AIX-MARSEILLE UNIVERSITE
FACULTE DE MÉDECINE DE MARSEILLE
ECOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE

T H È S E

Présentée et publiquement soutenue le 02 Décembre 2013

Par Richard OUEDRAOGO

LA SPECTROMETRIE DE MASSE :
APPLICATION A L'ETUDE DES CELLULES IMMUNITAIRES

Pour obtenir le grade de DOCTEUR d'AIX-MARSEILLE UNIVERSITE

SPECIALITE : MALADIES INFECTIEUSES

<u>Membres du Jury de la Thèse</u> :

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Professeur Jean-Louis MEGE	**Directeur de thèse**
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Et bien sûr à toi. Cela n'a pas été facile mais tu comprendras....

A vous tous mes chers amis.

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AVANT-PROPOS

La spectrométrie de masse MALDI-TOF est particulièrement adaptée à l'étude des macromolécules si bien qu'elle est devenue un outil couramment utilisée en biologie, surtout en protéomique (Reinders et al. 2004; Aebersold et Mann 2003; De Masi et al. 2013). Son usage résulte également du faible coût, hormis l'achat de l'appareillage, des analyses, de la rapidité de sa mise en œuvre et de la résolution des spectres obtenus (de l'ordre du dalton pour une protéine d'environ 10 kDa). Les biochimistes utilisent la spectrométrie de masse MALDI-TOF pour identifier des protéines isolées par électrophorèse sur gel (SDS-PAGE et électrophorèse sur gel à deux dimensions). Cette approche a été mise à profit par les biologistes cellulaires pour, par exemple, identifier la présence d'une hormone dans un lysat cellulaire ou le sécrétome d'un type cellulaire placé dans des conditions expérimentales bien définies (Ong et Mann 2005; Li et al. 2009; Daumas et al. 2013; Huang et al. 2013).

Les microbiologistes ont utilisé depuis quelques années la spectrométrie de masse MALDI-TOF pour identifier différentes espèces bactériennes (Carbonnelle et al. 2007; Seng, Drancourt, Gouriet, Scola, et al. 2009; Barbuddhe et al. 2008; Sogawa, Watanabe, et Nomura 2013). Il s'agissait pour eux non pas seulement d'identifier des protéines spécifiques de ces espèces mais d'utiliser des bactéries entières afin d'établir des profils de spectres, pouvant contenir plusieurs dizaines de pics, spécifiques de ces différentes espèces bactériennes. Cette approche sur bactéries entières présente un double intérêt, taxonomique et diagnostique. La spectrométrie de masse MALDI-TOF a permis en effet de ré-analyser la classification taxonomique des bactéries en confirmant ou en infirmant des données basées sur d'autres approches méthodologiques (Seng, Drancourt, Gouriet, Scola, et al. 2009; Sauer et Kliem 2010). Cette technique en pointe dans le domaine bactériologique permet ainsi une identification précise, rapide et sûre des espèces bactériennes. Déterminer la nature des agents pathogènes est souvent essentiel à l'adoption d'une gestion thérapeutique rapide et appropriée. A titre d'exemple, la spectrométrie de masse MALDI-TOF permet de différencier différentes souches de *Listeria* et même des variations clonales des souches de *L. monocytogenes* (Barbuddhe et al. 2008). Il est maintenant établi qu'une approche en spectrométrie de masse MALDI-TOF peut être utilisée pour l'analyse, l'identification, la caractérisation

et la classification de bactéries entières ou de microorganismes y compris les virus et les parasites (Parisi et al. 2008; Welker et Moore 2011; Wunschel et al. 2005; Krader et Emerson 2004; Pignone et al. 2006; Moura et al. 2008; Seng et al. 2010; Demirev et Fenselau 2008; Sauer et Kliem 2010; Böhme et al. 2010; Seng, Drancourt, Gouriet, La Scola, et al. 2009).

C'est dans ce contexte que mes travaux de thèse ont porté sur le développement la spectrométrie de masse MALDI-TOF appliquée à la recherche de signatures spécifiques pour l'identification et la caractérisation des cellules eucaryotes entières y compris celles impliquées dans les réponses immunitaires. Le présent mémoire sera divisé en trois parties.

Dans le premier article (Ouedraogo et al. PLoS One, 2010, 5:e13691), nous détaillons le principe de l'application de la spectrométrie de masse MALDI-TOF aux cellules eucaryotes intactes en général et des cellules immunitaires circulantes en particulier.

Dans le deuxième article (Ouedraogo et al. J. Proteomics, 2012, 75:5523-5532), nous montrons la capacité de cette nouvelle approche à caractériser différents états d'activation des macrophages humains *in vitro*.

Dans le chapitre publié dans la série Methods in Molecular Biology (Ouedraogo et al. 2013), nous avons détaillé les différentes étapes de notre méthode partant de la préparation des échantillons, de l'acquisition des spectres jusqu'à l'analyse des données. Nous avons reçu également une invitation à présenter notre approche en vidéo (Ouedraogo et al., JoVe, 2013).

INTRODUCTION

I - METHODES D'IDENTIFICATION DES CELLULES IMMUNES

Les méthodes d'identification de divers types des cellules de mammifères, en particulier des cellules de la réponse immune, sont essentielles pour la compréhension de leurs fonctions. On peut à l'heure actuelle les classer en deux grandes catégories.

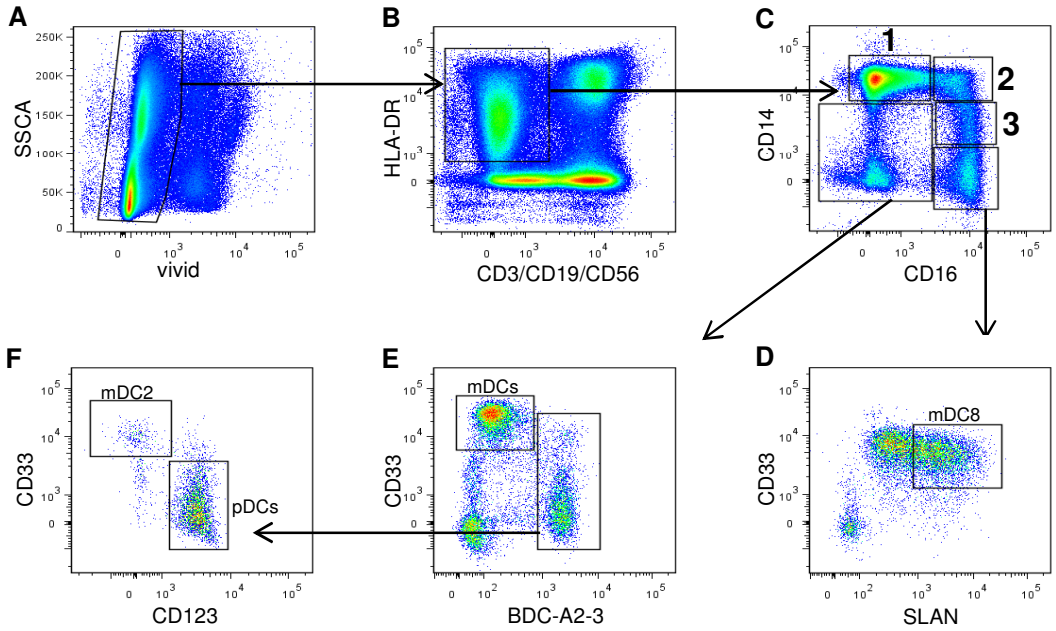
- Des méthodes basées sur les propriétés tinctoriales et cytochimiques des cellules

Connues depuis une centaine d'années, elles permettent d'identifier facilement les cellules circulantes telles que les lymphocytes, les monocytes et les neutrophiles. Elles ne permettent cependant pas de reconnaître les différents types de lymphocytes, les sous-populations monocytaires ou des populations cellulaires faiblement représentées telles que les cellules *natural killer* (NK) ou les cellules dendritiques (DCs). A fortiori, ces méthodes ne permettent pas d'isoler les différents types de cellules circulantes et donc d'évaluer l'état fonctionnel de ces cellules.

- La cytométrie en flux

Apparue dans son usage courant il y a une trentaine d'années, la cytométrie en flux est une technologie qui permet l'analyse sur cellules individuelles en suspension (Vignali 2000; Virgo et Gibbs 2012) de multiples paramètres tels que des caractéristiques physiques intrinsèques (propriété de diffusion de la lumière), ce qui permet d'apprécier leur taille et leur granularité. Elle permet également de détecter la fluorescence associée à ces cellules lorsqu'elles sont rendues fluorescentes (Recktenwald 1993; Brown et Wittwer 2000; Virgo et Gibbs 2012). C'est ainsi que l'on peut étudier différents paramètres en utilisant des sondes fluorescentes (analyse du cycle cellulaire, du pH intracellulaire, expression de la *green fluorescent protein* (GFP) (Ibrahim et Engh 2007). L'usage le plus courant de la cytométrie en flux consiste à utiliser des anticorps fluorescents dirigés contre des structures membranaires caractéristiques d'un type cellulaire donné : la sous-population cellulaire fluorescente peut alors être étudiée voire isolée par immunophénotypage (K Holmes et al. 2001). Il est maintenant possible de marquer en un seul prélèvement différents types de cellules circulantes grâce à l'utilisation d'un cocktail de douze anticorps spécifiques de chacune de ces populations cellulaires (Autissier et al. 2010; Virgo et Gibbs 2012). Malgré sa puissance analytique, la cytométrie en flux ne peut être utilisée qu'à la condition expresse de la disponibilité de plusieurs anticorps spécifiques. Autrement dit, il n'existe pas toujours de marqueur

Figure 1. Caractérisation des cellules myéloïdes circulantes par cytométrie en flux



d'après Ka M. (avec son autorisation)

Les leucocytes circulants vivants sont caractérisés par leur taille (A). Les cellules mononucléées qui expriment HLA-DR mais ni CD3, CD19 et CD56 (exclusion des lymphocytes T $CD3^+$, des lymphocytes B et des cellules NK) (B) sont analysées selon l'expression de CD14 et de CD16. Trois sous-populations de monocytes (1, 2, 3) sont observées (C). La sélection des cellules exprimant fortement CD16 mais pas CD14 permet d'observer la population des cellules dendritiques myéloïdes mDC8 (D). La sélection des cellules qui n'expriment ni CD14 ni CD16 permet de caractériser d'autres sous-populations de cellules dendritiques myéloïdes (E, F) et les cellules dendritiques plasmacytoïdes (F).

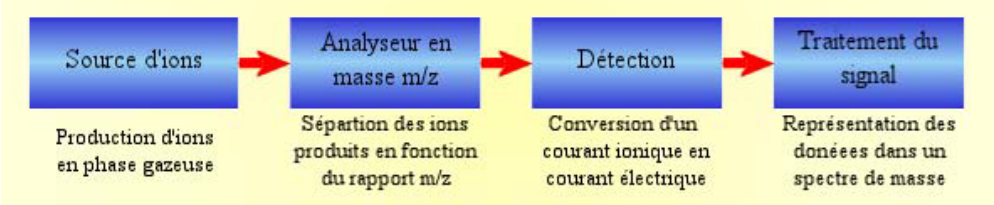
spécifique d'une population cellulaire donnée. Une combinatoire de marqueurs ou de niveau d'expression de ces marqueurs permet parfois de définir différentes sous-populations cellulaires : c'est le cas des monocytes, des cellules dendritiques et des lymphocytes circulants (**Figure 1**) mais rien ne dit que de nouveaux anticorps ou une autre combinatoire ne permettraient pas d'identifier de nouvelles sous-populations cellulaires. L'identification d'une nouvelle population cellulaire peut passer également par l'utilisation d'anticorps spécifiques de marqueurs de membrane et de marqueurs intracellulaires, ce qui nécessite des étapes supplémentaires de perméabilisation des cellules. C'est ainsi que l'identification de certaines cellules T régulatrices nécessite l'utilisation combinée de marqueurs membranaires (CD4 et CD25) et intracellulaires (Foxp3) (Fontenot, Gavin, et Rudensky 2003). Lorsque les marqueurs sont des molécules inductibles sécrétées (souvent des cytokines), il est nécessaire d'inclure dans le protocole expérimental des inhibiteurs de l'exportation afin d'augmenter la concentration intracellulaire dudit marqueur et sa détection. Ces approches, plus difficiles à mettre en œuvre, sont habituellement réservées à des protocoles de recherche.

II - LA SPECTROMETRIE DE MASSE, UNE NOUVELLE METHODE D'IDENTIFICATION DES CELLULES EUCARYOTES ?

La faible résolution des méthodes basées sur les propriétés tinctoriales des cellules et les contraintes associées à la cytométrie en flux, en particulier la nécessité de disposer de marqueurs fluorescents spécifiques, soulignent la nécessité de chercher de nouvelles méthodes d'identification et de caractérisation des cellules.

La spectrométrie de masse nous a semblé à cet égard prometteuse. C'est une méthode utilisée depuis plusieurs décennies pour détecter et identifier un très grand nombre de molécules (Link et al. 1999) par mesure de leur masse, ce qui en fait un outil universel qui s'applique aussi bien en astrophysique, en chimie, en médecine et en biologie (de Hoffmann 2000; Aebersold et Mann 2003). Elle permet également de caractériser les structures protéiques impliquées dans les interactions protéine-protéine (Sinz 2006). En ce qui concerne la biologie cellulaire, une telle approche nécessite d'analyser des lysats cellulaires peu compatibles avec une identification rapide des

Figure 2. Structure d'un spectromètre de masse



d'après (www.wikipedia.org)

cellules d'origine. Ces dernières années la spectrométrie de masse *Matrix-assisted laser desorption/ionozation time-of-flight* (MALDI-TOF) a été utilisée pour identifier différents types bactériens grâce à une approche sur bactéries entières (Wunschel et al. 2005; Welker et Moore 2011; Salvador et al. 2013). Il nous a alors semblé légitime d'utiliser une approche analogue afin de savoir si la spectrométrie de masse MALDI-TOF pourrait permettre d'identifier différents types de cellules eucaryotes.

III - LA SPECTROMETRIE DE MASSE

III - 1 - Structure générale d'un spectromètre de masse

Le spectromètre de masse comporte un système d'introduction de l'échantillon, une source d'ionisation, un ou plusieurs analyseurs qui séparent les ions produits selon leur rapport masse/charge (*m/z*), un détecteur qui comptabilise les espèces ionisées et, enfin, un système informatique pour traiter les signaux engendrés (Jurinke, Oeth, et Boom 2004; Mann, Hendrickson, et Pandey 2001) (**Figure 2**). Le résultat obtenu est un spectre de masse représentant les rapports *m/z*, où *m* représente la masse et *z* la valence (ou *m/q*, *q* représentant la charge) selon l'axe des abscisses et l'abondance relative de ces ions selon l'axe des ordonnées (Mann, Hendrickson, et Pandey 2001).

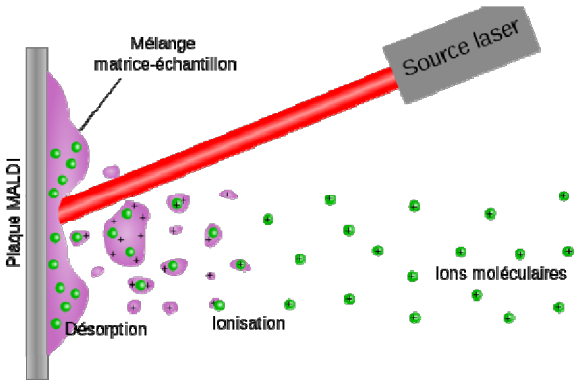
- Introduction de l'échantillon

L'échantillon peut être introduit directement dans la source, sous forme gazeuse, liquide (infusion directe), solide (canne d'introduction directe, dépôt sur la plaque MALDI etc...) ou par association avec une méthode séparative (chromatographie en phase liquide, chromatographie en phase gazeuse, électrophorèse capillaire etc...).

- Source d'ionisation

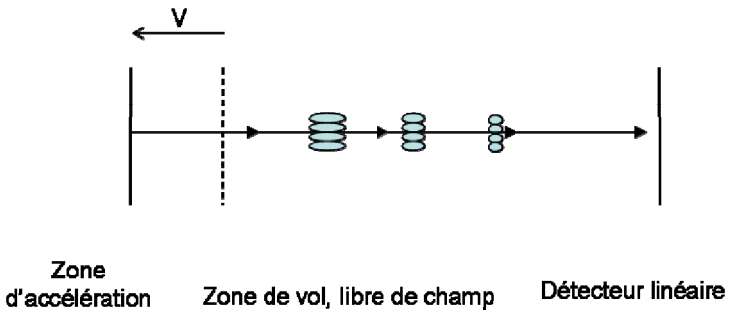
La source d'ionisation consiste à vaporiser les molécules et à les ioniser. Une source d'ionisation peut être utilisée soit en mode positif pour étudier les ions positifs, soit en mode négatif pour étudier les ions négatifs. Plusieurs types de sources existent et sont utilisés en fonction du résultat recherché et des molécules analysées. En effet, plusieurs types d'ionisation existent : l'ionisation électronique (*electron ionisation* : ESI),

Figure 3. Source d'ionisation MALDI



d'après (www.wikipedia.org)

Figure 4. Trajectoire d'un ion dans l'analyseur à temps de vol en mode linéaire



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l'ionisation chimique (*chemical ionization* : CI), l'ionisation par bombardement d'atomes rapides (*ionisation by fast atom bombardment* : FAB), l'ionisation par électronébulisation (*electrospray ionization*: ESI), l'ionisation chimique à pression atmosphérique (*atmospheric pressure chemical ionization* : APCI) et la désorption-ionisation par laser assistée par une matrice (*matrix assisted laser desorption ionization* : MALDI) (Ahmed 2008) (**Figure 3**). Les approches SELDI (*Surface Enhanced Laser Desorption Ionisation)* ou DIOS (*Desorption/ionization on silicon)* peuvent être directement couplées avec la méthode de détection par spectrométrie de masse en mode MALDI-TOF (Lewis et al. 2003; von Eggeling et al. 2001; Ahmed 2008).

- Analyseur

L'analyseur sépare les ions en fonction de leur rapport *m/z*. Il existe des analyseurs basse résolution : le quadripôle ou quadrupôle et le piège à ions 3D ou linéaire. Il existe également des analyseurs haute résolution, permettant de mesurer la masse exacte des analytes : le secteur magnétique couplé à un secteur électrique, le temps de vol (TOF) (**Figure 4**), la résonance cyclotronique ionique à transformée de Fourier (FT-ICR) et l'Orbitrap. Ces analyseurs peuvent être couplés entre eux pour réaliser des expériences de spectrométrie de masse en tandem. En général, un premier analyseur sépare les ions, une cellule de collision permet de fragmenter les ions et un second analyseur sépare les ions fragmentés. Certains analyseurs, comme les pièges à ions ou le FT-ICR, constituent plusieurs analyseurs en un et permettent de fragmenter les ions et d'analyser les fragments directement (Ahmed 2008).

- Détecteur

Le détecteur transforme les ions en signal électrique. Le signal électrique est d'autant plus important que les ions sont nombreux. L'amplification du signal permet son traitement par le système informatique du spectromètre (Ahmed 2008).

III - 2 - Le MALDI-TOF

Dans sa version MALDI, la spectrométrie de masse utilise un faisceau laser (laser à l'azote) pour désorber et ioniser de façon douce un mélange matrice/échantillon cocristallisé sur une surface métallique, appelée la cible. Cette désorption-ionisation sur

matrice permet l'analyse de biomolécules (comme les protéines, les peptides, les acides nucléiques et les sucres)**,** de grosses molécules organiques (comme les polymères, les dendrimères et autres macromolécules) qui tendent à devenir fragiles et à se fragmenter lorsqu'elles sont ionisées par des méthodes plus conventionnelles (Jurinke, Oeth, et Boom 2004; Li et al. 2009; Mellmann et al. 2008). En pratique, les molécules de la matrice absorbent l'énergie transmise par le laser et se transforment en ions. Cette ionisation de la matrice transfère des charges positives aux molécules de l'échantillon et provoque ainsi leur ionisation, leur dissociation et leur passage en phase gazeuse. La matrice sert aussi de protection de l'échantillon contre l'énergie disruptive du laser. L'ionisation de l'échantillon conduit à la formation d'ions essentiellement monochargés ($z = 1$). Il est à signaler qu'il existe plusieurs types de matrices composées de molécules cristallisées telles l'acide 3,5-diméthoxy-4-hydroxycinnamique (acide sinapique/acide sinapinique), l'acide α-cyano-4-hydroxycinnamique (alpha-cyano ou alpha-matrice) et l'acide 2,5-dihydroxybenzoïque : le choix de chacune d'elles est essentiellement empirique (Meyer et Ueland 2011).

L'analyseur à temps de vol (TOF) consiste à mesurer le temps que met une espèce moléculaire ionisée à parcourir une distance donnée lorsqu'elle est soumise à un champ électrique. Ce type d'analyseur se compose d'une zone d'accélération des espèces moléculaires ionisées (où s'applique la tension accélératrice) et d'une zone appelée tube de vol, libre de tout champ électrique. De ce fait, la séparation des différentes espèces moléculaires ionisées ne dépend ainsi que de la vitesse acquise lors de la phase d'accélération. Les espèces moléculaires ionisées ayant le plus petit rapport *m/z* parviendront en premier au détecteur et les espèces de plus haut rapport *m/z* leur succèderont. Les différents rapports *m/z* et l'intensité relative de ces signaux sont directement mesurés et intégrés dans un spectre en mode linéaire (Boom, Wjst, et Everts 2013).

EXPOSE DES TRAVAUX

ARTICLE 1

Global Analysis of Circulating Immune Cells by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

Richard Ouedraogo, Christophe Flaudrops, Amira Ben Amara, Christian Capo, Didier Raoult, Jean-Louis Mege

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Il a été montré ces dernières années que la spectrométrie de masse MALDI-TOF permet d'identifier différentes espèces bactériennes intactes, notamment celles présentant un intérêt médical (Welker et Moore 2011; Pignone et al. 2006; Moura et al. 2008; Seng et al. 2010). Fort de ce constat, je me suis demandé si une approche similaire pouvait permettre d'identifier différents types de cellules eucaryotes. En effet, les techniques actuelles d'identification des cellules par exemple humaines souffrent d'un certain nombre de défauts. La cytométrie en flux est une technique qui analyse en quelques minutes la fluorescence de cellules individuelles au sein de grands échantillons cellulaires. Sa principale contrainte consiste en ce qu'il est nécessaire de disposer d'anticorps spécifiques que l'on rend fluorescents après marquage direct ou indirect. On peut ainsi détecter au sein d'une population cellulaire donnée la présence de tel ou tel type cellulaire à la condition expresse qu'il existe un marqueur isolé qui soit spécifique d'une population cellulaire donnée. La caractérisation de différentes sous-populations lymphocytaires nécessite alors l'utilisation de différents marqueurs spécifiques de chacune d'entre elles. Dans ce contexte, des cytomètres multi-couleur (Holmes et al. 2001; Baumgarth et Roederer 2000; Nieto et al. 2009) sont indispensables puisqu'il faut associer chaque marqueur à un fluorochrome donné. L'approche en cytométrie en flux connaît un degré de complexité supplémentaire quand les marqueurs sont des moléculaires intracellulaires, ce qui nécessite une perméabilisation préalable des cellules (Kraan et al. 2003). Lorsque ces marqueurs sont des molécules inductibles

susceptibles d'être sécrétées (souvent des cytokines), il est nécessaire d'inclure dans le schéma expérimental des inhibiteurs de l'exocytose afin d'augmenter la concentration intracellulaire de ces marqueurs afin de permettre leur détection. Cette approche, délicate à mettre en œuvre, est habituellement réservée aux protocoles de recherche. En d'autres termes, l'identification d'un grand nombre de types cellulaires en cytométrie en flux est malaisée, voire impossible, hormis quelques cas bien particuliers. En outre, malgré une commercialisation ancienne (Pinkel 1982), cette technique demeure onéreuse, lourde à mettre en place, demande un personnel spécialisé et reste restreinte à un petit nombre de laboratoires spécialisés.

J'ai donc tenté d'identifier un certain nombre de cellules eucaryotes en spectrométrie de masse MALDI-TOF sur cellules entières selon une analyse combinatoire des pics obtenus et non pas après identification moléculaire de pics sensés être spécifiques de chaque type cellulaire. J'ai ainsi analysé la signature de 18 types cellulaires mammifères et 4 types d'amibes et j'ai montré une très forte divergence entre les cellules mammifères et les amibes. Au sein des cellules mammifères, j'ai utilisé des cellules primaires humaines (polynucléaires neutrophiles, monocytes, lymphocytes T, globules rouges, trophoblastes, DCs obtenues après culture de monocytes, macrophages dérivés de monocytes), des cellules primaires murines (macrophages dérivés de moelle osseuse) et des lignées cellulaires telles que les cellules J774, THP-1 et DH82, qui sont des lignées macrophagiques, JEG et BeWo, des lignées trophoblastiques, C8166 qui sont des lymphoblastes cancéreux, L929 qui sont fibroblastes murins, HeLa et 293T qui sont des cellules épithéliales et, enfin, XTC-2, une lignée de cellules d'œuf de xénope.

J'ai montré que les cellules mammifères sont comprises dans un cluster clairement séparé de celui constitué par les amibes et, de façon quelque peu surprenante, par les hématies. Les cellules du sang circulant, à l'exception des hématies, sont relativement proches les unes des autres. Les DCs et les macrophages humains, qui dérivent des monocytes sont aussi très distincts. En outre, les cellules des lignées placentaires BeWo et JEG sont proches les unes des autres mais sont largement séparées des trophoblastes primaires. Les deux lignées épithéliales sont elles aussi très proches l'une de l'autre.

J'ai ensuite utilisé un score qui permet d'identifier avec certitude ou vraisemblance une population cellulaire à partir d'une base de données créée pour la circonstance. J'ai pour ce faire purifié des monocytes et des lymphocytes à partir des cellules mononucléées du sang périphérique (PBMCs) : j'ai ainsi retrouvé les deux

signatures, monocytaire et lymphocytaire, au sein des PBMCs, ce qui montre le pouvoir discriminant d'une approche en spectrométrie de masse MALDI-TOF. J'ai enfin adapté un programme dédié à l'étude des transcriptomes, le programme MeV (http://www.tm4.org/mev/), pour comparer les signatures spécifiques fournies par les données de la spectrométrie de masse MALDI-TOF. Ce programme a permis de comparer les spectres des monocytes, des lymphocytes T, des PMNs et des hématies (RBCs). Nous avons ainsi montré que ces quatre types cellulaires donnent des signatures spécifiques pouvant être considérées comme des bio-marqueurs. Il est à noter que les cellules nucléées partagent nombre de pics à la différence des hématies.

En résumé, j'ai montré pour la première fois que l'approche en spectrométrie de masse MALDI-TOF sur bactéries entières peut s'appliquer aux cellules eucaryotes puisqu'elle permet d'obtenir des spectres spécifiques et reproductibles pour chacun des types cellulaires testés.

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Global Analysis of Circulating Immune Cells by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry

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Abstract

Background: MALDI-TOF mass spectrometry is currently used in microbiological diagnosis to characterize bacterial populations. Our aim was to determine whether this technique could be applied to intact eukaryotic cells, and in particular, to cells involved in the immune response.

Methodology/Principal Findings: A comparison of frozen monocytes, T lymphocytes and polymorphonuclear leukocytes revealed specific peak profiles. We also found that twenty cell types had specific profiles, permitting the establishment of a cell database. The circulating immune cells, namely monocytes, T lymphocytes and polymorphonuclear cells, were distinct from tissue immune cells such as monocyte-derived macrophages and dendritic cells. In addition, MALDI-TOF mass spectrometry was valuable to easily identify the signatures of monocytes and T lymphocytes in peripheral mononuclear cells.

Conclusions/Significance: This method was rapid and easy to perform, and unlike flow cytometry, it did not require any additional components such as specific antibodies. The MALDI-TOF mass spectrometry approach could be extended to analyze the cell composition of tissues and the activation state of immune cells.

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Introduction

Immune cells are characterized by specific morphologies and functions, which can be used to identify different immune cell types. This is illustrated by the use of flow cytometry to identify immune cell populations based on the recognition of increasing numbers of membrane antigens by specific antibodies. This method has been widely applied in the fields of immunology and hematology. The development of systems biology approaches (such as transcriptomics) has enabled cell subsets to be identified through their characteristic transcriptional signatures. For example, it has been recently reported that circulating lymphocytes and polymorphonuclear cells (PMNs) exhibit gene expression signatures reflecting the enrichment of genes encoding specific surface proteins that can be used as biomarkers for estimating the abundance of these cell types within complex tissues [1]. This approach enables discrimination between cells in the same lineage but at different stages and between cells that have differentiated, such as the differentiation of human monocytes into macrophages or dendritic cells (DCs) [2]. However, changes in mRNA levels do not necessarily reflect the altered expression of proteins [3]. A proteomic approach that analyzes signatures based on protein expression would provide a robust method with power similar to that of the transcriptomic approach.

Mass spectrometry (MS) is a key tool in cell proteomics [4–6]. This technique, based on mass determination [7], is currently used to identify proteins, their amino-acid sequences and their post-translational modifications [8,9]. This method can also be used for the identification and sequencing of DNA, RNA and sugars [9,10]. MALDI-TOF (matrix-associated laser desorption ionization/time of flight) MS is used to identify unknown protein or peptide sequences in fractionated cells [9]. Coupled with two-dimensional gels, MALDI-TOF MS can be used to create proteomic maps of cell types such as macrophages [4] and of intracellular compartments [11]. MALDI-TOF MS has been recently introduced into microbiology laboratories to identify [12,13] and classify bacterial species using intact bacteria [14,15]. In 2008 a large number of bacterial species present in clinical specimens were identified using databases established from isolated species [16,17]. In 2006, MALDI-TOF MS has been applied to mammalian cells from three cell lines after lysis in 2,5-dihydroxybenzoic acid matrix solution. In these conditions, it has been possible to discriminate the different mammalian lines [18]. Recently, MALDI-TOF MS has been applied to eukaryotic cell

lines to provide rapid characterization of cultured cells. However, the method used to analyze these cultured cells involved two steps of ethanol inactivation and formic acid/acetonitrile extraction [19]. To our knowledge, MALDI-TOF MS has not yet been directly applied to intact eukaryotic cells.

Our objective was to determine whether intact immune cells exhibited reproducible and specific signatures in MALDI-TOF MS. We found that this approach was useful for discriminating between immune cells. For example, circulating T lymphocytes, monocytes and PMNs as well as monocyte-derived macrophages and DCs all exhibited distinct spectra. We describe the first elements of a database that will be useful for studying cell subsets in tissues and possibly their activation state.

Methods

Ethics Statement

Healthy human placentas were collected after informed and written consent obtained from each subject, and the study was approved by the Ethics Committee of the Université de la Méditerranée, Marseille, France.

Human primary cells

Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated from leukopacks (Etablissement Français du Sang) by Ficoll gradient (MSL, Eurobio) and suspended in RPMI 1640 containing 20 mM HEPES (Invitrogen), as previously described [20]. Monocytes and T lymphocytes were isolated using CD14 and CD3 MicroBeads, respectively, and the MACS separation system (Miltenyi Biotec) according to the manufacturer's protocol. Monocytes were cultured for seven days in RPMI 1640 containing 10% human AB serum, 2 mM L-glutamine, 100 UI/mL penicillin and 100 µg/mL streptomycin to obtain monocyte-derived macrophages (MDMs), as previously described [21]. More than 90% of cells were macrophages as assessed by flow cytometry using CD68 as a specific marker. To obtain dendritic cells (DCs), monocytes were treated with 1,000 U/ml of human recombinant granulocyte macrophage-colony stimulating factor (Peprotech Inc.) and 500 U/ml of human recombinant interleukin 4 (Tebu-Bio) in RPMI 1640 containing 10% fetal calf serum (FCS), L-glutamine and antibiotics for seven days. The cells obtained expressed high levels of CD11c and CD1a, and low levels of CD14 and CD68. PMNs obtained after Ficoll gradient were prepared by sedimentation of red blood cells (RBCs) with 1.5% (w/v) dextran T500 (Pharmacosmos), as previously described [22]. Cell supernatants were centrifuged at 700× *g*, and a hypotonic shock of 30 s was applied to cell pellets to remove contaminating RBCs; more than 98% of the cells were PMNs. RBCs were diluted and suspended in RPMI 1640 containing 20 mM HEPES before use.

Human placentas were collected, and small pieces were rinsed several times and digested in Hank's balanced salt solution containing DNase I (Sigma-Aldrich, 300 units/mL) and 2.5% trypsin (Invitrogen) for 2×45 min, as previously described [23]. Isolated cells were filtered and centrifuged at 1,200× *g* on 25–60% Percoll gradients (GE Healthcare). Cells present at the interface were subjected to positive selection using MicroBeads coupled with rat anti-mouse IgG2 antibodies (Miltenyi Biotec) and mouse antibodies directed against epidermal growth factor receptor (Santa Cruz Biotechnology), a specific marker of trophoblasts. Primary trophoblasts were cultured in Dulbecco's Minimum Eagle's Medium (DMEM)-F12-Ham containing 10% FCS and antibiotics.

Mouse primary cells

Bone marrow-derived macrophages (BMDMs) were generated from six- to eight-week-old C57BL/6 mice killed by cervical dislocation, as previously described [24]. In brief, the bone marrow was flushed out from femurs and tibias in DMEM supplemented with 10% FCS, 2 mM L-glutamine and antibiotics. Cells were cultured in DMEM supplemented with 10% FCS, L-glutamine, antibiotics and 15% L929 cell supernatant rich in granulocyte macrophage-colony stimulating factor for seven days.

Cell lines

The human monocytic leukemia cell line THP1 (ATCC N° TIB-202) and the murine J774 (ATCC N° TIB-67) and canine DH82 (ATCC N° CRL-10389) macrophage cell lines were cultured in RPMI 1640 containing 10% FCS, L-glutamine and antibiotics. The human T cell leukemia cell line C8166 that stably expresses the CCR5 chemokine receptor [25] was kindly provided by Dr. G. Querat (Marseille). Murine L929 (ATCC N° CCL-1) is a fibroblastic-like cell line. The epithelial cells used were human HeLa (ATCC N° CCL-2) cells and 293T cells (ATCC N° CRL-1573). Fibroblast-like cells and epithelial cells were cultured in DMEM containing 10% FCS, L-glutamine and antibiotics. The human BeWo and JEG trophoblast cell lines were obtained from ATCC (N° CCL-98 and HTB-36, respectively) and were cultured in DMEM F-12 Ham medium in the same way as primary trophoblasts (Invitrogen). Confluent monolayers were trypsinized twice a week and used for a maximum of five passages.

Non-mammalian cells

The XTC-2 cell line, derived from *Xenopus laevis*, was cultured in Leibowitz-15 medium containing L-glutamine, amino-acids, 5% FCS and 2% tryptose phosphate (Invitrogen) at 28°C, as previously described [26]. Amoebae including *Acanthamoeba polyphaga* (ATCC N° 30461), *Acanthamoeba castellanii* (ATCC N° 30234), *Hartmannella vermiformis* (ATCC N° 50237) and *Poteriochromonas melhamensis* (ATCC N° 11532) were grown in peptone yeast-extract glucose (PYG) medium consisting of 20 g/L roteosepeptone, 1 g/L yeast extract, 1 g/L sodium citrate, 4 µM $MgSO_4$, 0.4 µM $CaCl_2$, 2.5 µM Na_2HPO_4, 2.5 µM KH_2PO_4, 5 µM $(NH_4)_2FeII(SO_4)_2$, and 0.1 M glucose for three days at 32°C, as previously described [27]. Harvested amoebae were washed twice in Page's amoeba saline (PAS) to remove most nutrients and diluted in sterile PBS.

