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Lipid Profiling of Polarized Human Monocyte-derived Macrophages

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Abstract

The highly orchestrated transcriptional and metabolic reprogramming during activation drastically transforms the main functions and physiology of human macrophages across the polarization spectrum. Lipids, for example, can modify protein function by acting remotely as signaling molecules but also locally by altering the physical properties of cellular membranes. These changes play key roles in the functions of highly plastic immune cells due to their involvement in inflammation, immune responses, phagocytosis and wound healing processes. We report an analysis of major membrane lipids of distinct phenotypes of resting (M0), classically activated (M1), alternatively activated (M2a) and deactivated (M2c) human monocyte derived macrophages from different donors. Samples were subjected to supercritical fluid chromatography-ion mobility-mass spectrometry analysis, which allowed separations based on lipid class, facilitating the profiling of their fatty acid composition. Different levels of arachidonic acid mobilization as well as other fatty acid changes were observed for different lipid classes in the distinct polarization phenotypes, suggesting the activation of highly orchestrated and specific enzymatic processes in the biosynthesis of lipid signaling molecules and cell membrane remodeling. Thromboxane A2

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production appeared to be a specific marker of M1 polarization. These alterations to the global composition of lipid bi-layer membranes in the cell provide a potential methodology for the definition and determination of cellular and tissue activation states.

Keywords

cytokines; immunology; mass spectrometry; phospholipases and phospholipids

Introduction

Macrophages are tissue resident innate immune cells that defend against infection and help maintain a homeostatic microenvironment [1, 2]. Traditionally, the term “macrophage” described a simple phagocytic leukocyte that existed essentially in two states: a resting/basal state and an activated, proinflammatory state. The latter, classically-activated form of the macrophage was thought to be the major cellular phenotype responsible for antimicrobial activities through the elaboration of inflammatory peptide and lipid mediators such as interleukins and prostaglandins, respectively. This paradigm has been upended by the growing appreciation of cellular plasticity of macrophages to move across many shades of both pro- and anti-inflammatory phenotypes, a process referred to as polarization. Akin to T lymphocyte activation, the emerging model illustrated that macrophages are not only activated towards a classical, proinflammatory (M1) state but can also exist in an alternatively-activated, anti-inflammatory (M2) state [3–5]. Most recently, macrophage polarization has gone far beyond the dichotomous M1/M2 model, to include subsets of alternatively activated cells referred to as M2a, M2c, M_{hem}, *etc.* [4–7]. Polarization alters the repertoire of secreted cytokines, phagocytic capacity, and surface proteins presented by macrophages, allowing the cell to carry out varying effector functions. A number of diseases and pathogenic conditions are associated with specific polarization states [8–11], making the understanding of macrophage polarization in the context of health maintenance and disease pathogenesis critical to improving the capacity to prevent macrophage-mediated diseases.

Peripheral blood monocytes, differentiated with macrophage colony stimulating factor (M-CSF) into macrophage-like cells, serve as a predominant human model cell type for characterizing macrophage polarization known as monocyte-derived macrophages (MDMs) [12–16]. Classical, M1 polarization is typically generated by stimulating these cells with interferon-gamma (IFN γ) in combination with bacterial lipopolysaccharide (LPS) [12, 14, 15]. The M2a and M2c phenotypes are generally modeled by incubating monocyte-derived macrophages with either interleukin-4 (IL-4) or interleukin-10 (IL-10), respectively [12, 14, 15].

Conventional approaches to define polarized activation states have been confined to gene expression profiles, the display of certain cell surface proteins, or the expression of characteristic intracellular or secreted proteins. Furthermore, marker overlap between polarized subsets is sometimes observed when using these approaches [14, 17]. This lack of marker specificity supports the notion of a spectrum of activation as opposed to distinct subsets and requires the use of several markers when defining similar phenotypic subtypes

[7, 18]. While both unbiased proteomic [19, 20] and transcriptomic [13, 15] approaches have been used to more fully characterize macrophage polarization, there are limited studies that have applied a lipidomics approach to this evolving paradigm [21], especially membrane lipid composition in human primary cells.

