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Primed mesenchymal stem cells package exosomes with metabolites associated with immunomodulation

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ABSTRACT

Mesenchymal stem cell (MSC) based therapies are currently being evaluated as a putative therapeutic in numerous human clinical trials. Recent reports have established that exosomes mediate much of the therapeutic properties of MSCs. Exosomes are nanovesicles which mediate intercellular communication, transmitting signals between cells which regulate a diverse range of biological processes. MSC-derived exosomes are packaged with numerous types of proteins and RNAs, however, their metabolomic and lipidomic profiles to date have not been well characterized. We previously reported that MSCs, in response to priming culture conditions that mimic the *in vivo* microenvironmental niche, substantially modulate cellular signaling and significantly increase the secretion of exosomes. Here we report that MSCs exposed to such priming conditions undergo glycolytic reprogramming, which homogenizes MSCs' metabolomic profile. In addition, we establish that exosomes derived from primed MSCs are packaged with numerous metabolites that have been directly associated with immunomodulation, including M2 macrophage polarization and regulatory T lymphocyte induction.

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1. Introduction

In recent years, the therapeutic potential and safety of mesenchymal stem/stromal cells (MSCs) has been investigated preclinically and clinically, in the context of immune regulation and regeneration in a variety of diseases [1–3]. The therapeutic effects of MSCs on tissue repair and regeneration are mediated by their paracrine activity via various secreted factors, including exosomes [4–11]. Exosomes are nano-sized vesicles that comprise a robust inter-cellular communication system [12,13]. There are now over

200 published reports indicating that exosomes are a critical active pharmaceutical ingredient that is responsible for mediating much of the therapeutic effects of MSCs, including their immunomodulatory properties [14–17]. The application of exosomes isolated and purified from MSC conditioned media holds several potential advantages of the use of cell-based therapies, including increased potency, greater consistency and lower cost. Previously we have reported that exosomes derived from primed MSC possess potent tissue healing properties, and producing a much higher yield of exosomal material, which has important implications for the translation of this technology [5]. Exosomes transport a variety of factors including proteins and RNAs [18]. To date, most studies have focused on the RNA, and to a lesser extent, the protein packaged into exosomes derived from MSCs [19–21]. Consequently, the metabolomic profile of MSC-derived exosomes, remains largely

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uncharacterized.

Metabolomics uses mass spectrometry to identify and quantify a variety of small molecules that are indicative of the metabolic, and physiological status of cellular subpopulations [22]. This analytical approach affords the analysis of a large number of samples and characterizes the molecular response of a biological system to any perturbation in its microenvironment [23]. Metabolomics enables the detection of differential abundance of metabolites between two conditions and adds value to translational studies focusing on elucidating the underlying the dynamic molecular processes within a given system [24]. We have previously established that pMEX are packaged with biologically active, regenerative proteins [4]. However, there are few metabolic or lipidomic studies of exosomes reported to date.

Here we report that exosomes derived from primed MSCs are packaged with a variety of metabolites and lipid membrane components. We compare the metabolomic and lipidomic profiles of classically cultured MSC vs primed MSCs demonstrating their metabolic reprogramming under such microenvironmental conditions. We establish that exosomes derived from primed MSCs (pMEX) are packaged with distinct profile of metabolites, the majority of which have been associated with immunomodulation. These findings suggest that further studies establishing the metabolite payload of MSC-derived exosomes may yield insights into their underlying physiological and cell signaling properties.

2. Materials and methods

2.1. Sample preparation for GC-TOF MS and LC-QTOF MS/MS analysis

Flash frozen cell pellets were ground using a GenoGrinder 2010 (SPEX SamplePrep) for 2 min at 1350 rpm. Cell pellets were then extracted with 225 μ L of methanol at -20°C containing an internal standard mixture described previously [25], and 750 μ L of MTBE (methyl tertiary butyl ether) (Sigma Aldrich) at -20°C containing cholesteryl ester 22:1. Samples were shaken for 6 min at 4°C with an Orbital Mixing Chilling/Heating Plate (Torrey Pines Scientific Instruments, Calsbad, CA). Then 188 μ L of LC-MS grade water (Fisher) was added. Samples were vortexed, centrifuged and the upper (non-polar) and bottom (polar) layers were collected (350 μ L and 125 μ L, respectively) and evaporated to dryness.

