Nontuberculous Mycobacteria Show Differential Infectivity and Use Phospholipids to Antagonize LL-37

Jennifer R. Honda1, Tamara Hess2†, Rachel Carlson3, Pitchaimani Kandasamy3, Luisa Maria Nieto Ramirez4, Grant J. Norton1, Ravleen Virdi1, M. Nurul Islam2, Carolina Mehaffy2, Nabeeh A. Hasan1, L. Elaine Epperson1, Danny Hesser2, Scott Alper1,5, Michael Strong1, Sonia C. Flores6, Dennis R. Voelker3, Karen M. Dobos2, and Edward D. Chan3,6,7

1Department of Biomedical Research, Center for Genes, Environment and Health, and 3Department of Medicine and Academic Affairs, National Jewish Health, Denver, Colorado; 2Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, Colorado; 4Facultad de Ciencias Básicas, Universidad Santiago de Cali, Cali, Colombia; 6Department of Immunology and Microbiology, and 6Division of Pulmonary Science and Critical Care Medicine, University of Colorado–Denver, Anschutz Medical Campus, Aurora, Colorado; and 7Department of Medicine, Denver Veterans Affairs Medical Center, Denver, Colorado

ORCID ID: 0000-0002-2411-2037 (S.A.).

Abstract

Comparisons of infectivity among the clinically important nontuberculous mycobacteria (NTM) species have not been explored in great depth. Rapid-growing mycobacteria, including Mycobacterium abscessus and M. porcinum, can cause indolent but progressive lung disease. Slow-growing members of the M. avium complex are the most common group of NTM to cause lung disease, and molecular approaches can now distinguish between several distinct species of M. avium complex including M. intracellulare, M. avium, M. marseillense, and M. chimaera. Differential infectivity among these NTM species may, in part, account for differences in clinical outcomes and response to treatment; thus, knowing the relative infectivity of particular isolates could increase prognostication accuracy and enhance personalized treatment. Using human macrophages, we investigated the infectivity and virulence of nine NTM species, as well as multiple isolates of the same species. We also assessed their capacity to evade killing by the antibacterial peptide cathelicidin (LL-37). We discovered that the ability of different NTM species to infect macrophages varied among the species and among isolates of the same species. Our biochemical assays implicate modified phospholipids, which may include a phosphatidylinositol or cardiolipin backbone, as candidate antagonists of LL-37 antibacterial activity. The high variation in infectivity and virulence of NTM strains suggests that more detailed microbiological and biochemical characterizations are necessary to increase our knowledge of NTM pathogenesis.

Keywords: nontuberculous mycobacteria; human macrophages; cathelicidin (LL-37) antibacterial peptide; phosphatidylinositol; cardiolipin
Clinical Relevance

Differences in virulence among the clinically relevant nontuberculous mycobacterial (NTM) species and the factors that promote host evasion are understudied. We show that macrophage infectivity varies among NTM species and even among isolates of the same species. Moreover, NTM-derived phospholipids may facilitate evasion from host innate antibacterial peptides such as cathelicidin.

More than 199 different species of nontuberculous mycobacteria (NTM) have been identified to date (1). NTM are common inhabitants of environmental water biofilms and soil, and can be aerosolized and inhaled into the lungs through daily activities such as showering and gardening (2, 3). Over the last 30 years, NTM lung disease (NTM-LD) has become increasingly prominent, even outnumbering tuberculosis cases in many regions of the world (4). Long-term therapy is often necessary to treat NTM-LD, and recurrence due to relapse or reinfection is often necessary to treat NTM-LD, and recurrence due to relapse or reinfection is common, resulting in substantial morbidity and mortality (5, 6).

Rapid-growing mycobacteria (RGM), such as Mycobacterium abscessus, are of great concern, especially with regard to patients with cystic fibrosis (CF). RGM require treatment with prolonged multidrug antibiotic regimes, and even then are often refractory to treatment, with a high likelihood of relapse (7). M. porcinum is another RGM that is increasingly recognized as a human pathogen (8). In patients without CF, the most common LD-causing NTM belong to the M. avium complex (MAC) and are slow-growing mycobacteria (SGM). In previous work, we applied rpoB gene sequencing to identify M. abscessus, M. porcinum, M. intracellulare, M. marseillense, and M. chimaera species from environmental and clinical samples (9). The differential ability of these NTM organisms to survive within host macrophages is an important observation that has furthered our understanding of NTM pathogenesis. Another barrier in the current field of NTM research is the lack of knowledge regarding the virulence mechanisms used by pathogenic NTM.

The well-characterized virulence factors of M. tuberculosis include a variety of lipid molecules; however, much less is known about the NTM lipid components that contribute to host immune evasion (10). Previously, we reported a novel virulence mechanism of NTM that is mediated by lipids and results in resistance to and inactivation of the cathelicidin antibacterial peptide (LL-37) by NTM cell membrane (CM) and/or cell wall (CW) lipids (11).

