Supplemental Methods and References

Materials and Methods for Global Metabolomics: courtesy of Robert Mohney

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Sample Preparation for Global Metabolomics

Spleens from twelve mice were collected and processed. Samples were pooled (4 mice/group) and stimulated on anti-CD3, anti-CD28, and ICAM as previously described for 36 hours before sorting undivided cells and first division c-Myc high and low cells ($1.6x10^6$ cells/sample). Samples were stored at –80°C until processed. Sample preparation was carried out as described previously [1] at Metabolon, Inc. Briefly, recovery standards were added prior to the first step in the extraction process for quality control purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills Genogrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: one for analysis by ultra high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS; positive ionization), one for analysis by UPLC-MS/MS (negative ionization), one for the UPLC-MS/MS polar platform (negative ionization), one for analysis by gas chromatography–mass spectrometry (GC-MS), and one sample was reserved for backup.

Three types of controls were analyzed in concert with the experimental samples: samples generated from a pool of human plasma extensively characterized by Metabolon, Inc. or generated from a small portion of each experimental sample of interest served as technical replicate throughout the data set; extracted water samples served as process blanks; and a cocktail of standards spiked into every analyzed sample allowed instrument performance monitoring. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to injection into the mass spectrometers (median RSD typically = 4-6%; $n \geq 30$ standards). Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled human plasma or client matrix samples (median RSD = 8-12%; n = several hundred metabolites). Experimental samples and controls were randomized across the platform run.

Mass Spectrometry Analysis

Non-targeted MS analysis was performed at Metabolon, Inc. Extracts were subjected to either GC-MS [2] or UPLC-MS/MS [1]. The chromatography was standardized and, once the method was validated no further changes were made. As part of Metabolon's general practice, all columns were purchased from a single manufacturer's lot at the outset of experiments. All solvents were similarly purchased in bulk from a single manufacturer's lot in sufficient quantity to complete all related experiments. For each sample, vacuum-dried samples were dissolved in injection solvent containing eight or more injection standards at fixed concentrations, depending on the platform. The internal

standards were used both to assure injection and chromatographic consistency. Instruments were tuned and calibrated for mass resolution and mass accuracy daily.

The UPLC-MS/MS platform utilized a Waters Acquity UPLC with Waters UPLC BEH C18-2.1×100 mm, 1.7 μm columns and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in acidic or basic LC-compatible solvents, each of which contained 8 or more injection standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic, positive ion-optimized conditions and the other using basic, negative ion-optimized conditions in two independent injections using separate dedicated columns (Waters UPLC BEH C18-2.1x100 mm, 1.7 μm). Extracts reconstituted in acidic conditions were gradient eluted using water and methanol containing 0.1% formic acid, while the basic extracts, which also used water/methanol, contained 6.5mM ammonium bicarbonate. A third aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate. The MS analysis alternated between MS and data-dependent MS^2 scans using dynamic exclusion, and the scan range was from 80-1000 m/z.

The samples destined for analysis by GC-MS were dried under vacuum desiccation for a minimum of 18 h prior to being derivatized under dried nitrogen using bistrimethyl-

silyltrifluoroacetamide. Derivatized samples were separated on a 5% diphenyl / 95% dimethyl polysiloxane fused silica column (20 m x 0.18 mm ID; 0.18 um film thickness) with helium as carrier gas and a temperature ramp from 60°C to 340°C in a 17.5 min period. All samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole MS using electron impact ionization (EI) and operated at unit mass resolving power. The scan range was from 50–750 m/z.

Compound Identification, Quantification, and Data Curation

Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments as well as associated MS spectra and curated by visual inspection for quality control using software developed at Metabolon [3]. Identification of known chemical entities is based on comparison to metabolomic library entries of purified standards. Commercially available purified standard compounds have been acquired and registered into LIMS for distribution to both the UPLC-MS/MS and GC-MS platforms for determination of their detectable characteristics. Peaks were quantified using area-under-the-curve. Raw area counts for each metabolite in each sample were normalized to correct for variation resulting from instrument inter-day tuning differences by the median value for each run-day, therefore, setting the medians to 1.0 for each run. This preserved variation between samples but allowed metabolites of widely different raw peak areas to be compared on a similar graphical scale. Missing values were imputed with the observed minimum after normalization.

