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Imaging mass spectrometry for assessing temporal proteomics: Analysis of calprotectin in *Acinetobacter baumannii* pulmonary infection

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Abstract

Imaging MS is routinely used to show spatial localization of proteins within a tissue sample and can also be employed to study temporal protein dynamics. The antimicrobial S100 protein calprotectin, a heterodimer of subunits S100A8 and S100A9, is an abundant cytosolic component of neutrophils. Using imaging MS, calprotectin can be detected as a marker of the inflammatory response to bacterial challenge. In a murine model of *Acinetobacter baumannii* pneumonia, protein images of S100A8 and S100A9 collected at different time points throughout infection aid in visualization of the innate immune response to this pathogen. Calprotectin is detectable within 6 h of infection as immune cells respond to the invading pathogen. As the bacterial burden decreases, signals from the inflammatory proteins decrease. Calprotectin is no longer detectable 96–144 h post infection, correlating to a lack of detectable bacterial burden in lungs. These experiments provide a label-free, multiplexed approach to study host response to a bacterial threat and eventual clearance of the pathogen over time.

Keywords

Acinetobacter baumannii; Calprotectin; Imaging MS; Microbiology; MALDI; Pulmonary infection

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1 Introduction

MALDI imaging mass spectrometry (IMS) is a label-free technology that allows researchers to map the spatial distribution of analytes in thin sections of tissue. Since the technology was introduced in 1997 [1], it has been applied to many biological systems to image small molecules [2, 3], lipids [4], peptides [5], and proteins [6, 7]. MALDI MS uses an energy-absorbing matrix, typically a low molecular weight organic acid, which absorbs the wavelength of the laser to aid the ionization and desorption of analytes [8]. Molecular images are collected by a raster of the laser beam over a thin section of tissue. Spectra are collected in either MS or tandem MS mode at each image pixel. Peaks of interest can be selected during data analysis, and ion intensity maps can be produced by integrating the area under a selected m/z peak or by selecting the maximum value within a peak-range and plotting these at each pixel location. Ion intensity maps are typically displayed with a false color scale to show relative abundance at each pixel [4]. The technology provides a multiplexed analysis that can easily be applied to many analytes, especially in situations where there is little a priori knowledge of the molecular content of the tissue [9,10].

As the field advances with improved sample preparation methods and instrumentation, more in-depth analyses of biological systems can be performed [11,12]. Sample preparation and matrix application methods have become more standardized, allowing for reliable and reproducible images [10, 13]. New laser systems are routinely capable of repetition rates of 1 kHz or greater, and commercial software is now available to facilitate data processing, normalization, peak selection, and ion map generation [14]. Due to these advancements, IMS is now successfully used for large-scale experiments, including studies with numerous biological and technical replicates. The expanded implementation of IMS has been applied to systems where analytes change their spatial distribution over time. Analysis of temporal dynamics has been pioneered to study secondary metabolites [15], pharmaceuticals [16], agrochemicals [17], lipids [18–20], and natural products [21, 22]. IMS has greatly increased the understanding of these systems, both spatially and temporally.

In general, the temporal study of proteins has typically been approached in a number of ways. Fluorescent approaches have been implemented successfully, yet still have certain caveats [23]. Molecules of interest must be tagged before experimentation with a fluorescent reporter, which should be specific for the species of interest and must exhibit spectroscopic characteristics that facilitate easy detection. However, altering the protein with a tag can impair or impede its normal function within the system [23]. Additionally, it is difficult to multiplex fluorescence-based approaches [24]. Protein microarrays and immunoblots have successfully studied differential expression, but they require homogenized samples and therefore sacrifice any information about spatial distribution of analytes within a sample [25–28]. Immunohistological staining can provide spatial information, but requires available antibodies or stains for proteins of interest and cannot easily be multiplexed [29]. For these reasons, MALDI IMS presents an attractive alternative to monitor relative changes in protein abundance over time.

Previous work in our laboratories has employed MALDI IMS of proteins to study the inflammatory response in animal models of bacterial infection. Such studies of mice systemically infected with *Staphylococcus aureus* determined that the host S100 proteins comprising the calprotectin heterodimer localize to abscesses [29, 30]. The same protein has been detected via MALDI IMS in lung tissue of mice infected with *Acinetobacter baumannii* [31]. Calprotectin is an abundant metal-binding protein; it accounts for an estimated 40% of the cytosolic content of neutrophils, the most abundant vertebrate immune cell [32, 33]. Calprotectin exists as a heterodimer of S100A8 and S100A9; it contains a calcium-binding domain and two distinct histidine-rich sites, which are necessary to bind

zinc and manganese, respectively [34–37]. Nutrient metal chelation of zinc and manganese contributes to the antimicrobial and antifungal properties of the protein by disrupting microbial processes and inhibiting superoxide defense, leading to enhanced neutrophil killing [34,38,39].