In the current study, we assessed the ability of different NTM species and isolates of the same species to survive exposure to human macrophages and to evade LL-37–mediated bacterial killing. We discovered that 1) the number of cell-associated NTM varied markedly among species and even among isolates of the same species after infection of human macrophages, suggesting intrinsic differences in infectivity even among closely related NTM isolates; 2) increased virulence correlated with heightened numbers of macrophage-associated NTM and resistance to and neutralization of LL-37 antibacterial activity; and 3) NTM-modified phospholipid molecules likely function as virulence factors by neutralizing the antibacterial activity of LL-37.

Portions of this manuscript have been previously published in abstract form (12).

Methods

Please see the data supplement for additional details regarding the methods used in this work.

NTM Isolates Used in this Study

Table 1 lists all of the isolates used in this study. These isolates were chosen because they are either well-described type strains with available genomic information or were previously cultured and identified via partial gene sequencing from environmental or clinical samples by our laboratory (9).

NTM Infection of Human Immune Cells and LL-37 Immunoblotting

Human THP-1–derived macrophages were used because they are a standard cell line that is commonly used in mycobacterial experiments and because the isogenic nature of this cell line limits experimental variability (13). The infection protocol used was adapted from our published work of mycobacteria-infected THP-1 macrophages (14–18). Isolation and infection of primary human monocyte-derived macrophage (MDM) and alveolar macrophage (AM) cell cultures, and LL-37 immunoblotting are described in the data supplement.

Monitoring the Antibacterial Activity of LL-37 against NTM

The methods used to monitor the antibacterial activity of LL-37 against NTM were previously described (11) and are also detailed in the data supplement.

Thin-Layer Chromatography of NTM Lipids and LL-37 Binding Immunoblotting

To define the NTM lipids that bind LL-37, CM lipids, total lipids from the CW, and cytosolic fractions were prepared from NTM isolates. To determine whether lipids present in these fractions bind LL-37, fractions were spotted in triplicate onto identical silica plates and subjected to TLC. The first two plates were sprayed with CuSO₄ and α-naphthol to visualize lipid species and glycolipids, respectively. To determine which NTM lipids LL-37 binds, the third plate was used for LL-37 binding IB as described in the data supplement.

Testing the LL-37 Inactivation Activity of Pure Phospholipids

Purified lipids were obtained from Avanti Polar Lipids and included soy L-α-lysophosphatidylinositol (PI; #840044), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC; #850457), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (PE; #850725), and 1,1’2,2’ tetraoleoyl cardiolipin (CL; #710335). The lipids were stored dry at −20°C, resuspended in 2:1 chloroform:methanol, and subjected to TLC and LL-37 binding immunoblots. For LL-37 bioassays, lipids were dissolved in absolute ethanol and stored at −20°C.

We previously demonstrated that Escherichia coli is susceptible to 25 µg/ml of LL-37, and NTM-exposed LL-37 loses its potent activity against E. coli (11). The E. coli bioassay was used to determine whether these purified lipids and total lipid extracts (TLEs) from M. chimaera 12-54-Sw-B-1 and 12-7-S-1-3 neutralize the antibacterial activity of LL-37. LL-37 at 25 µg/ml was added to 1 µg of lipid in 250 µl of LL-37 media (defined in the data supplement) and preincubated at 37°C.
Table 1. Nontuberculous Mycobacteria Isolates Used in This Study

| Species              | Identification          | Source         |
|----------------------|-------------------------|----------------|
| M. abscessus subsp. abscessus | 12-99-Sw-B-2 | Kitchen sink |
|                      | 12-45-Sw-A-1           | Kitchen sink |
|                      | 19977                  | ATCC type strain |
| M. porcinum          | KAU12-9-Sw-B-2         | Kitchen sink |
| M. intracellular      | AH2                    | Clinical, 90-yr-old woman |
|                      | AH9                    | Clinical, 70-yr-old  |
|                      | AH24                   | Clinical, 87-yr-old  |
|                      | 12-56-S-1-2            | Garden soil    |
|                      | 9141                   | Clinical       |
| M. avium             | Subsp. hominisuis H87  | ATCC type strain |
| M. interjectum       | 12-26-S-1-1            | Garden soil    |
| M. timonense         | AH20                   | Clinical, 50-yr-old  |
| M. colombense        | 12-29-S-1-2            | Garden soil    |
| M. marseillense      | AH17                   | Clinical, 38-yr-old  |
|                      | 12-2-S-1-1             | Garden soil    |
| M. chimaera          | AH6                    | Clinical, 73-yr-old  |
|                      | AH13                   | Clinical, 71-yr-old  |
|                      | AH25                   | Clinical, 79-yr-old  |
|                      | 12-54-Sw-B-1           | Kitchen faucet |
|                      | 12-42-Sw-A-3           | Showerhead     |
|                      | 12-40-Sw-A-1           | Showerhead     |
|                      | 12-35-Sw-A-1           | Kitchen faucet |
|                      | 12-43-Sw-A-1           | Showerhead     |
|                      | 12-29-Sw-A-1           | Kitchen faucet |
|                      | 12-44-Sw-A-1           | Showerhead     |
|                      | 12-49-Sw-B-1           | Kitchen faucet |
|                      | 12-25-Sw-B-2           | Showerhead     |
|                      | 12-2-Sw-B-2            | Refrigerator spout |
|                      | 12-22-Sw-A-1           | Showerhead     |
|                      | 12-7-S-1-3             | Fruit cannery soil |

