Norharmane Matrix Enhances Detection of Endotoxin by MALDI-MS for Simultaneous Profiling of Pathogen, Host, and Vector Systems

Short Title: NRM Matrix Improves Detection of Lipid A

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Abstract

The discovery of novel pathogenic mechanisms engaged during bacterial infections requires the evolution of advanced techniques. Here, we evaluate the dual polarity matrix norharmane (NRM) to improve detection of bacterial lipid A (endotoxin), from host and vector tissues infected with *Francisella novicida* (*Fn*). We evaluated NRM for improved detection and characterization of a wide range of lipids in both positive and negative polarities, including lipid A and phospholipids across a range of matrix assisted laser desorption-ionization (MALDI)-coupled applications. NRM matrix improved the limit of detection (LOD) for monophosphoryl lipid A (MPLA) down to picogram-level representing a ten-fold improvement of LOD versus 2,5-dihydroxybenzoic acid (DHB) and 100-fold improvement of LOD versus 9-aminoacridine (9-AA). Improved LOD for lipid A subsequently facilitated detection of the *Fn* lipid A major ion (*m/z* 1665) from extracts of infected mouse spleen and the temperature-modified *Fn* lipid A at *m/*z 1637 from infected *D. variabilis* ticks. Finally, we simultaneously mapped bacterial phospholipid signatures within an *Fn* infected spleen along with exclusively host-derived inositol-based phospholipid (*m/z* 933) demonstrating co-profiling for the host-pathogen interaction. Expanded use of NRM matrix in other infection models and endotoxin-targeting imaging experiments will improve our understanding of the lipid interactions at the host-pathogen interface.

Introduction

Lipopolysaccharide (LPS) is the major component of the outer leaflet of the outer membrane of most Gram-negative bacteria. The membrane anchor region, lipid A imparts endotoxin activity to LPS with specific lipid A structural configurations indicative of antimicrobial resistance (commonly terminal phosphate modifications) or local growth conditions (ex: acyl shortening observed in growth at low temperature).(Gunn 2001; Li *et al*. 2012; Needham and Trent 2013) Lipid A is substantially hydrophobic and is readily ionizable in the negative ion mode due to the terminal phosphate moieties. Matrix assisted laser desorption-ionization (MALDI) is commonly used for detection of diverse lipid A structures, but detection can be problematically matrix-dependent. In order to study low abundance and exotic lipid A structures, improved detection methods are necessary.(Heeren 2015) Due to the relationship between lipid A structure and virulence, *in situ* description of lipid A and its structure is crucial. (Gunn 2001; Hajjar *et al.* 2002; Pelletier *et al.* 2013; Hagar *et al.* 2013) Here we will describe an underutilized matrix for sensitive detection of lipid A including modified lipid A structures directly from infected vector and host extracts.

Norharmane (NRM; β-carboline, 9*H*-pyrido[3,4-*b*]indole]) is an indole alkaloid molecule commonly found in plants, including coffee and tobacco.(Schmeltz and Hoffmann 1977; Luxembourg *et al.* 2003; Wojtowicz *et al.* 2015) Norharmane was first reported as a matrix substance for MALDI in 1999 where it was used to facilitate ionization of sialyl oligosaccharides.(Yamagaki and Nakanishi 1999; Cerruti *et al.* 2012) Following this initial report, the use of NRM as a MALDI matrix for work in negative ion mode was systematically evaluated by Brown *et al* in 2001, alongside common matrices

such as 2,5-dihydroxybenzoic acid (DHB), sinapinic acid (SA), α-cyano-4-hydroxycinnamic acid (HCCA), and 9-nitroanthracene (9-NA).(Folch, Lees and Sloane Stanley 1957; Bligh and Dyer 1959; Brown *et al.* 2001) This work identified NRM and a related molecule, harmane, as ideal matrices for analysis of hydrophobic molecules. The historical use of DHB, HCCA, and 9-AA for lipid A and phospholipid characterization has resulted in an absence of well defined uses for NRM, though a growing number of reports have appeared including the use of NRM as a matrix for laser-induced post-ionization, notated MALDI-2.(Soltwisch *et al.* 2015)

