Mycobacterium abscessus Clearance by Neutrophils Is Independent of Autophagy

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ABSTRACT *Mycobacterium abscessus*, a rapidly growing nontuberculous mycobacterium, is increasingly prevalent in chronic lung disease, including cystic fibrosis, and infections are characterized by neutrophil-dominated environments. However, mechanisms of immune control are poorly understood. Azithromycin, a macrolide antibiotic with immunomodulatory effects, is used to treat *M. abscessus* infections. Recently, inhibition of macrophage bactericidal autophagy was described for azithromycin, which could be detrimental to the host. Therefore, we explored the role of autophagy in mycobactericidal neutrophils. Azithromycin did not affect *M. abscessus*-induced neutrophil reactive oxygen species formation, phagocytosis, or cytokine secretion, and neutrophils treated with azithromycin killed *M. abscessus* equally as well as untreated neutrophils from either healthy or cystic fibrosis subjects. One clinical isolate was killed more effectively in azithromycin-treated neutrophils, suggesting that pathogen-specific factors may interact with an azithromycin-sensitive pathway. Chloroquine and rapamycin, an inhibitor and an activator of autophagy, respectively, also failed to affect mycobactericidal activity, suggesting that autophagy was not involved. However, wortmannin, an inhibitor of intracellular trafficking, inhibited mycobactericidal activity, but as a result of inhibiting phagocytosis. The effects of these autophagy-modifying agents and azithromycin in neutrophils from healthy subjects were similar between the smooth and rough morphotypes of *M. abscessus*. However, in cystic fibrosis neutrophils, wortmannin inhibited killing of a rough clinical isolate and not a smooth isolate, suggesting that unique host-pathogen interactions exist in cystic fibrosis. These studies increase our understanding of *M. abscessus* virulence and of neutrophil mycobactericidal mechanisms. Insight into the immune control of *M. abscessus* may provide novel targets of therapy.

KEYWORDS azithromycin, cell trafficking, chloroquine, cystic fibrosis, innate immunity, phagocytosis, reactive oxygen species, wortmannin

Mycobacterium abscessus is a rapidly growing nontuberculous mycobacterium (NTM) that is emerging as an important pathogen in chronic lung disease, including cystic fibrosis (CF) (1–8). In CF, *M. abscessus* infection is associated with an accelerated decline in lung function and incomplete therapeutic options due to intrinsic antibiotic resistance (6, 9, 10). Two distinct colony morphologies, or morphotypes, are observed during culture on solid media. The smooth morphotype, characteristic of environmental isolates, is associated with biofilm formation, poor recognition by immune cells, sliding motility, and expression of a specialized cell envelope molecule, glycopeptidolipid. The rough morphotype is an adaptive change observed during *in vivo* growth, and is characterized by a loss of glycopeptidolipid, extensive clumping, enhanced recognition by immune cells, and increased virulence (11).

Citation Pohl K, Grimm XA, Caceres SM, Poch KR, Rysavy N, Saavedra M, Nick JA, Malcolm KC. 2020. *Mycobacterium abscessus* clearance by neutrophils is independent of autophagy. Infect Immun 88:e00024-20. <https://doi.org/10.1128/IAI.00024-20>.

Editor Igor E. Brodsky, University of Pennsylvania

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Received 5 February 2020

Returned for modification 27 April 2020

Accepted 14 May 2020

Accepted manuscript posted online 18 May 2020

Published 21 July 2020

Progression from infection to disease is poorly understood, but involves complex processes, including conversion to the rough morphotype, virulence factor expression, and host genetic background (11, 12). Similarities with *M. tuberculosis* have directed NTM research toward macrophages as the primary cell studied (13–17). Macrophage phagocytosis of both smooth and rough *M. abscessus* morphotypes occurs, but the smooth morphotype is readily cleared, while the rough morphotype eludes intracellular killing (18–23). Recent studies have described a role for macrophage autophagy in the clearance of NTM and *M. tuberculosis* (24–27).