A. baumannii is a pathogen of global public health concern. It is pervasive in the environment and capable of surviving for extended periods on hospital surfaces [40, 41]. *A. baumannii* can present clinically as a wide range of diseases, including wound infections, pneumonia, urinary tract infections, and sepsis [42]. This bacterium has two compounding factors that make it an especially significant threat. First, it often strikes immunocompromised patients and is therefore a major concern in the intensive care unit. Second, *A. baumannii* is quite adept at acquiring and maintaining resistance to antimicrobial therapies [43, 44]. Imipenimem-non-susceptible *A. baumannii* strains have been correlated to longer lengths of treatment in the intensive care unit [45]. In one setting, *A. baumannii* nosocomial pneumonia occurred more often than pneumonias caused by other bacteria, and those patients with *A. baumannii* pneumonia exhibited increased mortality [46]. Even with the increasing clinical importance, relatively little is known concerning the interactions between the host and this pathogen.

Human *A. baumannii* pulmonary infection is often characterized with lobar pneumonia, where one lobe experiences dense infection while the remaining lung is spared [47,48]. In response to *A. baumannii* pneumonia, neutrophils are quickly recruited to the lungs leading to a subsequent increase in the levels of proinflammatory cytokines and a heightened inflammatory response [49]. We have previously shown that calprotectin is recruited to *A. baumannii* infected lungs and enhances the clearance of bacteria from the alveolar spaces. Additionally, calprotectin-deficient mice exhibit increased bacterial burdens in the lung and higher levels of dissemination to the liver [31].

For these reasons, it is important to study how the host responds to *A. baumannii* during lung infections, both spatially and temporally. In this work, we sought to follow the proteins S100A8 and S100A9 throughout a time course of *A. baumannii* pulmonary infection using MALDI IMS. We report that calprotectin responds to *A. baumannii* infection, accumulates in areas of inflammation, and decreases abundance following pathogen clearance.

2 Material and methods

2.1 Bacterial strain and reagents

A. baumannii strain ATCC 17978 (Ab17978) was obtained from the American type culture collection and was used for all experiments.

Trifluoroacetic acid, formic acid, and sinapinic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). HPLC-grade ACN, ethanol, technical grade hexanes, and tissue protein extraction reagent were purchased from Fisher Scientific (Pittsburgh, PA, USA). Tissue-Tek optimal cutting temperature compound was purchased from VWR International (Suwanee, GA, USA). Dye-based drawing ink was purchased from Higgins Ink (Chartpak Inc. Leeds, MA, USA). PBS was purchased from Mediatech (Manassas, VA, USA).

2.2 *A. baumannii* infection

All animal experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee. For these infections, we used a previously established pneumonia model with minor modifications [31,50]. Briefly, cultures of Ab17978 were grown to logarithmic phase in Luria-Bertani medium, washed, and resuspended in PBS.

Seven-week-old, female Swiss Webster mice (Charles River Laboratories) were anesthetized and intranasally infected with 30 μ L bacterial suspension. The weights of the animals were measured daily to serve as an indicator of overall health. Mice were euthanized at the indicated time points post infection and lungs and livers were aseptically removed. Lungs for imaging experiments were perfused with 1 mL 1:1 optimal cutting temperature:water, marked for histological orientation using dye-based drawing ink, snap-frozen in a dry ice/hexane mixture, and stored at -80°C until analyzed. Lungs and livers for bacterial enumeration were homogenized in 1 mL PBS, serially diluted, and plated on Luria-Bertani medium agar.

For experiments to determine optimal dose of Ab17978 needed to cause infection, inocula ranging from 10^6 to 10^8 colony forming units (CFUs) were used. Mice mock-infected with 30 μ L of PBS served as controls. Seven mice were infected for each inoculum, with five mice used for CFU enumeration and two mice used for MALDI IMS. Lungs were harvested at 36 h post infection (hpi).

For time course studies, 30 μ L of Ab17978 at a concentration of 1×10^7 CFU/ μ L were administered and organs were harvested at 12 time points following infection (0, 6, 12, 18, 24, 36, 48, 72, 96, 144, 192, and 240 hpi). For each time point, five mice were used for CFU enumeration and two mice for MALDI IMS. Twelve mice were mock-infected with PBS to serve as controls, one at each time point.

Statistical analyses were performed using GraphPad Prism version 6.