MALDI-TOF MS

Primary cells and cell lines were obtained from at least ten different isolation procedures, and MALDI-TOF MS was performed at least in duplicate on each cell isolate. Isolated cells (10^6 cells per assay) were centrifuged at 300× *g* for 5 min, washed in sterile PBS without Ca^{2+} or Mg^{2+}, and again centrifuged to remove medium traces. Cell pellets were collected in 10 µL of sterile PBS without Ca^{2+} or Mg^{2+} and were frozen at −80°C for 2–3 days before analysis. When monocytes (10^6 cells) and T lymphocytes (10^6 cells) were mixed, cell pellets were collected in 20 µL of PBS. In some experiments, human monocytes were lysed with a RIPA buffer containing 25 mM Tris, 750 mM NaCl, 5% TritonX-100 (Sigma-Aldrich), 25 mM $MgCl_2$, 5 mM EDTA and 0.5% sodium dodecyl sulfate, or they were sonicated in the presence of complete protease inhibitor cocktail tablets (Roche Applied Science). MALDI-TOF MS was performed using an AutoflexII mass spectrometer and FlexControl software (Bruker Daltonics), as previously described [13,28]. In brief, after samples

were thawed, 1 µl was deposited on the MALDI target in which 1 µl of acid-α-cyano-4 hydroxy-cynnamic (HCCA) matrix was added. This matrix consisted of a 10 mg/mL solution of HCCA diluted in 50% acetonitrile and 25% Milli-Q grade water containing 10% trifluoroacetic acid. The evaporation that gradually took place at room temperature allowed the formation of HCCA crystals containing the dispersed samples. The crystals were illuminated with a nitrogen laser (337 nm, 3 ns pulse width), and released ions were extracted with an accelerating voltage of 20 kV in linear mode, and extraction delay times varied from 280 to 320 ns depending on the chosen mass range (m/z range of 2000–20,000). Each spectrum resulted from the sum of positive ions obtained after 525 laser shots performed in seven different regions of the analyzed sample. A signal-to-noise ratio of 3 was selected to define peaks, with a maximum of 100 peaks per spectrum.

Spectrum analysis and database acquisition

The ClinProTools version 2.2 software (Bruker Daltonics) was used to analyze the variability between samples from different blood donors. The Gel View representation displays two types of spectra arranged in a pseudo-gel format. The 2D Peak Distribution View automatically selects two peaks, and their relative intensities are expressed as a 2D representation. The Biotyper version 2.0 (Bruker Daltonics) software was used to create an averaged spectrum for each cell type corresponding to at least 20 individual spectra obtained from at least ten different cell cultures tested in duplicate. Baselines were automatically subtracted from spectra, and the background noise was smoothed during acquisition through the FlexControl software. This reference was validated by other samples from the same cell type. The Biotyper software realigns acquired spectra from each cell type and automatically creates an average spectrum using default Biotyper software settings provided by the manufacturer. These settings were the same than those used in routine bacteriology [13]. Briefly, the sensitivity or the maximum tolerated error on the values of mass spectra and spectrum shift was 8000 particles per million. The minimum frequency to benchmark selection of peaks was 25%, and only peaks with a signal/noise intensity above background were selected by the software. The cell-type reference consisting of 70 peaks was added to the database. The Biotyper software was also used to identify unknown spectra by comparison with reference spectra, as recently described for the identification and classification of microorganisms [13]. We used the score values proposed by the manufacturer for microorganisms: values between 0.000 and 1.699 did not allow reliable cell identification; values between 1.700 and 1.999 allowed probable cell identification; scores higher than 2.0 were considered statistically significant and allowed the confident identification of different cell species. Finally, we used Multiexperiment Viewer (MeV) version 4.3 software (http://www.tm4.org/) to perform hierarchical clustering with dendrogram representations of collected MALDI-TOF MS data. The mass values of peaks (with an area equal or greater than to 20) of the reference spectrum of each cell type were selected

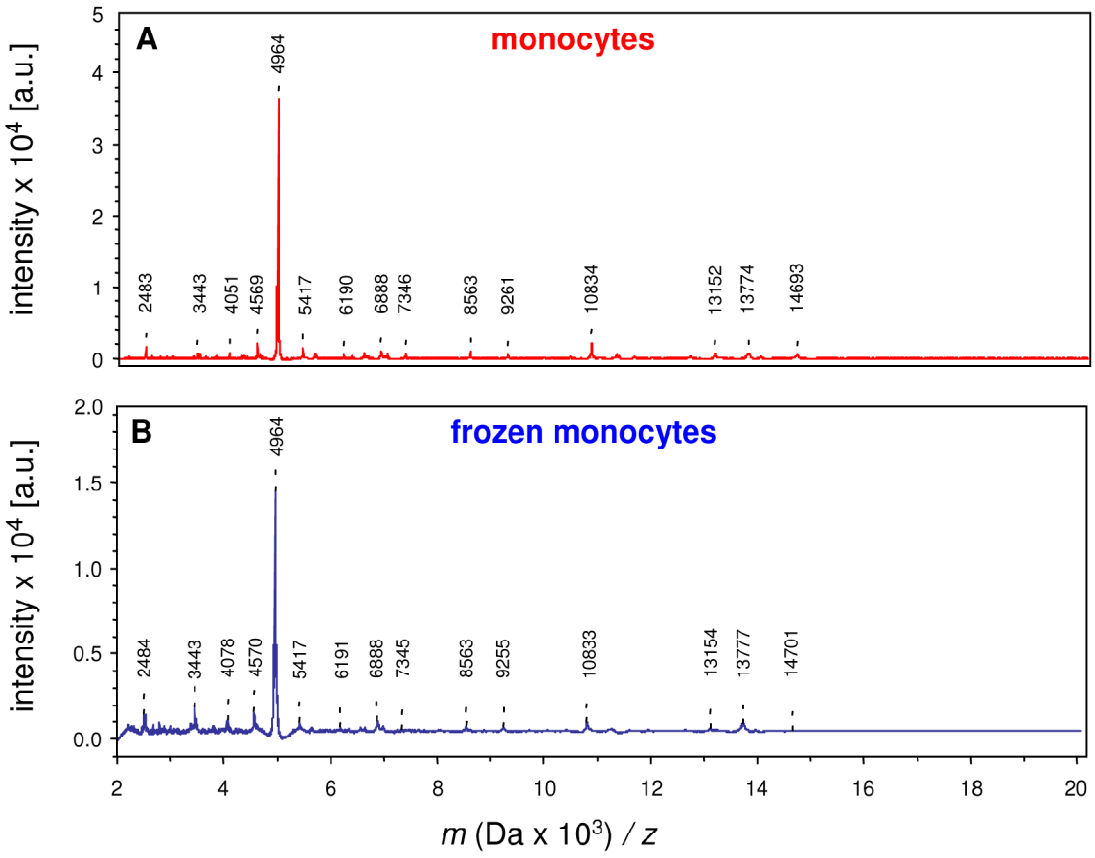
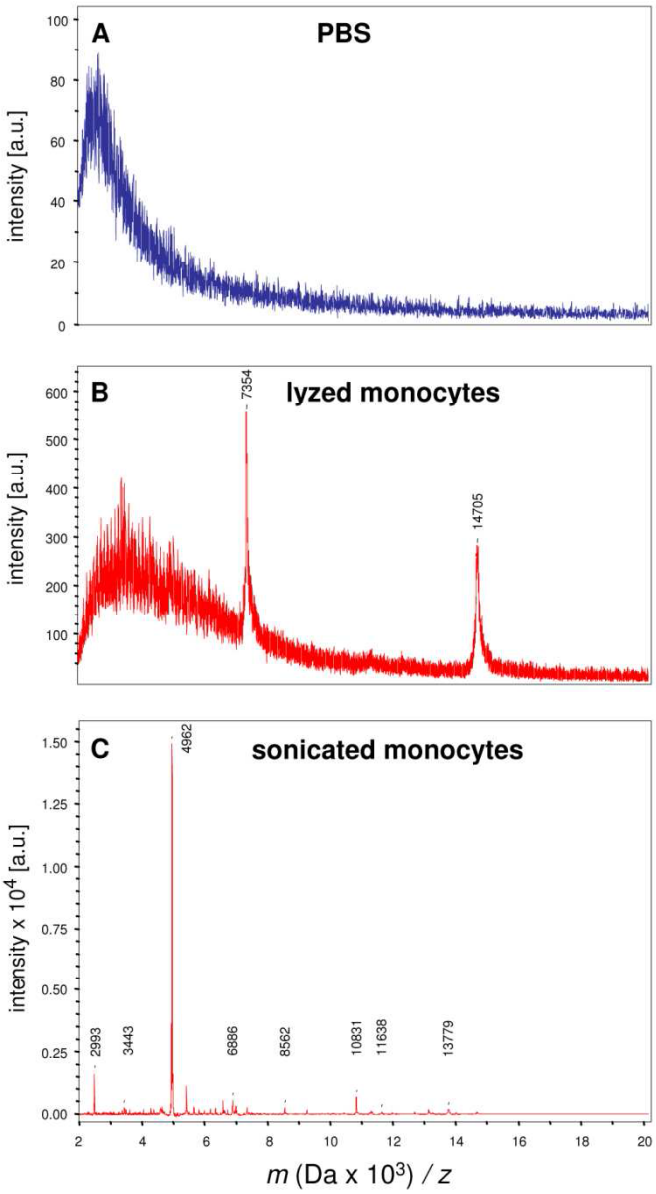


Figure 1. MALDI-TOF MS spectra of monocytes. Human monocytes (10^6 cells per assay) were collected in 10 µl of PBS, and 1 µl was deposited on the MALDI target. Representative MALDI-TOF MS spectra are shown for A, freshly isolated and B, frozen monocytes.
doi:10.1371/journal.pone.0013691.g001

Figure S1. MALDI-TOF MS spectra of monocyte preparations.



Human monocytes (106 cells per assay) were collected in 10 µl of PBS, and 1 µl was deposited on the MALDI target. Representative MALDI-TOF MS spectra are shown: A, in the absence of monocytes; B, lysed monocytes; C, sonicated monocytes.
doi:10.1371/journal.pone.0013691.s001

after treatment of these spectra (smoothing and subtraction of background noise) by the FlexAnalysis software. For a given spectrum, only the weight values with a gap strictly greater than 3 were selected; for spectra of different cell types, the gap must be greater than or equal to 3. The m/z values of peaks from given spectra were transferred into an Excel file with a value of +1. The value of −1 was assigned to m/z positions without a peak, and conventional color code was applied to hierarchical clustering representation.

Results

MALDI-TOF MS analysis of monocytes

In the first series of experiments, the MALDI-TOF MS signature of monocytes isolated with CD14 microbeads was analyzed. When 10^6 monocytes per assay were used, several peaks with different intensities were detected (Fig. 1A), whereas no peak was observed in the control matrix (supplementary Fig. S1A). A major peak was observed at 4964 m/z, and several minor peaks were detected between 2,000 and 15,000 m/z. Note that the software identified more than 100 peaks per spectrum. In a second series of experiments, monocytes were either frozen at −80°C for two days, lysed using a lysis buffer or sonicated. The freezing procedure did not alter the monocyte signature (Fig. 1B). In contrast, when the monocytes were lysed, diffuse spectra were obtained (supplementary Fig. S1B). When the monocytes were sonicated, the spectra showed peaks that did not correspond to those observed with viable monocytes (supplementary Fig. S1C). Consequently, only frozen samples were used in subsequent experiments.

The effect of monocyte concentration on the presence and positions of peaks was then assessed. Increasing the initial cell concentration (10^6 cells) by 5- or 10-fold did not modify the position or the intensity of detected peaks, but it did increase the background. Using 10^5 monocytes was insufficient to detect the full range of peaks that were detected with 5×10^5 or 10^6 monocytes. As a consequence, further experiments were performed using 10^6 frozen cells per assay. Taken together, these results showed that monocyte freezing was a very simple method that permitted delayed handling of samples.

MS analysis of circulating cells

We then compared the profiles of circulating cells isolated from a healthy blood donor. The MALDI-TOF MS signature of T lymphocytes was distinct from that of monocytes (compare Fig. 2A with Fig. 1B). The gel view representation created by the ClinProTools version 2.2 software allowed the comparison of individual spectra (Fig. 3). Peaks at 5418, 6577, 7345 and 10.836 m/z were present in monocytes but not in T lymphocytes. Conversely, a peak at 8412 m/z present in T lymphocytes was lacking in monocytes. Some peaks were common to both monocytes and T lymphocytes (e.g., the peak at 11360 m/z). Similarly, the MALDI-TOF MS spectrum of PMNs (Fig. 2B) differed from those of both monocytes and T lymphocytes. Indeed, the intensity (see the peak at 3445 m/z) and presence (see the peak at 4329 m/z) of certain peaks distinguished PMNs from both monocytes and T lymphocytes. Finally, the MALDI-TOF MS spectrum of RBCs (Fig. 2C) differed dramatically from those of monocytes, T lymphocytes and PMNs. First, the intensity of the detected peaks was lower by 1000-fold in RBCs. Second, the great

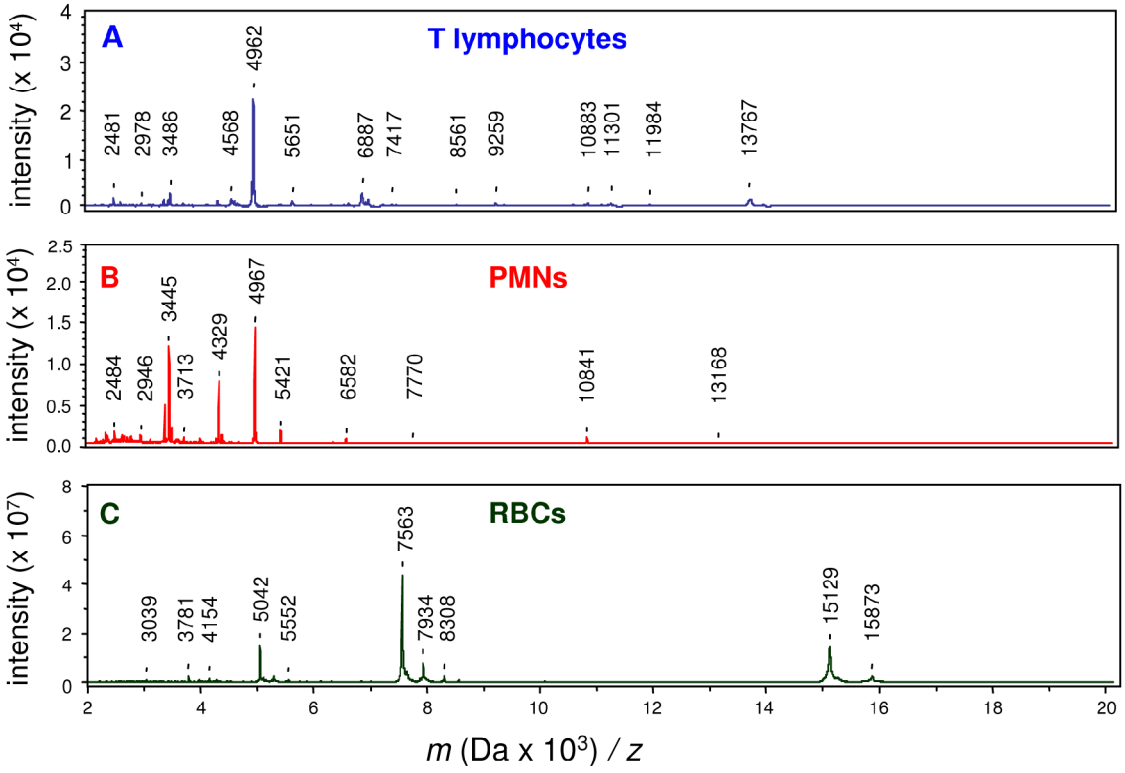


Figure 2. MALDI-TOF MS spectra of circulating cells. T lymphocytes (A), PMNs (B) and RBCs (C) were isolated from a healthy blood donor. Representative MALDI-TOF MS spectra are shown.
doi:10.1371/journal.pone.0013691.g002

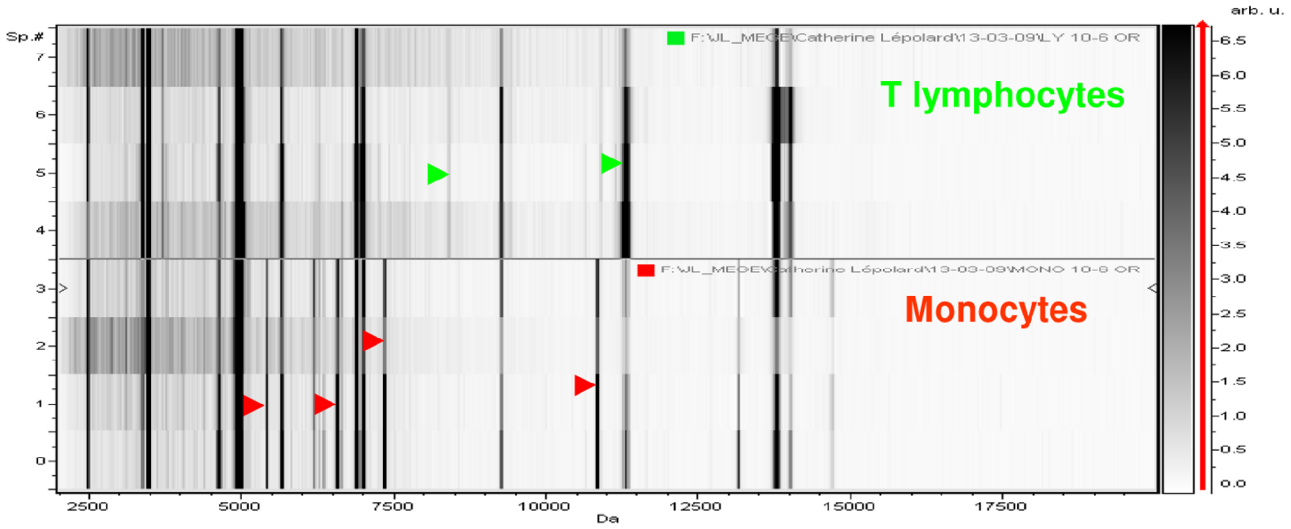


Figure 3. Gel view representation of monocytes and T lymphocytes. Monocytes and T lymphocytes were isolated from a healthy blood donor. MALDI-TOF MS spectra were analyzed using the ClinProTools software, and the spectra are presented in Gel View representation. Representative spectra are shown with *m/z* values on the x-axis and the peak intensity (in arbitrary units) on the y-axis. Major differences between monocytes and T lymphocytes are indicated by arrowheads.
doi:10.1371/journal.pone.0013691.g003

majority of peaks (see, for example, the peaks at 5042, 7563, 11129 and 15873 *m/z*) were present only in RBCs, whereas other peaks common to monocytes, T lymphocytes and PMNs, such as the peaks at 2484, 4964 m/z, were lacking in RBCs.

The reproducibility of the signatures of monocytes, T lymphocytes and PMNs was tested using ten different donors. The 2D representation provided by the ClinProTools version 2.2 software illustrates the differences between monocytes, T lymphocytes and PMNs, and it shows that their signatures are remarkably homogenous (Fig. 4). The MALDI-TOF MS data was then clustered hierarchically using the MeV software. The presence of peaks is represented in red (Fig. 5). Clearly, RBCs did not cluster with the other circulating cells. Monocytes clustered with PMNs while T lymphocytes were nearer to monocytes than PMNs. These results suggest that MALDI-TOF MS profiles of circulating cells are reproducible and specific.

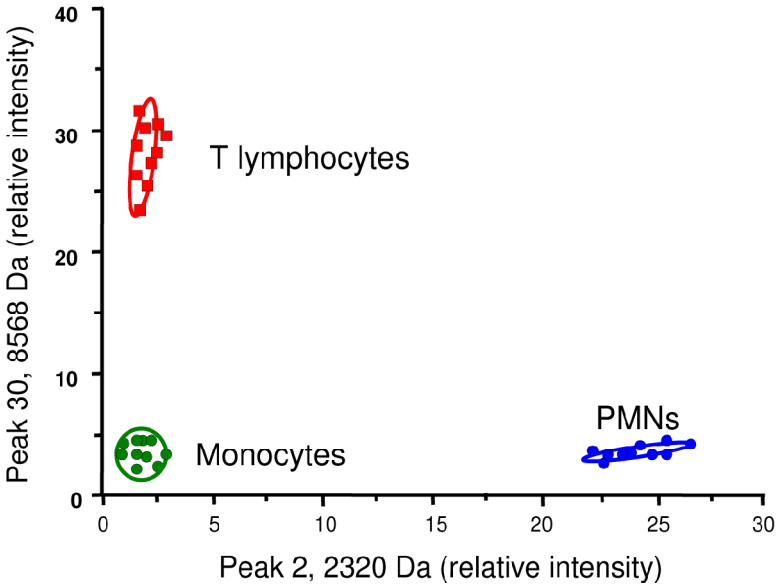


Figure 4. Reproducibility of MALDI-TOF MS signatures. Monocytes (in green), T lymphocytes (in red) and PMNs (in blue) were isolated from ten healthy blood donors. MALDI-TOF MS spectra were analyzed using the ClinProTools software and 2D Peak Distribution View. The relative intensities of the two peaks automatically selected were homogenous among blood donors, and the ellipses represent the standard deviation within each cell population (monocytes, T lymphocytes and PMNs, respectively).
doi:10.1371/journal.pone.0013691.g004

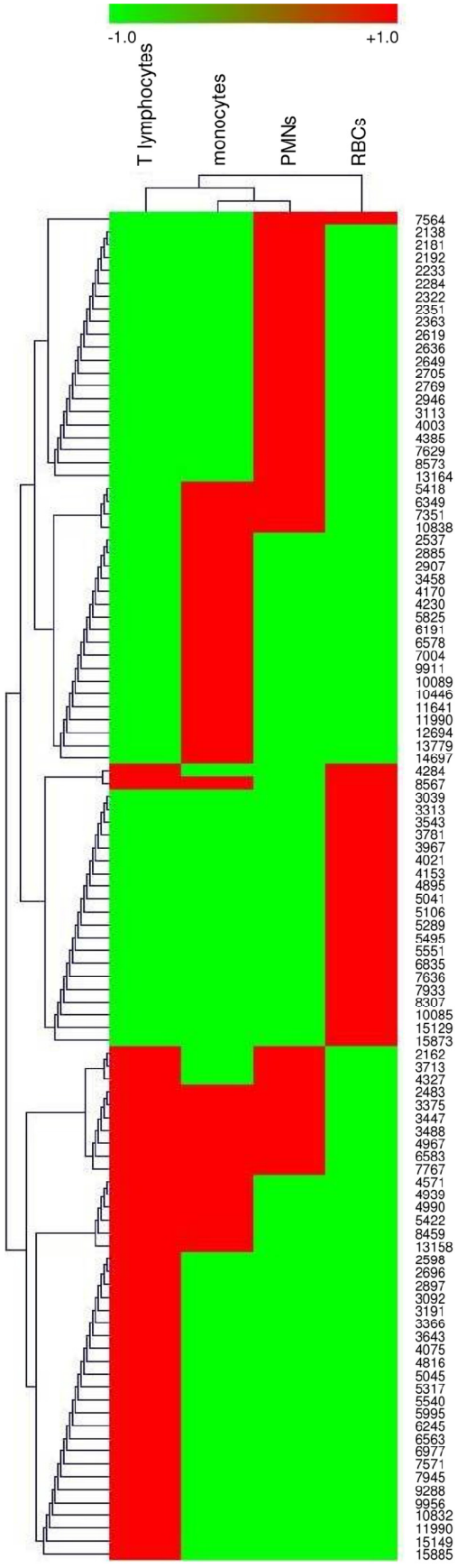


Figure 5. Hierarchical clustering of circulating cells. Monocytes, T lymphocytes, PMNs and RBCs were isolated from a healthy blood donor. MALDI-TOF MS spectra were analyzed using MeV software. A conventional value of +1 was assigned to the m/z values of spectra (in red) and −1 to m/z positions without peaks (in green).
doi:10.1371/journal.pone.0013691.g005

Development of a cell database

Because MALDI-TOF MS profiles seemed to be specific for different types of circulating cells, we created a cell database using the BioTyper version 2.0 software. We included primary myeloid cells such as human MDMs and murine BMDMs in the database. We also included human THP-1 myelomonocytic cells and the murine J774 and canine DH82 macrophage cell lines. Because circulating monocytes differentiate into MDMs or DCs depending on culture conditions, we also assessed the ability of MALDI-TOF MS to discriminate between MDMs, monocyte-derived DCs, and circulating monocytes. Similarly, circulating CD3 T cells were compared to a human CCR5-transfected leukemia cell line. We also analyzed one fibroblast-like cell line, murine L929 cells, and two epithelial cell lines, consisting of human HeLa and 293T cells. Additionally, we compared human placenta trophoblasts with the human BeWo and JEG trophoblast cell lines. Finally, we selected non-mammalian cells such as a *Xenopus* cell line and different types of amoebae for analysis.

The mean spectra of the 22 different cell types were introduced into the database, allowing for an accurate comparison. The cell database was then used to classify and clusterize the various primary cells and cell lines (Fig. 6). Two major clusters were found. The first one contained an invertebrate cell line, several amoebae and (surprisingly) human RBCs. The second cluster included mammalian immune cells and cell types. Among this latter cluster, several branches were identified. The branch that included circulating immune cells could be divided into three specific branches: T lymphocytes, monocytes and PMNs. It is noteworthy that monocyte-derived macrophages and DCs were present in distinct branches, along with several macrophage cell lines (J774, DH82, THP-1) and a T cell line (C8166 cells). Two trophoblast cell lines (i.e., JEG and BeWo cells) clustered together but were distant from primary trophoblasts. Epithelial cells also clustered together (HeLa and 293T cells). Finally, non-immune cell lines including epithelial, fibroblastic and trophoblast cells were located in a branch distinct from immune cells. These results show that MS profiles discriminated immune cells from other eukaryotic cells, and it positioned circulating immune cells (monocytes, T lymphocytes and PMNs) distantly from other immune cells.

An MS database as a tool for cell identification

We tested the efficiency of the database in three different ways. First, we compared a new monocyte sample with the mean spectrum of monocytes generated within the database (Fig. 7). The resulting score of 2.65 was highly significant and authenticated the tested cell population as monocytes. Second, we determined whether it is possible to identify cell populations within a cell mixture. Circulating monocytes and T lymphocytes (10^6 each cell type) were mixed and the spectra obtained were compared to the database. Monocytes and T lymphocytes were respectively identified with a correct score of 2.25 for both monocytes and T lymphocytes. Third, the MALDI-TOF MS profile of PBMCs was analyzed according to the same procedure. Although the proportion of monocytes was small compared to T lymphocytes (about 1/7), we identified both T lymphocytes (with a score of 2.22) and monocytes (with a score of 2.21). Taken together, these results demonstrate that we were able to identify monocytes and T lymphocytes in a complex

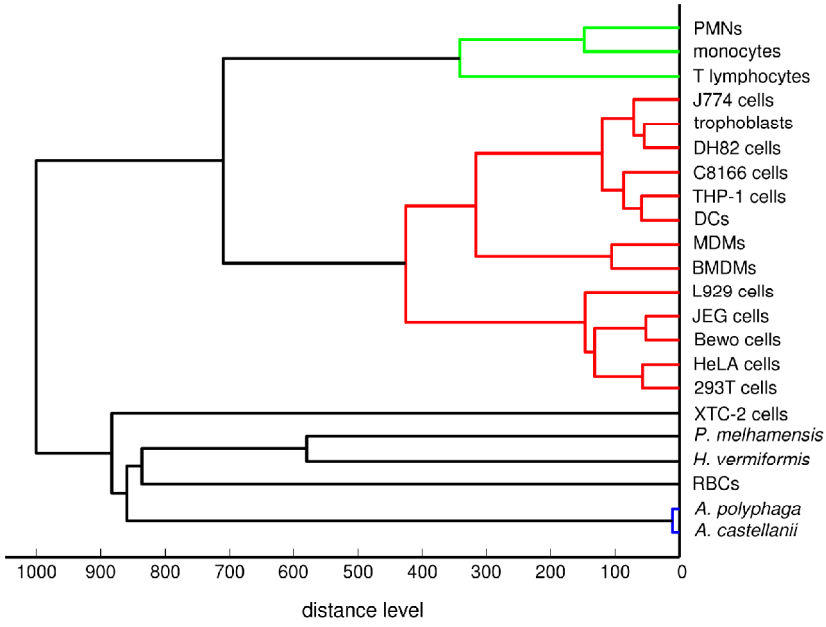


Figure 6. Dendrogram of 22 eukaryotic cell types. MALDI-TOF MS was performed on 22 cell types with at least 20 spectra per cell type. An averaged spectrum for each cell type was added to the database using the BioTyper software and the dendrogram creation method.
doi:10.1371/journal.pone.0013691.g006

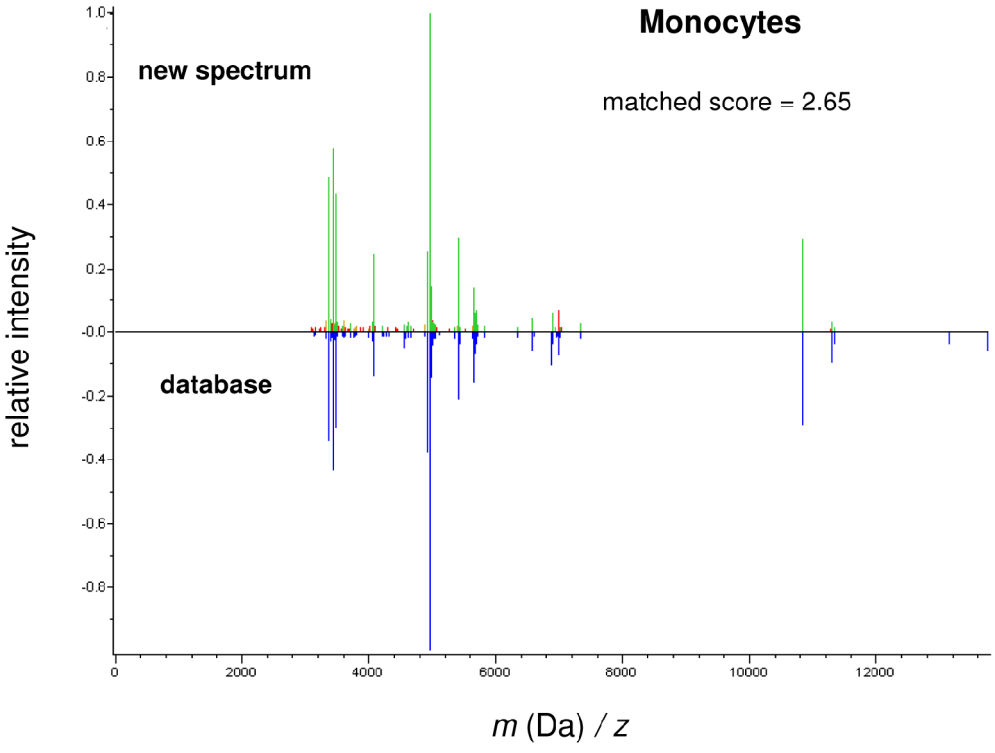


Figure 7. Efficiency of the database. A MALDI-TOF MS spectrum of unknown cells (here, monocytes from a blood donor) was compared to the averaged spectrum of monocytes generated from the database using the BioTyper software. The score indicates the identification of the tested cell population as monocytes.
doi:10.1371/journal.pone.0013691.g007

mixture, and they suggest that MALDI-TOF MS allows the confident identification of cell subsets in tissues.

Discussion

In this report, we showed that a MALDI-TOF MS approach was able to identify intact immune cells. This method was rapid and easy to perform and did not require any additional components (such as specific antibodies), in contrast to flow cytometry. In addition, the repertoire of analyzed molecules is different between MALDI-TOF MS and flow cytometry because MALDI-TOF MS is applicable to soluble molecules with a molecular weight ranging from 2 to 20 kDa, whereas flow cytometry detects surface markers or intracellular proteins through permeabilization procedures. Our MALDI-TOF MS approach extended to eukaryotic cells an approach previously used for bacterial identification [12–17,28]. In this study, intact primary or cultured cells were washed in saline to eliminate contamination by components such as cytokines or albumin contained in FCS, and thawed samples were deposited on the MALDI target in which HCCA matrix was added. Clearly, spectra were constituted by a collection of peaks, and their masses and relative intensities varied according cell origin. Other attempts have been performed to analyze MALDI-TOF MS profiles of whole eukaryotic cells. A recent report shows that MALDI-TOF MS typing is efficient to characterize 66 cell culture samples representing 34 species from insects to primates. Spectra of each cell type were composed of a variety of peaks with different masses and intensities, demonstrating the feasibility of our approach. However, as cell samples are treated by ethanol and formic acid/acetonitrile before assay [19], the two methods are not superimposable. Another report describes MALDI-TOF MS spectra from K562, BHK21 and GM15226 cell lines after lysis in 2,5-dihydroxybenzoic acid matrix solution. Again, obtained spectra show common peaks among the three cell types and specific peaks [18], demonstrating that MALDI-TOF MS performed on crude extracts of mammalian cell types may be useful to easily identify different cell types. In addition, we demonstrated that freezing cells at $-80^{\circ}C$ was sufficient to obtain high quality spectra, whereas cell lysis increased background noise in a non-interpretable way. Cell sonication led to spectra that were non-reproducible and peaks that were not entirely superimposable with those found in viable and frozen cells. The cell concentrations used to obtain significant spectra were relatively high, but were still less than those required for transcriptomics, another type of global method. In a prior study employing MALDI-TOF MS to analyze human macrophages, the extraction of membrane proteins requires specific protocols and a large quantity of cells [4].

We demonstrated that the spectra of immune cells were specific since they were markedly distinct from those of unrelated cell lines and differed between related immune cells. This specificity was supported by a set of peaks that represent the MS signature of each cell type. In addition, this study enabled us to develop a cell database comprised of 22 cell types representing diverse lineages of eukaryotic cells [29]. The database relies on the creation of a specific reference spectrum for each cell type and a score that validates the identification. These data have two major applications: first the establishment of a dendrogram of eukaryotic cells, and second, the analysis of mixed cell populations. The dendrogram revealed two major branches: one cluster of insect cells, amoebas and RBCs, and another cluster with immune cells and cell lines. Among the circulating leukocytes, the distance was smaller between monocytes and T lymphocytes, which are functionally distinct, than between monocytes and PMNs, although both are phagocytic cells. Monocytes, T lymphocytes and PMNs were in branches distinct from tissue immune cells such as macrophages and DCs. The divergence between monocytes and MDMs is consistent with previous transcriptomic studies in which each cell type had a specific program [30]. In addition, maturation from monocytes is a common feature of MDMs and DCs, and this accounts for the clustering of these two cell types. MDMs and DCs remained markedly distant in the dendrogram, which underlines their functional divergence. Interestingly, the clustering between cell types seemed independent of species origin. Indeed, human MDMs and murine BMDMs were close in the dendrogram. Similarly, the distance between the human THP-1, murine J774 and canine DH82 monocytic cells was low. We found that the position of primary trophoblasts was surprising: close to macrophage cell lines and distant from trophoblast cell lines (JEG and BeWo cells).

The use of the database enabled us to identify different cell populations among cell mixtures. The common signature of monocytes among individual donors was robust. Additionally, monocytes and T lymphocytes were accurately identified when they were mixed. Furthermore, the specific signatures of monocytes and T lymphocytes were found when PBMCs were studied. We suggest that the MALDI-TOF MS approach can be used to identify different cell types among tissue infiltrates. In addition, it is likely that its discriminative power is similar to that of genomic [29], proteomic and transcriptomic approaches [30]. Recent proteomic and transcriptomic approaches allow the discrimination between $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocyte subsets. Similarly, we preliminarily found that the activation of monocytes and macrophages resulted in specific spectra that correlated with their transcriptomic patterns (manuscript in preparation).