Infections with *M. abscessus* are highlighted by neutrophil accumulation and inflammatory conditions (28), suggesting that neutrophils play an important role in *M. abscessus* pathogenesis. Neutrophils use intracellular and extracellular mechanisms to kill *M. abscessus* (29), but these bactericidal pathways are inefficient compared to the ability to control other bacteria, and may exploit this limited control to its advantage (30). In neutrophils, intracellular mechanisms of *M. abscessus* killing are poorly understood.

Azithromycin (AZM) is a macrolide antibiotic, and it is one of the few therapeutic options for NTM infections (8). In addition to its antibiotic activity, AZM displays immunomodulatory effects to alter host responses to pathogens, including increased bactericidal activity but inhibition of inflammatory cytokines, reactive oxygen species (ROS), neutrophil extracellular trap formation, and eicosanoid synthesis (31–39). A novel immunomodulatory activity of AZM was described by Renna et al. during NTM infection, resulting in inhibition of macrophage autophagy and increased survival of *M. abscessus* (40), specifically by inhibiting lysosome acidification and reducing lysosome-phagosome fusion. The authors hypothesized that AZM treatment was associated with an increase in NTM infection in the CF population. While detailed clinical studies have not found an association of AZM therapy and NTM infections (41), the possibility that AZM alters autophagy-dependent killing suggests it may be an important molecular tool to dissect intracellular bactericidal processes (40, 42, 43).

The finding that AZM inhibits macrophage autophagy during NTM infection led us to explore the effects of AZM on neutrophil mycobactericidal activity. Surprisingly, neutrophil bactericidal activity was found to be largely independent of autophagy. However, wortmannin, an inhibitor of autophagy and intracellular trafficking, promoted *M. abscessus* survival, but did so through inhibition of *M. abscessus* uptake. These studies further define mycobactericidal pathways in neutrophils, and provide a framework for further studies to understand host and pathogen factors important in NTM clearance.

RESULTS

AZM does not inhibit neutrophil killing of *M. abscessus*. *M. abscessus* survival was determined in the presence and absence of AZM to test if AZM affected neutrophil mycobactericidal activity. The killing of the type strains of either morphotype (Sm-ATCC and R-ATCC) by neutrophils from healthy subjects was not affected by AZM (Fig. 1A). Similarly, killing of CF sputum isolates of *M. abscessus* by neutrophils from healthy subjects was mostly unaffected by AZM (Fig. 1B). The killing of the smooth morphotype of CF016 (Sm-CF016) was enhanced rather than inhibited in the presence of AZM. These data suggest that AZM does not inhibit neutrophil mycobactericidal activity against either morphotype, but it may instead promote neutrophil mycobactericidal activity in a strain-specific manner.

***M. abscessus* killing is autophagy-independent.** The lack of effect of AZM suggests that neutrophils do not use an AZM-sensitive autophagy pathway. However, neutrophils may use an AZM-insensitive autophagy pathway for intracellular killing. These findings led us to explore neutrophil autophagy pathways in greater detail. The mammalian target of rapamycin complex (mTORC1) regulates the unc-51-like kinase 1 (ULK1) complex that controls the class III phosphatidylinositol (PI3K) vacuolar protein sorting 34 (VPS34), the activation of which promotes autophagosome formation. Wortmannin inhibits VPS34, disrupting this process. Wortmannin inhibited neutrophil

FIG 1 No inhibition of *M. abscessus* killing by AZM. (A) Human peripheral neutrophils were incubated with AZM (closed bars; 25 μ g/ml) for 20 min and infected with *M. abscessus* smooth (Sm-ATCC) and rough (R-ATCC) morphotypes for 1 h. Surviving bacteria were compared to the initial inoculum to determine % killing; $n = 10$ independent experiments. (B) Infection with CF *M. abscessus* clinical isolates. Morphotypes are indicated below; $n = 4$ to 5 independent experiments. *, $P < 0.05$.

