SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 1.

Transcriptional analysis identifies a signature set of 100 genes regulated by DASA.

Gene expression analysis of RNA isolated from primary murine BMDM cells treated with or without DASA-58 (50μM) followed by 24 hours of LPS (100ng/ml) by Illumina Microarray. Transcriptional analysis identifies a signature set of 100 genes regulated by DASA-58.

Table S1, related to Figure 1.

Signature set of genes differentially regulated by LPS with and without PKM2 activation. Data shown is normalized expression value of each gene in log2 scale. Fold change and signal to noise ratio were estimate for DASA+LPS vs DMSO+LPS columns. FDR-adjusted p-values were estimated using Benjamini-Hochberg method.

Table S2, related to Figure 1.

LPS-induced pathways modulated by PKM2 activation by DASA-58.

Enrichment of pathway maps in signature set of 100 genes was estimated using MetaCore.

Table S3, related to Figure 1.

LPS-induced Process Networks modulated by PKM2 activation by DASA-58. Enrichment of process networks in signature set of 100 genes was estimated using MetaCore.

Table S4, related to Figure 1.

LPS-induced Metabolic Networks modulated by PKM2 activation by DASA-58. Enrichment of metabolic networks in signature set of 100 genes was estimated using MetaCore.

Table S5, related to Figure 1.

LPS-induced Transcription Factors modulated by PKM2 activation by DASA-58. Enrichment of target genes of transcription factors in signature set of 100 genes was estimated using MetaCore.

Table S6, related to Figure 1.

Set of metabolites differentially regulated by LPS with and without PKM2 activation. Table represents processed data for metabolites in absolute scale. p-values were estimated using a two-tailed t-test (assuming equal variance) and metabolites with fold-change > 10% were selected. Metabolites scoring with nominal p-value < 0.05 across any of the four comparisons were selected.

EXPERIMENTAL PROCEDURES

Reagents.

TEPP-46 and DASA-58 were synthesized in accordance with published methods (Boxer et al., 2010; Jiang et al., 2010). LPS used *in vitro* and *in vivo* studies were E. coli, serotype EH100 (Alexis), and 055:B5 (Sigma-Aldrich), respectively. 4-hydroxytamoxifen (H7904) was purchased from Sigma. Antibodies used were anti-PKM2 (3198), antiphospho-PKM2 (Tyr105)(Chen et al., 2014; Iqbal et al., 2013), β-actin (4267) (all from Cell Signaling Technologies), anti-IL-1β (R&D, AF401-NA), anti-HIF-1α (Novus, NB100-449), and anti-PKM1 (Novus, NBP2-14833). DASA-58 was predominantly used for the *in vitro* studies. TEPP-46, but not DASA-58, is pharmacokinetically validated *in vivo(Anastasiou et al., 2012)*. Therefore TEPP-46 efficacy was verified using key *in vitro* assays. Dimethyl sulfoxide (DMSO) was used as vehicle control for the PKM2 activators in all *in vitro* assays.

Mice and cell culture

BMDMs and peritoneal cells were isolated from C57BL/6 mice from Harlan UK. All experiments were carried out with prior ethical approval from Trinity College Dublin Animal Research Ethics Committee. Bone marrow from C57BL/6 mice was differentiated into macrophages for 7

days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin (P/S) and 20% L929 mouse fibroblast supernatant. Peritoneal cells were harvested by flushing the peritoneal cavity of a mouse with 6 ml sterile PBS. The macrophages from this lavage were allowed to adhere to tissue culture plates for 30 minutes in Dulbecco's Modified Eagles media supplemented with 10% FCS, after which the medium and non-adhered cells were removed and fresh medium added. 1 hour prior to relevant treatment and for the duration of each experiment the cells were incubated in Dulbecco's Modified Eagles media containing 1% fetal calf serum, and 1% L929 mouse fibroblast supernatant. Cells were used at a concentration of $1x10^6$ cells per ml unless otherwise stated. Each 'n' represents bone marrow/peritoneal macrophages from individual mice.