In conclusion, we developed a new method for identifying immune cells based on a MALDI-TOF MS approach. A major advantage of this method compared to the usual techniques is the lack of purification steps and staining procedures, which often lead to cell activation. The cell database we constructed was useful for identifying a cell type within a cell mixture, and it could potentially be used to identify different functional states of a cell population such as monocytes or macrophages.

Supporting Information

Figure S1 MALDI-TOF MS spectra of monocyte preparations. Human monocytes (106 cells per assay) were collected in 10 µl of PBS, and 1 µl was deposited on the MALDI target. Representative MALDI-TOF MS spectra are shown: A, in the absence of monocytes; B, lysed monocytes; C, sonicated monocytes.
Found at: doi:10.1371/journal.pone.0013691.s001 (0.30 MB TIF)

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Author Contributions

Conceived and designed the experiments: CC DR JLM. Performed the experiments: RO CF ABA. Analyzed the data: RO CF ABA CC DR JLM. Contributed reagents/materials/analysis tools: RO CF ABA. Wrote the paper: CC DR JLM.

References

1. Palmer C, Diehn M, Alizadeh AA, Brown PO (2006) Cell-type specific gene expression profiles of leukocytes in human peripheral blood. BMC Genomics 7: 115.
2. Lehtonen A, Ahlfors H, Veckman V, Miettinen M, Lahesmaa R, et al. (2007) Gene expression profiling during differentiation of human monocytes to macrophages or dendritic cells. J Leukoc Biol 82: 710–720.
3. Consortium, C. H. L. P. P. (2010) First insight into the human liver proteome from PROTEOME(SKY)-LIVER(Hu) 1.0, a Publicly available database. J Proteome Res 9: 79–94.
4. Dupont A, Tokarski C, Dekeyzer O, Guihot AL, et al. (2004) Two-dimensional maps and databases of the human macrophage proteome and secretome. Proteomics 2004 4: 1761–1778.
5. Srisomsap C, Sawangareetrakul P, Subhasitanont P, Panichakul T, Keeratichamroen S, et al. (2004) Proteomic analysis of cholangiocarcinoma cell line. Proteomics 4: 1135–1144.
6. Horlock C, Shakib F, Mahdavi J, Jones NS, Sewell HF, et al. (2007) Analysis of proteomic profiles and functional properties of human peripheral blood myeloid dendritic cells, monocyte-derived dendritic cells and the dendritic cell-like KG-1 cells reveals distinct characteristics. Genome Biol 8: R30.
7. Robertson JW, Rodrigues CG, Stanford VM, Rubinson KA, Krasilnikov OV, et al. (2007) Single-molecule mass spectrometry in solution using a solitary nanopore. Proc Natl Acad Sci U S A 104: 8207–8211.
8. Johnson RP, El-Yazbi AF, Hughes MF, Schriemer DC, Walsh EJ, et al. (2009) Identification and functional characterization of protein kinase A-catalyzed phosphorylation of potassium channel Kv1.2 at serine 449. J Biol Chem 284: 16562–16574.
9. Li X, Cowles EA, Cowles RS, Gaugler R, Cox-Foster DL (2009) Characterization of immunosuppressive surface coat proteins from *Steinernema glaseri* that selectively kill blood cells in susceptible hosts. Mol Biochem Parasitol 165: 162–169.
10. Mellmann A, Cloud J, Maier T, Keckevoet U, Ramminger I, et al. (2008) Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. J Clin Microbiol 46: 1946–1954.
11. Trost M, English L, Lemieux S, Courcelles M, Desjardins M, et al. (2009) The phagosomal proteome in interferon-γ-activated macrophages. Immunity 30: 143–154.
12. Lay JO, Jr., Holland RD (2000) Rapid identification of bacteria based on spectral patterns using MALDI-TOF MS. Methods Mol Biol 146: 461–487.
13. Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, et al. (2009) Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis 49: 543–551.
14. Dieckmann R, Helmuth R, Erhard M, Malorny B (2008) Rapid classification and identification of salmonellae at the species and subspecies levels by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 74: 7767–7778.
15. Petersen CE, Valentine NB, Wahl KL (2009) Characterization of microorganisms by MALDI mass spectrometry. Methods Mol Biol 492: 367–379.
16. Majcherczyk PA, McKenna T, Moreillon P, Vaudaux P (2006) The discriminatory power of MALDI-TOF mass spectrometry to differentiate between isogenic teicoplanin-susceptible and teicoplanin-resistant strains of methicillin-resistant *Staphylococcus aureus*. FEMS Microbiol Lett 255: 233–239.
17. Degand N, Carbonnelle E, Dauphin B, Beretti JL, Le Bourgeois M, et al. (2008) Matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of nonfermenting gram-negative bacilli isolated from cystic fibrosis patients. J Clin Microbiol 46: 3361–3367.
18. Zhang X, Scalf M, Berggren TW, Westphall, Smith LM (2006) Identification of mammalian cell lines using MALDI-TOF and LC-ESI-MS/MS mass spectrometry. J Am Soc Mass Spectrom 17: 490–499.
19. Karger A, Bettin B, Lenk M, Mettenleiter TC (2010) Rapid characterisation of cell cultures by matrix-assisted laser desorption/ionisation mass spectrometric typing. J Virol Methods 164: 116–121.
20. Benoit M, Ghigo E, Capo C, Raoult D, Mege JL (2008) The uptake of apoptotic cells drives *Coxiella burnetii* replication and macrophage polarization: a model for Q fever endocarditis. PLoS Pathog 4: e1000066.
21. Desnues B, Raoult D, Mege JL (2005) IL-16 is critical for *Tropheryma whipplei* replication in Whipple's disease. J Immunol 175: 4575–4582.
22. Lepidi H, Benoliel AM, Mege JL, Bongrand P, Capo C (1992) Double localization of F-actin in chemoattractant-stimulated polymorphonuclear leucocytes. J Cell Sci 103: 145–156.
23. Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF, 3rd (1986) Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. Endocrinology 118: 1567–1582.
24. Meghari S, Bechah Y, Capo C, Lepidi H, Raoult D, et al. (2008) Persistent *Coxiella burnetii* infection in mice overexpressing IL-10: an efficient model for chronic Q fever pathogenesis. PLoS Pathog 4: e23.
25. Fouchier RA, Meyer BE, Simon JH, Fischer U, Malim MH (1997) HIV-1 infection of non-dividing cells: evidence that the amino-terminal basic region of the viral matrix protein is important for Gag processing but not for post-entry nuclear import. EMBO J 16: 4531–4539.
26. Drancourt M, Jarlier V, Raoult D (2002) The environmental pathogen *Mycobacterium ulcerans* grows in amphibian cells at low temperatures. Appl Environ Microbiol 68: 6403–6404.
27. Greub G, Raoult D (2002) Crescent bodies of *Parachlamydia acanthamoeba* and its life cycle within *Acanthamoeba polyphaga*: an electron micrograph study. Appl Environ Microbiol 68: 3076–3084.
28. Fournier PE, Couderc C, Buffet S, Flaudrops C, Raoult D (2009) Rapid and cost-effective identification of *Bartonella* species using mass spectrometry. J Med Microbiol 58: 1154–1159.
29. Hartman H, Fedorov A (2002) The origin of the eukaryotic cell: a genomic investigation. Proc Natl Acad Sci U S A 99: 1420–1425.
30. Zhao C, Zhang H, Wong WC, Sem X, Han H, et al. (2009) Identification of novel functional differences in monocyte subsets using proteomic and transcriptomic methods. J Proteome Res 8: 4028–4038.

ARTICLE 2

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

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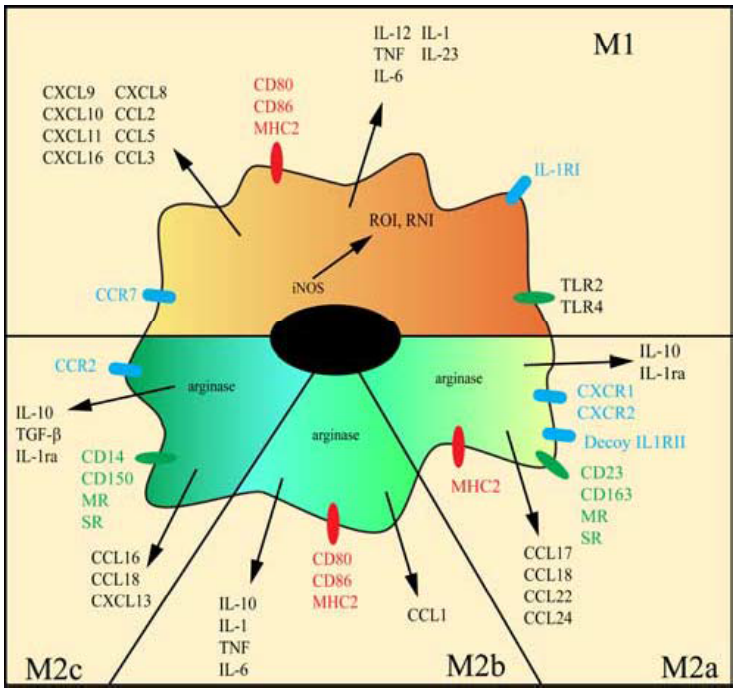
Dans cet article, nous avons voulu connaître le niveau de précision auquel peut prétendre la spectrométrie de masse MALDI-TOF sur cellules entières. Nous avons pour cela utilisé un seul type cellulaire, les macrophages dérivés de monocytes humains, dont une caractéristique majeure est la plasticité phénotypique et fonctionnelle. En effet, ces macrophages réagissent à leur microenvironnement en adaptant leurs propriétés (**Figure 5**). En présence de cytokines telles que l'interféron (IFN)-γ ou le *Tumor Necrosis Factor* (TNF) ou de produits microbiens tels que les lipopolysaccharides (LPS) ou les peptidoglycanes (PGN), ils deviennent des macrophages dits M1 caractérisés par un ensemble de marqueurs phénotypiques et l'acquisition d'un important potentiel inflammatoire et microbicide. En revanche, les macrophages soumis à des cytokines telles que l'interleukine (IL)-4 ou le *Transforming Growth Factor* (TGF)-β sont appelés M2, caractérisés eux aussi par un ensemble de marqueurs phénotypiques et la perte de leur potentiel inflammatoire et microbicide (Mantovani, Sica, et Locati 2005; Fernando O Martinez et al. 2006; Fernando Oneissi Martinez 2008; Benoit, Desnues, et Mege 2008a; Tugal, Liao, et Jain 2013). Il n'existe pas de marqueurs phénotypiques uniques des macrophages qui permettent de caractériser leurs réponses aux stimuli auxquels ils sont soumis. Il apparaît ainsi que l'état fonctionnel des macrophages ne peut être apprécié qu'en fonction de paramètres tels que données transcriptionnelles, phénotypiques, protéiques et fonctionnelles (Benoit, Desnues, et

Figure 5. Propriétés des macrophages polarisés



d'après Benoit, Desnues, et Mege. 2008

Mege 2008a; Mantovani et al. 2013; Tugal, Liao, et Jain 2013). La spectrométrie de masse MALDI-TOF sur cellules entières représente alors une alternative qu'il m'a semblé utile de tester dans la mesure où cette méthode ne nécessite aucun traitement préalable des cellules.

Nous avons donc stimulé les macrophages dérivés de monocytes par différents inducteurs tels que l'IFN-γ, le TNF, l'IL-4, l'IL-10, le TGF-β1, le LPS ou par différentes bactéries pathogènes telles que des streptocoques du groupe B, *Staphylococus aureus,* le BCG (*Mycobacterium bovis*, la souche vaccinale utilisée contre la tuberculose)*, Coxiella burnetii,* la bactérie responsable de la fièvre Q et *Orientia tsutsugamushi*, l'agent responsable du typhus des broussailles et j'ai cherché à savoir si la spectrométrie de masse MALDI-TOF sur cellules entières permet d'obtenir des spectres spécifiques de ces différentes voies d'activation. Nous avons en outre analysé les données de la spectrométrie de masse MALDI-TOF avec le logiciel R afin de nous affranchir des outils imposés par le constructeur des spectromètres de masse (Bruker Daltonics). R est en effet un logiciel gratuit dont le principal avantage est le traitement statistique des données. Nous avons ainsi créé notre propre script qui permet de normaliser, d'aligner et d'afficher graphiquement les résultats des spectres.

Nous avons montré que l'IFN-γ et l'IL-4 induisent des signatures spécifiques des macrophages, ce qui suggère qu'il est possible de déterminer l'existence d'une polarisation M1/M2 des macrophages par une approche simple et rapide qui est la spectrométrie de masse MALDI-TOF sur cellules entières. Fort de ce constat, nous avons ensuite stimulé les macrophages avec d'autres agonistes M1 tels que le TNF, le LPS et une combinaison IFN-γ + LPS. Un *clustering* hiérarchique montre que les agonistes M1 induisent un profil propre lorsqu'on compare les macrophages stimulés et les macrophages non stimulés. Au sein de la réponse M1, il est possible de différencier les spectres des macrophages selon qu'ils sont stimulés par telle ou telle cytokine. Il est possible également d'obtenir un cluster M2 lorsque les macrophages sont stimulés par l'IL-4, l'IL-10 ou le TGF-β1 et comparés aux macrophages non stimulés, chacune de ces cytokines donnant un profil propre. En résumé, nos résultats suggèrent que la spectrométrie de masse MALDI-TOF permet de détecter des macrophages selon qu'ils sont M1 ou M2.

Nous avons alors cherché à savoir si différentes bactéries induisent des réponses M1 ou M2 des macrophages ou si ces réponses sont spécifiques de chaque agent infectieux. Nous avons d'une part utilisé des bactéries extracellulaires inactivées par la

chaleur (streptocoques du groupe B, *S. aureus*) et d'autre part des bactéries connues pour infecter les macrophages (BCG, *C. burnetii, O. tsutsugamushi*). Les bactéries extracellulaires induisent des réponses proches du profil M2 des macrophages mais ces réponses sont quelque peu différentes selon que les bactéries utilisées sont les streptocoques du groupe B ou *S. aureus*. La réponse induite par le BCG est relativement comparable à celle des macrophages traités avec l'IL-4 (réponse de type plutôt M2) alors que *C. burnetii* et *O. tsutsugamushi* induisent une réponse qui leur est spécifique.

En résumé, la spectrométrie de masse MALDI-TOF sur cellules entières est suffisamment sensible pour détecter différents états d'activation des macrophages humains dérivés de monocytes. Notre approche a permis également de montrer que le répertoire d'activation de ces macrophages est extrêmement étendu et souligne la très grande sensibilité de ces cellules à leur microenvironnement.

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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.

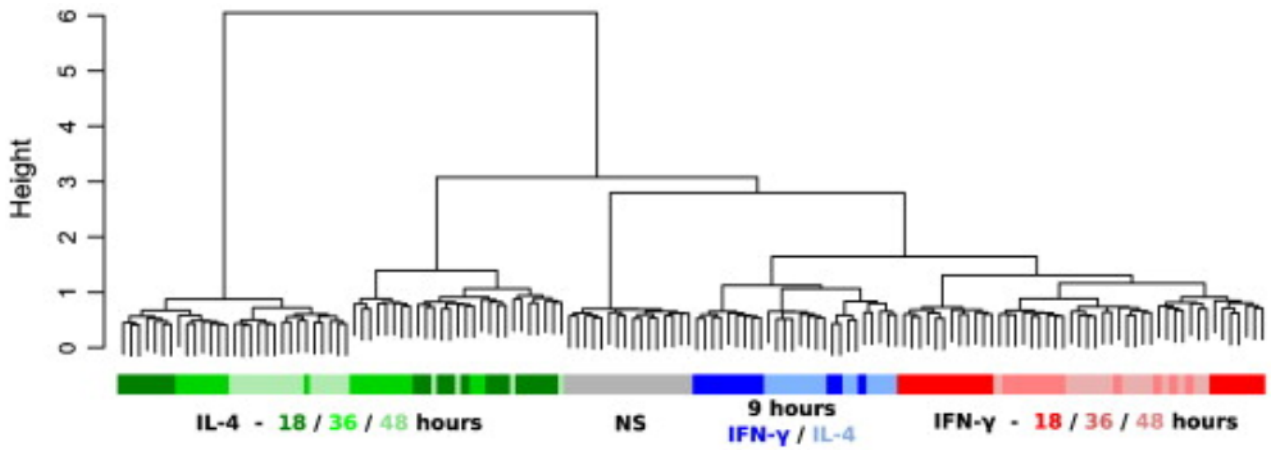
2.2. Activation of macrophages

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

Figure S1. Effet of time on the MALDI-TOF MS spectra



Macropahges from different samples were stimulated with IFN-γ or IL-4 for different periods of time. The resulting spectra were analysed using hierarchical clustering. The early responses of macrophages to IFN-γ and IL-4 overlapped, whereas the later responses were clearly unique.
http://dx.doi.org/10.1016/j.jprot.2012.07.046

and analysed in linear mode with time of flight. The resulting accuracy and resolution was 200 ppm. The generated spectra were a result of the sum of the positive ions that were obtained after 525 laser shots in different locations of the spot. We performed control acquisition with matrix only, with cytokines only or with heat-killed bacteria alone. The peaks identified in these control acquisitions were neither observed nor selected in macrophage acquisitions.

2.4. Spectrum analysis

All analyses and graphical outputs were performed using R (version 2.13). Raw data were loaded in R using the *readBrukerFlexData* library. The spectra were analysed using the *MALDIquant* library and specific algorithms. Briefly, the square root of the intensities was used to enhance the graphical visualisation of the spectra. The background was corrected using a statistic-sensitive non-linear peak-clipping algorithm for baseline estimation [24]. Peaks were detected using a signal-to-noise ratio of 6. The detected peaks were considered similar across spectra when the *m/z* values were within a 2000 ppm window. The x-axis of spectra represented the *m/z* ratio of ionised molecules, and the y-axis indicated the relative proportion of these molecules (in relative intensities). The gel view representation indicates the reproducibility of the spectra obtained from different samples that were arranged in a pseudo-gel format. A hierarchical clustering with a ward algorithm for agglomeration and a dissimilarity matrix based on the Jaccard distance were used to classify the spectra. The Jaccard index measures the similarity between Boolean sample sets. The Jaccard distance, which measures dissimilarity between sample sets, is complementary to the Jaccard index and is obtained by subtracting the Jaccard coefficient from 1 or by dividing the difference of the sizes of the union and the intersection of two sets by the size of the union. To obtain reference spectra, we first computed a distance matrix for the class and then obtained the mean distance of each spectrum versus all other spectra of the same class. The reference spectra for each class were defined as the spectra with the minimal mean distance to the class. The implementation of the analysis presented in this manuscript is provided online (Material S1).

3. Results

3.1. MALDI-TOF MS analysis of macrophages stimulated with IFN-γ and IL-4

Human monocyte-derived macrophages were stimulated with IFN-γ and IL-4 for 18 h; these treatments are known to polarise macrophages towards M1 and M2 profiles, respectively [9]. Whole cells were then analysed using MALDI-TOF MS, and the representative spectra are shown (Fig. 1A). The spectrum of unstimulated macrophages was composed of numerous peaks concentrated in the range of 2–6 kDa with a major peak at a mass/charge (*m/z*)=4965. The spectral representation of macrophages stimulated with IFN-γ and IL-4 showed dramatic differences between the treatments (Fig. 1A).

The reproducibility of the MALDI-TOF MS data obtained from different macrophage samples was assessed using a virtual gel view representation and hierarchical clustering based on common and specific peaks of spectra. All samples were grouped into three major clusters that were representative of unstimulated macrophages, IL-4-stimulated macrophages and IFN-γ-stimulated macrophages (Fig. 1B). Weak variations in *m/z* values and/or peak intensities were observed among the samples within the same cluster. These variations led us to define reference spectra. To this end, the spectra of the various samples were analysed by comparing the presence or absence of the peaks and by integrating the variability of the intensities of shared peaks. This approach allowed for the identification of specific fingerprints of IFN-γ- and IL-4-stimulated macrophages. When IFN-γ stimulated macrophages were compared with unstimulated macrophages, 20 peaks specifically appeared (see also Table 1) and 13 peaks that were present in the unstimulated macrophages were absent in the IFN-γ-stimulated samples (Fig. 1C). Furthermore, the intensity of the major peak at *m/z*=4965 in the unstimulated macrophages was clearly reduced in the IFN-γ-stimulated samples. When macrophages stimulated with IL-4 were compared with unstimulated macrophages, we found that 22 peaks appeared (see also Table 1) and 13 peaks that were present in the unstimulated macrophages disappeared in the IL-4-stimulated samples (Fig. 1C). The spectra obtained in response to IFN-γ and IL-4 were clearly different (Fig. 1C), demonstrating that the M1 and M2 agonists altered the MALDI-TOF MS fingerprints.

As macrophages are known to be versatile cells, we analysed the time course of their MALDI-TOF MS responses to IFN-γ and IL-4. Differences in time and stimulation were assessed by hierarchical clustering. After 9 h of stimulation, the spectra of macrophages stimulated with IFN-γ or IL-4 were similar and resembled those of unstimulated macrophages. In contrast, macrophages stimulated with IFN-γ or IL-4 for 18, 36 and 48 h clustered according to the agonist (Fig. S1). These results suggest that macrophages exhibited distinct patterns of activation only after 18 h of stimulation. All subsequent analyses were therefore conducted after 18 h of stimulation. Taken together, these results demonstrated that the whole-cell MALDI-TOF MS approach is useful for analysing the activation status of human macrophages.

3.2. MALDI-TOF MS analysis of macrophages stimulated with M1- and M2-related agonists

It is commonly accepted that IFN-γ, TNF and LPS induce an inflammatory (M1-type) response in macrophages whereas IL-4, TGF-β1 and IL-10 induce an immunoregulatory (M2-type) response [25]. We therefore sought to determine whether whole-cell MALDI-TOF MS could detect the M1 and M2 subtypes. We found that the spectra from the macrophages stimulated with M1 agonists clustered together and were separate from the unstimulated macrophages. In addition, we detected differences between the M1 agonists; the response to TNF was close to that of IFN-γ, and the response to the combination of IFN-γ and LPS was closer to that induced by LPS than to the response induced by IFN-γ (Fig. 2A). Second, the spectra of macrophages stimulated with M2 agonists were

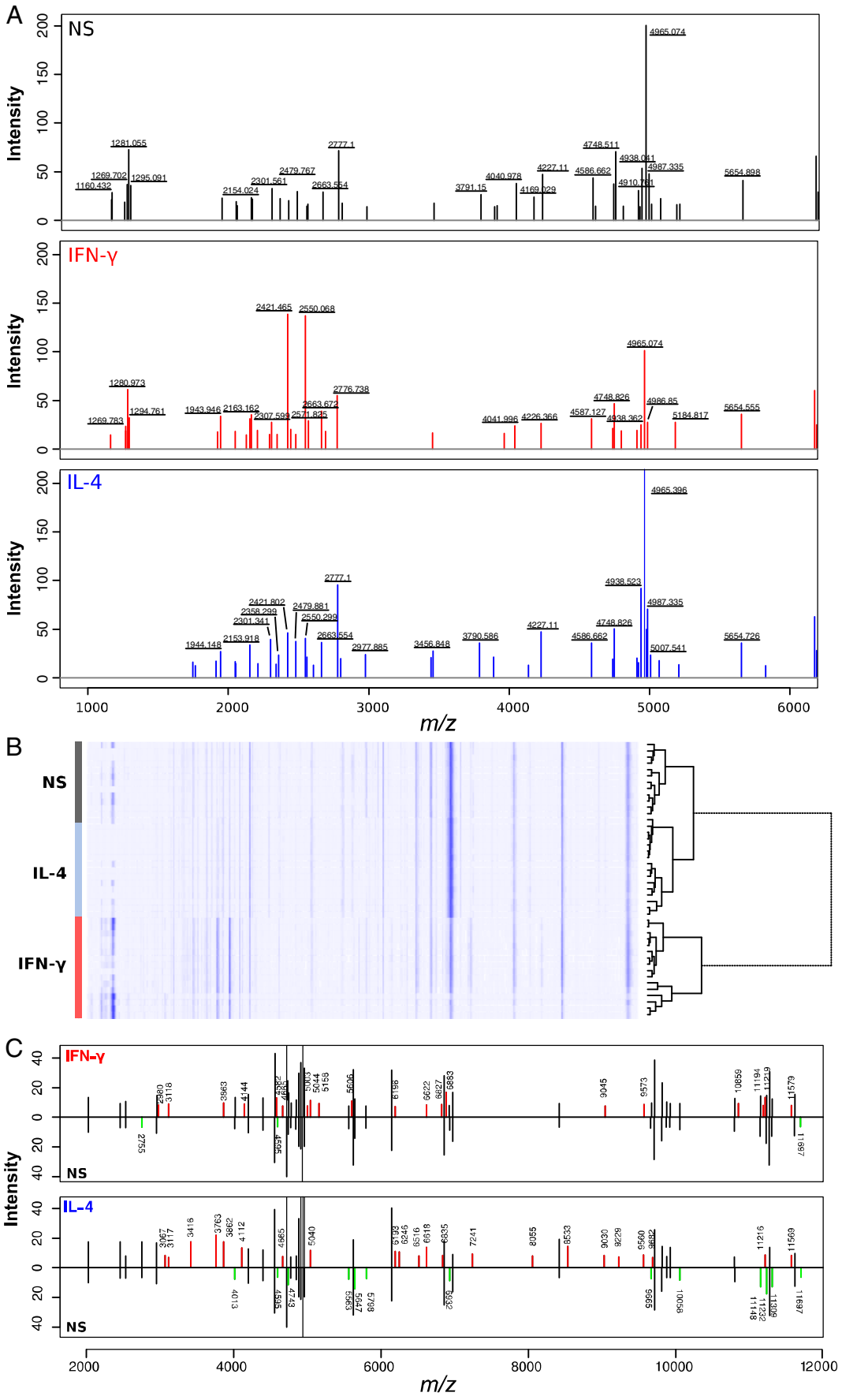

A
NS
IFN-γ
IL-4
Intensity
m/z
B
NS
IL-4
IFN-γ
C
IFN-γ
IL-4
NS
Intensity
m/z

Table 1 – Characteristic peaks of macrophages stimulated with cytokines.
The *m/z* ratio of specific peaks that resulted from the comparison of the reference spectra between stimulated and unstimulated macrophages is shown. Each row corresponds to peaks with different *m/z* values, and peaks that were considered identical are presented in the same row. The peaks that were specific for each experimental condition are underlined. *, M1-related peaks. **, M2-related peaks.

IFN-γ	TNF	IL-4	IL-10
2980	2978	–	2977
–	–	3067	–
3118	3116	3117	–
–	3417	3416	–
–	–	3763	–
3863	3863	3862	–
–	–	–	3962
–	–	4112	–
4144	–	–	4141
4582	–	–	–
4665	4662	4665	–
–	–	–	4978
5003	–	–	–
5044	–	5040	–
5158	–	–	–
–	5387	–	5387
5606	5601	–	–
–	–	–	6052
–	–	–	6080
–	6107	–	–
6196	–	6193	–
–	–	6246	–
–	–	6516	–
–	–	–	6545
6622	6618	6618	–
6827*	6826*	–	–
–	–	6835**	6833**
–	–	–	–
6883	–	–	–
–	–	–	6912
–	6920	–	–
–	7234	7241	7237
–	–	8055	–
–	–	8533	–
–	–	9030	–
–	9039	–	–
9045	–	–	–
–	–	9229	–
–	–	9560**	9561**
–	9565	–	–
9573	–	–	–
–	–	9682	–
10589	–	–	–
–	11187	–	–
11194	–	–	–
–	–	–	11206
11219	–	11216	–
-	11565	11569	–
11579	–	–	11579

Fig. 1 – MALDI-TOF MS spectra of macrophages. A, Monocytes from one healthy blood donor were differentiated into macrophages. Macrophages were stimulated with IFN-γ or IL-4 for 18 h. Macrophages were then collected in 10 μL of PBS, and 1 μL was deposited onto the MALDI target. Representative MALDI-TOF MS spectra are shown. B–C, Macrophages from different blood donors were stimulated with IFN-γ and IL-4 for 18 h. The spectra were arranged in a pseudo-gel format using a gel view representation (B). Spectrum classification resulted in reference spectra. The reference spectra from stimulated macrophages were plotted against the reference spectra from unstimulated macrophages (C). The peaks that were detected when macrophages were stimulated are in red, and the peaks that were present in unstimulated macrophages but not in stimulated macrophages are in green. NS: non-stimulated macrophages.

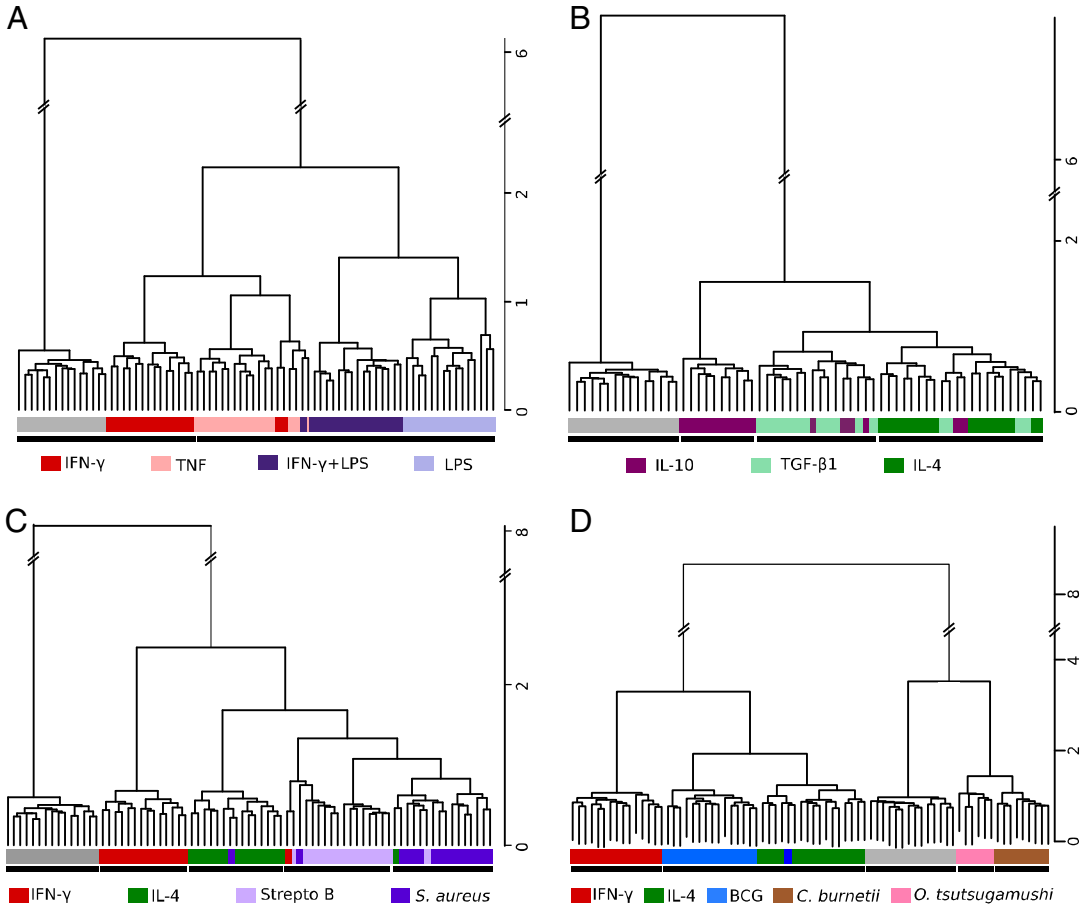


Fig. 2 – Hierarchical clustering of activated macrophages. Macrophages were stimulated with different agonists for 18 h. The results are shown as hierarchical clustering of the data. Macrophages were activated with M1-related agonists (A), M2-related agonists (B), extracellular bacteria (C) and intracellular bacteria (D). Unstimulated macrophages are presented in grey. Strepto B: group B *Streptococcus*.

distinct from those of unstimulated macrophages, and distinct responses to IL-4, TGF-β1 and IL-10 were identified (Fig. 2B), suggesting that MALDI-TOF MS discriminated M2 subtypes. When reference spectra were used to characterise common M1 and M2 peaks, we found that only one peak (*m*/z=6,826) was common to the spectra induced by IFN-γ and TNF and was absent in the spectra induced by IL-4 and IL-10 (Table 1). Similarly, two peaks (*m*/z=6835 and 9560) were common to the macrophages stimulated with IL-4 and IL-10 but were absent in the macrophages stimulated with IFN-γ and TNF. In contrast, 8 specific peaks characterised the response of macrophages to IFN-γ when compared with the responses to TNF, IL-4 and IL-10. The responses to TNF, IL-4 and IL-10 included 4, 10 and 7 specific peaks, respectively, compared with the other agonists. The list and the *m*/z values of these peaks are shown in Table 1. Taken together, these results show that whole-cell MALDI-TOF MS analysis can discriminate macrophages according to their subtypes.

3.3. *MALDI-TOF MS analysis of macrophages stimulated with bacterial pathogens*

To assess the ability of whole-cell MALDI-TOF MS to detect pathogen-associated fingerprints, we investigated the responses of macrophages to representative extra- and intracellular bacteria. The signatures induced by extracellular bacteria including group B *Streptococcus* and *S. aureus*, were highly reproducible and were specific to each type of bacteria. Analysis of the reference spectra showed that 5 and 6 peaks were specifically associated with the responses to *S. aureus* and group B *Streptococcus*, respectively, as compared with the unstimulated macrophages (Table 2). The responses to bacterial organisms were closer to those induced by IL-4 than to those induced by IFN-γ (Fig. 2C), suggesting that the responses were M2-related. Next, the responses of macrophages to intracellular bacteria, such as BCG, *O. tsutsugamushi* and *C. burnetii*, were investigated. Again, these bacteria induced specific responses. Analysis of the reference spectra obtained in response to intracellular bacteria showed that 6, 4 and 8 peaks were specifically associated with the responses to BCG, *O. tsutsugamushi* and *C. burnetii*, respectively (Table 3). The BCG-stimulated macrophages clustered with the IL-4-stimulated samples, suggesting that BCG induced an M2-type response. In contrast, the *O. tsutsugamushi* and *C. burnetii* spectra clustered together and were closer to the unstimulated macrophages than to the IFN-γ- or IL-4-stimulated macrophages (Fig. 2D). These data suggest that M1/M2 polarisation does not completely describe the multifaceted activation of macrophages. They also

Table 2 – Characteristic peaks of macrophages stimulated with extracellular bacteria.
The *m/z* ratio of specific peaks that resulted from the comparison of the reference spectra between stimulated and unstimulated macrophages is shown. Each row corresponds to peaks with different *m/z* values, and peaks that were considered identical are presented in the same row. The peaks that were specific for each experimental condition are underlined.

IFN-γ	IL-4	*S. aureus*	Group B *Streptococcus*
–	–	–	2135
–	–	–	2271
–	–	2641	2639
2980	–	–	–
–	3067	3066	–
3118	3117	–	–
–	–	3386	3386
–	3416	3415	3416
–	3763	3762	3762
3863	3862	3860	3860
–	4112	4110	4109
4144	–	–	–
–	–	–	4426
4582	–	–	4578
4665	4665	–	4660
–	–	4995	4993
5003	–	–	–
5044	5040	5039	5038
5158	–	–	–
5606	–	–	–
–	–	–	6010
–	–	6053	6052
–	–	6102	6099
6196	6193	6191	6192
–	6246	6244	6244
–	6516	6510	–
–	–	–	6538
6622	6618	6615	6615
6827	–	–	–
–	6835	–	–
6883	–	–	6885
–	–	7003	–
–	7241	7238	–
–	8055	–	–
–	–	8138	–
–	–	8178	–
–	8533	8528	8528
–	9030	9025	9027
9045	–	–	–
–	9229	9227	9226
–	–	9552	–
–	9560	–	–
9573	–	–	–
	9682	–	–
10589	–	–	–
–	–	10848	–
11194	–	–	–
–	–	–	11209
11219	11216	–	–
–	11569	–	11564
11579	–	–	–

indicate that whole-cell MALDI-TOF MS may be useful for assessing specific macrophage responses to bacterial pathogens.