We have previously reported the use of NRM for mass spectrometry imaging (MSI) and one dimensional thin layer chromatography (TLC)-MALDI experiments.(Nchoutmboube *et al.* 2013; Shirey *et al.* 2013; Scott *et al.* 2014) MSI is a technique used to characterize the spatial relationship of molecular targets to histological features and MALDI is the most commonly used ionization method for MSI. (Caprioli, Farmer and Gile 1997; Stoeckli *et al.* 2002; Cornett *et al.* 2007; Schwamborn and Caprioli 2010; Chaurand 2012; Heeren 2015) TLC-MALDI is a technique that couples traditional TLC separation with mass/charge identification by MALDI.(Gusev, Proctor and Rabinovich 1995; Nicola, Gusev and Hercules 1996; Fuchs *et al.* 2007) TLC-MALDI is another powerful tool for lipid profiling since it is rapid and offers improved lipid identification by virtue of the separation of lipid mixtures based on head group chemistry and it is especially useful for differential identification of isobaric species of phosphatidylcholine (PC) and phosphatidylethanolamine (PE).(Fuchs *et al.* 2009) Both techniques require a matrix capable of ionizing the molecular target(s) of interest; for example, lipids.(van Hove, Smith and Heeren 2010) Several matrices have

been reported for lipid-targeting MSI, including DHB, 1,5-diaminonapthalene (DAN), HCCA, and 9-aminoacridine (9-AA).(Fuchs, Süss and Schiller 2010; Zemiski Berry *et al.* 2011; Cerruti *et al.* 2012) DHB is a widely used matrix for MALDI-MSI and applicable for lipid analysis in positive-ion mode, whereas 9-AA is used in negative-ion mode. 9-AA offers the advantage that the mass spectra consist largely of deprotonated or molecular ions, which simplifies lipid identification.(Cerruti *et al.* 2012) Neither 9-AA nor DHB can serve as universal matrices for bacterial lipid analysis due to the need for large amounts of sample to produce mass spectra; one nanogram and 100 picograms of lipid A are currently required, respectively. To highlight the relative lack of sensitivity, approximately 10^9 colony forming units of bacteria will yield one microgram of LPS, only a fraction of which can be efficiently hydrolyzed and detected as the membrane anchor lipid A. Identifying an improved matrix, with near universal compatibility for a broader range of lipids in both positive- and negative-ion modes, including lipid A, would greatly benefit bacterial lipid research.

The first report of the use of NRM for MALDI analysis of lipid A was made by d'Hauteville *et al* in 2002 to describe the activity of two lipid A biosynthesis genes (*msbB1* and *msbB2*) active in *Shigella flexneri*.(d'Hauteville *et al.* 2002) Prior to this study lipid A was extracted from large-scale culture (>1 liter) and analyzed by MALDI using NRM matrix to assess *in vitro*-grown structures, but the sensitivity did not exist to analyze low input *in vivo*-grown structures. To advance the study of lipid A structural modifications in primary clinical samples, improved detection limits are necessary for direct observation in tissue.(Li *et al.* 2012; O'Hara *et al.* 2013; Pelletier *et al.* 2013) This required the development of advanced extraction methodologies, as well as sensitive

lipid A detection techniques. In 2005, a method for lipid A microextraction was reported by the Caroff group in which the authors achieved lipid A extraction from ten milligrams of lyophilized bacteria followed by mass spectrometric analysis.(Hamidi *et al.* 2005) This report revolutionized the study of the lipid A structure-function relationship by making lipid A analysis from low-level bacterial cultures possible. Here we present work combining these two advances to analyze the *in vivo* lipid A structure in both a mouse (host) and tick (vector) model of *Francisella novicida* (*Fn*) by extracting lipid A directly from burdened tissue.

Lipid A structure is influenced by a variety of factors including osmolarity, nutrient availability, presence of host/vector factors, and growth temperature. The precise conformation of lipid A in any given condition is one of many components contributing to membrane integrity, permeability, topology, and content. *Fn* lipid A, although primarily found with 18-carbon acyl chains at 37°C, is modified by 16-carbon acyl chains when grown at lower temperatures (to resemble the ambient conditions of a tick or the environment).(Shaffer *et al.* 2007; Gunn and Ernst 2007; Li *et al.* 2012; Needham and Trent 2013) This shortening alters membrane permeability and resistance to antimicrobial agents and is likely a reflection of *Fn* lipid A structure in ticks.(Li *et al.* 2012) To date, the detection levels necessary to evaluate lipid A shortening *in vivo* have not been identified. In this work, we present the findings of a lipid A extraction directly from whole hard-bodied *D. variabilis* (*Dv*) ticks infected with *Fn* confirming the predictions made from *in vitro* studies. Finally, by coupling the highly sensitive detection limit of NRM to MALDI-MSI, we can directly map phospholipids of bacterial origin (expected to be in low overall abundance versus host phospholipids) within infected

141 host tissue, expanding the utility of lipid MSI studies to improve our understanding of
142 bacterial pathogenic mechanisms. The results presented herein expand the fields of
143 pathogenesis, general microbiology, and lipid profiling by offering a versatile alternative
144 matrix for lipid analysis.
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146
147

Materials and Methods

Ethics Statement

All experiments were performed in accordance with the University of Maryland, Baltimore Institutional Animal Care and Use Committee (IACUC) protocol approval #0814005 in adherence with the Guide for the Care and Use of Animals (NIH), the Animal Welfare Act, and applicable US Federal laws.