Definition of abbreviations: *M.* = Mycobacterium; *NTM* = nontuberculous mycobacteria.
*“P”* or *“AH”* indicates isolates recovered from respiratory samples from patients suspected of having NTM lung disease. The number 12 indicates isolates recovered from environmental sources in Hawai‘i. *“Sw”* indicates NTM that were recovered from swabs used to sample biofilms on the surface of showerheads, kitchen sinks, and refrigerator spouts. *“S”* indicates NTM that were recovered from environmental sources of soil.

for 4 hours. Subsequently, $2 \times 10^5$ *E. coli* was inoculated into each of the samples, incubated for an additional 4 or 20 hours, and swabbed onto Luria-Bertani agar plates to assess *E. coli* growth. As an untreated control, *E. coli* was inoculated into 250 μL of LL-37 medium. As a positive control, *E. coli* was incubated with synthetic LL-37 peptide (25 μg/mL). The vehicle, 0.4% ethanol, was also incubated with and without LL-37 in the presence of *E. coli*.

**Results**

**NTM Phylogenomic Tree**
In our previously published work, we identified several NTM species using partial rpoB gene sequencing (9). Thirty-two NTM isolates from this earlier study were selected for the current experiments. Partial rpoB sequences were used to create an alignment matrix of 726 bp. Of the 726 sites in the alignment, 146 sites (~20%) were variable and contained no missing data. The phylogenetic relationships between the 32 isolates in Figure 1 show the delineation between the growth rates (rapid vs. slow growing) and the included species.

**NTM Demonstrate a Range of Inter- and Intraspecies Infectivity in Human Macrophages**
THP-1-derived macrophages were infected with a variety of RGM and MAC species. RGM isolates of *M. abscessus* and *M. porcinum* showed inter- and intraspecies variations in THP-1 cell-associated growth by 96 hours after infection (Figure 2A). Specifically, three different isolates of *M. abscessus* showed an increased cell-associated burden with time, and a fourth isolate (P2a) showed minimal change. In contrast, there was a marked temporal reduction in the macrophage-associated burden of *M. porcinum*.

The MAC species tested also demonstrated a range of infectivity in THP-1 macrophages. Among the five *M. intracellular* isolates examined, two (AH2 and 12-56-S-1-2) showed modest growth when cocultured with macrophages. In contrast, *M. intracellular* AH9, AH24, and 9141 demonstrated decreases in viable bacteria over time (Figure 2B). Macrophages also showed effective control of two isolates of *M. avium* (Chester and H87), *M. timonense*, *M. interjectum*, and *M. colombense* (Figure 2C). The three isolates of *M. marseillense* tested showed a progressive decline in bacterial colony-forming units (cfu) over time, with THP-1 macrophages significantly controlling the growth of isolate AH17 by 96 hours after infection (Figure 2D). The *M. chimaera* isolates tested showed a range of growth in macrophages, as demonstrated in Figure 2E, including significant differences between the cfu of *M. chimaera* isolate 12-54-Sw-B-1 (Figure 2E, red bars) and *M. chimaera* isolate 12-7-S-1-3 (Figure 2E, blue bars). Based on these findings, we considered *M. chimaera* 12-54-Sw-B-1 to be more virulent than *M. chimaera* 12-7-S-1-3. To investigate the status of LL-37 expression in THP-1 macrophages before and after infection with the more- or less-virulent *M. chimaera* isolates, we performed LL-37 IB. Less LL-37 was detected in lysates from cells infected with the more-virulent 12-54-Sw-B-1 *M. chimaera* isolate (red arrows) compared with lysates from cells infected with the less-virulent 12-7-S-1-3 *M. chimaera* isolate (blue arrows) at the 24- and 48-hour time points (Figure 2F).

To determine whether the cfu trends observed in the THP-1 macrophages could also be seen in primary immune cells, we compared the temporal cell-associated burden of *M. chimaera* 12-54-Sw-B-1 with that of *M. chimaera* 12-7-S-1-3 in infected human MDMs and AMs. Similar to what was seen in the THP-1 cells, a significantly
greater bacterial burden was observed with \textit{M. chimaera} 12-54-Sw-B-1 than with \textit{M. chimaera} 12-7-S-1-3 in both types of primary human macrophages (Figures 3A and 3B).

