

A multi-omics analysis reveals metabolic reprogramming in THP-1 cells upon treatment with the contact allergen DNCB

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ABSTRACT

Dendritic cell (DC) activation by contact allergens is one of the key steps in the development of allergic contact dermatitis (ACD). Recent evidence suggests that metabolic reprogramming is a prerequisite for the activation of DCs, macrophages and monocytes. Therefore, we used an integrated approach by combining proteomics and metabolomics to investigate the metabolism of human THP-1 cells in response to the strong contact allergen, 2,4-dinitrochlorobenzene (DNCB). Cells were treated with 5, 10 and 20 μ M DNCB for 4, 8, and 24 h, respectively. Using a targeted metabolomics approach, we quantified levels of 188 endogenous metabolites, among them phospholipids, acylcarnitines, amino acids and hexoses. In addition, proteomic changes were analyzed using an untargeted quantitative approach based on stable isotope labeling with amino acids in cell culture (SILAC). We detected several alterations in the metabolome and consistently in the proteome indicating metabolic reprogramming of THP-1 cells by DNCB. In particular, we found an increase in phospholipids that was accompanied by an up-regulation of fatty acid synthase (FAS), a key enzyme in lipid synthesis.

1. Introduction

Allergic contact dermatitis (ACD) is a widespread T cell-mediated hypersensitivity reaction of the skin which can be regarded as the most abundant immunotoxic disease affecting the general population (Kimber et al., 2002). Contact allergens can be contained in jewelry, fragrances, hair dyes and cosmetics. Furthermore, many materials and commodities used in occupational settings contribute to exposure with contact allergens (Peiser et al., 2012).

The development of ACD represents a complex multi-step process. The involvement of an adaptive immune response discriminates ACD against immediately induced contact dermatitis by irritants (Tan et al.,

2014). As professional antigen-presenting cells (APCs) dendritic cells (DCs) are needed for the priming of naïve T cells in the local lymph nodes during the sensitization phase and for the activation of memory T cells at the sites of further contact allergen exposure during the elicitation phase, leading to the occurrence of contact eczema (Martin et al., 2011).

DCs become activated by exogenous stimuli binding to pattern recognition receptors (PRRs) including toll-like receptors (TLRs) that recognize so-called pathogen-associated molecular patterns (PAMPs). After activation DCs undergo substantial phenotypical alterations (Banchereau and Steinman, 1998). The field of immunometabolism is currently gaining attention as knowledge is increasing on how

Abbreviations: AA, amino acid; AC, acylcarnitine; ACD, allergic contact dermatitis; ACN, acetonitrile; APC, antigen-presenting cell; BA, biogenic amine; BMDC, bone-marrow derived DC; CA, cinnamaldehyde; CD, cluster of differentiation; DC, dendritic cell; CPT1, carnitine palmitoyl transferase I; DMSO, dimethyl sulfoxide; DNCB, 2,4-dinitrochlorobenzene; DTT, dithiothreitol; ETC, electron transport chain; ESI, electrospray ionization; EURL ECVAM, European Union Reference Laboratory for alternatives to animal testing; ENPL, endoplasmic; FAO, fatty acid oxidation; FAS, fatty acid synthase; FASP, filter-aided sample preparation; FBS, fetal bovine serum; FIA, flow injection analysis; GARD, Genomic Allergen Rapid Detection; H1, sum of hexoses; h-CLAT, human cell line activation test; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HNRH1, heterogeneous nuclear ribonucleoprotein H; iNOS, inducible nitric oxide synthase; KPYM, Pyruvate kinase isozymes M1/M2; LC, liquid chromatography; LPC, lysophosphatidylcholine; MALDI, Matrix-assisted laser desorption/ionization; MANOVA, multivariate analysis of variance; MDHM, mitochondrial malate dehydrogenase; MPS, mononuclear phagocyte system; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; NAD + /NADH, Nicotinamide adenine dinucleotide; NO, nitric oxide; OECD, Organisation for Economic Co-operation and Development; OXPHOS, oxidative phosphorylation; PAMP, pathogen-associated molecular pattern; PBS, phosphate-buffered saline; PC, phosphatidylcholines; PCA, principal component analysis; PDIA1, protein disulfide-isomerase; PRR, pattern recognition receptor; SDS, sodium dodecyl sulfate; RT, room temperature; SILAC, stable isotope labeling with amino acids in cell culture; SM, sphingomyelin; RLA2, 60S acidic ribosomal protein P2; TCA, tricarboxylic acid; TOF, time of flight; TLR, toll-like receptor; UPR, unfolded protein response

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metabolic states co-decide about the fate and activity of immune cells like monocytes, macrophages, and DCs (O'Neill et al., 2016). Together, these cell types constitute the mononuclear phagocyte system (MPS) (Guilliams et al., 2014). Resulting from MPS cell activation, the alterations in the interplay of metabolic pathways such as glycolysis, tricarboxylic acid (TCA) cycle and amino acid metabolism is referred to as metabolic reprogramming. It seems that for cells of the MPS TLR-dependent induction of glycolysis to fuel lipid synthesis via intermediates of the TCA cycle represents a common mechanism (Arts et al., 2016). Accordingly, metabolic reprogramming could be detected in primary monocytes (Lachmandas et al., 2016), macrophages (Jha et al., 2015) and DCs (Rehman et al., 2013) derived from human donors and mice. In DCs, the TLR-mediated metabolic transition to glycolysis represents an essential step for DC maturation and function (Krawczyk et al., 2010). Consistent with this, we recently provided evidence for metabolic reprogramming in DCs activated by selected electrophilically reactive contact allergens (Mussotter et al., 2016).