4. Discussion

We have recently used MALDI-TOF MS to classify intact eucaryotic cells without the extraction of cellular proteins. This method has a high resolution and detects circulating cells, such as monocytes, lymphocytes and neutrophils. It also detects murine and human macrophages and diverse macrophage-related cell lines [6]. Here, we investigated whether the accuracy of the whole-cell MALDI-TOF MS approach enabled the assessment of multifaceted activation of macrophages and, particularly, the conditions leading to M1/M2 polarisation. Analysis of the MALDI-TOF MS profiles of

Table 3 – Characteristic peaks of macrophages stimulated with intracellular bacteria.
The *m/z* ratio of specific peaks that resulted from the comparison of the reference spectra between stimulated and unstimulated macrophages is shown. Each row corresponds to peaks with different *m/z* values, and peaks that were considered identical are presented in the same row. The peaks that were specific for each experimental condition are underlined.

IFN-γ	IL-4	BCG	*O. tsutsugamushi*	*C. burnetii*
–	–	–	2283	2282
–	–	–	–	2339
–	–	–	–	2429
–	–	–	2666	2668
–	–	–	2780	2779
2980	–	–	–	–
–	3067	3071	–	–
–	–	–	3087	3087
3118	3117	–	–	–
–	–	–	–	3153
–	3416	3417	–	–
–	3763	3766	3768	3768
–	–	–	3791	–
3863	3862	3862	3865	3866
–	4112	4111	–	–
4144	–	–	–	–
–	–	4430	4432	–
4582	–	–	4583	4584
–	–	–	4607	–
4665	4665	–	–	–
–	–	–	4984	–
5003	–	5002	5006	5006
5044	5040	–	–	–
–	–	–	5071	–
5158	–	–	–	–
5606	–	–	–	–
–	–	–	–	5779
–	–	6014	–	6017
–	–	6054	–	–
–	–	–	–	6061
6196	6193	6195	6196	6195
–	6246	6247	6251	–
–	6516	6516	–	–
–	–	–	–	6544
6622	6618	–	6621	6627
6827	–	–	–	–
–	6835	6832	–	–
6883	–	–	–	–
–	7241	7240	–	7244
–	–	7454	7458	–
–	8055	–	–	–
–	–	8181	–	–
–	–	–	–	8272
–	8533	–	–	–
–	9030	–	–	–
9045	–	–	–	–
–	9229	–	–	–
–	9560	–	–	–
9573	–	–	–	–
–	–	9661	–	–
–	9682	–	–	–
–	–	9877	–	–
–	–	9921	–	–
–	–	–	–	9932
10589	–	–	–	–
–	–	10855	–	–
11194	–	–	–	–
11219	11216	–	–	–
–	11569	–	–	–

macrophages showed that the responses to IFN-γ and IL-4, two canonical inducers of M1 and M2 macrophages, respectively, clustered into distinct groups. The resulting spectra of the M1/M2 polarised macrophages were highly reproducible. Hence, MALDI-TOF MS profiles of macrophages stimulated with IFN-γ and IL-4 may be considered fingerprints of M1 and M2 macrophages, respectively. To our knowledge, only two studies have explored the ability of whole-cell MALDI-TOF MS to assess various states of a unique cell type. Small differences in MALDI-TOF MS spectra are sufficient to assess the viability of CHO cells [26]. Marvin-Guy et al. demonstrated that the spectra from T84 epithelial cells in the growth phase are very different from those of confluent cells [27]. Our results suggested that whole-cell MALDI-TOF MS could be used to assess the plasticity of cells, such as macrophages.

The inflammatory cytokine, TNF, and the bacterial component, LPS, are considered to be M1 agonists; thus, we investigated whether whole-cell MALDI-TOF MS could be used to study the activation of macrophages by TNF and LPS. The TNF and LPS responses were close to the classical M1 response induced by IFN-γ although the TNF and LPS responses exhibited specific features. This underlines that the accuracy of the technique allowed to detect distinct subgroups in the M1 polarised macrophages. The combination of IFN-γ and LPS also led to an M1-type response, and was closer to the profile induced by LPS alone. This finding may be a result of the differences between the priming and activation of macrophages. Indeed, it is well known that IFN-γ does not induce cytokine release or promote the microbicidal activity of macrophages, but it amplifies these functions in the macrophage response to LPS [28]. IFN-γ and LPS differentially modulate gene expression in macrophages [29], and it has recently been demonstrated that proteome profiles are different in human macrophages stimulated with IFN-γ, LPS and the combination of IFN-γ+LPS [18]. We also asked whether whole-cell MALDI-TOF MS could be used to discriminate different types of M2 macrophages. It has been suggested that M2 macrophages include at least three subsets with different phenotypic characteristics, repertoires of gene expression, abilities to produce cytokines and functional activities [30,31]. We found that IL-10 and TGF-β1 induced spectra that were close to but distinct from those induced by IL-4. Taken together, these results demonstrated that MALDI-TOF MS approach was able to discriminate different subtypes of M1 and M2 macrophages.

Macrophages are known to play essential roles in host defence by recognising, engulfing and killing microorganisms. They respond to a broad variety of bacteria through a unique pattern of gene expression changes, referred to as the "common host response" [12,32–34]. Evidence also indicates that bacterial pathogens can interfere with macrophage activation. We found that the MALDI-TOF MS spectra of macrophages stimulated with the extracellular bacteria such as *S. aureus* and group B *Streptococcus* evoked M2 profiles. These M2-type profiles may be a result of the production of IL-10 by macrophages stimulated with *S. aureus* [35] and the inhibition of the NF-κB pathway by group B *Streptococcus* [36]. The MALDI-TOF MS approach discriminated the responses to *S. aureus* and group B *Streptococcus*. The MALDI-TOF MS profiles of macrophages stimulated with intracellular bacteria were clustered into a group that included BCG- and cytokine-stimulated macrophages and a distinct cluster composed of *O. tsutsugamushi*, the agent of scrub typhus, *C. burnetii*, the agent of Q fever, and unstimulated macrophages. Interestingly, BCG-stimulated macrophages exhibited a more similar response to macrophage stimulated with IL-4 than with IFN-γ, suggesting an M2-type response. It has been demonstrated that bacteria, such as *Mycobacterium tuberculosis*, induce an M2 phenotype *in vitro* [37], which is consistent with our findings. We previously demonstrated that *O. tsutsugamushi* replicates in human monocytes and upregulates the expression of genes that are associated with the M1 polarisation of macrophages [22]. This apparent discrepancy with our current results may be attributed to a variety of causes. For example, exocytosis may interfere with the fingerprints observed in the MALDI-TOF MS analysis, as macrophages are known to produce high levels of cytokines in response to pathogens. Moreover, the response to heat-killed *O. tsutsugamushi* is dramatically different from that of live organisms [22], suggesting that the use of macrophages and heat-killed pathogens instead of monocytes and live organisms may affect the cellular response to bacterial aggression. This is highlighted by the transcriptional responses of monocytes and macrophages to *C. burnetii*. Indeed, *C. burnetii* induces a transcriptional M1-type response in monocytes and an atypical M2-type profile in macrophages [12]. Taken together, these results show that an easy assay using whole-cell MALDI-TOF was able to detect specific fingerprints of macrophages stimulated with various bacteria. Whole-cell MALDI-TOF may therefore be suitable to explore the immune response.

In conclusion, the accuracy and reproducibility of the whole-cell MALDI-TOF MS approach enables the analysis of different types of macrophage activation. This fast and inexpensive method permits the classification of M1- and M2-associated macrophage phenotypes and reveals the diversity of proteomic responses to cytokines and bacterial pathogens. The specific fingerprints we found may be useful for studying the activation of macrophages in pathological conditions.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2012.07.046.

Conflict of interest

There is no financial/commercial conflict of interests.

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REFERENCES

[1] Huang Q, Huang Q, Chen W, Wang L, Lin W, Lin J, et al. Identification of transgelin as a potential novel biomarker for gastric adenocarcinoma based on proteomics technology. J Cancer Res Clin Oncol 2008;134:1219-27.
[2] Visentin M, Simula MP, Sartor F, Petrucco A, De Re V, Toffoli G. Identification of proteins associated to multi-drug resistance in LoVo human colon cancer cells. Int J Oncol 2009;34:1281-9.

[3] Fan Y, Wang J, Yang Y, Liu Q, Fan Y, Yu J, et al. Detection and identification of potential biomarkers of breast cancer. J Cancer Res Clin Oncol 2010;136:1243-54.
[4] Welker M. Proteomics for routine identification of microorganisms. Proteomics 2011;11:3143-53.
[5] Buchanan CM, Malik AS, Cooper GJS. Direct visualisation of peptide hormones in cultured pancreatic islet alpha- and beta-cells by intact-cell mass spectrometry. Rapid Commun Mass Spectrom 2007;21:3452-8.
[6] Ouedraogo R, Flaudrops C, Ben Amara A, Capo C, Raoult D, Mege JL. Global analysis of circulating immune cells by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. PLoS One 2010;5:e13691.
[7] Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S. Host innate immune receptors and beyond: making sense of microbial infections. Cell Host Microbe 2008;3:352-63.
[8] Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. Immunity 2005;23:344-6.
[9] Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. Front Biosci 2008;13:453-61.
[10] Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol 2008;8:958-69.
[11] Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol 2011;11:723-37.
[12] Benoit M, Desnues B, Mege JL. Macrophage polarization in bacterial infections. J Immunol 2008;181:3733-9.
[13] Biswas SK, Sica A, Lewis CE. Plasticity of macrophage function during tumor progression: regulation by distinct molecular mechanisms. J Immunol 2008;180:2011-7.
[14] Cassetta L, Cassol E, Poli G. Macrophage polarization in health and disease. Sci World J 2011;11:2391-402.
[15] Gordon S. Alternative activation of macrophages. Nat Rev Immunol 2003;3:23-35.
[16] Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 2004;25:677-86.
[17] Slomianny M-C, Dupont A, Bouanou F, Beseme O, Guihot AL, Amouyel P, et al. Profiling of membrane proteins from human macrophages: comparison of two approaches. Proteomics 2006;6:2365-75.
[18] Brown J, Wallet MA, Krastins B, Sarracino D, Goodenow MM. Proteome bioprofiles distinguish between M1 priming and activation states in human macrophages. J Leukoc Biol 2010;87:655-62.
[19] Kraft-Terry SD, Gendelman HE. Proteomic biosignatures for monocyte-macrophage differentiation. Cell Immunol 2011;271:239-55.
[20] Drancourt M, Roux V, Fournier PE, Raoult D. rpoB gene sequence-based identification of aerobic Gram-positive cocci of the genera *Streptococcus*, *Enterococcus*, *Gemella*, *Abiotrophia*, *and Granulicatella*. J Clin Microbiol 2004;42:497-504.
[21] Delaby A, Gorvel L, Espinosa L, Lépolard C, Raoult D, Ghigo E, et al. Defective monocyte dynamics in q Fever granuloma deficiency. J Infect Dis 2012;205:1086-94.
[22] Tantibhedhyangkul W, Prachason T, Waywa D, El Filali A, Ghigo E, Thongnoppakhun W, et al. *Orientia tsutsugamushi* stimulates an original gene expression program in monocytes: relationship with gene expression in patients with scrub typhus. PLoS Negl Trop Dis 2011;5:e1028.
[23] Ghigo E, Imbert G, Capo C, Raoult D, Mege JL. Interleukin-4 induces *Coxiella burnetii* replication in human monocytes but not in macrophages. Ann N Y Acad Sci 2003;990:450-9.
[24] Ryan CG, Clayton E, Griffin W, Sie S, Cousens D. SNIP, a statistics-sensitive background treatment for the quantitative analysis of PIXE spectra in geoscience applications. Nucl Inst Methods Phys Res B 1988;34:396-402.
[25] Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol 2010;11:889-96.
[26] Feng H, Sim LC, Wan C, Wong NSC, Yang Y. Rapid characterization of protein productivity and production stability of CHO cells by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 2011;25:1407-12.
[27] Marvin-Guy LF, Duncan P, Wagnière S, Antille N, Porta N, Affolter M, et al. Rapid identification of differentiation markers from whole epithelial cells by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry and statistical analysis. Rapid Commun Mass Spectrom 2008;22: 1099-108.
[28] Gifford GE, Lohmann-Matthes ML. Gamma interferon priming of mouse and human macrophages for induction of tumor necrosis factor production by bacterial lipopolysaccharide. J Natl Cancer Inst 1987;78:121-4.
[29] Yu SF, Koerner TJ, Adams DO. Gene regulation in macrophage activation: differential regulation of genes encoding for tumor necrosis factor, interleukin-1, JE, and KC by interferon-gamma and lipopolysaccharide. J Leukoc Biol 1990;48:412-9.
[30] Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002;23:549-55.
[31] Mège J-L, Mehraj V, Capo C. Macrophage polarization and bacterial infections. Curr Opin Infect Dis 2011;24:230-4.
[32] Nau GJ, Richmond JFL, Schlesinger A, Jennings EG, Lander ES, Young RA. Human macrophage activation programs induced by bacterial pathogens. Proc Natl Acad Sci USA 2002;99: 1503-8.
[33] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006;124:783-801.
[34] Jenner RG, Young RA. Insights into host responses against pathogens from transcriptional profiling. Nat Rev Microbiol 2005;3:281-94.
[35] Frodermann V, Chau TA, Sayedyahossein S, Toth JM, Heinrichs DE, Madrenas J. A modulatory interleukin-10 response to staphylococcal peptidoglycan prevents Th1/Th17 adaptive immunity to *Staphylococcus aureus*. J Infect Dis 2011;204:253-62.
[36] Fong CHY, Bebien M, Didierlaurent A, Nebauer R, Hussell T, Broide D, et al. An antiinflammatory role for IKKbeta through the inhibition of "classical" macrophage activation. J Exp Med 2008;205:1269-76.
[37] Rajaram MVS, Brooks MN, Morris JD, Torrelles JB, Azad AK, Schlesinger LS. *Mycobacterium tuberculosis* activates human macrophage peroxisome proliferator-activated receptor gamma linking mannose receptor recognition to regulation of immune responses. J Immunol 2010;185:929-42.

ARTICLE 3

Whole-cell MALDI TOF Mass Spectrometry: a Tool for Immune Cell Analysis and Characterization

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Les méthodes utilisées dans les deux articles précédents sont décrites en détail dans cet article. Y figurent en particulier les logiciels associés au spectromètre de masse. Le logiciel R qui permet de s'affranchir des logiciels dédiés associés au spectromètre de masse et son utilisation sont également présentés de façon succincte, le script utilisé étant, lui, présenté en annexe.

Chapter 12

Whole-Cell MALDI-TOF Mass Spectrometry: A Tool for Immune Cell Analysis and Characterization

Richard Ouedraogo, Julien Textoris, Aurélie Daumas, Christian Capo, and Jean-Louis Mege

Abstract

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is widely used in proteomics. It has been recently demonstrated that MALDI-TOF MS can be used to identify and classify numerous bacterial species or subspecies. We applied MALDI-TOF MS directly to intact mammalian cells, and we found that this method is valuable to identify human circulating cells and cells involved in the immune response including macrophages. As macrophages are characterized by a high degree of plasticity in response to their microenvironment, we stimulated human macrophages with cytokines, bacterial products, and a variety of bacteria. We found that MALDI-TOF MS discriminated unstimulated and stimulated macrophages, and also detected multifaceted activation of macrophages. We conclude that whole-cell MALDI-TOF MS is an accurate method to identify various cell types and to detect subtle modifications in cell activity.

Key words Mass spectrometry, Matrix-assisted laser desorption/ionization time-of-flight, Intact cell, Macrophage, Cell activation

1 Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a powerful tool for the analysis of ionized molecules (i.e., proteins) by measuring their mass/charge (m/z) ratio. This technique is currently used in biochemistry to identify peptides, proteins, posttranslational modifications of proteins [1, 2], and nucleic acids [2, 3]. Typically, in cell biology, using MALDI-TOF MS to analyze cellular protein composition requires a critical cell lysis step, as well as a variety of fractionation and separation steps, including affinity separation methods, gel electrophoresis, chromatographic separations [4]. Combined with gel electrophoresis, MALDI-TOF MS allows for the study of the proteome [5] and the identification of a large number of proteins

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in the proteome, the secretome, and membranes from activated macrophages [6]. Seemingly, MALDI-TOF MS can identify M1 responses of macrophages, such as the response to interferon (IFN)-γ and lipopolysaccharide (LPS) [5]. Using pulse stable isotope labeling of amino acids in cell culture, Kraft-Terry et al. identified a bioprofile in *macrophage colony-stimulating factor*-differentiated monocytes that is consistent with an M2 profile [6]. However, these approaches based on cell component separation require large sample quantities and cannot be used to analyze clinical specimens. Various attempts have been made to study single mammalian cells by MALDI-TOF MS, but, to date, these methods have been proven fastidious, and the biological information extracted is limited [7].

New applications of the MALDI-TOF MS method have been introduced recently in bacteriology laboratories. The fingerprints of intact bacteria allow rapid identification and taxonomic classification of numerous bacterial species and subspecies [8–10]. Using databases established from isolated bacterial species, the identification of many bacterial species in clinical samples [11] is fast, easy to perform, and inexpensive.

The MALDI-TOF MS procedure for the identification of bacterial species was expanded to three mammalian cell lines in 2006 [4]. More recently, 66 cell lines, representing 34 species from insects to primates, have been identified by MALDI-TOF MS [12]; but this method involves ethanol inactivation and formic acid–acetonitrile extraction. We applied MALDI-TOF MS directly to intact cells, and found this method highly valuable to the identification of human circulating cells, and cells involved in the immune response, including macrophages [13]. In addition, macrophages have a high degree of plasticity, and adapt quickly in response to their microenvironment. They sense microorganisms through receptors that bind conserved and ubiquitous microbial motifs, such as LPS. Macrophages stimulated with type I cytokines, i.e., IFN-γ, Tumor Necrosis Factor (TNF), or bacterial products, i.e., LPS, adopt an M1 macrophage phenotype which is inflammatory, tumoricidal, and microbicidal. Macrophages stimulated with interleukin (IL)-4 or IL-10, adopt an M2 macrophage phenotype, which regulates inflammatory and immune responses, and is only weakly tumoricidal and microbicidal [14]. Hence, macrophages constitute a model of choice to assess the accuracy of whole-cell MALDI-TOF MS to detect subtle modifications in cell activity [15].

We describe here the effective use of MALDI-TOF MS to identify many intact eukaryotic cell populations by creating a database of known samples. The experimental protocol, bioinformatics analysis of whole-cell MALDI-TOF MS spectra, and the comparison of unknown samples to the database allow the identification of various cell types within heterogeneous samples [13] or multifaceted activation of macrophages [15].

2 Materials

The different cell types or stimulated cells were prepared separately to establish databases. Experiments with mixed cell types (circulating cells) are indicated. Prepare sterile solutions for cell isolation and culture. Prepare and store all reagents at 4 °C. Prepare MALDI-TOF matrix just before use.

2.1 Human Circulating Cells

1. Isolate peripheral blood mononuclear cells (PBMCs) from leukopacks (or blood donors) by Ficoll gradient (MSL, Eurobio), as previously described [16].
2. Prepare $CD14^+$ monocytes using CD14 MicroBeads and the MACS separation system (Miltenyi Biotec), according to the manufacturer's protocol.
3. Prepare T $CD3^+$ lymphocytes using CD3 MicroBeads and the MACS separation system (Miltenyi Biotec), according to the manufacturer's protocol.
4. Obtain monocyte-derived macrophages (MDMs) by incubating monocytes (10^6 cells in 6-well plates) 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 for 4 days. Replace human serum with fetal calf serum (FCS) for 3 additional days. The obtained cell population was identified as macrophages (more than 95 %) by acquisition of membrane CD68 expression and CD14 down-modulation.
5. Obtain dendritic cells (DCs) by incubating monocytes with 1,000 U/mL human recombinant *granulocyte macrophage-colony stimulating factor* (GM-CSF) and 500 U/mL of human recombinant IL-4 in RPMI 1640 containing 10 % FCS, 2 mM L-glutamine, 100 IU/mL penicillin, and 50 µg/mL streptomycin for 7 days. Change the medium every 3 days and add again 1,000 U/mL GM-CSF and 500 U/mL IL-4. The obtained cells expressed high levels of CD11c and CD1a, and low levels of CD14 and CD68.
6. Obtain polymorphonuclear cells (PMNs) after Ficoll centrifugation, by sedimentation of red blood cells (RBCs) within dextran T500 (1.5 % (w/v), Pharmacosmos) (*see* **Note 1**).
7. Obtain red blood cells (RBCs) by 1/1,000 dilution of blood in Phosphate-buffered saline (PBS).

2.2 Noncirculating Cells

The preparation of several cell populations including mammalian primary cells and cell lines, one *Xenopus laevis* cell line (XTC-2 cells), and four types of amoebae (*Acanthamoeba polyphaga*, *Acanthamoeba castellanii*, *Hartmannella vermiformis*, *Poteriochromonas melhamensis*) is described in ref. 13.

2.3 Macrophage Stimulation

1. Stimulate MDMs with 20 ng/mL of human recombinant IFN-γ, IL-4, IL-10, or TNF (purchased from R&D Systems or other suppliers) for different time points. Also stimulate MDMs with 1 μg/mL LPS from *Escherichia coli* or heat-killed bacteria (50 bacteria per cell) including *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium avium*, *Rickettsia prowazekii*, and *Orientia tsutsugamushi* (*see* **Note 2**)
2. *M. tuberculosis* (CIP H37Rv strain 103471), *M. bovis* (Bacillus Calmette-Guérin, BCG CIP strain 671203), and a clinical isolate of *M. avium* subsp. *hominissuis* were obtained from the Laboratory of Microbiology of the Hospital La Timone (Marseille) [17].
3. *R. prowazekii* strain Breinl (ATCC VR-142), the agent of epidemic typhus [18], and *O. tsutsugamushi* strain Kato (CSUR R163) [19], the agent of scrub typhus, were propagated in L929 cells (*see* **Note 3**).
4. Heat-killed bacteria were obtained after heating microorganisms at 95 °C for 1 h.

2.4 Reagents and Materials for MALDI-TOF MS

1. The matrix solution consists of a 10 mg/mL solution of acid-α-cyano-4-hydroxy cinnamic (HCCA) diluted in 500 μL acetonitrile, 250 μL Milli-Q grade water, and 250 μL trifluoroacetic acid at 10 %. Mix and sonicate for at least 20 min. Centrifuge at 13,000 × *g* for 5 min. Discard the pellet and keep the supernatant. The matrix solution is ready for use (*see* **Note 4**).
2. AutoFlex II mass spectrometer (Bruker Daltonics).
3. Bruker MSP 384 software polished steel target (Bruker Daltonics).
4. FlexControl 3.0 software (Bruker Daltonics).
5. FlexAnalysis 3.3 software (Bruker Daltonics).
6. Biotyper 3.0 software (Bruker Daltonics).
7. ClinProTools 2.2 software (Bruker Daltonics).

3 Methods

3.1 MALDI-TOF Target Preparation

1. Moisten the Bruker MSP 384 polished steel target with hot tap water. Rub with KIMTECH paper. Rub with 70 % ethanol. Rinse with hot tap water by rubbing with KIMTECH paper. Rub finally with 70 % ethanol.
2. Immerse the target in 70 % ethanol and sonicate for at least 15 min.
3. Cover the target with 500 μL to 1 mL of trifluoroacetic acid. Rub with KIMTECH paper. Rinse with high performance liquid chromatography (HPLC) grade water without rubbing. Dry the target at room temperature (*see* **Note 5**).

3.2 Preparation of Deposits

1. Centrifuge cells (2×10^6 cells per assay) at $300 \times g$ for 5 min and wash them in PBS without Ca^{2+} or Mg^{2+} (*see* **Note 6**). Centrifuge to remove traces of culture medium. Collect cell pellets in 20 µL of PBS without Ca^{2+} or Mg^{2+}. Freeze cells for 2–3 days before analysis at –80 °C.
2. Thaw gently samples on ice (4 °C) (*see* **Note 7**).
3. Put the Bruker MSP 384 polished steel target on a horizontal support to obtain uniform deposits throughout the spot (*see* **Note 8**).
4. Homogenize cells in Eppendorf tubes before deposition of 1 µL on the MALDI target. Add 1 µL of the HCCA matrix to the sample on the target. Avoid mixing the spot with the pipette (*see* **Note 9**).
5. Drop 12–16 different spots of the same sample.
6. The evaporation takes place gradually at room temperature, and the formed HCCA crystals contain dispersed sample molecules.
7. Samples may be immediately analyzed or stored in the dark for several days before analysis.

3.3 Acquisition of Data

1. Insert the Bruker MSP 384 polished steel target containing samples in the Autoflex II mass spectrometer (Bruker Daltonics) outfitted with the Compass 1.2 software suite (consisting of FlexControl 3.0 and FlexAnalysis 3.3 from Bruker Daltonics). Run samples in positive mode, with 240 laser satisfactory shots in 40 shot steps intervals and 40 % laser power, performed in different regions of the analyzed sample spot. A signal-to-noise ratio of 3.0 was selected to define peaks, with a maximum of 100 peaks per spectrum. After the target plate calibration was complete, the AutoExecute command was used to analyze the samples. The processes described below are manufacturer or software defaults, and do not require adjusting.
2. Laser settings. Fuzzy Control, On; Weight, 2.00; Laser power, between 30 and 45 %; Matrix Blaster, 5.
3. Data Evaluation. Peak Selection Masses, 4,000–10,000; Mass Control List, Off; Peak Exclusion, ignore the largest peaks in the defined mass range; Peak Evaluation Processing Method, Default; Smoothing: On; Base-line Subtraction, On; Peak, Resolution higher than 400; Protein maximal resolution, ten times above the threshold.
4. Accumulation. Parent Mode, On; Sum up to 240 satisfactory shots in 40 shot steps; Dynamic Termination, On; Criteria Intensity, Early termination if reaching intensity value of 20,000 for ten peaks.

5. Movement. Spiral large, Maximal allowed number at one raster position; Parent Mode, 80; Quit sample after 25 subsequently failed judgments.
6. Processing. Flex analysis. Method, none; BioTools MS method, none.
7. Sample Carrier. Manual fine control speed, *x* (10,000 µm/s) *y* (20,000 µm/s); Relative *x* (5 µm) *y* (5 µm); Absolute *x* (−2,000 µm) *y* (−2,000 µm); State *x* (0) *y* (0); Random walk, 50 shots at raster spot; Mode, off.
8. Spectrometer. High voltage, switched On; Ion Source 1, 19,99 kV; Ion Source 2, 18,74 kV; Lens, 7 kV; Pulsed Ion Extraction, 330 ns; Polarity, Positive; Matrix Suppression, Mode Gaiting; Gaiting strength, height; Suppress up to, *m/z* 1,500 Da.
9. Detection. Mass range, 2,000–20,137; Mode, Medium Range; Detector Gain, Linear (18×); Sample Rate, 1.00 GS/s; Electronic Gain, Enhanced (100 mV); Real-time Smooth, High; Spectrum, Size (63,463 pts), Delay (29,412 pts).
10. Processing Method. MBT process.
11. Setup. Range, Medium; Laser Frequency, 25 Hz; Autoteaching, On; Instrument-specific Settings: Digitizer Trigger Level (2,000 mV), Digital off Linear (127 cnt), Digital off Reflector (127 cnt); Detector Gain Voltage Offset, Linear (1,300 V), Reflector, 1,400 V; Laser Attenuator, Offset (75 %), Range (15 %); Electronic Gain Button Definitions: Gain, regular (Offset Lin, 100 mV; Offset Ref 100 mV; 200 mV/full scale); enh. (Offset Lin, 51 mV; Offset Ref 51 mV; 100 mV/full scale); highest (Offset Lin, 25 mV; Offset Ref 25 mV; 50 mV/full scale).
12. Calibration. Calibration strategy, Interactive; Mass Control List, Bacterial test standard; Zoom Range, ±5 %; Peak Assignment Tolerance, User Defined (1,000 ppm); Mode, linear.

3.4 Data Analysis

3.4.1 Spectrum Analysis

1. The FlexAnalysis software 3.3 allows raw spectrum processing, baseline subtraction, smoothing, peak list editing, and displays several spectra into one window or superimposes spectra (for the comparison of different types of circulating cells, *see* Fig. 1).
2. The ClinProTools 2.2 software from Bruker Daltonics is used to analyze the variability between different samples. Load spectra of each cell category to create according classes.

   2D representation generated by ClinProTools 2.2. The software selects automatically two peaks that are present in each cell type but have different intensities (here, the peaks 2 and 30). This representation highlights the reproducibility of spectra between spectra of each class (here, ten spectra obtained from ten different blood donors), and the differences between

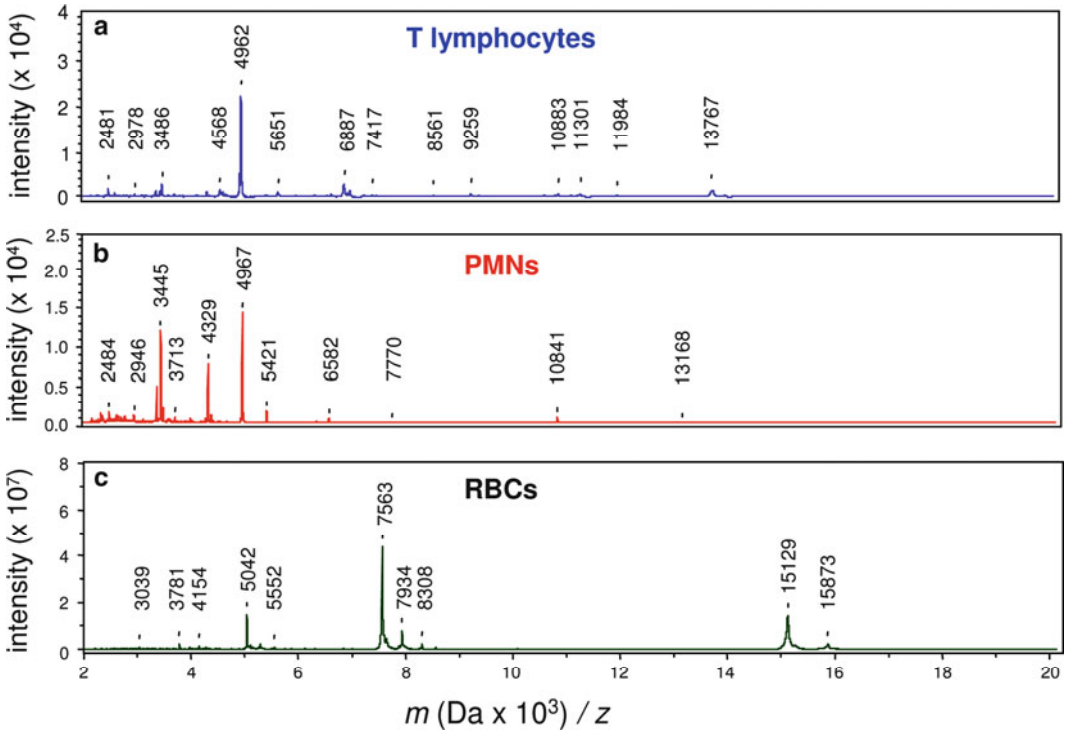

Fig. 1 MALDI-TOF MS spectra of circulating cells. T lymphocytes (**a**), PMNs (**b**), and RBCs (**c**) were isolated from a healthy blood donor. Representative MALDI-TOF MS spectra are shown. The figure is extracted from the ref. 13

the different classes (here, monocytes, T lymphocytes and PMNs) (*see* Fig. 2).

3. Gel-view representation generated by ClinProTools 2.2. This representation compares the reproducibility of spectra within the same class, and the differences between different classes. The different bands represent different peaks of each class. The intensity of bands corresponds to the intensity of detected peaks. This representation shows the reproducibility of spectra within each class (here, four spectra obtained from four different blood donors) and the peaks that are differentially expressed in different classes (here, monocytes and T lymphocytes isolated from each blood donor) (*see* Fig. 3).

3.4.2 Database Creation

1. The Biotyper 3.0 software from Bruker Daltonics is used to create and manage databases. An averaged spectrum for each cell category corresponds to at least ten individual spectra. Here, we created a database that includes 17 mammalian cell types, one *X. laevis* cell line (XTC-2 cells), and four types of amoebae (*A. polyphaga*, *A. castellanii*, *H. vermiformis*, *P. melhamensis*) (*see* **Note 10**).

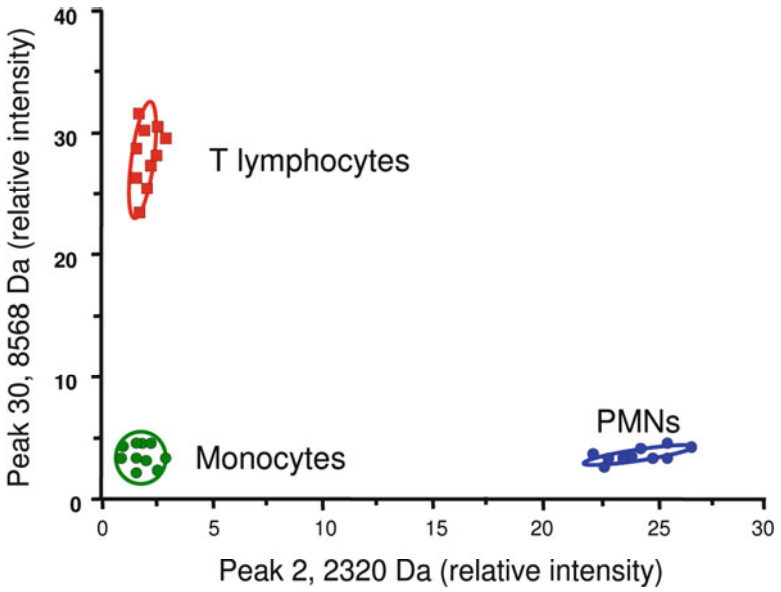

Fig. 2 Reproducibility of MALDI-TOF MS signatures. Monocytes, T lymphocytes, and PMNs were isolated from ten healthy blood donors. MALDI-TOF MS spectra were analyzed using 2D Peak Distribution View. The relative intensities of the two peaks automatically selected were homogenous among blood donors, and the *ellipses* represent the standard deviation within each cell population (monocytes, T lymphocytes, and PMNs, respectively). *See* ref. 13

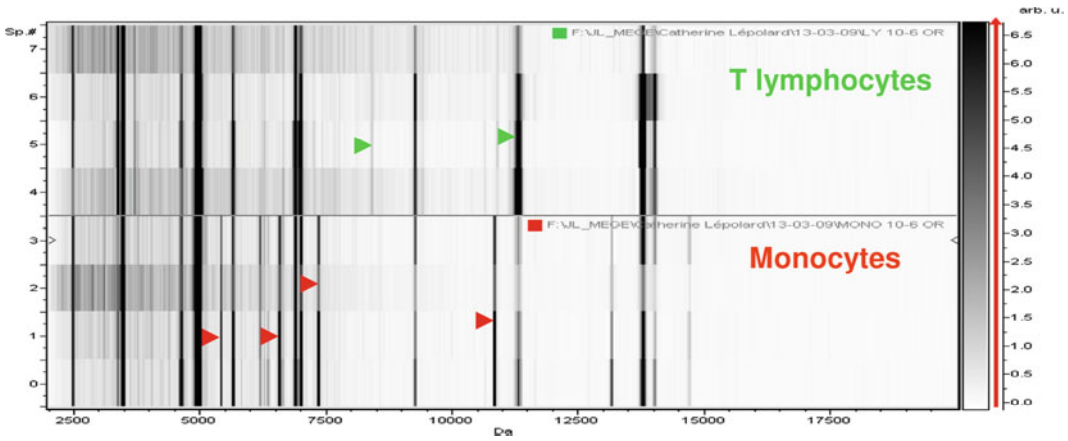

Fig. 3 Gel view representation of monocytes and T lymphocytes. Circulating cells were isolated from four different healthy blood donors. MALDI-TOF MS spectra are presented in Gel View representation. Spectra are shown with *m/z* values on the *x*-axis and the peak intensity (in arbitrary units) is coded with the grey scale presented on the *right*. Major differences between monocytes and T lymphocytes are indicated by *arrowheads*. *See* ref. 13

2. Baselines are automatically subtracted from spectra, and the background noise smoothed. An average spectrum is automatically created using default Biotyper method settings provided by the manufacturer. The sensitivity (the maximum tolerated

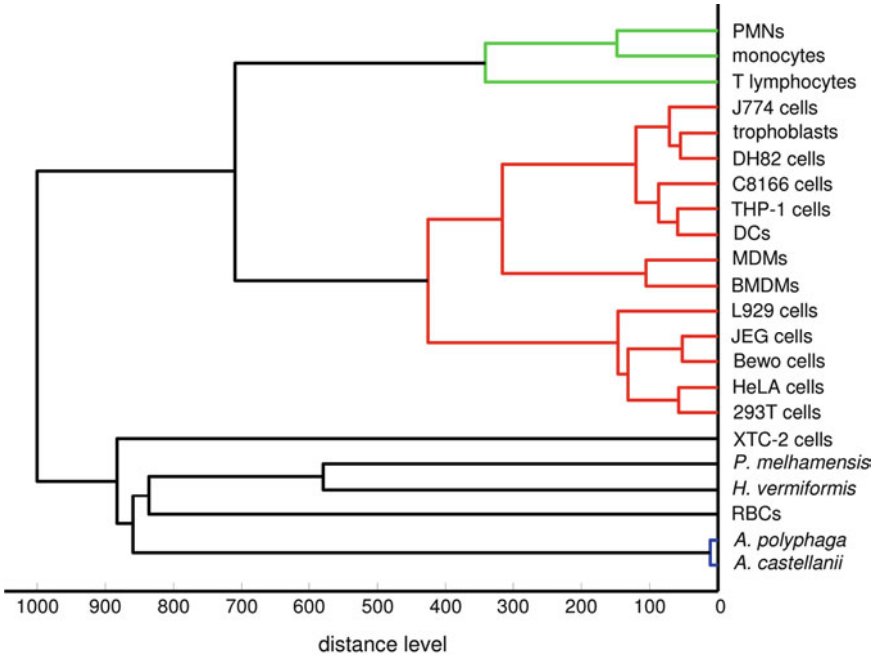

Fig. 4 Dendrogram of 22 eukaryotic cell types. MALDI-TOF MS was performed on 22 cell types with at least 20 spectra per cell type. A mean spectrum for each cell type was added to the database using the BioTyper 3.0 software and the dendrogram creation method. *See* ref. 13

error) of mass spectrum values and spectrum shifts was 200 particles per million. The minimum frequency to benchmark selection of peaks was 25 %, and only peaks with signal/noise intensity above background are automatically selected by the software. An average virtual spectrum consisting of 70 peaks is added to the database as a new reference.