**More-Virulent NTM Also Resist LL-37-mediated Bacterial Killing**

We previously reported resistance to and inactivation of the LL-37 antibacterial peptide as a virulence mechanism of NTM (11). Because \textit{M. abscessus} 12-45-Sw-A-1 showed significantly higher cfu than \textit{M. porcinum} in the THP-1 macrophage assay (Figure 2A), we tested these two isolates for their susceptibility to LL-37. \textit{M. abscessus} 12-45-Sw-A-1 completely resisted exposure to varying concentrations of LL-37 (Figure 4A), whereas the \textit{M. porcinum} isolate KAU12-9-Sw-B-2 exhibited significant sensitivity to 125 \(\mu\)g/ml of LL-37 (Figure 4B, blue line). Although 25 \(\mu\)g/ml of LL-37 effectively killed \textit{E. coli} (Figures 4C and 4D, inset boxes), 25 \(\mu\)g/ml and 125 \(\mu\)g/ml of LL-37 incubated in the 96-hour conditioned media from \textit{M. abscessus} lost its potent antibacterial activity against \textit{E. coli} (Figure 4C), suggesting that a component in the conditioned media from this more virulent isolate inactivated LL-37. In contrast, 125 \(\mu\)g/ml of LL-37 incubated in 96-hour conditioned media from \textit{M. abscessus} lost its potent antibacterial activity against \textit{E. coli} (Figure 4C), suggesting that a component in the conditioned media from this more virulent isolate inactivated LL-37.

In contrast, 125 \(\mu\)g/ml of LL-37 incubated in 96-hour conditioned media from \textit{M. abscessus} did not neutralize the antibacterial activity of LL-37 against \textit{E. coli} (Figure 4C). Boiling the conditioned medium from \textit{M. abscessus} before the addition of LL-37 did not abrogate the neutralizing activity of the medium (data not shown), indicating that a heat-stable, secreted NTM component was responsible for LL-37 inactivation.

The more-virulent \textit{M. chimaera} isolate, 12-54-Sw-B-1, and less-virulent isolate, 12-7-S-1-3, were also tested for resistance to LL-37. Although 12-54-Sw-B-1 showed a modest decrease in cfu in the absence of LL-37 at the 48-hour time point, bacterial cfu increased by the 96-hour time point (Figure 4E). Importantly, 12-54-Sw-B-1 was completely resistant to LL-37 (Figure 4E red lines). On the other hand, 25 and 150 \(\mu\)g/ml of LL-37 demonstrated significant antibacterial activity against the 12-7-S-1-3 isolate of \textit{M. chimaera} (Figure 4E, blue lines).

**Modified Phospholipids of NTM Bind LL-37**

To investigate the potential of specific NTM lipids that may be responsible for LL-37 inactivation, TLEs were prepared from \textit{M. chimaera} 12-54-Sw-B-1 and 12-7-S-1-3 and subjected to TLC (Figure 5A). To further dissect which NTM factor(s) bind LL-37, TLEs were also probed for lipids that bind to LL-37. The LL-37 binding immunoblot, in which a TLC plate was incubated with LL-37 peptide and then immunoblotted with anti-LL-37 antibody (19), revealed that LL-37 binds to \textit{M. chimaera} polar lipids (Figure 5B).

To examine whether the binding of LL-37 to NTM polar lipids extends to another species of MAC and to RGM, as well as to further dissect which lipids of NTM...
Figure 2. Differential infectivity of RGM and SGM species in human THP-1 macrophages. THP-1 macrophages were infected with environmental or clinical RGM or Mycobacterium avium complex species isolates. The change in \( \log_{10} \) cfu is shown per species. Three bars are shown for each species corresponding to the change in cfu determined after 24 hours (first bar), 48 hours (second bar), and 96 hours after infection (third bar) compared with cfu quantified at 1 hour after infection of macrophages. \( n=3-10 \) independent experiments. (A) M. abscessus \( (n=4 \) isolates) and an isolate of M. porcinum; \( \*P=0.01 \) and \( \**P=0.002 \). (B) M. intracellulare \( (n=5 \) isolates); \( \*P=0.003 \). (C) M. avium \( (n=2 \) isolates); \( \**P=0.003 \); M. timonense \( (n=1 \) isolate); M. interjectum \( (n=1 \) isolate); M. colombiense \( (n=1 \) isolate); \( \*P=0.02 \). (D) M. marseillense \( (n=3 \) isolates), \( \**P=0.003 \). (E) M. chimaera \( (n=15 \) isolates), \( \*P=0.004 \). (F) LL-37 immunoblot of cell lysates from THP-1 macrophages infected with the more-virulent M. chimaera isolate 12-54-Sw-B-1, as indicated by the red arrows, or the less-virulent M. chimaera isolate 12-7-S-1-3, as indicated by the blue arrows, at various time points after infection. \( \beta\)-actin was used as a loading control. cfu = colony-forming units; THP-1 = human leukemia monocytic cell line.