Mechanisms of DC activation have proven a promising target for the development of in vitro testing methods (Roggen, 2014). One prominent example is the human Cell Line Activation Test (h-CLAT) which uses the monocytic THP-1 cell line as a surrogate for DCs and measures the induction of the co-stimulatory molecule CD86 and the cellular adhesion molecule CD54 after treatment with potentially allergenic substances (EURL ECVAM, 2015; OECD, 2016). However, alternative testing methods for contact allergens currently still suffer from limitations. In particular, potency assessment and the prediction of contact allergens that need non-enzymatic or metabolic activation (i.e., pre- and prohaptens) remain challenging. Thus, existing testing strategies need to be improved, for instance by taking advantage of new biomarkers (Wong et al., 2015). To that end omics technologies can be used that increasingly became valuable tools in other areas of toxicology (Hartung, 2009). In recent years, transcriptomics, proteomics and metabolomics have been applied effectively in the research on ACD (Jakob et al., 2017; Johansson et al., 2013; Zwicker et al., 2016).

In our study, we addressed the hypothesis that metabolic reprogramming is a central event during THP-1 activation by a contact allergen. To that end, we employed a multi-omics approach to investigate the activation of THP-1 cells by the contact allergen 2,4-dinitrochlorobenzene (DNCB). THP-1 cells were exposed to three concentrations of the contact allergens DNCB (5, 10 and 20 μ M) for 4, 8 and 24 h, respectively. We used a targeted metabolomics approach to quantify alterations of lipids, amines and hexoses based on flow injection analysis (FIA) or liquid chromatography-tandem (LC) mass spectrometry (MS/MS). Additionally, we applied a complementary SILAC based proteomics approach to assess alterations on the proteome level.

2. Materials and methods

2.1. Cell line and routine cell culture

THP-1 cells were obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were grown in RPMI 1640 media supplemented with 10% (v/v) fetal bovine serum (FBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 10 mM), L-glutamine (2 mM), sodium pyruvate (1 mM) and penicillin/streptomycin (100 U/ml). FBS was obtained from Biochrom (Berlin, Germany). Medium and all other additives were purchased from PAN-Biotec (Aidenbach, Germany). For routine cell culture, cells were passaged every 3 to 4 days and seeded at 1×10^5 cells per ml in T75 flasks. Cells were incubated in a standard incubator at 37 °C, 5% CO₂ and 95% humidity.

For SILAC experiments, RPMI media were supplemented with 10% (v/v) dialyzed FBS obtained from Biochrom. Carbon-12 (¹²C) and Carbon-13 (¹³C) labeled L-lysine and L-arginine were obtained from Silantes (Munich, Germany) and added in the same concentrations as in standard RPMI medium composition.

2.2. Assessment of cytotoxicity

MTT assay was performed in regular and in SILAC RPMI medium to assess possible cytotoxicity. For this purpose, THP-1 cells were seeded in 96-well plates (1×10^5 cells per well at 1×10^6 per ml). Cells were incubated for 24 h with ascending concentrations of DNCB (both obtained from Sigma Aldrich, Munich, Germany) in three replicates per treatment. The analysis was repeated in three biological replicates in total. DNCB was first dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich, Munich, Germany) and then diluted into cell culture medium. The final DMSO concentration was kept at 0.2%. Untreated and vehicle controls were included. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added 24 h after treatment at 0.5 mg/ml final concentration and incubated for 2 h at 37 °C. Formazan crystals were dissolved in DMSO. Absorption at 595 nm was measured in a GENios microplate reader (Tecan, Männedorf, Switzerland).

2.3. Assessment of cell activation

Expression of cell surface markers CD86 and CD54 was observed by flow cytometry. THP-1 were treated as stated above for 24 h, then harvested by centrifugation (200 $\times$ g, 5 min, room temperature) and washed twice with PBS. The following antibodies and corresponding isotype controls were used (all obtained from Miltenyi Biotec, Bergisch Gladbach, Germany): CD86-FITC, human (clone: FM95); CD54-APC, human (clone: REA266). For discrimination of dead and living cells, the LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit was used (Life Technologies, Darmstadt, Germany). Staining was performed as stated by the manufacturers. Flow cytometry was performed using a FACSaria™ III (BD Biosciences, Franklin Lakes, NJ, USA). 10,000 events per sample were recorded. FITC chromophores were excited at 488 nm and detected using a 530/30 nm filter. APC chromophores and Live-Dead stain were excited at 633 and detected using a 660/20 nm filter and 780/60 nm filter, respectively. Analysis of flow cytometry data was performed using FlowJo (V10; FlowJo, LLC, Ashland, Oregon, USA).

2.4. Cell culture for metabolomics experiments

THP-1 cells were seeded and directly treated in T25 flasks in standard cell culture medium at 1×10^6 cells per ml. Cells were harvested by centrifugation 4, 8 and 24 h after treatment (200 $\times$ g, 3 min, 4 °C). In total five biological replicates per treatment were analyzed. Cell pellets were washed twice with ice-cold ammonium formate buffer solution (155 mM, pH 7.4). Supernatants were removed after centrifugation (200 $\times$ g, 3 min, 4 °C). Cell pellets were weighed, frozen in liquid nitrogen and stored at –80 °C. For extraction of metabolites, frozen cell pellets were dissolved by 3 freeze and thaw cycles (5 min freezing in liquid nitrogen; 5 min thawing at room temperature; 1 min ultrasonication in a water bath) in 0.5 ml UHPLC grade ice-cold methanol (J.T. Baker, Deventer, Holland). Supernatants were cleared by centrifugation (12,000 $\times$ g, 30 min, 4 °C) and analyzed immediately using the Biocrates AbsoluteIDQ® p180 Kit (Biocrates, Innsbruck, Austria).

2.5. Targeted metabolomics approach

In total, 188 metabolites were analyzed, including amino acids (AAs) and biogenic amines (BAs), acylcarnitines (ACs), the sum of hexoses (90–95% glucose; H1), sphingomyelins (SMs), as well as lysophosphatidylcholines (LPCs) and phosphatidylcholines (PCs). Lipid chain bonds to the glycerol core of LPCs and PCs were specified for ester (“a”) or ether (“e”) bonds (“a” = acyl, “aa” = diacyl, and “ae” = acyl-alkyl). Lipid side chains were denoted with “Cx:y” (“x” = number of carbons; “y” = number of double bonds). For detailed information on the metabolites analyzed and the method of metabolite quantification please refer to (Jourdan et al., 2012).