The non-polar layer was re-suspended in a methanol:toluene (9:1, v/v) mixture containing 50 ng/ml CUDA ((12-[[cyclohexylamino]carbonyl]amino]-dodecanoic acid) (Cayman Chemical, Ann Arbor, MI) vortexed, sonicated for 5 min and centrifuged and prepared for lipidomic analysis. Half the polar layer was re-suspended in an acetonitrile:water (4:1, v/v) mixture with 5 μ g/ml Val-Try-Val (Sigma) sonicated for 5 min and centrifuged and prepared for HILIC-QTOF MS/MS analysis. The second polar phase aliquots were derivatized with 10 μ L of methoxyamine hydrochloride in pyridine (40 mg/mL) by shaking at 30°C for 90 min followed by trimethylsilylation with 90 μ L of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA, Sigma-Aldrich) by shaking at 37°C for 30 min containing C8–C30 fatty acid methyl esters (FAMES) as internal standards. Derivatized samples were subsequently submitted for analysis by GC-TOF MS (0.5 μ L injection). Method blanks and pooled human plasma (BioIVT) were included as quality control samples for all platforms and prepared in same manner.

2.2. Chromatographic and mass spectrometric conditions for lipidomic LC-QTOF MS analysis.

For analysis of the non-polar phase, re-suspended samples were

injected at 1 μ L ESI (+) and 5 μ L for ESI(–), onto a Waters Acquity UPLC CSH C18 (100 mm length x 2.1 mm id; 1.7 μ m particle size) with a Waters Acquity VanGuard CSH C18 pre-column (5 mm \times 2.1 mm id; 1.7 μ m particle size) at 65°C coupled to an Agilent 1290 Infinity UHPLC (Agilent Technologies, Santa Clara, CA). For positive mode 10 mM ammonium formate and 0.1% formic acid were used and 10 mM ammonium acetate (Sigma–Aldrich) was used for negative mode. Both positive and negative modes used the same mobile phase composition of (A) 60:40 v/v acetonitrile:water (LC-MS grade) and (B) 90:10 v/v isopropanol:acetonitrile. The gradient started at 0 min with 15% (B), 0–2 min 30% (B), 2–2.5 min 48% (B), 2.5–11 min 82% (B), 11–11.5 min 99% (B), 11.5–12 min 99% (B), 12–12.1 min 15% (B), and 12.1–15 min 15% (B). A flow rate of 0.6 mL/min was used. For data acquisition an Agilent 6550 QTOF with a jet stream electrospray source with the following parameters was used: mass range, m/z 50–1700; capillary voltage, ± 3 kV; nozzle voltage, ± 1 kV; gas temperature, 200°C ; drying gas (nitrogen), 14 L/min; nebulizer gas (nitrogen), 35 psi; sheath gas temperature, 350°C ; sheath gas flow (nitrogen), 11 L/min; acquisition rate, 2 spectra/s. For lipid identification, MS/MS spectra were collected at a collision energy of 20 eV with an acquisition rate MS1 of 10 spectra/s (100 ms) and an acquisition rate for MS/MS of 13 spectra/s (77 ms) with 4 precursor ions per cycle... Mass accuracy was maintained by constant reference ion infusion (purine and HP-0921 in an acetonitrile:water mixture).

2.3. Chromatographic and mass spectrometric conditions for polar metabolite HILIC-QTOF MS analysis

Five microliters of re-suspended sample was injected onto a Waters Acquity UPLC BEH Amide column (150 mm length x 2.1 mm id; 1.7 μ m particle size) with an additional Waters Acquity VanGuard BEH Amide pre-column (5 mm \times 2.1 mm id; 1.7 μ m particle size) maintained at 45°C coupled to an Agilent 1290 Infinity UHPLC. The mobile phases were prepared with 10 mM ammonium formate and 0.125% formic acid (Sigma–Aldrich) in either 100% LC-MS grade water for mobile phase (A) or 95:5 v/v acetonitrile:water for mobile phase (B). Gradient elution was performed from 100% (B) at 0–2 min to 70% (B) at 7.7 min, 40% (B) at 9.5 min, 30% (B) at 10.25 min, 100% (B) at 12.75 min, isocratic until 16.75 min with a column flow of 0.4 mL/min. Spectra were collected using a 5600 + TripleTOF MS (SCIEX, Framingham, MA, USA) using data dependent mode for MS/MS spectra acquisition. Data was collected in ESI(+) mode with the following parameters: m/z 50–1700, curtain gas: 35, ion source gas 1 and 2: 60, temperature: 350°C , ion spray voltage floating: +4.5 kV, declustering potential: 80 V, MS1 accumulation time 100 ms, MS2 accumulation time 50 ms, dependent product ion scan number 8, intensity threshold 1000, active precursor exclusion after 2 spectra for 5 s, collision energy 20 eV with 15 eV collision energy spread. Mass accuracy was maintained through calibration every 10 injections using APCI positive calibration solution delivered using a calibration delivery system.