killing of *M. abscessus* (Fig. 2A). However, chloroquine, which similarly inhibits autophagy, but by disrupting phagosome-lysosome fusion, had no effect (Fig. 2B). As these agents inhibit autophagy by distinct mechanisms, but have different effects on neutrophil bactericidal activities, we used rapamycin to clarify if autophagy is a mechanism of *M. abscessus* killing in neutrophils (44, 45). Rapamycin promotes the activation of mTORC1 and VPS34 to stimulate autophagy. Pretreatment of cells with rapamycin had no effect on neutrophil killing of *M. abscessus* (Fig. 2C), suggesting that autophagy is not involved. To confirm that wortmannin is effective in inhibiting neutrophil killing, a CF clinical isolate was used. Killing of Sm-CF016 was also inhibited by wortmannin (Fig. 2D).

No effect of AZM on *Pseudomonas aeruginosa* killing. Bacterial species may be processed through different intracellular pathways. To test the specificity of AZM and wortmannin on neutrophil bactericidal activity, *P. aeruginosa* killing was assessed. AZM treatment did not affect *P. aeruginosa* killing (Fig. 3). It is important to note that extracellular antibiotic activity of AZM is minimized in this system due to efficient uptake of free AZM by neutrophils (46). In contrast to AZM, wortmannin-treated neutrophils demonstrated reduced killing of *P. aeruginosa*, while other regulators of

FIG 2 Wortmannin inhibits neutrophil killing of *M. abscessus*. Neutrophils were incubated with wortmannin (WT; 10 μ M) (A), chloroquine (Cq; 5 μ g/ml) (B), and rapamycin (500 nM) (C) for 20 min and infected with smooth (Sm-ATCC) and rough (R-ATCC) isolates of the type strain; $n = 7$ to 10 independent experiments. (D) Neutrophil killing of Sm-ATCC is compared to a smooth clinical isolate (Sm-CF016) in cells treated with wortmannin (WT); $n = 6$ to 7. *, $P < 0.05$; †, $P < 0.001$.

FIG 3 Wortmannin inhibits neutrophil killing of *P. aeruginosa*. Neutrophils were incubated with AZM (closed bar; 25 μ g/ml) or wortmannin (WT) (hatched bar; 10 μ M) for 20 min and infected with *P. aeruginosa* PAO1 for 1 h; $n = 6$ independent experiments. Ctl, control nontreated cells; **, $P < 0.01$.

autophagy and intracellular trafficking, including rapamycin and chloroquine, did not affect *P. aeruginosa* killing (not shown).

AZM does not affect neutrophil ROS generation and phagocytosis. Although *M. abscessus* is not susceptible to neutrophil ROS (29), AZM may modify ROS generation and indirectly affect immune responses or tissue injury. As shown previously, Sm-ATCC and R-ATCC induced neutrophil intracellular ROS (29), but AZM had no effect on *M. abscessus*-induced ROS (Fig. 4). The requirement for intracellular targeting was confirmed using cytochalasin D, which inhibited *M. abscessus*-induced intracellular ROS (not shown) (29). In addition, chloroquine and wortmannin also inhibited *M. abscessus*-induced intracellular ROS (Fig. 4C and D), supporting an intracellular trafficking effect

FIG 4 Azithromycin does not affect intracellular ROS production. (A) Time course of intracellular ROS production; RFU, relative fluorescence units. Neutrophils were incubated with the ROS detection reagent CM-H₂DCFDA and infected with Sm-ATCC (closed circle) or R-ATCC (closed square). (B) Area under the concentration-time curve (AUC) of ROS production over 120 min in the absence or presence of AZM (closed bars; 25 μ g/ml). (C) AUC of ROS production in the absence or presence of chloroquine (Cq, 5 μ g/ml; closed bars). (D) AUC of ROS production in the absence or presence of wortmannin (WT, 10 μ g/ml; closed bars); $n = 3$ to 8. *, $P < 0.05$; **, $P < 0.01$.

for these agents. No change in the level of phagocytosis between morphotypes was observed (Fig. 5). Furthermore, AZM did not affect the extent of neutrophil phagocytosis, or loss of cell surface CD16 expression, a marker for neutrophil activation, of either morphotype.