The AbsoluteIDQ® p180 Kit was employed as described by the manufacturer. In short, samples were prepared as follows. Isotope labeled standard mixtures were pipetted onto filter paper inserts before addition of cell extracts. Samples were dried with nitrogen using an EVA LS evaporator (VLM, Bielefeld, Germany). AAs were derivatized with phenylisothiocyanate (Sigma Aldrich, Munich, Germany). Metabolites were extracted with 5 mM ammonium acetate in methanol and centrifuged subsequently. Eluates were diluted with solvent depending on the following MS method. For LC-MS/MS analysis, eluates were diluted with 40% methanol in water (1:10, v/v). For flow injection analysis-tandem mass spectrometry (FIA-MS/MS) analysis, Biocrates' kit running solvent was used (1:50, v/v). Extracts were analyzed by electrospray ionization-tandem mass spectrometry (ESI-MS/MS) using an Agilent 1200 Series HPLC (Agilent, Waldbronn, Germany) coupled to a QTRAP 5500 triple quadrupole mass spectrometer (AB Sciex, Darmstadt, Germany) controlled by the Analyst 1.6 software package (AB Sciex, Darmstadt, Germany). AAs and BAs were analyzed using LC-MS/MS in positive ion mode after metabolite separation on a Zorbax Eclipse XDB-C18 column (3 × 100 mm, 3.5 µm; Agilent, Waldbronn, Germany). The FIA-MS/MS procedure was used for the quantification of ACs, PCaas, PCaes, LPCs, SMs and H1 in positive and negative ion mode. The MetIDQ™ software package was used for compound identification and quantification by multiple reaction monitoring (MRM). Metabolite concentrations were calculated in micromolar units.

2.6. Statistical analysis of metabolomics data

Only metabolites with concentrations above the respective limits of detection in each sample were included for statistical analysis, which restricted the analysis to 123 metabolites. Each sample was normalized to the pellet weight and standardized to the mean of the untreated control of the respective time point, which was set to 100%.

Heatmaps of log₂-transformed relative metabolite levels were generated using the function heatmap() from the R package stats. Hierarchical clustering was performed using Pearson correlations as similarity measure and the average linkage method to calculate the distance between clusters.

For multivariate analysis of variance (MANOVA) metabolomics data were processed using IBM SPSS Statistics (V21; IBM Analytics, Ehningen, Germany) including Z-score normalization and Bonferroni correction (Potratz et al., 2016). The data set was split by time points. Metabolites with a significant group difference ($p < 0.001$) in at least one exposure group compared to untreated or vehicle controls were regarded significantly changed. Such metabolites were selected for principal component analysis (PCA). PCA was conducted using SIMCA (V14; MKS Data Analytics Solutions, Umeå, Sweden).

Significance of up- or down-regulation after DNCB treatment was assessed using the Jonckheere-Terpstra test for trends comparing cells treated with 5 µM DNCB, 10 µM DNCB and 20 µM DNCB as well as untreated controls and vehicle controls. The analysis of log₂-transformed relative metabolite levels was performed separately for each of the time points 4 h, 8 h and 24 h, respectively. Multiple testing correction for the 123 analyzed metabolites was performed using the Bonferroni method for control of the family-wise error rate. p -Values < 0.05 were considered significant.

2.7. Cell culture for proteomics experiments

THP-1 cells were grown in SILAC media for 7 days before treatments yielding incorporation rates of $> 95\%$. Controls were grown in ¹³C medium, treatment groups in ¹²C medium. THP-1 cells were seeded and directly treated in T25 flasks in standard cell culture medium at 1×10^6 cells per ml. Cells were harvested by centrifugation 4 h, 8 h and 24 h after treatment (200 × g, 5 min, 4 °C). In total 3 biological replicates per treatment were analyzed. Cell pellets were washed twice

in ice-cold PBS and counted using a CASY® Modell TTC cell counter (Roche, Basel, Switzerland). Control and treatment cell suspensions were mixed 1:1.

2.8. Proteomics analyses

Sample preparation followed the filter-aided sample preparation (FASP) protocol (Wiśniewski et al., 2009). In short, cells were lysed in 4% (w/v) sodium dodecyl sulfate (SDS), 100 mM Tris/HCl pH 7.6, 0.1 M dithiothreitol (DTT) at 95 °C for 5 min. DNA was sheared by sonication and lysates clarified by centrifugation (16,000 × g; 5 min; RT). Lysates were mixed with UA buffer (8 M urea in 0.1 M Tris/HCl, pH 8.5) on Microcon-30 kDa centrifugal filter units from Merck Millipore (Darmstadt, Germany). Proteins were loaded on the filter unit by centrifugation (14,000 × g; 15 min) and washed with UA buffer. Iodoacetamide solution (0.05 M iodoacetamide in UA) was incubated for 20 min at room temperature. Filter units containing alkylated proteins were washed three times with UA buffer and three times with ABC buffer (0.05 M NH₄HCO₃). Trypsin (2 µg per sample) was added. Filter units were incubated overnight at 37 °C. Tryptic peptides were eluted into new collection tubes adding 0.5 M NaCl. Tryptic reaction was stopped by adding 0.1% trifluoroacetic acid (TFA; ratio 1:25). Peptide yields were monitored using the Protein A280 method of a NanoDrop 1000 Spectrophotometer v3.7 (Thermo Fisher Scientific, Wilmington, DE).

Peptides were purified using C18 ZipTip® pipette tips (Merck Millipore; Darmstadt, Germany). In short, ZipTip® pipette tips were prepared by sequential washing with acetonitrile (ACN), 7:3 ACN/0.1% TFA and 0.1% TFA. Subsequently, peptides were loaded on ZipTip® pipette tips and washed with 0.1% TFA. Peptides were eluted with 7:3 ACN/0.1% TFA.