2.3 IMS

Frozen lungs were identically oriented based on histological ink and sectioned at -20°C to 10 μ m thickness using a Leica CM3050S cryostat (Leica Microsystems, Bannockburn, IL, USA). Tissue sections were thaw-mounted onto indiumtin oxide coated glass slides (Delta Technologies, Loveland, CO, USA). Excess lipids and salts were removed by washing the slides in sequential 30 s washes of chilled 70, 90, and 95% ethanol. Sinapinic acid matrix was prepared at a concentration of 20 mg/mL in 60% ACN with 0.1% TFA. A Portrait 630 (Labcyte, Sunnyvale, CA, USA) acoustic robotic microspotter was used to apply matrix to samples. The microspotter was operated in flyby mode at 300 μ m lateral resolution. Technical replicates were performed at 500 μ m lateral resolution. Matrix was applied in four passes of 15 droplets, with each droplet having an approximate volume of 170 pL.

Samples were analyzed using a Bruker Autoflex Speed TOF mass spectrometer with a modified Swiss Cross source (Bruker Daltonics, Billerica, MA, USA) equipped with a SmartBeamTM laser (Nd:YAG, 355 nm) in linear positive-ion mode. Two hundred laser shots were collected at each matrix spot in 50 shot increments with a random walk pattern within the spot. The laser repetition rate was 1000 Hz; extraction and acceleration voltages were 19.67 kV and 18.22 kV, respectively, with a delayed extraction time of 350 ns and a lens voltage of 8.5 kV. The measured mass range was from m/z 3000–25 000. Within each trial, lungs were imaged at the same time in a random order selected by an unaffiliated and unbiased researcher. Images were collected at 300 μ m lateral resolution. Technical replicates were performed at 500 μ m lateral resolution to reduce data size and to show similar spatial distributions from additional sections. Images were viewed using Fleximaging 3.0 Software (Bruker Daltonics) and were normalized to total ion current. Ion density maps were extracted and scaling was constant for each ion of interest. To assess differences in images, regions of interest were selected from areas of inflammation and correlating control tissue using Fleximaging. Spectral filter data were exported and average pixel intensity was calculated.

2.4 Histology

For each section analyzed, a serial section was collected for histological analysis. Serial sections were hematoxylin and eosin (H&E) stained. Slides were scanned using a MIRAX Scan slide scanner (Carl Zeiss MicroImaging, Göttingen, Germany) in manual bright field mode and optical images were generated using MIRAX Viewer version 1.11.22.0 (Carl Zeiss MicroImaging). Forty times magnification images were generated by an experienced pathologist to assess biological and technical replicates for gross pathology and similarity. Images were obtained using an Olympus BX41 microscope (Olympus, Melville, NY, USA) equipped with a Spot Insight Camera (Diagnostics Instruments, Sterling Heights, MI, USA).

2.5 Protein purification and identification

Tissue samples with high abundance of S100A8 and S100A9 (samples 48 hpi) were used for protein identification. Adjacent sections of tissue from those imaged were collected and homogenized using tissue protein extraction reagent. Extracts were fractionated by RP HPLC using a Waters 2690 Separations Module and C8 column (VYDAC, Grace Davison Discover Sciences). Proteins were eluted over 75 min using the following gradient: initial flow of 95% solvent A (0.1% TFA), 5% solvent B (ACN, 0.1% TFA), ramped to 25% B over 15 min, ramped to 60% B over 50 min, ramped to 95% over 10 min, held for 5 min, ramped down to 5% B over 10 min and held for 20 min. Fractions were collected each minute at a rate of 0.5 mL/min. MALDI spectra were collected from each fraction to determine fractions of interest.

Fractions corresponding to S100A8 and S100A9 were dried down and reconstituted in 100 mM ammonium bicarbonate, pH 8.0. Aliquots corresponding to 2 µg of protein (measured by Bradford assay) were digested with endoproteinase trypsin (Promega Madison, WI, USA) at 1:20 enzyme:protein overnight at 37°C. The resulting peptides were acidified and bomb-loaded onto a self-packed pre-column (4 cm × 0.1 mm, Jupiter 5 µm, 300 Å, C18) (Phenomenex, Torrance, CA, USA) fritted with an M520 inline microfilter union (IDEX, Lake Forest, IL, USA). Following equilibration, this column was attached to a self-packed analytical column (20 cm × 0.1 mm, Jupiter 3 µm, 300 Å, C18) (Phenomenex) equipped with a laser-pulled, 1 µm nanospray emitter tip (P-2000, Sutter Instruments, Novato, CA, USA) and coupled directly to an LTQ (Thermo Scientific, Waltham, MA, USA) with a nanoelectrospray source.