In control infected neutrophils, intracellular localization of Sm-FITC occurred in zones that excluded elastase (Fig. 6) (29). AZM-treated neutrophils similarly resulted in intracellular localization of Sm-FITC in zones that excluded elastase (not shown). Interestingly, immunofluorescence analysis in wortmannin-treated neutrophils demonstrated a peripheral localization of *M. abscessus* (Fig. 6), similar to that seen for cytochalasin D, indicating reduced *M. abscessus* uptake. Therefore, any intracellular effects of wortmannin appear secondary to inhibition of uptake.

Effect of AZM on cytokine production. To assess the ability of AZM to modulate a secondary immune response, the levels of interleukin-8 (IL-8) and CCL4/MIP1 β were measured after exposure to *M. abscessus*. AZM had a modest inhibitory effect on CCL4 production by smooth *M. abscessus*-treated neutrophils, but had no effect on IL-8, or after exposure to rough *M. abscessus* (Fig. 7). These data indicate that AZM does not have a profound secondary immune effect on *M. abscessus*-exposed neutrophils.

Neutrophils from patients with CF kill *M. abscessus*. Immunocompromised patients are at risk for NTM disease (6, 7). The bactericidal activity of CF neutrophils was determined using isogenic smooth (Sm-CF016) and rough (R-CF016) morphotypes isolated from CF sputum. Both clinical morphotypes were killed with similar kinetics (Fig. 8A). As observed in neutrophils from healthy subjects, AZM increased killing of Sm-CF016 by CF neutrophils, while chloroquine had no effect on killing (Fig. 8B). R-CF016 killing was unaffected by either agent (Fig. 8B). For these agents, this particular isogenic pair of isolates had similar responses as the ATCC morphotypes. In contrast to neutrophils from healthy subjects exposed to Sm-ATCC, wortmannin had no effect on the ability of CF neutrophils to kill Sm-CF016 (Fig. 8C), while wortmannin did inhibit killing by CF neutrophils of R-CF016.

DISCUSSION

The use of AZM in the CF population has increased in recent years (40). AZM has proven to have profound effects on the health status in chronic lung diseases, including decreases in the frequency of pulmonary exacerbations (47–49). Recent studies indicate that AZM inhibits autophagy (40, 42, 43) and mediates increased NTM survival in CF (40). However, the effect of AZM on neutrophils in response to mycobacteria is not well studied. Previous reports indicate that AZM modulates pathogen-exposed neutrophil inflammatory mediator production, ROS, phagocytosis, and killing (35–39). However, classic neutrophil innate immune processes in *M. abscessus* in the absence of opsonization, such as phagocytosis, ROS generation, and cytokine secretion, were unaffected by acute AZM treatment.

In contrast to the inhibitory effects of AZM reported in macrophages (40), mycobactericidal activity of neutrophils from healthy subjects and subjects with CF was not inhibited by AZM. As dysregulated neutrophilic inflammation is a hallmark of CF lung disease, these data predict that AZM treatment would not be detrimental to the host response to *M. abscessus*, although CF-specific effects could exist. Furthermore, AZM enhanced, rather than inhibited, killing of a CF isolate of *M. abscessus* by neutrophils isolated from healthy donors and CF subjects. The finding that AZM enhanced the killing of Sm-CF016, but not other CF isolates, suggests instead that a complex interaction between host and pathogen mediates *M. abscessus* clearance. Importantly, both Sm-ATCC and Sm-CF016 are similarly susceptible to AZM in *in vitro* kill assays and by drug susceptibility testing (not shown). While CF016 was isolated from a subject diagnosed with NTM disease, other isolates were isolated from subjects with indolent infections. It may be informative to screen a large number of clinical isolates to establish the extent of this apparent neutrophil-dependent AZM sensitivity, and to determine *M. abscessus* genetic elements associated with AZM-sensitive and -insensitive strains.