2.4. LC-MS/MS data processing using MS-DIAL and statistics

Both lipidomic and HILIC data processing was performed using MS-DIAL²⁶¹³ for deconvolution, peak picking, alignment, and identification. For both data sets, in house m/z and retention time libraries were used in addition to MS/MS spectra databases in msp format as previously described [27]. Features were reported when present in at least 50% of samples in each group. Statistical analysis was done by first normalizing data using the sum of the knowns, or mTIC normalization, to scale each sample. Peak heights were then submitted using R to DeviumWeb and normalized further by log transformation and Pareto scaling. ANOVA analysis was performed

with FDR correction and post hoc testing.

2.5. Chromatographic and mass spectrometric conditions for GC-TOF MS analysis

Primary metabolite data was collected using a Leco Pegasus IV time-of-flight (TOF) MS (Leco Corporation, St. Joseph, MI) coupled to an Agilent 6890 GC (Agilent Technologies, Santa Clara) equipped with a 30 m long 0.25 mm id Rtx-5 Sil MS column (0.25 μ m film thickness) and a Gerstel MPS2 automatic liner exchange system (Gerstel GMBH & Co. KG, Ruhr, Germany). The chromatographic gradient used a constant flow of 1 ml/min with following gradient: 50 °C (1 min), 20 °C/min to 330 °C, hold 5 min. Mass spectrometry data was collected using 1525 V detector voltage at m/z 85–500 with 17 spectra/s, electron ionization at –70 eV and an ion source temperature of 250 °C. QC injections, blanks and pooled human plasma were used for quality assurance throughout the run. Data was processed by ChromaTOF for deconvolution, peak picking, and BinBase²⁸³ for metabolite identifications.

3. Results

3.1. Primed MSCs undergo metabolic lipidomic reprogramming

MSCs are typically cultured and expanded in high serum

containing media and with atmospheric oxygen tension (~21%) [29]. Upon administration, MSCs experience a remarkable shift in the microenvironmental niche, including much lower exposure to growth factors, as well as substantially lower oxygen tension (eg 1–5% O₂) [30]. Most published reports to date have focused on elucidating MSC physiology while under classic expansion culture conditions [31]. Upon serum deprivation and hypoxia, MSC undergo metabolic reprogramming [32]. We sought to determine how the metabolic reprogramming of MSCs exposed to serum deprivation and hypoxia influenced their metabolomic and lipidomic profiles. MSCs were isolated from human bone aspirates using canonical methods, as previously described [5]. The resulting cells were verified to express established MSC surface markers via flow cytometry, as previously reported [4]. At passage 5, expansion media was removed, and cells were washed 3 times with 1X PBS. MSCs either received expansion and were cultured at atmospheric oxygen tensions (~21%), or priming conditions consisting of serum deprivation and culturing under hypoxic conditions, 1% O₂. Following 48 h of incubation, cells were processed for metabolomic and lipidomic profiling using mass spectrometry.

Primed MSCs (pMSC) exposed to hypoxia and serum deprivation resulted in the increased expression of dipeptides, suggesting that hypoxic MSCs increase their pool of free amino acids to fulfill the energetic demands that cannot be sufficiently provided from the glycolytic flux (Fig. 1A). Ingenuity Pathway Analysis (IPA) of

