAO1. Before infection, MH-S cells were transfected with siNC, siRNA against AnxA2 (with and without Tre), or siRNA against Fam13A for 24 h. (D) MH-S cells were transfected with siAtg5, siAtg7, siBecn1, and pcDNA3.1-Fam13A for 24 h. Expression of Atg5, Atg7, Becn1, and Fam13A in MH-S cells was determined by immunoblotting. (E) Association of AnxA2 and Fam13A in siNC-, siAtg5-, siAtg7-, and siBecn1-transfected MH-S cells was determined by immunoblotting with AnxA2 Ab for IP and detection 2 h postinfection with PAO1. Total Rho GTPases and activated Rho GTPases in Fam13A-silenced MH-S cells (F), Fam13A-overexpressed MH-S cells (G), anxa2−/− AMs (H), and control cells were determined by immunoblotting, with or without PAO1 infection for 2 h. (I) Diagram delineating a pathway in AnxA2-deficient macrophages against PAO1 infection. After PAO1 infection, AnxA2 binds Fam13A to activate Rho GTPase and subsequently inactivates the Akt1–mTOR–ULK1/2 signaling pathway to induce autophagosome formation. siAtg5, siAtg7, siBecn1, and siFam13A indicate siRNA specifically against Atg5, Atg7, Becn1, and Fam13A, respectively. Data are representative of three independent experiments.
overexpression was strongly associated with rapid recurrence after gemcitabine-adjuvant chemotherapy in patients with resected pancreatic cancer. In GEM-MIA PaCa-2 cells, in which AnxA2 is highly expressed, the levels of p-Akt and p-mTOR were significantly increased (56). Thus, AnxA2 acts as a dual regulator in the Akt-mTOR signaling pathway that may be independent of cell nutrition.

Another important finding in this work is that Fam13A is involved in the regulation of PAO1-induced autophagy by activating Rho GTPase. Previously, variants in Fam13A were found in genome-wide association studies to be associated with lung functions in the general population, as well as in several common chronic lung diseases, such as COPD, asthma, and idiopathic interstitial pneumonias (57). Sequence analysis indicated the presence of a Rho GTPase–activating protein domain on exons 2–5 (58). We observed an induction of Fam13A and consistent induction of activated Rho GTPase in AMs after PAO1 infection, suggesting that Fam13A may mediate Rho GTPase activation instead of inactivation (Figs. 5B, 6H, Suppemental Fig. 2G). Further data determined that overexpression of Fam13A could directly induce Rho GTPase activation; on the contrary, Fam13A deficiency inhibited Rho GTPase activation in AMs, in the presence or absence of PAO1 infection (Fig. 6F, 6G, Suppemental Fig. 2E, 2F), indicating that Fam13A directly mediates Rho GTPase activation. In addition, we observed a stable association between AnxA2 and Fam13A after PAO1 infection (Fig. 6A, 6B), and expression of activated Rho GTPase did not increase in AnxA2-depleted AMs (Fig. 6H). These results indicated that Rho GTPase regulator function of Fam13A may be strictly limited to AnxA2.

Rho GTPases are normally identified as key regulators of cytoskeletal dynamics (59), and they affect several vascular processes, such as endothelial permeability, cell motility, angiogenesis, NO production, smooth muscle contractility, cell proliferation, differentiation, and apoptosis (60, 61). Previous studies also indicated that Rho GTPases play roles in the defense against bacterial pathogen invasion. For example, Rho GTPases are involved in the regulation of pulmonary vascular barrier function (62) and macrophagosome formation and maturation (63), and they act as regulators of immunity in plants and animals (64). However, the role of Rho GTPases in the regulation of autophagy, especially bacteria-induced autophagy, is still unclear. The only report described that Rho GTPase RhoA positively regulates starvation-induced autophagy through a mechanism that is likely dependent on actin, whereas Rac1 has the opposite effect (65). We determined that Rho GTPases are involved in regulation of PAO1-induced autophagy, because reduced Rho GTPase activation is correlated with dampened autophagosome formation (Fig. 6C, 6F). In the process of autophagosome formation induced by starvation, previous studies established that RhoA is a Rho GTPase, triggers a series of signals leading to actin polymerization (65). However, our data demonstrate that, as potential upstream signals, Rho GTPases regulate autophagosome formation via the Akt-mTOR signaling pathway. Gordon et al. (66) supported a model in which RhoA GTPase represses mTOR signaling upstream of Akt1, which is consistent with our observation.

