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Whole-cell MALDI-TOF MS: A new tool to assess the multifaceted activation of macrophages

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ABSTRACT

Whole-cell MALDI-TOF MS is routinely used to identify bacterial species in clinical samples. This technique has also proven to allow identification of intact mammalian cells, including macrophages. Here, we wondered whether this approach enabled the assessment human macrophages plasticity. The whole-cell MALDI-TOF spectra of macrophages stimulated with IFN-γ and IL-4, two inducers of M1 and M2 macrophage polarisation, consisted of peaks ranging from 2 to 12 kDa. The spectra of unstimulated and stimulated macrophages were clearly different. The fingerprints induced by the M1 agonists, IFN-γ, TNF, LPS and LPS+IFN-γ, and the M2 agonists, IL-4, TGF-β1 and IL-10, were specific and readily identifiable. Thus, whole-cell MALDI-TOF MS was able to characterise M1 and M2 macrophage subtypes. In addition, the fingerprints induced by extracellular (group B *Streptococcus*, *Staphylococcus aureus*) or intracellular (BCG, *Orientia tsutsugamushi*, *Coxiella burnetii*) bacteria were bacterium-specific. The whole-cell MALDI-TOF MS fingerprints therefore revealed the multifaceted activation of human macrophages. This approach opened a new avenue of studies to assess the immune response in the clinical setting, by monitoring the various activation patterns of immune cells in pathological conditions.

1. Introduction

MALDI-TOF is a MS technique that combines a soft, matrix-assisted, ionization process and a TOF analyzer to separate the generated ions. In MALDI-MS, the mixture of a biological sample with an energy-absorbing matrix allows the genesis of mostly intact-single-charged biomolecules. This property has promoted the technique as one of the most popular in biology and medicine to explore various proteomes.

MALDI-TOF has been extensively used in biology to search biomarkers and to monitor post-translational modifications [1–3]. These approaches usually require large amounts of material, and several steps of fractionation or separation, which are not compatible in the daily monitoring of clinical samples. Another successful application of MALDI-TOF is the identification of microorganisms in clinical samples [4], which is now used in routine in the clinical setting. The spectrum is used as a fingerprint of the bacterial species, without peak identification. Recently, it has been shown that MALDI-TOF MS may also be used to identify whole eukaryotic cells. Buchanan et al. showed that cultured pancreatic islet alpha and beta cells are easily discriminated by MALDI-TOF MS based on the fingerprint

Abbreviations: BCG, Bacille Calmette-Guérin; IFN-γ, Interferon-gamma; IL, Interleukin; LPS, Lipopolysaccharide; M-CSF, Macrophage Colony Stimulating Factor; TGF-β1, Tumor Growth Factor beta 1; TNF, Tumor Necrosis Factor.

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derived from the different hormone peptides produced by the cells [5]. MALDI-TOF MS has been employed to identify intact mammalian cells including lymphocytes, monocytes, polymorphonuclear cells and erythrocytes. In a similar way, different types of macrophages such as monocyte-derived-macrophages or macrophage cell-lines from different species were easily distinguished [6].

Tissue macrophages play a pivotal role in mounting an immune response to microbial pathogens. They sense infectious agents through receptors that bind conserved and ubiquitous microbial motifs, such as LPS and peptidoglycan and they produce microbicidal compounds [7]. Macrophages present microbial antigens to T cells, thus contributing to the development of adaptive immune response. T cells release cytokines that in turn activate macrophages and either reinforce or regulate their microbicidal activity. Such responses have been classified into M1 and M2 macrophage responses that are analogous to the model of Th1/Th2 lymphocyte polarisation [8–10]. Macrophages activated by the classical pathway (type I cytokines such as IFN-γ and TNF, or bacterial products, such as LPS) are considered M1 macrophages, which are inflammatory, microbicidal and tumouricidal [9,11–14]. Macrophages activated by alternative pathways (IL-4, IL-10, TGF-β1) are considered M2 macrophages, which are poorly microbicidal and tumouricidal and regulate inflammatory and immune responses [10,15,16]. Whole proteome studies have been made using gel-based separation systems and such approach have allowed the identification of a large number of proteins in the proteome, the secretome and in membranes from activated macrophages [17]. Brown et al. showed that 80% of proteins represent the core macrophage proteome, and 20% of proteins define the response-specific proteome. Among the latter proteins, it is possible to identify two unique M1-related signatures induced by either IFN-γ or LPS [18]. Using pulse stable isotope labeling of amino acids in cell culture, Kraft-Terry et al. identified a bioprofile in M-CSF-differentiated monocytes that is consistent with an M2 profile [19].