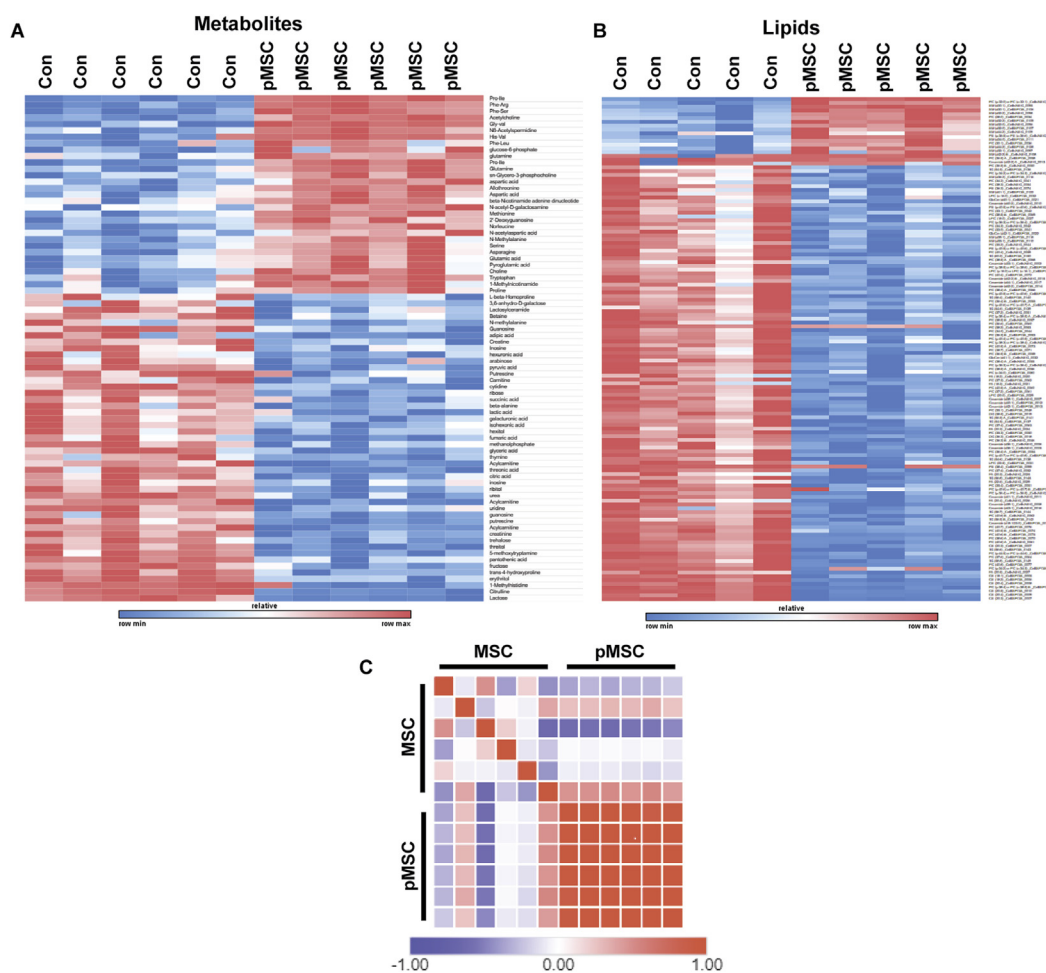


Fig. 1. Metabolomic and lipidomic profiling of primed MSCs demonstrated significant metabolic reprogramming, increased dipeptide expression, and substantial modulation of lipid membrane components (A, B). Similarity analysis using Pearson's correlation determined that priming conditions greatly increased the metabolic homogeneity of MSC biological replicates, as compared to classically cultured MSCs (C). N = 6 per condition, significance calculated with T-tests with a multi-testing correction analysis using a false discovery rate of 1%. Similarity matrix was calculated using the Pearson correlation function of the Broad Institute's Morpheus tool.