BMDM cells isolated from mice carrying a $PKM2^{fl/fl}$ allele allowed for Cre-recombinase-mediated deletion of the PKM2 specific *Pkm* exon 10, selectively disrupting PKM2 but not PKM1 expression. LoxP sites flanking PKM2 specific exon 10 were introduced into the *Pkm* locus of mouse embryonic stem cells using homologous recombination using standard protocols as previously described(Israelsen et al., 2013). Resulting $Pkm2^{fl/fl}$ mice were crossed with mice carrying Tamoxifen-inducible Cre recombinase (Cre-ER) as described by Israelsen et al

(Israelsen et al., 2013). Ablation of PKM2 was achieved by adding 600nM 4-hydroxytamoxifen (Sigma H7904) on day 4 of macrophage differentiation, until 24 hours prior to experiment.

Western blotting

SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis was carried out as previously described (Fitzgerald et al., 2001). Western blots were developed using autoradiographic film, or electronically using a Gel Doc™ EZ System gel imaging system alternatively.

RNA Isolation and Gene Expression.

Total RNA was isolated using the RNeasy mini kit (Qiagen) and transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For mRNA, 18s ribosomal RNA or RPS18 gene were used as housekeeping controls. Target gene expression using taqman probes or normal primers was normalized to the housekeeping gene and relative quantitation values were calculated using the 2(-Delta Delta C(T)) method(Livak and Schmittgen, 2001).

Nuclear and cytosolic fractionation.

Nuclear Extract Kit (Active Motif, 40010) was used according to manufacturers recommendations in order to separate nuclear and cytosolic fractions. 20 µl of each fraction was separated by SDS-Page prior to western blotting.

Crosslinking

For crosslinking of PKM2, bone marrow derived macrophages or RAW 264.7 macrophages were treated with 50µM TEPP-46, 20µM DASA-58 or DMSO. After 24 hours, cells were washed three times with PBS (pH8) and crosslinking was performed using 500µM disuccinimidyl suberate (Thermo Scientific Pierce) in PBS (pH 8.0) for 30 minutes at ambient temperature. The reaction was quenched by addition of Tris-HCl pH 7.5 to 100mM final. Lysates were analysed by 8% SDS-PAGE and Western Blot.

Size exclusion chromatography

Mouse leukaemic monocyte RAW 264.7 macrophage cells were cultured in 1% FCS 24 hours prior to the experiment. The cells were pretreated with 10µM TEPP-58 or DMSO for one hour, followed by stimulation with 100ng/ml LPS. After 24 hours cells were lysed hypotonically. 2-3 mg of resulting protein was loaded on a Superdex 200 10/300GL column (GE Healthcare) and eluted with 50mM sodium phosphate, 150mM

NaCl, pH7.5. 250μL fractions were taken and analysed by SDS-PAGE and Western Blot.

ELISA

$1x10^6$ BMDMs per ml were pretreated for 1 hour with DASA-58 as indicated, followed by 100ng/ml LPS for 24 hours. Supernatants were collected and ELISAs were performed according to manufacturers' instructions. Kits used were IL-1β (Quantikine, MLB00C), IL-1β (DuoSet DY401), IL-6 (DuoSet DY406), IL-10 (Duoset DY417), IL-10 (Quantikine M1000B) and TNFα (Duoset DY410) (all from R&D). Results presented as mean±SEM. Two-tailed t-tests were carried out to measure significance of expression of cytokines with different treatments.

Microarray Profiling

Quantification of RNA concentration and purity was measured using the NanoDrop spectrophotometer (Thermo Scientific). A total of 500ng RNA was amplified and labeled using the Illumina TotalPrep RNA Amplification kit (Ambion) as per the manufacturer's instructions. A total of 1.5μg of labelled cRNA was then prepared for hybridization to the Illumina Mouse WG-6 chip. The Illumina microarray was performed by Partners HealthCare Center for Personalized Genetic Medicine (PCPGM)

Core (Boston, USA). The BeadChips were scanned according to the protocol described in the Illumina Whole Genome Gene Expression for BeadStation Manual v3.2, Revision A using scanning software BeadScan 3.5.31. The GenomeStudio® Data Analysis Software (Illumina) was used for data collection. A GenomeStudio Probe-level Final Report was generated by combining the Sample Probe Profile and Control Probe Profile tables. The Final report comprising the full dataset was initially processed using the Bioconductor package lumi by employing a background correction estimate. Subsequently, signal intensities were VST transformed (variance-stabilizing transformation) and RSN normalized (robust spline normalization) using the Lumi package.