Peptides were eluted over 75 min on a nanoAcquity UPLC system (Waters, Manchester, UK) using the following gradient: initial flow of 98% solvent A (0.1% formic acid), 2% solvent B (ACN, 0.1% formic acid), ramped to 25% B over 45 min, ramped to 90% B over 15 min, held for 5 min, ramped down to 2% over 2 min, and held for 8 min. Throughout the entire run, a full scan was taken followed by five data dependent collisionally activated dissociation MS/MS scans with dynamic exclusion enabled with a repeat count of 1, a repeat duration of 30 s, and an exclusion duration of 60 s. The resulting MS/MS spectra were searched via SEQUEST against a UniprotKB database of canonical mouse proteins. Identifications were filtered and collated at the protein level using Scaffold (Proteome Software, Portland, OR, USA). Peptide hits corresponding to S100A8 and S100A9 were manually verified at both the full MS level and MS/MS level using Xcalibur (Thermo Scientific, Waltham, MA, USA).

3 Results

3.1 Inflammatory response to *A. baumannii* correlates to bacterial burden

In order to successfully visualize protein accumulation and dissemination, the bacterial burden necessary to establish a detectable inflammatory response in immunocompetent mice

was determined. Higher doses of *A. baumannii* resulted in increased inflammation, as seen in the histological staining in Fig. 1A and B. This is consistent with the idea that the magnitude of the immune response is impacted by bacterial burden. IMS analysis of S100A8 and S100A9, Fig. 1C and D, showed increased intensity correlating with bacterial burden. Figure 1E shows bacterial enumeration from lungs at 36 hpi, further emphasizing a correlation of dose to bacterial burden. Biological and technical replicates are presented in Supporting Information Fig. 1. It was determined that bacterial burden of approximately 10^8 CFUs, delivered in 30 μ L of a 1×10^7 CFU/ μ L inoculum, is necessary to cause inflammation and robust calprotectin accumulation detectable by MALDI IMS.

3.2 Calprotectin protein dynamics correlate with bacterial burden

To define the kinetics of the inflammatory response to *A. baumannii* pulmonary infection, a time course study with the time points specified in section 2.2 was employed. Animal weight was used to monitor overall health (Fig. 2A). Bacterial burden was evaluated from the primary site of infection in the lungs (Fig. 2B) and livers (Fig. 2C), a site of secondary dissemination. These data indicate that the majority of the bacterial burden is cleared by 96–144 hpi.

MALDI IMS was used to study temporal dynamics of calprotectin subunits at these time points. These experiments revealed a strong correlation between calprotectin expression and bacterial burden during the time course of infection (Fig. 3). Signal for S100A8 and S100A9, appearing at m/z 10 165 and 12 999, respectively, is first detected at 6 hpi, showing a rapid response of the innate immune system to *A. baumannii*. The signals continue to accumulate in a similar manner that corresponds to the bacterial burdens. A maximal accumulation of the antimicrobial proteins occurs at 36–48 hpi and can be correlated to the decrease in bacterial burden shown in Fig. 2B. Signals from S100A8 and S100A9 begin to decrease near 96–144 hpi corresponding to the clearance of *A. baumannii* from lungs (Fig. 2B). These experiments were performed in both biological and technical replicates (Supporting Information Figs. 2–4). Additionally, S100A8 or S100A9 were not detected in the PBS control tissue (Supporting Information Fig. 5). For all images, an ion map of m/z 14 050, an abundant peak in collected spectra belonging to histone H2A.1, was generated to demonstrate that changes in calprotectin abundance were not an effect of ubiquitous changes in protein signal. The histone signal may also be used to monitor variations in tissue density caused by atelectasis of inflamed lungs or perfusion effects. Figure 4 shows two tissues from the 96 h time point stained for neutrophils using an immunohistochemical stain and correlating with MALDI IMS data. This demonstrates histological differences introduced by pneumonia and confirms the presence of neutrophils in infected lungs.

To further assess signal differences in the time course (Fig. 3) compared to the uninfected controls (Supporting Information Fig. 5), average pixel intensity was determined by selecting regions of interest using FlexImaging. Average pixel intensities for S100A8 and S100A9 were calculated and plotted in Supporting Information Fig. 6. This provides a descriptive assessment of protein changes with time.

The ability of this technology to provide multiplexed analysis of bacterial infections without a priori knowledge of the proteins present can also be demonstrated in this analysis. Supporting Information Fig. 7 shows the analytical power of a signal analysis using MALDI IMS. These signals of interest potentially represent other proteins involved in response to bacterial infection.

3.3 S100A8 and S100A9 identified from murine lung tissue infected with *A. baumannii*

To confirm the m/z values of interest correspond to S100A8 and S100A9, tissue from lungs at 48 hpi was homogenized and proteins were separated by offline HPLC fractionation. Recovery of fractions of interest was verified by MALDI MS analysis. Figure 5A shows an in situ spectrum from a single pixel in the IMS image data. Figure 5B shows spectra taken from the fractions enriched in S100A8 and S100A9 following offline fractionation. These fractions were subjected to tryptic digestion and analyzed by LC-MS/MS. Figure 5C shows the sequence coverage obtained for both proteins. Upon manual investigation of peptides from S100A8, near complete sequence coverage was obtained, allowing for its confident identification. The abundance of hemoglobin proteins (m/z 14 980 and 15 710) within the fraction did not preclude identification of peptides corresponding to S100A8. For the fraction containing S100A9, manual investigation revealed a total of four peptides from S100A9, allowing for its confident assignment as well.