Matrices and Solvents

9-AA, DHB, and NRM were all purchased from Sigma-Aldrich (St. Louis, MO). Solvent solutions were volumetric parts as follows: 1:1 E:W (ethanol:water); 1:2:0.8 C:M:W (chloroform:methanol:water); 1:1 C:M (chloroform:methanol); 2:1 C:M chloroform:methanol. Ethanol, methanol, and chloroform were obtained from Sigma-Aldrich (St. Louis, MO). Phosphate buffered saline (PBS) and certified endotoxin-free water were sourced from Gibco (Grand Island, NY). Matrix application was technique-specific, as given.

Lipid A Limit of Detection

A commercial preparation of monophosphoryl lipid A (MPLA) from *Salmonella enterica* serovar *minnesota* (R595) was purchased from Sigma-Aldrich (St. Louis, MO). For spot analysis, a concentrated stock solution of 1 mg mL^{-1} MPLA was made in 1:1 C:M. A 10-fold dilution series (1 µg $µL^{-1}$ through 10 ng $µL^{-1}$) was made in the same solvent. One microliter of each dilution in the series was spotted onto a stainless steel MALDI target plate, air dried, and followed by 1 µL of matrix (NRM: 20 mg mL^{-1} in 2:1 C:M, 9-AA 20 mg mL^{-1} in 2:1 C:M, DHB 40 mg mL^{-1} in 2:1 C:M, concentrations optimized for MPLA

detection). MPLA spotted for the limit of detection study was analyzed on a Bruker Daltonics (Billerica, MA) solariX XR (MALDI FT-ICR,12T) calibrated to 1 ppm using sodium trifluoroacetic acid in negative-ion mode. Limit of detection was defined as the quantity of MPLA spotted in the last detectable spot in the dilution series, minimum criterion for detection was defined as the presence of at least two isotopic peaks in addition to the corresponding, monoisotopic peak. LOD data were analyzed in DataAnalysis software (Bruker Daltonics, Billerica, MA) using the Sophisticated Numerical Annotation Procedure (SNAP).

Bacterial Strains and Growth Conditions

Francisella novicida (*Fn*) was grown in tryptic soy broth containing 0.1 g/L L-cysteine [TSBC] (Broth, Becton-Dickenson, Hunt Valley, MD; L-cysteine, Sigma-Aldrich, St. Louis, MO) in shaking liquid culture (225 RPM) to mid-log phase. Agar plates were the same broth formulation with the addition of 1.5% (w/v) agar (Becton-Dickenson, Hunt Valley, MD). For lipid A microextraction: one milliliter of liquid culture was harvested into a microfuge tube, pelleted (8000 x g, 5 minutes), and supernatant discarded. The remaining pellet was processed by the microextraction method described below. For rodent infections: an overnight, shaking liquid culture (300 µL) was used to inoculate a large volume of fresh, warmed (37°C) TSBC (15 mL). These early-log phase subcultures (3 hour culture) were prepared as follows for injection.

Mice, Ticks, and Infection

Uninfected and infected solid organs (kidney, spleen) were collected from female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME), 6-8 weeks of age. Briefly, mice were housed in biosafety level 2 (BSL2) microisolator cages and provided food and water *ad libitum*. Infectious doses of *Fn* were prepared as follows: one milliliter of the1:50 (v:v) large volume subculture was pelleted (3500 x g, 5 minutes) and resuspended in PBS. This solution was diluted further in PBS to contain approximately 300 colony forming units (CFU) in 50 µL (injection volume). Doses were administered subcutaneously, control groups received sterile PBS injections. Colony forming units in duplicate 50 µL doses were assessed on TSBC-agar plates (above). Mice were euthanized by carbon dioxide narcosis prior to tissue collection, followed by secondary thoracotomy. Spleens were collected for lipid A extraction forty-eight hours post infection along with uninfected, matching control tissue. Tissues were excised then snap frozen by floating on foil in a pool of liquid nitrogen and stored at -80°C for sectioning or lipid A extraction. *D*. *variabilis* (*Dv*) ticks were kindly provided by Daniel Sonenshine (Department of Biological Sciences, Old Dominion University). *Fn* U112 (10,000-30,000 CFU) and PBS were injected into the emargination cavity of unfed male and females using pulled glass capillaries attached to a Nanoject II pump (Drummond Scientific, Broomall, PA). Ticks were incubated overnight at 23°C with 95% humidity then were washed with 3% hydrogen peroxide, sterile water, and 70% ethanol in succession, dried, and placed in a sterile 5 ml conical for processing (counting or microextraction)