Statistical analysis of microarray data

Post-processing and statistical analysis of microarray data was carried out in MATLAB. Normalized data was first checked for correlation between replicates, which was found to be >0.96 on average within different treatment groups. Of the 45,281 probes on the chip, we removed probes without any gene assignments and further filtered them on the basis of having a variance, signal or entropy less than 10th percentile of the entire dataset. Remaining probes were analyzed for statistically significant differences (fold-change > 25%) between three groups using a two-tailed t-test assuming equal variance between two samples. The three groupings

were: a) LPS+DMSO vs. DMSO control, b) LPS+DASA vs. DASA control, c) LPS+DMSO vs. LPS+DASA. 107 probes (100 genes) were identified as statistically significant across the three groups. p-values were corrected for multiple hypothesis testing by Benjamini-Hochberg's method to control false discovery rate at 25%.

Co-Immunoprecipitation.

BMDMs treated with LPS for 24 hours were lysed as previously described (Fitzgerald et al., 2001). PKM2 immune complexes were precipitated using anti-PKM2 antibody precoupled to protein A/G PLUS agarose beads (Santa Cruz Biotechnology) for 2 h at 4 °C allowing precipitation of the immune complexes. For control reactions, non-specific Rabbit IgG antibody was used. Immunoprecipitated proteins were eluted by adding 5xLaemmlii sample buffer and resulting lysates were fractionated by SDS–PAGE and visualised by immunoblotting using anti-PKM2 or Hif-1α antibodies respectively. Lysates were also immunoblotted for PKM2 and Hif-1α to control for input.

Affinity Purification with Biotinylated Oligonucleotides

Oligonucleotides for the HIF1α binding site on the IL1β promoter were annealed 90 –95 °C for 3–5 min and then heat block was allowed to cool

to room temperature (forward, 5'BIO-ggt agg cac gta gat gca cac c-3'; reverse, 5'ggt gtg cat cta cgt gcc tac c-3').

Primary BMDM cells were seeded at 0.5×10^6 cell/ml and treated with DASA (50µM) or TEPP (100µM) for one hour prior to LPS treatment (24 hours, 100ng/ml). Oligonuceotide pulldown was performed as previously described ((Doyle et al., 2013; Quinn et al., 2014)). PKM2 and HIF1α protein levels were detected by Western blotting.

Chromatin Immunoprecipitation

Primary BMDMs were plated at 0.5×10^6cell/ml in DMEM, treated with DASA (50µM) or TEPP (100µM) for one hour prior to LPS treatment (100ng/ml) for 24 h. ChIP was performed as previously described ((Quinn et al., 2014; Tannahill et al., 2013)). Lysates (700µl) were incubated with primary antibodies; Anti-HIF-1-alpha antibody – (Abcam ChIP Grade, ab2185), negative control anti-IgG (Sigma, I5006), and positive control Pol II Antibody (Santa Cruz Biotechnologies (N20) sc-899). For Sequential ChIP, the pulled down HIF1α sample was reprobed for binding of PKM2. The sample was resuspended in elution buffer (100µl TrisHCL PH7.5; 10nM EDTA, 1% SDS) for 10 min at 68°C, centrifuged at 2000rpm for 2min and 10µl of this sample was removed as 1% PKM2 input. The remaining sample was diluted in 600µl Buffer TE

and probed for PKM2 binding (2 hours incubation, 30µl pre-blocked Protein A/G beads, 30µl PKM2 D78AXP antibody (4053)). Quantitative RT-PCR was carried out using primers for either the IL1β promoter consensus HIF1α binding site (-408), or the β-actin promoter as a positive control for Pol II binding (data not shown). Data are calculated as percent of input, and represented by one experiment expressed as fold binding (n=3, ±SD).

Extracellular acidification.

XF24 Extracellular Flux analyzer (Seahorse Biosciences) was used to determine the bioenergetic profile of LPS stimulated BMDMs. BMDMs were plated at 200,000 cells/well in XF24 plates overnight before pretreatment with 50µM TEPP-46 or DASA-58, followed by stimulation with LPS for 24 hours. OCR was assessed in glucose containing media (Seahorse Biosciences). Results were normalized to cell number and are represented as mean ± SEM.