4 Discussion

Temporal protein dynamics has been studied using a variety of technologies, including fluorescence microscopy, protein microarrays, and immunoblotting. However, these technologies are not label-free and easily multiplexed, and in some cases fail to provide spatial information about proteins of interest. Here, MALDI IMS was used to monitor the spatial distribution of calprotectin over time following infection with *A. baumannii*. We observed that areas of high intensity for S100A8 and S100A9 correlate with areas of inflammation, shown in Fig. 3. Furthermore, S100A8 and S100A9 are present in the lungs within 6 h following infection and dissipate after bacterial clearance at 96–144 hpi. As a significant protein in the cytoplasm of neutrophils and as a proinflammatory molecule, calprotectin is rapidly recruited to infected areas and aids in the recruitment of an amplified immune response [51,52].

In addition to being proinflammatory, calprotectin has antimicrobial properties stemming from the chelation of the nutrient metals manganese and zinc. These metals serve as important cofactors in many bacterial processes [36,53]. A decrease in their bioavailability due to increased concentrations of calprotectin would likely lead to many physiological changes in the bacterium, including increased expression of dedicated metal import systems, decreased levels of nonessential zinc- and manganese-dependent enzymes, and alterations in metabolic pathways which require these metals [36]. Additionally, zinc and manganese chelation inhibits superoxide defense, allowing for enhanced neutrophil killing [34]. Taken together, calprotectin functions to both inhibit the growth of invading pathogens and assist in the signaling required to recruit the next line of defense. In light of this, monitoring calprotectin during pulmonary infection provides important new information regarding *A. baumannii* pathogenesis.

Applying MALDI IMS analyses to a time-course study of *A. baumannii* pulmonary infection successfully defined spatial and temporal components of calprotectin response to bacterial infection. As the field of MALDI IMS advances, the ability to study larger and more diverse sample sets becomes a reality. Application of methodologies similar to those reported here could greatly contribute to the study of many other analytes' spatial distributions and their changes over time. Such analyses are the basis for additional imaging capabilities, especially 3D studies of protein distribution in models of infection over time. These findings further show that MALDI IMS is a valuable label-free, multiplex technology that preserves the spatial location of analytes within the sample.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CFU	colony forming unit
H&E	hematoxylin and eosin
hpi	hours post infection
IMS	imaging mass spectrometry
SA	sinapinic acid

5 References

- [1]. Caprioli RM, Farmer TB, Gile J. Molecular imaging of biological samples: Localization of peptides and proteins using MALDI-TOF MS. *Anal. Chem.* 1997; 69:4751–4760. [PubMed: 9406525]
- [2]. Nilsson A, Fehniger TE, Gustavsson L, Andersson M, et al. Fine mapping the spatial distribution and concentration of unlabeled drugs within tissue micro-compartments using imaging mass spectrometry. *PLOS ONE.* 2010; 5:e11411. [PubMed: 20644728]
- [3]. Hsieh Y, Casale R, Chen J, Knemeyer I, et al. Matrix-assisted laser desorption/ionization imaging mass spectrometry for direct measurement of clozapine in rat brain tissue. *Rapid Commun. Mass Spectrom.* 20:965–972. [PubMed: 16470674]
- [4]. Cornett DS, Reyzer ML, Chaurand P, Caprioli RM. MALDI imaging mass spectrometry: molecular snapshots of biochemical systems. *Nat. Methods.* 2007; 4:828–833. [PubMed: 17901873]
- [5]. Clemis EJ, Smith DS, Camenzind AG, Danell RM, et al. Quantitation of spatially-localized proteins in tissue samples using MALDI-MRM imaging. *Anal. Chem.* 2012; 84:3514–3522. [PubMed: 22356211]
- [6]. Cazares LH, Troyer D, Mendrinos S, Lance RA, et al. Imaging mass spectrometry of a specific fragment of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 2 discriminates cancer from uninvolved prostate tissue. *Clin. Cancer Res.* 2009; 15:5541–5551. [PubMed: 19690195]
- [7]. Chaurand P, Norris JL, Cornett DS, Mobley JA, Caprioli RM. New developments in profiling and imaging of proteins from tissue sections by MALDI mass spectrometry. *J. Proteome Res.* 2006; 5:2889–2900. [PubMed: 17081040]
- [8]. Chaurand P, Sanders ME, Jensen RA, Caprioli RM. Proteomics in diagnostic pathology: Profiling and imaging proteins directly in tissue sections. *Am. J. Pathol.* 2004; 165:1057–1068. [PubMed: 15466373]
- [9]. MacAleese L, Stauber J, Heeren RMA. Perspectives for imaging mass spectrometry in the proteomics landscape. *Proteomics.* 2009; 9:819–834. [PubMed: 19212956]
- [10]. Goodwin RJ. Sample preparation for mass spectrometry imaging: small mistakes can lead to big consequences. *J. Proteom.* 2012; 75:4893–4911.