Lipid A Microextraction

Microextraction of lipid A from 1 mL of turbid shaking culture (*in vitro*), ticks (*in vivo*) mouse spleen (*in vivo*) was performed as previously described with the following deviations: ticks or mouse spleens were extracted in a double volume of the initial extraction solution (800 µL total) starting with a tissue shredding step consisting of three, 10-15 second full speed pulses of the spleen in the extraction solution (Tissue Tearor Homogenizer, Cole Parmer, Vernon Hills, IL).(Hamidi *et al.* 2005) Briefly, 1 mL of mid-log phase *Fn* grown in TSBC was pelleted and supernatant discarded. The pellet was extracted in 400 µL of a solution containing 5 parts isobutyric acid : 3 parts 1M ammonium hydroxide and heated at 100°C for one hour followed by a fifteen minute incubation on ice and centrifugation at 2000 x g for fifteen minutes. Supernatant was collected and mixed in equal parts with water then frozen and lyophilized. Contaminants were washed from the dried material by two rounds of methanol washes: 1 mL of methanol, vortexing, and pelleting at 10,000 x g for five minutes. The final product was reconstituted in 2:1 C:M (100 µL) along with 4-8 grains of Dowex ion exchange resin (Fisher Scientific, Pittsburgh, PA), incubated with vortexing for at least five minutes. One milliliter of the extraction was spotted with 1 µL of NRM as above for MALDI analysis on a Bruker AutoFlex Speed in negative-ion mode calibrated with Agilent Tuning Mix (Santa Clara, CA) and data was processed in flexAnalysis (Bruker Daltonics, Billerica, MA). All microextraction chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Total Lipid Extraction

Total lipids were extracted from *Fn* (10 mL mid-log shaking culture at 37°C, pelleted) as previously described.(Bligh and Dyer 1959) Briefly, pellets were extracted on ice in 11.4 mL of the single phase extraction solution - 1:2:0.8 C:M:W - for thirty minutes with vigorous agitation (stirbar). Insoluble product was pelleted at 1000 x g for 10 minutes at 4°C. The supernatant was converted into two phases by adding 3 mL of water and 3 mL chloroform, shaken vigorously until the solution turned cloudy (~30 second to 1 minute) and allowed to separate for five minutes at room temperature followed by a fifteen minute separation at 1500 x g at room temperature. The organic phase was collected and dried under a stream of nitrogen. Aqueous phases were washed with 3 mL chloroform and separated as above to recover remaining lipids, processed as above, and pooled with the first organic phase collection. Total lipids were reconstituted in 100 µL of 2:1 C:M solution. 12 µL of total lipid extract was spotted for two-dimensional thin layer chromatography (2D-TLC), below.

Two-Dimensional TLC-MALDI

2D-TLC-MALDI was performed on *Fn* lipid extracts grown at 37°C in TSBC as described above. Lipid extracts were spotted onto aluminum-backed TLC silica gel 60 F_{254} plates (20cm x 20cm, EMD Chemicals Inc., Germany) that were pre-run (wash) in an equilibrated chamber of 1:1 C:M in the direction of the first dimension and dried. Loaded TLC plates were run in an equilibrated chamber of 65:25:3.6:0.4 (v:v:v:v) chloroform:methanol:water:ammonium hydroxide, air dried, turned 90° and separated in the second dimension for acyl complexity in an equilibrated chamber of 60:60:10 (v:v:v)

toluene, pyridine, water. Solvent fronts were marked and lipid migration spots were determined using water exclusion (dried plates were sprayed with water to define lipid spot pattern). For MALDI analysis, the appropriate region of the TLC plate was excised (approximately 5 cm x 7.5 cm), spray coated with matrix (NRM matrix solution, 12 mg mL^{-1} in 2:1 C:M applied with a TLC sprayer) and scanned in negative ion mode at 500 µm spatial resolution on a Bruker AutoFlex Speed, data was processed in flexImaging (Bruker Daltonics, Billerica, MA). Unless otherwise noted, all reagents were sourced from Sigma-Aldrich (St. Louis, MO).

