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Characterization of a SILAC method for proteomic analysis of primary rat microglia

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Abstract

Microglia play important and dynamic roles in mediating a variety of physiological and pathological processes during the development, normal function and degeneration of the central nervous system. Application of SILAC-based proteomic analysis would greatly facilitate the identification of cellular pathways regulating the multifaceted phenotypes of microglia. We and others have successfully SILAC-labeled immortalized murine microglial cell lines in previous studies. In this study, we report the development and evaluation of a SILAC-labeled primary rat microglia model. Although the isotope labeling scheme for primary microglia is drastically different from that of immortalized cell lines, our de novo and uninterrupted primary culture labeling protocol (DUP-SILAC) resulted in sufficient incorporation of SILAC labels for mass spectrometry-based proteomic profiling. In addition, label incorporation did not alter their morphology and response to endotoxin stimulation. Proteomic analysis of the endotoxin-stimulated SILAC-labeled primary microglia identified expected as well as potentially novel activation markers and pro-inflammatory pathways that could be quantified in a more physiologically relevant cellular model system compared to immortalized cell lines. The establishment of primary microglia SILAC model will further expand our capacity for global scale proteomic profiling of pathways under various physiological and pathological conditions. Proteomic data are available via ProteomeXchange with identifier PXD002759.

Keywords

Cell biology; Mass spectrometry; Microglia; Neuroimmune; Pathway profiling; SILAC

Research on microglia, discovered nearly a century ago [1], had historically focused on their role as resident immune cells in the CNS in response to neuronal injury and invading pathogens and their contribution to the pathogenesis of various neurological disorders

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including Parkinson's and Alzheimer's disease [2, 3]. Representing >10% of the cells in the CNS, microglia have increasingly been recognized as performing a highly dynamic role in early life CNS remodeling, formation of neuronal plasticity and maintenance of cellular homeostasis in an adult brain under physiologic and pathological conditions, in addition to their classical immune-related functions [4]. Underpinning their dynamic involvement in various cellular processes is a spectrum of "staged" and distinct microglial morphological and functional phenotypes [5]. The molecular mechanisms underlying the various microglial activation stages and the factors/pathways that regulate their shift from one stage to the next are not well understood.

MS-based proteomic analysis enables expression profiling of thousands of proteins in a single sample preparation, making it a powerful tool to identify cellular pathways associated with specific microglial activation stages. Highly desired, but currently not systematically evaluated for mass spectrometric pathway profiling, is a SILAC-based model with primary microglia which more closely resemble microglia in vivo than immortalized microglial cell lines. We have previously reported the establishment of an immortalized murine microglial cell line-based SILAC system where a near complete SILAC label incorporation (>98%) is conveniently achieved by propagating the immortalized cells in label-containing media through multiple and consecutive passaging [6, 7]. Primary microglia, however, may pose a particular challenge to achieving a sufficient isotopic label incorporation due to how primary microglia are prepared in culture [8–10]: a mixed glia culture (microglia and astroglia) are first established from newborn pup's brain cells and microglia are then separated from astroglia two weeks later. No passaging is involved in the two-week time span during which various cell types including microglia undergo their in vitro maturation process.

To prepare SILAC-labeled primary microglia, we employed our de novo and uninterrupted primary culture SILAC labeling strategy (DUP-SILAC) of making light or heavy labeled amino acid pairs (arginine and lysine) available to microglial cells throughout their in vitro maturation process without the introduction of any disruptive steps that may interfere with the maturation of various cell types in the mixed culture system. Brains were harvested from newborn Sprague Dawley rat pups (Charles River), cortical and midbrain tissues were mechanically triturated and dispersed cells (8×10^6) were seeded to poly-D-lysine coated 75-cm^2 culture flasks [10] and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in either the "Light" or "Heavy" SILAC media (10 mL/flask). Light SILAC media consisted of arginine and lysine-free DMEM supplemented with 10% dialyzed FBS, 2 mM L-glutamine, 1% MEM non-essential amino acids, 1 mM sodium pyruvate, 50 units/mL penicillin, 50 μg/mL streptomycin, 10 mM HEPES, pH 7.4 and 100 μg/mL each of L-arginine and L-lysine (Life Technologies). The Heavy SILAC media was made up of the same ingredients except that the light L-arginine and L-lysine were replaced with heavy labeled $^{13}C_6$-$^{15}N_4$-L-arginine and $^{13}C_6$-$^{15}N_2$-L-lysine (Cambridge Isotope) [7]. Four days later, fresh Light or Heavy SILAC media (5 mL/flask) were added to respective cultures. Two weeks after the start of the cultures, the flasks were placed on an orbital shaker and shaken at 120 rpm for 60 min. Media containing the detached microglia were collected and seeded in Light or Heavy SILAC media to poly-D-lysine coated 6-well culture plates (10^6 cells/3 mL media/well) or Nunc 8-well glass chamber slides (2.5×10^4 cells/0.3 mL media/chamber) and grown overnight before treatment.