Measurement of metabolites by LC-MS

Endogenous metabolic profiles for polar metabolites was obtained using liquid chromatography tandem mass spectrometry (LC-MS) method. Analyses of polar metabolites using 32 negative ion mode MS were performed using an ACQUITY UPLC (Waters Corp.; Milford, MA)

coupled to a 5500 QTRAP triple quadrupole mass spectrometer (AB SCIEX; Framingham, MA). MultiQuant software (version 1.2; AB SCIEX; Framingham, MA) was used to process all raw LC-MS data and integrate chromatographic peaks. The processed data were manually reviewed for quality of integration and compared against known standards to confirm metabolite identities. Performance of the machine was evaluated using external reference samples, however internal standards were not used at the time of sample preparation. Therefore the processed mass-spec data reported represent relative abundance as quantified by integration of LC-MS peaks, which are correlated to absolute concentrations of metabolites.

Statistical analysis of metabolomics data

Processed data was log transformed (base 2) and first checked for correlation between replicates, which was found to be >0.94 on average within different treatment groups. To identify significantly different metabolites, LPS+DMSO and LPS+DASA-58 treated samples were first compared to their respective controls. The fold-change induced in these two different treatments, as well as the absolute metabolite levels, were also directly compared. In each instance, significance was assessed using a two-tailed t-test (assuming equal variance) and metabolites with fold-change > 10% were selected. Metabolites scoring with nominal p-value <

0.05 across any of the four comparisons were selected for further analysis.

Mycobacterium tuberculosis assays

Mycobacterium tuberculosis H37Ra was grown to log phase at 37°C in 5% CO_2 in Middlebrook 7H9 broth (Difco) and made up in endotoxin-free water. Prior to use a single cell suspension of *Mtb* H37Ra was obtained and multiplicity of infection (MOI) was determined using Auramine O staining to assess intracellular bacterial load by fluorescence microscopy. Infections were carried out at an MOI of 5 bacteria/macrophage. Bone marrow derived macrophages were prepared as described previously. Prior to *Mtb* infection media was changed and cells were washed to remove antibiotics. Serum was reduced to 0.5% prior to treatment with TEPP-46. At defined times cells were lysed in 0.1% Triton, serial dilutions prepared and plated out on Middlebrook agar and incubated at 37°C in 5% CO_2 for 14 days. For RNA analysis, cells were lysed in Trizol Reagent (Invitrogen) and RNA extracted according to manufacturers instructions. ELISA results depicted as means +/- SEMs of results from triplicate wells for one representative experiment n=2. *P<0.05, **P<0.01, ***P<0.001 (two-way analysis of variance with post-hoc Bonferroni correction).

In vitro uptake assay

BMDM pre-treated with TEPP-46 were infected with *Salmonella typhimurium* strain UK-1 at an MOI of 10 bacteria/cell. 15 min post-infection extracellular bacteria were removed and cells were washed with media containing gentamycin at 100 μg/ml. Cells were then incubated in media supplemented with TEPP-46 for 20 min (to confirm equal uptake of bacteria) or 4h post-infection to measure bacterial viability within each macrophage. Bacterial levels were assessed by lysing cells in cold H_20 and plating out serial dilutions on LB agar and quantified as CFU/ml. Data shown represents mean ± SEM for three independent infections.

Endotoxin-induced and *S. typhimurium in vivo* model of sepsis.

Mice were treated ± TEPP-46 (50mg/kg) or vehicle (20% 2-Hydroxypropyl-β-cyclodextrin) i.p. for 60 min. Sepsis was induced by injecting 15mg/kg of LPS i.p., alternatively mice were infected with $1x10^6$ CFU *S. typhimurium* i.p. Mice were culled after 2 hours. Serum was isolated from whole blood which was collected, left to coagulate at room temperature, and spun at 1000rpm for 10 min at 4°C. Peritoneal cells were harvested by flushing out the peritoneal cavity of a mouse with 2ml sterile PBS. This lavage typically yields 30% macrophages equivalent to $0.5-1x10^6$ macrophages per mouse(Avijit Ray, 2010; Zhang

et al., 2008). Whole cell population was then spun down and the pellets used for protein analysis.

To measure bacterial dissemination mice were intraperitoneally infected with $1x10^6$ CFU of *S. Thyphimurium* and sacrificed 24h post infection. Livers and spleens were extracted and Log CFU/organ was determined.

All experiments had previous ethical approval from Trinity College Research Ethics Committee.

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