bind LL-37, various lipid-containing fractions were separated from M. intracellulare and M. abscessus. These lipid fractions were subjected to TLC and stained with CuSO\(_4\) to detect glycolipids, or with \( \alpha\)-naphthol to detect glycolipids, and also probed for lipid–LL-37 complexes. LL-37 bound avidly to polar lipids in the CM and CW fractions (outlined by red box) of both species, but not to any of the nonpolar lipids (Figures 5C and 5D). Moreover, LL-37 did not bind to any component in the cytosolic fraction, indicating that the products responsible for this biological activity were present as surface molecules in M. intracellulare and M. abscessus. It is plausible that the cytosolic fraction contained less lipids than the CM and CW fractions; however, the bands in the upper portion of the lipid species and glycolipid TLC plates were similar in amount and intensity. Thus, we reasoned that the fractions contained comparable amounts of lipids, and thus the active component was primarily resident in the aforementioned fractions.

The current literature provides little information regarding detailed biochemical studies of NTM polar lipids (glycosylated or charged species decorated on smaller C8-C20 branched or single fatty acyl moieties) versus nonpolar lipids (wherein macromolecules include longer-chain branched or single fatty acyl backbones). Despite the scarcity of information, all mycobacteria are known to possess numerous phospholipids, including many PI derivatives, notably the PI mannosides (PIMs). To determine whether LL-37 binds these and/or other phospholipids of NTM, mass spectrometry (MS) was used to probe for NTM-specific phospholipids in BAL fluid samples from M. intracellulare–infected patients.

A representative spectrum of the molecular ions detected shows a region of PI in which the most prominent species were PI(18:1/18:1), PI(16:0/18:1), and PI(18:0/20:4) (Figure E1 in the data supplement). Next, biotinylated LL-37 peptide was incubated with M. abscessus–conditioned medium for 24 hours in a pull-down assay. Results from the MS analyses support our conjecture that M. abscessus phospholipids interacted with LL-37 (the pulled-down phospholipids are identified in Figure E2 and Table E1).

Putative NTM Candidates as LL-37 Antagonists

Because NTM-specific phospholipids are not commercially available, we used soy PI and synthetic CL, PE, and PC as substitues to test their LL-37 neutralizing activity. TLC and LL-37 binding immunoblot analyses revealed that among the four phospholipids
tested, LL-37 interacted most strongly to CL (Figure 6A). When PI and CL controls were subjected to TLC and LL-37 immunoblots alongside TLEs from M. chimaera 12-54-Sw-B-1 and M. chimaera 12-7-S-1-3, LL-37 bound more strongly to lipids that comigrated with CL and less strongly to lipid components that comigrated with PI (Figure 6B). Next, using the E. coli bioassay, we tested PI, CL, PE, and PC for their capacity to inactivate LL-37. As previously observed, E. coli was effectively killed when it was incubated with fresh LL-37 alone (inset boxes, Figures 4C and 4D). However, the bactericidal activity of 25 μg/ml of LL-37 against E. coli was completely lost after coincubation with CL (Figure 6C, no. 6). In stark contrast, LL-37 retained potent antibacterial activity against E. coli despite incubation with PI, PE, and PC (Figure 6C, nos. 5, 7, and 8). LL-37 antibacterial activity for E. coli was also abrogated when the peptide was incubated with TLEs of 12-54-Sw-B-1 or 12-7-S-1-3, but not with the vehicle control (Figure 6C, nos. 4, 9, and 10).

**Discussion**

In this study, we used our collection of environmental and clinical isolates of RGM and SGM that we previously identified to the species and subspecies levels using gene sequencing to further explore NTM infectivity and virulence. Based on our data, we believe that *in vitro* assays of cell-associated NTM survival in the presence of macrophages and evasion of LL-37 bactericidal activity are useful for distinguishing more- and less-infective NTM isolates. Our data provide additional evidence supporting our hypothesis that more-virulent NTM isolates are more capable of neutralizing the antibacterial activity of LL-37 than less-virulent isolates. In this study, we took additional steps to identify the NTM-derived antagonist(s). First, TLC and LL-37 binding assays (Figure 5) demonstrated that LL-37 binds to polar NTM lipids. The MS data presented in Figures E1 and E2 support our TLC data, and further implicate LL-37 interactions with CL, PE, and PC. In Figure 6, we demonstrated that LL-37 inactivation, was beyond the scope of this study and will be the focus of future studies.

In our experience, mycobacterial CL and PIM are notoriously difficult to separate and purify. Moreover, in the absence of robust, enriched fractions for NTM-derived CL, PIM, and other phospholipids, our MS analyses were unable to distinguish between CL and PIM (data not shown), or among the phospholipids phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (Figure 5 and Table E1). For this purpose, high-quality fragmentation data generated via tandem MS will be needed. Purified phospholipid fractions from various NTM species should also be tested in *vitro* along with dose titration and kinetic assays to validate whether NTM-derived CL or PIM inactivate LL-37 to a higher degree than other phospholipids. Ideally, endogenous LL-37 peptide and NTM bioactive lipids should also be purified directly from patient samples and tested *ex vivo* for LL-37 antibacterial activity. Alternatively, specific neutralization of the bioactive phospholipid using enzymatic treatments in relevant biological material, such as BAL fluid from NTM-infected patients, would validate whether NTM-derived CL or PIM inactivate LL-37 to a higher degree than other phospholipids.