Tissue Preparation and Imaging

Tissue profiling experiments were performed on uninfected, unfixed, frozen mouse kidney and spleen. Twelve micron sections were cut using a ThermoFisher cryostat (Waltham, MA), mounted onto a cold glass slide and incubated at 37°C until visibly dry. For spot profiling and MSI matrices were applied with a Bruker ImagePrep (Billerica, MA) device except where noted. Matrix crystal characterization was performed on 12 µm sections of mouse brain tissue, applied with a Suncollect automated pneumatic sprayer device (Sunchrom GmbH, Friedrichsdorf, Germany) as follows: DHB – 18 layers (6 mg mL^{-1}), 9-AA – 13 layers (6 mg mL^{-1}), and NRM – 13 layers (6 mg mL^{-1}). All matrices were solvated in 1:2:0.8 C:M:W for crystal sizing and description, matrices were applied to a level of comparably similar coverage. For the lipid-depleting experiment, methanol washing steps were performed as follows: one minute wash in 70% methanol followed by a one minute wash in 100% methanol, after which the sections were allowed to dry under ambient conditions and then prepared for imaging

using the ImagePrep as above. Tissue spot profiling and washing MSI data were collected on a Synapt G1 from Waters (Milford, MA) using MassLynx Software (Waters, Milford, MA), calibrated with a polyethylene glycol (PEG) mixture. Spectra were processed for image construction in BioMap 3.7.5.5 software (Novartis, Basel, Switzerland, www.maldi-msi.org). Simultaneous mapping tissue mapping experiments were prepared as described, with matrix deposition on the Sunchrom device and analysis on a Bruker solariX 12T MALDI-FTICR calibrated to 1ppm in negative ion mode using infused sodium trifluoroacetate clusters. Root-mean-square normalization was performed in flexImaging version 4.0 and ion identities were predicted in the Lipid Maps database (Lipid Maps Consortium, www.lipidmaps.org) along with the support of previously published lipid identities.

Results and Discussion

Physiological Range Detection of Lipid A Made Possible Using NRM

We first sought to establish the detection limits for common MALDI matrices used for lipid A, making future analysis of primary samples possible. First, we evaluated the dynamic range and limit of detection (LOD) of lipid A using NRM as a matrix. For this analysis, we used synthetically derived monophosphoryl lipid A (MPLA), a representative lipid A molecule derived from the lipopolysaccharide (LPS) of *Salmonella enterica* serovar *Minnesota* (*Se*). MPLA was 10-fold serially diluted from 1 μg μL^{-1} (1 μg spotted) through 10 pg μL^{-1} (10 pg spotted) followed by addition of 1 μL of DHB, 9-AA, or NRM (concentrations given in Methods). This analysis showed a two-log improvement of the LOD of MPLA with NRM compared to 9-AA and a log improvement compared to DHB. All three matrices resulted in detection of the singly charged lipid A in negative ion mode, where the monoisotopic peak is *m/*z 1744 from 1000 pg MPLA, the total amount in a single spot (Fig. S1). MPLA was undetectable below 100 pg when spotted with DHB and 1000 pg when spotted with 9-AA. Only NRM yielded the monoisotopic peak and at least two additional isotopic peaks from 10 pg MPLA (Fig. S1C). To evaluate performance in a narrow range of concentration near and below LOD, signal-to-noise (S:N) ratios were calculated from triplicate samples (Table 1) diluted to an intermediate range bracketing 10pg MPLA. MPLA spotted at 12.5 pg and 6.3 pg total results in an average S:N ratios of 7.0 and 3.9, respectively. For the purposes of defining an endpoint value S:N≥3 (for *m/*z 1744) was established as a detection cutoff. MPLA applied at 5.0 pg yielded an average S:N<3 (2.5), establishing

