***Leishmania mexicana* Promotes Pain-reducing Metabolomic Reprogramming In Cutaneous Lesions**

METHODS:

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Female C57BL/6 mice were purchased from Envigo (Harlan laboratories) Indianapolis, IN, USA, and housed at The Ohio State University animal facility, following approved animal protocols and University Laboratory Animal Resources (ULAR) regulations (2010A0048-R3 Protocol). All the experiments were performed using 5 age-matched 5-8 week old female mice per group. 129S6/SvEvTac mice were purchased from Taconic Biosciences, Inc.

Parasites

129S/SvEvTac mice were used to maintain L. mexicana (MNYC/B2/62/m379) parasites via subcutaneous inoculation into the shaved back rumps. Amastigotes were then obtained from the draining lymph nodes of infected mice and grown at 26°C in M199 medium supplemented with 1% Penicillin/Streptomycin, and 1% HEPES 10% fetal bovine serum (FBS), to generate stationary phase promastigotes.

BMDMs and RAW cells for in vitro studies we used the immortalized RAW 264.7 macrophage cell line, as well as primary bone marrow-derived macrophages (BMDMs). The RAW 264.7 cell line was purchased from American Type Culture Collection (ATCC), and cell identity was verified regularly based on cell morphology. Both RAW 264.7 and BMDMs were cultured with RPMI medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% HEPES at 37°C with 5% CO2. BMDMs were obtained from the femur and tibias of C57BL/6 mice. After isolation, the bone marrow was cultured with

complete RPMI supplemented with 20% supernatant from L-929 cells for 7-10 days until differentiation was complete.

METHOD DETAILS

***In vitro* cell culture and infection**

RAW 264.7 macrophages and BMDMs were plated in a 24-well plate at a density of 0.5x106 per well and infected overnight with stationary phase *L. mexicana* promastigotes at a ratio of 10:1 (parasite to macrophages). The controls were treated with media alone. Then, the extracellular parasites were removed by washing with PBS and new media was applied. After a 24hrs incubation, the supernatant and cell pellet were collected and processed for mass spectrometry.

Mass spectrometry

For in vitro experiments, the culture supernatant was collected and cell debris was removed by centrifugation according to SOP 5 of the Laboratory Guide for Metabolomics Experiments. The attached cells were be scraped, washed with PBS and quenched with 80% methanol. Then they were snap-frozen, centrifuged, and lyophilized according to SOP 4 of the Laboratory Guide for Metabolomics Experiments. For in vivo studies, the ears were collected, snap frozen, and processed for mass spectrometry analysis according to SOP 7 of the Laboratory Guide for Metabolomics Experiments. Samples were then incubated with 500 uL of 100% MeOH and sonicated. The tissue was weighed and homogenized at 40 mg/mL of 50% MeOH solution for 3 cycles in a Precellys homogenizer. The supernatant was collected, dried down, and reconstituted in ½ of the original volume in 5 % MeOH with 0.1 % formic acid. For metabolite candidates found via untargeted analysis, pure standards purchased from Sigma-Aldrich were diluted in 100 % MeOH stocks to 10 ug/mL, dried down, and reconstituted in 5 % MeOH with 0.1 % formic acid and run in the same conditions described below to match features for identification. Untargeted analysis was performed on a Thermo Scientific Q-Exactive Plus Orbitrap mass spectrometer (MS) with HPLC separation on a Poroshell 120 SB-C18 (2 x 100 mm, 2.7 µm particle size) with a Thermo

