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

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ONLINE DATA SUPPLEMENT

Online Supplemental Information

NTM isolates used in this study

M. abscessus subsp*. abscessus* #19977 and *M. avium* Chester #700737 (11) were obtained from the American Type Culture Collection (ATCC). *M. avium* subsp*. hominissuis* H87 was recovered from hospital water by our laboratory and its complete genome sequence reported (32, 33). *M. intracellulare* 9141 is a clinical isolate from National Jewish Health (NJH) previously described (11, 34). Additional environmental and clinical NTM isolates published in our prior work were also included; partial *rpoB* gene sequencing was used for species identification (9). Frozen glycerol stocks of each isolate were used in this study.

NTM phylogenetic tree

For phylogenetic analysis, the multi-sequence alignment of partial *rpoB* sequences (9) from the NTM isolates used in the current study (n = 32) and phylogenetic reconstruction were performed in the SeaView software package (35)**.** The phylogeny was constructed based upon the variable sites observed in the alignment of the 723 bp *rpoB* sequences using the neighbor-joining method using 100 bootstrap replicates. Node support values greater than or equal to 70% were considered reliable and visualized on the phylogeny. Since the genome of *M. intracellulare* 9141 was not published or available, it was omitted from this phylogenetic analysis.

***In vitro* NTM infection of human THP-1 macrophages**

Briefly, $1x10^6$ THP-1 monocytes in 6-well plates were differentiated into macrophages with 160 nM phorbol myristate acetate (PMA) for 48 hours. Media containing PMA was removed prior to infection, the cell monolayer was washed with 1X PBS, and fresh media added. Frozen stocks of NTM were thawed and 10^7 NTM colony forming units (CFU)/ml were added per well for a starting multiplicity of infection (MOI) of 10:1 (NTM:macrophage). At the one-hour timepoint, unphagocytosed bacteria were thoroughly removed by washing the monolayer with 1X PBS and the number of viable RGM that remained either attached onto the THP-1 cell surface or taken up intracellularly was determined. Wells apportioned to the one-hour time point were lysed using 0.25% sodium dodecyl sulfate (SDS) solution and the number of cell-associated NTM were determined by culturing serially diluted cell lysates onto Middlebrook 7H10 agar in duplicate. To determine changes in CFU at the 24, 48, and 96-hour time points, the macrophage

cell lysates were prepared at the indicated times by the same aforementioned method. CFU were manually scored. Three or more independent experiments were completed for all THP-1 assays.

Change in $\log_{10}$ CFU was calculated by subtracting the CFU counted at the 24, 48 and 96-hour timepoints from the CFU counted after one-hour of infection. Three bars are shown for each species corresponding to the change in CFU determined after 24 hours (first bar in series), 48 hours (second bar in series), and 96 hours after infection (third bar in series) compared to the CFU counts at the one-hour timepoint. Bars that bracket zero indicate the NTM neither increased or decreased growth in the presence of THP-1 macrophages and remained similar to the starting inoculum number.

LL-37 immunoblotting

Mammalian proteins were extracted from THP-1 macrophages by rotating the cells at room temperature in mammalian protein extraction reagent (M-PER, Thermo Fisher) supplemented with sigma protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), and dithiothreitol (DTT) for 15 minutes as described by the manufacturer. The samples were centrifuged at 13,000 x *g* for 5 minutes at 4°C to pellet cell debris and the supernatants collected. Protein concentrations were determined using Nanodrop spectrophotometer. 22μg of protein were separated using 4-12% Bis-Tris SDS PAGE (Invitrogen) and transferred onto PVDF membranes (iBlot, Invitrogen). Membrane were rocked in 5% non-fat milk – 1X PBS 0.25% TWEEN 20 (PBST) overnight at 4°C, washed once in PBST followed by two additional washes in 1X PBS. 1:250 dilution of primary monoclonal antibody for LL-37 (Hycult Biotech) and 1:2,500 dilution of monoclonal anti β-actin antibody (Sigma) in 5% non-fat milk – PBST was used. 1:1,000 dilution of goat anti-mouse antibody (Thermo Scientific) in 5% non-fat milk-PBST was used. Bands were visualized using the iBright Imager (ThermoFisher).