In conclusion, the similarities between human and NTM-derived CL are unknown at this time. Higher amounts of CL are associated with more pathogenic mycobacteria, including *M. tuberculosis* (21–23), but little is known about its functional role in the pathogenesis of NTM lung infections. Mycobacterial PI is another phospholipid that is abundantly present in the membrane and plays an essential role in viability, growth, and infectivity (24, 25). PI is the basic subunit for the synthesis of PIM, lipomannan, and lipoarabinomannan—higher-order glycolipids and lipoglycans, and major constituents of the mycobacterial CW (26–28). The diversity and high amounts of phospholipids in the mycobacterial CM suggest the possibility that subtle and overt differences in the resistance to and inactivation of LL-37 among the different NTM species and strains may be due to differences in the strength of LL-37 antagonism as well as the relative abundance of these different phospholipids on the surface of these mycobacteria. Determining the differential amounts of CL, PI, and PIM in the CMs and CWs of more- and less-virulent NTM species, and their stochastic effects needed for LL-37 inactivation, was beyond the scope of this study and will be the focus of future studies.
Figure 4. Differential susceptibility to LL-37 of more- and less-virulent NTM isolates. (A and B) The direct antibacterial activity of LL-37 against M. abscessus 12-45-Sw-A-1 (A) or M. porcinum KAU12-9-Sw-B-2 (B) was assessed by monitoring changes in cfu over time in LL-37 bacterial-killing assays. (C and D) Next, the LL-37 neutralization capabilities of the RGM were determined by incubating fresh LL-37 (25 or 125 µg/ml) with 96-hour conditioned medium from a liquid culture of M. abscessus 12-45-Sw-A-1 (C) or M. porcinum KAU12-9-Sw-B-2 (D). The bioactivity of LL-37 against Escherichia coli was also assayed to assess whether NTM was capable of neutralizing LL-37. Also shown is the growth of E. coli incubated alone (no LL-37) or with neat LL-37 (i.e., LL-37 not exposed to NTM-conditioned medium [both surrounded by a black box]). (E) The direct effects of 25 or 150 µg/ml of LL-37 on the viability of M. chimaera 12-54-Sw-B-1 and 12-7-S-1-3 were assessed by cfu counts after the indicated times. n = 3 independent experiments. *P < 0.04, **P = 0.008, ***P = 0.01, ****P = 0.001, and ####P = 0.0004.

further support the notion that specific NTM-derived lipids neutralize LL-37. Further studies using purified or synthetic NTM-derived phospholipids have the potential to lead to the development of novel, targeted therapeutics to alleviate NTM infections.

We acknowledge that NTM species may possess unique virulence attributes and structural features, such that the aforementioned phospholipids may not be the only molecules capable of inactivating LL-37, and these lipid species may even act as molecular decoys. We do not discount the possibility that other neutral lipid species (e.g., methylmannose polysaccharides) coexist within this phospholipid-enriched polar lipid fraction and may also influence the antibacterial activity of host-protective innate immune peptides (29). Robust chemical classifications will be needed in future work to define and identify possible LL-37–interacting NTM bioactive products. Detailed lipidomic studies may also help to identify additional differences in lipids between more- and less-virulent NTM isolates.

In this study, we also show a wide range of viability and growth in THP-1 macrophages among the different isolates of M. abscessus subsp. abscessus tested (Figure 2A). The single isolate of M. porcinum tested demonstrated low infectivity in THP-1 macrophages, but we anticipate that examining a larger number of M. porcinum isolates will reveal a wide range of interspecies infectivity similar to that seen with M. abscessus. Additionally, differences in inter-NTM-species infectivity were noted for MAC (Figures 2B–2D). Overall, we found that two of the five M. intracellulare isolates tested (40%) showed a numerical increase in macrophages in a temporal fashion. However, all of the M. avium, M. timonense, M. interjectum, and M. marseillense isolates tested showed decreased numbers of cell-associated NTM in the presence of macrophages. Regarding M. chimaera, three of 15 isolates tested (20%) (12-54-Sw-B-1, 12-42-Sw-A-3, and 12-40-Sw-A-1) showed positive growth in the number of cell-associated NTM in the presence of THP-1 macrophages, suggesting that these isolates are better equipped to survive in macrophages than other isolates of the same species. Importantly, these data indicate that although all were identified as M. chimaera, they showed an interspecies variability in their ability to survive in human immune cells, including human MDMs and AMs (Figures 3A and 3B).