FIG 5 Azithromycin does not affect phagocytosis. Neutrophils were incubated with FITC-labeled Sm-ATCC and R-ATCC (FITC-Mab) for the indicated times. (A) Representative flow cytometry plots showing forward scatter (FSC) and side scatter (SSC) of nonstimulated neutrophils, and gating of Alexa

(Continued on next page)

FIG 6 Wortmannin inhibits *M. abscessus* phagocytosis. Neutrophils were incubated with FITC-Mab (green; Sm-ATCC) for 1 h in the presence of wortmannin (WT; 10 μ M) or cytochalasin D (cytoD; 5 μ g/ml). Following cytocentrifugation, neutrophils were stained for neutrophil elastase (red). Images are presented with (upper) and without (lower) FITC-Mab staining to clarify localization. Pictures are representative of 5 independent experiments.

Although AZM had little effect on bactericidal activity, AZM-insensitive autophagy may still participate in neutrophil *M. abscessus* killing. However, the finding that chloroquine and rapamycin do not modify neutrophil killing suggests that classical autophagy is independent of *M. abscessus* intracellular processing (Fig. 9). Bactericidal activity in neutrophils that is independent of autophagy is in contrast to autophagy activation in macrophages to many pathogens, including *M. abscessus* (24, 25, 50–53). Autophagy is bactericidal in macrophages, and escape from autophagy is a strategy to promote virulent intracellular growth (24, 54, 55). Interestingly, autophagy is associated only with macrophage killing of the rough *M. abscessus* morphotype (51). However, in our hands autophagy pathways were not implicated in neutrophil killing of either *M. abscessus* morphotype. Activation of neutrophil autophagy has been demonstrated in response to rapamycin, *Escherichia coli*, and *Burkholderia pseudomallei* (44, 45), although we were unable to detect LC3B expression or localization by *M. abscessus* or rapamycin using several antibodies.

Wortmannin inhibits intracellular trafficking pathways, including autophagy, through its inhibition of VPS34. Neutrophil extracellular trap formation by soluble activators has also been reported to be sensitive to wortmannin (56, 57). Interestingly, wortmannin prevented *M. abscessus* killing (Fig. 2 and 8), and these observations were extended to *P. aeruginosa* killing (Fig. 3). In macrophages and other cell types, wortmannin inhibits phagocytosis (58–62). However, the role of wortmannin-sensitive pathways in pathogen phagocytosis in neutrophils is less clear. Nakayama et al. demonstrated inhibition of *M. intracellulare* and *M. goodii* uptake by wortmannin in human neutrophils (63). However, other studies failed to associate wortmannin with inhibition of phagocytosis (64, 65). *Staphylococcus aureus* killing is attenuated by wortmannin, but the effect on phagocytosis was not explored (66). At the concentration used (10 μ M), wortmannin is likely targeting PI3Ks other than VPS34 to block uptake. Overall, our studies support a role of wortmannin-sensitive pathway(s) in *M. abscessus* uptake and killing.