Ethics Statement

Peripheral blood mononuclear cells (PBMC) were obtained from blood collected from healthy volunteers and differentiated into monocyte-derived macrophages (MDM) under a protocol approved by the Colorado Multiple Institutional Review Board (Protocol #HS-2651). Alveolar macrophages (AM) from bronchoalveolar lavage were procured from the National Jewish Health (NJH) Human Cell Core who regularly obtains de-identified donor lungs from non-smokers through the International Institute for the

Advancement of Medicine and the National Disease Research Interchange and Donor Alliance. These lung samples are not suitable for transplantation and donated for medical research. The Human Cell Core has obtained IRB exemption to allocate AM to NJH investigators. Deidentified BAL samples came from NTM patients under care at NJH who donated their samples for research purposes which were then deposited into the NJH Biobank. Honest Broker that determined the use of its samples did not involve research on human subjects and de-identified patient samples prior to distribution.

***Ex vivo* NTM infection of primary human cell cultures**

$1x10^6$ PBMC from three healthy, non-smoking donors were seeded into 6-well plates. PBMC's were allowed to differentiate into MDM by adding 20 pg/mL of macrophage colony stimulating factor (M-CSF) for seven days. Media containing M-CSF was removed on day 4, the cell monolayer washed with 1X PBS, fresh media added and the culture was allowed to differentiate for three additional days. $1x10^6$ alveolar macrophages (AM) were seeded into 6-well plates and allowed to adhere for 48 hours prior to infection. Frozen stocks of NTM were thawed and 10^7 (CFU)/ml was added per well for a starting multiplicity of infection (MOI) of 10:1 (NTM:macrophage). At the one-hour timepoint, the cells were lysed, serial dilution performed, and CFU quantified as described above. Three independent experiments were completed for all human cell culture assays.

Direct antibacterial activity of LL-37 against NTM

We refer to the bioactive 4.5 kDa cathelicidin antibacterial peptide as "LL-37". Synthetic human LL-37 (NH_2 -LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-COOH) used in this study was the same as published (11). Briefly, LL-37 was synthesized by the University of Colorado Anschutz Medical Campus Peptide Core, purified by preparative reversed-phase high performance liquid chromatography (HPLC), and verified by analytical reversed-phase HPLC. LL-37 molecular mass was determined by electrospray mass spectrometry; purity was >98%. LL-37 was lyophilized and stored at -20°C and resuspended in 0.1% trifluoroacetic acid (final pH 2.0) before use.

The culture medium used for LL-37 antibacterial kill assays was previously optimized (RPMI-1640 supplemented with sodium bicarbonate pH 7.3; diluted 1:4 in distilled water) (36) and referred to herein as the "LL-37 medium." For bacterial killing assays, $2x10^5$ to $2x10^6$ bacteria per 250 μl of LL-37 medium (pH 7.0) in low-bind microfuge tubes were incubated with various concentrations of LL-37 (*i.e.,* 0, 25, and 125

μg/mL of LL-37) . Tubes were vigorously vortexed, rotated, and incubated at 37°C up to 96 hours. Killing efficacy was analyzed by performing serial dilutions of bacterial-peptide cocktail. Each dilution was plated in duplicate onto Middlebrook 7H10 plates that were incubated at 37°C. RGM and SGM were incubated for 3-5 days or 10-14 days, respectively. 0.1% trifluoroacetic acid was used as the vehicle control. CFU were manually scored. Three or more independent experiments were completed for all LL-37 kill assays.

***Escherichia coli* bioassays to assay LL-37 antibacterial activity**

We apply the *E. coli* bioassay as a readout for NTM-mediated inactivation of LL-37. Specifically, at the completion of the LL-37 bacterial killing assays (96 hours after the culture was first inoculated), samples were centrifuged for five minutes at 6,000 *x g* to pellet the bacteria, leaving LL-37 in the supernatant. The conditioned media were transferred into new microfuge tubes to which $2x10^5$ *E. coli* was inoculated and incubated for four hours. After incubation, *E. coli* was spread onto duplicate LB agar plates, incubated for 24 hours, and *E. coli* growth assessed. Three or more independent experiments were completed for all bioassays.