3. The Biotyper 3.0 software is used to generate a dendrogram representation of cell categories according to their protein fingerprint (*see* Fig. 4). This figure shows that mammalian cells (in red and green) and nonmammalian cells (in black and blue) were in two distinct branches of the dendrogram. Circulating nucleated cells (in green) clustered within a subbranch distinct of primary cells (trophoblasts, DCs, MDMs, murine bone marrow-derived macrophages (BMDMs)) and cell lines. Note that human RBCs, which are unnucleated cells, clustered with nonmammalian cells.
4. The Biotyper 3.0 software is used to identify unknown spectra by comparison with database references. The sensitivity and the maximum error tolerated to determine the mean m/z values are 1 Da. Score values between 0.000 and 1.699 indicate that the unknown spectra did not match with known references. Values between 1.700 and 1.899 indicate probable cell identification.

Table 1
Identification of subpopulations by MALDI-TOF MS

Multiple cell population	Identification	Scores
Monocytes + T lymphocytes (equal concentration)	Monocytes	2,250
	T lymphocytes	2,247
PBMCs	Monocytes	2,078
	T lymphocytes	2,024
Whole blood (after hypotonic shock)	PMNs	2,049
	Monocytes	1,585
	T lymphocytes	1,654

The fingerprints of monocytes and T lymphocytes were identified in a mixed population (50 % monocytes, 50 % T lymphocytes) and in PBMCs. In blood, it was possible to identify PMNs (that represent about 70 % of total leukocytes), but not monocytes or T lymphocytes

Scores between 2.000 and 3.000 are considered statistically significant, and allow effective identification of the unknown spectra [13]. This procedure is currently used to identify bacterial species in clinical samples [11]. Here, we extend this method and the scores provided by Bruker Daltonics to identify the different cell populations present in a complex tissue. First, isolated monocytes and T lymphocytes were mixed, and the resulting fingerprint shows that monocytes and T lymphocytes are identified by MALDI-TOF MS (Table 1) The Table 1 also shows that the fingerprints of monocytes and lymphocytes T, respectively, are identified in peripheral blood mononuclear cells. In whole blood that contains leukocytes essentially composed of PMNs, the fingerprint of PMNs is identified, but not those of monocytes and T lymphocytes.

3.5 Macrophage Activation Analysis with R Software

The analysis of MALDI-TOF MS spectra described above is performed using Bruker Daltonics software. We present here a similar analysis performed with an open-source software (R), and specific algorithms that are presented as supplementary material in a recently published manuscript [15].

1. Load raw spectra in R (version 2.14) using the readBrukerFlexData library.
2. Analyze spectra using the MALDIquant library and specific algorithms. The square root of the intensities is used to enhance graphical visualization of the spectra.
3. Correct background using Statistics-sensitive Nonlinear Peak-clipping algorithm for baseline estimation [20]. Peaks are detected using a Signal-to-Noise Ratio of 6.0. The detected

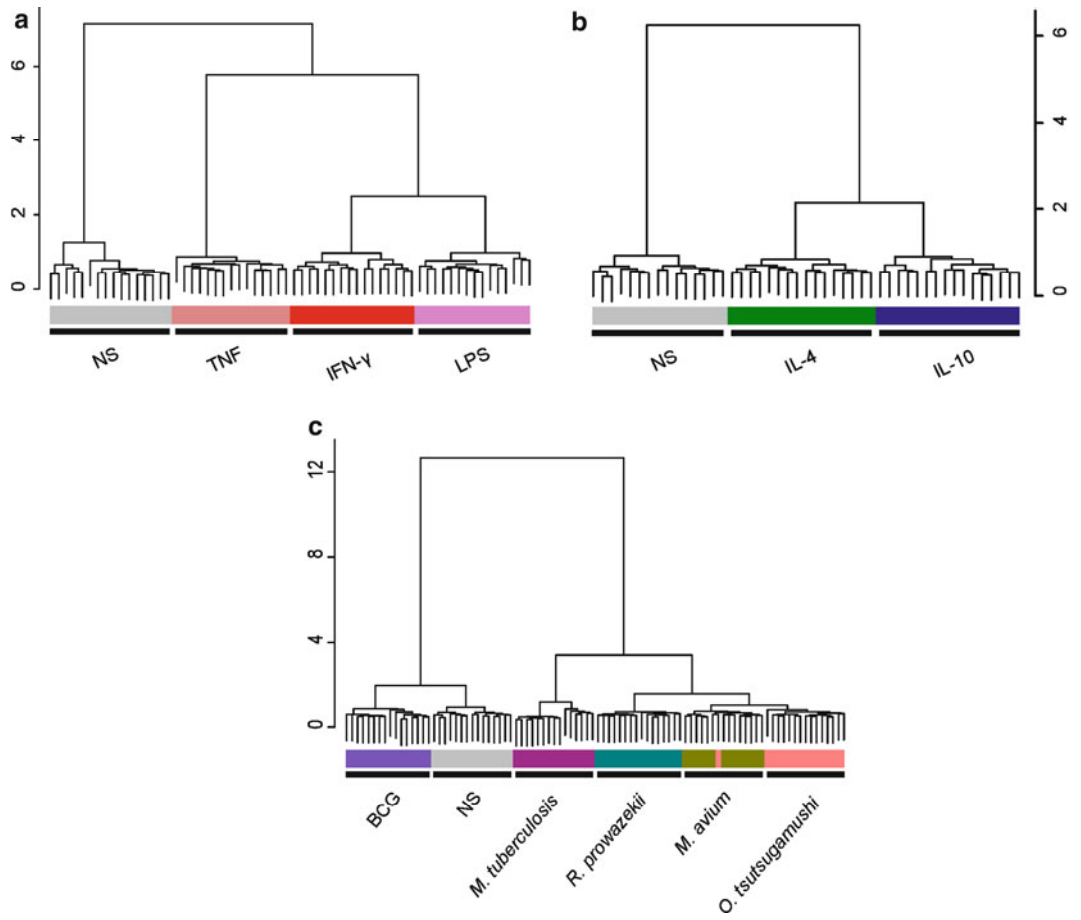

Fig. 5 Hierarchical clustering of activated macrophages. Monocyte-derived macrophages were stimulated with M1-related agonists (**a**), M2-related agonists (**b**), and intracellular bacteria (**c**) for 18 h. Unstimulated macrophages are presented in *grey* (NS)

peaks are considered similar across spectra when the m/z values are within a 2,000 ppm window.

4. Use hierarchical clustering to classify the spectra, ward algorithm for agglomeration, and a dissimilarity matrix based on the Jaccard distance. The Jaccard index measures similarity between boolean sample sets. The Jaccard distance, which measures dissimilarity between sample sets, is complementary to the Jaccard index and is obtained by subtracting the Jaccard coefficient from 1 or by dividing the difference of the sizes of the union and the intersection of two sets by the size of the union. This procedure was used to discriminate unstimulated (in grey) and stimulated macrophages (in colors) (*see* Fig. 5). Note that the responses of macrophages to different M1 agonists clustered but were not superimposable (*see* Fig. 5a). Similarly, the responses of macrophages to IL-4 and IL-10, two M2 agonists,

clustered compared to unstimulated macrophages but were distinct (*see* Fig. 5b). Different intracellular bacteria induced specific signatures (*see* Fig. 5c). Taken together, these results show that MALDI-TOF MS detected the multifaceted activation of macrophages.

4 Notes

1. PMNs must be isolated from remaining RBCs after dextran T500 sedimentation. Lyse RBCs by a 30 s hypotonic shock to obtain pure PMNs. In the absence of lysis, RBC signals were detected in MALDI-TOF MS and masked the detection of PMNs.
2. The stimulation of human MDMs is usually performed in RPMI 1640 supplemented with 10 % FCS, 100 UI/mL penicillin and 50 μg/mL streptomycin.
3. Wash bacteria with PBS to remove the components contained in growth media (such as serum proteins) that may interfere with MALDI-TOF MS spectra.
4. A matrix solution containing crystals does not allow a good ionization of sample molecules, and may affect the quality of spectra.
5. A target improperly cleaned may bias the results. It is therefore very important to take the time to carefully clean targets.
6. Cells may agglutinate in the presence of Ca^{2+} and Mg^{2+}. In addition, salts may interfere with MALDI-TOF MS.
7. Rapid and vigorous thawing alters samples, thus affecting the MALDI-TOF MS analysis.
8. Homogeneous deposits are necessary to obtain reproducible and high-quality spectra.
9. Mixing spots with pipettes alters spectrum quality. It is therefore important to respect the proportions and indicated details.
10. Each laboratory needs to construct its own databases before comparing cell populations and looking at unknown samples. We may export our databases to other laboratories.

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References

1. Johnson RP, El-Yazbi AF, Hughes MF, Schriemer DC, Walsh EJ, Walsh MP et al (2009) Identification and functional characterization of protein kinase A-catalyzed phosphorylation of potassium channel Kv1.2 at serine 449. J Biol Chem 284:16562–16574
2. Li X, Cowles EA, Cowles RS, Gaugler R, Cox-Foster DL (2009) Characterization of immunosuppressive surface coat proteins from *Steinernema glaseri* that selectively kill blood cells in susceptible hosts. Mol Biochem Parasitol 165:162–169
3. Mellmann A, Cloud J, Maier T, Keckevoet U, Ramminger I, Iwen P et al (2008) Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. J Clin Microbiol 46:1946–1954
4. Zhang X, Scalf M, Berggren TW, Westphall MS, Smith LM (2006) Identification of mammalian cell lines using MALDI-TOF and LC-ESI-MS/MS mass spectrometry. J Am Soc Mass Spectrom 17:490–499
5. Brown J, Wallet MA, Krastins B, Sarracino D, Goodenow MM (2010) Proteome bioprofiles distinguish between M1 priming and activation states in human macrophages. J Leukoc Biol 87:655–662
6. Kraft-Terry SD, Gendelman HE (2011) Proteomic biosignatures for monocyte-macrophage differentiation. Cell Immunol 271: 239–255
7. Rubakhin SS, Churchill JD, Greenough WT, Sweedler JV (2006) Profiling signaling peptides in single mammalian cells using mass spectrometry. Anal Chem 78:7267–7272
8. Sauer S, Freiwald A, Maier T, Kube M, Reinhardt R, Kostrzewa M et al (2008) Classification and identification of bacteria by mass spectrometry and computational analysis. PLoS One 3:e2843
9. Dieckmann R, Helmuth R, Erhard M, Malorny B (2008) Rapid classification and identification of salmonellae at the species and subspecies levels by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 74:7767–7778
10. Lay JO Jr, Holland RD (2000) Rapid identification of bacteria based on spectral patterns using MALDI-TOF MS. Methods Mol Biol 146:461–487
11. Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, Rolain JM et al (2009) Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis 49:543–551
12. Karger A, Bettin B, Lenk M, Mettenleiter TC (2010) Rapid characterisation of cell cultures by matrix-assisted laser desorption/ionisation mass spectrometric typing. J Virol Methods 164:116–121
13. Ouedraogo R, Flaudrops C, Ben Amara A, Capo C, Raoult D, Mege JL (2010) Global analysis of circulating immune cells by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. PLoS One 5:e13691
14. Benoit M, Desnues B, Mege JL (2008) Macrophage polarization in bacterial infections. J Immunol 181:3733–3739
15. Ouedraogo R, Daumas A, Ghigo E, Capo C, Mege JL, Textoris J (2012) Whole-cell MALDI-TOF MS: a new tool to assess the multifaceted activation of macrophages. J Proteomics 75:5523–5532
16. Delaby A, Gorvel L, Espinosa L, Lépolard C, Raoult D, Ghigo E et al (2012) Defective monocyte dynamics in Q fever granuloma deficiency. J Infect Dis 205:1086–1094
17. Mba Medie F, Ben Salah I, Henrissat B, Raoult D, Drancourt M (2011) *Mycobacterium tuberculosis* complex mycobacteria as amoeba-resistant organisms. PLoS One 6:e20499
18. Meghari S, Bechah Y, Capo C, Lepidi H, Raoult D, Murray PJ et al (2008) Persistent *Coxiella burnetii* infection in mice overexpressing IL-10: an efficient model for chronic Q fever pathogenesis. PLoS Pathog 4:e23
19. Tantibhedhyangkul W, Prachason T, Waywa D, El Filali A, Ghigo E, Thongnoppakhun W et al (2011) *Orientia tsutsugamushi* stimulates an original gene expression program in monocytes: relationship with gene expression in patients with scrub typhus. PLoS Negl Trop Dis 5:e1028
20. Ryan CG, Clayton E, Griffin WL, Sie SH, Cousens DR (1988) SNIP, a statistics-sensitive background treatment for the quantitative analysis of PIXE spectra in geoscience applications. Nucl Inst Meth Phys Res B 34:396–402

ARTICLE 4

Whole-cell MALDI-TOF mass spectrometry is an accurate and rapid method to analyze different modes of macrophage activation

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Journal of Vizualized Experiments, sous presse

Le *Journal of Visualized Experiments* (JoVE) publie des protocoles scientifiques innovants sous forme de vidéos afin d'accélérer la recherche chimique, physique, médicale et biologique. La technique que j'ai utilisée pour étudier en spectrométrie de masse MALDI-TOF sur cellules entières est détaillée dans l'article et la vidéo qui sera accessible sur Internet ultérieurement.

Whole-cell MALDI-TOF mass spectrometry is an accurate and rapid method to analyze different modes of macrophage activation

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Keywords: MALDI-TOF, mass spectrometry, fingerprint, macrophages, activation, IFN-γ, TNF, LPS, IL-4, bacterial pathogens

Short abstract

This protocol describes the use of whole-cell MALDI-TOF mass spectrometry on eukaryotic cells. Here, we illustrate the accuracy of this technique by analyzing the multiple activation states of macrophages in response to their microenvironment.

Long abstract

MALDI-TOF is an extensively used mass spectrometry technique in chemistry and biochemistry. It has been also applied in medicine to identify molecules and biomarkers. Recently, it has been used in microbiology for the routine identification of bacteria grown from clinical samples, without preparation or fractionation steps. We and others have applied this whole-cell MALDI-TOF mass spectrometry technique successfully to eukaryotic cells. Current applications range from cell type identification to quality control assessment of cell culture and diagnostic applications. Here, we describe its use to explore the various polarization phenotypes of macrophages in response to cytokines or heat-killed bacteria. It allowed the identification of macrophage-specific fingerprints that are representative of the diversity of proteomic responses of macrophages. This application illustrates the accuracy and simplicity of the method. The protocol we described here may be useful for studying the immune host response in pathological conditions or may be extended to wider diagnostic applications.

INTRODUCTION

Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) is a popular mass spectrometry technique to study biological samples. Using a laser beam and an energy-absorbing matrix allows a soft ionization process: the evaporation and genesis of mostly single-charged biomolecules. This process is called desorption/ionization, justifying the acronym MALDI. These ions are then accelerated by a magnetic field and enter a TOF analyzer that allows the separation of these ions and the quantification of their respective masses[1].

MALDI-TOF MS has been extensively used in biology, chemistry and medicine to identify molecules and biomarkers[2–4] or to monitor post-translational modifications on proteins[5,6]. Recently, several groups applied MALDI-TOF MS to the identification of microorganisms from clinical samples[7,8]. This microbiological application, which is now used in routine in clinical settings, has many advantages compare to classical applications of MALDI-TOF MS: samples containing whole cells are directly processed, avoiding time consuming steps to fractionate or separate large amounts of material. Moreover, no characterization of the various peaks is needed: the whole spectrum is considered as a fingerprint of the sample, and matching algorithms compare the tested spectrum with a database of reference spectra.

We and others have applied this whole-cell analysis technique to eukaryotic cells. Many applications may be derived from this technique: (1) identify the main cell types from a mixed sample[9–11]; (2) assess the viability of cell cultures over time (including quality control industrial applications)[12]; (3) monitor activation states of a single cell type[13]; (4) assess the malignant transformation of a clinical sample[14,15].

Here, we describe the use of whole-cell MALDI-TOF MS to explore the various polarization phenotypes of macrophages in response to cytokines or heat-killed bacteria. Macrophages play a pivotal role in the immune response to microbial pathogens. They detect infectious agents in the tissues through pattern recognition receptors able to detect conserved microbial patterns, such as lipopolysaccharide (LPS)[16]. Macrophages are professional antigen-presenting cells that interact with T cells to mount the adaptive immune response. T cells influence macrophages by releasing cytokines that either reinforce or regulate the microbicidal activity of macrophages. By analogy to the Th1/Th2 lymphocyte polarization, inflammatory, microbicidal and tumoricidal macrophages have been classified into M1 macrophages and immunoregulator macrophages as M2 macrophages[17-19]. The term M1 refers to the classical activation of macrophages by type I cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF), or bacterial products, such as LPS[18,20–23], whereas macrophages activated by alternative pathways (interleukin (IL)-4, IL-10, Transforming Growth Factor-β1 are considered M2 macrophages[19,24,25]. The high phenotypic and functional plasticity of macrophages in response to their microenvironment renders these macrophages useful to analyze subtle changes by a MALDI-TOF MS approach.

PROTOCOL

In the present protocol, we used the whole-cell MALDI-TOF technique to obtain a mass spectrum considered as a fingerprint of the sample. A bioinformatic analysis allowed the comparison and the classification of these fingerprints. There were three main parts in this protocol:

1. The preparation of the biological samples: control macrophages and macrophages stimulated with different agonists,

2. The analysis of each type of samples with technical replicates by whole-cell MALDI-TOF MS,

3. The bioinformatics analysis of raw data.

1. PREPARATION OF BIOLOGICAL SAMPLES

Prepare **sterile** solutions for cell isolation and culture. Prepare and store all reagents at 4°C

1.1) Preparation of human monocytes

1.1.1) Prepare cell culture medium. Add 55 mL of human serum AB^+ or fetal bovine serum (FBS) and 5 mL of the antibiotic solution (penicillin at 10,000 UI/mL and streptomycin at 10,000 µg/mL; final concentration of 100 UI/mL for penicillin and 100 µg/mL for streptomycin) to RPMI 1640 medium (500 mL)

1.1.2) Isolate peripheral blood mononuclear cells (PBMCs) from leukopacks (leukocyte concentrates)

1.1.2.1) Prepare 50-mL tubes containing 15 mL Ficoll. Dilute PBMCs in saline (vol/vol, 1/10). Depose 30 mL diluted PBMCs on Ficoll as previously described[26]

1.1.2.2) Centrifuge at 700 x *g* for 20 min. Recover PBMCs at the interface between Ficoll (density of 1.077) and diluted plasma. Dilute PBMCs in culture medium and centrifuge at 300 x *g* for 5 min

1.1.3) Prepare $CD14^+$ monocytes from PBMCs using magnetic beads coated with anti-CD14 antibodies. Note: use manual separation (it is also possible to use automated separation). Keep products and cells at 4°C until monocyte obtention

1.1.3.1) Prepare running buffer consisting of phosphate-buffered saline (PBS) pH 7.2, 0.5% bovine serum albumin (BSA) and 2 mM ethylenediaminetetraacetic acid (EDTA)

1.1.3.2) Gently dissociate pelleted PBMCs (10^7 cells per assay) into 80 µL of running buffer

1.1.3.3) Add 20 µL CD14 MicroBeads to PBMCs**.** Mix and incubate PBMCs for 15 min**.** Wash PBMCs with 5 mL of running buffer and centrifuge at 300 x *g* for 5 min. Gently dissociate pelleted PBMCs into 500 µL of running buffer for 10^8 PBMCs.

Note: use 500 µL of running buffer for PBMC concentrations lower than 10^8 PBMCs

1.1.3.4) Proceed to magnetic separation. Note: for less than 2 x 10^8 PBMCs, use MS column; for more than 2 x 10^8 PBMCs and less than 2x10^9 PBMCs, use LS column

1.1.3.5) Rinse pre-column and column with running buffer (500 µL for MS column or 3 mL for LS column). Add PBMCs into the pre-column (wait that PBMCs pass throughout the column). Rinse 3 times with running buffer (500 µL for MS column or 3 mL for LS column). Remove the pre-column

1.1.3.6) Place the column on 15-mL tubes. Add running buffer (1 mL for MS column or 5 mL for LS column) on the column and elute $CD14^+$ cells by applying a pressure on the column

1.1.3.7) Centrifuge the eluate at 300 x *g* for 5 min. Discard the supernatant. Wash monocyte pellet with 10 mL of culture medium. Note: analyze the purity of monocyte preparation using anti-CD14 antibodies and flow cytometry (classically higher than 95%)

1.2) Differentiation of monocytes into macrophages

1.2.1) Incubate monocytes (10^6 monocytes per well in 6-well plates) in 3 mL of culture medium containing 10% human serum AB^+ at 37°C. After 4 days, replace the culture medium containing human serum by 3 mL culture medium containing 10% FBS for 3 additional days

1.2.2) Identify the obtained cell population as monocyte-derived macrophages (MDMs) by flow cytometry (see figure 1)

1.2.2.1) Replace the culture medium by 3 mL PBS. Scrape MDMs with a rubber policeman and collect MDM suspensions. Centrifuge at 400 x *g* for 5 min. Add PBS containing 2% bovine BSA to pelleted MDMs and gently agitate cell suspension. Adjust the cell concentration (10^6 MDMs in 200 µL PBS) and incubate at 4°C

1.2.2.2) Add 10 µL of anti-CD14 antibodies and 10 µL of anti-CD68 antibodies to MDM suspension and incubate at 4°C for 20 min in the dark. Note: these antibodies must be labeled with two different fluorochromes consistent with flow cytometry analysis. For example, anti-CD14 antibodies may be conjugated with phycoerythrin coupled (for monocytes staining) and anti-CD68 antibodies with Alexa Fluor 647 coupled (for macrophages staining)

1.2.2.3) Centrifuge MDMs at 400 x *g* for 5 min. Remove supernatants. Gently dissociate the cell pellet and incubate MDMs in 250 µL 3% paraformaldehyde (PFA) for 15 min at room temperature. Centrifuge PFA-fixed MDMs at 400 x *g* for 5 min. Wash MDMs with 3 mL PBS and centrifuge MDMs at 400 x *g* for 5 min. Gently dissociate MDM pellet in 400 µL PBS

1.2.2.4) Analyze the differentiation of monocytes (that express CD14 but not CD68) into monocytes (that express CD68 but not CD14). Note: classically more than 95% of cells are MDMs

1.3) 18 hour-stimulation of MDMs

1.3.1) Replace the culture medium of adherent MDMs by fresh 3 mL culture medium (containing 10% FBS)

1.3.2) To induce a M1 or M2 polarization, use the following human recombinant cytokines: IFN-γ and TNF for M1 polarization, and IL-4 for M2 polarization. M1 macrophages are also obtained by stimulation with LPS. Note that stock-solutions of cytokines are conserved at -80°C and that the stock-solution of LPS is conserved at -20°C

1.3.2.1) Dilute the stock-solution of IFN-γ (1 μg/μL) at 1/100 in culture medium to obtain a dilution of 10 ng/μL. Add 6 μL of this IFN-γ dilution to MDMs to obtain a final IFN-γ concentration of 20 ng/mL

1.3.2.2) Dilute the stock-solution of TNF (1 μg/μL) at 1/100 in culture medium to obtain a dilution of 10 ng/μL. Add 6 μL of this TNF dilution to MDMs to obtain a final TNF concentration of 20 ng/mL

1.3.2.3) Dilute the stock-solution of IL-4 (1 μg/μL) at 1/100 in culture medium to obtain a dilution of 10 ng/μL. Add 6 μL of this IL-4 dilution to MDMs to obtain a final IL-4 concentration of 20 ng/mL

1.3.2.4) Add 3 μL of the stock-solution of LPS (1 mg/mL) to MDMs to obtain a final LPS concentration of 1 μg/mL

1.3.3) To stimulate MDMs with bacteria, use heat-killed bacteria. Wash living bacteria with PBS and heat them at 95°C for 1 hour. Use *Orientia tsutsugamushi* (strain Kato (CSUR R163), *Mycobacterium bovis* (Bacillus Calmette-Guérin, BCG CIP strain 671203) and *Coxiella burnetii* (Nine Mile in phase I) were because these pathogens are known to infect macrophages

1.3.3.1) Prepare a *O. tsutsugamushi* suspension at 10^9 bacteria/mL. Add 50 μL to MDMs to obtain a concentration of 50 bacteria per MDM

1.3.3.2) Prepare a *M. bovis* suspension at 10^9 bacteria/mL. Add 50 μL to MDMs to obtain a concentration of 50 bacteria per MDM

1.3.3.3) Prepare a *C. burnetii* suspension at 10^9 bacteria/mL. Add 50 μL to MDMs to obtain a concentration of 50 bacteria per MDM

1.4) Preparation of biological samples for MALDI-TOF MS

1.4.1) Wash stimulated MDMs with PBS without Ca^{2+} or Mg^{2+} (cells may agglutinate in the presence of Ca^{2+} or Mg^{2+}). Scrape MDMs with a rubber policeman and collect MDM suspensions. Centrifuge MDMs. Wash again MDMs in PBS without Ca^{2+} or Mg^{2+} at 400 x *g* for 5 min to discard FBS contamination

1.4.2) Adjust the cell concentration (2 x 10^6 MDMs per assay). Centrifuge MDMs at 400 x *g* for 5 min and discard supernatants. Collect cell pellets in 20 μL of PBS without Ca^{2+} or Mg^{2+}

1.4.3) Analyze samples immediately or store them at -80°C before analysis

2. ANALYZIS OF MACROPHAGES BY WHOLE-CELL MALDI-TOF MS

2.1) Preparation of CHCA matrix

2.1.1) Add 500 µL of acetonitrile, 250 µL 10% trifluoroacetic acid and 250 µL of high-performance liquid chromatography (HPLC) water to a vial. Dilute 10 mg of CHCA in this solution to a final concentration of 10 mg/mL. Mix and sonicate for at least 20 min

2.1.2) Centrifuge at 13,000 x *g* for 5 min. Discard the pellet and keep the supernatant. Note: prepare the matrix solution just before use. A matrix solution that contains crystals does not allow a good ionization of sample molecules, and this may affect the quality of the spectra

2.2) Preparation of MALDI steel target

2.2.1) Moisten the polished steel target with hot tap water. Rub with precision wipe paper. Add 70% ethanol and rub. Rinse with water by rubbing. Add 70% ethanol and rub with precision wipe paper

2.2.2) Immerse the target in 70% ethanol and sonicate for at least 15 min. Cover the target with 500 µL to 1 mL of trifluoroacetic acid at 80%. Rub with paper precision wipe. Rince with HPLC water without rubbing. Dry the target at room temperature. Note: an improperly cleaned target may affect the quality of the spectra

2.3) Preparation of deposits

2.3.1) Place the clean target on a horizontal support to obtain uniform deposits

2.3.2) Gently thaw MDM samples on ice. Note: rapid and vigorous thawing may alter samples, thus affecting the quality of the spectra. Homogenize MDMs before deposition of 1 µL (containing approximately 1 x 10^5 cells) on the MALDI target. Add 1 µL of the MALDI matrix solution on the sample. Avoid mixing spot with the pipette. Mixing spots with pipettes alters the quality of the spectra. Depose 12 to 16 samples per assay (technical replicates)

2.3.3) Evaporate spontaneously at room temperature. Note: evaporation takes place gradually and leads to the formation of matrix/sample crystals. The deposits may be immediately analyzed or stored in the dark for several days before analysis

2.4) Data acquisition and mass spectrometer tunning

2.4.1) Insert the steel target containing samples in the mass spectrometer

2.4.2) Configure the mass spectrometer and run data acquisition. Use the default configuration for automated acquisition of the data. Note: a detailed view of the configuration of flexControl software is given in the appendix / table 1. This may vary according to used mass spectrometer and software.

3. BIOINFORMATIC ANALYSIS

Note: the bioinformatic analysis was performed using the free and open source statistical analysis software R, along with specific analysis libraries (*MALDIquant*). R can be downloaded freely from its website http://cran.r-project.org/. A detailed description of the script is given as supplementary material

3.1) Loading and pre-treatment of raw data

3.1.1) Store raw data on the computer associated with the mass spectrometer in multiple files and folders. Retrieve the root folder of the experiment with all subfolders. Copy root folder to personal computer for analysis

3.1.2) Use *ReadBrukerFlexData* and *MALDIquant* librairies to load and analyze raw data. Note: *ReadBrukerFlexData* allows the loading of raw data from the mass spectrometer into a specific object in R for further analysis. See *MALDIquant* description for further information[27]

3.1.3) Analyze generated spectra. Note: each spectrum consists of a list of peaks with their respective masses and intensities (relative abundance). Use the square root of the intensities to enhance the graphical visualization of the spectra. Correct the background using a statistic-sensitive non-linear peak-clipping algorithm for baseline estimation[27]. Use a signal-to-noise ratio of 6 to detect peaks. Consider that the detected peaks are similar across spectra when the mass/charge (*m/z*) values are within a 2000 ppm window

3.2) Score definition and computation. Classify the spectra using a hierarchical clustering with a ward algorithm for agglomeration and a dissimilarity matrix based on the Jaccard distance. Note: the Jaccard index measures the similarity between boolean sample sets (*i.e.* the presence/absence of a list of peaks)

3.3) Comparison of spectra and viewing

3.3.1) Analyze raw spectra plots. Note: x-axis represents the *m/z* ratio (in daltons) and the y-axis represents the intensity (relative abundance)

3.3.2) Assess the similarity between spectra by hierarchical clustering. Represent similarity (or divergence) as dendrogram

3.3.3) Assess the reproducibility by the mean of virtual gel-view representation. Note: virtual gel-view representation is a modified heatmap plot where relative abundance is color-coded with increasing intensities of blue

Representative results

The aim of the present protocol is to demonstrate the accuracy of whole-cell MALDI-TOF MS to assess the responsiveness of macrophages to their microenvironment.

The figure 1 represents the analysis of monocytes and MDMs by flow cytometry. Note that monocytes expressed CD14 but not CD68 (figure 1A). Conversely, MDMs expressed CD68 but not CD14 (figure 1B).

The figure 2 describes the principle of whole-cell MALDI-TOF MS. Cells are deposited with matrix on the target plate. Within the mass spectrometer, a laser beam induces the desorption and ionization of molecules by shooting multiple times on the sample (250 shots). The produced ions are accelerated by a magnetic field and separated according to their *m/z* ratio in the tube. The TOF analyzer records the impact of the various ions at the end of the tube. According to the time of flight, each impact is converted into a *m/z* ratio, and impacts corresponding to the same *m/z* ratio are summed up to generate the full raw spectrum.

The figure 3 illustrates the role of sample preparations in the interpretation of MALDI-TOF MS results. A good quality spectrum is represented in Figure 3A. It usually contains a major peak around 5 kD (*m/z* = 4965). A minimum cell concentration is required to obtain good samples: figure 3B shows a poor quality spectrum obtained with a low cell concentration. However, raising MDM concentration above 1 x 10^5/µL does not improve the quality of spectra. Similar poor results are obtained when the sample is mixed with matrix before deposition on the target plate. If mixing is done on the target plate, it may also result in heterogeneous crystallization, as shown in figure 3C. Hence, deposition of the samples on the target is a tricky and critical step in this protocol.

The reproducibility of the spectra is shown in Figure 4. Here, spectra from various samples are represented as a heatmap. Relative abundance (intensity) is color-coded by intensities of blue. This virtual gel-view representation illustrates the reproducibility of the samples within each class. The normalization and alignment of the spectra is a critical step to obtain such results. An unsupervised analysis by hierarchical clustering is summarized as a dendrogram on the right hand side of the figure. It illustrates that all samples clustered within three different groups: unstimulated MDMs (NS), IFN-γ-stimulated or IL-4-stimulated MDMs.

The figure 5 illustrates the discrimination of M1 macrophages (MDMs stimulated with IFN-γ) from M2 macrophages (MDMs stimulated with IL-4) and unstimulated MDMs. Indeed, the peak representation of a reference spectrum for IFN-γ, IL-4 or unstimulated MDMs shows specific peaks for each class. This representation is obtained using the R *MALDIquant* library27.

The figure 6 illustrates the specific fingerprints induced by several agonists. It is commonly accepted that IFN-γ, TNF and LPS induce an inflammatory (M1-type) response in macrophages. We used MDM samples stimulated with these cytokines alone or in combination to illustrate the accuracy of whole-cell MALDI-TOF MS. Indeed, spectra from all stimulated samples were clearly separated from those of unstimulated macrophages (Figure 6A). However, we obtained a specific fingerprint from each type of stimulation, as illustrated by the clustering of the samples

according to the stimuli. Interestingly, MDMs also exhibited specific fingerprints induced by heat-killed bacteria (Figure 6B). These results highlight the hypothesis that MALDI-TOF MS may be used to analyze circulating cells to assess the host-response to infection or inflammatory diseases in the clinical setting.

Figure legends

Figure 1: Assessment of CD14 and CD68 expression by flow cytometry. Monocytes (left panel) or MDMs (right panel) were labeled with anti-CD14-PE and anti-CD68-AF647 antibodies to assess membrane expression of these molecules. The differentiation of monocytes into MDMs is accompanied by the down-modulation of CD14 expression and the up-modulation of CD68 expression.