Purified peptides were separated using a Dionex UltiMate 3000 RSLCnano system equipped with an Acclaim® PepMap100 C18, 3 µm, 100 Å, Separation Bed 75 µm i.d. × 2 cm trap column and an Acclaim® PepMap™ RSLC C18, 2 µm, 100 Å, 75 µm i.d. × 50 cm separation column. Sample loading was performed with loading solvent (2% ACN, 0.05% TFA) at a flow rate of 7 µl/min. Gradient elution was performed with mobile phases A (0.1% TFA) and B (80% ACN, 0.08% TFA) at a flow rate of 300 nl/min. The gradient profile was as follows: 4% B for 5 min; 4–10% B for 1 min; 10–50% B for 164 min; 50–95% B for 1 min; 95% B for 20 min; 95–4% B for 1 min; 4% B for 20 min. 15 s fractions were collected and mixed with matrix solution (90% ACN; 4.5% of a solution saturated with α-cyano-4-hydroxycinnamic acid; 0.1% TFA; 1 mM ammonium dihydrogen phosphate). 420 nl droplets were spotted on MTP AnchorChip™ Targets BC (Bruker; Billerica, MA) using a Probot™ microfraction collector. For calibration, Bruker's "Peptide Calibration Standard II" was diluted 1:200 in calibrant matrix solution (80% ACN; 4.5% of a solution saturated with α-cyano-4-hydroxycinnamic acid; 0.1% TFA; 1 mM ammonium dihydrogen phosphate) and spotted manually on calibrant spots.

Fractions were analyzed using an ultrafleXtreme™ MALDI-TOF-TOF mass spectrometer (Bruker Daltonik; Bremen, Germany) in positive ionization and reflector mode. MS data were obtained within a mass range of 700–3500 Da. Precursors with a signal-to-noise ratio of > 10 were selected for MS/MS analysis.

Bruker's ProteinScape™ 4.0 platform was used for protein identification and quantitation. Protein searches were performed using the MASCOT Daemon against the SwissProt database. Taxonomy was set to *Homo sapiens* (human). Enzyme was set to trypsin. Search parameters allowed one missed cleavage. Carbamidomethyl residues (Cys), oxidation (Met) and label:¹³C(6) (Lys, Arg) were set as variable as variable modifications. Peptide tolerance was set to ± 50 ppm. MS/MS tolerance was set ± 0.8 Da. Peptide decoy and percolator functions were used, false discovery rate (FDR) was adjusted to 1.0%, significance threshold was set to $p < 0.05$. Mascot's ion score threshold for significant peptide identification was set to 15.0. At least two significant

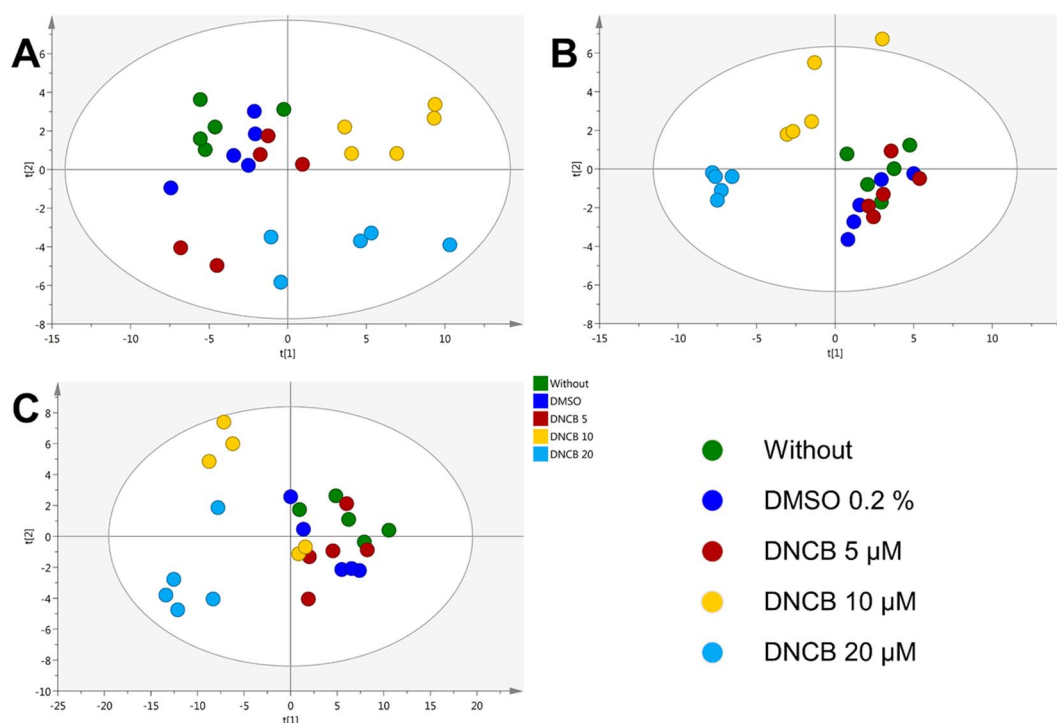


Fig. 1. Principal component analysis (PCA) of metabolomics data. Metabolites that were significantly affected by one of the treatments, i.e. those with a p -value ≤ 0.001 according to a multivariate analysis of variance (MANOVA) using IBM SPSS Statistics (V21), were subjected to PCA performed with SIMCA-P+ (V14). PCA was performed for each time point individually with 4 h depicted in A; 8 h in B; 24 h in C. Abbreviations: DMSO, dimethyl sulfoxide; DNCB, 2,4-dinitrochlorobenzene; PLS-DA, partial least squares discriminant analysis; WO, without treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

peptide IDs were needed for reliable protein identification. Quantitation was performed with Bruker's WARP-LC software and the implemented quantitation function of ProteinScape™ using the peptide median for protein ratio calculation and interquartile range for outlier detection and the normalization function. Outlier detection was surveyed manually.

2.9. Statistical analysis of proteomics data

Proteins were regarded as significantly regulated when reliably identified in all 3 replicates as well as up- or down-regulated by > 1.3 (standard deviation (SD) of < 0.1). Heatmaps of log2-transformed relative protein levels were generated using the function heatmap() from the R package stats. For proteins that could be detected in at least 24 of the 27 measured samples hierarchical clustering was performed using Pearson correlations as similarity measure and the average linkage method to calculate the distance between clusters.