323 the refined LOD at 6.3 pg total material, the lowest concentration tested resulting in
324 S:N≥3.
325 Low-level detection of lipid A is central to structural analysis from biological extracts,
326 including infected tissues and primary clinical isolates. Given that $10^{9\text{-}10}$ CFU,
327 representing a small colony of bacteria on an agar plate will yield approximately 10
328 micrograms of LPS (~3-5 µg hydrolyzed lipid A, *E. coli* for reference), this improved
329 LOD for lipid A represents a detectable signal from 6 orders of magnitude fewer
330 bacteria.(Watson *et al.* 1977) Detection of lipid A from less than 10^3 CFU puts detection
331 by MALDI on a clinically relevant scale, for example in a typical urinary tract infection
332 $10^{4\text{-}5}$ CFU mL^{-1} are present.(Schmiemann *et al.* 2010; Kwon *et al*. 2012) Therefore, we
333 sought to detect lipid A directly from infected tissues.
334 For lipid A detection from biological samples, we extracted lipid A from two sources:
335 cultures (*in vitro*) and infected mouse spleen (*in vivo*). *Francisella novicida* (*Fn*) lipid A is
336 a tetra-acylated, mono-glycosylated structure and is readily detectable as a negative ion
337 at *m/z* 1665 (Fig. 1A). The *m/z* 1637 species represents a shortening of one of the fatty
338 acids by two carbon units (Fig. S2). We infected mice subcutaneously with *Fn*,
339 harvested infected spleens (2.8×10^6 CFU mL^{-1}, blood) and extracted lipid A using the
340 microextraction method.(Hamidi *et al.* 2005) *Fn* lipid A was extracted from liquid culture
341 using the Caroff isobutyric acid/ammonium hydroxide microextraction method, solvated
342 in 2:1 chloroform:methanol (50 µL) from which 1 µL was spotted onto a MALDI target
343 plate with 1 µL NRM matrix and analyzed using MALDI.(Hamidi *et al.* 2005) As
344 expected, extracted *Fn* lipid A is readily detectable (Fig. 1A) from a 1 mL *in vitro* culture
345 (>10^8 CFU mL^{-1}) as two major species, *m/z* 1665 and *m/z* 1637. The previously

described structure of the larger molecule (*m/z* 1665) is given in Figure S2 and is the dominant structure when *Fn* is grown at 37°C.(Shaffer *et al.* 2007) Both of the *Fn* lipid A products detected *in vitro* were robustly detected *in vivo*, and in similar relative abundances (Fig. 1B) to the *in vitro* lipid A profile; the dominant lipid A species (*m/z* 1665) along with the minor species at (*m/z* 1637) were both observed *in vivo*. These results represent the first lipid A analysis by MALDI-MS from directly extracted infected tissue, as opposed to lipid A extraction from expanded cultures, *ex vivo.* This approach will be a powerful tool to study the effect of host influences on bacterial lipid A structures.

Given the low input necessary for lipid A detection using the combination of NRM matrix and the Caroff microextraction method, we aimed to confirm the major lipid A ions of *Fn* growing in ticks, one of the arthropod vectors of multiple *Francisella* subspecies.(Nano 2006) We posited that an intermediate (vector, tick) temperature-controlled structure of *Fn* lipid A should increase in relative abundance compared to the warm (host, mouse) temperature-controlled structure as demonstrated previously, *in vitro*.(Li *et al.* 2012) *D. variabilis* (*Dv*) ticks were injected with *Fn* and maintained at 23°C for 48 hours. Lipid A was extracted from whole ticks (1 x 10^7 CFU per tick) by microextraction and spotted with NRM matrix for analysis. Compared to the relative abundance of the warm temperature structure of *Fn* lipid A (*m/z* 1665) we observed a nearly equal balance (Fig. 1C) with a previously described intermediate-length structure (*m/z* 1637) in the whole tick extracts.(Li, Wang and Ernst 2011; Shaffer *et al*. 2007) These results taken together support the hypothesis that *Fn* lipid A structure is

368 modulated in response to local environmental cues such as growth at alternative
369 temperatures or in an arthropod vector.(Shaffer *et al.* 2007; Li *et al.* 2012)
370 The LOD to detect lipid A was compared using NRM, 9-AA, and DHB revealing a two-
371 log improvement in LOD between 9-AA (100-fold) and NRM and a log improvement
372 between DHB (10-fold) and NRM. The expansion of the working range of lipid A LOD,
373 from routine analysis of nanogram quantities to routine analysis of picogram quantities,
374 makes NRM a powerful tool for characterizing low-yield lipid A extractions from clinical
375 or environmental samples, including bacteria isolated from biofilms on implanted
376 devices or directly from infected wound sites. Overall, NRM will have wide applications
377 for the detection and study of complex lipids.
378
379 **Simultaneous Bacterial Lipid A and Phospholipid Profiling from Lipid Extracts**
380 After growth at environmental (≤25°C) and mammalian (37°C) temperatures, bacteria
381 rapidly remodel their membrane lipids to maintain proper membrane function and
382 fluidity.(Li *et al.* 2012) Profiling these changes at both the phospholipid and lipid A
383 levels, simultaneously, will yield a greater understanding of the relationship between
384 temperature and global membrane remodeling. Harnessing the improved lipid A
385 detection level conferred by NRM, we profiled both lipid A and phospholipids from a *Fn*
386 lipid extract using two-dimensional (2D) thin layer chromatography (TLC) coupled to
387 MALDI (2D-TLC-MALDI). Due to the complexity of bacterial lipid preparations, we
388 wanted to first resolve lipid classes and subsequently visualize the discrete acyl length
389 variants of two major *Fn* lipid components: lipid A and phosphatidylglycerol (PG). For
390 this analysis, we separated total *Fn* lipid extracts in a 2D-TLC format followed by MALDI