Gene expression analysis by real time quantitative reverse transcription PCR (Real Time qRT-PCR)

RNA was isolated using RNeasy kit (Qiagen) according to the manufacturer's instructions. Total RNA (3.5-4 μg for experiments presented in Ext. Fig. 5H, 9A or 5 μg for experiments presented in Ext. Fig. 9B) was reverse-transcribed using the M-MLV reverse transcriptase, RNAse OUT recombinant ribonuclease inhibitor (10777-019; Invitrogen) and oligodT primers. Quantitative real-time-PCR was performed using the SYBR Green PCR Master Mix (4309155; Life Technologies). Gene expression was analyzed using the 7500 Real-Time PCR Systems and Sequence Detection Software, Applied Biosystems, v. 1.4.

Samples were run in triplicate. The relative abundance of a given mRNA was determined by normalization to that of β-2-microglobulin mRNA. The results for an induction of a given gene are presented as the average fold difference over the abundance of this gene's mRNA in ''Myc Low" cells, which was set to 1. The relative levels of transcript abundance were calculated as described before [4] and according to the $2^{-\Delta\Delta Ct}$ method. The sequences of primers used were reported before. [5]. In general, primer concentration applied to analyze expression of metabolic or transporter genes was 200 nM, however other primer concentrations (100 nM or 400 nM) were also used due to differences in optimal primer dynamic range for a specific gene and cDNA template ("Undivided" cells *vs*. "1st Division" cells).

SLC3A2 Imaging

OT-I Tg T cells (isolated and enriched as described) were stimulated in #1.5 polymer coverslips (80426; ibidi) coated with CD3, CD28, and ICAM for 2hrs and fixed with 4% paraformaldehyde for 10 min. Cells were permeabilized with TBS containing 0.1% (v/v) Triton-X100 for 3 min and blocked with TBS containing 2% bovine serum albumin prior to labeling of alpha tubulin (1:1000; MA1-19162; Thermo Fisher) and CD98 (1:500; eBioscience; 12-0981-81). Primary antibodies were detected using donkey anti-mouse AF488 (1:1000, Jackson Immunoresearch; 715-545-151). Three-dimensional spinning-disc confocal images were acquired with a step size of 0.3 μm, and analyzed using Imaris x64 8.1.2 (Bitplane). Surfaces were created to define proximal and distal half of the cell related to the site of activation and the sum fluorescent intensity of the GFP channel was assessed for both halves. Three independent experiments were performed and 5 images with at least 10 cells were analyzed per experiment.

Electrophoresis and Western Blotting

Cells were lysed on ice with RIPA lysis buffer supplemented with the protease inhibitor cocktail Complete Mini (Roche) for 30 min and cleared from debris by centrifugation at 14.000 rpm for 10 min at 4°C. Samples were prepared with 4x denaturing sample buffer (Biorad) and submitted to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) using Criterion XT precast gels (Biorad). Proteins were transferred to a nitrocellulose membrane at 40 V for 90 min using a wet blotting system. Blocking and probing with antibodies was performed according to manufacturers protocols. The following antibodies from Cell Signaling were used: c-myc (5605), p-

mTOR Ser2481 (2974), p-mTOR Ser 2448 (5536), mTOR (4517), p-P70S6K Thr389 (9234), P70S6K (2708), HK2 (2876), and actin (sc-4778; Santa Cruz). Proteins were visualized by standard enhanced chemiluminescence reaction.

c-Myc Heterozygous Mice

C-Myc (flox) conditional knockout mice were given by Fred Alt (Boston Children's Hospital)[6]. The c-Myc null allele was generated by breeding c-Myc flox animals with EIIa-cre mice (JAX003724). Offspring that were EIIa-cre positive and c-myc null/wt were identified by PCR of genomic DNA from tail biopsy. For Cre genotyping, primers and procedures were obtained from Jackson Laboratories. For c-Myc, primers used to amplify floxed undeleted (flox, 500 bp) and deleted (Δ, 450 bp) c-myc alleles, respectively, were myc-fl S (GCCCCTGAATTGCTAGGAAGACTG), myc-fl-A (CCGACCGGGTCCGAGTCCCTATT), myc-Δ-S (TCGCGCCCCTGAATTGCTAGGAA), and myc-Δ-A (TGCCCAGATAGGGAGCTGTGATACTT). These founder animals were then crossed with OT1/CD45.1 animals to generate experimental animals.

Supplemental References

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