Located on many cellular compartments, particularly membranes, lipids play critical roles in energy storage, membrane structure, and signaling [22, 23]. Many autocrine and paracrine signaling molecules are lipids, including, for example, the eicosanoids, which have substantial immunoregulatory capabilities [24, 25]. While lipids can serve as a reflection of cellular metabolism, few data have characterized this in the context of human macrophage polarization [26, 27]. In the current study, we sought to characterize and annotate the distinct alterations of membrane lipid categories in human polarized monocyte-derived macrophages for different polarization phenotypes (M1, M2a, M2c) by applying mass spectrometric and non-conventional chromatographic techniques. These findings introduce new technologies that can be used to deepen our current understanding of the molecular biology of macrophage polarization.

Materials and Methods

Reagents

Chloroform and methylene chloride were CHROMOSOLV-grade (Sigma-Aldrich, St. Louis, MO) and Methanol was Optima® LC/MS-grade (Fisher Scientific, Fair Lawn, NJ). Lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Lipid standards include phosphatidylcholines (PC, chicken egg), phosphatidylethanolamines (PE, porcine brain), phosphatidylserines (PS, porcine brain), phosphatidylglycerols (PG, chicken egg), phosphatidylinositols (PI, bovine liver), sphingomyelins (SM, porcine brain). Recombinant Human M-CSF and IFN γ (PeproTech, Rocky Hill, NJ). Recombinant Human IL-4 and IL-4 (R&D Systems, Minneapolis, MN). HyClone Charcoal/Dextran Treated FBS (GE Healthcare Life Sciences HyClone Laboratories, Logan, Utah). RPMI 1640 Medium 1X without HEPES supplemented with L-Glutamine and Phenol Red (Gibco by Life Technologies, Carlsbad, CA). Ultra-pure LPS from *Escherichia coli* K12 in endotoxin-free water (Invivogen, San Diego, CA).

Human subjects

This study was reviewed and approved by the Vanderbilt University Institutional Review Board (protocol #140699). Subjects included three healthy, non-pregnant adults (age 18–60). Exclusion criteria included: fever >101°F in preceding 48 h, antibiotic therapy currently, or within the prior 2 weeks, current immunosuppressive therapy or short-term steroid therapy within the prior 2 weeks, chronic viral infection, including, but not limited to, HIV infection (per self-report), and/or currently diagnosed with or undergoing treatment for cancer

Isolation and cultivation of monocyte-derived macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated from human peripheral blood by density gradient centrifugation using Ficoll-PaqueTM as outlined by the manufacturer Miltenyi (Auburn, CA). Following centrifugation, monocytes were isolated from PBMCs via

negative selection using the Miltenyi Monocyte Isolation Kit II® as instructed by the manufacturer. Monocytes were cultured in 6 well plates at a concentration of 1.35×10^6 monocytes per well for seven days in 100ng/mL M-CSF in RPMI+/- with 20% FBS to differentiate into MDM as previously described [15]. Following differentiation, cells were allowed to rest for 24 hours by replacing M-CSF+ RPMI+/- with M-CSF- RPMI+/- prior to carrying out downstream experiments.

Macrophage polarization

After monocyte differentiation (7 days), cells were treated for 24 hours in RPMI+/- with 5% FBS with 20 ng/mL of polarizing stimuli, IFN γ , IL-4 and IL-10 for M1, M2a and M2c respectively. Additionally, 100 ng/mL LPS were added to M1 cells. Cultures destined for both PCR and Lipidome analysis were treated equally.