3. Results

3.1. Dose selection for omics studies and monitoring of THP-1 cell activation

To select appropriate doses of DNCB, the cytotoxicity was assessed using the MTT assay in regular and in SILAC RPMI medium (Fig. S1). For DNCB two clearly non-toxic doses, i.e. 5 μ M (cell viability comparable to controls), 10 μ M (90% cell viability compared to controls), and one slightly cytotoxic dose, i.e. 20 μ M (75% cell viability compared to controls) have been selected.

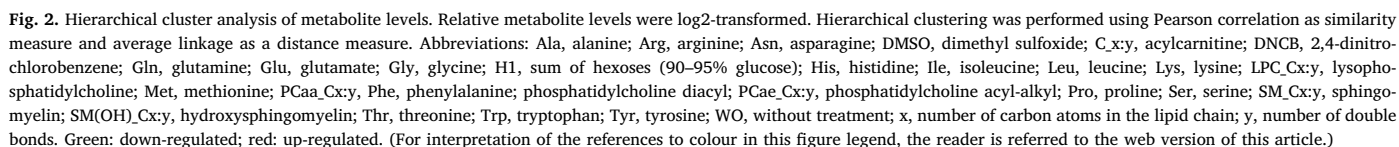
THP-1 cell activation was monitored by flow cytometry. Treatment with 10 μ M and 20 μ M DNCB resulted in significant up-regulation of the surface activation markers CD86 and CD54, whereas untreated cells, vehicle controls or 5 μ M DNCB treatment did not modulate surface marker expression (Fig. S2).

3.2. Metabolomics analysis

Metabolic changes during THP-1 activation were analyzed using a targeted metabolomics approach after 4, 8 and 24 h of treatment, respectively.

In a first step, we used MANOVA analyses to identify the number of significantly changed metabolites ($p \leq 0.001$). This resulted in 56, 22 and 64 metabolites after 4 h, 8 h and 24 h, respectively. Subsequent PCA analysis revealed a sound separation of samples treated with 20 μ M DNCB at all time points, whereas controls without treatment and vehicle controls clustered together. Samples treated with 10 μ M DNCB were completely separated after 4 h and 8 h. Despite a partial overlap with controls and 5 μ M DNCB, the 10 μ M DNCB sample group still separated reasonably well after 24 h. Cells treated with 5 μ M DNCB mainly clustered with the control groups (Fig. 1).

Hierarchical cluster analysis revealed grouping of different metabolite classes in prominent clusters (Fig. 2). In general, amino acids and short-chain acylcarnitines (clusters A and C) showed opposing trends in terms of treatment dependency compared to long-chain acylcarnitines, lysophosphatidylcholines, phosphatidylcholines and sphingomyelins (cluster B). Amino acids and short-chain acylcarnitine levels decreased following treatment with DNCB in a concentration- and time-dependent manner starting after 4 or 8 h (cluster 2) and revealing lowest levels after 24 h at DNCB 20 μ M (cluster 5). Conversely, long-chain acylcarnitines, lysophosphatidylcholines, phosphatidylcholines and to some extent sphingomyelins increased upon treatment with DNCB in a concentration- and time-dependent manner and peaked after 24 h at higher DNCB concentrations (clusters 4 and 5). Untreated samples, vehicle controls and 5 μ M DNCB did not induce frequent significant alterations of metabolite levels (cluster 3) although some exceptions were detected. For instance, a decrease of phosphatidylcholines and lysophosphatidylcholines led to clustering of samples mainly belonging to these treatment groups (cluster 1). In-differences in sample clustering of the complete data set were crucially dependent on time points. Time-point-separated hierarchical cluster analysis revealed more distinctive sample clustering (Fig. S3).



XY plots were generated to further assess the ability of selected significantly regulated metabolites to separate differentially treated samples (Fig. 4).

4. Discussion

as significantly regulated. However, only treatment with 20 μ M DNCB resulted in a significantly altered regulation of proteins. After 4 h, 60S acidic ribosomal protein P2 (RLA2) was up-regulated. After 24 h, significantly up-regulated proteins were fatty acid synthase (FAS), mitochondrial malate dehydrogenase (MDHM), protein disulfide-isomerase (PDIA1), heterogeneous nuclear ribonucleoprotein H (HNRH1) and endoplasmic reticulum protein (ENPL). Pyruvate kinase isozymes M1/M2 (KPYM) were down-regulated after 24 h. Complete protein expression data is given in the supplementary section (Table S2). Due to the low total number of regulated proteins, the attempt of hierarchical clustering of regulated proteins resulted in no significant clusters (Fig. S5). Selected protein profiles are depicted in Fig. 5. Complete protein expression data are given in the Supplementary section.

Table 1

Top metabolites after Jonckheere-Terpstra testing for each time point.

Rank	4 h	p-Value	8 h	p-Value	24 h	p-Value
1	C2	1.29E – 08	Spermine	5.65E – 08	PCae_C44:4	6.95E – 07
2	Taurine	3.52E – 08	C2	1.38E – 07	LPCa_C16:1	1.01E – 06
3	C4	4.06E – 06	Glu	1.05E – 05	LPCa_C18:1	1.45E – 06
4	PCaa_C36:0	2.54E – 05	Taurine	3.35E – 05	PCaa_C42:2	1.45E – 06
5	PCaa_C34:4	3.35E – 05	C14	3.35E – 05	PCae_C42:1	1.45E – 06
6	PCaa_C38:0	5.76E – 05	H1	7.47E – 05	PCaa_C28:1	2.06E – 06
7	PCae_C36:4	5.76E – 05	C3	9.64E – 05	Spermine	4.06E – 06
8	PCae_C38:6	5.76E – 05	C5	0.00012372	C14:1	4.06E – 06
9	PCae_C38:5	7.47E – 05	PCaa_C32:0	0.00012372	LPCa_C17:0	4.06E – 06
10	PCae_C36:5	9.64E – 05	C4	0.000157885	LPCa_C18:2	4.06E – 06

The Bonferroni method was used for multiple testing correction. p-Values < 0.05 were considered significant. For complete lists, refer to Table S1. Abbreviations: C_x:y, acylcarnitine; Glu, glutamate; H1, sum of hexoses (90–95% glucose); LPC_x:y, lysophosphatidylcholine; PCaa_{Cx}:y, phosphatidylcholine diacyl; PCae_{Cx}:y, phosphatidylcholine acyl-alkyl; x, number of carbon atoms in the lipid chain; y, number of double bonds.