- [11]. Watrous JD, Alexandrov T, Dorrestein PC. The evolving field of imaging mass spectrometry and its impact on future biological research. *J. Mass Spectrom.* 2011; 46:209–222. [PubMed: 21322093]
- [12]. Seeley EH, Oppenheimer SR, Mi D, Chaurand P, Caprioli RM. Enhancement of protein sensitivity for MALDI imaging mass spectrometry after chemical treatment of tissue sections. *J. Am. Soc. Mass Spectrom.* 2008; 19:1069–1077. [PubMed: 18472274]
- [13]. Kaleta BK, van der Wiel IM, Stauber J, Dekker LJ, et al. Sample preparation issues for tissue imaging by imaging MS. *Proteomics.* 2012; 9:2622–2633. [PubMed: 19415667]
- [14]. Spraggins JM, Caprioli RM. High-speed MALDI-TOF imaging mass spectrometry: rapid ion image acquisition and considerations for next generation instrumentation. *J. Am. Soc. Mass Spectrom.* 2011; 22:1022–1031. [PubMed: 21953043]
- [15]. Yang Y-L, Xu Y, Straight P, Dorrestein PC. Translating metabolic exchange with imaging mass spectrometry. *Nat. Chem. Biol.* 2009; 5:885–887. [PubMed: 19915536]
- [16]. Khatib-Shahidi S, Andersson M, Herman JL, Gillespie TA, Caprioli RM. Direct molecular analysis of whole-body animal tissue sections by imaging MALDI mass spectrometry. *Anal. Chem.* 2006; 78:6648–6456.
- [17]. Anderson DMG, Carolan VA, Crosland S, Sharples KR, Clench MR. Examination of the distribution of nicosulfuron in sunflower plants by matrix-assisted laser desorption/ionisation mass spectrometry imaging. *Rapid Commun. Mass Spectrom.* 2009; 23:1321–1327. [PubMed: 19337978]
- [18]. Burnum KE, Cornett DE, Puolitaival SM, Milne SB, et al. Spatial and temporal alterations of phospholipids determined by mass spectrometry during mouse embryo implantation. *J. Lipid Res.* 2009; 50:2290–2298. [PubMed: 19429885]
- [19]. Burnum KE, Frappier SL, Caprioli RM. Matrix-assisted laser desorption/ionization imaging mass spectrometry for the investigation of proteins and peptides. *Ann. Rev. Anal. Chem.* 2008; 1:689–705.
- [20]. Sugiura Y, Konishi Y, Zaima N, Kajihara S, et al. Visualization of the cell-selective distribution of pufa-containing phosphatidylcholines in mouse brain by imaging mass spectrometry. *J. Lipid Res.* 2009; 50:1776–1788. [PubMed: 19417221]
- [21]. Esquenazi E, Yang Y-L, Watrous J, Gerwick WH, Dorrestein PC. Imaging mass spectrometry of natural products. *Nat. Product Res.* 2009; 26:1521–1534.
- [22]. Esquenazi E, Coates C, Simmons L, Gonzalez D, et al. Visualizing the spatial distribution of secondary metabolites produced by marine cyanobacteria and sponges via MALDITOF imaging. *Mol. Biosyst.* 2008; 4:562–570. [PubMed: 18493654]
- [23]. Bastiaens PLH, Pepperkok R. Observing proteins in their natural habitat: the living cell. *Trends Biochem. Sci.* 2000; 25:631–637. [PubMed: 11116191]
- [24]. McDonnell LA, Heeren RMA. Imaging mass spectrometry. *Mass Spectrom. Rev.* 2007; 26:606–643. [PubMed: 17471576]
- [25]. Phizicky E, Bastiaens PI, Zhu H, Snyder M, Fields S. Protein analysis on a proteomic scale. *Nature.* 2003; 422:208–215. [PubMed: 12634794]
- [26]. Ciaccio MF, Wagner JP, Chuu C-P, Lauffenburger DA, Jones RB. Systems analysis of EGF receptor signaling dynamics with microwestern arrays. *Nat. Methods.* 2010; 7:148–155. [PubMed: 20101245]
- [27]. Crompton PD, Kayala MA, Traore B, Kayentao K, et al. A prospective analysis of the Ab response to *Plasmodium falciparum* before and after a malaria season by protein microarray. *Proc. Natl. Acad. Sci.* 2010; 107:6958–6963. [PubMed: 20351286]
- [28]. Lee MV, Topper SE, Hubler SL, Hose J, et al. A dynamic model of proteome changes reveals new roles for transcript alteration in yeast. *Mole. Syst. Biol.* 2011; 7:1–12.
- [29]. Attia AS, Schroeder KA, Seeley EH, Wilson KJ, et al. Monitoring the inflammatory response to infection through the integration of MALDI IMS and MRI. *Cell Host & Microbe.* 2012; 11:664–673. [PubMed: 22704626]
- [30]. Corbin BD, Seeley EH, Raab A, Feldmann J, et al. Metal chelation and inhibition of bacterial growth in tissue abscesses. *Science.* 2008; 319:962–965. [PubMed: 18276893]