Reverse-transcriptase PCR

RNA was isolated from polarized macrophages using the Trizol method followed by RNA washing using the Qiagen® RNeasy plus mini kit (Bio-Rad). RNA quality and quantity was assessed using Nanodrop®. 1 μ g total RNA was used as template for cDNA synthesis. cDNA was generated in the BIO-RAD® MJ Mini Personal Thermal Cycler using BIO-RAD® iScript™ Reverse Transcription supermix (Bio-Rad Laboratories, Hercules, CA) for RT-qPCR as outlined by the manufacturer. Following cDNA synthesis, samples were diluted 1:10 in ultrapure water. ThermoFisher Scientific Taqman® primers specific for M1 and M2 polarization transcripts were used in conjunction with BIO-RAD® iQ™ Supermix to carry out qPCR using the ThermoFisher Scientific StepOnePlus™ real-time PCR system (Waltham, MA). 20 μ L reactions were generated comprised of 10 μ L iQ™ Supermix, 4 μ L H₂O, and 1 μ L Taqman® primer. Reactions were placed in Applied Biosystems StepOnePlus™ real-time PCR system using the thermal cycling protocol as outlined by the manufacturer (3min 95° for activation; 12sec 95° denaturation step; 45sec at 60° annealing; 40cycles). The C_t formula was used to obtain fold change relative to untreated cells and normalized to GAPDH.

Lipid Extraction

500 μ L of 5% HCl and 750 μ L of Folch solution (2:1, CHCl₃:MeOH with 17mg/L butylated hydroxytoluene) were added to cell pellets for lipid extraction. Samples were vortexed and centrifuged briefly at 15,000 rpm. The organic layer was removed and dried under vacuum. Samples were stored at -80°C until analysis.

Supercritical Fluid Chromatography-Ion Mobility-Mass Spectrometry (SFC-IM-MS)

For membrane lipid analysis a supercritical fluid chromatography-ion mobility-mass spectrometry (SFC-IM-MS) system from Waters (Milford, MA) was used. The SFC (UPC²) was coupled fluidly with IM-MS (Synapt G2-S) via an electrospray ionization (ESI) source. The SFC was used with an ethylene bridged hybrid (BEH) silica column (1.7 μ m, 3.0 x 100 mm) held at 60 °C, which separates lipids based on head group polarity. Samples were re-constituted in 100 μ L of methylene chloride. 2 μ L were injected in triplicates for all MDMs biological replicates. The flow rate of 1 mL/min was measured in the liquid state of CO₂

with MeOH (10 mM ammonium formate) as modifier. Initially the mobile phase composition consisted of 10% modifier in CO₂ and after injection, modifier concentration increased to 40% in 7 min. Then the concentration was increased to 80% in one min and held at 80% for 4 min. The positive-mode (+) ESI conditions were as follows: capillary, +2.28 kV; sampling cone, 40 V; source temperature, 100 °C; desolvation temperature, 150 °C; desolvation gas flow, 500 L/h; and cone gas flow, 20 L/h, respectively. The traveling wave velocity and height for IM separation were 652 m/s and 40 V, respectively. Leucine–enkephalin (m/z 556.2771) was used for lock mass correction. The negative-mode (–) ESI conditions were the same as that of the positive-mode except for the capillary voltage source temperature and desolvation temperature (–2.52 kV, 125 °C, 150 °C). The data acquisition was carried out in MS^E mode with transfer collision energy ramping from 40 to 50V in both positive- and negative-mode.

The combination of SFC with MS^E in lipid profiling provides the necessary information for the identification of the lipid classes and their respective fatty acid composition (Supplementary Figure 1). The separation of phospholipids classes is achieved efficiently based solely on the polarity of their respective head group, with less polar classes (*e.g.* PGs) eluting before more polar classes (*e.g.* PCs) [28]. Subsequently, low- and high-energy spectra collected in MS^E mode provides accurate mass and fragments for lipid class identification and fatty acid composition in positive and negative ion-mode respectively. Despite the fact that lower intensities observed for longer chain lengths and higher degrees of unsaturation [29], the separation of lipid classes prior to ionization minimizes ionization suppression effects and permits direct comparison of the distribution of individual phospholipids species within its class across samples without the need of cell count or protein quantitation normalization processes. This capability combined to the speed of analysis makes this methodology amenable for large-scale lipid profiling studies.