Pathogen recognition and intracellular processing are important steps in controlling infections, but these mechanisms are not well understood for *M. abscessus*. Mannose-capped lipoarabinomannans (ManLAMs) may be important in blocking *M. intracellulare*-containing phagosome maturation (63). LAMs and lipomannans are found in mycobacteria, and ManLAM was also identified as a factor that promotes intracellular survival by redirecting *M. tuberculosis* away from autophagic killing (54). The possibility that a

FIG 5 Legend (Continued)

Fluor-450-conjugated anti-CD16. (B) Flow cytometric analysis of FITC-Mab association with CD16⁺ neutrophils, indicated by the boxed region, at 5 and 60 min in the absence and presence of AZM. Examples of smooth (Sm-Mab; upper panels) and rough (R-Mab; lower panels) *M. abscessus* uptake are shown. (C) Summary of the effect of AZM (closed bars) on neutrophil uptake of *M. abscessus*; *n* = 5.

FIG 7 Azithromycin does not affect cytokine secretion. Neutrophils were incubated with AZM (closed bars) and infected with Sm-ATCC or R-ATCC for 2 h, and secretion of IL-8 (A) and CCL4/MIP1 β (B) was determined by ELISA; $n = 3$. *, $P < 0.05$.

similar pathway regulates *M. abscessus* recognition or clearance was explored. However, ManLAM or mannan from *Saccharomyces cerevisiae* did not block neutrophil uptake or killing of *M. abscessus* (not shown), suggesting different processing pathways for *M. abscessus*. Lipomannans and LAMs were not identified in *M. abscessus* (15), although unique structural features of lipomannan and LAM found in the closely related *M. chelonae* suggest that detection and recognition may differ among mycobacterial species or isolates (67, 68). The role of surface lipids in neutrophil *M. abscessus* recognition and uptake is a focus of ongoing studies.

Finally, AZM had little effect on release of the proinflammatory cytokines IL-8 and CCL4/MIP1 β , which are strongly induced. Although AZM is known to inhibit inflammasome activation and subsequent IL-1 β release in other cell systems (33, 69, 70), we have observed only minimal IL-1 α and IL-1 β release from *M. abscessus*-infected neu-

FIG 8 CF neutrophil killing of *M. abscessus*. (A) CF neutrophils were incubated with Sm-ATCC or R-ATCC for the indicated times. (B) CF neutrophils were incubated with AZM (closed bars; 25 μ g/ml) or chloroquine (Cq) (gray bars; 5 μ g/ml) before infection with clinical smooth and rough morphotypes of CF016 for 1 h; $n = 3$. (C) CF neutrophils were incubated with wortmannin (WT) (closed bars; 10 μ M) before infection; $n = 5$ to 7. *, $P < 0.05$; **, $P < 0.01$.

FIG 9 Different neutrophil processing pathways for *M. abscessus* and autophagic cargo. Phagocytic uptake and intracellular processing of *M. abscessus* are independent of classical autophagy. Rapamycin (rapa) enhances autophagic vesicle formation (open arrow), while azithromycin (AZM) and chloroquine (Cq) prevent autophagosome-lysosome fusion; these agents fail to modify neutrophil killing of *M. abscessus*. Wortmannin (WT) disrupts autophagic vesicle formation (not shown), but also inhibits *M. abscessus* killing by reducing phagocytosis. Specific vesicles are indicated in italics; *M. abscessus* (closed ovals) and autophagic targets (gray squares) are depicted.

trophils (29, 30). Therefore, AZM does not appear to indirectly modify neutrophil immune responses by cytokine release.