391 mass spectrometry imaging (MSI) to visualize discrete lipid bands. Figure 2 illustrates
392 the separation approach, as well as co-detection of lipid A and several PG lipids. The
393 previously observed improvements in LOD of lipid A from primary extracts using NRM
394 was also apparent in the TLC silica plate format, with three lipid A variant structures
395 detectable: *m/z* 1665, 1637, and 1609, a minor constituent at mammalian growth
396 temperature (37°C) representing a further shortening of one of the fatty acids by 2
397 carbon units. The reference *Fn* lipid A structures (Fig. S2), their expected temperature-
398 controlled abundance ratios, and the major ions within the peaks have been previously
399 described. (Shaffer *et al.* 2007; Li *et al.* 2012) TLC-MALDI is an established method for
400 rapid phospholipid profiling. Here, we have demonstrated the use of a single matrix to
401 profile both lipid A and phospholipids simultaneously from a single TLC-MALDI
402 experiment. The approach can be readily translated to multiple infection model systems.
403 The spleen is a dynamic secondary lymphoid organ responsible for clearance of
404 compromised red blood cells in the red pulp and immune response and surveillance in
405 the white pulp. During the course of an immune response, splenic architecture
406 undergoes dramatic restructuring, forming germinal centers and follicles. To determine if
407 NRM could be effective as a lipid matrix for MSI of the host response, naïve spleen
408 sections were prepared with NRM matrix dissolved in a single-phase lipid extraction
409 solution (1:2:0.8 chloroform:methanol:water) and spot analyzed in positive- and
410 negative-ion modes.(Cerruti *et al.* 2012; Scott *et al.* 2014) We sought to establish a
411 baseline profile of lipids in the spleen in both polarities, including those containing
412 polyunsaturated fatty acids (PUFAs), such as arachidonic acid (AA), released from
413 membrane phospholipids and required for the production of specific classes of

414 inflammatory lipids. The most abundant ion detected in negative-ion mode was *m/z*
415 885.6, an arachidonic acid-containing lipid phosphatidylinositol (PI) described in
416 numerous other tissues (Fig. 3).(Murphy, Hankin and Barkley 2008) Higher molecular
417 weight lipid ions were also detected in the spleen, again highlighting increased NRM
418 efficiency in higher mass ranges (Figs. 3 and S3, Supplemental Text). Together, these
419 data highlight the dual polarity application of NRM for efficient lipid profiling experiments
420 on-tissue and widen the available methods to study lipid-mediated inflammation.
421 Profiling the extent of incorporation of PUFAs in the phospholipid repertoire may have
422 interesting implications for host lipid based inflammatory response, including our
423 understanding of the overall potential for a tissue to absorb damage from reactive
424 oxygen species. Additionally, release of specific PUFAs from the parent phospholipid is
425 a critical initiation point for production of lipid signaling molecules that can be potent
426 modulators in the context of inflammation and immunity. Prostaglandins, produced from
427 liberated AA have an important, yet poorly defined role in *Francisella* infections.
428 Woolard *et al* demonstrated in 2007 that the T cell blocking mechanism observed in
429 *Francisella tularensis* LVS infected macrophages was due to production of
430 prostaglandin E2 (PGE_2).(Woolard *et al.* 2007) Mapping the AA-containing
431 phospholipids upstream of these immunomodulatory effects will be the focus of further
432 study.
433
434 **Simultaneous Mapping of Unique Bacterial and Host Lipids**
435 In an effort to harness the improved performance of our lipid detection and mapping
436 experiments, we sought to use NRM to further describe the host-pathogen interaction