Measurement of eicosanoids

To quantify eicosanoids in media samples, 100uL of media was placed in a microcentrifuge tube containing 25% methanol in water (500uL) and internal standard (d₄-PGE₂ and d₄-LTB₄, 1ng each). The sample was vortexed and then extracted on an Oasis MAX uElution plate (Waters Corp., Milford, MA) as follows. Sample wells were first washed methanol (200uL) followed by 25% methanol in water (200uL). The sample was then loaded into the well and washed with 600uL 25% methanol. Eicosanoids were eluted from the plate with 30uL 2-propanol/acetonitrile (50/50, v/v) containing 5% formic acid into a 96-well elution plate containing 30uL water in each well.

Samples were analyzed on a Waters Xevo TQ-S micro triple quadrupole mass spectrometer connected to a Waters Acquity I-Class UPLC (Waters Corp., Milford, MA USA). Separation of analytes was obtained using an Acquity PFP column (2.1 x 100mm) with mobile phase A being 0.01% formic acid in water and mobile phase B acetonitrile. Eicosanoids were separated using a gradient elution beginning with 30% B going to 95% B over 8 minutes at a flow rate of 0.250mL/min.

Data Analysis

Lockspray mass correction was applied after acquisition using the vendor supplied software, followed by batch conversion to mzXML format. After peak filtering, detecting and aligning with XCMS [30] the features (discrete retention time and m/z values) were assigned to a phospholipid class by their retention time and accurate mass. For further analysis, features only found in standards or databases such as METLIN [31] and LIPIDMAPS [32, 33] with mass tolerance below 10 ppm were taken into account.

For qRT-PCR data analysis, One-Way ANOVA using Geisser-Greenhouse correction was applied using GraphPad software followed by Dunnett's multiple comparisons test with individual variances computed for each comparison with corrections with P values <0.05 considered significant. For SFC-IM-MS data analysis, t-test and Levene's test (SPSS software) were used in binary comparisons (P values <0.05 considered significant).

Results

Membrane phospholipid composition of MDM

After validating the appropriately polarization of macrophage into M1, M2a and M2c directions by canonical transcription polarization markers (Supplementary Figure 2), the analysis of membrane phospholipid composition, supercritical fluid chromatography (SFC) was combined with ion mobility-mass spectrometry (IM-MS). These have the capability of providing the necessary information for the identification of the lipid classes and their respective fatty acid composition.

Six major membrane phospholipid (PL) classes were identified, phosphatidylcholines (PCs), phosphatidylethanolamines (PE), phosphatidylserines (PS), phosphatidylglycerols (PG), phosphatidylinositols (PI) and sphingomyelins (SM). Their total mass spectrometry signal (intensity) was calculated and individual species abundancies are reported as the percent (percent contribution or distribution) of the total signal for each class. This relative quantitation lipidome or "ratiomics analysis" [34, 35] was able to detect changes in species distribution within each phospholipid class in all 4 activation states. It is evident from the 3D principal component analysis plot in Figure 1 that controls and M1 MDMs are the furthest apart in principal component 1. Furthermore, both M2 groups are separated from controls and M1 but group closely in principal components 1 and 2. However, in principal component 3, substantial differences were recognized between M2a and M2c MDM, identifying them as unique "phenotypes" in the three-dimensional macrophage polarization space.

We analyzed the membrane lipid composition of each class independently and compared them across resting, M1, M2a and M2c MDMs. Given the different lipid composition within different organelles and plasma membranes as well as asymmetric membrane lipid distribution, the localization of lipid changes is not surveyed in this lipid profiling study [23, 36]. PL classes mainly located in the cytosolic leaflet or luminal side of plasma membranes are shown in Figures 2 and 3, respectively. During the polarization of macrophages, distinct phospholipase A₂ enzymes are involved in the arachidonic acid (AA) mobilization for consequent eicosanoid production [37]. Figure 2A–C shows the percent composition of