In conclusion, we demonstrated a typical phenotype of *P. aeruginosa* infection in *anxa2<sup>-/-</sup>* mice, suggesting an important role for AnxA2 in bacterial defense in mice. AnxA2 deficiency impaired autophagosome formation and bacterial clearance capacity, as well as intensified inflammatory responses. Importantly, we revealed a previously unidentified mechanistic role for AnxA2, in association with Fam13A, in the activation of Rho GTPases. These activated Rho GTPases, in turn, inactivate Akt1 and mTOR to release ULK1/2 to form autophagosomes during *P. aeruginosa* infection. We supposed that AnxA2 affects autophagy-mediated bacterial clearance during *P. aeruginosa* infection, as well as some other pathways, because autophagy plays diverse important roles, such as inflammasome activation (67, 68). Overall, our findings provide insight into the role of AnxA2 in the regulation of autophagy against *P. aeruginosa* and may identify a novel therapeutic approach to combat bacterial infection.

**Acknowledgments**

We thank S. Rolling (University of North Dakota Imaging Core Facility) for help with confocal imaging. We also thank Dr. K. Hajjar for kindly providing *anxa2<sup>-/-</sup>* mice and Dr. Xiao-min Yin (Indiana University) for providing LC3-RFP G120A plasmids.

**Disclosures**

The authors have no financial conflicts of interest.