Figure 2: Principle of MALDI-TOF MS technology. This drawing describes the principle of the MALDI-TOF mass spectrometry.

Figure 3: Whole-cell MALDI-TOF MS spectra. A zoomed view of spots deposited on the MALDI target (left panels) with corresponding spectra (right panels). Note that a good quality spot leads to accurate spectra (A) and that spots with bad quality leads to defective spectra (B, C).

Figure 4: Reproducibility of MALDI-TOF MS spectra. Virtual gel-view of the whole spectra obtained from control and IL-4- or IFN-γ-stimulated MDMs are presented as a heatmap. Horizontal axis refers to the *m/z* ratio. Spectra are classified according to the presence/absence of peaks. NS: non stimulated; IFN-γ: interferon-gamma; IL-4: interleukin 4. This figure was reproduced from [13] with permission.

Figure 5: Reference spectra for M1 and M2 macrophages. The reference spectra for IFN-γ- and IL-4-stimulated MDMs are compared to the reference spectrum of non-stimulated (NS) MDMs. The peaks that are shared by stimulated and NS MDMs are in black. The peaks that are induced by stimulation are in red, whereas peaks that are detected only in NS MDMs are in green. *m/z*: mass/charge ratio; IFN-γ: interferon-gamma; IL-4: interleukin 4. This figure was reproduced from [13] with permission.

Figure 6: Hierarchical clustering of activated MDMs. MDMs were stimulated with different agonists for 18 h. The results are shown as hierarchical clustering of the data. MDMs were activated with M1-related agonists (A) and intracellular bacteria or IL-4 (B). Unstimulated MDMs are presented in grey. IFN-γ: interferon-gamma; LPS: lipopolysaccharide from *Escherichia coli*; TNF: tumor necrosis factor, IL-4: interleukin 4; BCG; bacillus Calmette-Guérin; *C. burnetii*: *Coxiella burnetii*; *O. tsutsugamushi*: *Orientia tsutsugamushi*. This figure was reproduced from [13] with permission.

Discussion

This protocol describes the use of MALDI-TOF-MS on eukaryotic whole cells. Here, we illustrate the accuracy of the method by analyzing the multiple activation states of macrophages in response to their microenvironment.

The success of the protocol relies on few critical steps. First, any solution contaminant may alter the spectra. For example, it is important to wash cells in PBS to remove culture medium and serum proteins before deposition on the target. A cell concentration of 1 x 10^5 cells per µL is also needed to ensure reproducible results. Second, the crystallization is the most sensible step in the protocol. To ensure good quality results, the target plate must be carefully washed and the matrix should be prepared before the deposition of samples on the target. The best results are obtained when the samples are deposited on the target just before the matrix solution (avoid mixing the samples with the matrix before the deposition on the target). Correct spontaneous mixing between samples and the matrix solution needs homogeneous deposits. Third, whole-cell MALDI-TOF-MS is a high-throughput technique, which can rapidly result in high amounts of raw data. Bioinformatics analysis is thus a major tool to systematically analyze the data in a reasonable amount of time. Quality assessment, background correction and normalization can be automated. The selection of relevant peaks (e.g. above a given signal-to-noise ratio) and the comparison of spectra based on the presence/absence of these peaks require important computation steps. These methods are described in details in the supplementary material of this article.

Although the acquisition of a mass spectrometer may represent a significant investment, daily running costs are low, and a high number of biological and technical replicates may be easily obtained in one run. For example, our university hospital is able to routinely identify bacteria in 200 clinical samples each day with a similar whole-cell technique. The cell concentration may be a limit for specific clinical applications such as the analysis of needle biopsies or cells harvested from broncho-alveolar lavages. A recent article described an automated approach of whole-cell MALDI-TOF analysis that allowed the robust classification of samples with as few as 250 cells on each spot[11]. A proof of concept of the clinical application of this technique to the diagnosis of oral cancer has also been recently published[15]. The matrix choice may limit the type of analyzed molecules. Some matrices will favor the ionization of specific type of molecules (proteins, lipids, sugars…) and of a given mass range. In our conditions, we were not able to retrieve good quality spectra with ions above 20 kDa. In our protocol, we focused of the analysis of whole spectrum as a fingerprint of a given activation state of cells. Therefore, we did not try to identify the proteins that form the main peaks of the spectrum. The identification of specific biomarkers requires an alternative use of mass spectrometry.

In conclusion, we describe here one application of the accuracy and reproducibility of the whole-cell MALDI-TOF MS approach for the analysis of macrophage activation. This method allowed the identification of macrophage-specific fingerprints that are representative of the diversity of proteomic responses to cytokines and bacterial pathogens. The protocol we described here may be useful for studying the immune host response in pathological conditions or may be extended to wider diagnostic applications.

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Disclosures:

The authors have no conflict of interest to declare.

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References

1. Gross, J. *Mass Spectrometry: A Textbook*. Springer-Verlag, Berlin, (2011).
2. Villanueva, J., *et al.* Serum Peptide Profiling by Magnetic Particle-Assisted, Automated Sample Processing and MALDI-TOF Mass Spectrometry. *Analytical Chemistry* **76**(6), 1560–1570 (2004).
3. Diamandis, E. P. Mass Spectrometry as a Diagnostic and a Cancer Biomarker Discovery Tool Opportunities and Potential Limitations. *Molecular & Cellular Proteomics* **3**(4), 367–378 (2004).
4. Pusch, W. & Kostrzewa, M. Application of MALDI-TOF Mass Spectrometry in Screening and Diagnostic Research. *Current Pharmaceutical Design* **11**(20), 2577–2591 (2005).
5. Aebersold, R. & Mann, M. Mass spectrometry-based proteomics. *Nature* **422**(6928), 198–207 (2003).
6. Domon, B. & Aebersold, R. Mass Spectrometry and Protein Analysis. *Science* **312**(5771), 212–217 (2006).
7. Carbonnelle, E., *et al.* Robustness of two MALDI-TOF mass spectrometry systems for bacterial identification. *Journal of Microbiological Methods* **89**(2), 133–136 (2012).
8. Bizzini, A., Jaton, K., Romo, D., Bille, J., Prod'hom, G. & Greub, G. Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry as an Alternative to 16S rRNA Gene Sequencing for Identification of Difficult-To-Identify Bacterial Strains. *Journal of Clinical Microbiology* **49**(2), 693–696 (2011).
9. Buchanan, C. M., Malik, A. S. & Cooper, G. J. S. Direct visualisation of peptide hormones in cultured pancreatic islet alpha- and beta-cells by intact-cell mass spectrometry. *Rapid Communications in Mass Spectrometry: RCM* **21**(21), 3452–3458 (2007).
10. Ouedraogo, R., Flaudrops, C., Ben Amara, A., Capo, C., Raoult, D. & Mege, J.-L. Global analysis of circulating immune cells by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *PloS One* **5**(10), e13691 (2010).
11. Munteanu, B., Reitzenstein, C. von, Hänsch, G. M., Meyer, B. & Hopf, C. Sensitive, robust and automated protein analysis of cell differentiation and of primary human blood cells by intact cell MALDI mass spectrometry biotyping. *Analytical and Bioanalytical Chemistry* **404**(8), 2277–2286 (2012).
12. Feng, H., Sim, L. C., Wan, C., Wong, N. S. C. & Yang, Y. Rapid characterization of protein productivity and production stability of CHO cells by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry: RCM* **25**(10), 1407–1412 (2011).
13. Ouedraogo, R., Daumas, A., Ghigo, E., Capo, C., Mege, J.-L. & Textoris, J. Whole-cell MALDI-TOF MS: A new tool to assess the multifaceted activation of macrophages. *Journal of Proteomics* **75**(18), 5523–5532 (2012).
14. Amann, J. M., *et al.* Selective profiling of proteins in lung cancer cells from fine-needle aspirates by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* **12**(17), 5142–5150 (2006).
15. Maurer, K., Eschrich, K., Schellenberger, W., Bertolini, J., Rupf, S. & Remmerbach, T. W. Oral brush biopsy analysis by MALDI-ToF Mass Spectrometry for early cancer diagnosis. *Oral Oncology* **49**(2), 152–156 (2013).
16. Ishii, K. J., Koyama, S., Nakagawa, A., Coban, C. & Akira, S. Host innate immune receptors and beyond: making sense of microbial infections. *Cell Host & Microbe* **3**(6), 352–363 (2008).

17. Mantovani, A., Sica, A. & Locati, M. Macrophage polarization comes of age. *Immunity* **23**(4), 344–346 (2005).
18. Martinez, F. O., Sica, A., Mantovani, A. & Locati, M. Macrophage activation and polarization. *Frontiers in Bioscience: A Journal and Virtual Library* **13**, 453–461 (2008).
19. Mosser, D. M. & Edwards, J. P. Exploring the full spectrum of macrophage activation. *Nature reviews. Immunology* **8**(12), 958–969 (2008).
20. Murray, P. J. & Wynn, T. A. Protective and pathogenic functions of macrophage subsets. *Nature Reviews. Immunology* **11**(11) 723–737 (2011).
21. Benoit, M., Desnues, B. & Mege, J.-L. Macrophage polarization in bacterial infections. *Journal of Immunology (Baltimore, Md.: 1950)* **181**(6), 3733–3739 (2008).
22. Biswas, S. K., Sica, A. & Lewis, C. E. Plasticity of macrophage function during tumor progression: regulation by distinct molecular mechanisms. *Journal of Immunology (Baltimore, Md.: 1950)* **180**(4), 2011–2017 (2008).
23. Cassetta, L., Cassol, E. & Poli, G. Macrophage polarization in health and disease. *TheScientificWorldJournal* **11**, 2391–2402 (2011).
24. Gordon, S. Alternative activation of macrophages. *Nature reviews. Immunology* **3**(1), 23–35 (2003).
25. Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A. & Locati, M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends in Immunology* **25**(12), 677–686 (2004).
26. Delaby, A., Gorvel, L., *et al.* Defective monocyte dynamics in Q fever granuloma deficiency. *The Journal of infectious diseases* **205**(7), 1086–1094 (2012).
27. Gibb, S. & Strimmer, K. MALDIquant: a versatile R package for the analysis of mass spectrometry data. *Bioinformatics* **28**(17), 2270–2271 (2012).

Figure 1

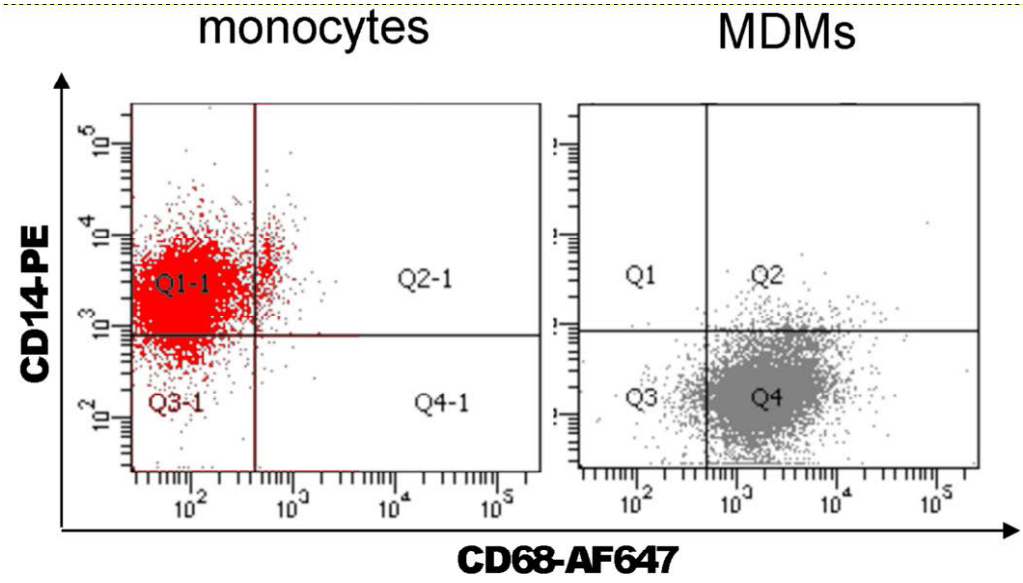


Figure 2

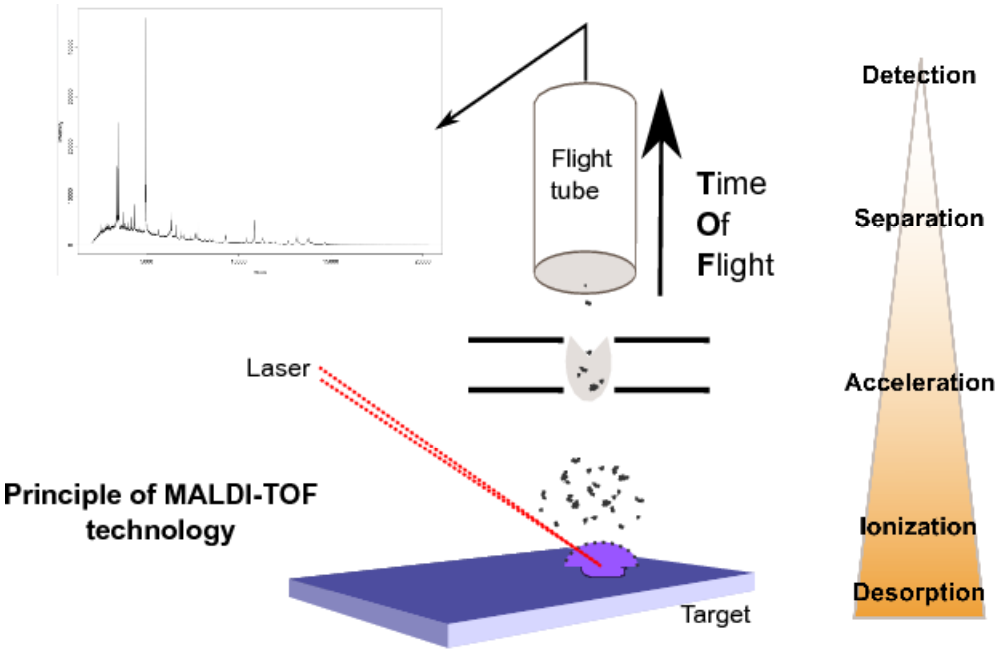


Figure 3

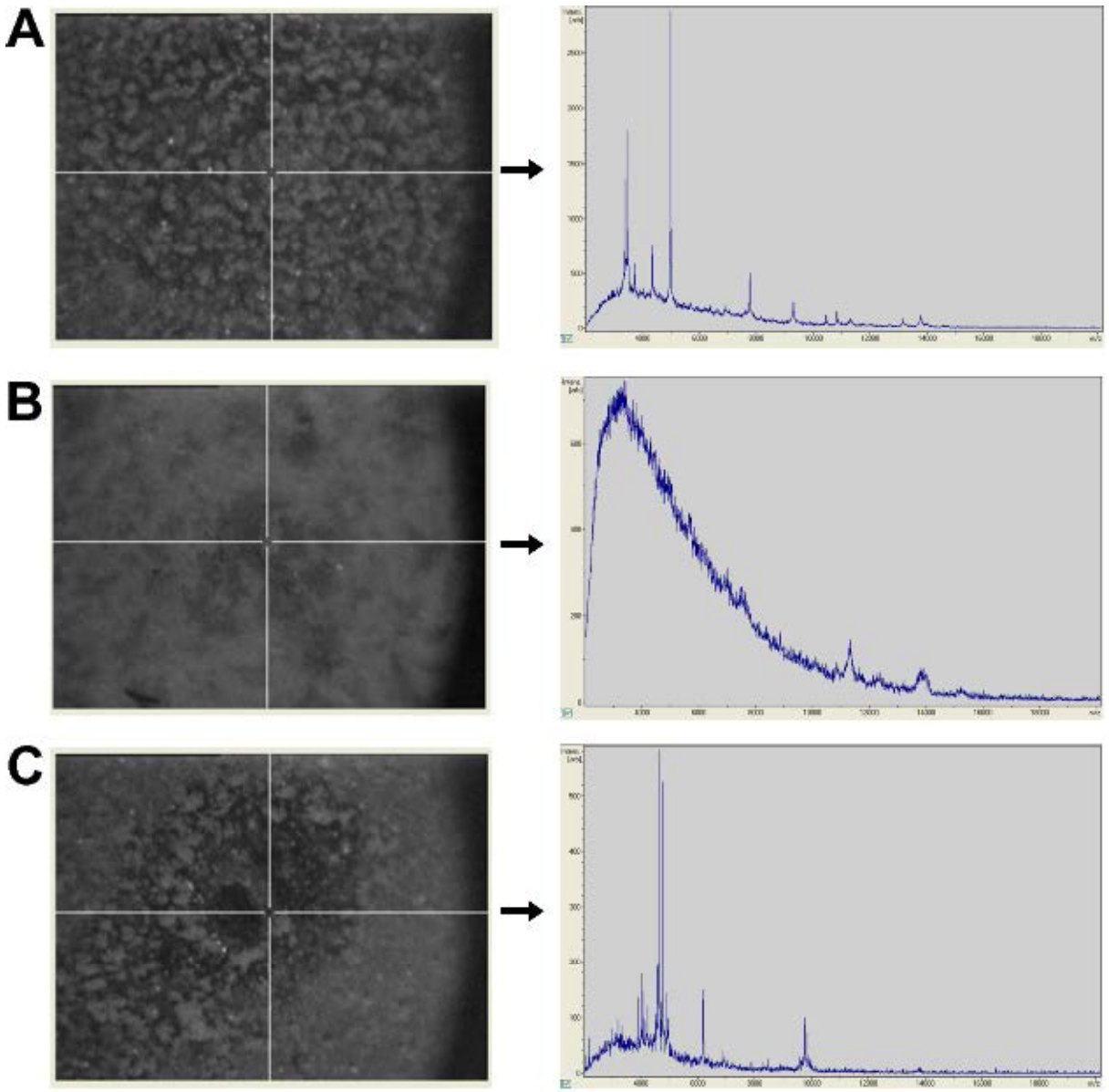


Figure 4

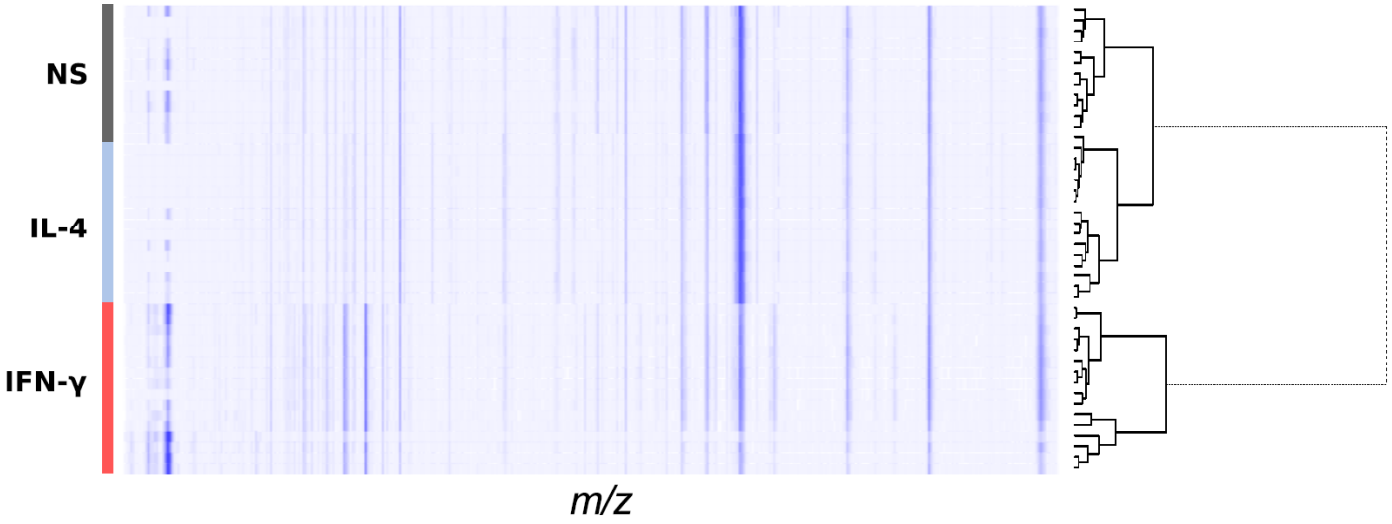


Figure 5

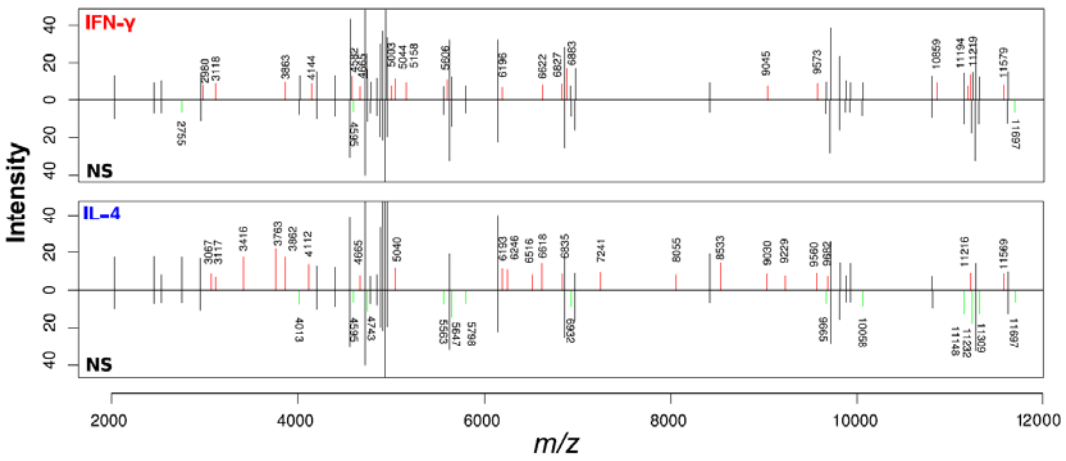
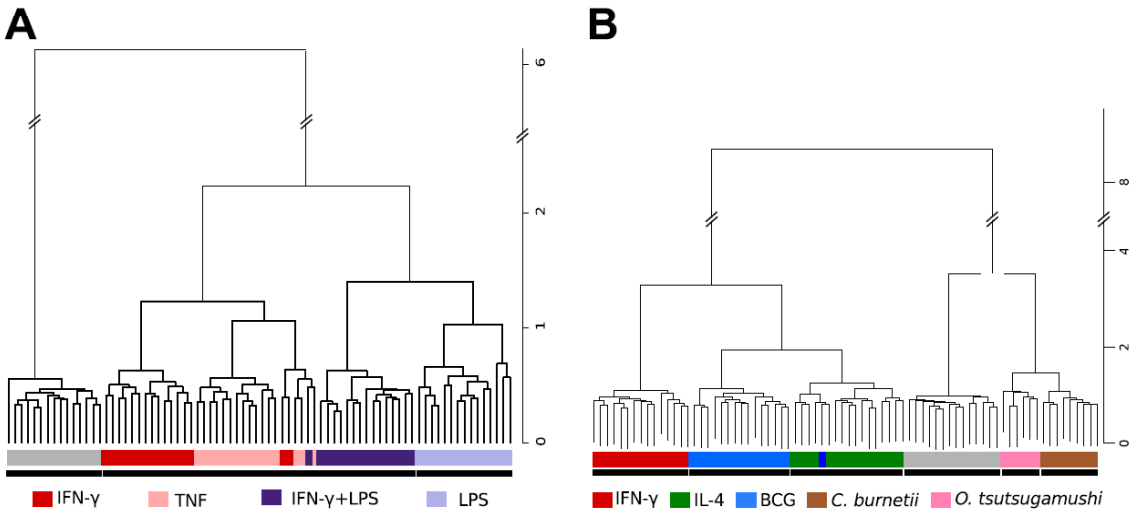


Figure 6


A
6
2
1
0
IFN-γ
TNF
IFN-γ+LPS
LPS
B
8
4
2
0
IFN-γ
IL-4
BCG
C. burnetii
O. tsutsugamushi

CONCLUSIONS ET DISCUSSION

La spectrométrie de masse MALDI-TOF sur bactéries entières est utilisée depuis plusieurs années pour identifier rapidement différentes espèces bactériennes, voire pour identifier différents mutants d'une même espèce bactérienne (Demirev et Fenselau 2008; Carbonnelle et al. 2011). Mon premier objectif a donc été de savoir si la spectrométrie de masse MALDI-TOF pouvait s'appliquer à l'analyse de cellules eucaryotes entières. J'ai tout d'abord utilisé des monocytes humains et j'ai montré que les spectres obtenus contiennent de nombreux pics de différentes intensités. Ces spectres sont hautement reproductibles qu'ils proviennent de différents échantillons du même donneur ou de différents donneurs. J'ai ensuite comparé ces spectres à ceux des lymphocytes T, des neutrophiles et des hématies provenant des mêmes donneurs de sang. Les spectres obtenus sont largement différents puisque chacun d'entre eux est caractérisé par des pics spécifiques et/ou par des pics d'intensités différentes selon le type cellulaire dont ils proviennent. Ces spectres comportent également un nombre important de pics et d'intensité identiques. Il n'est donc pas possible d'identifier les différentes populations cellulaires sur la base de quelques pics considérés comme spécifiques de telle ou telle population cellulaire. Seule une approche combinatoire des pics contenus dans les spectres est opérationnelle. C'est la raison pour laquelle nous avons utilisé un logiciel initialement dédié à l'étude des microarrays pour établir un clustering hiérarchique en assignant la valeur +1 aux pics présents dans un spectre donné et la valeur -1 lorsque le pic est absent de ce spectre. Il apparaît ainsi que les hématies sont contenues dans un cluster complètement différent de celui correspondant aux autres cellules circulantes. Les monocytes sont compris dans le même sous-cluster que les neutrophiles et que les lymphocytes T sont plus proches des monocytes que des neutrophiles.

Le fait que chaque type de cellules circulantes soit caractérisé par une empreinte différente nous a incités à créer une base de données comprenant 17 types de cellules mammifères et 5 types de cellules non-mammifères. La comparaison sous forme de dendrogramme des empreintes associées à chaque type cellulaire a révélé deux branches majeures. La première regroupe les quatre types d'amibes, les cellules de xénope et de façon inattendue les hématies ; la seconde regroupe l'ensemble des cellules mammifères nucléées. Il semble que ce dendogramme fasse sens. En effet, un sous-cluster est composé des cellules immunitaires circulantes (monocytes, lymphocytes T et neutrophiles). Les lignées de macrophages quelle qu'en soit l'espèce d'origine (cellules J774 et THP-1) sont regroupées dans un autre cluster. Les macrophages en culture

(macrophages dérivés de monocytes et macrophages dérivés de la moelle osseuse) sont proches les uns des autres et distincts des lignées de macrophages. De même, les empreintes en spectrométrie de masse MALDI-TOF MALDI-TOF des lignées de trophoblastes (cellules JEG et BeWo) sont très largement différentes de l'empreinte des trophoblastes natifs.

J'ai ensuite étudié la validité de cette base de données, et des outils développés par Bruker Daltonics pour l'identification bactérienne. Pour cela, j'ai comparé des spectres de monocytes avec la référence « monocyte » contenu dans la banque de données. Le score obtenu reflète l'identification certaine de ces nouveaux spectres comme provenant bien de monocytes. J'ai également étudié la possibilité d'identifier une population cellulaire au sein d'un mélange de types cellulaires. J'ai ainsi mélangé à parts égales des monocytes et des lymphocytes T et j'ai retrouvé leurs signatures respectives au sein de ce mélange. J'ai enfin identifié les deux signatures, monocytes et lymphocytes T, dans les PBMCs. La spectrométrie de masse MALDI-TOF pourrait ainsi être utilisée pour identifier différents types cellulaires au sein d'un mélange complexe de cellules et pourrait servir d'alternative à la cytométrie en flux. En effet, nous avons fait le choix de travailler avec environ cent mille cellules par échantillon mais il a été montré que la spectrométrie de masse MALDI-TOF nécessite un nombre bien moins important de cellules alors que la cytométrie en flux nécessite des échantillons bien plus importants (travailler avec moins de cent mille cellules est illusoire). Enfin et surtout, la cytométrie en flux nécessite l'utilisation d'anticorps spécifiques, les critères de taille et de granulosité étant insuffisamment discriminants, alors que la spectrométrie de masse MALDI-TOF sur cellules entières se passe de traitement préalable des échantillons. A titre d'exemple, lorsque l'on veut préparer différents types de cellules placentaires, s'il est possible de caractériser les macrophages et les cellules dendritiques placentaires grâce à l'utilisation d'anticorps ou de cocktails d'anticorps spécifiques, il n'est pas facile de caractériser les trophoblastes pour lesquels il n'existe pas d'anticorps complètement spécifiques. On pourrait donc utiliser la spectrométrie de masse MALDI-TOF comme moyen d'étudier l'isolement de cette population cellulaire.

Puisque la spectrométrie de masse MALDI-TOF permet d'identifier différents types cellulaires, y compris les macrophages dérivés de monocytes, je me suis demandé si elle peut être utilisée pour étudier différents états d'activation de ces macrophages. En

effet, les macrophages se polarisent en macrophages M1 sous l'action d'une cytokine telle que l'IFN-γ et en macrophages M2 sous l'action d'une cytokine telle que l'IL-4, les caractéristiques transcriptionnelles, phénotypiques et fonctionnelles de ces macrophages étant très largement différentes (Fernando O. Martinez et al. 2006; Benoit, Desnues, et Mege 2008a; Fernando Oneissi Martinez 2008). Les outils utilisés pour le traitement des données dans l'article précédent appartenant au fabricant du spectromètre de masse MALDI-TOF, Bruker Daltonics. Bruker Daltonics refusant de communiquer ses méthodes d'analyse, nous avons fait le choix d'utiliser nos propres méthodes d'analyse et de les rendre publiques (pour le détail, cf article 3). J'ai montré dans le deuxième article que les spectres des macrophages quiescents, des macrophages M1 et des macrophages M2 sont différents les uns des autres. Les macrophages M1 sont compris dans un cluster différent de celui des spectres des macrophages quiescents et des macrophages M2. Ce résultat est conforme à la réalité biologique puisque les macrophages natifs sont considérés comme plutôt des macrophages M2, les uns et les autres étant peu microbicides à la différence des macrophages M1. Différents agonistes sont capables d'induire une polarisation M1, en particulier une cytokine telle que le TNF et un ligand bactérien tel que le LPS. J'ai donc testé ces molécules et il apparaît qu'elles induisent une empreinte peptidique/protéique de type M1. On peut cependant noter que ces signatures sont quelques peu différentes les unes des autres, ce qui montre qu'il existe probablement plusieurs états M1, ce qui est de nouveau conforme à la variabilité des états M1. J'ai procédé de la même façon avec des agonistes de la réponse M2 tels que l'IL-10 et le TGF-β. J'ai trouvé que ces deux molécules induisent une signature M2 mais que les signatures M2 sont différentes les unes des autres, ce qui était attendu puisqu'il existe plusieurs états M2 (M2a, M2b, M2c) ou un continuum d'états M2 (Benoit, Desnues, et Mege 2008a).

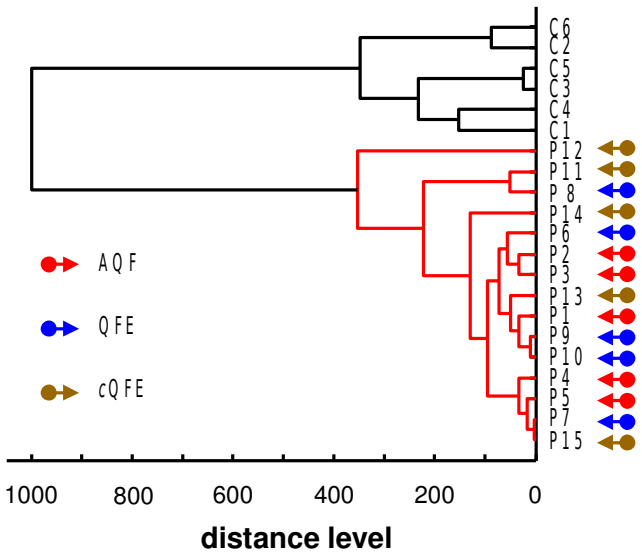
Les macrophages constituent une ligne de défense importante contre les agents infectieux puisqu'ils possèdent une puissante machinerie microbicide. Ils sont cependant la cible de différents agents pathogènes qui utilisent différents mécanismes moléculaires pour contourner cette activité microbicide (Flannagan, Cosío, et Grinstein 2009). Je me suis alors demandé si la spectrométrie de masse MALDI-TOF permet de détecter la réponse des macrophages à différentes bactéries. Les signatures induites par des bactéries extracellulaires telles que les streptocoques du groupe B ou les staphylocoques dorés sont reproductibles et spécifiques de chacune de ces bactéries. Elles sont plus proches de la signature IL-4 que de la signature IFN-γ, suggérant qu'il

s'agit dans les deux cas d'une signature plutôt M2. J'ai ensuite étudié la réponse des macrophages à des bactéries intracellulaires telles que le BCG, une souche vaccinale contre la tuberculose, *Orientia tsutsugamushi*, l'agent du typhus des broussailles et *Coxiella burnetii*, l'agent de la fièvre Q. Chacune de ces bactéries induit une réponse spécifique. Le BCG induit une réponse de type M2 puisque cette réponse est dans le même cluster que la réponse à l'IL-4. En revanche, les réponses à *O. tsutsugamushi* et *C. burnetii* sont plus proches de celle des macrophages quiescents que de celles des macrophages M1 et M2. En d'autres termes, la notion de polarisation M1/M2 des macrophages ne décrit pas complètement les différents modes d'activation des macrophages. Mes résultats indiquent également que la spectrométrie de masse MALDI-TOF pourrait permettre d'étudier la réponse des macrophages à de nombreux agents pathogènes.

Puisque la spectrométrie de masse MALDI-TOF semble utilisable pour analyser la réponse de cellules isolées telles que les macrophages, je me suis demandé s'il était possible de l'utiliser pour déterminer l'état d'activation des cellules circulantes de différents types de patients. Dans un travail préliminaire, j'ai fait le choix d'étudier l'ensemble des cellules mononucléées du sang périphérique pour trois raisons principales. La première est que seules les cellules circulantes sont aisément accessibles au lit du malade. La deuxième est que les PBMCs contiennent la quasi-totalité des cellules de la réponse immune, en particulier les monocytes dont le spectre de réponse aux agents infectieux et aux cytokines est comparable à celui des macrophages, ces derniers dérivant des monocytes. La troisième est que les PBMCs peuvent être obtenus rapidement (moins d'une heure) après la prise de sang avec peu de matériel de laboratoire alors que l'isolement des monocytes ou des lymphocytes demande bien plus de temps (plusieurs heures), du matériel et du personnel spécialisés.

Nous avons choisi des pathologies infectieuses et inflammatoires puisque ce sont elles qui sont en priorité susceptibles d'induire des modifications structurelles et fonctionnelles des PBMCs. Nous les avons également choisis selon un critère de disponibilité (accessibilité à des cohortes). La pathologie infectieuse consiste en la fièvre Q qui présente l'avantage pour notre étude de se présenter soit sous une forme aiguë le plus souvent spontanément résolutive soit sous une forme chronique dont la manifestation principale est une endocardite. La maladie inflammatoire aiguë est, elle, représentée par des patients traumatisés admis en soins intensifs.