- [31]. Hood MI, Mortensen BL, Moore JL, Zhang Y, et al. Identification of an *Acinetobacter baumannii* zinc acquisition system that facilitates resistance to calprotectin-mediated zinc sequestration. *PLOS Pathogens*. 2012; 8:e1003068. [PubMed: 23236280]
- [32]. Edgeworth J, Freemont P, Hogg N. Ionomycin-regulated phosphorylation of the myeloid calcium-binding protein p14. *Nature*. 1989; 342:189–192. [PubMed: 2478889]
- [33]. Steinbakk M, Naess-Andresen C-F, Fagerhol MK, Lingaas E, et al. Antimicrobial actions of calcium binding leucocyte L1 protein, calprotectin. *Lancet*. 1990; 336:763–765. [PubMed: 1976144]
- [34]. Damo S, Chazin WJ, Skaar EP, Kehl-Fie TE. Inhibition of bacterial superoxide defense: a new front in the struggle between host and pathogen. *Virulence*. 2012; 3:325–328. [PubMed: 22546907]
- [35]. Loomans HJ, Hahn BL, Li QQ, Phadnis SH, Sohnle PG. Histidine-based zinc-binding sequences and the antimicrobial activity of calprotectin. *J. Infect. Dis*. 1998; 177:812–814. [PubMed: 9498472]
- [36]. Kehl-Fie TE, Skaar EP. Nutritional immunity beyond iron: a role for manganese and zinc. *Curr. Opin. Chem. Biol*. 2010; 14:218–224. [PubMed: 20015678]
- [37]. Damo SM, Kehl-Fie TE, Sugitani N, Holt ME, et al. Molecular basis for manganese sequestration by calprotectin and roles in the innate immune response to invading bacterial pathogens. *Proc. Nat. Acad. Sci*. 2013; 110:3841–3846. [PubMed: 23431180]
- [38]. Sohnle PG, Hunter MJ, Hahn B, Chazin WJ. Zinc-reversible antimicrobial activity of recombinant calprotectin (migration inhibitory factor—related proteins 8 and 14). *J. Infect. Dis*. 2000; 182:1272–1275. [PubMed: 10979933]
- [39]. Kehl-Fie TE, Chitayat S, Hood MI, Damo S, et al. Nutrient metal sequestration by calprotectin inhibits bacterial superoxide defense, enhancing neutrophil killing of *Staphylococcus aureus*. *Cell Host & Microbe*. 2011; 10:158–164. [PubMed: 21843872]
- [40]. Giamarellou H, Antoniadou A, Kanellakopoulou K. *Acinetobacter baumannii*: a universal threat to public health? *Int. J. Antimicro. Agents*. 2008; 32:106–119.
- [41]. Müller-Redetzky H, Suttrop N, Witzernath M. Experimental models of pneumonia-induced sepsis. *Drug Discovery Today: Dis. Models*. 2012; 9:e23–e32.
- [42]. Peleg AY, Seifert H, Paterson DL. *Acinetobacter baumannii*: emergence of a successful pathogen. *Clin. Microbiol. Rev*. 2008; 21:538–582. [PubMed: 18625687]
- [43]. Vincent JL, Rello J, Marshall J, Silva E, et al. International study of the prevalence and outcomes of infection in intensive care. *J. Am. Med. Assoc*. 2009; 302:2323–2329.
- [44]. Rice LB. Challenges in identifying new antimicrobial agents effective for treating infection with *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *Clin. Infect. Dis*. 2006; 43(Suppl 2):S100–S105. [PubMed: 16894511]
- [45]. Jean SS, Hsueh PR, Lee WS, Chang HT, et al. Nationwide surveillance of antimicrobial resistance among nonfermentative Gram-negative bacteria in intensive care units in Taiwan: SMART Programme data 2005. *Int. J. Antimicrob. Agents*. 2009; 33:266–271. [PubMed: 19091522]
- [46]. Werarak P, Waiwarawut J, Tharavichitkul P, Pothirat C, et al. *Acinetobacter baumannii* nosocomial pneumonia in tertiary care hospitals in Thailand. *J. Med. Assoc. Thailand*. 2012; 95(Suppl 2):S23–S33.
- [47]. Lee YT, Kuo SC, Yang SP, Ling YT, Chiang DH, Tseng FC, et al. Bacteremic Nosocomial Pneumonia Caused by *Acinetobacter baumannii* and *Acinetobacter nosocomialis*: a single or two distinct clinical entities? *Clin. Microbiol. Infect*. 2013; 19:640–645.
- [48]. Ketani, L.; Busby, H. Clinically Oriented Pulmonary Imaging. Kanne, JP., editor. Humana Press; New York: 2012. p. 41-60.
- [49]. Faassen, H. v.; KuoLee, R.; Harris, G.; Zhao, X., et al. Neutrophils play an important role in host resistance to respiratory infection with *Acinetobacter baumannii* in mice. *Infect. Immunol*. 2007; 75:5597–5608. [PubMed: 17908807]
- [50]. Jacobs AC, Hood MI, Boyd KL, Olsen PD, et al. Inactivation of phospholipase D diminishes *Acinetobacter baumannii* pathogenesis. *Infect. Immunol*. 2010; 78:1952–1962. [PubMed: 20194595]