selected species (higher composition and higher changes) for PE, PI and PS respectively. Phospholipid species are abbreviated (lipid class, number of carbon atoms and double bonds of both acyl chains combined; O- and P- denotes ether and vinyl ether linkage, respectively) due to the fact that fragmentation of individual m/z species result in fragments corresponding to various acyl groups, making the assignment of individual structures not possible. Drops in the composition of species with an unsaturation degree of 4 or higher (possibility of containing AA) were expected for polarized MDMs [38]. However, small overall changes were observed for statistically significant (p -value <0.05) lipids. The highest changes were detected for PE(38:4) and PI(38:4), where the composition decreased after activation by 1.6–2.5 and 2.3–3.8% respectively. Interestingly, the opposite profile was observed for PI(38:5). Given the nature of the percent distribution of all species within a lipid class, the decrease of one species is commensurately compensated by the increase of other species. Thus, lipids with lower degrees of unsaturation show an increase in polarized MDMs such as, PI(36:2), PS(36:2) and PE(34:1). Similarly, on the non-cytosolic (luminal side), changes in the fatty acid distribution of PCs and SMs were observed (Fig. 3). The increase of PC(30:1), PC(32:0) and PC(34:0) and the decrease of PC(36:4), PC(38:4) and PC(40:5) indicate AA mobilization. However, it is not clear if this mobilization is entirely for lipid mediator synthesis or the release of AA surpasses the eicosanoid production. Another possibility is that the released AA is simply being exchanged with serum proteins as it has been previously reported for RAW 264.7 cells [35]. Figure 3C illustrates the changes observed in PG. These are consistent with the other classes, particularly PE and PC. Higher distribution is determined for species not containing AA (PG(34:1), PG(34:2), PG(36:1) and PG(36:2)) for polarized MDMs compared to resting. Statistically different lipid species, not only between polarized and resting macrophages but also between similar phenotypes such as M2a and M2c are of great interest for the determination of the involvement of the different signaling pathways in the distinct polarization states. Six species, PC(32:1), PC(O-32:1/P-32:0), PC(34:3), PI(38:3), PI(38:5) and PS(36:2) are statistically different in the different polarization states. It is worth noting that only PI(38:5) potentially contains AA. The largest change for species containing AA was observed in PG(40:7). As shown in Figure 4, the abundancy of this PG(40:7) decreased by 57, 49 and 39% ($P<0.005$) after M1, M2a and M2c polarization. Furthermore, other species show statistically different distribution profiles between different treatments. PI(36:2), PC(34:3) and PC(33:1) have a specific profile for M1, M2a and M2c polarized MDMs (Figure 4). The mechanisms of this particular changes is still unclear however, the fact that macrophages were derived from 3 different donors suggests a higher degree of orchestration in the polarization process.

It should be noted that in spite of little difference in relative distributions, many species showed significantly different profiles across all groups, and the low variabilities (error bars) between biological replicates have an average standard deviation of 3.1% for lipids in all treatments plotted in Figures 2 and 3. The standard deviations for all species detected and for species with odd-numbered chain fatty acids are 4.2 and 6.7% respectively. The slightly higher deviation for odd-numbered chain fatty acids distribution could be caused by differences in the donor's diet since they are not endogenous [39].