Taken together, we have identified an intracellular *M. abscessus* clearance pathway in neutrophils that is independent of autophagy. Furthermore, our data indicate that AZM has little anti-inflammatory or immunomodulatory activity in *M. abscessus*-infected neutrophils, either by direct regulation of innate immune responses or by modulating regulatory cytokine secretion. Therefore, nonantibiotic immunomodulatory activities of AZM that act on other cells do not appear to be present in neutrophils (33, 34, 71). The lack of autophagy activation may explain, in part, the limited ability of neutrophils to kill *M. abscessus*, but the molecular details of putative *M. abscessus* processing pathways await future experiments. These studies enhance our understanding of *M. abscessus* clearance and suggest that enhancing specific intracellular trafficking pathways may be therapeutically useful.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. *M. abscessus* subsp. *abscessus* (ATCC strain 19977/CIP104356T) was propagated from frozen aliquots in 7H9 broth supplemented with 0.5 g/liter bovine albumin fraction V, 0.2 g/liter dextrose, 0.3 mg/liter catalase (ADC; BD Biosciences), 2% glycerol, and 0.05% Tween 80 at 37°C with shaking at 200 rpm for 3 to 5 days. The smooth (Sm-ATCC) and rough (R-ATCC) morphotypes were isolated from the mixed ATCC 19977 stock. Clinical isolates were obtained from the Culture, Biorepository, and Coordinating Core of the Colorado Cystic Fibrosis Foundation Research Development Program at National Jewish Health. Washed cultures were sonicated using six 1-s bursts of a Fisher Sonic Dismembrator 100 at approximately 4 W output to obtain single cells and smaller aggregates, and adjusted to an optical density at 600 nm (OD_{600}) of 1.0 in PBS containing Ca^{2+} and Mg^{2+} , corresponding to approximately 1×10^9 CFU/ml. *Pseudomonas aeruginosa* (strain PAO1) was grown in LB overnight, washed in PBS, and the OD_{600} adjusted to 1.0, corresponding to approximately 2×10^9 CFU/ml, and further diluted before use.

Neutrophil isolation. Neutrophils were isolated from healthy volunteers by the plasma Percoll method as previously described (72). Isolated neutrophils were washed and resuspended in Krebs-Ringer phosphate-buffered dextrose (154 mM NaCl, 5.6 mM KCl, 1.1 mM $MgSO_4$, 2.2 mM $CaCl_2$, 0.85 mM NaH_2PO_4 , 2.15 mM Na_2HPO_4 , and 0.2% dextrose). Cells were confirmed to be >98% pure by visual inspection of cytopins. CF neutrophils were isolated from patients without regard for clinical status. Blood was collected into heparin-containing tubes, and neutrophils were separated from peripheral blood mononuclear cells using Lymphoprep. Briefly, blood tubes were centrifuged for 20 min at $350 \times g$ with no brake to isolate platelet-rich plasma, and the remaining sample diluted 1:1 with 0.9% NaCl. One milliliter of prewarmed 6% dextran was added per 10 ml of diluted blood and allowed to sediment for 30 to 45 min. The top layer of cells from the dextran sedimentation was layered carefully onto 15 to 20 ml of Lymphoprep, and centrifuged at $800 \times g$ for 20 min at room temperature with no brake. The cell pellet was resuspended in 9 ml water for 15 sec, 1 ml $10\times$ PBS was added, and immediately centrifuged at $300 \times g$ for 6 min. The cells were washed two times with PBS before experiments.

These studies were approved by the National Jewish Health Institutional Review Board, and written informed consent was obtained from all neutrophil donors. The study was conducted in accordance with the Declaration of Helsinki.

Killing assay. Neutrophils were suspended in RPMI supplemented with 10 mM HEPES, pH 7.4, and 2% pooled heat-inactivated platelet-poor plasma (complete RPMI). Sample tubes contained cells (1×10^6) suspended in complete RPMI and the respective bacteria at a multiplicity of infection (MOI) of approximately 10:1 in a 0.1 ml volume. Neutrophils were preincubated with inhibitors for 20 to 30 min prior to adding bacteria. The following inhibitors were used: azithromycin (AZM; 25 $\mu\text{g/ml}$), wortmannin (10 μM), chloroquine (5 $\mu\text{g/ml}$) (56), and rapamycin (500 nM) (44, 45). Tubes were initially centrifuged at $4,000 \times g$ for 1 min and pelleted cells were incubated at 37°C for 5 min to promote bacterium-neutrophil interaction, followed by resuspension and incubation for up to 2 h, as indicated. These experimental conditions promote synchronous, close contact of mycobacteria and neutrophils. Triton X-100 (0.1%) in 0.9% NaCl was added to inactivate neutrophils and aid in mycobacterial dispersion. Following vortex mixing and serial dilutions in saline, samples were plated on 7H10 agar supplemented with OADC and incubated at 37°C. Colonies were counted 3 to 5 days after plating, and compared to colony counts at initiation of infection. *P. aeruginosa* killing was performed at an MOI of 1:1.