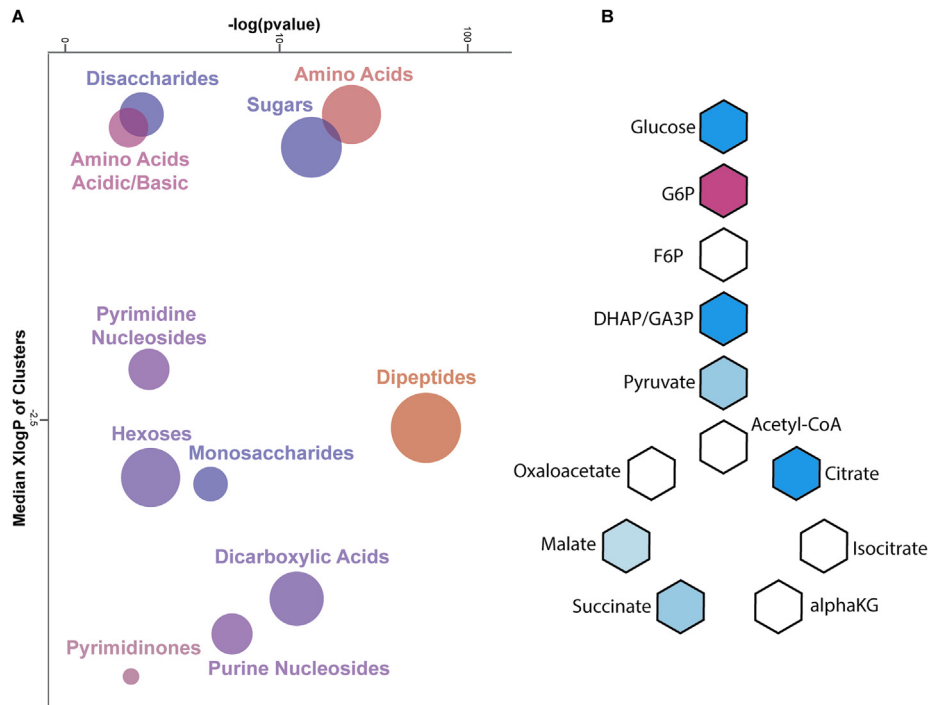


Fig. 2. Mass spectrometry metabolic analysis determined that MSCs exposed to hypoxia and serum deprivation significantly modulated amino acid, nucleoside, and carbohydrate metabolism based on chemical ontology analysis using the ChemRICH tool developed by Barupal et al. (A). Analysis of metabolites associated with carbohydrate metabolism determined that primed MSCs downregulate several pathways related to aerobic respiration. $N = 6$ per condition. For ChemRICH analysis enrichment p -values are given by the Kolmogorov–Smirnov-test. For Krebs' cycle analysis significance calculated with T-tests with a multi-testing correction analysis using a false discovery rate of 1%.