Fatty acid composition

In previous studies, the macrophage fatty acid composition in murine cells lines has been investigated and absolute quantitative amounts have been reported [40, 41]. In our study, we focused on differences between relative fatty acid compositions (ratios) for different lipid classes among different groups. It is worth emphasizing that with collision-induced dissociation, fragmentation yields are indirectly proportional to the mass of the ion, so these relative signal intensities do not provide absolute quantitation data as hydrolysis followed by GC-MS analysis would [42]. However, profile comparisons of these relative intensities allow for estimations of fatty acid distribution within the same lipid class without compromising throughput (short separation times and no further sample preparation is needed). Figure 5 shows the changes in AA composition of 6 different phospholipid classes for all 4 groups (these values were selected using Tables S1–S6 in Supplementary material). AA composition values decreased after M1 polarization as it would be expected in all lipid classes. The largest changes compared to resting macrophages ($P < 0.005$) are observed in SM, PE and PI with 2.2 and 2.6 and 3.5% decrease respectively. Furthermore, the low AA levels observed in PC and SM could be explained by the low AA and linoleic acid (LA) concentrations in FBS (~7% and 5.5% of fatty acid composition respectively), especially considering the long culture time during derivatization and polarization [43–47]. A previous fatty acid comparison determined that the AA content of total lipids was three times lower in macrophages derived from bone marrow to lipids of peritoneal macrophages [48]. The smallest changes for all 4 groups are observed in PG, which is consistent with a previous study of classically activated murine resident peritoneal macrophages (RPM) cells [35]. Additionally, the AA levels for each lipid class are lower in M1 than in both M2 activation states, supporting the predominant role of cytoplasmic phospholipase A₂ (cPLA₂) in AA mobilization during M1 polarization [37]. Interestingly, AA composition is significantly different (p -value < 0.01) between M2a and M2c MDMs for good substrate lipid classes highly abundant in AA such as PE and PI, with M2c having higher AA levels. Some lipid mediators detected in human MDMs such as resolvins, maresins and protectins are synthesized from docosahexaenoic acid (DHA) [21]. Small DHA changes in the fatty acid composition of PG's are observed between resting macrophages, M1 and both M2 subtypes. A decrease from 3.9% composition in resting macrophages to 2.1 and 2.5% was measured after M2a and M2c polarization respectively (Tables S1–S6 in Supplementary material). However, the biggest change was observed in classical activation with a decrease to 1.6%.

Eicosanoid analysis

The concentration of several biologically relevant eicosanoids, including prostaglandin (PG)E₂, PGD₂, PGF_{2α} and thromboxane (Tx)A₂ (estimated through measurement of its stable metabolite, TxB₂), in the cell culture media was measured with the purpose of determining the fate of AA mobilization during the polarization process. As illustrated in Figure 6, no statistically significant changes were observed for PGE₂ and PGD₂, although a slight increase of PGE₂ can be appreciated in M1 in comparison to controls and both M2 subgroups. Production of TxB₂ (a surrogate metabolic marker of TxA₂) was significantly increased when macrophages were polarized towards an M1 phenotype and decreased in M2c macrophages. Furthermore, significant differences were observed between M2a and M2c polarization states. Trends in the generation of PGF_{2α} were also observed, as PGF_{2α}

generation was elevated in M1 polarization and greatly reduced when macrophages took on an M2 activation state, though these changes did not reach statistical significance.

Discussion

These studies demonstrate that macrophage polarization was associated with significant and specific changes in the lipidome of MDMs as evidenced by alterations in various classes of membrane phospholipids. Moreover, we have established an innovatively applied methodology for rapid lipid profiling, which allowed for the identification of a wide range of lipid classes for specific macrophage polarization phenotypes. Furthermore, the monitoring of several lipid species at the end of the “omics” cascade has the potential of providing insight into the different polarization subsets with overlapping phenotypes (Figure 1). The methods used in this study advance the field of macrophage polarization research by demonstrating that state-of-the-art lipidomic techniques can be used to further interrogate polarized human macrophages, a class of cells that are still not well characterized.

After confirming our *in vitro* polarization using gene expression, we sought to explore the extensive macrophages lipidome and its dynamic metabolism after polarization. By simultaneously collecting low- and high-energy spectra, both PL and acyl chain composition of the different lipid groups was determined. This methodology was able to depict the global membrane lipidomic picture of macrophage polarization and reveal characteristic lipid species for resting, classic, alternative and deactivated human monocyte derived macrophages.