Preparation of total lipid extracts (TLE) and thin layer chromatography (TLC)

To generate total lipid extracts (TLE), 1 mL aliquots of NTM glycerol stocks were thawed and inoculated into 50 mL of the chemically-defined growth medium Proskauer Beck (PB) (37) and incubated without shaking at 37°C for 5-28 days. Once turbid, the cultures were centrifuged at 1,700 x *g* for 30 minutes at room temperature to pellet the bacteria. Supernatants were discarded and the bacterial pellets were resuspended in 5 mL of 10:10 HPLC grade chloroform:methanol, vortexed, transferred into 13 x 100 mm glass tubes, and incubated on a tube rocker for 2 hours at room temperature. The tubes were then centrifuged at 2,600 x *g* for 5 minutes at room temperature and the supernatants were transferred into new 13 x 100 mm glass tubes and evaporated under N_2. This process was repeated two additional times to maximize lipid extraction from the bacterial pellets. Methods used for TLC have been described previously (38, 39). Three identical TLC plates were spotted and the plates were developed simultaneously in 60:30:6 chloroform:methanol:water solvent system. $CUSO_4$ charring spray was used to visualize lipid species on the first plate. The second plate was sprayed with α-naphthol to visualize glycolipids and the third was used for Far Eastern Immunoblotting. Herein, we refer to far eastern blots specifically as “LL-37 binding immunoblot.”

LL-37 binding Immunoblotting

The third TLC plate was incubated with 2.5 µg/mL of LL-37 peptide in 3% BSA-PBS blocking solution for 45 minutes. After incubation, the plate was washed five-seven times with 1X PBS to remove any unbound LL-37 and blocked for 1 hour at room temperature in 3% BSA-PBS. After blocking, the plate was incubated for 1 hour at room temperature with 1:250 LL-37 primary monoclonal antibody (Hycult Biotech) in 10% BSA-PBS, washed five-seven times in 1X PBS, followed by incubation with 1:1000 goat-anti-mouse IgG HRP in 1X PBS for 30 minutes at room temperature. Detection was performed using SuperSignal™ West Femto chemiluminescent substrate kit (ThermoFisher, #34095).

Preparation of NTM cell membrane and cell wall lipids

To better define the NTM lipids that bind LL-37, cell membrane (CM) lipids, total lipids from the cell wall (CW), and cytosolic fractions were prepared from NTM isolates. Briefly, *M. abscessus* or *M. intracellulare* were inoculated into 250 ml of 7H9 Middlebrook broth, incubated at 37°C, and after reaching logarithmic growth, cell pellets were prepared. Cells were lysed using a combination of water bath sonication for ten minutes and four rounds of zirconia/silica bead beating (4M/s for 20 seconds). The samples were centrifuged and the pellets separated from the supernatants. The pellet fraction was labeled as the CW fraction. The supernatants were then subjected to ultracentrifugation at 100,000 x *g* for one hour at 4°C; after ultracentrifugation, the supernatant was labeled as the cytosolic fraction. 500 µl sterile ultrapure water was added to the pellets and this fraction was labeled as the CM fraction. For lipid extraction, five ml of 2:1 chloroform/methanol was added to the CW, cytosolic, and CM fractions. To determine whether lipids present in these fractions bind LL-37, the fractions were spotted in triplicate onto identical silica plates and subjected to TLC. The plates were developed simultaneously in 60:30:6 chloroform:methanol:water solvent system. The first two plates were sprayed with $CuSO_4$ and alpha-naphthol to visualize lipid polymers and glycolipids, respectively. The third plate was used for LL-37 binding immunoblots.