Due to their high degree of plasticity in response to their micro-environment, macrophages constitute a model of choice to assess the accuracy of whole-cell MALDI-TOF MS to detect subtle modifications. In the current study, we demonstrated that MALDI-TOF MS accuracy is sufficient to study the multifaceted activation of macrophages. When various M1 agonists (IFN-γ, TNF, LPS, LPS+IFN-γ) and M2 agonists (IL-4, TGF-β1, IL-10) were used to stimulate human macrophages, MALDI-TOF MS fingerprints revealed the M1 and M2 subtypes. When macrophages were activated by extracellular bacteria, specific signatures were obtained that were similar to those induced by IL-4. In contrast, intracellular bacteria induced signatures that did not fit with either of the M1 or M2 polarization profiles.

2. Materials and methods

2.1. Bacterial species

Staphylococcus aureus (CIP strain 7625) and group B *Streptococcus* (CIP strain 103227) were obtained from the Laboratory of Microbiology of the Hospital La Timone (Marseille). Briefly, *S. aureus* and group B *Streptococcus* were grown on sheep blood Colombia agar for 2 days, and the purity of the cultures was assessed as previously described [20]. The *Mycobacterium bovis* Bacille Calmette-Guerin (BCG) strain was provided by the Institute Pasteur (CIP strain 105050), and the bacteria were subcultured on Middlebrook 7H10 agar (Becton Dickinson, le Pont de Claix, France) for 2–3 weeks as previously described [21]. *Orientia tsutsugamushi*, strain Kato (CSUR R163), and *Coxiella burnetii*, strain Nine Mile (RSA 493), were cultured on mouse L929 cells in MEM containing 5% FCS and 2 mM L-glutamine as previously described [22]. The L929 cells were infected for approximately 7 days. Infected cells were sonicated and centrifuged at 300 ×*g* for 10 min to discard cellular debris. The supernatants were then centrifuged at higher speed and bacteria were collected. The collected bacteria were washed in PBS (pH 7.2) and stored at −80 °C. Bacterial concentrations were determined by indirect immunofluorescence and/or quantitative PCR using specific primers. The bacterial viability was assessed using the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Life Technologies, Saint Aubin, France). Heat-killed bacteria were obtained by incubating the bacteria at 95 °C for 1 h.

2.2. Activation of macrophages

Peripheral blood mononuclear cells were isolated from five buffy coats (Etablissement Français du Sang, Marseille) and three healthy blood donors by Ficoll density gradient as previously described [23]. Monocytes were obtained from mononuclear cells using magnetic beads coated with anti-CD14 Abs (Miltenyi Biotech, Paris, France) according to the manufacturer's instructions. This procedure resulted in more than 95% monocyte purity as assessed by flow cytometry, and monocyte viability was greater than 98% as determined by trypan blue exclusion. Monocytes (10^6 cells in 6-well plates) were incubated in 3 mL of RPMI 1640 containing 20 mM HEPES, 10% human serum AB^+, 2 mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin (Life Technologies) for 4 days, and FCS replaced human serum for an additional 3 days. The obtained cell population was identified as macrophages (more than 95% pure) by flow cytometry using CD68 expression as a positive marker of macrophages. Macrophages were stimulated with 20 ng/mL human recombinant IFN-γ (PeproTech, Neuilly-sur-Seine, France), IL-4 (AbCys, Paris, France), IL-10, TGF-β1 (R&D Systems, Lille, France) and TNF (Euromedex, Mundolsheim, France) for different periods of time. Macrophages were also stimulated with 1 μg/mL LPS from *Escherichia coli* (Sigma–Aldrich, Saint Quentin Fallavier, France) or heat-killed bacteria (50 bacteria per cell).

2.3. MALDI-TOF MS

Stimulated macrophages (10^6 cells) were centrifuged for 5 min at 300 ×*g*. The cell pellets were suspended in 10 μL of sterile PBS and frozen at −80 °C for 2 to 3 days. After thawing, 1 μL of the cell suspension was added to 1 μL of α-cyano-4-hydroxy-cynnamique acid matrix and the mixture was deposited onto the MALDI target using an AutoFlexII spectrometer and FlexControl software (Bruker Daltonics, Wissembourg, France) as previously described [6]. The ions that resulted from a 170 ns pulse ion extraction of the laser emitting at 337 nm were subjected to an electric field of 20 kV