**References**

null
SI Figure 1. (A-D) Bacterial burdens in blood (A), liver (B), spleen (C) and kidney (D) were significantly increased after 24 h post-PAO1 infection in anxa2−/− mice compared with WT mice. (E) Viabilities of siNC- and siAnxA2-transfected MH-S cells after 2 h post-infection of PAK, PA14, KP and E. coli, by an MTT assay at a wavelength of 570 nm. (F) Bacterial killing in siNC- and siAnxA2-transfected MH-S cells after 2 h post-infection of PAK, PA14, KP and E. coli, were determined by plate count. Data are representative of three reproducible experiments expressed as means ± SEM (one-way ANOVA with Tukey’s post hoc; *p ≤ 0.05; **p ≤ 0.005).
SI Figure 2. (A) Viabilities of siNC-, siAtg5-, siAtg7- and siBeclin1-transfected MH-S cells after 2 h post-infection of PAO1, by an MTT assay at a wavelength of 570 nm. (B) Bacterial killing in siNC-, siAtg5-, siAtg7- and siBeclin1-transfected MH-S cells after 2 h post-infection of PAO1, were determined by plate count. (C) Viabilities of pcDNA3.1-Fam13a- and empty pcDNA3.1- transfected MH-S cells after 2 h post-infection of PAO1, by an MTT assay at a wavelength of 570 nm. (D) Bacterial killing in pcDNA3.1-Fam13a- and empty pcDNA3.1- transfected MH-S cells after 2 h post-infection of PAO1, were determined by plate count. (E-G) Densitometric quantification of the immunoblotting gel data presented in Fig. 6 (in text) using Quantity One software, respectively for Fig. 6F (E), Fig. 6G (F) and Fig. 6H (G). Data are representative of three reproducible experiments expressed as means ± SEM (one-way ANOVA with Tukey’s post hoc; *p ≤ 0.05; **p ≤ 0.005).
Table S1. Primers used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOB F</td>
<td>CGGTCTTCAGTGGAGCTACA</td>
</tr>
<tr>
<td>APOB R</td>
<td>GCTCCCATGTGGTGAGATG</td>
</tr>
<tr>
<td>Brd4 F</td>
<td>AGTGTTCCACCTGTTCTTCC</td>
</tr>
<tr>
<td>Brd4 R</td>
<td>TTAAGGAGAGAGCGCCCTCA</td>
</tr>
<tr>
<td>Cd1d F</td>
<td>TTTGCAAAACAGAAGCTGGTC</td>
</tr>
<tr>
<td>Cd1d R</td>
<td>TGCTGGTTACTCAACTTGCC</td>
</tr>
<tr>
<td>EYA4 F</td>
<td>TTCGCTCTCCTTTGAACACAC</td>
</tr>
<tr>
<td>EYA4 R</td>
<td>AAGACAGGAGACACACAAATGC</td>
</tr>
<tr>
<td>Fam13A F</td>
<td>GCCAGGAGAGAGAAGATTC</td>
</tr>
<tr>
<td>Fam13A R</td>
<td>TGAGACTTTTCCATGCTCAGG</td>
</tr>
<tr>
<td>Malt1 F</td>
<td>GACACTGTGGAGAGAAGCA</td>
</tr>
<tr>
<td>Malt1 R</td>
<td>AGATGGAGACGCTGGAACAG</td>
</tr>
<tr>
<td>Ndusf4 F</td>
<td>TTCAGTGCCAAAGAAGATGC</td>
</tr>
<tr>
<td>Ndusf4 R</td>
<td>AGACTTGGACCTGGGTTC</td>
</tr>
<tr>
<td>NSF F</td>
<td>GGGTCATAAGTTGCCAGAT</td>
</tr>
<tr>
<td>NSF R</td>
<td>GGCCTCTGACTGAGATGTT</td>
</tr>
<tr>
<td>PINK1 F</td>
<td>TGGCTCTGTGTCAGCTTAC</td>
</tr>
<tr>
<td>PINK1 R</td>
<td>CCCTCTACTCCAGCTTGTC</td>
</tr>
<tr>
<td>RBPJ F</td>
<td>TGTGCCACCTTTGAATCATT</td>
</tr>
<tr>
<td>RBPJ R</td>
<td>CAATACAGTCGGCTCTGAA</td>
</tr>
<tr>
<td>ScgB F</td>
<td>TTCTTCATGGAGCTCATGGC</td>
</tr>
<tr>
<td>ScgB R</td>
<td>GGCCAAGTGCTTAAATGTA</td>
</tr>
<tr>
<td>Sgk1 F</td>
<td>GTTGCCAGCTGACAGAACAT</td>
</tr>
<tr>
<td>Sgk1 R</td>
<td>GAGAGGAGGGTGAGCTCCTTC</td>
</tr>
<tr>
<td>Sirga F</td>
<td>CACAAGCAGTCCAGAGCACAT</td>
</tr>
<tr>
<td>Sirga R</td>
<td>CCAGGGTCTGTCCAGGTG</td>
</tr>
<tr>
<td>Sirt1 F</td>
<td>TCCTCTAGTTCCTGTGGCAGT</td>
</tr>
<tr>
<td>Sirt1 R</td>
<td>GGCCTCTCTGGATCATCTC</td>
</tr>
<tr>
<td>Tob1 F</td>
<td>AATGCCAGCTTTATGGAAACC</td>
</tr>
<tr>
<td>Tob1 R</td>
<td>ACCACTGTCAAGCTCTTCCCT</td>
</tr>
<tr>
<td>pFam13a F</td>
<td>CCAGCTTATGGCTTGAAATCATG</td>
</tr>
<tr>
<td>pFam13a R</td>
<td>CGGATCCATCTCTTTTGCTGATGAG</td>
</tr>
</tbody>
</table>