The primary rat microglia harvested from mixed glia cultures that had been grown for two weeks in the DMEM-based Light (D-Light) and Heavy (D-Heavy) SILAC media were first compared with those from mixed glia cultures grown in the traditional DMEM/F12-based media (D/F12) [10] prepared from same pool of rat brains. As shown in Fig. 1A, the D-Light and D-Heavy microglia morphologically resembled the D/F12 microglia, exhibiting the characteristic morphology of primary microglia in vitro [9,11]. The culture was highly pure (>98%) for microglia as revealed by immunostaining for Iba-1 (ionized calcium-binding adaptor molecule 1), a microglia-specific marker (Fig. 1B), consistent with our previous reports [12,13]. Only a tiny fraction of cells were immune-positive for astroglial marker glial fibrillary acidic protein (D/F12: 0.41%, $n = 980$; D-Light: 0.14%, $n = 690$; D-Heavy: 0.38%, $n = 792$). Functionally, D/F12, D-Light, and D-Heavy microglia were equally responsive to endotoxin lipopolysaccharide (LPS, 0.2–5 ng/mL)-induced production of nitric oxide (NO), a reporter for the M1 microglial activation marker, inducible NO synthase (iNOS, [14]) (Fig. 1C).

We next determined label incorporation efficiency, a key determinant of whether these microglia would be suitable for SILAC-based proteomic profiling. Microglia (10^6) were harvested from D-Heavy mixed glia cultures at 9 days (the earliest in vitro microglia maturation time point under the condition), 2 and 3 weeks following initial seeding [8–10], washed 3× with PBS, and then resuspended in 40 μL of lysis buffer (100 mM Tris-HCl, pH7.6, 4% SDS, and 100 mM dithiothreitol) [7]. Microglia were lysed at 95°C for 5 min followed by a brief sonication and centrifuged for 5 min at 17 000 × *g*. For MS, 50 μg of protein were processed using the FASP method [15, 16], digested with trypsin-LysC, desalted using HYPER-SEP C18 columns as described [6], concentrated by vacuum centrifugation, and resuspended in 0.1% formic acid. For samples with a lower protein concentration, 12 μg of protein were processed using the GASP method [17]. Peptides were separated on an Acclaim PepMap C18 (75 μm × 50 cm) UPLC column (Thermo) using an EASY-nLC 1000 with gradient time ranging from 60 to 240 min (2–40% acetonitrile in 0.1% formic acid). Mass spectrometric analysis was performed by a hybrid quadrupole-Orbitrap instrument (Q Exactive Plus, Thermo), using a top 10 data-dependent acquisition method with a dynamic exclusion time of 20 s. Full scan and MS/MS resolution was 70 000 and 17 500, respectively. Protein identifications were assigned through MaxQuant (version 1.5.0.30) using the UniProt *Rattus norvegicus* database (November 2014, 38 912 entries). Carbamidomethyl (C) was set as a fixed modification and acetyl (protein N-terminus) and oxidation (M) were set as variable modifications. Additional specifications included designating trypsin/P as the enzyme used with the possibility of two missed cleavages, Lys8 and Arg10 as the heavy SILAC labels, 20 ppm (first search)/4.5 ppm (recalibrated second search) mass tolerance for precursor ions and 20 ppm mass tolerance for fragment ions. The mass spectrometric raw data have been deposited to the ProteomeXchange Consortium [18] via the PRIDE partner repository with the dataset identifier PXD002759.