We did not uniformly observe that clinical isolates were more infective to macrophages than environmental isolates; rather, we found that infectivity varied among the different NTM species and among different strains within the same species. By comparison, in a study performed over 20 years ago, Pedrosa and colleagues reported that animal-derived...
MAC isolates were more virulent in mice, human MAC isolates were less virulent in mice, and environmental isolates were unable to infect mice (30). However, that study was limited in that it was published before the advent of high-throughput sequencing, and consequently MAC was treated as a single species. Thus, it is probable that the authors compared different MAC species rather than different isolates of the same species. After applying variable-number tandem-repeat typing,
Tatano and colleagues identified M. avium isolates from patients with either nodular bronchiectasis or cavitary LD (n = 5 for each group), and compared their survival in THP-1 macrophages (31). They observed no significant difference in growth between the two groups, suggesting that M. avium isolates show similar intraspecies virulence. More recently, Rindi and colleagues demonstrated that infection with a M. avium subsp. hominisuis sequesvar A (MAH-A) or B (MAH-B) resulted in similar bacterial burdens 2 hours after infection in peripheral blood mononuclear cells and THP-1 macrophages (32). However, MAH-A showed significantly higher cfu by day 5 after infection than MAH-B, which suggests that MAH also exhibits intraspecies differences in virulence.

In summary, we show that NTM infectivity correlates with resistance to and antagonism of LL-37, and that the leading candidate for this immune evasive mechanism is a modified phospholipid such as CL or PIM, both of which are major components of the NTM CM. We also provide evidence that macrophage infectivity varies among different species of related NTM and among different isolates of the same NTM species. Future work should also correlate in vitro NTM infectivity and LL-37 evasion with clinical and radiographic assessments of disease severity and prognosis in patients with NTM-LD, identification of point-of-care biomarkers of infectivity, and the design of novel therapeutics that target NTM-derived pathogenic determinants (e.g., CL and PIM) as an immunoenhancer by, for example, preventing the antagonism of LL-37.

Author disclosures are available with the text of this article at www.atjsjournals.org.

Acknowledgment: The authors thank Pearleanne Zelanez and John Yang, Ph.D., for their assistance in downloading some of the data used in this study from the National Jewish Health (NJH) Research Database and providing access to stored samples in the NJH Biobank, respectively. They also thank Dr. Hong Wei Chu and Nicole Pavelka (NJH Human Cell Core) for providing alveolar macrophages and expertise and assistance with the ex vivo human cell experiments. They acknowledge Diagnostic Laboratory Services (Aiea, Hawai’i) for providing some of the clinical isolates used in this study.