During the polarization process, different lipid mediators’ biosynthetic pathways involving several enzymes are expressed and activated to different extents. Studies have shown that different expression profiles of the major participants in fatty acid mobilization (hydrolysis), cPLA₂ and sPLA₂, have been observed in human monocyte-derived macrophages treated with LPS + IFN γ , IL-4 and IL-10 [49]. These phospholipases differ in specificity for fatty acid species and position in the glycerol backbone, affinity to different PL classes and localization within the cell upon activation [37]. Thus, different PL pools are substrates in this highly orchestrated and not fully understood enzymatic process at different times. Furthermore, some crosstalk between both phospholipases has been observed in mouse macrophages (P388D₁) and other studies in human neutrophils showed the ability of sPLA₂ to induce leukotriene biosynthesis by activating cPLA₂ [50, 51]. Additionally, the formation of lysophosphatidylcholine by sPLA₂ in the outer membrane causes an increase of Ca²⁺ concentration, which subsequently causes cPLA₂ to be activated and translocated through activating protein kinases needed for the phosphorylation of Ser⁵⁰⁵ [37, 52, 53]. On the other hand, in human eosinophils, sPLA₂ induced eicosanoid production at plasma membranes and perinuclear membranes, independently of cPLA₂ activation [54]. These examples show the complexity of interconnectivity in fatty acid release in innate immunity. However, the combination of the localization and characteristics (specificity and activity) of these phospholipases and the different PL composition of organelle membranes suggest a specific course in which these changes occur in membrane PL composition (Figures 2 and 3). Generally, lipid species capable of containing AA in either *sn*-1 or *sn*-2 positions were of lower abundance in M1 compared to resting macrophages. Alternatively, lipid species with

no AA were more abundant in M1 compared to resting macrophages. On the contrary, PI(38:5) is considerably lower in resting macrophages compared to M1 and both M2 phenotypes. Brown and co-workers observed a similar but less drastic trend in murine resident peritoneal macrophages [35]. Anti-inflammatory M2 activation states showed similarities to both resting and classically activated groups. Interestingly, the largest changes between M2a and M2c were detected in lipid classes with high affinity to cPLA₂ (PE, PC and PI) but in AA-free species. The characteristic upregulation of sPLA₂ in M2 macrophages and its lack of *sn*-2 AA specificity suggest that sPLA₂ is most likely the enzyme responsible for the hydrolysis of these species. It should be noted that due to the large size of human macrophages (diameter > 30µm) the amount of PL is significantly high [49]. Therefore, the changes detected in our study between the different treatments are relatively small.

Analysis of fatty acid composition of distinct PL classes allowed us to track global acyl changes and assign them to a region in the polarization spectrum. As mentioned above, the *sn*-2 AA specificity of cPLA₂ is responsible for the majority of the AA release. Across the 6 major PL classes the AA composition was lowest in M1 compared to resting, M2a and M2c macrophages indicating higher AA release in proinflammatory macrophages. This is consistent with previous studies comparing lipid mediators for M1 and M2a polarized human macrophages after IFNγ + LPS and IL-4 treatment respectively, where the biosynthesis of inflammation associated prostaglandins was higher for M1 macrophages compared to M2a [21]. On the contrary, relevant to resolution-associated lipids (resolvins and maresins), we observed a higher release of docosahexaenoic acid (DHA) in M1 compared to M2a (Supplementary Tables S1–S6). However, we measured DHA bound to different PL classes and not the lipid mediators directly. The lipid composition of the cytoplasmic face of Golgi and ER is poorly defined (known cPLA₂ locations) [55]; hence, it is not possible to determine the origin and the amount of the AA released for lipid mediator production. Furthermore, the differences in activation and translocation of phospholipases can alter AA mobilization not only by regulating the hydrolysis efficiency but also by inducing translocation to other lipid membranes with different composition. PI and SM had the highest percentage of AA mobilization, with PI showing the largest changes between resting, M1, M2a and M2c macrophages (Figure 5). Two interesting observations in the AA metabolism of M2 MDMs are apparent. (i) M2c had the lowest AA release in all PL classes and (ii) in PE, a substrate to cPLA₂, no statistical difference was detected between both M2 and resting macrophages, though M2a and M2c are statistically different ($P < 0.01$). Furthermore, PG showed no change in AA composition between resting and both M2 groups. The small composition of AA in SM distributed among different species limited the probabilities of being detected as a SM species during the low-energy spectra collection. Thus, the SM species plotted in Figure 3 lack AA.