Figure 6. Dendrogramme représentant les spectres de référence de patients atteints d'une fièvre Q et de sujets sains



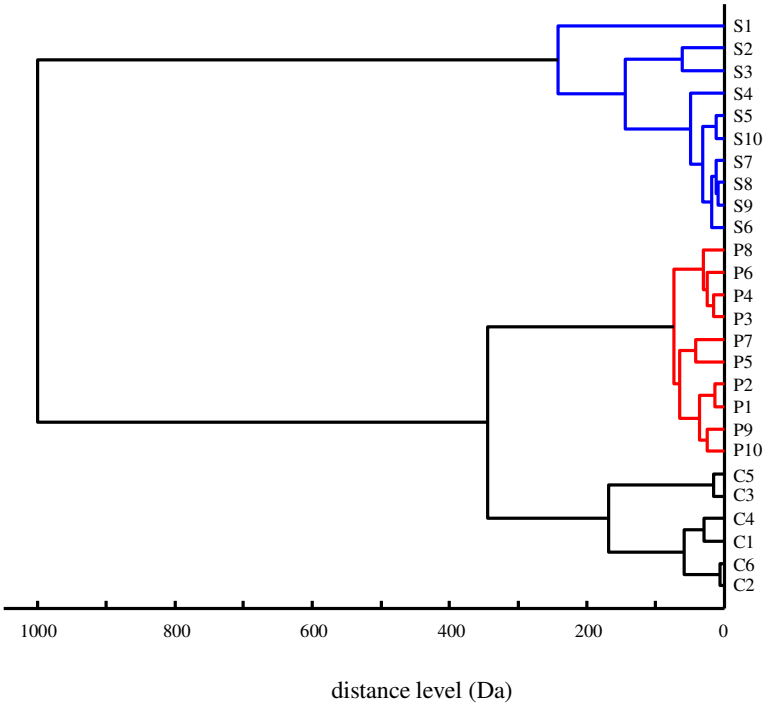
Douze spectres des PBMCs de 6 sujets contrôles (C1-C6) et de 15 patients atteints d'une fièvre Q (P1-P15) ont été obtenus par spectrométrie de masse MALDI-TOF. Un spectre moyen pour chaque individu a été créé en utilisant le logiciel Biotyper. Deux clusters apparaissent clairement, le premier regroupant les sujets contrôles et le second les patients atteints d'une fièvre Q. On peut noter qu'il n'est pas possible de différencier fièvre Q aiguë et endocardite de la fièvre Q.
AQF: fièvre Q aiguë; QFE: endocardite de la fièvre Q considérée comme active; cQFE: endocardite de la fièvre Q considérée comme guérie.

Nous avons tout d'abord comparé l'empreinte en spectrométrie de masse MALDI-TOF des PBMCs des patients souffrant d'une maladie infectieuse telle que la fièvre Q à celle de contrôles sains. Les PBMCs des patients appartiennent à un cluster différent de celui des donneurs sains mais il n'est pas possible de discriminer fièvre Q aiguë et endocardite de la fièvre Q (**Figure 6**). Les raisons qui peuvent expliquer ce résultat demeurent inconnues. Du matériel bactérien persiste de longues années dans les PBMCs des malades atteints d'une fièvre Q chronique (Marmion et al. 2005), ce qui peut expliquer une activation des PBMCs somme toute similaire que les patients souffrent d'une fièvre Q aiguë ou chronique. Il a également été montré que les PBMCs des patients atteints d'une fièvre Q aiguë ou chronique produisent en excès un certain nombre de cytokines (Capo et al. 1999; Honstettre et al. 2003) Quoiqu'il en soit, notre approche, si elle a permis de différencier infection à *C. burnetii* et absence d'infection, n'a pas permis de discriminer entre infection aiguë et infection chronique.

Nous avons utilisé la même approche pour comparer l'empreinte des PBMCs de patients traumatisés en soins intensifs, de patients atteints d'une fièvre Q et de donneurs sains. Nos résultats montrent clairement que la spectrométrie de masse MALDI-TOF permet de caractériser le trauma. De façon intéressante, l'empreinte des patients en soins intensifs est très différente de celles des patients atteints d'une fièvre Q (**Figure 7**). Ce résultat n'est pas complètement inattendu puisqu'ils sont dans un état hyper-inflammatoire conjuguant de nombreuses caractéristique spécifiques : signaux de danger (LPS, peptidoglycane, protéines de choc thermique (Gentile et Moldawer 2013; Cognasse, Hamzeh-Cognasse, et Garraud 2008)), composants immunorégulateurs, choc hypovolémique et septique (Basilia Zingarelli MD 2009), syndrome de dysfonction multiviscérale (E. J. Seeley, Matthay, et Wolters 2012; Lamkanfi et Dixit 2010; Harlan 2010). En résumé, notre approche en spectrométrie de masse sur cellules entières s'est révélée assez sensible pour discriminer l'empreinte des PBMCs de patients infectés et de patients dans un état hyper-inflammatoire.

Ces résultats demandent à être étendus à un plus grand nombre de malades. C'est ainsi que le pronostic vital des patients en soins intensifs peut être engagé sans que l'on sache prédire lesquels survivront et lesquels succomberont. Il serait intéressant de déterminer dans une étude rétrospective si ces deux catégories de malades présentent deux types d'empreintes en spectrométrie de masse MALDI-TOF. Il serait également intéressant de comparer différentes pathologies infectieuses entre elles ou différentes

Figure 7. Dendrogramme représentant les spectres de référence de patients atteints d'une fièvre Q, de patients septiques et de sujets sains



Douze spectres des PBMCs de 6 sujets contrôles (C1-C6), 10 patients atteints d'une fièvre Q (P1-P10) et 10 patients traumatisés en unité de soins intensifs (S1-S10) ont été obtenues par spectrométrie de masse MALDI-TOF. Un spectre moyen pour chaque individu a été créé en utilisant le logiciel Biotyper. Les patients en réanimation sont regroupés dans un cluster spécifique alors que les patients atteints d'une fièvre Q et les contrôles sont regroupés dans un autre cluster. Deux sous-clusters permettent de différencier patients atteints d'une fièvre Q et contrôles sains.

pathologies inflammatoires entre elles. Une étude menée sur des personnes âgées atteintes ou non d'une maladie inflammatoire suggère des différences importantes de l'empreinte protéique de leurs PBMCs (A. Daumas, résultats personnels), ce qui suggère que la spectrométrie de masse MALDI-TOF est suffisamment performante pour détecter des phénomènes inflammatoires.

Mes résultats ouvrent la porte à deux nouvelles perspectives. On peut tout d'abord se demander quelles sont les molécules détectées dans les différents spectres observés. Ce ne sont probablement pas les protéines de la membrane cellulaire détectées classiquement en cytométrie en flux puisque la spectrométrie de masse MALDI-TOF détecte des protéines d'une part solubles et d'autre part de faible poids moléculaire. Des premières tentatives d'identification des pics présents dans les spectres se sont révélées infructueuses mais il n'est pas certain que cette approche d'identification soit efficace, notre procédure étant d'essence combinatoire.

De nouvelles approches en spectrométrie de masse sont apparues récemment, en particulier dans le cadre du diagnostic précoce du cancer mais, de nouveau, il est nécessaire de prétraiter les prélèvements par des agents chimiques (Maurer et al. 2013). La spectrométrie de masse MALDI-TOF s'applique maintenant à des coupes de tissus, essentiellement dans un cadre d'imagerie MALDI. Cette approche permet de révéler le profil de diverses protéines exprimées dans le tissu et de les identifier par fragmentation (E. H. Seeley et al. 2008; Nicklay et al. 2013; Fonville et al. 2012). Dans l'optique du développement d'un outil de diagnostic tissulaire, une approche sur tissus équivalente à celle sur cellules entières que nous avons développée pourrait être testée. Elle impliquerait d'abord de comparer des tissus d'origine différente et d'analyser la combinatoire des empreintes obtenues. Elle pourrait permettre également de comparer tissus sains et tissus pathologiques avec comme idée sous-jacente qu'il serait possible d'obtenir le spectre spectrométrie de masse MALDI-TOF des cellules tumorales au sein d'un tissu sain. A cet égard, j'ai observé que les cellules leucémiques provenant de malades atteints d'une leucémie lymphoïde aiguë, d'une leucémie lymphoïde chronique ou d'une leucémie myéloïde aiguë donnent un signal en spectrométrie de masse MALDI-TOF sur cellules entières différent de celui des lymphocytes de donneurs sains. Ce signal est en outre totalement différent de celui des PBMCs provenant de sujets sains, de sujets atteints d'une fièvre Q et de sujets en soins intensifs (**Figure 7**). Il semble ainsi que la spectrométrie de masse MALDI-TOF sur cellules entières puisse être utilisée en clinique humaine.

REFERENCES

Aebersold R, Mann M. 2003. Mass spectrometry-based proteomics. *Nature* 422:198-207.

Ahmed FE. 2008. Utility of mass spectrometry for proteome analysis: part I. Conceptual and experimental approaches. *Expert Rev Proteomics* 5:841-864.

Autissier P, Soulas C, Burdo TH, Williams HC. 2010. Evaluation of a 12-color flow cytometry panel to study lymphocyte, monocyte, and dendritic cell subsets in humans. *Cytometry A.*;77:410-9.

Barbuddhe SB, Maier T, Schwarz G, Kostrzewa M, Hof H, Domann E, Chakraborty T, Torsten Hain T. 2008. Rapid identification and typing of *Listeria* species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Appl Environ Microbiol.*74 (17):5402-5407.

Basilia Z. 2009. Ischemia-reperfusion injury. *In science and practice of pediatric critical care medicine*; Derek S. Wheeler; Hector R. Wong; Thomas P. Shanley, Eds.; Springer London; 1–12.

Baumgarth N, Roederer M. 2000. A practical approach to multicolor flow cytometry for immunophenotyping. *J Immunol Methods* 243:77-97.

Ben Amara A, Bechah Y, Mege JL. 2012. Immune response and *Coxiella burnetii* invasion. *Adv Exp Med Biol.* 984:287-298.

Benoit M, Desnues B, Mege JL. 2008. Macrophage polarization in bacterial infections. *J Immunol.* 181:3733-3739.

Böhme K, Fernández-No IC, Barros-Velázquez J, Gallardo JM, Calo-Mata P, Cañas B. 2010. Species differentiation of seafood spoilage and pathogenic gram-negative bacteria by MALDI-TOF mass fingerprinting. *J Proteome Res.* 9:3169-3183.

Boom D van den, Wjst M, Everts R E. 2013. MALDI-TOF Mass Spectrometry. *In pharmacogenomics*; Innocenti F, Schaik R. H. N. van, Eds.; *Methods mol biol.* humana press; 71–85.

Brown M, Wittwer C. 2000. Flow cytometry: principles and clinical applications in hematology. *Clinical Chem.* 46:1221-1229.

Capo C, Amirayan N, Ghigo E, Raoult D, Mege JL. 1999. Circulating cytokine balance and activation markers of leucocytes in Q fever. *Clin Exp Immunol.* 115: 120-123.

Carbonnelle E, Mesquita C, Bille E, Day N, Dauphin B, Beretti JL, Ferroni A, Gutmann L, Nassif X. 2011. MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. *Clin Biochem.* 44:104-109.

Cognasse F, Hamzeh-Cognasse H, Garraud O. 2008. L'engagement des Toll-like receptors stimule la libération de facteurs plaquettaires à capacité immunomodulatrice. *Transfus Clin Biol.;*15:139-47.

Daumas A, Textoris J, Ouedraogo R, Capo C, Mege JL. 2013. Identification de différents états d'activation des cellules mononucléées du sang par spectrométrie de masse de type MALDI-TOF: application en pathologie humaine. *Revue Médecine Interne,* 34, A158-A159.

Daumas A, Textoris J, Ouedraogo R, Capo C, Mege JL. 2013. Identification de différents états d'activation des cellules mononucléées du sang par spectrométrie de masse de type MALDI-TOF: application en pathologie humaine. *Revue Médecine Interne,* 34, A158-A159.

De Hoffmann E. 2000. Mass spectrometry. In: *Kirk-othmer encyclopedia of chem Technology.* 1002. John Wiley & Sons, Inc.

De Hoffmann E. Mass Spectrometry. *Kirk-Othmer Encyclopedia of Chemical Technology.*; 2000.

De Masi R, Pasca S, Scarpello R, Idolo A, De Donno A. 2013. The clinical potential of blood-proteomics in multiple sclerosis. *BMC Neurol.* 13:45.

Delaby A, Gorvel L, Espinosa L, Lépolard C, Raoult D, Ghigo E, Capo C, Mege JL. 2012. Defective monocyte dynamics in Q fever granuloma deficiency. *J Infect Dis.* 205:1086-1094.

Demirev PA, Fenselau C. 2008. Mass spectrometry for rapid characterization of microorganisms. *Annu Rev Anal Chem.* 1:71-93.

Flannagan RS, Cosío G, Grinstein S. 2009. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nat Rev Microbiol.* 7:355-366.

Fontenot JD, Marc A. Gavin MA, Rudensky AY. 2003. Foxp3 programs the development and function of $CD4^{+}CD25^{+}$ regulatory T cells. *Nat Immunol.* 4:330-336.

Fonville JM, Carter C, Cloarec O, Nicholson JK, Lindon JC, Bunch J, Holmes E. 2012. Robust data processing and normalization strategy for maldi mass spectrometric imaging. *Anal Chem* 84:1310-1319.

Gentile, Lori F., et Lyle L. Moldawer. 2013. DAMPs, PAMPs, and the origins of SIRS in bacterial sepsis. *Shock* 39: 113-114.

Harlan John M. 2010. Endothelial activation and dysfunction in sepsis. In *Endothelial dysfunction and inflammation*, eds.: Dauphinee S, Karsan A. Progress in inflammation research. Springer Basel. 1-13.

Holmes K, Lantz LM, Fowlkes BJ, Schmid I, Giorgi JV. 2001. Preparation of cells and reagents for flow cytometry. *Curr Protoc Immunol.* Chapter 5: Unit 5.3.

Honstettre A, Imbert G, Ghigo E, Gouriet F, Capo C, Raoult D, Mege JL. 2003. Dysregulation of cytokines in acute Q fever: role of interleukin-10 and tumor necrosis factor in chronic evolution of Q fever. *J Infect Dis.* 187:956-962.

Huang TL, Cho YT, Su H, Shiea J. 2013. Principle component analysis combined with matrix-assisted laser desorption ionization mass spectrometry for rapid diagnosing the sera of patients with major depression. *Clin Chim Acta* 424C:175-181.

Ibrahim SF, van den Engh G. 2007. Flow cytometry and cell sorting. *Adv Biochem Eng Biotechnol.*;106:19-39.

Jurinke C, Oeth P, Boom DVD. 2004. MALDI-TOF mass spectrometry. *Mol Biotech* 26:147-163.

Kraan J, Gratama JW, Keeney M, D'Hautcourt JL. 2003. Setting up and calibration of a flow cytometer for multicolor immunophenotyping. *J Biol Regul Homeost Agents* 17:223-233.

Krader P, Emerson D. 2004. Identification of archaea and some extremophilic bacteria using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. *Extremophiles* 8:259-268.

Lamkanfi M, Dixit VM. 2010. Manipulation of host cell death pathways during microbial infections. *Cell Host Microbe* 8:44-54.

Lewis WG, Shen Z, Finn MG, Siuzdak G. 2003. Desorption/ionization on silicon (DIOS) mass spectrometry: background and applications. *Int J Mass Spectrom.* 226:107-116.

Li X, Cowles EA, Cowles RS, Gaugler R, Cox-Foster DL. 2009. Characterization of immunosuppressive surface coat proteins from *Steinernema glaseri* that selectively kill blood cells in susceptible hosts. *Mol Biochem Parasitol.* 165:162-169.

Link AJ, Eng J, Schieltz DM, Carmack E, Mize GJ, Morris DR, Garvik BM, Yates JR. 1999. Direct analysis of protein complexes using mass spectrometry. *Nature Biotechnol.* 17:676-682.

Mann M, Hendrickson RC, Pandey A. 2001. Analysis of proteins and proteomes by mass spectrometry. *Ann Rev Biochem.* 70: 437-473.

Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. 2013. Macrophage plasticity and polarization in tissue repair and remodelling. *J Pathol.* 229:176–185.

Mantovani S, Massimo L. 2005. Macrophage polarization comes of age. *Immunity*: 344-346.

Marmion BP, Sukocheva O, Storm PA, Lockhart M, Turra M, Kok T, Ayres J, Routledge H, Graves S. 2009. Q fever: persistence of antigenic non-viable cell residues of Coxiella burnetii in the host--implications for post Q fever infection fatigue syndrome and other chronic sequelae. *QJM:* 673-684.

Martinez FO, Gordon S, Locati M, Mantovani A. 2006. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol.* 177 :7303-7311.

Martinez FO, Sica A, Mantovani A, Locati M. 2008. Macrophage activation and polarization. *Front Bioscience* 13:453.

Maurer K, Eschrich K, Schellenberger W, Bertolini J, Rupf S, Remmerbach TW. 2013. Oral brush biopsy analysis by MALDI-TOF mass spectrometry for early cancer diagnosis. *Oral Oncology* 49:152-156.

Maurin M, Raoult D. 1999. Q Fever. *Clin Microbiol Rev* 12:518-553.

Mellmann A, Cloud J, Maier T, Keckevoet U, Ramminger I, Iwen P, Dunn J, et al. 2008. Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. *J Clin Microbiol.* 46:1946-1954.

Meyer K, Ueland PM. 2011. Use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for multiplex genotyping. *Adv Clin Chem.* 53:1-29.

Moura H, Woolfitt AR, Carvalho MG, Pavlopoulos A, Teixeira LM, Satten GA, Barr JR. 2008. MALDI-TOF mass spectrometry as a tool for differentiation of invasive and noninvasive streptococcus pyogenes isolates. *FEMS Immunol Med Microbiol* 53:333–342.

Nicklay JJ, Harris GA, Schey KL, Caprioli RM. 2013. MALDI imaging and in situ identification of integral membrane proteins from rat brain tissue sections. *Anal Chem.* 85:7191-7196.

Nieto WG, Almeida J, Romero A, Teodosio C, López A, Henriques AF, Sánchez ML, et al. 2009. Increased frequency (12%) of circulating chronic lymphocytic leukemia–like B-cell clones in healthy subjects using a highly sensitive multicolor flow cytometry approach. *Blood* 114:33-37.

Ong SE, Mann M. 2005. Mass spectrometry–based proteomics turns quantitative. *Nature Cheml Biol.* 1:252-262.

Parisi D, Magliulo M, Nanni P, Casale M, Forina M, Roda A. 2008. Analysis and classification of bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and a chemometric approach. *Anal Bioanal Chem.* 391:2127-2134.

Pignone M, Greth KM, Cooper J, Emerson D, Tang J. 2006. Identification of mycobacteria by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. *J Clin Microbiol* 44:1963-1970.

Pinkel Dan. 1982. Flow cytometry and sorting. *Anal Chem.* 54: 503A-519A.

Raoult D. 2012. Chronic Q fever: expert opinion versus literature analysis and consensus. *J infect.* 65:102-8.

Recktenwald DJ. 1993. Introduction to flow cytometry: principles, fluorochromes, instrument set-up, calibration. *J Hematother.* 2:387-394.

Reinders J, Lewandrowski U, Moebius J, Wagner Y, Sickmann A. 2004. Challenges in mass spectrometry-based proteomics. *Proteomics* 4:3686–3703.

Salvador F, Porte L, Durán L, Marcotti A, Pérez J, Thompson L, Noriega LM, Lois V, Weitzel T. 2013. Breakthrough bacteremia due to *Clostridium tertium* in a patient with neutropenic fever, and identification by MALDI-TOF mass spectrometry. *Int J Infect Dis.* 17:e1062-3.

Sauer S, Kliem M. 2010. Mass spectrometry tools for the classification and identification of bacteria. *Nature Rev Microbiol.* 8:74-82.

Seeley EJ, Matthay MA, Wolters PJ. 2012. Inflection points in sepsis biology: from local defense to systemic organ injury. *Amer J Physiol.* 303:L355-L363.

Seeley EH, Oppenheimer SR, Mi D, Chaurand P, Caprioli RM. 2008. Enhancement of protein sensitivity for MALDI imaging mass spectrometry after chemical treatment of tissue sections. *J Amer Soc Mass Spect.*19:1069-1077.

Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, Rolain JM, Raoult D. 2009. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin Infect Dis.* 49:543-551.

Seng P, Rolain JM, Fournier PE, La Scola B, Drancourt M, Raoult D. 2010. MALDI-TOF-mass spectrometry applications in clinical microbiology. *Future Microbiol.* 5:1733-1754.

Sinz A. 2006. Chemical cross-linking and mass spectrometry to map three-dimensional protein structures and protein–protein interactions. *Mass Spectrom Rev.* 25: 663–82.

Sogawa K, Watanabe M, Nomura F. 2013. Rapid identification of microorganisms using maldi-tof mass spectrometry. *Rinsho Byori.*:44-51.

Tantibhedhyangkul W, Prachason T, Waywa D, El Filali A, Ghigo E, Thongnoppakhun W, Raoult D, et al. 2011. *Orientia tsutsugamushi* stimulates an original gene expression

program in monocytes: relationship with gene expression in patients with scrub typhus. *PLoS Negl Trop Dis.* 5:e1028.

Tugal D, Liao X, Jain MK. 2013. Transcriptional control of macrophage polarization. *Arterioscler Thromb Vasc Biol.*33:1135-1144.

Van den Boom D, Wjst M, Everts RE. 2013. MALDI-TOF mass spectrometry. *Methods mol biol.* 1015:71-85.

Vignali DA. 2000. Multiplexed particle-based flow cytometric assays. *J Immunol Methods* 243:243-55.

Virgo PF, Gibbs GJ. 2012. Flow cytometry in clinical pathology. *Ann Clin Biochem.* 49:17-28.

Von EF, Junker K, Fiedle W, Wollscheid V, Dürst M, Claussen U, Ernst G. 2001. Mass spectrometry meets chip technology: a new proteomic tool in cancer research? *Electrophoresis* 22:2898-2902.

Welker M. 2011. Proteomics for Routine Identification of Microorganisms. *Proteomics* 11: 3143–53.

Welker M, Moore ER. 2011. Applications of whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry in systematic microbiology. *Syst Appl Microbiol* 34:2-11.

Wunschel SC, Jarman KH, Petersen CE, Valentine NB, Wahl KL, Schauki D, Jackman J, Nelson CP, White E. 2005. Bacterial analysis by MALDI-TOF mass spectrometry: An inter-laboratory comparison. *J Am Soc Mass Spectrometry* 16:456-462.

ANNEXE

Tutorial script for whole-cell MALDI-TOF analysis

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June 19, 2013

Contents

Abstract

The present document is intended to be a tutorial to use R to analyze whole-cell MALDI-TOF data in the way it is described in the accompanying publication "Whole-cell MALDI-TOF mass spectrometry is an accurate and rapid method to analyze different modes of macrophage activation", published in the Journal of Visualized Experiments. The document will guide the reader to load, explore and analyze MALDI-TOF data.

The present tutorial will guide the reader through the analysis with R of MALDI-TOF data. To keep a reasonable computational time, this tutorial comes along with a reduced dataset of 48 spectra obtained from macrophages either unstimulated or stimulated with IFN gamma (to induce a M1 polarization) or IL-4 (to induce a M2 polarization).

1 Required libraries

In order to load and analyze MALDI-TOF data, you will need to install at least three specific libraries written by *Sebastian Gibb*: `readBrukerFlexData`, `MALDIquant`, and `MALDIquantForeign`.

```
## Loading libraries used in analysis
library(readBrukerFlexData)  # Allow to load Bruker raw data in R
## Contains most of pre-processing functions as well as
## alignment functions
library(MALDIquant)

library(MALDIquantForeign)
```

2 Data loading

Once you have extracted the archive, you should use this pdf tutorial, along with the script `.Rnw` that generated it, and a data folder: `data_18h`. This folder holds all raw MALDI-TOF data. These data were generated by a Bruker Autoflex II. First, you will set the directory to the current directory, so the script will find the data easily. This is done by the command `setwd()`. If you have extracted the archive in `/home/myuser/tutorialMALDI/`, you will use this as an argument for `setwd("/home/myuser/tutorialMALDI/")`. Then, raw data will automatically be imported from the data folder into an object we chose to name `spectra` by the command `importBrukerFlex()`. The `spectra` object is a list. You can find how many raw spectra it contains by executing the command `lenght(spectra)`. If you type `is(spectra[[1]])`, you'll see that it is a special `MALDIquant` object (see `MALDIquant` library doc for more details).

```
## set working directory to the root of the tree folders
## that contains raw data obtained from Bruker MALDI-TOF
setwd("/data/partage/MALDI/JoVE/data_18h/")

## Load raw data into MassSpectrum objects. All spectra are
## concatenated into a list
spectra = importBrukerFlex("./")


length(spectra)

## [1] 48
```

```
is(spectra[[1]])

## [1] "MassSpectrum"       "AbstractMassObject"
```

Sometimes, there is no spectra obtained from a given run. If you keep this "empty" spectra, it will end up with errors later. Empty spectra, if they exists, are then removed by the following code.

```
v.empty = lapply(spectra, function(y) {
    return(min(y@intensity) == max(y@intensity))
})
length(which(unlist(v.empty) == T))

## [1] 0



## If length of empty spectra is > 0, uncomment the
## following lines to remove the empty spectra spectra =
## spectra[-c(which(unlist(v.empty)==T))] length(spectra)
```

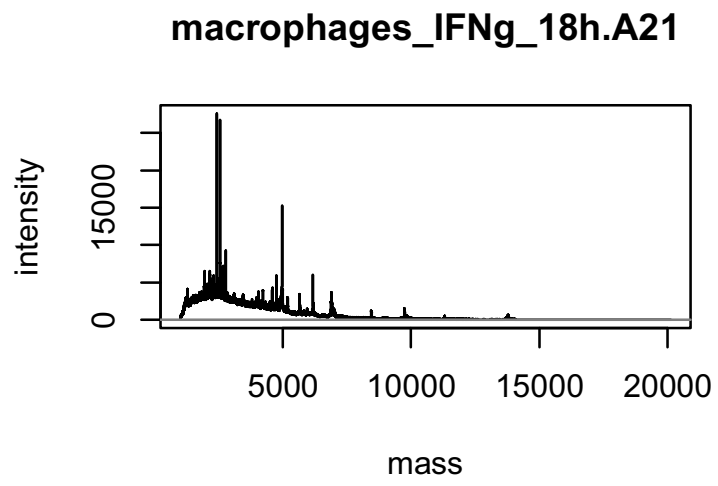
The following lines are used to compute "label" vectors for figures.

```
# sampleNames are the names of the main folders under the
# root
sampleNames = lapply(spectra, function(y) {
    y@metaData$sampleName
})
sampleNames = as.factor(unlist(sampleNames))
levels(sampleNames)

## [1] "macrophages_IFNg_18h"
"Macrophages_IL_4_18h"
## [3] "macrophages_NS_18h"


# all other vectors are computed from complete filename
# (with folder path)
group = lapply(spectra, function(y) {
    y@metaData$file
})
group[grep(" IFNg ", group)] = "IFNg"
group[grep("IL-4", group)] = "IL4"
group[grep("NS", group)] = "NS"
group = as.factor(unlist(group))
levels(group)

## [1] "IFNg" "IL4"  "NS"
```

3 Spectrum visualization and pre-processing

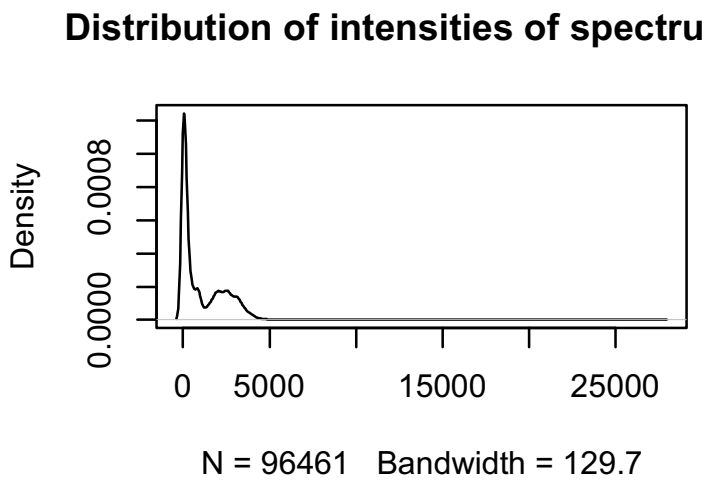
To get a graphical representation of a given spectrum, you can type `plot(spectra[[1]])`. You'll see one major peak close to 5000 m/z, and a few other peaks of much lower intensities. To improve visualization, you can transform the intensities (*i.e.* the abondance of each ion a a given mass) by applying a mathematical transformation such as log or square root. Square root transformation may also result in variance stabilization. Variance stabilization is used to overcome the dependancy of the variance from the mean. After square root transformation, the variance is nearly constant and the data are approximately Gaussian distributed. The later is important for various statistical analysis. Here, the distribution is still not Gaussian, but visualization of peaks with lower intensities is improved.

```
plot(spectra[[1]])
```

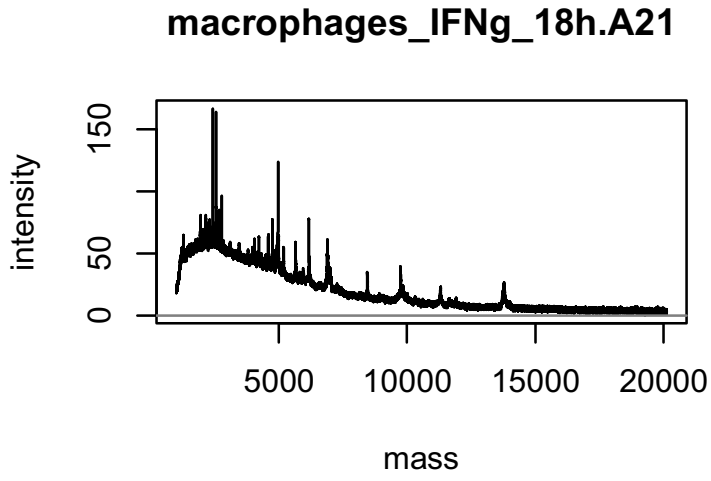


/data/partage/MALDI/JoVE/data_18h/macrophages IFNg 18h/0_A21/1/1

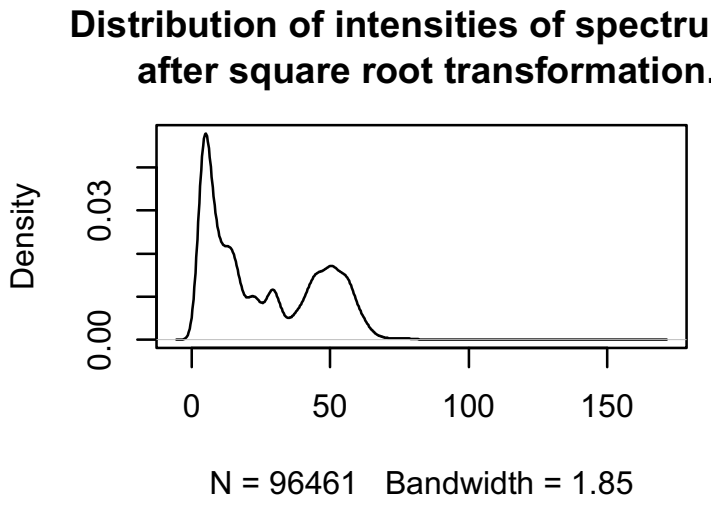
```
plot(density(intensity(spectra[[1]])),
main = "Distribution of intensities of spectrum 1.")
```




```
spectra = lapply(spectra, transformIntensity, fun = sqrt)
plot(spectra[[1]])
```




```
plot(density(intensity(spectra[[1]])),
main = "Distribution of intensities of spectrum 1\n
after square root transformation.")
```

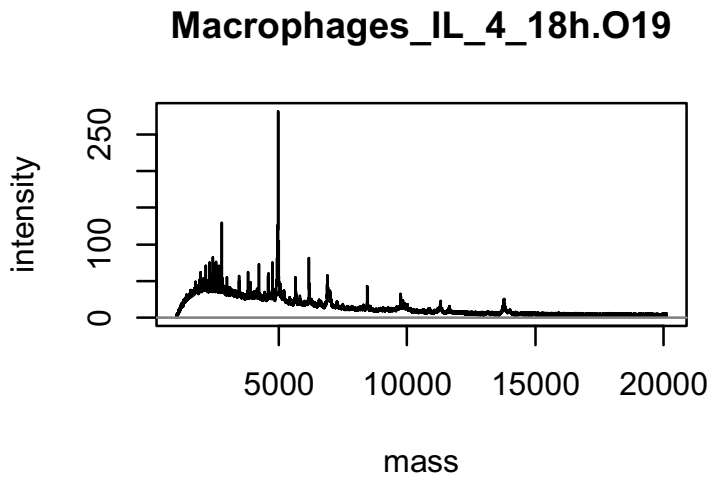


Then we apply a moving average with a window size of 5 m/z values to smooth the data.

```
## This function perform a smoothing of the intensities by
## using a moving average window
movAvg = function(y) {
    return(filter(y, rep(1, 5)/5, sides = 2))
}
spectra = lapply(spectra, transformIntensity, fun = movAvg)
```

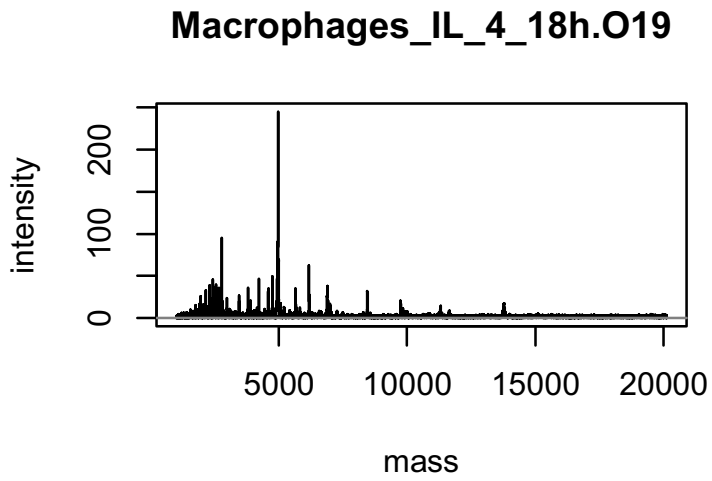
For some spectrum, there is a baseline deviation that we must remove before starting the analysis. This can be done by various algorithms (see MALDIquant documentation for details). Here we use SNIP algorithm.

```
plot(spectra[[27]])
```



/data/partage/MALDI/JoVE/data_18h/Macrophages IL−4 18h/0_O19/1/1

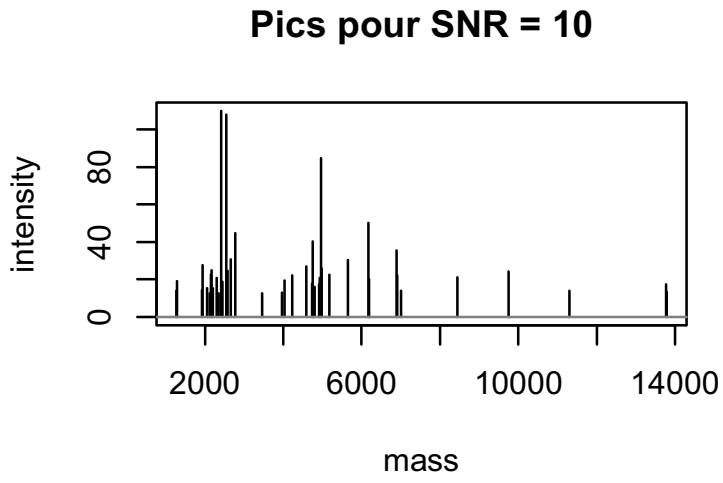
```
## Baseline correction (Best method is SNIP)
spectra = lapply(spectra, removeBaseline, method = "SNIP")

plot(spectra[[27]])
```