- [51]. Chen GY, Nuñez G. Sterile inflammation: sensing and reacting to damage. *Nat. Rev. Immunol.* 2010; 10:826–837. [PubMed: 21088683]
- [52]. Striz I, Trebichavsky I. Calprotectin—a pleiotropic molecule in acute and chronic inflammation. *Physiol. Res.* 2004; 53:245–253. [PubMed: 15209531]
- [53]. Jakubovics NS, Jenkinson HF. Out of the iron age: new insights into the critical role of manganese homeostasis in bacteria. *Microbiology.* 2001; 147:1709–1718. [PubMed: 11429449]

Figure 1.

Levels of calprotectin in the lung increase as the bacterial challenge is increased. Seven-week-old mice were intranasally infected with varying doses of *A. baumannii*. (A) hematoxylin and eosin (H&E) stained serial sections of lung, (B) 40 $\times$ magnification of areas with inflammation, (C) ion intensity map of m/z 10 165, S100A8, collected at 300 μ m spatial resolution, (D) ion intensity map of m/z 12 999, sodiated S100A9, collected at 300 μ m spatial resolution, (E) ion intensity map of m/z 14 050, histone H2A.1, (F) bacterial burden in the lungs of mice at each inoculum. The dashed line represents the detection limit. * $p < 0.05$ by ANOVA.

Figure 2.

A. baumannii is rapidly cleared from sites of infection. Seven-week-old mice were intranasally infected with 10^8 CFUs *A. baumannii* and monitored over time in hours post infection (hpi) for (A) weight change. Values from 6 to 216 hpi were statistically decreased ($p < 0.05$) from uninfected animals by two-way ANOVA. (B) Bacterial burden in the lung, $n = 5$. Values from 0 to 72 hpi were statistically different ($p < 0.05$) from uninfected animals by Student's t test. (C) Bacterial dissemination to the liver, $n = 5$. Values from 18 to 36 hpi were statistically different ($p < 0.05$) from uninfected animals by Student's t test.

Figure 3.

Calprotectin is recruited to sites of infection and signal decreases following bacterial clearance. Columns, left to right: (A) H&E stained serial sections of lung, (B) 40× magnification of areas with inflammation, (C) ion intensity map of m/z 10 165, S100A8 collected at 300 μm spatial resolution, (D) ion intensity map of m/z 12 999, sodiated S100A9 collected at 300 μm spatial resolution, and (E) ion intensity map of m/z 14 050, histone H2A.1 collected at 300 μm spatial resolution.

Figure 4.

Histological comparison of atelectasis in inflamed lungs versus uninfected at 96 hpi. Lungs from 96 h time point. From left, PBS mock-infected and *A. baumannii* infected. (A) Ion intensity map of m/z 10 165, S100A8 at 300 μm lateral resolution. (B) Ion intensity map of m/z 12 999, sodiated S100A9 at 300 μm lateral resolution. (C) H&E stained sections of lung. (D) Immunohistochemistry stain for neutrophils. (E) 10 $\times$ magnification of immunohistochemistry stain for neutrophils.

Figure 5. Identification of S100A8 and S100A9 from murine lung. Identification of S100A8 and S100A9: (A) Spectrum extracted from a single pixel of IMS data from the 48 hpi lung. (B) Spectra taken from selected fractions of HPLC fractionated homogenate, showing S100A8 (left) and S100A9 (right). (C) Sequence coverage for mouse S100A8 and S100A9 are shown. Underlined peptides were detected following recovery of the species of interest by offline HPLC fractionation, tryptic digestion, and subsequent LC-MS/MS analysis.