Using a more targeted approach for analyzing specific AA downstream metabolites of the cyclooxygenase cascade, we observed a significant increase in TxB₂ (a surrogate for TxA₂) in M1 cells and a decrease in this same metabolite in M2c macrophages. This is not entirely unexpected, because TxA₂ is known to be a proinflammatory mediator [56]. It is interesting too that our data suggest a decrease in PGF_{2α} accompanies macrophage M2a and M2c activation states while a tendency to increase release of this mediator was observed in the

proinflammatory M1 state. These data are among the first to characterize the prostanoid shifts associated with macrophage polarization along the M1–M2 continuum.

These experiments were carried out at a single 24 hour time point after treatment. Thus, we are unable to examine the plasticity of polarized profiles at earlier time points over time and identify lipids that initially contained AA and served as substrate for prostaglandin biosynthesis and were later replenished. Full time course studies with sufficient temporal resolution will be necessary to understand the flux of AA and other fatty acids. Additionally, a global picture of the localization of the AA pools perturbed during macrophage polarization can be determined by fractionation of major organelles /compartments prior to the methodology described herein [36]. Furthermore, it should be noted that various *in vitro* methods are used to differentiate human peripheral blood monocytes into macrophages. Alternative methods of differentiating monocytes into macrophages have been shown to alter downstream activation states [57–59]. Translating *in vitro* polarization studies can also be difficult when factors such as cytokines, cell maturation, cell origin, chemokines, and adhesion that are omitted in the *in vitro* systems play major roles *in vivo* [60, 61]. The nomenclature and methodologies used for modeling macrophage polarization *in vitro* vary greatly within the field and have recently come under fire [62]. We instead chose the most common method of differentiating monocytes, using M-CSF, in an effort to maintain a reproducible experimental standard for the discovery/exploration of phenotypic small molecules changes.

This methodology in combination with the lipid profiling data of both PL and fatty acid composition might be of utility to better understand and quantify the lipidome dynamic changes in the human monocyte derived macrophage polarization spectrum.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Three-dimensional PCA plot of lipid profiles for control, M1, M2a and M2c MDM. PC1 separates controls, M1 and both M2 groups (M2a and M2c have similar grouping in PC1). In both PC2 and PC3 M2a and M2c are grouped separately.

Figure 2.

Comparison of relative distribution of PE, PI and PS for controls, M1, M2a and M2c MDM. A), B) and C) illustrate the changes for PE, PI and PS respectively. Lipids with highest contribution and changes were plotted. (* indicates p-value<0.05 for control-M1 comparison, ** indicates p-value<0.05 for control-M2a comparison, *** indicates p-value<0.05 for control-M2c comparison and **** indicates p-value<0.05 for M2a-M2c comparison). (1.5-column fitting image)

Figure 3.

Comparison of relative distribution of PC, SM and PG for controls, M1, M2a and M2c MDM. Classes associated to the luminal side are illustrated in A) and B) (PC and SM respectively). Panel C) represents the lipid distribution for PG Lipids with highest contribution and changes were plotted. (* indicates p-value < 0.05 for control-M1 comparison, ** indicates p-value < 0.05 for control-M2a comparison, *** indicates p-value < 0.05 for control-M2c comparison and **** indicates p-value < 0.05 for M2a-M2c comparison). (1.5-column fitting image)

Figure 4.

Comparison of selected individual lipid species for controls, M1, M2a and M2c macrophages. The percent total lipid class refers to percent of total lipid class signal. PG(40:7) corresponds to 9.8% of the total signal of PGs in controls. Its distribution within GPs changes to 4.2%, 5.3 and 6.1% for M1, M2a and M2c macrophages respectively (* indicates p-value<0.05, ** indicates p-value<0.01, *** indicates p-value<0.005).

Figure 5. Percent changes in arachidonic acid composition for different lipid classes. AA macrophages mobilization is illustrated for different polarization states (controls, M1, M2a and M2c) (* indicates p-value<0.05, ** indicates p-value<0.01, *** indicates p-value<0.005). (1.5-column fitting image)

Figure 6.

Eicosanoid concentration in cell culture media for different polarization states (controls, M1, M2a and M2c) (* indicates p-value < 0.05, ** indicates p-value < 0.01, LOD for PGF_{2α} = 0.1 ng/mL).