Using an abundant cellular protein beta actin (actin, cytoplasmic 1) as an example, the percentage of the label incorporation for the peptide VAPEEHPVLLTEAPLNPK was approximately 55 and 78% in microglia isolated from 9 day and 2-week old mixed glia cultures, respectively (Fig. 2A). The global-scale incorporation efficiency of heavy-labeled amino acids was determined by Heavy:Light ratios reported in MaxQuant for all identified

peptides where percent efficiency at the peptide level was calculated as 1-(1/ratio(H/L)) [19]. Average labeling efficiency was approximately 53.4 ± 6.1%, 67.6 ± 4.4% and 72.5 ± 3.1% (mean ± SEM) at the 9 day, 2 and 3 week timed intervals, respectively (Fig. 2B). The labeling efficiency of ~70% achieved in primary rat microglia cultures in this study are similar to that observed for primary murine neuronal cultures [20, 21], but lower than the 95% efficiency observed for 20-day old primary astroglial cultures [22]. As reported for primary neuron SILAC labeling studies, two different sets of heavy SILAC labels (sometimes referred to as “Heavy” and “Medium”) may be used to exclude unlabeled proteins from the analysis [23,24]. This approach would bypass the need to account for labeling efficiency and could potentially reduce the risk of introducing variation, making it worthy of consideration in future experiments involving primary neural cells with limited proliferation capacity.

Next, we performed proteomic analysis of SILAC-labeled primary microglia after exposure to the M1 stage activator LPS, in order to test the quantitation accuracy using normalized ratios (which accounts for labeling efficiency) from MaxQuant through bioinformatic analysis of the SILAC datasets. D-Light and D-Heavy microglia in 6-well plates were treated in duplicate for 24 h with Light SILAC media (control) and Heavy SILAC media containing 1 ng/mL LPS, respectively. Afterwards, cells were rinsed with PBS, collected in lysis buffer and equal amounts of proteins from D-Light and D-Heavy microglia were combined and processed for mass spectrometric analysis.

Using a peptide and protein false discovery rate of 1.0% in MaxQuant, 2299 protein groups and 12 442 peptides were identified across replicates ($n = 2$ biological replicates with two experimental replicates). Numerous hallmarks of classical microglial activation were observed in response to LPS treatment, including up-regulation of STAT1, CD14, and TLR-2. As an example, raw data representing lower fold changes of the up- and down-regulated proteins, SOD2 and LRP1, respectively, as well as induction of iNOS are shown in Fig. 3A–C. LPS-induced iNOS up-regulation in microglia was independently confirmed by indirect immunofluorescence analysis of the iNOS protein (Fig. 3D). Additionally, based on the resulting relative quantitation from SILAC-labeled primary microglia, bioinformatic analysis (IPA version 01-01) revealed LPS exposure to be a predicted upstream event (Fig. 3E). The expression level of only a few targets was inconsistent with the Ingenuity Knowledge Base, which included the downregulation of LRP1 (low density lipoprotein receptor-related protein 1). LRP1 has been shown, however, to be involved in the anti-inflammatory response of murine macrophages where LRP1 deficiency causes downregulation of alternative (anti-inflammatory) activation markers and enhances the M1 response [25]. Given the importance of LRP1 in macrophage polarization, differential expression of LRP1 in microglia could be time-dependent where downregulation occurs at early time points (post-M1 stimulation) to prevent transition to an alternative activation phenotype. Future work is needed to investigate LRP1 regulation in the context of microglial phenotypic changes.

It should be noted that single amino acid (arginine) based-SILAC labeling of primary microglia has been reported previously [26, 27]. However a comprehensive characterization of the singly labeled cells in terms of possible phenotypic alterations post labeling, labeling

efficiency time course, and quantitation accuracy using high-resolution mass spectrometry were not reported.