References
1. LPSN. List of prokaryotic names with standing in nomenclature; 2018 [accessed 2019 Oct 21]. Available from: http://www.bacterio.net/mycobacterium.html.
2. Falkinham JO III, Iseman MD, de Haas P, van Soolingen D. Mycobacterium avium in a shower linked to pulmonary disease. J Water Health 2008;6:209–213.
3. De Groote MA, Pace NR, Fulton K, Falkinham JO III. Relationships between Mycobacterium isolates from patients with pulmonary mycobacterial infection and potting soils. Appl Environ Microbiol 2006;72:7602–7606.
4. Griffith DE, Aksamit TR. Understanding nontuberculous mycobacterial lung disease: it’s been a long time coming. F1000 Res 2016;5:2797.
5. Vinnard C, Longworth S, Mezochov A, Patrawalla A, Kreiswirth BN, Hamilton K. Deaths related to nontuberculous mycobacterial infections in the United States, 1999-2014. Ann Am Thorac Soc 2016; 13:1951–1955.
6. Fleschner M, Olivier KN, Shaw PA, Adjemian J, Strollo S, Claypool RJ, et al. Mortality among patients with pulmonary nontuberculous mycobacteria disease. Int J Tuberc Lung Dis 2016;20:582–587.
7. Park IK, Olivier KN. Nontuberculous mycobacteriosis in cystic fibrosis and non-cystic fibrosis bronchiectasis. Semin Respir Crit Care Med 2015; 36:217–224.
8. Wallace RJ Jr, Brown-Elliott BA, Wilson RW, Mann L, Hall L, Zhang Y, et al. Clinical and laboratory features of Mycobacterium porcinum. J Clin Microbiol 2004;42:5689–5697.
9. Honda JR, Hasan NA, Davidson RM, Williams MD, Epperson LE, Reynolds PR, et al. Environmental nontuberculous mycobacteria in the Hawaiian Islands. PLoS Negl Trop Dis 2016;10:e005088.
10. Tran T, Bonham AJ, Chan ED, Honda JR. A paucity of knowledge regarding nontuberculous mycobacterial lipids compared to the tubercle bacillus. Tuberculosis (Edinb) 2019;115:96–107.
11. Honda JR, Hess T, Malcolm KC, Otvotsky AR, Bai X, Irani VR, et al. Pathogenic nontuberculous mycobacteria resist and inactivate cathelicidin: implication of a novel role for polar mycobacterial lipids. PLoS One 2015;10:e0126994.
12. Honda JR, Ntio L, Zhou Y, Hesser D, Hasan NA, Mehaffy C, et al. Mycobacterium avium complex species virulence and lipid profiles. Am J Respir Crit Care Med 2017;195:A5060.
13. Theus SA, Cave MD, Eisenach KD. Activated THP-1 cells: an attractive model for the assessment of intracellular growth rates of Mycobacterium tuberculosis isolates. Infect Immun 2004;72:1169–1173.
14. Bai X, Oberley-Deegan RE, Bai A, Otvotsky AR, Kinney WH, Weaver M, et al. Curcumin enhances human macrophage control of Mycobacterium tuberculosis infection. Respirology 2016;21:951–957.
15. Bai X, Kinney WH, Su WL, Bai A, Otvotsky AR, Honda JR, et al. Caspase-3-independent apoptotic pathways contribute to interleukin-32γ-mediated control of Mycobacterium tuberculosis infection in THP-1 cells. BMC Microbiol 2015;15:39.
16. Bai X, Feldman NE, Chmura K, Otvotsky AR, Su WL, Griffin L, et al. Inhibition of nuclear factor-κB activation decreases survival of Mycobacterium tuberculosis in human macrophages. PLoS One 2013;8:e61925.
17. Bai X, Kim SH, Azam T, McGibney MT, Huang H, Dinarello CA, et al. IL-32 is a host protective cytokine against Mycobacterium tuberculosis in differentiated THP-1 human macrophages. J Immunol 2010;184:3830–3840.
18. Oberley-Deegan RE, Lee YM, Motley GE, Cook DM, Chan ED, Crapo JD. The antioxidant mimetic, MnTE-2-PyP, reduces intracellular growth of Mycobacterium abscessus. Am J Respir Cell Mol Biol 2009;41:170–178.
19. Taki T, Gonzalez TV, Goto-Inoue H, Hayasaka T, Setou M. TLC blot (far-eastern blot) and its applications. Methods Mol Biol 2009;536:545–556.
20. Schlaime M, Hostetter KY. Cardiolipin synthase from mammalian mitochondria. Biochim Biophys Acta 1997;1348:207–213.
21. Sarma PV, Srikanth L, Venkatesh K, Murthy PS, Sarma PU. Isolation, purification and characterization of cardiolipin synthase from Mycobacterium tuberculosis (PRIVATE). Bioinformation 2013:9:690–695.
22. Akamatsu Y, Nojima S. Separation and analyses of the individual phosphatidylinositol mannosyltransferase PimA from Mycobacterium phlei (PIM). Glycobiology 1995;5:439–449.
23. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 1998;393:537–544.
24. Brennan PJ, Nkaido H. The envelope of mycobacteria. Annu Rev Biochem 1995;64:29–63.
25. Guerin ME, Korudakova J, Schaeffer F, Svetlikova Z, Buschiazzo A, Giganti D, et al. Molecular recognition and interfacial catalysis by the essential phosphatidylinositol mannosyltransferase PimA from mycobacteria. J Biol Chem 2007;282:20705–20714.
26. Khoo KH, Della A, Morris HR, Brennan PJ, Chatterjee D. Structural definition of acylated phosphatidylinositol mannosides from Mycobacterium tuberculosis: definition of a common anchor for lipomannan and liparabinomannan. Glycobiology 1995;5:117–127.
27. Nigou J, Gilleron M, Puzo G. Lipoarabinomannans: from structure to biosynthesis. *Biochimie* 2003;85:153–166.

28. Briken V, Porcelli SA, Besra GS, Kremer L. Mycobacterial lipoarabinomannan and related lipoglycans: from biogenesis to modulation of the immune response. *Mol Microbiol* 2004;53:391–403.

29. Mendes V, Maranha A, Alarico S, Empadinhas N. Biosynthesis of mycobacterial methylglucose lipopolysaccharides. *Nat Prod Rep* 2012;29:834–844.

30. Pedrosa J, Flórido M, Kunze ZM, Castro AG, Portaels F, McFadden J, et al. Characterization of the virulence of *Mycobacterium avium* complex (MAC) isolates in mice. *Clin Exp Immunol* 1994;98:210–216.

31. Tatano Y, Yasumoto K, Shimizu T, Sano C, Sato K, Yano S, et al. Comparative study for the virulence of *Mycobacterium avium* isolates from patients with nodular-bronchiectasis- and cavitory-type diseases. *Eur J Clin Microbiol Infect Dis* 2010;29:801–806.

32. Rindi L, Lari N, Garzelli C. Virulence of *Mycobacterium avium* subsp: *hominissuis* human isolates in an in vitro macrophage infection model. *Int J Mycobacteriol* 2018;7:48–52.

33. Ovrutsky AR, Chan ED, Kartalija M, Bai X, Jackson M, Gibbs S, et al. Cooccurrence of free-living amoebae and nontuberculous mycobacteria in hospital water networks, and preferential growth of *Mycobacterium avium* in *Acanthamoeba lenticulata*. *Appl Environ Microbiol* 2013;79:3185-3192.