437 within infected tissue. *Fn* is a Gram-negative species with two membranes, the inner
438 membrane consisting of phospholipids and the asymmetrical outer membrane.
439 Comprising the outer membrane are phospholipids on the inner leaflet of the outer
440 membrane and LPS (the lipid A anchor component) on the outer leaflet of the outer
441 membrane. Bacterial membranes contain a dominant fraction of PG and PE, though
442 generalizations about individual bacterial backgrounds are difficult since the unique lipid
443 composition is linked to taxonomy.(Ratledge and Wilkinson 1988) We have previously
444 characterized the *in vitro* phospholipid and lipid A populations of *Fn* using standard lipid
445 extraction methods and determined that phosphatidylglycerol (PG) 32:0 was a major
446 component of the bacterial membrane.(Zhang and Rock 2008; Li, Wang and Ernst
447 2011) Using MSI we sought to map the distribution of PG 32:0 in *Fn* infected tissue.
448 Figure 4 demonstrates the distribution of PG 32:0 (*m/z* 721.5) within *Fn* infected spleen
449 versus naïve spleen, with high relative abundances observed in the red pulp following
450 infection. At 48 hours post-infection organisms are present in the spleen, especially the
451 red pulp.(Conlan *et al.* 2003; Elkins, Cowley and Bosio 2007; Kanistanon *et al.* 2008;
452 Ojeda *et al.* 2008; Rasmussen *et al.* 2012) Although PG 32:0 is not exclusively a
453 bacterial phospholipid, the relative abundance in the naive spleen profile (Fig. 3) is low
454 and near the detection threshold. PG levels in mouse tissues (liver, <5%) are modest
455 compared to other phospholipid classes.(White 1973) It is worth noting that PG 32:0
456 was not reported present in human plasma samples analyzed by the Lipid Maps
457 Consortium; however, absent complete comparative descriptions of the mouse and
458 human splenic lipidomes, it is impossible to directly compare PG
459 content.(Quehenberger *et al.* 2010) For the purposes of this work, PG was considered

an abundant bacterial marker, though the possibility exists that the presence of bacteria or simply of an activated immune response may be sufficient to stimulate production of host-borne PG 32:0.

In contrast, several classes of lipid are not made by *Fn* including the PIs, thus they are exclusively host lipids in this infection.(Li, Wang and Ernst 2011) To demonstrate the simultaneous host-pathogen lipid monitoring made we mapped PI 42:8 (*m/z* 933.5), an extensively polyunsaturated PI with 42 total acyl carbons (Table S1), throughout the infected spleen with some bias toward a red pulp distribution. Similarly, PE 38:4 (*m/z* 766.5) is another exclusively host lipid as it is not found in lipid extracts of *Fn*. Curiously, PE 38:4 was found in the white pulp with high relative intensity organized puncta (Fig. 4) suggesting that this may be a marker for a specific cell type or a highly localized immune process. By co-localizing components of the immune response to *Fn* in tandem with the host and pathogen lipid distributions we aim to further describe the basic pathogenic mechanisms of this infection. Achieving higher sensitivity for a wide variety of pathogen lipids is crucial to the success of this approach and the studies herein implicate its feasibility for numerous infection models. Further studies will be necessary to define imaging parameters for exclusive bacterial lipids, such as lipid A; however, highly expressed bacterial lipids can serve as proxy markers to map bacterial infection from a new perspective while simultaneously mapping the host response. This is a valuable combination for future host-pathogen interaction studies.

Conclusions

When used in MALDI-coupled techniques for bacterial, vector, and host lipid analysis, NRM is a powerful and versatile matrix allowing picogram-level detection of MPLA and enabling analysis of lipid A from primary extracts of *in vitro* and *in vivo* infection model systems. Beyond MALDI spot analysis, the use of NRM in alternative MALDI-coupled techniques resulted in the detection of a wide range of lipids and facilitated analysis of both lipid A and phospholipids directly from 2D-TLC-MALDI plates making it possible to survey many lipid components of bacterial membranes in a single scan. It also increased the presence of higher mass lipids, extending the useable information range from a single experiment. The overall utility of NRM is underlined by improved LOD of lipid A, robust performance in positive- and negative-ion modes, and versatility across multiple MALDI applications. Finally, we demonstrated the capability of coupling bacterial lipid mapping to host lipid mapping using this simultaneous monitoring approach. Lipids exclusive to the host were found in the same regions as specific bacterial lipids as well as in unique tissue structures (organized splenic white pulp) involved in the immune response to *Fn* infection. Future studies will focus on the direct mapping of lipid A within infected tissues, including optimization of the on-tissue LPS hydrolysis steps that will be necessary for robust MSI of lipid A signal from bacterial infections bearing smooth LPS. Together, our results establish a path to describe novel lipid-based mechanisms of microbial pathogenesis that will find wide utility within the infection and immunity fields.

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Graphical Abstract Sentence

Simultaneous profiling of *Francisella novicida* lipid A and phospholipids from mammalian host tissue and whole arthropod vectors using the dual polarity matrix, norharmane.

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