\(Hind\ II)\n
\(BamH I)\n
Table S2. Significant change of host autophagy-related factors in AnxA2 or Fam13A deficient MH-S cells (over 5-fold).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene description</th>
<th>siAnxA2 vs CTRL Fold change</th>
<th>siFam13A vs CTRL Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actb</td>
<td>Actin, beta</td>
<td>1.01171</td>
<td>-11.63180</td>
</tr>
<tr>
<td><strong>Akt1</strong></td>
<td>Thymoma viral proto-oncogene 1</td>
<td>20.09237</td>
<td>13.13340</td>
</tr>
<tr>
<td>Atg10</td>
<td>Autophagy-related 10 (yeast)</td>
<td>-3.21075</td>
<td>-5.81637</td>
</tr>
<tr>
<td>Atg7</td>
<td>Autophagy-related 7 (yeast)</td>
<td>-3.06084</td>
<td>-37.80196</td>
</tr>
<tr>
<td>Bcl2</td>
<td>B cell leukemia/lymphoma 2</td>
<td>-1.90966</td>
<td>-7.92708</td>
</tr>
<tr>
<td>Cdkn1b</td>
<td>Cyclin-dependent kinase inhibitor 1B</td>
<td>-2.45685</td>
<td>-6.07543</td>
</tr>
<tr>
<td>Cxcr4</td>
<td>Chemokine (C-X-C motif) receptor 4</td>
<td>-2.03963</td>
<td>-15.25398</td>
</tr>
<tr>
<td>Dapk1</td>
<td>Death associated protein kinase 1</td>
<td>-1.38929</td>
<td>-29.69519</td>
</tr>
<tr>
<td>Dram2</td>
<td>DNA-damage regulated autophagy modulator 2</td>
<td>-2.57016</td>
<td>-5.72730</td>
</tr>
<tr>
<td>Eif2ak3</td>
<td>Eukaryotic translation initiation factor 2 alpha kinase 3</td>
<td>-3.06101</td>
<td>-5.43009</td>
</tr>
<tr>
<td>Esr1</td>
<td>Estrogen receptor 1 (alpha)</td>
<td>-1.15217</td>
<td>-12.39905</td>
</tr>
<tr>
<td>Hdac1</td>
<td>Histone deacetylase 1</td>
<td>-5.33757</td>
<td>-1.98670</td>
</tr>
<tr>
<td>Igf1</td>
<td>Insulin-like growth factor 1</td>
<td>-5.44704</td>
<td>-2.66451</td>
</tr>
<tr>
<td>Map1lc3b</td>
<td>Microtubule-associated protein 1 light chain 3 beta</td>
<td>-3.51274</td>
<td>-6.02464</td>
</tr>
<tr>
<td>Mapk14</td>
<td>Mitogen-activated protein kinase 14</td>
<td>-2.40791</td>
<td>-5.12917</td>
</tr>
<tr>
<td><strong>Mtor</strong></td>
<td>Mechanistic target of rapamycin (serine/threonine kinase)</td>
<td>17.33539</td>
<td>12.79797</td>
</tr>
<tr>
<td>Nfkb1</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B cells 1, p105</td>
<td>5.75894</td>
<td>32.52907</td>
</tr>
<tr>
<td>Npc1</td>
<td>Niemann Pick type C1</td>
<td>-3.16719</td>
<td>6.42205</td>
</tr>
<tr>
<td>Rb1</td>
<td>Retinoblastoma 1</td>
<td>-2.04819</td>
<td>-5.07060</td>
</tr>
<tr>
<td>Snca</td>
<td>Synuclein, alpha</td>
<td>1.57903</td>
<td>-40.75448</td>
</tr>
<tr>
<td><strong>Ulk1</strong></td>
<td>Unc-51 like kinase 1 (C. elegans)</td>
<td>-14.10357</td>
<td>-96.45157</td>
</tr>
<tr>
<td><strong>Ulk2</strong></td>
<td>Unc-51 like kinase 2 (C. elegans)</td>
<td>-25.53407</td>
<td>-157.82231</td>
</tr>
</tbody>
</table>