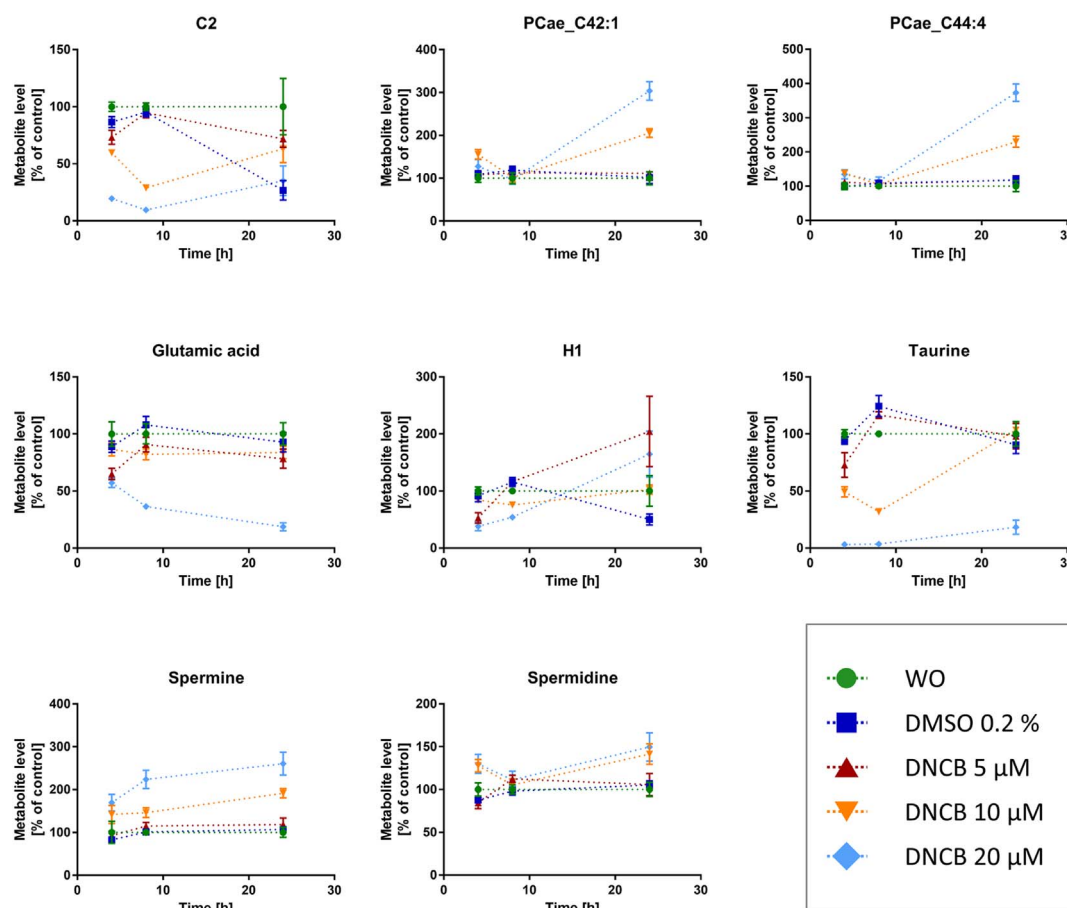


Fig. 3. Selected metabolite profiles. Values represent percentage of untreated controls, which were set as 100%. Error bars represent standard errors of the mean (SEM) (n = 5). Abbreviations: DMSO, dimethyl sulfoxide; DNCB, 2,4-dinitrochlorobenzene; H1, sum of hexoses (90–95% glucose); PCae_{Cx}:y, phosphatidylcholine acyl-alkyl; WO = without treatment; x, number of carbon atoms in the lipid chain; y, number of double bonds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

been reported for the activation of MPS cells in general (O'Neill and Pearce, 2016), but has yet not been investigated in the context of ACD. Our study provides evidence for metabolic reprogramming of THP-1 cells upon activation by the contact allergen DNCB. Based on the changes in the metabolome we could largely separate the 10 and 20 μM DNCB treatments from control treatments using PCA.

We could identify characteristic profiles of regulated metabolites after treatment with DNCB. For example, the biogenic amines spermine and taurine as well as the long-chain phosphatidylcholines PCae_C42:1 and PCae_C44:4 responded concentration- and/or time-dependently to DNCB treatment (Fig. 3 and Fig. S4). XY plotting of these metabolites

confirmed their capability to separate these samples (Fig. 4). Therefore, we conclude from the results of our study that especially long-chain phosphatidylcholines and the biogenic amines spermine and taurine may be regarded as valuable biomarker candidates for the detection of contact allergens in vitro. The potential of these biomarker candidates should be further assessed in future studies using additional contact allergens and irritants.

Compared to samples treated with 20 μM DNCB, alterations on the metabolome level were comparatively less pronounced following treatment with 10 μM DNCB. However, induction of the surface markers CD86 and CD54 confirmed THP-activation at both DNCB doses.

