Mass Spectrometric Quantification of Enriched Microglia Using a Metabolically-labeled Immortalized Cell Mix

Harris Bell-Temin, Christina Carlson, Anne-Marie Carpenter, Stanley M. Stevens

Department of Cell Biology, Microbiology, and Molecular Biology, University of South Florida, Tampa, FL

Metabolic labels, such as SILAC, are an excellent choice for immortalized cell work, but are difficult to apply to in vivo tissues or enriched cells. Often in these cases, an isobaric labelling system such as TMT or iTRAQ is preferable. Both systems, however, require a high rate HCD MS/MS scan for the quantification of reporter ions, which is not possible on many first or second generation high resolution instruments in service. In order to quantify ethanol induced changes to microglial activation, we created a simulated proteome of enriched microglia using immortalized cells treated with multiple agonists estimated to approximate the expression of proteins within an order of magnitude of in vivo microglia via spectrum counting. Following a density gradient enrichment, microglia from chronically ethanol exposed mice and control mice were mixed with equal amounts of the immortalized cell labeled mix and were analyzed on a high resolution Orbitrap XL mass spectrometer. Over 2,500 proteins and 9,000 peptides were identified from the microglia, and of these 81% of proteins and 62% of peptides were successfully quantified in the control and ethanol treated groups. 157 proteins were found to have significantly altered expression, and several novel pathways involved in ethanol induced activation of microglia were uncovered.

Microglia act as the frontline immune defense in the brain, and have responses that can be either neurotoxic or neurotrophic depending on the activating stimuli. Their neurotoxic responses include inflammatory cytokines such as TNF-α and IL-6, as well as production of reactive oxygen and nitrogen species, while the neurotrophic response is hallmarked by production if anti-inflammatory cytokines such as TGF-β and fibrinogenic and other growth factors. Microglial inflammatory response has been implicated as a cause of neuronal damage observed due to alcohol use disorders. Our previous work in immortalized microglial cells,

however, suggests microglia have a non-inflammatory activating response to ethanol, more akin to a neurotrophic response than a neurotoxic, as it is devoid of inflammatory cytokine markers.

Ultimately, however, data derived from immortalized cell lines can only suggest what is occurring *in vivo*. In order to quantify protein expression of microglia in mice that have been on a 30 day chronic feeding, microglia were enriched using a Percoll density gradient, yielding approximately 3 ug of microglial protein following lysis. The enriched microglia from ethanol treated and control animals were then mixed with immortalized BV2 cells that had been treated with a wide variety of activators to stimulate all known microglial activation states in order to produce as broad a proteome as possible. Samples were then separated on a 10 cm, 75 µm ID reversed phased C18 column inline with an Orbitrap XL mass spectrometer. Raw files were analyzed on MaxQuant and ratio of ratio combination was performed at the peptide level prior to recombination to produce protein levels. The ratio of ratio combination can be seen in Figure 1. Proteins with significantly ($p<.05$) altered expression levels in two or more biological samples were input into Ingenuity Pathway Analysis for functional and regulatory analysis.

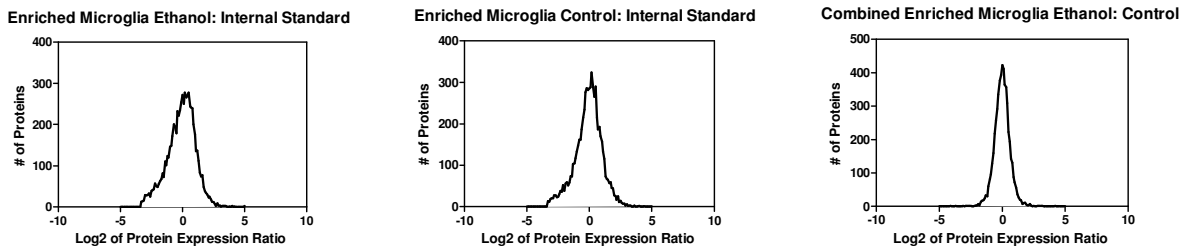

Figure 1. Ratio of Ratio Combination for Enriched Microglia. Ratio of ratio combination at the peptide level between enriched microglia and internal standards for control and ethanol treated animals.

Mass spectrometric analysis identified 2,513 protein groups across the six biological samples. In addition, 9,051 unique peptides were identified. 6,645 peptides were quantified between the control enriched microglia and internal standard mix within an order of magnitude, and 6,867 peptides were quantified between the ethanol enriched microglia and internal standard mix within an order of magnitude. When ratio of ratio combination was performed at the individual level, 5,494 peptides were quantified, representing 62% of all identified peptides. After combining peptide levels, 1,928 protein groups were able to be quantified, representing quantification of 81% of all protein groups identified.

Pathway analysis yielded several novel findings regarding the activating effect of ethanol upon microglial activity, bolstering our previous *in vivo* work. The use of a metabolically labeled standard of immortalized cells, despite being sourced from a single immortalized cell line, was able to accurately quantify over 80% of all proteins identified. The methodology describes provides accurate global scale quantification of *in vivo* materials when isobaric labeling methods are unfeasible due to instrument availability.