In summary, we report a comprehensive (morphological, biochemical and proteomic) characterization of a SILAC-based model for primary rat microglia. Even though it was not possible to consecutively passage the primary glial cultures, which is the approach used for SILAC labeling of immortalized microglial cell lines, the two-week de novo and uninterrupted primary culture SILAC (DUP-SILAC) labeling strategy achieved a label incorporation ratio of ~70%, a value comparable to that observed for murine primary neuron cultures [20, 21]. The SILAC-labeled primary rat microglia were suitable for MS-based proteomic analysis with nearly 2300 proteins identified. Furthermore, proteomic analysis revealed that the SILAC labeled primary rat microglia responded in a similar fashion as immortalized rat microglial cells to stimulation with LPS as defined by expression changes of known proteins and related pathways associated with LPS-induced classical activation. In light of their multifaceted phenotypic stages, the SILAC-based proteomic model with primary microglia, which are characteristically more closely related to microglia in vivo compared to immortalized cell lines and are more suitable for studies involving co-cultures of multiple neural cell types, should further facilitate the identification of distinct cellular pathways and unique regulators mediating the dynamic microglial responses to various activity-modulating agents under physiological and pathological conditions.

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Abbreviations

DMEM/F12	DMEM/Ham’s nutrient mixture F12 (1:1)
iNOS	inducible nitric oxide synthase
SILAC	stable isotope labeling by amino acids in cell culture

References

1. del Rio-Hortega P. Art and artifice in the science of histology. 1933. Histopathology. 1993; 22:515–525. [PubMed: 8354484]
2. Block ML, Zecca L, Hong JS. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. Nat Rev Neurosci. 2007; 8:57–69. [PubMed: 17180163]
3. Liu B. Modulation of microglial pro-inflammatory and neurotoxic activity for the treatment of Parkinson’s disease. AAPS J. 2006; 8:E606–E621. [PubMed: 17025278]
4. Delpech JC, Madore C, Nadjar A, Joffre C, et al. Microglia in neuronal plasticity: Influence of stress. Neuropharmacology. 2015; 96:19–28. [PubMed: 25582288]
5. Raivich G, Bohatschek M, Kloss CU, Werner A, et al. Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. Brain Res Brain Res Rev. 1999; 30:77–105. [PubMed: 10407127]
6. Bell-Temin H, Barber DS, Zhang P, Liu B, Stevens SM Jr. Proteomic analysis of rat microglia establishes a high-confidence reference data set of over 3000 proteins. Proteomics. 2012; 12:246–250. [PubMed: 22121004]