Detection of NTM PI by mass spectrometry

Bronchoscopy is occasionally performed on patients with or suspected of having NTM-LD to obtain bronchoalveolar lavage fluid (BALf) for culture. 9 mL of BALf from three different patients with culture-confirmed NTM lung infection were obtained to detect NTM-lipids. Lipids were extracted and the lipid classes separated by TLC. These bands were excised and the lipids were analyzed by mass spectrometry using a QSTAR XL; Applied Biosystems/MDS Sciex (Thornhill, Ontario, Canada). Samples were spotted onto a matrix-assisted laser desorption ionization (MALDI) plate utilizing 2'6'-dihydroxyacetophenone as a matrix for negative ion analysis. The most abundant ions from each sample were subjected to collision-induced dissociation to determine structural composition.

LL-37 pull-down assays

To capture NTM lipids that interact with LL-37, biotinylated LL-37 was incubated with *M. abscessus*-conditioned medium (supernatant collected after *M. abscessus* was incubated in LL-37 medium for 96 hours) for 24 hours. NTM-derived lipids bound to LL-37 were captured using streptavidin-linked magnetic beads (Invitrogen Streptavidin Dynabead kit™). To release the lipids from the beads, magnetically separated complexes were resuspended in 2:1 chloroform/methanol solution. Using a shotgun approach, lipids were then subjected to gas chromatography mass spectrometry (GC-MS). Putative LL-37 interacting lipids of *M. abscessus* are shown in the boxes.

Pure phospholipid TLC

~50 µg of either CL, PI, PE or PC were suspended in 2:1 chloroform:methanol and spotted onto 8 cm x 10 cm silica plates and subjected to TLC and LL-37 binding immunoblot as described above.

Statistical analyses

CFU data was analyzed with GraphPad 7 using paired *t*-tests to determine statistical significance. Values with $p<0.05$ were considered statistically significant. Data are expressed as means ± S.E.M. for three or more independent determinations for each experimental point. P-values were adjusted for multiple tests using the FDR method (40).