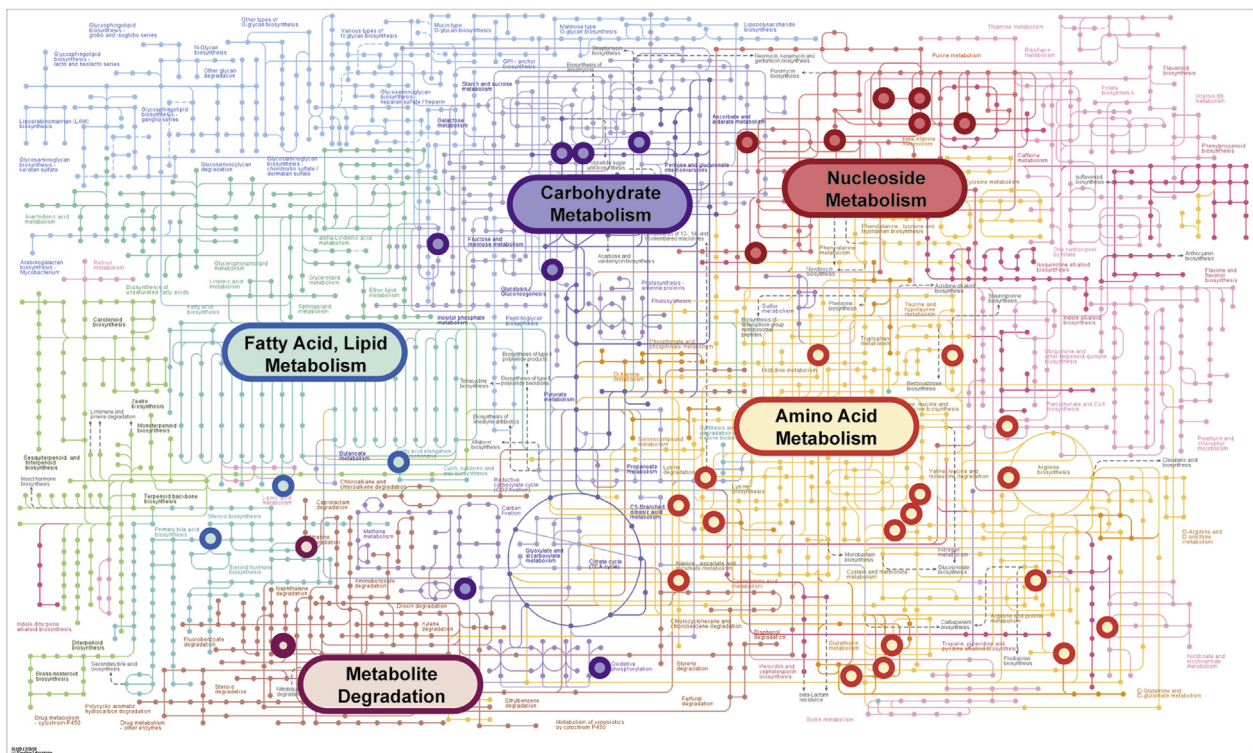


Fig. 3. Exosomes derived from MSCs exposed to serum deprivation and hypoxia are packaged with clustered networks of metabolites associated with amino acid, nucleoside, and carbohydrate metabolic pathways. Pathways associations determined using KEGG Metabolic Mapper function using high confidence metabolites with peak intensities significantly above background, $n = 6$.

metabolomics data also determined that pMSC shifted toward a prosurvival metabolic profile (Fig. S1). Lipidomics profiling determined that pMSC decreased expression of numerous lipid membrane components, consistent with previous reports of their differential morphology (Fig. 1B). Biological replicates of pMSC were also metabolically more similar to each other and displayed significantly less batch to batch variation than MSCs cultured under expansion conditions (Fig. 1C). Metabolic examination using the ChemRICH analysis, which creates chemical clusters based on

structural similarity [33], demonstrated that pMSC modulated expression of numerous nucleosides, biosynthetic and glycolytic associated metabolites, indicating their metabolic reprogramming (Fig. 2A). Significant alterations in glycolysis and TCA cycle intermediates in pMSC as compared to MSC, suggest a conversion from aerobic respiration to glycolytic metabolism (Fig. 2B). Further IPA analysis established that pMSC metabolites reflected a low carbohydrate environment (Fig. S2). Collectively, these data demonstrate that MSCs undergo substantial metabolic