Cytokine and chemokine release. Secretion of IL-8 and CCL4/MIP1 β was determined from cell supernatants after 2 h stimulation with *M. abscessus* at an MOI of 10:1. Products were quantified by enzyme-linked immunosorbent assay (ELISA) (ELISATech).

Intracellular reactive oxygen species assays. Intracellular reactive oxygen species (ROS) was measured using neutrophils loaded with 10 μM CM-H2DCFDA for 20 min; labeled cells were washed and resuspended in complete RPMI and 2×10^5 cells were allowed to settle for 20 min in wells of a black 96-well plate at 37°C in the presence of inhibitors. Cells were incubated with dimethyl sulfoxide (DMSO), AZM, chloroquine, or wortmannin. Bacteria were added at an MOI of 10:1, centrifuged at $110 \times g$ for 1 min and placed in a BioTek FL800 plate reader; reads were performed every 5 min for up to 2 h at 37°C at excitation/emission of 485/528. Area under the concentration-time curve (AUC) values were generated using GraphPad Prism 4.0.

Phagocytosis of *M. abscessus*. Fluorescein isothiocyanate (FITC)-labeled *M. abscessus* (FITC-*Mab*) was prepared by incubating washed *M. abscessus* with 30 $\mu\text{g/ml}$ FITC in 0.1 M sodium bicarbonate buffer, pH 9.6, for 30 min at room temperature in the dark. FITC-*Mab* was sonicated, as described above, washed two times, and resuspended in PBS (29). For analyzing bacterial localization by flow cytometry, neutrophils were incubated with FITC-*Mab* at an MOI of 5:1. Samples were processed as described above in the killing assay with a brief centrifugation and incubation before resuspending the cells, and incubated over a period of 5 to 60 min. Reactions were stopped with an equal volume of ice-cold complete RPMI. Neutrophils were stained with eFluor 450-labeled anti-CD16 (CB16) to identify neutrophils, and fixed in 1% formaldehyde/3% sucrose. To distinguish intracellular and extracellular staining, flow analysis was compared in duplicate samples in both the absence (total staining) and presence (internal staining) of 0.4% trypan blue to quench external FITC; results of total staining are reported, as external staining was minimal (29). Analysis was performed on an LSRII flow cytometer (BD Biosciences) and FlowJo software (TreeStar). Neutrophils were gated to exclude cellular debris and planktonic and aggregated bacteria.

Immunofluorescence analysis. For analyzing bacterial localization by microscopy (29), neutrophils were incubated with inhibitors, as indicated, and infected with FITC-*Mab* for 1 h at an MOI of 10:1; cells were adhered to microscope slides by cytocentrifugation and air dried. Cells were permeabilized in 0.5% Triton X-100 for 30 min, stained overnight with anti-elastase (abcam; ab-21595; 1:100), and visualized by confocal fluorescence microscopy after binding of Alexafluor 555-conjugated goat anti-rabbit antibody.

Statistical analysis. Data are presented as mean $\pm$ standard error of the mean (SEM) and analyzed by *t* test. Significance was set at a *P* value of 0.05. All data sets were normally distributed.

ACKNOWLEDGMENTS

This research was funded in part by grants from the Cystic Fibrosis Foundation to K.C.M. (MALCOL14G0), J.A.N. (NICK14G0), and K.P. (POHL16F0 and NCATS Colorado CTSA), and was supported by resources provided by the Cystic Fibrosis Foundation Colorado Cystic Fibrosis Research and Development Program.

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