Scientific Ultimate WPS 3000 UHPLC system. The gradient consisted of solvent A, H2O with 0.1% Formic acid, and solvent B 100% acetonitrile at a 200 µL/min flow rate with an initial 2% solvent B with a linear ramp to 95% B at 15 min, holding at 95% B for 1 minutes, and back to 5% B from 17 min and equilibration of 5% B until min 30. For each sample, 5 µL was injected for each sample with ionization in the MS on a HESI electrospray ionization source using a capillary voltage of 4.5 kV in positive and 4.0 kV in negative mode at 320 °C capillary temperature and 100 °C probe temperature. Gas settings were set to 15 for sheath gas and 5 auxiliary gas while the S-Lens was set to 50 V. The top 5 ions were selected for data dependent analysis with a 10 second exclusion window, with a mass range of 80-1200 m/z and a resolution of 70,000 for MS scans and 17,5000 for MSMS scans and fragmentation normalized collision energies of 30 V. For feature selection in the untargeted results analysis, including database comparison and statistical processing, samples were analyzed in Progenesis QI and the pooled sample runs were selected for feature alignment. Using the pooled QC samples, features with above 30% CV and max abundances below 5000 intensity were filtered out and Anova p-value scores between the groups were calculated with a cutoff of < 0.05. With database matching using the Human Metabolome Database, selecting for adducts M+H, M+Na, M+2H, and 2M+H for positive mode and M-H, M+Cl, M-2H, and 2M-H and less than 10 ppm mass error, unique features were tentatively identified as potential metabolites. Metabolites were only annotated with MSMS fragmentation matching scores above 20 % with Progenesis Metascope.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis of mass spectrometry datasets

Peak intensity data tables from the mass spectrometry experiment were formatted into comma-separated values (CSV) files conforming to MetaboAnalyst's requirements and uploaded into the one-factor statistical analysis module. Each analysis passed MetaboAnalyst's internal data integrity check and additional data filtering was performed based on interquartile range. On the normalization overview

page, sample normalization was performed based on the median of the data and the auto-scaling option was chosen to perform data scaling; No transformation of the data was performed. For dimensionality reduction, both principal component analysis (PCA) and partial least-squares discriminant analysis (PLSDA) were employed. Cross-validated sum of squares (Q2) performance measures were used to determine if PLSDA models were overfitted. Visualization of significant, differentially regulated metabolites was done by generating volcano plots with cutoffs of < 0.05 false-discovery rate (FDR) and > 2-fold change (FC). Clustering of samples and features were analyzed by creating dendrograms and hierarchical heatmaps, respectively.

Pathway analysis of mass spectrometry datasets

We have used two different techniques in order to identify enriched pathways in our data sets. First we used the Functional Analysis Module (MS peaks to pathways) in MetaboAnalyst 4.0. Detected peaks (mass-to-charge ratios + retention times) from positive and negative analytical modes of the mass spectrometer for each sample were organized into four column lists along with calculated FDR-corrected *p*-values and t-scores from univariate t-tests. These peak list profiles were uploaded to the functional analysis module and passed the internal data integrity checks. The ion mode in MetaboAnalyst was set to the appropriate type depending on the analytical mode that was used to generate the data. For each analysis the mass tolerance was set to 10 ppm, the retention time units were set to minutes, and the option to enforce primary ions was checked. In parameter settings, the mummichog algorithm (version 2.0) and the modified gene set enrichment algorithm were used for all analyses. The *p*-value cutoff for the mummichog algorithm was left at the default (top 10% of peaks). Currency metabolites and adducts were left at default settings. Lastly, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway library for *Mus musculus* was selected as the metabolic network that the functional analysis module would use to infer pathway activity and predict metabolite identity; only pathways/metabolite sets with at least three entries were allowed.

Integrative Network Analysis:

The Metscape 3.1.3 App from the Cytoscape 3.9.1 software was used in order to build integrative network analysis of our in-vivo dataset using Metscape's internal database which incorporates KEGG and EHMN data. The IDs of the metabolomic dataset were converted from HMDB IDs to KEGG IDs recognized by the Cytoscape software via the Chemical Translation Service (CTS) and verified by the Metaboanalyst Compound ID Conversion tool. The metabolomic dataset was then uploaded as a compound file to Metscape and the FDR-adjusted *p*- Value and FC Ratio cutoff points were set at 0.05 and 1.5 respectively. 810 from a total of 2771 metabolites were not accepted as input compounds by Metscape and were removed from the dataset. A Compound-compound network was created for the detected purine Metabolism pathway of our dataset, along with an integrative network build from the combination of arachidonic acid Metabolism, glycerophospholipid metabolism, linoleate metabolism, and prostaglandin formation from arachidonate. The two created networks allowed us to visualize the integrated relationship among the metabolites and endocannabinoids involved in purine metabolism and arachidonic acid metabolism respectively.

Statistical analysis

All in vitro and in vivo data show a representative experiment with N =3 per group, with 4 experimental replicates in each group. N represents different biological replicates. For the mass spectrometry data all statistical analysis were performed with MetaboAnalyst software.