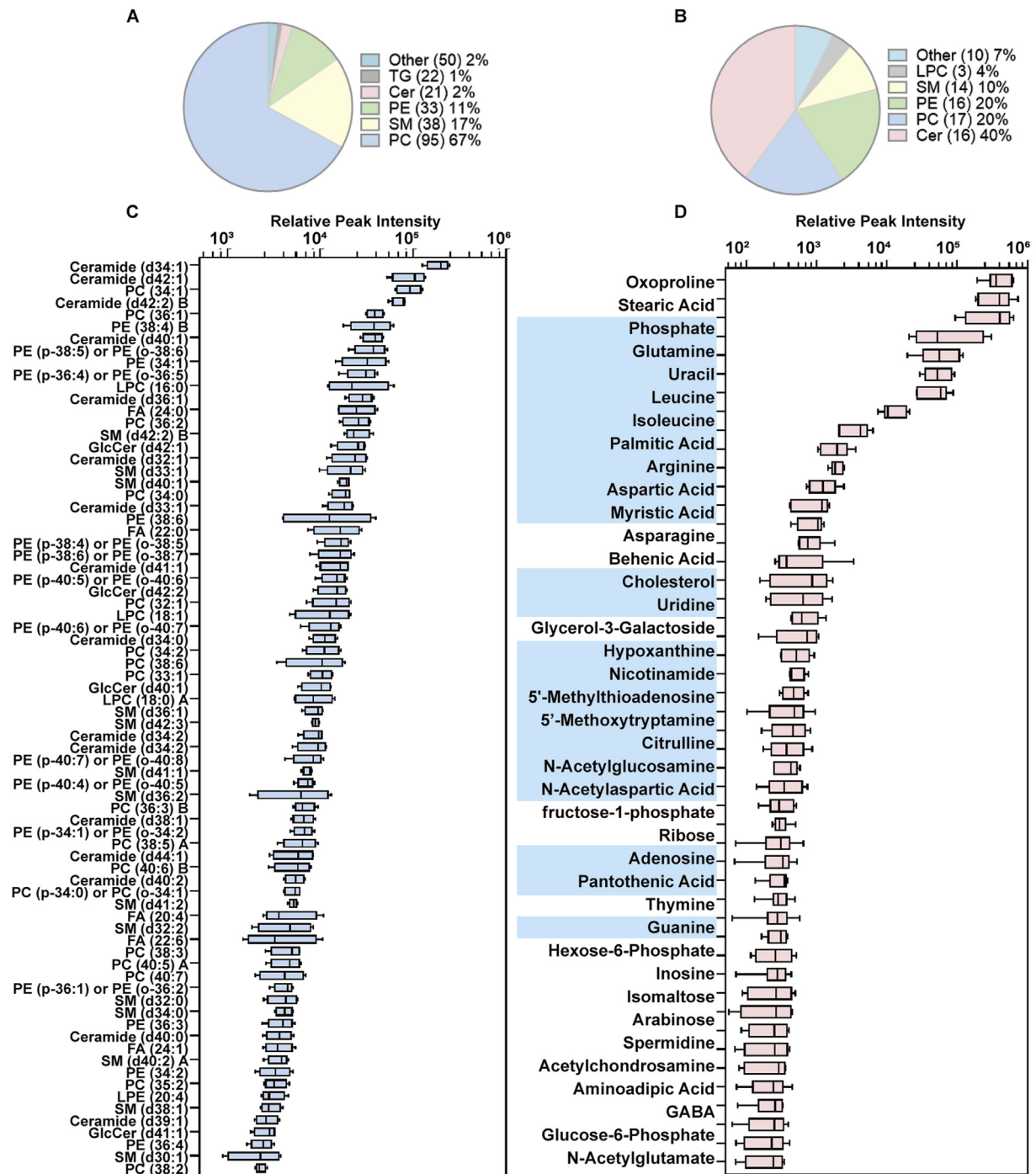


Fig. 4. Mass spectrometry based lipidomic analysis established exosomes derived from primed MSCs are packaged with higher fractions of lipid membrane components, ceramide, lysophosphatidylcholines, and phosphatidylethanolamine phospholipids, as compared to pMSCs (A–C). pMEX are packaged with numerous metabolites, including 21 metabolites established to possess immunoregulatory properties (D), using metabolites identified with high confidence above background, $n = 6$.

reprogramming once they are transitioned from canonical expansion culture conditions, to conditions that mimic the microenvironmental they experience upon administration.

3.2. Exosomes derived from primed MSCs are packaged with metabolites associated with immunomodulation

Over 200 studies have now been published establishing the therapeutic properties of MEX in numerous animal models of disease [34–36]. Several reports have also established the immunomodulatory properties of MEX [37–39]. Here we demonstrate that exosomes derived from pMSC (pMEX) are packaged with numerous primary metabolites, clustered in metabolic networks associated with amino acids, carbohydrates and nucleosides biosynthetic pathways. (Fig. 3). Comparison of the metabolomic profile with our previous proteomics data determined that pMEX are packaged with numerous proteins that correspond to the enzymatic and signaling pathways associated with several of the metabolites detected in pMEX (Fig. S3). Using lipidomic analysis we established that pMEX ceramide, lysophosphatidylcholines, and phosphatidylethanolamine phospholipids, are present at substantially higher ratios as compared to their parental cells of origin (pMSC) (Fig. 4A and B). Many of these factors are associated with lipid rafts and ceramide platforms that have been established to be central communication hubs of receptor mediated inter-cellular signaling. In addition, pMEX are packaged with 21 distinct metabolites that have been directly associated with immunoregulation, including: adenosine, arginine, aspartic acid, cholesterol, glutamine, nicotinamide, UDP-N-acetylglucosamine, 5'-deoxy-5'-methylthioadenosine, palmitic acid and isoleucine. Collectively, these metabolites have been directly associated with the modulation of anti-inflammatory responses, M2 macrophage polarization and induction of regulatory T lymphocytes (Fig. 4C) [40–53]. These data establish that pMEX are packaged with numerous metabolites that have been associated with anti-inflammatory and immunoregulatory functions.

4. Discussion

MSCs and their derived exosomes possess therapeutic potential, but little is currently known about their associated metabolic and lipid membrane profiles [54]. Historically, metabolites and cellular lipids have been viewed as relatively inert by-products of various cellular activities [55–57]. However, a growing body of evidence indicates that many metabolites possess functional properties capable of influencing cellular physiology, and immunological properties [58–61] which we define as epimetabolites [61]. Here, we report that priming conditions greatly decreases the variability of MSC batches, on a metabolic level. We also demonstrate for the first time that pMEX are package with numerous metabolites that may underly some of their immunomodulatory properties.

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Transparency document

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Appendix A. Supplementary data

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