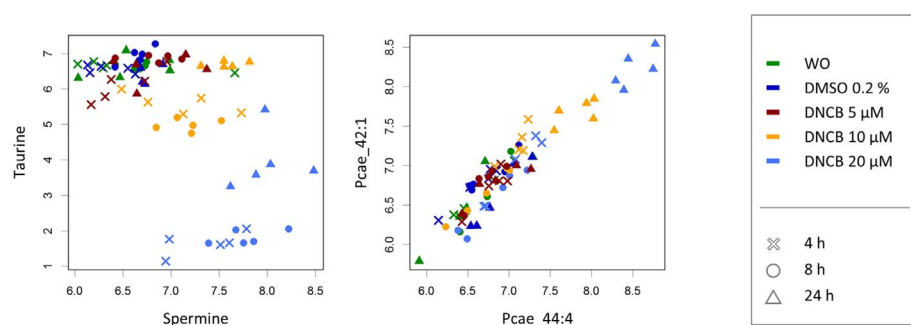


Fig. 4. XY plots of significantly regulated metabolites. Plots of all time points were generated using the R programming language. Values represent log₂-transformed metabolite levels. Abbreviations: DMSO, dimethyl sulfoxide; DNCB, 2,4-dinitrochlorobenzene; H1, sum of hexoses (90–95% glucose); PCae_Cx:y, phosphatidylcholine acyl-alkyl; WO = without treatment, x, number of carbon atoms in the lipid chain; y, number of double bonds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

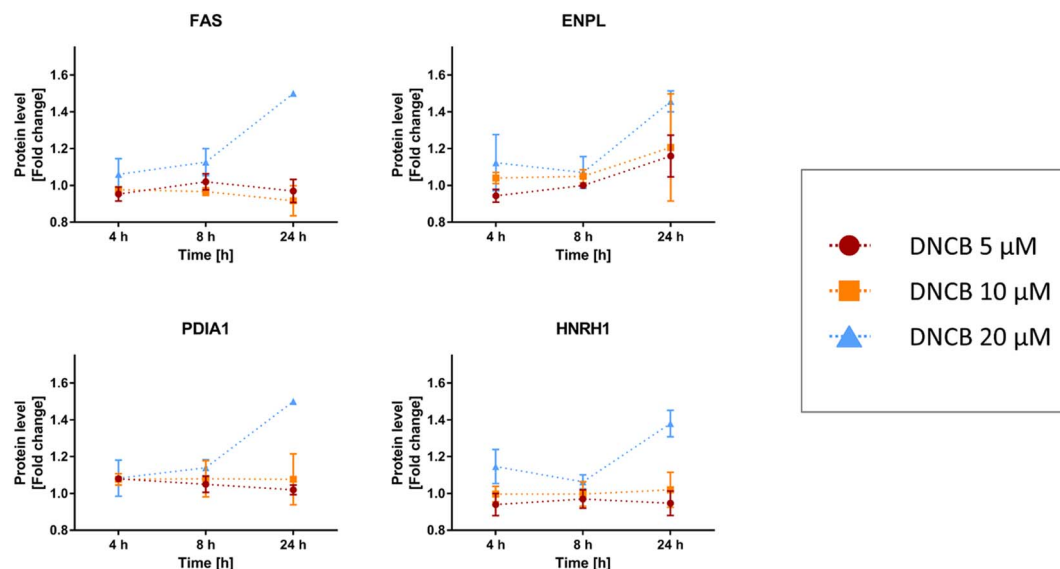


Fig. 5. Expression profiles of selected regulated proteins. Values represent fold changes compared to controls. Error bars represent standard deviations (SD). Proteins with a regulation > 1.3-fold (SD < 0.1) were regarded as significantly regulated. Abbreviations: DNCB, 2,4-dinitrochlorobenzene; ENPL, endoplasmic; FAS, fatty acid synthase; HNRH1, heterogeneous nuclear ribonucleoprotein H; PDIA1, protein disulfide isomerase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Additionally, phospholipid levels increased at the medium DNCB dose, indicating the capability of THP-1 cells to perform the necessary metabolic reactions. Significant changes on the proteome level only occurred after treatment with 20 μ M DNCB. Possibly, THP-1 cells in the steady state provide sufficient metabolic enzymes to elicit the less pronounced changes on the metabolome level after treatment with 10 μ M DNCB. Notably, weak responses of THP-1 cells on the proteome level after treatment with contact allergens was reported before (Zwicker et al., 2016). Sub-cellular fractionation of THP-1 cells, especially enrichment of plasma membranes or mitochondria, could represent a promising strategy to improve the detection of significantly regulated proteins involved in metabolic reprogramming and DC functioning.

Activation of THP-1 cells by treatment with DNCB had an impact on the overall intracellular hexose (H1) levels. Especially incubation with 20 μ M DNCB led to a significant drop in H1 levels after 4 h, which remained low 8 h after treatment but seemed to have recovered after 24 h. In contrast, no reduction of hexose levels after 4 h was observed in control samples. We only detected some reduction in hexose levels in vehicle controls after 24 h, an effect that might be due to the missing activation by an external stimulus. Reduced glucose levels are consistent with our previously published findings showing up-regulation of glycolytic enzymes in bone-marrow derived DCs (BMDCs) after 8 h of treatment with the contact allergens DNCB and cinnamaldehyde (CA) indicating an induction of glycolysis (Mussotter et al., 2016). During

MPS cell activation, immediate induction of glycolysis is an essential step in metabolic reprogramming. Rapidly increased glucose consumption is essential for fully functional activation especially of DCs and M1 macrophages (Lachmandas et al., 2016; O'Neill and Pearce, 2016). The switch to glycolysis is accompanied by the decline of oxidative phosphorylation (OXPHOS), which occurs independently from oxygen or nutrient concentrations in the surrounding tissues. This is mediated by an increase of nitric oxide (NO) via TLR-dependent induction of inducible nitric oxide synthase (iNOS). NO inhibits OXPHOS by nitrosylation of electron transport chain (ETC) complexes (Kelly and O'Neill, 2015). Notably, contact allergens can contribute additional protein modifications to ETC complexes (Jeong et al., 2008) and might therefore enhance the breakdown of the ETC. In our study, short-chain acylcarnitines decreased immediately after treatment with DNCB. Acylcarnitines in general consist of fatty acids bound to carnitines and represent the transport form of fatty acids into the mitochondria. The generation of acylcarnitines via carnitine palmitoyl transferase I (CPT1) happens at the outer mitochondrial membrane. Availability of fatty acids in the mitochondria is essential for fatty acid oxidation (FAO). The production of acylcarnitines via CPT1 represents a rate-limiting step in FAO (Houten and Wanders, 2010). Therefore, a decrease of short-chain acylcarnitines indicates diminished FAO and provides further evidence for a diminished OXPHOS after THP-1 activation.