7. Liu B, Barber DS, Stevens SM Jr. Stable isotope labeling with amino acids in cell culture-based proteomic analysis of ethanol-induced protein expression profiles in microglia. Methods Mol Biol. 2012; 829:551–565. [PubMed: 22231838]
8. McCarthy KD, de Vellis J. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J Cell Biol. 1980; 85:890–902. [PubMed: 6248568]
9. Giulian D, Baker TJ. Characterization of ameboid microglia isolated from developing mammalian brain. J Neurosci. 1986; 6:2163–2178. [PubMed: 3018187]
10. Liu B, Hong JS. Primary rat mesencephalic neuron-glia, neuron-enriched, microglia-enriched, and astroglia-enriched cultures. Methods Mol Med. 2003; 79:387–395. [PubMed: 12506711]
11. Zhang P, Wong TA, Lokuta KM, Turner DE, et al. Microglia enhance manganese chloride-induced dopaminergic neurodegeneration: role of free radical generation. Exp Neurol. 2009; 217:219–230. [PubMed: 19268665]
12. Gao HM, Jiang J, Wilson B, Zhang W, et al. Microglial activation-mediated delayed and progressive degeneration of rat nigral dopaminergic neurons: relevance to Parkinson's disease. J Neurochem. 2002; 81:1285–1297. [PubMed: 12068076]
13. Liu B, Du L, Hong JS. Naloxone protects rat dopaminergic neurons against inflammatory damage through inhibition of microglia activation and superoxide generation. J Pharmacol Exp Ther. 2000; 293:607–617. [PubMed: 10773035]
14. Dutta G, Zhang P, Liu B. The lipopolysaccharide Parkinson's disease animal model: mechanistic studies and drug discovery. Fundam Clin Pharmacol. 2008; 22:453–464. [PubMed: 18710400]
15. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods. 2009; 6:359–362. [PubMed: 19377485]
16. Manza LL, Stamer SL, Ham AJ, Codreanu SG, Liebler DC. Sample preparation and digestion for proteomic analyses using spin filters. Proteomics. 2005; 5:1742–1745. [PubMed: 15761957]
17. Fischer R, Kessler BM. Gel-aided sample preparation (GASP)–a simplified method for gel-assisted proteomic sample generation from protein extracts and intact cells. Proteomics. 2015; 15:1224–1229. [PubMed: 25515006]
18. Vizcaino JA, Deutsch EW, Wang R, Csordas A, et al. ProteomeXchange provides globally coordinated proteomics data submission and dissemination. Nat Biotechnol. 2014; 32:223–226. [PubMed: 24727771]
19. Biochemistry, M. P. I. o., http://www.biochem.mpg.de/221777/SILAC 2003–15.
20. Spellman DS, Deinhardt K, Darie CC, Chao MV, Neubert TA. Stable isotopic labeling by amino acids in cultured primary neurons: application to brain-derived neurotrophic factor-dependent phosphotyrosine-associated signaling. Mol Cell Proteomics. 2008; 7:1067–1076. [PubMed: 18256212]
21. Liao L, Park SK, Xu T, Vanderklish P, Yates JR 3rd. Quantitative proteomic analysis of primary neurons reveals diverse changes in synaptic protein content in fmr1 knockout mice. Proc Natl Acad Sci U S A. 2008; 105:15281–15286. [PubMed: 18829439]
22. Greco TM, Seeholzer SH, Mak A, Spruce L, Ischiropoulos H. Quantitative mass spectrometry-based proteomics reveals the dynamic range of primary mouse astrocyte protein secretion. J Proteome Res. 2010; 9:2764–2774. [PubMed: 20329800]
23. Schwanhausser B, Gossen M, Dittmar G, Selbach M. Global analysis of cellular protein translation by pulsed SILAC. Proteomics. 2009; 9:205–209. [PubMed: 19053139]
24. Zhang G, Deinhardt K, Chao MV, Neubert TA. Study of neurotrophin-3 signaling in primary cultured neurons using multiplex stable isotope labeling with amino acids in cell culture. J Proteome Res. 2011; 10:2546–2554. [PubMed: 21370927]
25. May P, Bock HH, Nofer JR. Low density receptor-related protein 1 (LRP1) promotes anti-inflammatory phenotype in murine macrophages. Cell Tissue Res. 2013; 354:887–889. [PubMed: 23963646]
26. McLaughlin P, Zhou Y, Ma T, Liu J, et al. Proteomic analysis of microglial contribution to mouse strain-dependent dopaminergic neurotoxicity. Glia. 2006; 53:567–582. [PubMed: 16419087]
27. Liu J, Zhou Y, Wang Y, Fong H, et al. Identification of proteins involved in microglial endocytosis of alpha-synuclein. J Proteome Res. 2007; 6:3614–3627. [PubMed: 17676786]

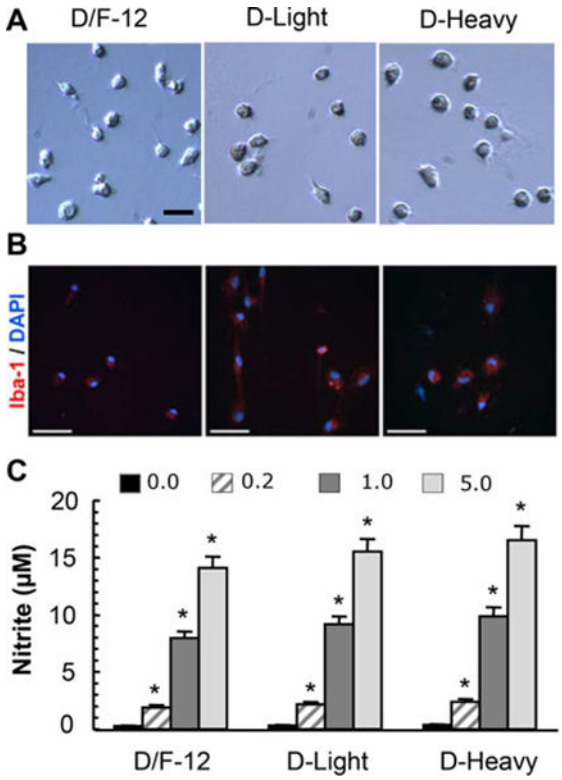

Figure 1.