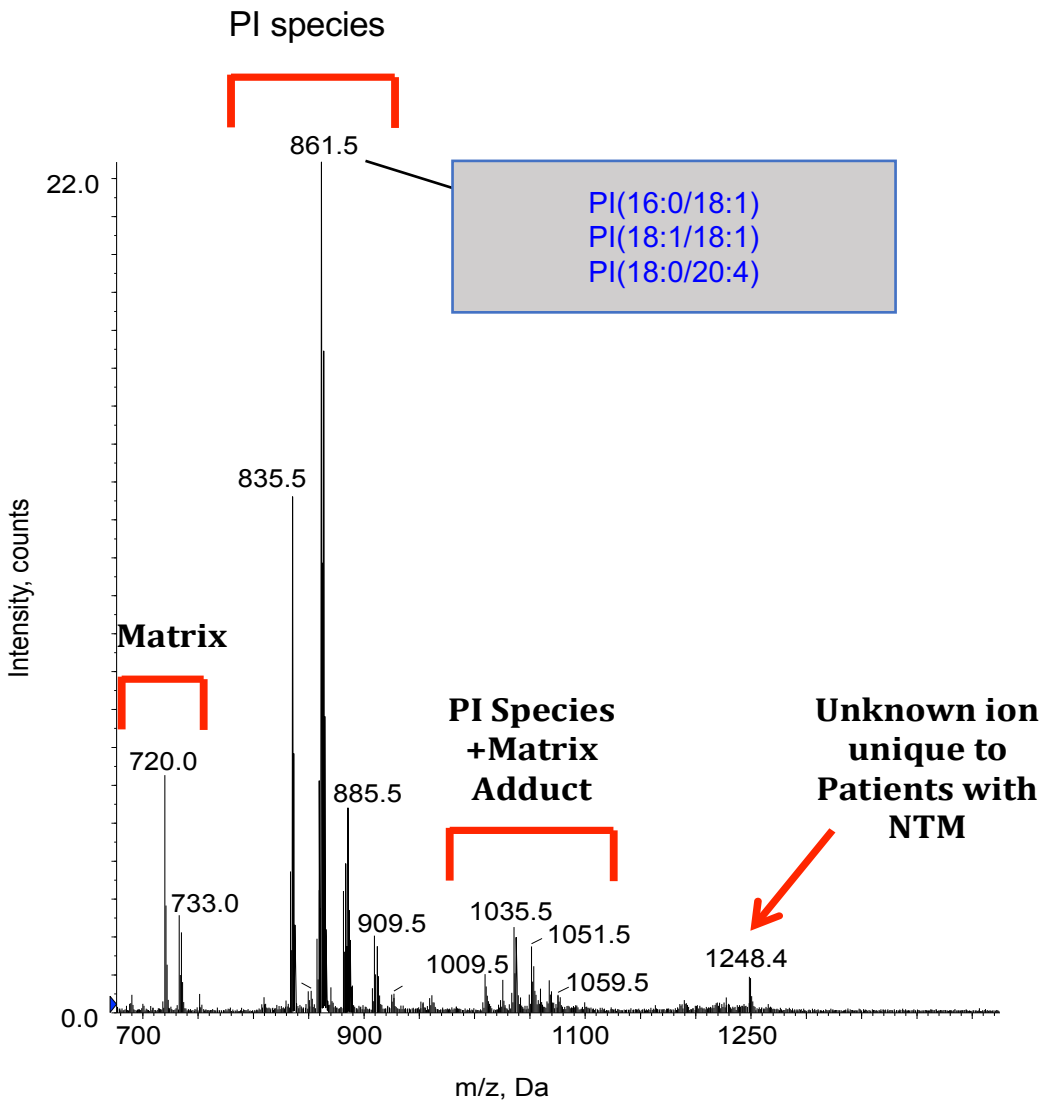
Supplemental Figure Legends

Supplemental Figure E1. PI species are detected in human lung lavage samples. MALDI-TOF MS analysis of the BALf samples from three *M. intracellulare*-infected patients was used to detect NTM phospholipid species. Representative spectrum is shown.

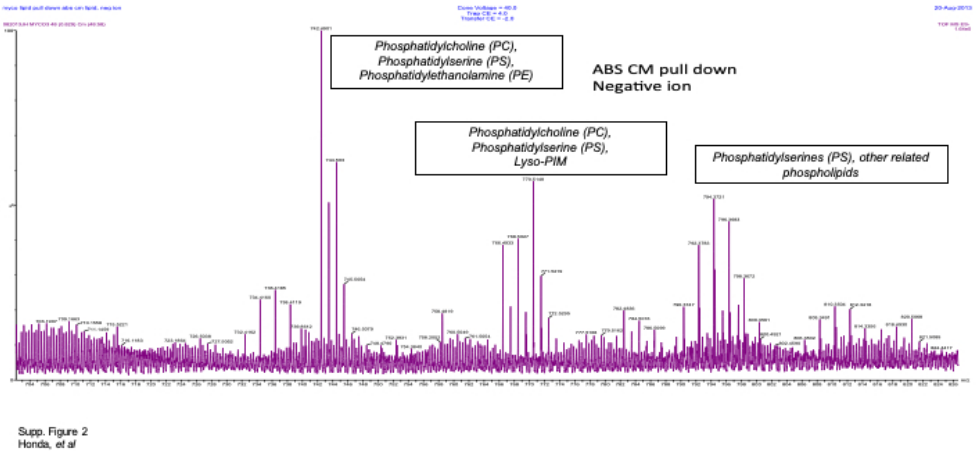
Supplemental Figure E2. LL-37 binds to NTM lipids. Biotinylated LL-37 was incubated with *M. abscessus* ATCC 19977 conditioned medium for 24 hours. Streptavidin-linked magnetic beads (Invitrogen Streptavidin Dynabead kit™) and magnetic separation was used to capture NTM-derived lipids bound to LL-37. Magnetically separated complexes were resuspended in 2:1 chloroform/methanol solution and subjected to gas chromatography mass spectrometry (GC-MS).

Supplementary Table Legend

Supplementary Table E1 Legend: List of molecular features (m/z) matched with LipidMaps database (https://www.lipidmaps.org/) with mass tolerance (+/- m/z) 0.05. Data were acquired in negative mode. The table provides putative identification of phospholipid species from M. abscessus CM pull down experiment.



Supplemental Figure E1



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