In addition, we provide evidence that activation of THP-1 cells by DNCB leads to an induction of lipid synthesis. Phospholipid levels,

especially levels of phosphatidylcholines and lysophosphatidylcholines, increased over time following treatment with DNCB. These data support the observation on glucose levels. The immediate switch to glycolysis serves the production of pyruvate, which is fed into the TCA cycle as citrate. Citrate itself serves as a precursor for the synthesis of fatty acids, which are needed to synthesize phospholipids (Everts et al., 2014). Indeed, we also found FAS up-regulated after DNCB treatment. FAS, the key enzyme of fatty acid synthesis, catalyzes the synthesis of palmitate, which then can be elongated to form longer fatty acids that are needed for lipid synthesis. Induced fatty acid synthesis has already been reported during the differentiation of monocytes into phagocytic macrophages. Here, augmented phospholipid synthesis coincided with the enlargement of filopodia pointing to cellular motility (Ecker et al., 2010). BMDCs and macrophages exhibited an up-regulation of FAS and other proteins involved in lipid metabolism during differentiation (Becker et al., 2012). FAS is also included in the genomic biomarker signature of the Genomic Allergen Rapid Detection (GARD) assay to predict contact allergens in vitro using a transcriptomics approach (Johansson et al., 2011). Other recent studies also reported a dominance of phosphatidylcholines in MPS cell models (Ecker et al., 2010; Martins et al., 2016; Santinha et al., 2012). During MPS cell activation, phospholipids are needed as a source for the synthesis of prostaglandins, which are potent mediators of inflammation during immune system activation (Kelly and O'Neill, 2015) and for the expansion of the endoplasmic reticulum (ER) and Golgi apparatus to facilitate enhanced synthesis and secretion of proteins (Everts et al., 2014).

Activation of DNCB-treated THP-1 cells was accompanied by decreasing amino acid levels. This effect, especially observed after treatment with the highest DNCB concentration, further supports the assumption that the TCA cycle in activated THP-1 cells is not primarily used to produce reducing equivalents and for ATP production by OXPHOS anymore. Instead, the TCA cycle seems rather be used to produce citrate to fuel fatty acids synthesis. Additionally, citrate may be converted to itaconic acid, which inherits anti-microbial properties (Michelucci et al., 2013). Citrate also supports NO production (Infantino et al., 2011), which has been shown to be linked to the reduction of OXPHOS. However, elevated consumption of citrate would soon lead to an interruption of the TCA cycle. Thus, conversion of glutamate to α -ketoglutarate can be anapleurotic to refill the TCA cycle (Jha et al., 2015). Indeed, in our study we found glutamate levels reduced in a concentration-dependent manner after DNCB treatment.

In addition, we detected an increased synthesis of spermidine and spermine after DNCB treatment. This may further account for the decline in amino acid levels as amino groups are needed for the synthesis of these polyamines. Especially spermine was up-regulated in a time- and concentration-dependent manner. Both biogenic amines can serve manifold functions including stimulation of protein synthesis (Igarashi and Kashiwagi, 2015) and protection of macromolecules against ROS (Seiler and Raul, 2005). Taken together, the degradation of amino acids presumably serves the anapleurosis of the TCA cycle as well as for synthesis of NO and polyamines. Of course, elevated protein synthesis may also account for a decline in levels of free amino acids. We found several lines of evidence for an enhanced protein synthesis after activation of THP-1 cells by DNCB. The translation-related proteins RLA2 and HRNH1 were up-regulated after 4 h and 24 h, respectively. Furthermore, the chaperones ENPL and PDIA1 were induced after 24 h of DNCB treatment. Both proteins are involved in the unfolded protein response (UPR). Increased protein synthesis during DC activation can result in an induction of the UPR (Pearce and Everts, 2015). UPR can also influence lipid synthesis and antigen presentation (Grootjans et al., 2016). PDI isoforms have also been found induced by the contact allergens DNCB and CA during BMDC activation in our previous study (Mussotter et al., 2016).

However, after 24 h we found evidence for an induction of mitochondrial activity by DNCB. At this time point, on the metabolome level, long-chain acylcarnitines increased and H1 levels normalized. On

the proteome level, the mitochondrial protein MDHM was up-regulated time-dependently peaking after 24 h. At the same time, KPVM - the key enzyme of glycolysis - was down-regulated. This is consistent with findings in LPS-activated THP-1 cells. Here, the high NAD^+/NADH ratio caused by the increased glycolysis rate later mediates a switch from the pro-inflammatory state to a more anti-inflammatory state, which is characterized by increased fatty acid oxidation (Liu et al., 2012, 2011). THP-1 cells represent a well-established model system for studying contact allergens. However, this model is limited compared to the in vivo situation as immune relevant factors derived from keratinocytes, the microbiome or other immune cells are missing. Possibly, the absence of external stimuli in an inflammatory milieu prevents a prolonged activation of isolated THP-1 in cell culture. Such limitations may be addressed in future studies by using co-culture models, for instance.

5. Conclusion

We identified several characteristic metabolic changes that indicated metabolic reprogramming in THP-1 cells during activation by DNCB. In particular, we found up-regulation of phospholipids, which is consistent with diminished levels of glucose and amino acids and which indicates a consumption of TCA cycle intermediates to facilitate lipid synthesis. On the proteome level, the induction of FAS - the key enzyme of fatty acid synthesis - supported our findings on phospholipid metabolism. In summary, our study provided further evidence to the hypothesis that activation of immune cells by contact allergens is linked to metabolic reprogramming. It also revealed that analyzing metabolic alterations in THP-1 cells can be very useful to find novel biomarker candidates that support the identification of contact allergens in vitro. Based on our data we suggest long-chain phosphatidylcholines (e.g. PCae_C42:1 and PCae_C44:4) and biogenic amines (e.g. spermine and taurine) as promising biomarker candidates to be assessed in future studies.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.taap.2017.12.016>.

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