Comparison of primary rat microglia prepared from mixed glia cultures grown for 2 weeks in regular and SILAC media. (A) Photomicrograph of microglia prepared in DMEM/F12 (D/F12), DMEM light SILAC (D-Light) and DMEM heavy SILAC (D-Heavy) media. (B) Immunostaining. Microglia cultures were immunostained for microglial marker Iba-1 (Abcam) and counter-stained with DAPI for cell nuclei (Invitrogen) [12]. (C) LPS-induced nitrite production. Microglia were stimulated with 0 (control), 0.2, 1 and 5 ng/mL LPS for 24 h and amounts of nitrite released were measured and expressed as mean ± SEM as previously described [11]. *p<0.05 compared to control (n = 3, ANOVA with Bonferroni/Dunn post hoc analysis). Scale bar: 20 μm in A and 16 μm in B.

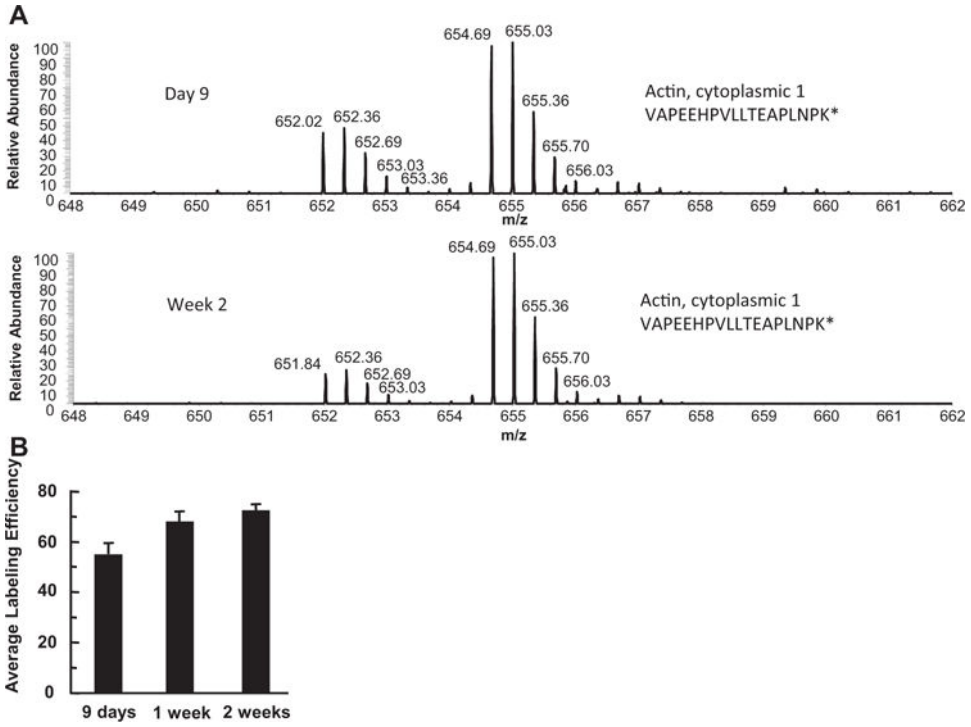

Figure 2.

SILAC labeling efficiency. (A) Representative full scan mass spectra showing the peptide pair with the sequence VAPEEHPVLL-TEAPLNPK* from beta actin in microglia following 9 days (top panel) and 2 weeks (bottom panel) of SILAC labeling. B. Overall labeling efficiency calculated based on heavy label incorporation in all identified peptides (n = 9780 peptides).