/data/partage/MALDI/JoVE/data_18h/Macrophages IL−4 18h/0_O19/1/1

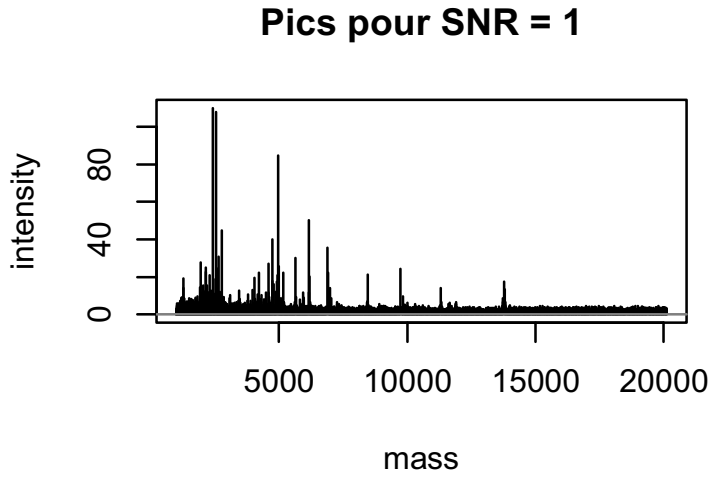
Then, we want to detect peaks in a given spectrum. For this, we have to choose a signal to noise ratio to discriminate between peaks and noise background. A SNR of 6 is usually a good compromise (between the number of peaks obtained and the specificity of these peaks). Peaks are stored in a second object. The plot function will now display only peaks. Many visualization possibilities are available within the MALDIquant library, for example to label the peaks with their respective m/z value.

```
pk = lapply(spectra, detectPeaks, SNR = 10, halfWindowSize = 20)

plot(pk[[1]], main = "Pics pour SNR = 10")
```



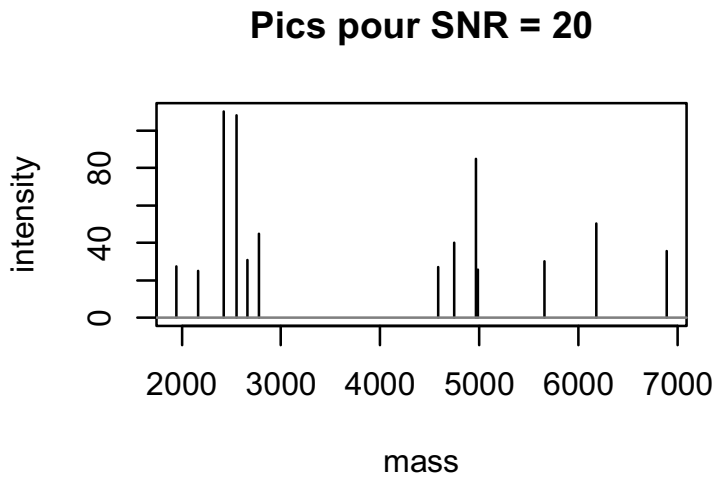
/data/partage/MALDI/JoVE/data_18h/macrophages IFNg 18h/0_A21/1/1

```
plot(lapply(spectra, detectPeaks, SNR = 1, halfWindowSize = 20)[[1]],
    main = "Pics pour SNR = 1")
```



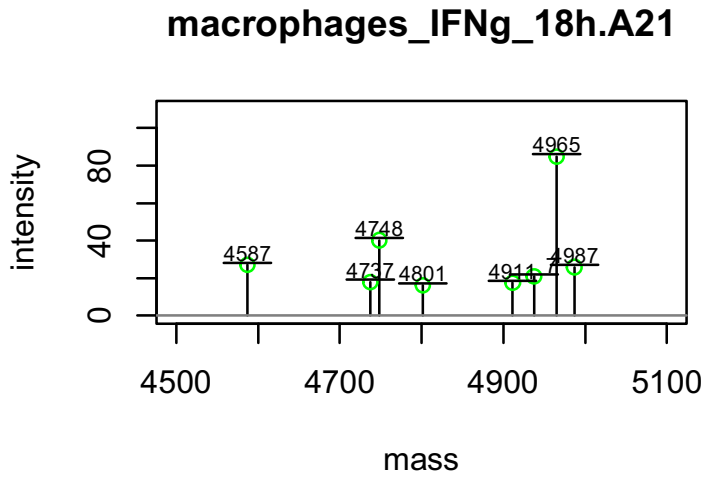
/data/partage/MALDI/JoVE/data_18h/macrophages IFNg 18h/0_A21/1/1

```
plot(lapply(spectra, detectPeaks, SNR = 20, halfWindowSize = 20)[[1]],
    main = "Pics pour SNR = 20")
```



/data/partage/MALDI/JoVE/data_18h/macrophages IFNg 18h/0_A21/1/1

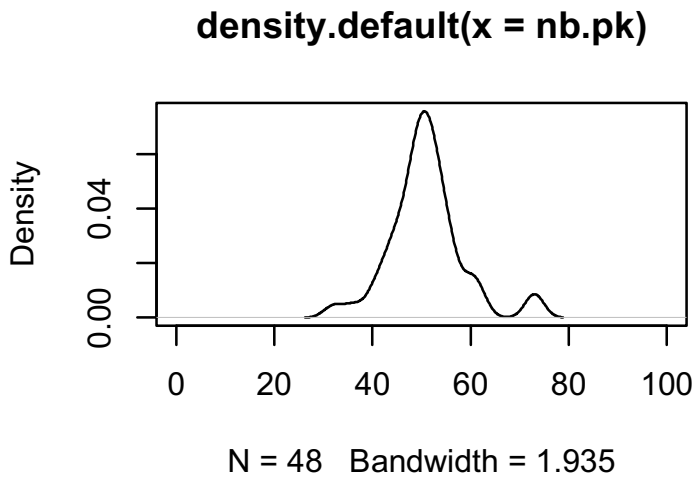
```
## To label peaks
plot(pk[[1]], xlim = c(4500, 5100))
points(pk[[1]], col = "green")
labelPeaks(pk[[1]])
```



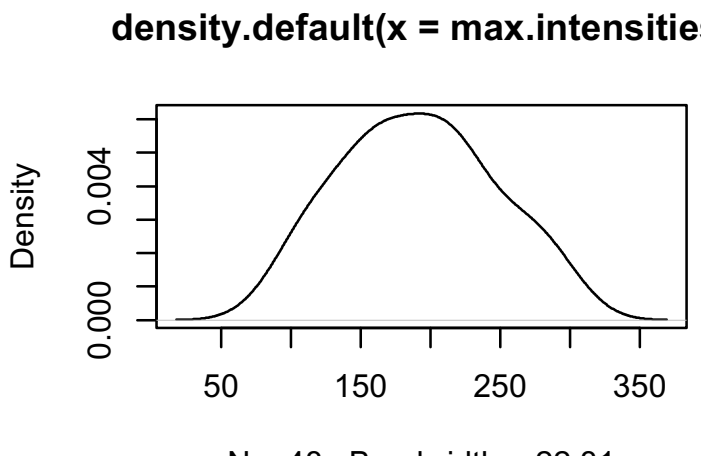
/data/partage/MALDI/JoVE/data_18h/macrophages IFNg 18h/0_A21/1/1SLin/fid

Here, we use peak detection to automatically check the quality of the samples. We want a given sample to have at least 50 peaks, and we want the maximal intensity of the main peak to be at least above 50. To determine these values, we plot the distribution of the number of peaks in each spectrum, and the distribution of maximal intensities.

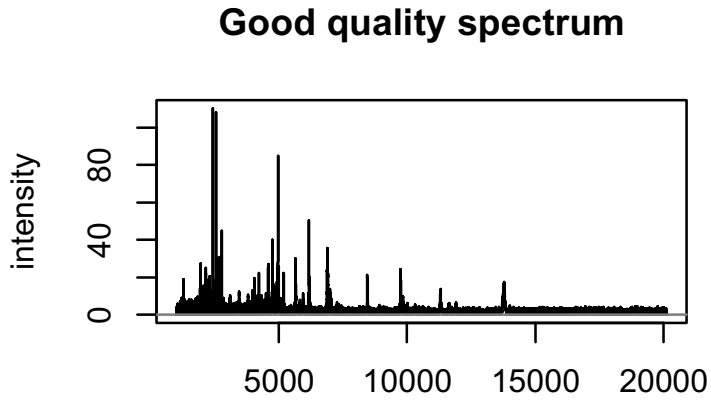
```
## The following steps check if spectra quality is
## sufficient for the analysis. We use two criteria : a
## sufficient number of peaks detected with a sufficient
## SNR (usually 50) the maximal intensity of all peaks is
## at least i = 40

nb.pk = unlist(lapply(pk, function(y) {
    length(y@mass)
}))
plot(density(nb.pk), xlim = c(0, 100))
```




```
length(which(nb.pk < 40))

## [1] 2


max.intensities = unlist(lapply(spectra, function(y) {
    as.numeric(y@intensity)[order(as.numeric(y@intensity), decreasing = T)[1]]
}))
plot(density(max.intensities))
```

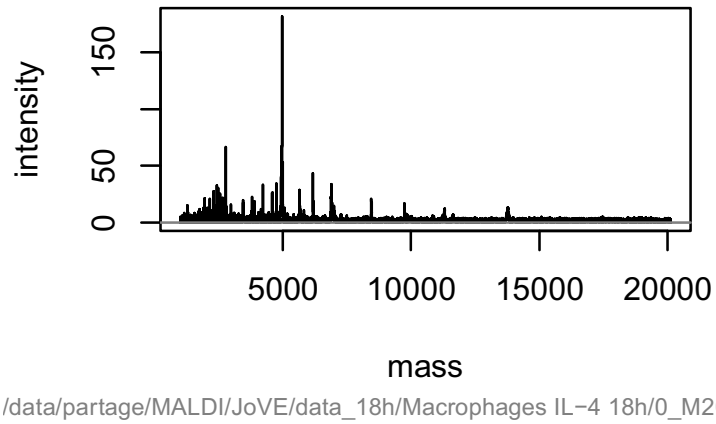



```
length(which(max.intensities < 50 | nb.pk < 40))

## [1] 2

# 2 spectrum will be removed for poor quality, this is how
# they look like :
which(max.intensities < 50 | nb.pk < 40)

## [1] 4 8


# good quality spectrum
plot(spectra[[1]], main = "Good quality spectrum")
```



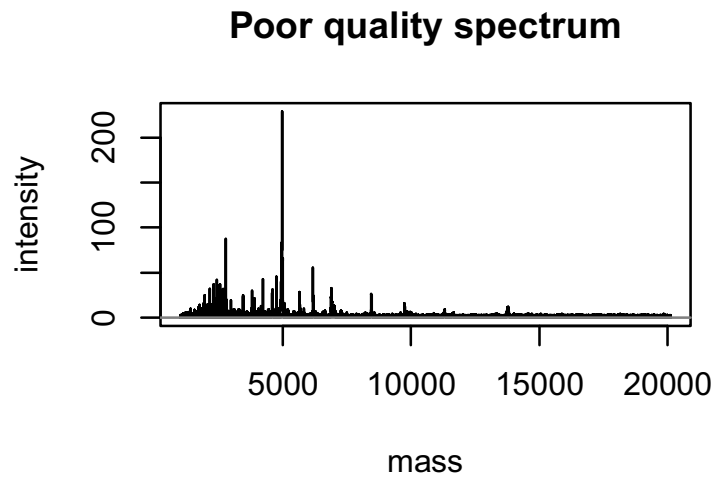
/data/partage/MALDI/JoVE/data_18h/macrophages IFNg 18h/0_A21/1/1

```
plot(spectra[[20]], main = "Poor quality spectrum")
```



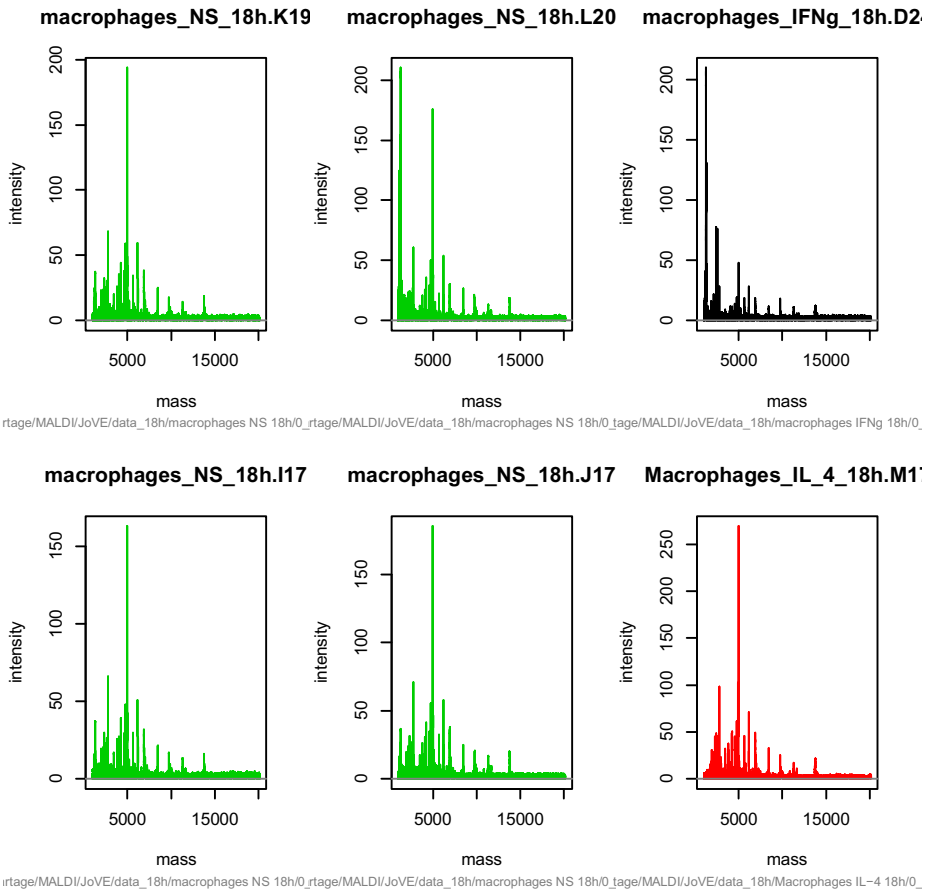
/data/partage/MALDI/JoVE/data_18h/Macrophages IL-4 18h/0_M20/1/1

```
plot(spectra[[24]], main = "Poor quality spectrum")
```




```
## If you want to eliminate some spectra, uncomment the
## following lines
spectra = spectra[-c(which(max.intensities < 50 | nb.pk < 40))]
sampleNames = as.factor(as.vector(sampleNames[-c(which(max.intensities <
    50 | nb.pk < 40))]))
group = as.factor(as.vector(group[-c(which(max.intensities <
    50 | nb.pk < 40))]))
```

```
## Just to check visually some spectra sampled into the
## whole dataset
par(mfrow = c(2, 3))
ind = sample(1:length(spectra), 6)
for (i in ind) {
    plot(spectra[[i]], col = group[i])
}
```



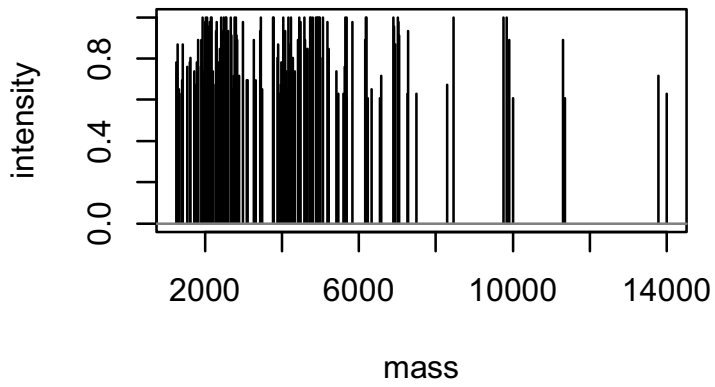
Then, we will normalize the dataset by "total ion current".

```
## Calibrate/Normalize intensity values by 'total ion
## current'
spectra <- standardizeTotalIonCurrent(spectra)
```

Finally, as we will compare spectrum for presence/absence of a given peak, we must first align the spectra.

```
## Alignment.  To perform alignement, we first create a
## reference spectra for whole dataset with a low SNR, and
## a low minimal frequency This reference spectra is a
## MassPeak object, and will contain a list of peaks used
## to aligned all spectra together
pk = lapply(spectra, detectPeaks, SNR = 4, halfWindowSize = 20)

refPeaks <- referencePeaks(pk, "strict", 0.6, 0.002)

## Check that the reference spectra contains sufficient
## peaks and that these peaks are distributed across the
```

```
## whole range of m/z values to obtain a good alignment
par(mfrow = c(1, 1))
plot(refPeaks)
```




```
## If necessary, recompute PeakLists with the SNR wanted
## for the analysis
pk = lapply(spectra, detectPeaks, SNR = 6, halfWindowSize = 20)

## This function from MALDIquant computes all warping
## functions for the alignment
warpingFunctions <- determineWarpingFunctions(pk, reference = refPeaks)

pk.aligned <- warpMassPeaks(pk, warpingFunctions)
sp.aligned = warpMassSpectra(spectra, warpingFunctions)


## As aligned spectra may have different minimum and
## maximum masses we computed the max of the minimal values
## and the min of maximal values This gives us the range
## that is common for all aligned spectra
mins = unlist(lapply(sp.aligned, function(y) {
    min(y@mass)
}))
maxs = unlist(lapply(sp.aligned, function(y) {
    max(y@mass)
}))
lim1 = round(max(mins, na.rm = T), 0) + 1
lim2 = round(min(maxs, na.rm = T), 0) - 1
lim1
```

```
## [1] 1002

lim2

## [1] 20132
```

4 Analysis and comparison of spectra

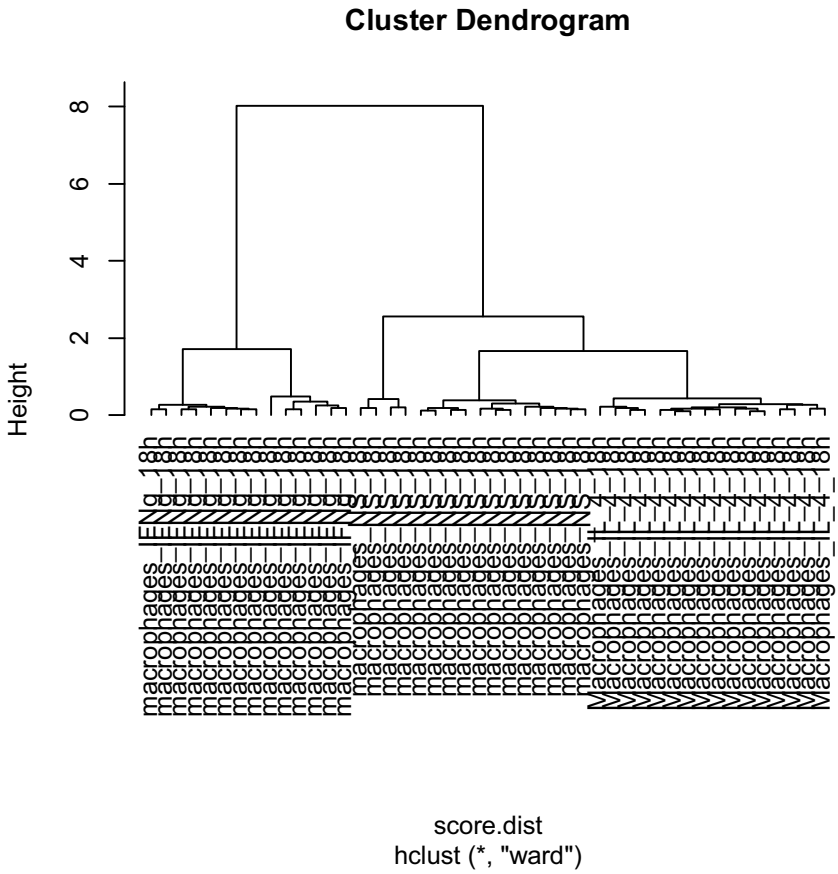
See below the functions that we will use to compute a score to compare spectra. The idea of that is to use two criteria : the presence / absence of a given peak in the two spectra that are compared (as it is a boolean criteria, we will compute the Jaccard Index), and then a correlation coefficient to adjust for the intensities of a given peak in both spectra. The second criteria is less important and may be avoided.

```
### Some functions we will need to compute scores (modified
### by S. Gibb)
as.binary.matrix <- function(x) {
    return(ifelse(is.na(x), 0, 1))
}

jaccard <- function(x) {
    n11 <- tcrossprod(x)
    n01 <- tcrossprod(1 - x, x)
    n10 <- tcrossprod(x, 1 - x)
    # return(n11/(n01+n10+n11))
    return(2 * n11/(n01 + n10 + 2 * n11))
}

## This function computes the 'Score'.  This version
## computes a score based on the Jaccard indice and a
## pearson correlation coefficient based on the intensities
## of common peaks between the two spectra : S = jac*cor ;
## jac is Jaccard indice (between 0-1) and cor is Pearson
## correlation coefficient (between 0-1).
computeModJacScoreOnPeaks <- function(p, tolerance = 0.002, range = c(0,
    20000)) {
    trimmedPeaks <- trim(p, range[1], range[2])
    binnedPeaks <- binPeaks(trimmedPeaks, method = "relaxed",
        tolerance = tolerance)
    ## remove peaks occuring only once
    filteredPeaks <- filterPeaks(binnedPeaks, minFrequency = 2/length(binnedPeaks))
    ## to run a groupwise filtering use filteredPeaks <-
    ## filterPeaks(binnedPeaks, labels=group, minFrequency=2/3)

    peakMatrix <- intensityMatrix(filteredPeaks)

    ja <- jaccard(as.binary.matrix(peakMatrix))
```

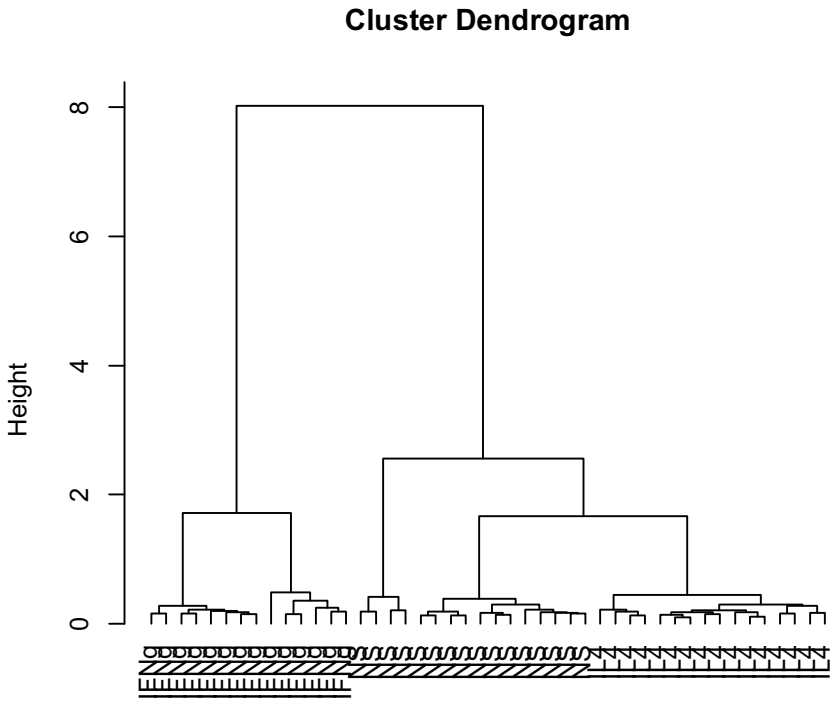
```
    co <- cor(t(peakMatrix), method = "pearson", use = "pairwise.complete.obs")

    return(ja * co)
}
```

In order to draw a virtual gelview of the dataset, the code below will compute a matrix with a given resolution for m/z, and for each value, we will keep a median intensity value. We will end with a numerical data matrix which will be plot as a heatmap. In the heatmap, m/z value order will be conserved, but samples will be reorganized according to a clustering based on the score that compare the spectra.

```
## To build a virtual gel-view representation of the
## spectra, we first summarize the dataset by keeping only
## one value for each m/z value We take the median
## intensity of the n values in a given m/z value
system.time({
    m3 <- trim(sp.aligned, lim1, lim2)

    m3 <- lapply(m3, function(x) {
        m <- round(mass(x))
        i <- unlist(lapply(split(intensity(x), m), median))
        x@intensity <- i
        x@mass <- unique(m)
        return(x)
    })

    m3 <- intensityMatrix(m3)
})

## Check that you have 1 on the diagonal
score.mat <- computeModJacScoreOnPeaks(pk.aligned)

# Computes distance matrix to draw dendrogram
score.dist = as.dist(1 - score.mat)

# Plot dendrogram, label leafs with various vectors
# (sampleNames, group)
plot(hclust(score.dist, method = "ward"), labels = sampleNames,
    hang = -1)
```




```
plot(hclust(score.dist, method = "ward"), labels = group, hang = -1)
```

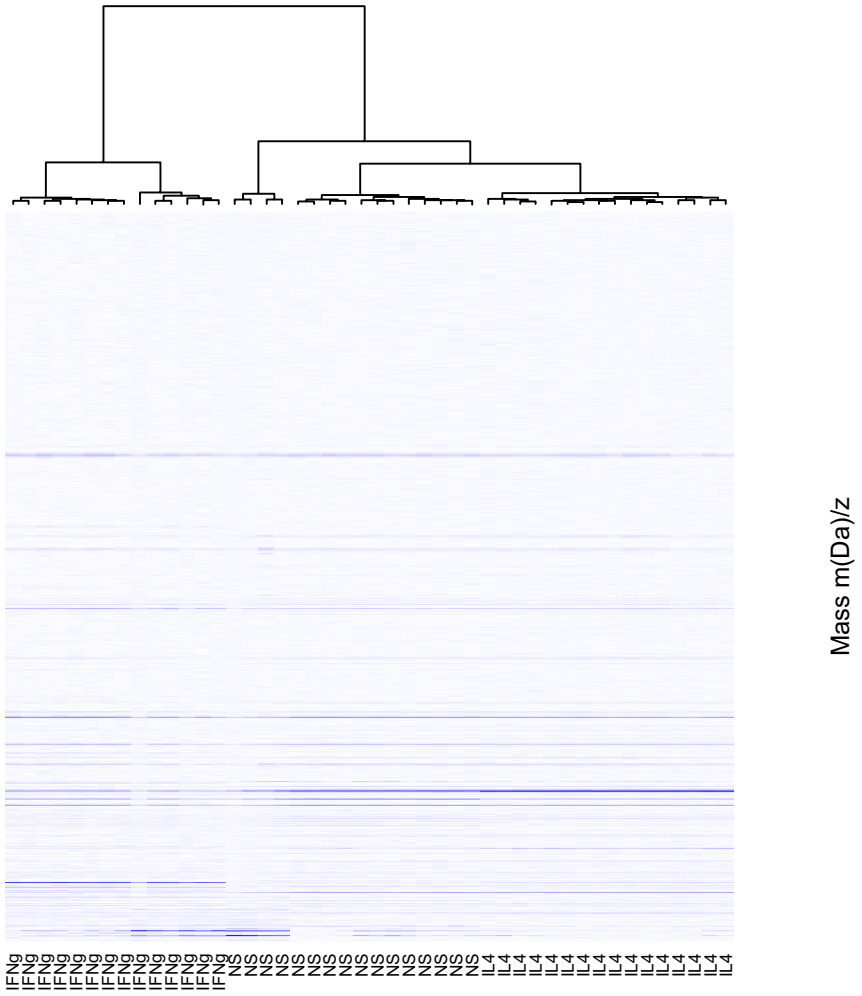


Of note, it is important to understand that clustering implies an agglomerative method to display as a dendrogram the distance matrix. The distances presented in the dendrogram are not the true distances of the initial matrix (`score.dist`). They may differ depending on the agglomerative function used (textttaverage / complete / ward ...). Here, we chose to use the Ward algorithm. It favorize clusters of equal number of samples by variance stabilization. However, the heigh displayed along the dendrogram is not comprised between 0 and 1, because it does not represent the distance, but the Ward's crierion for agglomeration. To illustrate the difference between the intra-class distance (which is low) and the inter class distance (which is high), we proide below a chunk of code which comptes the mean distance of each class.

```
# Mean intra-class distance is low
mean(as.vector(as.matrix(score.dist)[which(group == "IFNg"),
    which(group == "IFNg")]))
mean(as.vector(as.matrix(score.dist)[which(group == "IL4"), which(group ==
    "IL4")]))
mean(as.vector(as.matrix(score.dist)[which(group == "NS"), which(group ==
    "NS")]))
# Mean inter-class distance is high
mean(as.vector(as.matrix(score.dist)[which(group == "IFNg"),
```

```
    which(group == "IL4")]))
mean(as.vector(as.matrix(score.dist)[which(group == "IFNg"),
    which(group == "NS")]))
mean(as.vector(as.matrix(score.dist)[which(group == "NS"), which(group ==
    "IL4")]))
```

```
## To draw the gel-view representation : As score.dist is
## used to compute the spectra dendrogram, you have to
## compute score.mat and score.dist first
heatmap(as.matrix(t(m3)), Rowv = NA, scale = "col",
Colv = as.dendrogram(hclust(score.dist,
method = "ward")), col = colorRampPalette(c("white",
"blue", "blue"))(1000), labCol = group, labRow = FALSE, ylab =
"Mass m(Da)/z", xlab = "Samples")
```



PUBLICATIONS

1. PATENT: N° WO 2011/154650.
Inventors: **Richard Ouedraogo,** Christian Capo, Amira Ben Amara, Didier Raoult, Jean-Louis MEGE.

2. **Richard Ouedraogo**, Christophe Flaudrops, Amira Ben Amara, Christian Capo, Didier Raoult, Jean-Louis Mege. Global Analysis of Circulating Immune Cells by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry. *PLoS One,* 2010, 5:e13691.

3. **Richard Ouedraogo**, Aurélie Daumas, Eric Ghigo, Christian Capo, Jean-Louis Mege, Julien Textoris. Whole-Cell MALDI-TOF MS: A New Tool to Assess the Multifaceted Activation of Macrophages. *Journal of Proteomics*. 2012, 75:5523-5532.

4. **Richard Ouedraogo**, Julien Textoris, Aurélie Daumas, Christian Capo, Jean-Louis Mege. Whole-cell MALDI -TOF Mass Spectrometry: a Tool for Immune Cell Analysis and Characterization. Book: *Methods in Molecular Biology Series, Immunoproteomics*. (eds, K. Fulton & S. Twine), Humana Press, New York, 2013, p. 197-209

5. A Daumas, J Textoris, **R Ouedraogo**, C Capo, J-L Mege. Identification de différents états d'activation des cellules mononuclées du sang par spectrométrie de masse de type MALDI-TOF : application en pathologie humaine. *La revue de medecine interne*, 34 (06.2013)

7. **Richard Ouedraogo**, Aurélie Daumas, Christian Capo,Jean-Louis Mege, Julien Textoris. Whole-cell MALDI-TOF mass spectrometry is an accurate and rapid method to analyze different modes of macrophage activation. Journal of Visualized Experiments. *In press*

6. **Richard Ouedraogo**, Julien Textoris, Christian Capo, Didier Raoult, Jean-Louis Mege. Q fever: An analysis of host response by MALDI-TOF mass spectrometry. *In preparation*

8. **Richard Ouedraogo**, Claude Villard, Jean-Louis Mege, Daniel Lafitte, Eric Ghigo. Comparative mapping and functional proteomics of proteomes of phagosomes between microbicidal cells (BMDM) and non microbicidal cells (L929). *In preparation*

Résumé

L'invention de la spectrométrie de masse MALDI-TOF a énormément contribué à la compréhension de la chimie des protéines, de la biologie cellulaire et moléculaire. L'identification et la caractérisation des microorganismes intacts par la spectrométrie de masse MALDI-TOF ont été une révolution dans les laboratoires de microbiologie clinique lors de ces dernières années. Au regard de ses nombreux avantages en terme de rapidité, de coût, de sensibilité et de fiabilité, nous avons cru pouvoir l'appliquer à l'étude des cellules eucaryotes intactes, en particulier à l'étude des cellules immunitaires. La comparaison des monocytes circulants, des lymphocytes T et des polynucléaires neutrophiles a révélé des profils de pics spécifiques. Les macrophages et les cellules dendritiques dérivés des monocytes sont distincts des monocytes circulants. En outre, la spectrométrie de masse MALDI-TOF a permis d'identifier facilement les signatures spécifiques des monocytes et des lymphocytes T dans les PBMCs. Vingt deux types cellulaires présentent des profils spécifiques, permettant l'établissement d'une base de données « cellules entières ». Nous nous sommes ensuite demandé si l'approche en spectrométrie de masse MALDI- TOF pourrait évaluer la plasticité des macrophages humains en analysant les données avec le logiciel R, la librairie « MALDIquant » et des algorithmes spécifiques. Les profils de spectres sont différents dans les macrophages quiescents ou stimulés par l'IFN-γ (macrophages M1) ou par IL-4 (macrophages M2). Les empreintes peptidiques/protéiques induites par les agonistes M1, IFN-γ, TNF, LPS et LPS + IFN-γ ou les agonistes M2, IL-4, TGF-β1 et IL-10, sont distinctes des macrophages non stimulés et spécifiques de chaque agoniste. La spectrométrie de masse MALDI -TOF peut ainsi être utilisée pour caractériser les sous-types de macrophages M1 et M2. En outre, les empreintes induites par des bactéries extracellulaires (streptocoque du groupe B, *Staphylococcus aureus*) sont spécifiques et similaires à celles induites par l'IL-4. Les réponses des macrophages à des bactéries intracellulaires (BCG, *Orientia tsutsugamushi*, *Coxiella burnetii*) sont également uniques. La spectrométrie de masse MALDI-TOF sur cellules entières a ainsi révélé donc les multiples facettes d'activation des macrophages humains. Enfin, des résultats préliminaires montrent que notre approche pourrait être utilisée en clinique en analysant les cellules circulantes de la réponse immune.

***Mots clés* : SM MALDI-TOF, protéomique, macrophages polarisés, cytokines, bactéries, maladies infectieuses.**

Summary

The invention of MALDI-TOF mass spectrometry has greatly contributed to the understanding of protein chemistry, cell and molecular biology. The identification and characterization of intact microorganisms by MALDI-TOF mass spectrometry has been a revolution in clinical microbiology laboratories in recent years. The many advantages in terms of rapidity, low cost, sensitivity and reliability have led us to apply MALDI-TOF mass spectrometry to the study of intact eukaryotic cells, in particular the study of immune cells. Circulating monocytes, T lymphocytes and neutrophils showed specific peak profiles. Macrophages and dendritic cells derived from monocytes were distinct to circulating monocytes. In addition, MALDI -TOF mass spectrometry may be used to identify specific signatures of monocytes and T lymphocytes in PBMCs. We also found that twenty two cell types have specific profiles, allowing the establishment of a cell database. We then asked if the whole cell MALDI -TOF mass spectrometry approach could evaluate the plasticity of human macrophages. For that purpose, we analyzed data with the R software, the library "MALDIquant" and specific scripts. The spectral profiles were different in unstimulated macrophages or stimulated with IFN-γ (M1 macrophages) or IL-4 (M2 macrophages). The fingerprints induced by M1 agonists such as IFN-γ, TNF, LPS and LPS + IFN-γ or M2 agonists such as IL-4, TGF- β1 and IL-10 were distinct from that of non-stimulated macrophages and were specific for each agonist, demonstrating that MALDI -TOF mass spectrometry can be used to characterize the M1 and M2 macrophages subtypes. The fingerprints induced by extracellular bacteria (group B *Streptococcus*, *Staphylococcus aureus*) were specific and closed to that induced by IL-4. The responses of macrophages to intracellular bacteria (BCG, *Orientia tsutsugamushi*, *Coxiella burnetii*) were also specific. We concluded that the whole-cell MALDI-TOF mass spectrometry showed the multifaceted activation of human macrophages. Preliminary results showed that our approach could be useful to study circulating immune cells from patients with an infectious or inflammatory disease.

***Key words*: MALDI-TOF MS, proteomics, macrophages polarization, cytokines, bacteria, infectious diseases.**