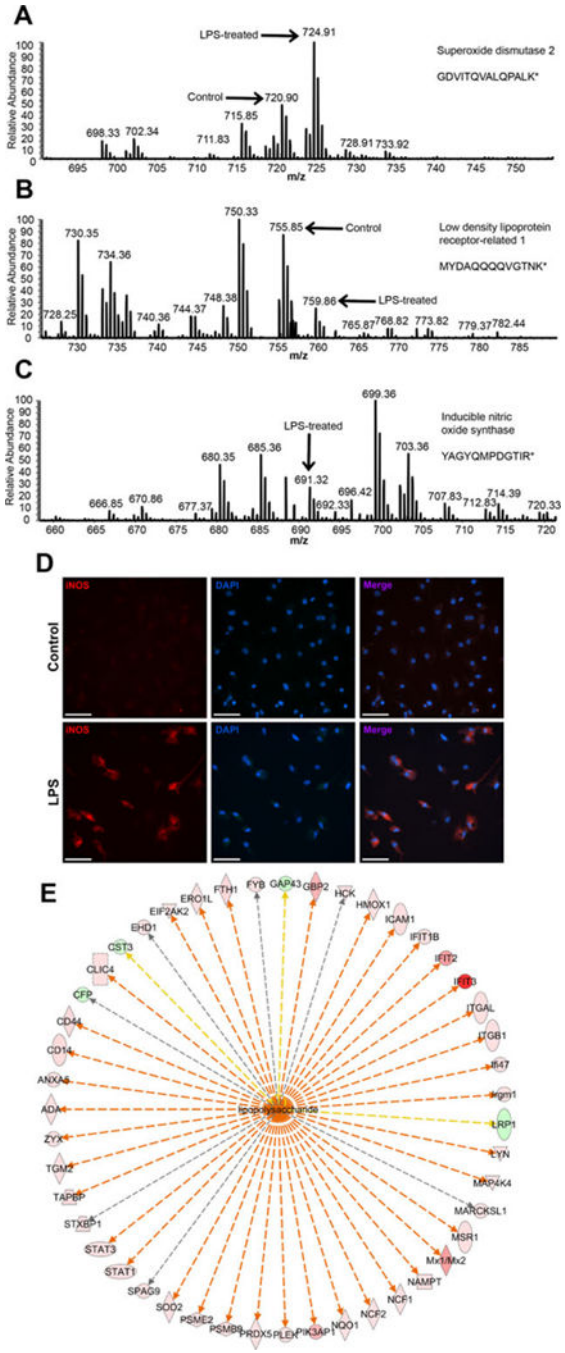

Figure 3.

LPS-induced microglial differential expression and upstream regulator prediction. (A–C) Full scan, high resolution mass spectra of SILAC pairs. The peptide GDVITQVALQPALK* from superoxide dismutase (SOD2), is shown in A. The heavy:light ratio, generated by the MaxQuant, was 1.98, or 3.40 after normalization. The peptide MYDAQQQQVGTNK* from low density lipoprotein receptor-related 1 (LRP1) is shown in B. The heavy:light ratio was 0.33, or 0.41 after normalization. The peptide YAGYQMPDGTIR* from iNOS is shown in C. A heavy:light ratio could not be calculated for this inducible protein since it was not

present in control cells. (D) Immunostaining analysis of LPS-induced iNOS upregulation in microglia. Cells were counter-stained with DAPI for cell nuclei. Scale bar: 16 m. (E) Upstream regulator analysis using Ingenuity Pathway Analysis predicts LPS to be an activated upstream regulator (central node) where known downstream targets are shown in the outer nodes. Outer nodes represent enzymes (diamond), kinases (triangle), transporters (trapezoid), and other proteins (circle) where red and green node color represents up- and down-regulation, respectively. Orange indicates predicted activation for LPS as an upstream regulator, while orange, yellow and grey arrows indicate predicted activating effect, inconsistent relationship, or reported effect with limited information on downstream target expression change. For biological replicates 1 and 2, the *p*-values were 3.52×10^{-22} and 6.22×10^{-14}, respectively, and the *Z*-scores were 5.267 and 4.358, respectively.